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Contents of Volume 28

No. 1. *January 1948*

INTERNAL SECRETIONS AND TOXEMIA OF LATE PREGNANCY *George Van S. Smith and O. Watkins Smith*. 1

LOCAL ACTION OF SEX HORMONES *Harold Speert*. 23

PTEROYLGLUTAMIC ACID AND RELATED COMPOUNDS *T. H. Jukes and E. L. R. Stokstad*. 51

CONDITIONING FACTORS IN NUTRITIONAL DISEASE *Benjamin H. Ershoff*. . 107

No. 2. *April 1948*

NEURAL CONTROL OF THE PITUITARY GLAND *G. W. Harris*. 139

FERTILIZATION AND IMMUNITY *Albert Tyler*. 180

CHEMORECEPTION IN INSECTS *V. G. Dethier and L. E. Chadwick*. 220

USE OF MICROORGANISMS FOR ASSAY OF VITAMINS *E. E. Snell*. 255

No. 3. *July 1948*

SOME THERMODYNAMIC AND KINETIC ASPECTS OF METABOLIC PHOSPHORYLATION *A. G. Ogston and O. Smithies*. 283

ALLOXAN DIABETES *F. D. W. Lukens*. 304

RENAL FUNCTION IN EARLY LIFE *R. A. McCance*. 331

CHEMICAL, PHYSICAL AND MORPHOLOGICAL PROPERTIES OF ANIMAL VIRUSES *J. N. Beard*. 349

THE RELATION OF NUTRITIONAL DEFICIENCIES TO GRAYING *Douglas V. Frost*. . 368

No. 4. *October 1948*

ORIGIN OF MELANOPHORES AND THEIR ROLE IN DEVELOPMENT OF COLOR PATTERNS IN VERTEBRATES *Mary E. Rawles*. 383

ANTICONVULSANTS *James E. P. Toman and Louis S. Goodman*. 409

GASTRIC ABSORPTION *Leonard Karel*. 433

THE PHYSIOLOGY OF ADIPOSE TISSUE *E. Wertheimer and B. Shapiro*. 451

PHYSIOLOGICAL REVIEWS

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INTERNAL SECRETIONS AND TOXEMIA OF LATE PREGNANCY

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CRITICAL, COMPREHENSIVE PUBLICATIONS concerning the toxemias of pregnancy have been written by Berkeley (15), Holland (76), Hinselmann (73), Stander (174), Herrmann (71), Kosmak (83), McIlroy (102), Dieckmann (44), Dexter and Weiss (43) and Browne (26). These authors have made exhaustive studies of the literature on this subject and included their own investigations. The present review was prompted by the increased knowledge of internal secretions in relation to this diseased state, chiefly as regards the hormones elaborated by the placenta. Unfortunately, detailed morphologic studies of the glands of internal secretion in toxemia as well as in normal pregnancy are lacking (43), with the exception of the placenta and only recently has this organ been receiving more thorough histologic attention (*v.i.* under Placental Hormones).

Anterior lobe of the hypophysis. Gonadotropic hormones. No follicular activity was found in the ovaries of hypophysectomized rats following the injection of sera from patients with toxemia (5, 140). The effect noted was the same as that produced both by sera from normally pregnant women and by an extract of human placenta (140). The results supported Philipp's (112) demonstration that the hypophyses of pregnant women are gonadotropically inactive, so far as the ovaries of rodents are concerned, and indicated further that patients with toxemia do not differ in this respect from normal. The anatomical studies reviewed by Dieckmann (44) did not suggest that the anterior lobe is primarily at fault in eclampsia.

Thyrotropic hormone. Bonilla and Kramann (19) extracted a substance with properties similar to those of the thyrotropic hormone from the urine of patients with eclampsia. They did not obtain it from the urine of normal pregnant women.

Adrenotropic hormones. Fauvet and Münzner (58) reported subnormal corticotropic hormone levels in seven of eight women with severe toxemia.

Posterior lobe of the hypophysis. From a perusal of the literature and their own studies, Dieckmann (44), Dexter and Weiss (43) and Browne (26) concluded that the secretions of this gland are not implicated etiologically in pre-eclampsia

and eclampsia. Their conclusion holds for pressor as well as antidiuretic activity. Krieger and Kilvington (85) have more recently surveyed the conflicting literature concerning the antidiuretic property of blood and toxemia and tested the blood of 303 women. They found blood antidiuretic activity in 29 to 41 per cent of the groups of patients studied and that the activity in both normal and toxemic cases was related to labor and the early puerperium. Therefore they suggested that the contradictory results in the literature might be explained if considered in relation to the onset of labor.

Antidiuretic substance in the urine. Concentrates prepared from the urine of patients with toxemia were discovered to exert a marked antidiuretic effect which was not obtained with similar extracts from normal women in late pregnancy (18, 43, 67, 84, 125, 183). According to some authors, this effect was not due to pitressin (67, 125). Furthermore, a principle resembling the urinary substance was found in larger amounts in the placentas of toxemic women than in those of normal pregnancy (67).

Melanophore-expanding principle. Küstner and Dietel (87) reported a substance with this property in the blood and placentas of patients with eclampsia. Ehrhardt (51) confirmed this. Others have demonstrated this effect in the blood of toxemic women (6, 104).

Effects of administered posterior pituitary extracts. Chipman (192), Dieckmann and Michel (45) and Hofbauer (74) stated that the use of pituitary extracts in toxemia occasionally precipitates convulsions; Stander (174), referring to reports in which eclampsia appeared to ensue or become aggravated upon the injection of pituitrin, was of the opinion that the routine use of pituitary extracts in many clinics, without appreciably increased incidence of eclampsia, indicated that such observations were coincidental. In this connection, a number of patients with diabetes insipidus have been treated throughout pregnancy with posterior-lobe extracts (44, 121, 138) without any alarming developments. The following is quoted from Dexter and Weiss (43): "Because patients with diabetes insipidus are especially sensitive to the antidiuretic effect of the posterior pituitary hormone, it is worth noting here that usually such patients show no striking improvement during pregnancy and that there have even been instances in which diabetes insipidus appeared during pregnancy and disappeared after delivery."

Dieckmann and Michel (45), however, and others (25, 88, 104, 128, 187, 208) demonstrated an increased susceptibility of toxemic individuals to posterior pituitary extracts, a much higher and longer-lasting rise of blood pressure resulting. Moreover, Browne (25), using the cold pressor test and injecting tonephrin (pitressin), noted that the hypersensitivity of pre-eclamptic women to this hormone persists during the puerperium, being found as late as the two hundred thirty-fifth day. He also noted a similar cold pressor test hypersensitivity to pitressin in normal puerperal women as late as the twentieth day. From further studies he concluded (26) that the high reaction to pressor substances in pre-eclamptic toxemia is not due to predisposition but to something acquired during pregnancy and seemingly after the third month.

Inactivating power of the blood of pregnant women upon pitocin and pitressin.

Fekete and others (59, 195–197) found that posterior-lobe hormones were inactivated when mixed with serum from pregnant women. Woodbury *et al.* (208) confirmed this and noted, moreover, that the hypersensitivity of patients with toxemia to these hormones (pitressin and pitocin) was not associated with a diminished ability of their blood to inactivate them. Plasma pitocinase studies were made by Page (105). After establishing a curve of values through normal pregnancy, he found the enzyme within normal range in only 3 of 16 women with toxemia; in 7 the values were high, in 6, low. There was no correlation between the amount of enzyme and the severity of the disease.

THE THYROID GLAND. Anselmino and Hoffmann (75) presented evidence which they interpreted as demonstrating increased thyroid hormone, or a material with the same action, in the blood in normal pregnancy, the amount being enormously increased in eclampsia. No toxemia occurred in Davis' (41) eight patients with hyperthyroidism complicated by pregnancy, but five of Kibel's (82) nine similar cases developed pre-eclampsia.

It has been suggested that hypothyroidism may play a part in the production of toxemia of pregnancy (37, 38, 78, 109). Since, according to Dexter and Weiss (43), the clinical picture does not indicate hypothyroidism, the basal metabolic rate in this condition is not at myxedematous levels, and the protein content of the edema fluid is extremely low, hypofunction of the thyroid gland cannot play a significant rôle in its causation.

THE PARATHYROID GLANDS. "Many studies have been made of serum calcium in toxemic patients, but the consensus of opinion is that there are no significant changes in the total calcium or in any of its fractions in eclampsia or any of the other toxemias" (44). Patients with eclampsia and pre-eclampsia have been treated with parathyroid extracts (23, 24, 91) but without results sufficiently impressive to indicate any specificity of the therapy.

PANCREAS. *Diabetes.* The incidence of toxemia is greatly increased in diabetic patients (17, 44, 61, 63, 89, 199, 204). No one has elucidated the significance of this.

THE ADRENAL GLANDS. Fauvet and Münzer (58) found the adrenals small in six women who died of eclampsia, the weight of the two glands being less than 10.0 grams in all. In normal pregnancy the adrenal cortex is markedly increased, due to an increase in size and in fat content of the cells of the zona fasciculata, so that the adrenals are larger than in non-pregnant individuals, in whom these glands weigh 12 to 15.5 grams (8).

The adrenal medulla. The injection of epinephrin into patients with toxemia was found to cause a reversal of the usual blood pressure response, *viz.*, a drop instead of a rise (33, 92). Macchiarulo (95) recorded that an excess of epinephrin in the blood was usually present in eclamptic women and to this he attributed in part convulsions, vascular contractions and hypertension.

The adrenal cortex. The results of research on the adrenal have been reviewed by Swingle and Remington (176) but little has been done on any possible relation between adrenal function and toxemia of pregnancy. Over-activity of the adrenals has been considered (26, 43, 181). Taylor *et al.* (181) found in two

cases of toxemia after delivery "a pronounced divergence of the sodium and potassium lines which might indicate a return to normal from a previous condition of relatively low potassium and high sodium storage." "This could on theoretical grounds have been produced by hyperactivity of the adrenal cortex." Against this view, however, they pointed out their observation of potassium as well as sodium retention in toxemia, whereas lowered serum potassium and a high excretion rate are characteristic of adrenal hyperactivity. Interesting in this connection are reports concerning two patients with Addison's disease complicated by pregnancy (127, 211) who went through essentially uneventful gestations while under treatment with sodium chloride and cortical extract.

Although urinary corticoids and ketosteroids have been determined during normal pregnancy (188, which includes references to the work of others on ketosteroid excretion in pregnancy), they have not been studied in relation to toxemia.

THE OVARIES. Toxemia has not been stated to have occurred in patients whose pregnancies continued after the early removal of the corpus luteum or of both ovaries (2, 4, 8, 22, 28, 44, 49, 50, 79, 118, 123, 167, 177, 191). This may not be interpreted to mean that toxemia has never developed in such patients. On the other hand, since a fairly large number of such cases has been reported, it may be significant.

In presenting their case of eclampsia associated with ovarian pregnancy, Pride and Rucker (117) stated that it was the seventh recorded instance of toxemia in patients with pregnancies outside of the uterus.

THE PLACENTA. For decades authors have ascribed toxemia of pregnancy to abnormal placental function. The literature concerning this organ has been thoroughly covered by the writers referred to in the first paragraph of this review. Knowledge of the placental hormones, however, was only beginning to be acquired during the period they reviewed. The following statements made by Dexter and Weiss (43) summarize their own conclusions and those of others on the probable significance of findings published before 1940. "The main histological changes in toxemia are found in the placenta, liver, kidneys and retina. The placental changes consist of a premature degeneration of the syncytium. These lesions develop early and may precede even the appearance of albuminuria. The present evidence points to the placenta as the 'intrauterine factor' responsible for toxemia of pregnancy. Although the mechanism by which toxemia is produced is not known in its details, a chemical (hormonal) mechanism . . . may be suspected with some justification. The clinical predisposing factors . . . disturb the placental circulation, causing secondary degenerative changes (premature ageing). The observations reported by us and others regarding the origin of the edema of pregnancy indicate that . . . the abnormalities in water metabolism during pregnancy are not due to causes usually found in the non-pregnant state. A consideration of the various possible explanations . . . suggests specific factors which retain fluid in cells and tissue spaces. By exclusion, endocrine factors such as exist in premenstrual edema and Cushing's syndrome can be suspected. It is possible that the placenta produces a water-retaining hormone and also a vasoconstrictor substance. . . ."

PLACENTAL HORMONES. *Chorionic gonadotropin.* A gonadotropic factor in the urine of pregnant women was discovered by Ascheim and Zondek (7). That it differs from gonadotropic hormones of anterior pituitary origin was demonstrated by Evans *et al.* (56, 57). By tissue culture technic, Gey *et al.* (62, 81) showed it to be a placental product, probably from the cytotrophoblast. The histochemical studies of Wislocki and Bennett (206) also indicated that the cytotrophoblast of the chorionic villi is responsible for its secretion. These latter investigators also pointed out that the growth and activity of the cytotrophoblast are roughly in accord with the previosuly established curves of gonadotropic content in blood and urine throughout gestation (27, 55, 134, 142, 161, 209). Chorionic gonadotropin, or CG, is now the generally accepted appellation for the hormone.

Ehrhardt (52) reported positive tests for gonadotropic hormone in the cerebrospinal fluid of eclamptic and pre-eclamptic women, but normal titers in their blood and urine, and concluded therefrom that the gonadotropic activity of the anterior pituitary was high in toxemia of late pregnancy. Excessive gonadotropic hormone in the blood and urine of patients with toxemia was first reported by Smith and Smith (137–139), who demonstrated also that the placentas of of toxemic patients contained more than could be recovered from those of normal pregnancy and that the high gonadotropic titer of blood and placenta in toxemia was not accountable to pituitary gonadotropes (140). High CG in the blood and urine of women with toxemia has been confirmed (5, 21, 70, 119, 134, 185), as well as the evidence for its placental origin (5). It has been shown, however, not to be demonstrable in all cases, not to bear any direct relation to the severity of the disease and to be associated also with stillbirth and premature delivery in the absence of toxic signs (120, 145, 148, 171, 193). Browne, Henry and Venning (29) could make no correlation between elevated CG and late pregnancy toxemia. Taylor and Scadron (180) and Cohen, Wilson and Brenan (34) found only a slight correlation. The latter investigators studied 119 pregnancies and based their conclusion on the fact that, although 44 per cent of toxemic patients had high serum CG, elevated levels were also present in 15 per cent of normal deliveries. Other studies (120, 148, 161, 171) have indicated that elevation of CG during the last month before labor is a normal phenomenon. This may explain the high values in normal late pregnancy which disturbed Taylor and Scadron and Cohen *et al.*

Smith and Smith, in 1934 (138), noted a marked rise of serum CG early in the third trimester in a woman who later developed pre-eclampsia. They have since performed repeated analyses for serum CG upon 117 women during the last trimester to determine whether or not accidents of late pregnancy could be predicted by an abnormal rise in this factor (139, 144–146, 148, 161, 171). (Since the incidence of late pregnancy accidents was known to be high in patients with diabetes, 68 of the above number were chosen for study because they had diabetes.) In all of 42 women whose pregnancies progressed and terminated normally, serum CG was at a low and uniform level between the twenty-fourth and thirty-sixth weeks, any rise after the thirty-sixth week being considered normal. Of 56 patients in whom a progressive elevation of CG was observed

prior to the thirty-sixth week, 45 developed toxemia, eight delivered prematurely and three had their babies die *in utero*. On the other hand, 19 patients in whom no abnormal elevation of serum CG was detected developed late pregnancy complications. Nine had stillbirth or premature delivery and 10 developed toxemia. They concluded that, although an abnormal rise of this hormone in the blood prior to the last month of pregnancy warrants the prediction of later trouble, it gives no indication of the type or severity of the accident and, furthermore, that accidents may occur without any warning so far as a rise of CG is concerned.

High serum CG prior to the appearance of toxic signs was reported by Rakoff (119) in two of three patients. White *et al.* (200–203) performed repeated analyses for serum CG as a prognostic test in patients with diabetes complicated by pregnancy. In 1945 she (200) summarized results concerning 181 such women. The studies on the first 33 of her patients were performed by Smith and Smith (144, 145, 161); therefore these cases were included with the 117 just analysed (*v.s.*). Her results agreed with the Smiths' in that an abnormal rise of serum CG was always followed by clinical abnormalities and the level of hormone was consistently low in uncomplicated pregnancies. She failed to confirm, however, the fairly high incidence of late pregnancy accidents observed by the Smiths in patients whose serum CG did not become abnormally elevated. Only one of her 52 patients with normal CG levels developed toxemia, none delivered prematurely and only 2 lost their babies. Rubin, Dorfman and Miller (120) followed serum CG during the last trimester in 5 diabetic patients, one of whom had normal CG levels and an uncomplicated pregnancy, 2 of whom developed toxemia, 1 with and 1 without high CG, and 2 of whom had fetal death, 1 with and 1 without elevated CG.

Progesterone. Progestational activity has been repeatedly detected in extracts of human placenta (1, 53, 54, 65, 101, 152). Since histochemical studies (42, 206) indicate that the chorionic syncytium elaborates steroids, progesterone is probably secreted by these cells. The demonstration that pregnanediol glucuronidate is an excretory product of progesterone (190) and the development of a method of quantification (189) led to extensive studies concerning the urinary excretion of this compound in normal and toxemic pregnancy. In normal pregnancy the amount increases from around 10.0 mgm. daily at one month to between 60.0 and 130.0 mgm. daily shortly before term and practically disappears within 24 hours of delivery (10, 12, 28–30, 32, 66, 80, 144, 146–148). The corpus luteum has been removed in pregnancy without more than slight and temporary alteration in the excretion of pregnanediol (28, 79). Browne and his associates (30) noted peaking of pregnanediol excretion at approximately four-week intervals during pregnancy. This was also observed by Smith and Smith (146), who found in individual curves the final and highest peak about two weeks before term, this being followed by decreasing values before and during labor (147). A prepartum drop in pregnanediol was also noted by others (10, 12, 13, 66). Studies by Stover and Pratt on 5 patients (175), by Wilson, Randall and Osterberg on 1 patient (205) and by Bachman on 6 women (9)

failed to reveal any decline of pregnanediol before labor, but Lyon (94), reporting results on 68 patients whose labors were spontaneous, determined a peak of pregnanediol excretion about two weeks before delivery, followed by decreasing values and then a precipitous drop for five to seven days prepartum, regardless of whether labor was premature, at term or postmature.

Smith and Smith (135, 136), in their earliest studies of estrogen and progesterone metabolism, discovered that progesterone decreased the destruction of both administered and secreted estrogen. Their finding of low estrogens in toxemia (*v.i.*) led them to suspect, therefore, that progesterone might also be deficient (139, 161). Smith and Kennard (152) attempted to compare the progesterone content of normal and toxemic placentas. Although there was evidence of deficient progesterone in half of the placentas from untreated toxemic patients, the method used was not sufficiently accurate to warrant definite conclusions. In 1938 three groups of investigators, working independently, reported low values for urinary pregnanediol in toxemia of late pregnancy (29, 144, 194). This has been confirmed (11, 13, 146, 148, 180). There is general agreement, however, that the abnormality is revealed only by comparing the averages of normal with those of toxemic pregnancy. Cope (39, 40) did not find it in 10 women with late pregnancy toxemia and Hain (66) observed excessively high urinary pregnanediol in 1 patient with severe pre-eclampsia.

Smith and Smith (148) pointed out the wide range of normal values and concluded that the curve of pregnanediol excretion as pregnancy advances is more important than the actual level at any given time. In 14 normal pregnancies they found progressively increasing values, with minor fluctuations, between the twenty-eighth and thirty-sixth weeks; whereas, in 21 patients with toxemia the values steadily decreased during this time, with one exception, in a patient whose toxemia became self-corrected during the period of observation. In two women studied prior to the onset of toxic signs, urinary pregnanediol progressively diminished for four weeks before any clinical abnormality was apparent. These investigators have studied the relationship between progesterone and the urinary excretion of estrogen metabolites (*v.i.* Metabolism of the estrogens) and believe that measurement of the latter, though more laborious, provides more information concerning secreted progesterone than does the determination of urinary pregnanediol. In all of 50 patients studied by them prior to the development of toxemia, premature delivery or intrauterine death, evidence for a progressive deficiency of progesterone before as well as during the clinical abnormality was acquired (144, 146, 148, 149, 171, 172). White and Hunt (202) utilized pregnanediol determinations as a prognostic test in 60 diabetic women of whom 43 were receiving therapy with estrogen and progesterone. Of the untreated patients, 12 had normal curves of pregnanediol excretion and normal pregnancies with live births, though two of the babies died after delivery. Of the five untreated women whose pregnanediol excretion was decreasing between the twenty-fifth and thirty-sixth weeks, three developed toxemia, one delivered prematurely and the fifth showed no clinical abnormality before being delivered by cesarean section in the thirty-fourth week. Rubin, Dorfman and Miller (120)

noted diminished excretion of pregnanediol prior to toxemia in two of their diabetic patients but failed to find this abnormality in two others whose babies died, one in utero and the other after delivery.

The estrogens. Estrogenic substance was first demonstrated in the human placenta by Fellner (60). This has been adequately confirmed (3). In 1927, Ascheim and Zondek (7) discovered the great concentration of estrogenic material in the urine of pregnant women, and Margaret Smith (157) found increasing amounts of it in the blood throughout human pregnancy. Removal of the corpus luteum or of all ovarian tissue as early as the sixth to eighth week of pregnancy has been shown not to interrupt gestation (2, 4, 8, 22, 28, 49, 50, 79, 118, 123, 167, 177, 191) or the continued elaboration of estrogen (2, 4, 22, 118, 123, 167, 191). The concentration of estrogens in the placenta, their continued excretion after ovariectomy,and the prompt cessation of excretion after delivery contribute to the generally accepted tenet that the placenta forms estrogens; the site of formation being likely in the syncytial cells of the chorionic villi (42, 206).

Estrogenic activity in the serum is relatively low until after the second month of normal pregnancy. It increases rapidly thereafter, the highest values being acquired during the last month (138, 139, 157, 161). Quantification of blood estrogen is not satisfactory because of still unsolved technical difficulties. The discovery that all but a small part of urinary estrogen is in combined forms, relatively inactive biologically and insoluble in water-immiscible organic solvents (20, 35, 90, 141, 210) made earlier studies of estrogen excretion only roughly significant. Subsequent investigations, in which hydrolysis and improved methods of extraction were used, yielded more accurate and significant results and established that the total estrogenic potency of the urine rises before the first missed period (100, 142), increases gradually and then markedly during pregnancy, reaching a peak in the last month, and diminishes rapidly prior to the onset of labor, becoming negligible within three days after delivery (9, 27, 29, 36, 46, 66, 93, 98, 134, 144, 146–148, 154, 161).

Low levels of estrogen in the blood, urine and placentas of most patients with toxemia of late pregnancy were first reported by Smith and Smith (137–140, 161). This finding, as regards the urine, has been confirmed by nearly all investigators (29, 93, 119, 120, 124, 134, 144, 146, 148, 149, 170, 171, 180, 193). There is, however, general agreement that in individual cases values within or even above the normal range often are obtained. Heim (70) reported high and Hain (66), in one case, very high urinary estrogen in toxemia, and normal blood values have been described (16). Shute (129–133), using antiproteolytic power of serum as a gauge of its estrogen content, claimed both high and low estrogen in toxemia. As with urinary pregnanediol, the curve of excretion in each individual is thought by the Smiths to be more important than the actual level at any one time. This is apparent in their curves for total estrogen of blood and urine in diabetic and non-diabetic women (161). Seven who developed toxemia or had premature delivery failed to show the marked increase of estrogen in blood and urine between the sixth and eighth months that characterized the 17

normal curves. In their later studies, in which urinary estrogen was separated into estradiol, estrone and estriol and the values converted from rat units into milligrams, a progressive decrease of total estrogen was demonstrated before and during toxemia, premature delivery and intrauterine death in all of 50 patients studied (144, 146, 148, 149, 171, 172).

That decreasing excretion of estrogens and pregnanediol before labor and late pregnancy toxemia actually reflects decreasing secretion of placental steroids is indicated by histological and histochemical studies of human placentas. The syncytial degeneration which characterizes normal term placentas involves an almost complete disappearance of the lipoidal droplets associated with secretion of steroid hormones (42, 206, 207). In 1936, Tenney (184) discovered that syncytial degeneration similar to but more pronounced than that of term placentas is of consistent occurrence in toxemic placentas. He and Parker later (186), from an examination of 100 toxemic placentas, correlated the amount of syncytial degeneration with the severity of the disease and concluded that "placenta damage begins before clinical signs of the condition appear." Wislocki and Dempsey (207) confirmed these observations in two toxemic placentas delivered in the fifth month, finding histochemical, syncytial changes typical of the organ at term. (They also noted a decrease of cytoplasmic basophilia and a premature increase of phosphatases suggesting a possible disturbance of nucleoprotein metabolism in this disease.) Histological and histochemical evidence, therefore, substantiates the above hormonal evidence, pointing to a premature aging of the placenta in toxemia and therefrom a premature deficiency in the secretion of progesterone and estrogen. An actual deficiency, then, of estrogen and progesterone, rather than any renal retention or change in conjugation to account for the low urinary levels, appears to be well established. The theory introduced by a number of workers (12, 29, 66, 179, 180, 182) that toxic signs, particularly changes in electrolyte balance and water retention, are due to high levels of these steroid hormones must, therefore, be discarded.

Smith and Smith (145) in summarizing and evaluating their findings on CG in the blood and urine during normal and toxemic pregnancy, postulated utilization of this hormone in the placenta for the production of estrogen and progesterone. They had noted that the precipitous decrease in CG of blood and urine at around the twelfth week of normal pregnancy was accompanied by a marked increase in estrogen excretion (142) and that the prepartum decrease in estrogen excretion coincided with a rise in serum CG (154, 161). This same reciprocal relationship between CG and estrogens had been observed in late pregnancy toxemia (144, 161), suggesting to them that an abnormal elevation of serum CG in this condition and at term reflected failing utilization. To explain the absence of elevated serum CG in some patients with toxemia, they postulated an actual decrease in the secretion of this factor from placental damage. In support of these hypotheses is the following more recent evidence. The cytotrophoblast and its more differentiated derivatives, the cells of Langhans, are the likely source of CG (81, 206). It is well known that the Langhans cells become fewer in number as pregnancy advances and are practically absent in

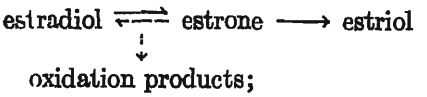
term placentas. There is no increase of cytotrophoblast in toxemic placentas (72, 207). And, finally, in two such placentas delivered in the second trimester Wislocki and Dempsey (207) found premature regression of the Langhans cells as well as the syncytium. As pregnancy continues, therefore, whether normal or abnormal, there is no proliferation of the cells which probably secrete CG to account for high values at term and in toxemia but rather, if anything, a degeneration. In view of the histochemical and hormonal findings pointing to decreased sex steroid production at term and in toxemia, then, it seems possible that failing utilization of CG accounts for the larger amounts of it in the circulation at these times and that decreased elaboration as well as failing utilization account for the normal serum values sometimes acquired. This concept was further corroborated recently (168) by a comparison of hormonal values and placental pathology in the same patients.

Metabolism of the estrogens. Certain changes in the partition of urinary estrogens and in the excretion of what appear to be estrogen metabolites have been found to precede and accompany the onset of labor and of late pregnancy toxemia. In order to evaluate the significance of these, a brief review on the subject of estrogen metabolism is in order.

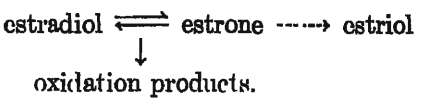
Three estrogens have been identified in the urine of pregnant women: estrone (31, 48), estriol (47, 96) and α estradiol (77, 153). Of these, α estradiol is commonly regarded as the primary estrogen in humans from which the others are derived. Marrian (97) suggested that estriol may also be secreted by the human placenta. In 1937 Pincus and Zahl (116) injected the three estrogens separately into rabbits under varying conditions and determined their subsequent urinary excretion by separation and colorimetric assay. They concluded, as had been previously demonstrated both in rabbits and women (135, 136) that progesterone increases the amount of estrogen excreted, probably by inhibiting destruction. Moreover, their results indicated the following scheme of conversion within the animal body: α estradiol $\leftrightharpoons$ estrone $\rightarrow$ estriol, the estrone to estriol reaction being facilitated by the presence of progesterone. That the same metabolic relationships pertain in women seems likely from the studies of Smith and Smith and their associates (144, 146–149, 155, 156, 170, 173) and those of Pincus and his associates (113–115). These conclusions are based entirely upon colorimetric (Pincus) and biological (Smith and Smith) assay of the three estrogens separated by admittedly imperfect methods. Chemical confirmation of them in man, however, was at least partially acquired by the isolation of α estradiol and estriol in the urine following estrone administration (110, 111) and of estrone after the administration of α estradiol (68, 69).

All investigators have been impressed by the small percentage of injected estrogen recoverable in the urine under any conditions. Numerous attempts have been made to isolate compounds in human urine which could be identified as products of the endogenous inactivation of the estrogens but none of them has been successful. Smith and Smith (143) evolved a method of Zn-HCl hydrolysis of human urine which results in much more estrogenic activity than can be accounted for by the known estrogens present. The additional activity appears

to be derived from reduction into active forms of estrogenically inactive oxidation products of the estrogens. Since the precursors of this additional potency have not yet been identified, this deduction is based largely upon circumstantial evidence. The procedure, however, seems to provide a gauge of the rate of oxidation of estrogens within the body (155, 156, 162, 173). Using this method in conjunction with the separate assay of estradiol, estrone and estriol fractions, they strengthened the evidence that progesterone decreases the rate of estrogen inactivation, by facilitating the conversion of estrone, which is rapidly inactivated *in vivo*, to estriol, which is much less readily destroyed, according to recovery experiments (126, 156). Their results also indicated that the destructive mechanism inhibited by progesterone is an oxidative one related largely to the reversible estradiol to estrone reaction. Thus in the presence of adequate progesterone:



whereas, when progesterone is deficient,



In applying these methods to the study of estrogen and progesterone metabolism in both pregnant and non-pregnant women, they were led to the concept that estrogenically inactive oxidation products of the reversible estradiol to estrone reaction, rather than estrogens *per se*, stimulate the production of estrogen and progesterone, this being accomplished in the non-pregnant woman through release of pituitary gonadotropes and in pregnancy through increased utilization of CG (147, 148, 150, 156, 166, 171, 173). This concept was strengthened by the experimental demonstration, 1, that a lactone produced from crystalline estrone by oxidative inactivation (198) causes release of pituitary gonadotropes; whereas, estrone has this property only under conditions involving rapid inactivation *in vivo* (158-160); 2, that diethylstilbestrol behaves like the estrone-lactone (163) and, 3, causes increased excretion of pregnanediol together with a drop in serum CG in pregnant women (168, 172).

In 38 untreated pregnancies the Smiths performed repeated urinalyses for estradiol, estrone, estriol and the additional estrogenic potency recoverable after Zn-HCl hydrolysis (148, 150, 171). Biological assay was used and the Zn-HCl activity interpreted as a gauge of the rate of oxidative inactivation of the estrogens *in vivo*. All of these women were studied from early in the third trimester to delivery. Of the 23 (see second paragraph below) who developed pre-eclampsia or eclampsia or had intrauterine death or premature delivery during the period of observation, three had been followed during the second trimester also. In 6 of the 15 normal pregnancies analyses were performed from the twelfth week to term. In normal pregnancy the percentage of total estrogens accountable to estriol was found to increase steadily, with minor

fluctuations, reaching a peak at approximately two weeks before term. At this time estrone was also high, whereas the proportion of activity accountable to estradiol was low, and the Zn-HCl values indicated the lowest rate of oxidative inactivation found at any time of pregnancy. These findings, together with the thirty-eighth-week peak in pregnanediol excretion were interpreted as reflecting a maximum rate of conversion of estradiol to estrone to estriol and a minimum rate of oxidative inactivation of the estrogens due to a high and balanced rate of secretion of estrogens and progesterone. The subsequent prepartum drop in estriol and estrone accompanied by an increase in estradiol and in the rate of estrogen oxidation was interpreted as reflecting decreased conversion and increased destruction of the estrogens due to progesterone withdrawal as well as reduced secretion of estrogen. According to their concept, *the syncytial degeneration of the placenta during these last weeks with decreasing secretion of steroids has its incipience at the time of the peak of estrogen and progesterone production and is due to the deficiency at that time of oxidation products of the estrogens and hence a deficient utilization of CG.* Once syncytial degeneration is under way, they believe, the process cannot be reversed, despite the increasing rate of estrogen inactivation (150, 168).

Bachman (9), using colorimetric assay, is the only other investigator who separately measured estradiol, estrone and estriol in the urines of pregnant women. No one has performed Zn-HCl hydrolysis in conjunction with such studies, and Bachman's investigations were limited to six normal pregnancies followed during the last trimester. His findings agree with the Smiths' as regards estradiol and estriol, but he failed to observe any prepartum drop in estrone.

Changes in estradiol, estrone and estriol and in the rate of estrogen inactivation similar to those during the last two weeks of normal pregnancy were consistently found by the Smiths to precede and accompany late pregnancy toxemia and associated accidents in the 23 abnormal cases studied during the last trimester the only difference being that when they take place prematurely they progress over a longer period and become exaggerated to a degree normally attained only after labor is well advanced. In the three patients followed from earlier in pregnancy there was urinary evidence of a premature peak in estrogen and progesterone secretion around the twenty-eighth week and a comparative *deficiency of estrogen oxidation products throughout the second trimester.* These patients were diabetic and, as pointed out, the earlier hormonal abnormality may not precede the premature degenerative changes in the placental syncytium in all cases of late pregnancy toxemia. Such a deficiency of oxidation products during the second trimester, however, may be the predisposing factor in a certain percentage of patients just as, according to their concept, a similar situation during the last weeks of normal pregnancy is responsible for failing utilization of CG and the prepartum withdrawal of placental steroids.

THEORIES OF ETIOLOGY AND ATTEMPTS AT TREATMENT. *The thyroid gland.* On the theory that hypothyroidism accompanied by hypercholesteremia produces placental arterial disease predisposing to thrombosis, infarcts and degene-

ation with resultant absorption of toxins from degenerated placental tissue, Patterson *et al.* (109) administered thyroid as a preventive measure. The clinical results reported by them and others (38, 64, 78, 86) with thyroid or iodine have not indicated specificity of therapy. As stated above, Dexter and Weiss (43) concluded that hypofunction of this gland cannot play a significant rôle in the causation of late pregnancy toxemia.

The adrenal glands. Parks (106, 107), assuming that the upset of sodium and potassium in toxemia is due to excessive hormones from the fetal adrenal glands, reported the control of edema by potassium in patients who had not responded to a low intake of sodium chloride.

Progesterone. Robson and Paterson (108, 122), from experiments with rabbits, concluded that the toxic condition from the uterus might be due to a failure of placental nutrition following removal of luteal secretion and described encouraging clinical results from small amounts of progesterone given early in the disease. These were not consistently achieved by others (14, 99, 103).

Estrogens. Shute (129–133), on the basis of a blood estrogen test devised by himself, classified toxemic patients into two groups, those with low estrogen (true pre-eclampsia and eclampsia) and those with high estrogen. The former he treated with estrogen and the latter with vitamin E, with satisfactory control of both. There are not reports in the literature of attempts to confirm the specificity of his test or his therapeutic results.

Testing their hypothesis that oxidation products of the estrogens cause utilization of CG for the placental secretion of its steroids, Smith and Smith (168, 172) investigated the effect of preventive treatment with diethylstilbestrol, this drug being employed because oxidation products such as estrone-lactone (*v.s.*) were not available in sufficient amounts and because experiments (163) had indicated a superiority of diethylstilbestrol for this purpose. Two diabetic patients with bad obstetrical histories were studied both during treatment and control periods and the placenta of one examined histologically. From the results, utilization of CG was enhanced and a premature aging of the placenta in steroid secretion, as well as late pregnancy complications, were averted. They (168) demonstrated that, once toxemia is evident, therapy with diethylstilbestrol is not likely to stimulate secretion of placental steroids or have any clearly favorable effect on the disease, since an excess of endogenous oxidation products pertains by this time and is unable itself to bring on renewed secretory activity in the already degenerate syncytium.[1]

[1] Since this paper went to press, Davis and Fugo (Davis, M. E., and N. W. Fugo. Proc. Soc. Exper. Biol. and Med. 65: 283, 1947) have reported experiments in which 50 to 200 mgm. of diethylstilbestrol were administered daily by mouth starting at the fourth to tenth week of pregnancy and continued for 6 to 8 weeks. This dosage is greatly in excess of the amounts recommended by Smith and Smith for early pregnancy (168, 172). In normal untreated pregnancy pregnanediol excretion increases steadily as pregnancy progresses; whereas the results presented by Davis and Fugo show no increase during the period of stilbestrol administration. It seems possible that such large doses early in pregnancy might inhibit progesterone secretion through depressing the utilization of CG, just as the prolonged administration of large doses to the non-pregnant woman are known to inhibit ovarian secretion through depressing pituitary gonadotropic activity.

Progesterone and estrogen. On the basis of their evidence for a progressive deficiency of these two hormones before and during late pregnancy toxemia and associated accidents, the clinical and hormonological effect of replacement therapy was investigated by Smith and Smith (144, 146, 149, 170). As a preventive measure in patients in whom studies of blood and urine warranted the prediction of later trouble, the therapy was considered promising but impractical. The first 10 trials of preventive therapy with estrogen and progesterone in diabetic patients at the George F. Baker Clinic of the New England Deaconess Hospital were carried out by the Smiths and reported by them (144, 146), as well as by White and her associates (200–203). The latter continued with this form of therapy and described good results in 91 patients, particularly as regards reduction of fetal mortality (200). There are no other publications concerning the use of such therapy prophylactically in either diabetic or non-diabetic patients, probably because of the expense involved. The prophylactic stimulation of placental secretion of sex steroids by diethylstilbestrol administration starting prior to any evidence of hormonal deficiency, now being investigated by Smith and Smith, would appear to offer a more physiological and practical approach.

Definitive therapy of toxemia with estrogen and progesterone has been disappointing even when seemingly large amounts were given (144, 146, 149, 170 179). Taylor (179) considered this to be evidence against steroid deficiency as solely responsible for the disease. Smith and Smith (149) demonstrated some temporary alleviation of pre-eclampsia with estrogen and progesterone, but their hormonal findings indicated that once toxic signs appear there is a rapid increase in the rate of destruction of administered as well as secreted hormones. This phenomenon, as well as decreased secretion, they believe, contributes to the low levels in toxemia. With Taylor they consider the deficiency of estrogen and progesterone not to be the final precipitating cause of the toxemic syndrome. Its universal occurrence in all cases, however, as shown by hormonal and histological studies (*v.s.*) and the evidence that it precedes toxic signs implicate it as an intermediary and contributory factor.

Menstruation and toxemia. In the publications covered in this review, the Smiths repeatedly referred to similarities between the phenomenon of menstruation and toxemia of late pregnancy, the analogy having been suggested to them primarily because of their evidence from urinalysis for a hormonal situation before and during menstruation entirely like that before and during late pregnancy accidents. The similarity between premenstrual changes and toxemia were observed clinically by Dexter and Weiss (43), who stated further that so far as types of edema and symptoms are concerned there is a certain similarity in menstruation, normal pregnancy and toxemia. The Smiths (150) recently summarized their evidence that withdrawal of hormonal support from the pregnant as from the non-pregnant uterus results in the formation and release of a toxic metabolite of tissue catabolism like the toxin they found in the menstruating endometrium. The pathological effects of this toxin upon experimental animals are such as to warrant the assumption that it is directly responsible for the local

changes resulting in menstruation and for the similar but generalized damage of late pregnancy toxemia. This hypothesis is strengthened by their evidence for certain properties in the circulating blood of women with toxemia similar to those of menstrual discharge, *viz.*, pyrogenic and fibrinolytic activity,[2] precipitation by anti-canine necrosin rabbit serum and a pseudoglobulin fraction capable of prolonging the survival time of rats given a lethal dose of the toxic euglobulin fraction of menstrual discharge (151, 165, 169), these properties being absent in normal pregnancy except during prolonged labor. According to their theory, the maternal portion of the placenta, *i.e.*, the decidua, being endometrium, should be the source of the toxin. (As such it could also be the cause of postpartum eclampsia.) A limited number of tests with specimens of decidua removed at the time of cesarean section have revealed marked toxicity in those from pre-eclamptic and eclamptic patients (150). In their publications, they have emphasized the extreme lability of the menstrual toxin (169). Failure to consider this possibility may explain the failures of the past to demonstrate any specific or consistent toxicity in placentas from toxemic women. Hertig (72) finds decidual necrosis and vascular thrombosis in relation to delivery and toxemia.

Certain known interrelationships between vascular supply, hormone production and tissue metabolism suggest that various conditions would bring about a menstrual-like phenomenon in the pregnant uterus. In the section on the metabolism of the estrogens (*v.s.*) the Smiths' data, suggesting that a deficiency of estrogen oxidation products during the second trimester might lead to premature syncytial degeneration was reviewed. Decidual, like endometrial, catabolism would be expected to result from the consequent withdrawal of estrogen and progesterone, with the production of toxin. The toxin in turn, to judge from its action in experimental animals, would be expected to augment degeneration of the syncytium and even cause degeneration of the Langhans cells and cytotrophoblast, thus disturbing hormone production even further.

Page (105-A) pointed out the possible rôle of placental ischemia in the pathogenesis of toxemia. An adequate vascular supply to the placenta is contingent upon an adequate production of estrogen and progesterone. Conversely, the Smiths (147) showed that decreased blood supply to the placenta through uterine contractions depresses the secretion of the steroid hormones. Decreased blood-supply to the uterine contents, such as might result from local mechanical conditions or generalized vascular situations, would be expected to result in degeneration not only of the syncytium but also of the decidua. Furthermore, since the pathological effect of menstrual toxin reflects vasoconstriction (151, 169), its release would in itself interfere with vascular supply.

In the course of their studies, the Smiths (151, 164, 169) deduced that types of injury not related to withdrawal of hormonal support cause the release of a similar if not identical toxin and acquired evidence for its presence in human exudative material and in the circulating blood of women with damaged tissues.

[2] Fibrinolytic activity in the circulating blood of patients with late pregnancy toxemia has been confirmed (203-A).

On this basis, damage of the chorionic epithelium might contribute to the production of toxin.

Any one of a number of processes, therefore, might be primarily responsible for setting off a chain of events leading to an overwhelming production of toxin and to toxemia of late pregnancy, unless delivery of the uterine contents intervened. Whatever the primary etiology, the final syndrome would be the same, a vicious circle in which decrease of vascular supply, hormonal deficiency and toxin formation were augmenting one another.

Even in cases wherein a deficiency of oxidation products of the estrogens does not pertain during the second trimester, the prophylactic use of adequate dosages of stilbestrol, now being investigated by Smith and Smith may prove effective through increasing uterine vascularity, both *per se* and by stimulating steroid secretion in the placenta. Once the disease is clinically manifest, according to their findings, the vicious circle of the final syndrome is already established and the best hope of cutting in upon it, aside from delivery of the products of conception, lies in neutralization of the toxin. To investigate this possibility, they (168) studied a few cases of pre-eclampsia to whom a crude preparation of their protective pseudoglobulin was experimentally administered. Clinically the results could be considered no more than promising. Their principal interest lies in the theoretical implications which appear to uphold the above concepts.

The presence of a toxin in toxemia has always offered attractive possibilities for explaining the clinical syndrome. Complete studies on the effects of menstrual toxin remain to be performed but experiments reported show that it causes edema and vasoconstriction and indicate that it produces damage of capillary endothelium as well as of tissues in general and has an antidiuretic effect (169). Dexter and Weiss (9) offered evidence for the existence of generalized damage of the capillary endothelial system in toxemia. That the toxin may be responsible for the hypertension and proteinuria of toxemia remains to be investigated. Damage of the renal glomerular capillaries would explain the proteinuria. The hypertension may be the consequence of a number of effects of the toxin, renal, suprarenal, cerebral and direct upon blood vessels. The antidiuretic substance in the placentas of toxemic patients (67) may be the toxin working directly or through pituitary stimulation (*v.i.*) in the experimental animal. The same explanation may apply as regards the melanophore-expanding principle in the blood and placentas of patients with toxemia (87). That patients with toxemia are hypersensitive to injected posterior-lobe hormones (*v.s.* Effects of Administered Posterior Pituitary Extracts) suggests an effect superimposed on that of the toxin. Smith and Smith (169) showed in rats that menstrual toxin causes the release of pituitary gonadotropic and adrenotropic hormones and believe that it produces an "alarm" reaction involving quite possibly the release of other pituitary hormones. Epinephrinemia in eclampsia (95) may reflect such an "alarm" reaction, and pituitary stimulation may explain a number of observations made in preeclamptic and eclamptic patients, *viz.*, urinary thyrotropic hormone (19), possibly the urinary antidiuretic substance (*v.s.* Antidiuretic substance in the urine), thyroid hormone (75) and, particularly, the possibility considered by some of over-

activity of the adrenal cortex (26, 43, 181). None of these abnormalities has been demonstrated prior to the clinical onset of the disease.

SUMMARY

From the above it is apparent that the primary etiology of toxemia of late pregnancy has yet to be determined, that no definitive treatment has been rewarded with spectacular cure, such as usually occurs following delivery of the products of conception, and that prevention of the disease by the administration of hormones has yet to be proven. Certain aberrations from the normal in hormonal or hormonal-like findings and in placental morphology appear to be established in women with pre-eclampsia and eclampsia: 1, excessive antidiuretic substance in urine and placenta; 2, a melanophore-expanding principle in the blood and placenta; 3, a hypersensitivity to the injection of posterior pituitary secretions; 4, high titers of chorionic gonadotropin in a large percentage of cases; 5, decreased excretion of estrogens and pregnanediol, and 6, histological and histochemical changes in the placenta similar to but more marked than those of the normal placenta at term and primarily involving degeneration of the syncytial cells which probably secrete the steroid hormones. Of these established abnormalities, the last three are underway prior to clinical manifestations and occur normally at term.

Synthesis. That the primary etiology of toxemia may consist of a number of causes is suggested by the already familiar predisposing factors such as essential hypertension, diabetes, primigravidity, hydatidiform mole, twins and hydramnios. The evidence at hand appears to establish premature senility of the placental syncytium and premature withdrawal of the placental steroid hormones as the final intermediary pathology. This disturbance, which occurs normally at term, must be brought about prematurely by the working of the primary etiology, which probably involves either an intrinsic metabolic abnormality affecting the placenta or a decrease in blood supply to the placenta or both. Since the syncytial-steroid aberration from the normal characterizes all cases, it may logically be assumed to be contributory factor to the development of toxemia in all cases. From the above discussion (*v.s. Theories of etiology and attempts at treatment*), however, it cannot be assigned the rôle of the sole precipitating cause of the toxemic syndrome. Recent incomplete work suggests, 1, that withdrawal of hormonal support from the pregnant as from the non-pregnant uterus may result in the formation of a menstrual-like toxin in the placenta (? maternal portion, *i.e.*, decidua); 2, that the primary etiology may, in conjunction with the steroid deprivation it causes, do the same thing, and 3, that release of this toxin may prove to be the final cause of toxemia of late pregnancy.

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LOCAL ACTION OF SEX HORMONES

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THE HYPODERMIC SYRINGE was, until recently, the standard medium for the administration of sex hormones to experimental animals and to human patients. The discoveries that some sex hormones are active orally and that sexual stimulation can be effected by hormonal pellets implanted under the skin (Deanesly and Parkes, 1937) or placed under the tongue are products of only the past few years. It is also possible to elicit the characteristic tissue response to a hormone by bringing it into direct contact with the target organ. Many demonstrations have been made of the effectiveness of sex hormones when applied locally in this manner. This paper is a review of the published reports of laboratory and clinical experiences with local organotherapy with the so-called male and female hormones.

VAGINA. *Experimental.* The intravaginal instillation of estrogens and biological materials containing estrogens has shown the feasibility of inducing vaginal estrus by this means. When the instilled quantities of hormone are sufficiently small their action is strictly a local one. Robson and Adler (1940) demonstrated this fact by means of a cleverly planned experiment in which they separated the upper part of the mouse vagina from the lower part. The latter is known to be more responsive to estrogens; but when estradiol, estriol glucuronide, or stilbestrol were injected into the upper vaginal sac, a distinctly greater estrogenic effect was produced there. It was concluded, therefore, that the action was local rather than systemic. In castrated female rats vaginally administered stilbestrol, in amounts up to 1 gamma daily, produced vaginal cornification but was without effect in modifying the appearance of castration cells in the pituitary (Morrell and Hart, 1941). Estrous changes in strips of castrated rat vagina *in vitro* were reported by Berger (1937) following the addition of estrogen to the culture medium. These experiments conclusively eliminated the possibility of systemic influences.

Interest in the local vaginal action of estrogens in experimental animals is based largely upon its usefulness for assay techniques. Castrated adult rats, and to a lesser extent mice, have been used for this purpose. Experiments have shown that bioassay methods for estrogens based on the intravaginal route of administration are simple, reliable and in general more sensitive than other biological methods of assay. The intravaginal method offers the further

advantage that it permits the analysis of estrogens in a variety of vehicles including pessaries (Powers, Varley and Morrell, 1929), cotton pledgets (Palmer and Zuckerman, 1939), blood (Albrieux, 1941), oil and other solvents. It also results in an economy of animals, since the same individuals can be used over and over again.

Using cotton wool pledgets soaked with stilbestrol in oil, Palmer and Zuckerman (1939) observed estrous changes in the vagina of mice within 24 hours after the administration of as little as 0.05 gamma of stilbestrol. Lyons and Templeton (1936) and Yerby (1937) assayed estrogens by instilling urinary extracts vaginally in castrated adult rats and observing the degree of cornification 24 hours after the second of 2 daily doses. Blood estrogens were assayed intravaginally in rats by Albrieux (1941), using pellets made from desiccated blood. Markee and Berg (1942), employing mice for the assay of blood estrogens during the menstrual cycle, found that extracts containing as little as 0.015 I.U. of estrone, when instilled vaginally, induced macroscopically detectable vaginal mucus.

Estimates of the relative effectiveness on the vaginal mucosa of estrogens applied locally as compared with estrogens administered by subcutaneous injection vary widely. Differences resulting from these two methods of administration depend upon the species and age of the test animal as well as the specific estrogenic compound and its vehicle, and are accentuated by variations in the dosage, frequency of treatment and time of observation. Powers, Varley and Morrell (1929) reported a ratio of effective subcutaneous dose to effective vaginal dose of 1:3, Pratt and Smeltzer (1929) 1:2, in spayed rats treated with estrogen. Subsequent investigators, however, have obtained markedly different quantitative results. In rats receiving estradiol this ratio was computed to be 12:1 by Berger (1935) and 4:1 by Freud (1939). The latter author found a ratio of 2:1 for estrone, 1:1 for estradiol benzoate, and 1:12 for stilbestrol. Under somewhat different experimental conditions but using castrated rats also, Stadler and Lyons (1938) calculated a ratio of 50:1 for estrone and a ratio varying between 100:1 and 200:1 for estradiol benzoate. The latter hormone was found to be 30 times as potent as estrone when both were administered vaginally. The high degree of sensitivity to which this method of estrogenic assay can be brought is shown by the figures of Mühlbock (1940), who instilled the hormones in 50 per cent glycerine intravaginally in castrated mature mice. The effective estrogenic doses reported by Mühlbock were: for estrone, 0.00025 gamma; for estradiol, 0.0005 gamma; for estriol, 0.00075 gamma. The same order of sensitivity has been attained by Hartman and Littrell (1945) in a new test for blood estrogens. The test sample, in volume of 0.01 or 0.02 cc., is injected subcutaneously in immature rats in the region of the future vaginal orifice. A positive reaction consists of a transverse dimpling of the skin, usually within 24 hours, followed by the opening of the vagina within 4–5 days. In adapting this test to guinea-pig weanlings, Littrell, Tom and Hartman (1946) have been able to establish an exponential curve correlating the time of opening

of the vagina with the amount of estrogen injected locally into the perineum. When 20 gamma of estradiol dipropionate was injected vaginal opening occurred in 10 hours, while with a dose of 0.004 gamma opening was delayed to 96 hours.

Emmens (1941) has determined the relative minimal effective estrogenic dose by subcutaneous as compared with local (vaginal) administration (S/L ratio) for a large number of natural and synthetic estrogens as well as other compounds of questionable estrogenic status and has cleverly applied these data toward an analysis of the mode of action of estrogens. The compounds investigated by Emmens fell into 2 distinct groups on the basis of their S/L ratios; namely, those having a high S/L ratio, greater than 50, and those with a low S/L ratio, usually less than 2. The substances with a high S/L ratio, listed

TABLE 1

Substances having a high S/L ratio (Estrogens)

(after Emmens, 1941)

	S/L
Estrone	260
Estrone methyl ether	60
Estradiol	50
Ethinyl estradiol	120
Estriol	2000
Ethinyl-dihydro-equilin	90
Diethylstilbestrol	320
ψ-diethylstilbestrol	450
Diethylstilbestrol dimethyl ether	400
Ethyl-propyl-stilbestrol	170
Di-*iso*-propyl-stilbestrol	310
Di-*n*-butyl-stilbestrol	310
Hexestrol (meso)	180
Hexestrol (racemic)	360
4:4-Dihydroxy-γ:δ-diphenyl-β:δ-hexadiene	170
Triphenylchloroethylene	65
3:3′:4:4′-Tetrahydroxy-γ:δ-diphenyl-*n*-hexane	63
1-Ethyl-2(*p*-hydroxyphenyl)-6-hydroxy-1:2:3:4-tetrahydronaphthalene	ca. 125

in table 1, comprise the natural estrogens, many compounds of the stilbestrol series, hexestrol, and a few other synthetic estrogens. The substances with a low S/L ratio are listed in table 2. The sharp dividing line between these two groups results from the fact that the synthetic compounds listed in table 2 are scarcely if at all more active when applied directly to the vagina than when injected. Yet some of such synthetic compounds, after injection into female rabbits, result in the excretion of phenolic metabolic products which are more active estrogenically than the parent compounds (Stroud, 1940). Emmens concluded that these synthetic compounds are probably inactive themselves and must be transformed into active substances in the body. He therefore called them pro-estrogens. Their action upon the vagina was presumed to depend upon their systemic absorption, whether applied locally or by injection.

Thus the active metabolites were visualized returning to the vagina in no greater concentration or amount after vaginal than after subcutaneous administration.

In a later experiment Emmens (1942) proffered additional evidence that pro-estrogens are metabolized to estrogens in the body. Using the technique of Robson and Adler, he divided the vagina of spayed mice into 2 compartments, with the anterior one opening suprapubically and the posterior one retaining its normal perineal aperture. Estrogens, when applied locally to one sac, even in many times the minimal effective dose, produced a strictly local reaction without affecting the other sac. Pro-estrogens, on the other hand, stimulated cornification in both sacs when given in only minimal effective doses into one

TABLE 2

Substances having a low S/L ratio (Pro-estrogens)
(after Emmens, 1941)

	S/L
4-Hydroxydiphenyl	<12
4:4′-Dihydroxydiphenyl	<7
4-Hydroxydiphenyl ether	<7
4:4′-Dihydroxydiphenyl ether	<15
Stilbene	1.6
4-Hydroxystilbene	<1.0
4-4′-Dihydroxystilbene	<1.0
4-Hydroxy-α:β-diethylstilbene	<0.6
α-Phenyl-stilbestrol	1.5
α-Phenyl-β:β-di(p-hydroxyphenyl)-ethylene	0.4
Dihydroxyhexahydrochrysene	ca. 0.7
9:10-Dihydroxy-9:10-di-cyclopentyl-9:10-dihydro-1:2:5:6-dibenzanthracene	1.6
9:10-Dihydroxy-9:10-di-n-propyl-9:10-dihydro-1:2:5:6-dibenzanthracene	0.9
Dihydroxy-di-α-naphthyl-acenaphthene	ca. 0.33
p-Hydroxypropiophenone	ca. 4.0
p-Hydroxypropiophenone pinacol	0.5
p-*tert*-Amyl-phenol	<2.0
4-Hydroxy-triphenylmethane	<3.0
trans-Androstenediol	0.9
Anhydro-hydroxy-progesterone	1.2
α-Ethylstilbestrol	<4.0

of them. It seems reasonable to conclude from these experiments that pro-estrogens do not become estrogenic until they have entered the systemic circulation.

The vaginal mucosa seems capable of hydrolyzing estrogenic esters with a high degree of efficiency. Emmens (1941) found that in instances where esterification of the true estrogens produced an increase in the S/L ratio, it did so by raising the minimal effective subcutaneous dose, while causing only small differences, on a molar basis, in the dose required vaginally. The effect of esterification on the S/L ratio is shown in table 3.

Clinical. Local hormonal therapy has found its greatest clinical usefulness in the treatment of vulvovaginitis, particularly in children. The histologic

character of the vaginal and vestibular mucosa of prepuberal girls and its concomitant low hydrogen ion concentration make this delicate membrane easy prey for the gonococcus as well as for non-specific bacterial invaders. After the discovery by Lewis in 1933 that systemic estrogenic therapy could produce hyperplasia of the vulvovaginal tissues and bring about clinical cure of gonococcal vulvovaginitis in young girls, several studies convincingly demonstrated that at least as high a degree of therapeutic success could be achieved by the vaginal administration of estrogenic hormone in gelatin capsules of Amniotin or suppositories of estradiol benzoate or stilbestrol (Te Linde and Brawner, 1935; Te Linde, 1938; Lewis and Weinstein, 1936; Lewis and Adler, 1936, 1937; Jacoby, Madonia, Till and Wood, 1939; Mazer and Shechter, 1939; Woodruff and Te Linde, 1942). Te Linde attributed the curative effects of this form of treatment to the increase in thickness of the vaginal mucosa, which was seen by biopsy; whereas Lewis and Weinstein, who discovered a reduction in vaginal pH follow-

TABLE 3

Effect of esterification on S/L ratio

(after Emmens, 1941)

	S/L
Estrone	260
Estrone butyrate	230
Estrone caproate	500
Estradiol	50
Estradiol benzoate	160
Diethylstilbestrol	320
Diethylstilbestrol dipropionate	190
Diethylstilbestrol dicaproate	300
Diethylstilbestrol dipalmitate	3000

ing local estrogenic treatment both in normal children and in others with vaginitis, regarded the increased acidity of the vaginal fluid as the important therapeutic factor in eradicating infection. Clinical cure occurred independently of systemic absorption of the hormone, for in only an occasional instance were side effects of the estrogen, such as slight mammary enlargement, observed. This contrasts with the relatively high incidence of precocious breast development, pubic hair growth, and uterine bleeding which occurred when estrogens were administered to children by hypodermic injection. Alternate methods of treatment for gonococcal vulvovaginitis, preferred now by most gynecologists, include the sulfonamides and penicillin.

Vaginal suppositories containing estrogens together with glucose and sodium phosphate (Kolpon Inserts, Roche-Organon) have also been used with success in the treatment of non-specific vaginitis in sexually mature women (Neugarten and Steinitz, 1941).

The atrophic changes which usually occur in the genital organs of postmenopausal women result in a lowered resistance of the vaginal epithelium to infection. Superficial ulceration and bacterial invasion are common. Sympto-

matic relief from the discharge, bleeding, itching, burning, soreness, and dyspareunia caused by senile vaginitis has been obtained by local treatment with Amniotin suppositories (Lewis and Adler, 1937) and with an ointment containing estriol, and the specific trophic effect on the vaginal mucosa has been demonstrated by biopsy (Mishell and Motyloff, 1940). Hamblen (1945) recommends the insertion nightly of a vaginal suppository containing 0.5 mgm. of diethylstilbestrol for 4–6 weeks, in order to avoid recurrences. The rationale for estrogenic therapy in patients with senile vaginitis is similar to that for vulvovaginitis in children. The results are similarly gratifying, but the relative importance of epithelial hyperplasia and the restoration of a low vaginal pH is still a matter of uncertainty.

Hamblen has also recommended prophylactic vaginal administration of diethylstilbestrol suppositories, both preoperatively and postoperatively, to postmenopausal women who require vaginal plastic operations. This treatment, it is claimed, together with application of diethylstilbestrol ointment to the vulva, renders the tissues less friable at the time of operation and favors per primum healing.

Uterus. *Experimental.* Cyclic hormonal control of the uterus is a biphasic phenomenon. A follicular, estrogenic, or proliferative phase of uterine development, characterized predominantly by endometrial growth and glandular proliferation, is associated with the follicular phase of the ovarian cycle and is reproducible in castrated animals by the injection of estrogenic hormones. A luteal, progestational, pregravid or (in primates) premenstrual phase, which occurs in association with the corpus luteum stage of the cycle, is marked by differentiation and secretory activity of the endometrial glands, and can be reproduced experimentally by the administration of progesterone to castrated animals which have been appropriately primed with estrogen. The biphasic nature of myometrial contractility has also been clearly related to hormonal control in certain species such as the rabbit, but in the human this relationship is still subject to differences of opinion and remains to be clarified.

The local action of estrogens on the uterus has been demonstrated in several species. Loewe and Voss (1926) produced localized hypertrophy of the guinea pig's uterus by injecting an estrogenic placental extract into a ligated segment of one uterine horn, using the other horn of the uterus as a control. This result was confirmed in the guinea pig and extended to young rabbits and castrated rats by Grumbrecht and Loeser (1938), using small doses of estrone, estradiol, and estradiol benzoate. The same authors introduced estrogen pellets into the uterus of amenorrheic women and demonstrated a proliferative effect on the endometrium by means of biopsies before and after treatment. Proliferation and hypertrophy of the endometrial cells are not inhibited by atropine; it is probable, therefore, that they represent specific effects of estrogen rather than the result of its vasodilating action (Hechter, Lev and Soskin, 1940). Perloff and Kurzrok (1941) succeeded in causing the formation of uterine fibromyomas in guinea pigs by the local implantation of 3 mg. pellets of estradiol into the uterus. This quantity of the hormone, however, might well have resulted in

tumor formation following systemic administration; tumorigenesis, therefore, cannot be attributed to its local action. In an *in vitro* experiment Gaillard and De Jongh (1938) failed to observe growth in thin slices of mouse uterus which were kept alive in a medium of plasma and salt solutions to which various concentrations of estrone were added. Using cover slip preparations of rat uterus Emmens and Ludford (1940) also found no response of the tissue to stilbestrol or to estradiol. In their hands, however, the vaginal tissues also failed to respond to the local action of estrogen by this technique.

McGinty, Anderson and McCullough (1938, 1939) were the first to demonstrate a local action of progesterone on the endometrium. These authors injected an oily solution of the crystalline hormone into a ligated uterine segment in immature rabbits which had been primed previously with estrogen. By this technique 0.5–5 gamma of progesterone was as effective in producing progestational transformation of the endometrium as was 0.5 mgm. of the hormone injected intramuscularly in divided doses over a period of 6 days; and threshold endometrial reactions were obtained with as little as 0.125 gamma of progesterone applied locally. These experiments have been confirmed in immature rabbits by Mussio-Fournier, Albrieux and Grosso (1939) and by Mennega and Tausk (1940), and in castrated rabbits by Sammartino and Nogués (1945).

The practical importance of the McGinty technique would seem to lie in its adaptability for the assay of blood or other biological fluids for their progestogenic activity. Results obtained by this method have been reasonably satisfactory from a qualitative standpoint, but the quantitative inaccuracies seem sufficiently great as to make the test unsatisfactory for better than very rough measurements of progesterone. Haskins (1939) obtained a positive test for progesterone with as little as 0.2 cc. of serum of a pregnant guinea pig. A smaller amount of serum failed to produce a progestational reaction in the rabbit's endometrium, and serum from a non-pregnant guinea pig was likewise ineffective. De Allende (1940) attempted to establish a curve of the normal progesterone content of the blood of the rhesus monkey during the menstrual cycle, by making assays of the serum of 3 animals every 3 days. While demonstrating progestogenic activity in the monkey's blood, her results failed to follow a consistent cyclic pattern. The amount of progesterone in the blood serum of the non-pregnant monkey was estimated to vary between a minimum of 0.06–0.12 gamma per cc. and a maximum of 0.25–2.5 gamma per cc. Using the same technique Haskins (1941) demonstrated progestational activity in 20 out of 21 samples of blood serum obtained from women at various stages of pregnancy up to 7 months. All the reactions in the rabbit's uterus were minimal, however, indicating, according to Haskins' calculations, that the serum probably contained less than 0.13 gamma of progesterone per cc.

Clinical. Karnaky (1942) has reported dramatic and immediate cessation of uterine contractions following the injection into the anterior lip of the cervix of large amounts of stilbestrol (25–100 mgm.) in oil in cases of threatened abortion and premature labor and has claimed excellent results with this method of treatment. There is considerable evidence that estrogens stimulate the pro-

duction of progesterone by the corpus luteum in the rabbit; and an increase in pregnanediol excretion has been observed following the treatment of a pregnant woman with stilbestrol (Smith, Smith and Hurwitz, 1946). So rapid was the therapeutic effect obtained by Karnaky, however, that it seems more likely that the administered medication acted directly on the uterus rather than through a metabolic process involving the mediation of progesterone. Whether the action was exerted primarily on the myometrium or whether primarily on a nerve plexus, whether it resulted from the estrogen or whether from the oil, are questions which remain to be answered. Hormonal injections into the cervix have not yet received general acceptance in the treatment of obstetrical complications (Hamblen, 1945).

Mammia. *Experimental. Nipple.* The nipple area is a specialized part of the integument which is particularly sensitive to hormonal stimulation. Tingling of the nipple, widening and hyperpigmentation of the areola, and hypertrophy of the tubercles of Montgomery are well known concomitants of pregnancy. Similar reactions may occur in non-pregnant women in response to appropriate endocrine therapy. In experiments on rats, mice, rabbits and guinea pigs, growth of the nipples is always observed when the animals are treated with adequate amounts of estrogenic hormone.

It has been a relatively simple matter to demonstrate the direct action of hormones, particularly estrogens, on the nipple and areola. By carefully regulating the dosage of the topically applied hormone, several experimenters have been able to produce unilateral growth of the treated nipple with little or no growth on the control side. Zondek (1935) was the first to show that percutaneous application of estrogen to the guinea pig nipple resulted in enlargement of this structure within 8 days. With the concentration of hormone used by Zondek, however, sufficient systemic absorption occurred so that soon the untreated nipple also began to enlarge and after 4 weeks there was no difference between the two. The guinea pig's nipple is remarkably sensitive to estrogenic stimulation. Several subsequent investigators have exploited this fact, using estrogens in a variety of vehicles including several oils, ointment bases, and alcohol. Mussio-Fournier, Albrieux and Buno (1937) found that 25 I.U. of an estrogen applied locally to the base of the nipple of male guinea pigs daily for 15 days produced an increase in length of 12+ per cent, as compared with an 81 per cent increase from the subcutaneous administration of 50 I.U. of the hormone daily for the same period of time. These results were abundantly confirmed in subsequent reports by Nelson (1941) and by Jadassohn and his co-workers, using various estrogens (Jadassohn, Uehlinger and Zurcher, 1937; Jadassohn, Uehlinger and Margot, 1938; Fierz, 1939; Jadassohn, Uehlinger and Fierz, 1941). Stilbestrol or estrone in a solution containing 1 gamma per cc., when applied to one nipple of male guinea pigs in the amount of 1 drop daily, caused in 30 days a tripling in the length of the treated nipple, a doubling of its width, hyperpigmentation of the nipple and areola, and an increase in the epithelial layer from a thickness of 2–4 cells to one containing approximately 30 layers of nuclei. Similar but less pronounced localized growth of the guinea-

pig nipple was obtained by topical application of testosterone propionate, androsterone, androstenedione, androstanedione, andrenosterone, and corticosterone. The pigmentary reaction in the guinea-pig nipple received special attention by Davis, Boynton, Ferguson and Rothman (1945). They found in castrated males that a local pigmentary response could be elicited by the topical application of diethylstilbestrol to the nipple area, thereby demonstrating that the action of estrogen on the melanoblasts is a direct one. A differential growth of the nipples of male rabbits was reported by Lyons and Sako (1940) and Lewis and Turner (1942) following unilateral local treatment with estrone or with diethylstilbestrol, the row of nipples receiving the estrogen growing at a faster rate than those on the control side. Similar results were obtained in toy fox terriers by Williams, Gardner and De Vita (1946) with estrone.

In the rhesus monkey, also, a topically applied solution of estrone resulted in unilateral nipple growth in young males (Speert, 1940; Chamberlin, Gardner and Allen, 1941). Painting one nipple of each of these animals daily with alcoholic solutions of estrone containing 0.05 mgm. per cc. and 0.5 mgm. per cubic centimeter for periods up to 75 days resulted in a 14-fold increase in the calculated volume of the nipple, while little or no growth occurred in the control nipple (Speert, 1948). After the cessation of treatment the experimental nipples involuted rapidly, but even 3 months later their average size was still 5 times as large as before treatment. A similar disparity occurred between the areolas on the treated and untreated sides. The same treatment of pregnant monkeys and a group of young females undergoing intensive systemic estrogenic therapy resulted in no demonstrable effect on the nipples or areolas. Failure to respond to an extraneous estrogenic stimulus is undoubtedly attributable to the state of estrogenic saturation which already existed in the tissues of these animals.

Supernumerary nipples are also sensitive to estrogenic stimulation, as is suggested clinically by their increased prominence and pigmentation in women during pregnancy. This suggestion was extended to the monkey by Zuckerman's observation (1935) of the changes in the supernumerary nipple of a male macaque following injection with estrogen. The topical application of estrone to a small supernumerary nipple in a young male monkey resulted in a 36-fold increase in its calculated volume, but identical treatment of a large accessory nipple in a mature female produced no change (Speert, 1942a; 1948). Here too the disparity in responsiveness of the supernumerary nipples can be ascribed to the differences in the growth stimuli to which they had already been subjected and their resultant varied capacity for further growth. This hypothesis will serve also to explain the failure of a supernumerary nipple in a recently pregnant guinea pig to increase in size after local treatment with estrone for 147 days (Speert, 1942b).

Mammary gland. The mammary gland proper, as well as the nipple structures, is capable of response to direct hormonal stimulation. Estrone in alcoholic or oily solution applied directly to the mammary area has resulted in glandular growth in male mice (Gardner and Chamberlin, 1941), castrated male and female guinea pigs (Nelson, 1941), male rabbits (Lyons and Sako,

1940; Lewis and Turner, 1942), and virgin heifers (Petersen, 1943). In virgin female goats inunction of the udder with estradiol benzoate produced complete mammary development, equivalent to that seen in the latter part of pregnancy (De Fremery, 1936), and similar treatment with an ointment containing 1 per cent diethylstilbestrol dipropionate resulted in copious lactation (Folley, Watson and Bottomley, 1940). In the latter experiments a daily yield of 1500 cc. of milk of normal composition was obtained, after a 30-day period of treatment which consisted of estrogenic inunction of the udder 3 times a week accompanied by daily milking. Since milking, combined with inunction of the udder with an ointment not containing estrogen, was also capable of initiating lactation, it is difficult to know how much to credit to the estrogen.

Unilateral growth of the mammary gland has been demonstrated in male monkeys after local treatment of one side with an alcoholic solution of estrone, the other side receiving alcohol alone as a control (Speert, 1940, 1948; Chamberlin, Gardner and Allen, 1941). In the monkey experiments the area of the estrogen treated mammary glands was greater than that of the controls and the duct system was better developed. Histologic sections revealed hyperplastic ducts and increased periductal connective tissue in the glands treated with estrone, in contrast to fewer, smaller, and more poorly developed ducts in the control glands.

These experiments have special significance because of their bearing on the problem of estrogenic control of the mammary gland. The mode of action by which estrogens produce their trophic effect on the mammary gland has been a matter of dispute for several years. Injected estrogenic hormones have commonly been found incapable of producing mammary development in hypophysectomized laboratory animals. This observation led to a large amount of experimental work and numerous publications, principally by Turner and his students, culminating in the theory that estrogens produce mammary development through the mediation of the anterior pituitary. It was postulated that this gland, under estrogenic stimulation, produces mammotrophic hormones called "mammogens", which in turn exert their trophic influence on the mammary gland (Gomez and Turner, 1938; Lewis and Turner, 1939; Mixner and Turner, 1943). Estrogens were held to be incapable of direct mammary stimulation. The evidence for and against this theory has been critically reviewed elsewhere (Speert, 1948). It will suffice here to point out that the demonstration in several species of unilateral mammary growth resulting from local application of estrogen cannot be reconciled with the mediation of this effect through the pituitary and is therefore regarded as critical evidence against this theory. Pituitary mediation, by requiring first systemic absorption of the estrogen, would be expected to result in bilateral, symmetrical mammary growth. It must be concluded, therefore, that estrogens can act directly on the mammary gland.

A report by Lyons (1942) has indicated that lactogenic hormone can also act locally upon the mammary gland. When this substance was injected intraductally, through the nipples of rabbits, localized lactation ensued in the injected parts of the gland while the control sectors remained unaffected.

Earlier studies had shown that the sensitivity of the crop-sac method of assay for lactogenic hormone could be greatly increased by the intradermal injection of the substance over the crop-sac. This method of administration permitted the detection of the lactogenic hormone in a dose of 1 gamma, in contrast to 100 gamma required by the intramuscular injection (Lyons and Page, 1935; Bates and Riddle, 1936). An improved method of preparation allowed for a positive local response with only 0.1 gamma (Lyons, 1937a). By injecting the hormone locally in divided doses over a period of 4 days Lyons (1937b) attained a further ten-fold increase in sensitivity. When lactogenic hormone is injected intradermally over the crop-sac of the pigeon the histological effects of stimulation are limited to the area immediately subjacent to the site of injection (Lyons, 1937a), the rest of the crop-sac wall being unaffected.

Clinical. Gratifying but temporary cosmetic improvement has been obtained in cases of hypomastia treated with estrogenic hormones. This condition is frequently associated with general sexual hypoplasia; in some cases, however, genital structure and function are normal and the developmental defect appears to be localized in the breasts. In the treatment of patients in the latter category it is important not to disturb normal ovarian function by systemic hormonal therapy. Local treatment with estrogenic ointment provides a means of concentrating effective amounts of the hormone in the breast with minimal effect on the other organs of the body. MacBryde (1939) treated a small series of patients with generalized sexual underdevelopment and hypoplastic breasts by daily inunction of one breast with 5 grams of an ointment containing 5,000 I.U. of estradiol or estradiol benzoate per gram. The treated breast enlarged more rapidly than that on the untreated side, although sufficient systemic absorption occurred to produce enlargment of the uterus and changes in the vaginal mucosa. Good results with this method of treatment were also reported by Cernea (1940), who had his patients rub 5 grams of an ointment containing 0.1 per cent stilbestrol into each breast nightly. Mammary enlargement was obtained in 30 days but treatment had to be repeated from time to time.

Ointments containing therapeutically effective concentrations of estrogen must be differentiated from fraudulent products which occasionally appear on the market and are advertised to the public as "bust developers" (J. A. M. A., 1940, 1943, 1944).

Puerperal mastitis has been treated by local inunction of the affected breast with stilbestrol salve and Leinzinger and Bayer (1940) claim excellent results with this method of treatment. In a series of 64 cases of acute puerperal mastitis suppuration occurred only twice, resulting in 90.6 per cent cure attributable to the stilbestrol inunctions, as compared with 71.4 per cent cure attained in a control series which received x-ray therapy. Local androgenic treatment in the form of testosterone or testosterone propionate inunctions was used by Spence (1940) for the relief of pain in cases of so-called chronic mastitis. Mammary pain was reported alleviated in 8 out of 10 patients so treated. The rationale of such types of therapy for either inflammatory or proliferative mastopathies remains so obscure that acceptance of hormonal inunctions as a treatment of choice must await confirmatory reports by other observers. Dunn (1944) has reported

negative results in the local as well as the systemic treatment of gynecomastia with androgenic hormone. Local treatment consisted of the injection into the substance of the affected breast of 50 mg. of testosterone propionate in 1 cc. of sesame oil, such injections being repeated at weekly or bi-weekly intervals for a total of 200 mgm. of the hormone.

VULVA. Perineal itching or pruritus vulvae is a rather common affliction of elderly women. Success has been reported by Klaften (1937), Zondek (1938), and Lubowe (1941) in treating this condition by the local application of ointments containing natural or synthetic estrogen. Sustained symptomatic relief, however, usually requires prolonged or repeated treatment, the safety of which has been challenged because of the danger of carcinogenesis. For the same reason even stronger objection has been raised against the use of estrogens in the treatment of krausosis vulvae, an atrophic lesion of the vulvar tissues with which pruritus is usually associated. Clinical cure of patients with this disease by local treatment with estrogenic ointment has been reported in a few instances (Jaffé, 1937; Tscherne, 1938) and the proliferative changes induced in the atrophic skin of the vulva have been demonstrated by biopsies before and after treatment. After many years of trial, however, Frank (1940) has found estrogenic therapy for kraurosis vulvae to be purely palliative and the improvement transient. Because of the high incidence of malignancy which results from the leukoplakic changes frequently associated with kraurosis, most gynecologists now regard excision or vulvectomy rather than hormonal therapy as the treatment of choice.

SKIN. A variety of dermatologic disorders involving other parts of the body's surface have been treated topically with hormones. In reviewing the results of treatment of skin affections, it is important to remember that here perhaps to a greater extent than in most of the other clinical entities reviewed, spontaneous improvement is common, psychologic factors are often of great importance, and that a tendency exists among clinicians to report favorable experiences with new therapeutic methods to the exclusion of equivocal or frankly negative results.

Experimental. In a series of old rats which were losing their hair and in another group of infantile rats with alopecia, Kun (1937) found estrogens to be effective in causing hair growth. After rubbing an ointment containing estradiol benzoate into the hairless parts of these animals for 1–2 months, complete growth resulted, while the control rats showed no change. A direct effect of the hormone on the hair follicles is suggested by the observation that growth occurred first at the sites where the ointment was applied. In apparent contrast to these findings is the recent report of Williams, Gardner and De Vita (1946). These authors observed a distinct inhibition of the regrowth of hair in three toy fox terriers after a solution of estrone was applied locally to shaved areas of the lateral body walls, whereas control areas which were treated with the solvent alone showed a normal rate of regrowth.

Estrogens are capable of increasing the water content of the skin. Selye (1944) treated the so-called hairless mouse, whose skin is affected by a congenital

anomaly, with topical applications of a solution of estradiol containing 250 gamma of the hormone per cubic centimeter. The treated animals responded with a non-pitting cutaneous edema, similar histologically to that which occurs in the sexual skin of monkeys. This phenomenon is probably similar to the premenstrual edema which develops in many normal women and the physiological edema of pregnancy, both of which are believed to be estrogenic effects. Another effect of estrogens in the rhesus monkey consists of reddening of the sex skin. Chamberlin, Gardner and Allen (1941) produced such reddening unilaterally by the local application of an alcoholic solution of estrone.

Plumage pigmentation in birds is intimately dependent upon the sex hormones. In the brown Leghorn, for example, the breast feathers are normally light colored in the hen and black in the capon. If a capon is treated with estrogen while its feathers are growing, a light brown bar makes its appearance in each feather, the width of the bar corresponding to the duration of estrogenic activity. Greenwood and Blyth (1935b) found that the local intradermal injection of small amounts of estrone, too small to cause a plumage response when injected into the pectoral muscles, induced local female pigmentary changes, the replacement of black by red, in the breast feathers nearest the point of injection. 'Espinasse (1939), who confirmed these observations, noted that the side of the feather toward the site of injection tended to react more and sooner than the distal side. Deanesly and Parkes (1937) obtained similar results after the inunction of one feather tract with estradiol and estradiol benzoate.

The direct action of sex hormones on the differentiation of melanophores in birds was demonstrated by Hamilton (1940) by means of tissue culture experiments. He explanted bits of dorsal skin from 6-day-old New Hampshire Red and Rhode Island Red chick embryos. Differentiation of red melanophores and increased production of pigment were stimulated by estrone, estradiol benzoate, estradiol dipropionate, and testosterone propionate, which were added to the culture medium in concentrations between 20 gamma and 300 gamma per cc.

Clinical. Increased pigmentation of the nipples, areolas, and linea alba occurs during pregnancy and can be produced in non-pregnant women and in experimental animals by treatment with large amounts of estrogen. Davis, Boynton, Ferguson, and Rothman (1945) demonstrated a direct stimulating action of estrogen on melanoblasts. It is therefore difficult to understand with what rationale Rocca (1942) treated his patient, although of menopausal age, with estrogenic hormone for pigmentary patches on her face. Yet after combined treatment with injections and local inunctions of estradiol for several months the facial pigmentation diminished and Rocca attributed the happy result, perhaps gratuitously, to the hormone.

In a better controlled series of observations, consisting of biopsies before and after treatment, Goldzieher (1946) observed a trophic effect of estradiol ointment and diethylstilbestrol ointment on the skin of the forearm of five elderly women. His published photographs of sections of skin obtained after six weeks of daily inunction with these hormones show distinct regeneration of the previously atrophic epidermis.

Testosterone propionate has been used with success in the treatment of senile dermatoses in men, but in the reported cases the results cannot be attributed to a local action of the hormone on the skin. In a series of Holander and Vogel (1942) improvement was obtained in 8 cases after testosterone ointment was applied to the *unaffected* parts of the skin; and in the patients treated by Dobes, Jones, and Franks (1945) for pruritus, injections of the hormone were given simultaneously with the inunctions.

Jaffé (1937) and Zondek (1938), the latter a pioneer in so many of the new uses for estrogens, claimed a salutary effect of estrogenic ointment on acne vulgaris in young girls and in menopausal women. This treatment was studied more critically in a well controlled series of cases by Lynch (1941), who found no better results among the girls using an ointment containing 4,000–5,000 R.U. of estradiol per ounce than in the control patients who used the ointment base alone. Lynch emphasized the natural tendency of acne to improve when treatment consists of only soap and water. Jaffé also reported beneficial results from percutaneously administered estrogen in the treatment of seborrheic dermatitis and a case of chronic leg ulcer with surrounding eczema.

Hormones have been used locally in attempts to treat ringworm of the scalp, since they have been found to possess fungistatic activity in vitro; but Lewis, Hopper and Reiss (1946) reported disappointing results after applying diethylstilbestrol and testosterone ointments locally to the areas of infection.

Estrogens have been incorporated in some commercial cosmetics, the advertising of which has often been unrestrained and the public exploited to the limit of its gullibility. An editorial in *The Journal of the American Medical Association* (1938) quoted the following advertising copy, which bore the approval of Good Housekeeping Bureau: "Endocreme contains a counterpart of the natural bodily element which helps account for that pink freshness of a youthful skin. The substance is known to the medical world as es-tra-diol, and Endocreme is the only cosmetic which contains es-tra-diol. It helps replenish the supply of the very substance which your skin may lack! Endocreme, therefore, is more than 'just a face cream'. It's an active, vital cosmetic which begins to work with the substructure of the skin. In later life the skin tends to fall into lines and wrinkles because the tissues underneath the skin sag. When Endocreme firms and tones these underlying tissues, the skin is smoothed out again . . . pores decrease in size . . . and lines begin to disappear." According to the manufacturer's claim, Endocreme contained 0.625 mgm. of estradiol per ounce, and the purchaser was instructed to apply one-half teaspoonful to the face, neck, arms and hands every night.

As recently as May 12, 1946 The New York Times carried the following advertisement: "Your birthday confidante . . . Gourielli's 'Look Younger' Cream ESTROLAR. Trust Gourielli Estrolar *implicitly* to keep each birthday a secret . . . to deny your age discreetly . . . defy it to show on your face. You *can* . . . for this 'Look Younger' Cream contains an Estrogenic Complex . . . the equivalent of a precious body substance which is abundant in youth but diminishes with every passing year. This Estrogenic Complex is an important aid in attain-

ing a younger appearance of face, throat and hands. 30-day supply, 5.50. Twin jars, 10.00". This cream is still being advertised.

Protection of the public from similar products will result from orders such as that recently issued by the Louisiana State Board of Health (J. A. M. A., 1946a): "The manufacture, processing, packing, sale or distribution of any cosmetic or beauty preparation containing estrogenic hormone, any of its chemical derivatives or any synthetic chemical products possessing properties similar to estrogenic hormone is hereby prohibited in Louisiana."[1]

PENIS. In a letter to the editor of *The Journal of the American Medical Association* published August 9, 1941 Schiller referred to a novel "The Golden Lotus" by Wang Shih-Cheng (d. 1593) which described Chinese life and customs, including some details of medical practice, during the Sung dynasty in the reign of Hui Tsung (1100–1126). Quotations from the English and German translations of this novel indicated that modern therapy based on the local application of sex hormones had its counterpart in oriental medicine centuries ago. An exchange of gifts is related between a Hindu monk and a wealthy pharmacist in which the latter was presented with "a little box with a red ointment, a little more than one-fifth of an ounce to anoint the glans penis, sufficient for one hundred and ten applications." The androgenic properties of the remedy were described as follows: "New strength will be given to limbs and belly, it will refresh the testicles, invigorate the penis . . . in a hundred days, hair and beard will be black once more, in a thousand days, your body will know its power. Your teeth will be stronger, your eyes more bright, your manhood made rigid . . . .".

In a modern experiment by Wigodsky and Greene (1940) it was found that 7.5 gamma of testosterone, when applied daily in alcohol or an ointment to the penis of castrated immature male rats, caused distinct growth of that organ in 10–22 days; estrone and estradiol, on the other hand, were without effect. Schiller mentioned similar treatment of human patients, designed to produce more frequent and more persistent erections, and local estrogenic applications to the clitoris of frigid women, but the results of these experiments have not yet been published.

SEMINAL VESICLE. Androgens can act directly on the epithelium of the seminal vesicle. This has been demonstrated in two independent experiments on rats. Lacassagne and Raynaud (1937) found that when testosterone propionate is injected into the interior of the seminal vesicle of a castrated animal, the vesicular epithelium can indicate the presence of as little as 2.5 gamma of the hormone. This is less than the amount required when testosterone propionate is administered by any other channel. Cells of the rat's seminal vesicle, which were grown in tissue culture by Demuth (1940), were seen to enlarge following the addition of testosterone to the culture medium.

[1] This regulation was later modified as follows: "No cosmetic or beauty preparation containing as one of its ingredients estrogenic hormone, any of its chemical derivatives or any synthetic chemical product may be manufactured, processed, packed, sold or distributed in Louisiana unless its label bears adequate directions for use and its label bears the number of international units per ounce of such ingredient" (J. A. M. A., 1946b).

CHICK'S COMB. The male sex hormones cause growth of the chick's comb. When androgens, either pure or in urinary extracts, are injected into chicks of either sex, stimulation of the comb occurs. For assay purposes capons or young cockerels are usually used. Fussgänger discovered in 1934 that the sensitivity of this test for androgens could be increased 50 times if the hormone was applied directly to the comb with a paint brush instead of being injected elsewhere into the body. If the androgenic substance was applied percutaneously to the back, however, it was only $\frac{1}{15}$ as effectual as when applied directly to the comb. Dessau (1937) also found that only $\frac{1}{15}$ as much male urinary extract was required for a positive response when smeared on the capon's comb as when the same extract was injected intramuscularly. The increased effectiveness of androgens administered locally to the capon's comb was confirmed for androsterone by Greenwood and Blyth (1935b), who demonstrated a greater response when the hormone was injected into the comb than when the same dose was injected into the pectoral region; and for testosterone and testosterone propionate by Deanesly and Parkes (1937), who produced a 200-fold increase in effectiveness by applying the hormones to the comb by inunction in propylene glycol. Ruzicka (1935) found that as little as 1–2 gamma of androsterone daily, applied locally in oil, could produce great growth in the comb of either sex.

Subsequent workers discovered that the sensitivity of the test could be increased further by appropriate choice of a vehicle. For example, alcohol was shown to be superior to sesame oil (Klempner, Frank and Hollander, 1940). The volume in which the androgen was dissolved was also important. The smaller the volume, and hence the greater the concentration, the greater the response in comb growth (Klempner, Hollander and Frank, 1940). In ascertaining the amount of growth some investigators have relied on the comb's weight (Danby, 1938; Frank and Klempner, 1937) after sacrificing the chicks; while others, using capons, have resorted to measurements of comb area (Oesting and Webster, 1938). Ruzicka (1935) observed an increase from 400 sq. mm. to 2700 sq. mm. in the combs of capons treated locally with an oily solution of 1 per cent androsterone for a week. After the cessation of treatment the comb usually continues to grow for about a week, then gradually returns to its normal size during the next 2–3 months.

Androgens to which the capon's comb has been found responsive after local treatment include testosterone, testosterone acetate, testosterone propionate, methyl testosterone, androsterone, androstenedione, adrostanedione, dehydroandrosterone, androstanediol and iso-androstanediol. In contrast to the different potencies which the various androgens have when injected intramuscularly, androstenedione, androstanedione, androstanediol, and androsterone were found to be about equally effective when applied directly to the comb (Dessau, 1937). A general correlation exists between the dose of the locally applied androgen and the percentage increase in the size of the comb (Dessau, 1937). Not all authors are in agreement, however, concerning the adequacy of this method for the assay of androgens. McCullagh and Guillet (1941), for example, regarded the inunction of the capon's comb as the most satisfactory of androgenic assay

methods, but admitted that the results were only roughly quantitative at best. Hoskins, Beach, Coffman and Koch (1941), who injected oily solutions of testosterone propionate into the comb of day-old cockerels, concluded that this method was of doubtful value for assay purposes. A more frankly pessimistic view of the problem was taken by Duff and Darby (1941). These workers found that the variation in response of the comb of day-old and 7-day-old chicks to androgens, either locally by inunction or by injection, was so large as to make quantitative assay impossible.

Nevertheless, despite its shortcomings as a quantitative test, the comb test is apparently quite specific for androgens. No response occurred to estrone, estriol, estradiol (Parkes, 1937) or estradiol benzoate (Frank and Klempner, 1937). The local application of progesterone, even in one hundred times the effective dose of androsterone, was without effect on the capon's comb (Dessau, 1937). On the contrary, Mühlbock (1938) found that the addition of estrogen or progesterone to testosterone ointment inhibited the action of the latter on the cock's comb. Inhibition of the capon comb's response to androsterone, whether administered locally or intramuscularly, was produced by estrone, estradiol, and diethylstilbestrol (Emmens and Bradshaw, 1939). The estrogens were more effective when applied directly to the comb. The ratio of the dose of estrogen required by inunction to the dose required by injection to produce this inhibition was 1:470 for estrone and 1:180 for estradiol, but only 1:2 for diethylstilbestrol. The stimulating activity which was demonstrated in extracts of pigs' ovaries applied locally to the capon's comb apparently resulted from an unidentified androgenic substance in the former (Parkes, 1937).

Sparrow's bill. One of the distinguishing sexual characteristics of the male English sparrow (Passer domesticus) is the black pigmentation in its bill. This pigmentation can be produced in the bills of female birds by treatment with androgens and seems to represent a specific effect of the male hormone. When the hormone is injected, however, the series of color changes is such as to make recognition of the end point difficult. Seeking a specific and quantitative assay method for androgens, Kirschbaum and Pfeiffer (1941) applied testosterone propionate locally to the angle of one side of the bill of sexually inactive female sparrows. Two gamma of the hormone administered daily in this manner resulted in a heavy deposition of melanin within 10 days on the treated side but none on the untreated side. In later and more extensive experiments by the same workers (Pfeiffer, Hooker and Kirschbaum, 1944), castrated males were found to be more uniform and more sensitive in their pigmentary response to locally applied androgen. One alcoholic drop containing as little as 0.063 gamma of testosterone, applied daily to the skin-bill junction, was capable of producing a unilateral pigment line in the bill. By systemic administration, on the other hand, 8 gamma of the hormone was required daily in order to elicit a pigmentary response, and this was bilateral and diffuse.

Testosterone and androsterone were about equally potent in their effect upon the sparrow's bill. Neither estrogens, progesterone, nor desoxycorticosterone acetate, however, was capable of producing pigmentation. Estradiol benzoate,

even in the relatively large dose of 10 gamma daily, failed to modify the bill's response to 0.25 gamma of testosterone when both were applied locally. This contrasts with the inhibitory effect of estrogen on the response of the capon's comb to androgen.

Because of its simplicity, specificity, low cost, and greater sensitivity, Pfeiffer, Hooker, and Kirschbaum regard this test as superior to the chick's comb for the quantitative assay of androgens. Values obtained by them in assaying crude extracts of testicular tissues of pigs and bulls by the two methods were in good agreement.

NASAL MUCOSA. *Experimental.* The existence of a naso-genital relationship has been suspected for over half a century. Recent physiologic observations afford some basis for the presumed clinical relationship between the nasal mucosa and genital function. Some of these experimental findings have been reviewed by Rosen (1942), who was able to demonstrate an uninterrupted nervous pathway from the nasal mucosa to the anterior pituitary, via the sphenopalatine and Vidian ganglia. Pseudopregnancy, it was learned, could be produced in the rat by excision of the sphenopalatine ganglion, which interrupted the non-olfactory innervation of the nose. The nasal mucosa has been associated with ovarian function in the rhesus monkey by the observation of Mortimer, Wright, and Collip (1936) that peaks of nasal activity, vascular in nature and consisting of reddening and swelling of the conchae, occur predominantly in menstruating monkeys, usually during the premenstruum. In order to determine the effect of estrogens on ciliated mucosa, Boyd, Clark, and Perry (1941) applied various concentrations of them topically to the buccoesophageal mucosa of the frog. Stimulation of ciliary movements was produced by solutions containing 0.07 – 0.2 mgm. of estrone per 100 cc. of saline, 0.002 – 0.02 mgm. of estriol per 100 cc. and 0.00005 – 0.0002 mgm of estradiol per 100 cc. Higher concentrations exerted an inhibitory effect on the cilia. These reactions occurred independently of the blood supply, for the mucosa was examined after being stripped from the frog's body.

Clinical. Following their observations on monkeys Mortimer, Wright, and Collip (1937) were led to attempt the treatment of patients with atrophic rhinitis by means of locally applied estrogen. After spraying oily solutions of estrone and estradiol (0.1 mgm. per cc.) into each nostril twice daily, 31 out of 38 patients experienced a significant degree of improvement in 2 – 6 weeks. Using similar treatment, coupled sometimes with local swabbing or packing of the nose with cotton pledgets dipped in estrogenic solution, Blaisdell (1938), Eagle, Baker, and Hamblen (1939), and Safer (1942) also reported good results in the treatment of atrophic rhinitis. Biopsy specimens, however, which Eagle, Baker, and Hamblen obtained from the middle turbinates in several cases before and after treatment, failed to reveal any consistent histological changes.

The same technique of treatment was used later by Mortimer, Wright, Thomson, and Collip (1939) for patients with so-called constitutional deafness. The daily nasal insufflation of 1 cc. of oil containing 1000 I.U. of estrogen was reported to result in "marked improvement of the aural defect and significant amelioration of the hearing level" in certain patients with this disease.

Multiple benign papillomata of the larynx occur typically in young children and tend to stop growing at puberty. Suspecting a relationship between this alteration in the course of the disease and the laryngeal changes which occur under gonadal influence, and stimulated by the success of Te Linde and Brawner in their treatment of gonococcal vaginitis by the direct application of estrogen to the vaginal mucosa, Broyles (1940) attempted to treat laryngeal papillomas in children by applying the hormone directly to the larynx. Whereas other forms of therapy for this condition have been unsatisfactory, Broyles reported distinct improvement, with regression of the growths, in 6 cases. Treatment consisted of a weekly spraying of the larynx, through a direct laryngoscope, with about 0.1 cc. of an oily solution of Amniotin containing 10,000 I. U. per cc.

COMMENT

There is abundant evidence in various species of animals and in man that the steroid and synthetic sex hormones can be absorbed in functional quantities from several of the body surfaces. To illustrate, growth of the prostate and seminal vesicles resulted from the application of testosterone to the penis of the rat (Wigodsky and Greene, 1940). Pratt and Smeltzer (1929) described the nasal absorption of estrogen in the human and the nasal and conjunctival absorption of estrogen in the rat. Experiments on the absorption of steroid hormones from the mucous membranes of the mouth were reviewed in a recent paper by Corner (1944). Vaginal absorption of estrogen, sufficient to induce menstruation in castrated monkeys, was reported by Powers, Varley and Morrell (1929).

Most impressive, both because of their large number and variety and because of their practical bearing on human therapeutics, are the experiments which demonstrated the percutaneous absorption of the sex hormones. Estrone applied to the skin in an oily vehicle caused vaginal estrus in spayed albino rats (Baer, 1939) and mice (Zondek, 1938). When the hormone was dissolved in benzol, ether, or alcohol it was just as effective administered percutaneously as when injected. Burrows (1945) has found that one drop of an estrogenic solution, applied locally to the skin of the interscapular region of mice twice a week, produces all the recognized estrogenic effects in the treated animals, including mammary cancer.

The androgen "Enarmon", rubbed into the skin of castrated immature male rats, caused development of the accessory sex organs (Ito, Hajazu and Kon, 1937). Sexual receptivity was induced in castrated female guinea pigs by the percutaneous administration of estrogens followed by progestin (Leighty, Wick and Jeffries, 1940). In males of the same species Moore, Lamar, and Beck (1938) produced growth of the nipples by rubbing an estradiol cream into the dorsal skin of shaved animals, and Ruinen (1932) produced mammary growth in rats, even in hypophysectomized males, by rubbing estrogen into the ventral skin. In a later report Zondek (1941), using estrogens percutaneously again, confirmed the observation of Bloch and Schrafl (1932) that these hormones increased the pigmentation and resulted in a strongly positive "dopa" reaction in the guinea pig's nipple and areola. According to an editorial in The Lancet (1938) a group of males employed in packing ampules of estrogenic material developed

tender swelling of the breasts as a result of handling the estrogenic compound. More recently 28 additional cases of frank gynecomastia, confirmed in 3 by histological examination, were reported among a group of 38 men engaged in the manufacture of stilbestrol (Fitzsimons, 1944). In the experiments of Ito, Hajazu, and Kon an estrogenic cream rubbed into the skin of infantile female monkeys caused within 2 weeks reddening and swelling of the sex skin, enlargement of the nipples, and an increase in uterine size. Loeser (1937) reported turgescence of the perineal region of castrated baboons and enlargement of the breasts of castrated and amenorrheic women after rubbing estradiol benzoate into the skin. He also found it possible to relieve menopausal flashes in 2 patients by this method of giving estrogen. Salmon (1938) observed full estrogenic changes in the vaginal smears of patients with little or no ovarian function, following the nightly inunction of their skin with an ointment containing estradiol. So effective is percutaneous absorption of estrogens in the human that Zondek (1938) reported satisfactory results in the treatment of menopausal arthritis as well as cases of primary and secondary amenorrhea with an estrogenic ointment.

The literature cited in this review has shown that localized tissue response to the sex hormones can be elicited by the direct application of these compounds to some of the body surfaces and internal organs. The concentration and amount of hormone required for the production of its effect locally without at the same time allowing significant systemic absorption, necessitates careful regulation such as is usually possible only in laboratory animals, several of which can be used in an experiment and be subjected to different doses. The fine adjustment of dosage necessary to permit a local action without systemic effects cannot be achieved consistently in the treatment of human patients. In attempting local therapy with either estrogens or androgens one must accept the fact that the amount of hormone applied, if it is to accomplish the desired local effect, will probably result in systemic absorption also. What, then, are the risks associated with the systemic absorption of these hormones?

Clinical experience has shown that adequate local estrogenic treatment for vulvovaginitis can be given to young girls with a relatively low incidence of undesired side effects. Slight mammary enlargement and occasional uterine bleeding, when they do occur, can reasonably be regarded as mild and transient complications. Their importance lies primarily in their psychological effect. The prescription of prolonged or repeated courses of estrogenic inunctions for mature women requires more circumspection. The continuous administration of estrogens interferes with normal ovarian function. In a large proportion of women with inadequate breast development, for whom estrogenic inunctions seem indicated for cosmetic reasons, ovarian function is already at a low level, making this consideration unimportant. In others, however, in whom the mammary defect exists despite the presence of normally functioning ovaries, a depression of the latter is liable to result from treatment adequate for breast development. As in the various species of animals, even among different strains of the same species, so in the human being the mammary tissue of different indi-

viduals varies in its sensitivity to estrogenic stimulation. If normal ovarian activity has failed to produce adequate mammary development, large amounts of estrogen would seem to be required for a reasonable expectation of a favorable response. In women of the childbearing age the possible impairment of their fertility, even though it be temporary, might well be a more important consideration than an augmented bust measurement, and hence be an effective deterrent against estrogenic therapy.

A stronger objection against prolonged estrogenic treatment is based upon its possible relation to carcinogenesis. In mice, and to a lesser degree in a few other species, hormonal factors are of fundamental importance in the production of cancer, especially mammary cancer. Castration dramatically reduces the incidence of mammary cancer in females of a susceptible strain (Lathrop and Loeb, 1916); and estrogenic treatment of male mice, which normally do not get the disease, results in tumor formation in them (Lacassagne, 1932). Uterine cancers have also been produced in experimental animals by means of estrogens (review by Greene and Brewer, 1941).

The relationship between cancer and estrogens is by no means as clearly established for man as for mice. Suspicion is aroused, however, by case reports like those of Allaben and Owen (1939), Auchincloss and Haagensen (1940), and Parsons and McCall (1941), in which breast cancer developed in women who had been receiving estrogenic hormone for 1–4 years; and those of Henry (1945) in which a definite adenocarcinoma of the endometrium was discovered in one patient and a "precancerous" endometrial hyperplasia in another, after 3 years of treatment with stilbestrol and estradiol, respectively. Fremont-Smith, Meigs, Graham and Gilbert (1946) have recently reported another case in which endometrial cancer appeared in a patient during a prolonged period of estrogenic therapy. The reviewer has personal records of several similar cases. The observations of Herrell (1937) have also suggested the importance of the continuous action of endogenous estrogens for the development of carcinoma of the endometrium, but Taylor and Millen (1938) indicated that there is reason for questioning this concept. Mention has already been made of the possible danger of estrogenic treatment of kraurosis and leukoplakia vulvae.

In the absence of more precise knowledge on this subject, a properly conservative attitude demands that prolonged estrogenic therapy, either local or systemic, be withheld from patients with a personal history of cancer of the breast or uterus, with a strong familial disposition to cancer, or with a precancerous genital lesion. The same type of reasoning leads to the conclusion that, until it can be definitely shown that they have no relation to the development of prostatic tumors, androgens should be administered to men with caution and for short periods of time, whether by injection or by inunction.

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PTEROYLGLUTAMIC ACID AND RELATED COMPOUNDS

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IT IS A COMMON OBSERVATION that the interpretation of scientific studies often becomes possible in the light of knowledge that is subsequently acquired. This observation has a strong application to an evaluation of the early work in the field of nutritional deficiencies. Descriptions of the specific lesions arising from the lack of a vitamin have commonly preceded alike the experimental definition of the uncomplicated deficiency and the chemical understanding of the missing nutrient. Particularly is this to be noted in the case of the vitamin B factors. The recognition and identification of the "newer" members of the vitamin B complex was preceded by early work which described nutritional lesions. With the progress of knowledge, more complete basal diets became possible as one by one the B vitamins were made available in pure form. In the case of pteroylglutamic acid, the interpretation of biological results was complicated by the existence of several naturally occurring derivatives of a parent molecule and by the variation which was encountered among different species of test organisms in response to these derivatives.

The biological effects of pteroylglutamic acid have been observed under a variety of circumstances which have given rise to a number of different names for the vitamin. The more commonly used names are listed in table 1. The name "folic acid" is widely used, doubtless due to its brevity and euphony, but since this name refers to an unidentified factor which was measured with *Streptococcus lactis* R, it is preferable to adopt the somewhat more cumbersome but chemically defined term, pteroylglutamic acid. Not included in table 1 are two conjugates which have been isolated in pure form; "fermentation *L. casei* factor" and "vitamin B_c conjugate". For "fermentation *L. casei* factor" (1) the name pteroyltriglutamic acid has been used (2), and in keeping with this nomenclature we have used the term pteroylheptaglutamic acid in preference to the longer name pteroylhexaglutamylglutamic acid (3) for "vitamin B_c conjugate".

The complexity of the nutritional relationships of the pteroylglutamic acid "family" of compounds has been to some extent lessened by recent studies in the fields of enzymology and structural chemistry. The following observations bear on the subject.

1. The parent molecule, pteroylglutamic acid, consists of a pteridine grouping linked through para-aminobenzoic acid to a single glutamic acid residue (fig. 1).

2. Successive molecules of glutamic acid may be attached in peptide linkage to the first glutamic acid radicle. Compounds with respectively three and seven glutamic acid groups have been isolated. These compounds have been termed "conjugates".

3. The growth of the test organism *S. fecalis* R is not markedly promoted by either of the two conjugates; the conjugate with seven glutamic acid groupings does not appreciably stimulate the growth of *Lactobacillus casei*.

4. Certain animals, including chicks, rats and, probably, monkeys, can utilize the conjugates as sources of pteroylglutamic acid when fed.

5. The conjugates are split with the liberation of pteroylglutamic acid by specific enzyme systems which are present in mammalian liver and kidney, avian pancreas, and other animal tissues. Such a system is also present in

TABLE 1

Nomenclature of preparations having biological activity presumably due to pteroylglutamic acid

NAME	DESCRIPTION	DATE	REFERENCE
None	Yeast extract, effective in the treatment tropical macrocytic anemia	1931	(4)
Vitamin M	Yeast and liver extract, effective against nutritional cytopenia in monkeys	1938	(5)
Factor U	Yeast extract, promoted growth in chicks	1938	(6)
Vitamin B_s	Adsorbed on fullers' earth, prevented nutritional anemia in chicks	1939	(7)
Norite eluate factor	From yeast and liver, active for *L. casei*	1940	(8)
Folic acid	Active for *S. lactis* R; concentrates prepared from spinach	1941	(9)
L. casei factor	Isolated from liver and yeast	1943	(10)

In addition to the above, less clearly defined fractions have received separate names, including vitamins B_{10} and B_{11} (11) and factors R and S (12).

certain vegetable materials, including almonds and potatoes, but is perhaps absent from *S. fecalis* R. These observations are thought to account for the finding that certain preparations were active when fed to deficient animals but were relatively inert in the microbiological assay. Treatment of such preparations with the specific enzyme system usually increases their potency in the microbiological assay.

6. The erythrocyte maturation factor of U.S.P. concentrated liver extracts is not identical with pteroylglutamic acid, although the factor and pteroylglutamic acid both produce identical hemopoietic responses in patients with addisonian pernicious anemia.

7. An "incomplete" molecule, pteroic acid, has been synthesized by omitting glutamic acid. It consists of a pteridine grouping linked to para-aminobenzoic

acid, and is biologically active for *S. fecalis* R but not for *L. casei* or for chicks or rats (table 2).

Tropical macrocytic anemia and its counterpart in monkeys. A description of "tropical macrocytic anemia" appeared in 1931 (4). The disease was found to have a blood picture similar to that in addisonian pernicious anemia but the other symptom-complexes associated with pernicious anemia were not present. The condition was observed in women patients in Bombay and was at times complicated by pregnancy. The administration of "Marmite", a concentrated extract of autolyzed yeast, 4 grams 2 to 4 times daily, was found to relieve the anemia. In a further communication (13) a similar condition was described in monkeys, the use of which animals had been undertaken to investigate the

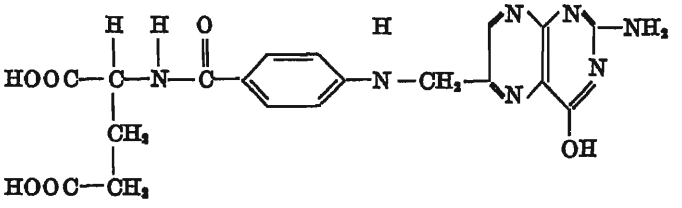


Fig. 1. Pteroylglutamic acid; N—[4—{[(2-amino-4-hydroxy-6-pteridyl)methyl]amino}-benzoyl]glutamic acid

TABLE 2

Biological activities of pteroylglutamic acid and related substances

NAME	RELATIVE ACTIVITY		ACTIVITY FOR	
	S. Fecalis R	*L. Casei*	Chick	Rat
Pteroic acid	50	0.01	−	−
Pteroylglutamic acid	100	100	+	+
Pteroyltriglutamic acid (Fermentation *L. casei* factor)	7.5	80	+	+
Pteroylheptaglutamic acid (Vitamin B_c conjugate)	0.3	0.2	+	+

nature of the missing dietary factor needed in tropical macrocytic anemia. The animals were placed on a diet similar to that consumed by women in Bombay who developed the anemia and a macrocytic anemia was produced in the monkeys which could be cured by feeding Marmite or by oral or parenteral administration of liver extracts which were active in the cure of tropical macrocytic anemia in human patients.

Studies of the bone marrow in the anemic monkeys showed a preponderance of megaloblastic cell types, and photographs of bone marrow preparations indicated a contrast between the marrow pictures respectively in monkeys with the nutritional anemia, in normal monkeys and in monkeys rendered anemic by massive hemorrhage (13). Further experiments (14) were concerned with studies of the active factor in liver; the erythrocyte and reticulocyte responses in de-

ficient monkeys were used as criteria in the assay. It was possible to differentiate the factor from the anti-pernicious-anemia factor of concentrated liver extracts; two preparations were encountered which were active for pernicious anemia in human subjects yet which produced no response in the experimental monkeys. In another article (15), the fractionation of liver extract by treatment with saturated ammonium sulfate was described; it was found that the anti-pernicious-anemia factor was precipitated, while the factor effective in monkey anemia was not precipitated. An extract prepared from yeast was subjected to similar treatment, and again the active factor for monkeys remained in solution. The factor was differentiated from thiamine, riboflavin and nicotinic acid (14).

The above observation that several types of purified liver extract which were potent in the treatment of pernicious anemia had no effect in curing the anemia in monkeys even when administered in "enormous doses" was paralleled by similar observations with human subjects with tropical macrocytic anemia. It was found that seven cases did not respond to a batch of "Anahaemin" which had been demonstrated to be effective in the treatment of pernicious anemia (16). There was no reticulocytosis, no clinical improvement, and no appreciable rise in red cell count during the 10-day period of treatment. Anahaemin has been described (17) as "a concentrated preparation of the erythrocyte maturation factor prepared by the method of Dakin and West. During precipitation it is completely precipitated with 99 per cent alcohol, taken through two ammonium sulfate fractionations, and one reineckate precipitate. Anahaemin in 2 cc. contains a fraction derived from 450 grams of fresh liver".

It has been postulated that the factor of Wills differs from both pteroylglutamic acid and the erythrocyte maturation factor (18). The evidence for the differentiation from pteroylglutamic acid was not striking; a patient failed to respond to the very small dose of 1.3 milligrams of pteroyltriglutamic acid for 10 days, following which a response was obtained to large doses of a crude liver preparation. It has been established (19, 20) that pteroylglutamic acid will cure a deficiency symptom in monkeys which appears to be similar in all respects to the condition first described by Wills and co-workers and it is also evident that pteroylglutamic acid will produce a prompt remission in nutritional macrocytic anemia and in the macrocytic anemia of pregnancy (21, 22, 23), indeed there is one report (24) describing remission of macrocytic anemia of pregnancy following treatment with pteroylglutamic acid in India under conditions which would appear to relate to the earlier observations (4). The pteroylglutamic acid content of Marmite is not known, but yeast, from which Marmite is prepared, is a good dietary source of pteroylglutamic acid and its conjugates. The preponderance of the evidence indicates that the first observation of pteroylglutamic acid deficiency was made by Wills in human subjects and that her experiments were the first to differentiate pteroylglutamic acid from the erythrocyte maturation factor of concentrated liver extracts.

Other studies of blood dyscrasias of nutritional origin in monkeys; "vitamin M". From time to time, reports of experiments with monkeys on restricted

diets have revealed the occurrence of a nutritional deficiency syndrome which was characterized by oral, gastrointestinal and hemopoietic disturbances. It was pointed out in a recent review (25) that observations were made in 1919 (26) which described diarrhea and dysentery in monkeys on diets consisting largely of autoclaved rice. Attention was also drawn (25) to the reported appearance of the syndrome in several laboratories and under various dietary regimes.

A separate series of investigations with monkeys was started in 1935 (27). In an attempt to produce riboflavin-deficiency cataract, a cooked diet of polished rice, ground wheat and purified casein was used, supplemented with cod liver oil, salt mixture and orange. A deficiency syndrome was encountered which was marked by anemia and leucopenia, ulceration of the gums, diarrhea, and susceptibility to bacillary dysentery. The disease terminated fatally within 26 to 93 days if allowed to progress. It was prevented by brewers' dried yeast, 10 grams daily or by a liver-stomach preparation, 2 grams daily (28).

It was reported (29) that riboflavin did not appreciably alter the course of the disease, termed "nutritional cytopenia", and that nicotinic acid, 10 mgm. or 50 mgm. daily, did not prevent the devlopment of the syndrome and did not postpone its fatal termination. The term "vitamin M" was proposed for the factor which prevented nutritional cytopenia in the monkey. Other features of the disease included edema (30) and susceptibility to various experimental infections (31, 32). The resistance of normal monkeys and the susceptibility of "vitamin-M-deficient" monkeys to bacterial dysentery was strikingly demonstrated (33).

An article from another laboratory (34) established the important point that leucopenia developed in monkeys which received a purified diet which was supplemented with certain vitamins, the effect of some of which had not been previously reported. The list included thiamine, riboflavin, pyridoxine, niacinamide, calcium pantothenate and ascorbic acid. The additional administration of 50 mgm. each of choline and para-aminobenzoic acid was ineffective. This report differentiated the protective factor from the known members of the vitamin B complex, with the possible exception of biotin. In addition, a new light was cast upon the possible nature of the unidentified factor; it was reported that a "folic acid concentrate" produced a white-cell response. As a result of assays of various supplements with *Streptococcus fecalis* R, doubt was later cast upon the possible identity of vitamin M with "folic acid" (25); this was, however, before studies with enzyme preparations had revealed the presence of microbiologically-inert conjugates of pteroylglutamic acid in natural foods. With the use of such enzyme preparations, prepared from rat liver, it was found that materials which were good sources of "vitamin M" were also good sources of "bound folic acid" which was liberated in the free form upon treatment with the liver preparation (35, 36) and the concept of the possible identity of the "*S. lactis* R stimulating substances together with the substances enzymatically convertable to such factors" with "vitamin M" was advanced (37). Finally it was demonstrated that pteroyltriglutamic acid, 4 or 4.5 mgm. per monkey in divided doses over a period of a few days, produced prompt and complete remis-

sion of nutritional cytopenia in monkeys, with a return of the granulocyte and total counts to normal levels, and with marked reticulocyte crises (19). Attention was drawn to the observation that the reticulocyte responses of the monkeys to pteroyltriglutamic acid were greater than the corresponding responses of pernicious anemia patients to adequate therapy (38). Monkeys on purified diets supplemented with all the known vitamin B factors except pteroylglutamic acid developed clinical and hematological signs of nutritional deficiency (20). Two of the animals were treated with pteroylglutamic acid, 0.1 to 0.35 mgm., at various intervals. Clinical and hematological remissions were observed.

Monkeys were fed a purified diet and a gradual loss of weight was observed followed by a syndrome which included anorexia, leucopenia and lowered resistance to secondary infections and which had a fatal termination. Feeding a norite eluate fraction of liver, which contained "folic acid", was found to promote growth and to alleviate leucopenia (39). More recently, studies from the same laboratory have indicated that even when a "folic acid" concentrate was supplied, further addition of whole liver was necessary for optimum growth and "blood regeneration" in monkeys (40). The activity of the liver preparation was very easily destroyed by heating (41).

Monkeys were maintained on a purified diet deficient in pteroylglutamic acid and the effect of the deficiency on experimental poliomyelitis was studied (42). An increased resistance to the disease was observed when a subacute nutritional deficiency was produced by maintaining the animals on a suboptimal level of a liver "folic acid" concentrate, but no resistance was observed in "acute" pteroylglutamic acid deficiency.

It was stated (43) that a concentrate "showing high B_{10} and B_{11} activity" for the chick was inadequate as a source of folic acid for the monkey. Presumably "B_{10} and B_{11} activity" is an expression of the presence of conjugated pteroylglutamic acid, in which case this finding may be contrasted with the parallel drawn (35, 36) between vitamin M activity and the presence of "bound folic acid" which was liberated into a microbiologically active form by enzymic digestion.

Biological effects of xanthopterin. Rats three to four weeks old when fed exclusively on goats' milk were found to develop an anemia which did not respond to supplementation with iron. The erythrocyte count fell to around 1 million per cu. mm. and the blood picture was of the macrocytic type (44). Typical reticulocyte responses were obtained with liver preparations and the condition was used as a method of testing such preparations (45). The animals showed sprue-like symptoms, so that the condition was termed "rat sprue". In another investigation of goats' milk anemia (46) fractionation of liver and human urine indicated that the active material had properties resembling those of uropterin (xanthopterin). Accordingly, a sample of uropterin was obtained from Koschara. This was injected at various levels, the effect on the erythrocyte counts was followed and graded responses were obtained over a rage of 0.5 to 1.0 microgram; 10 micrograms did not appear to cause a greater response than did 1 microgram and levels of 1 or 10 micrograms produced an elevation of 2 to 3

million in the erythrocyte count in 14 days. This experiment was the first clue to the chemical nature of pteroylglutamic acid. In contrast to the preceding report (44), the anemia was stated to be also relieved by iron and copper. Failure on the part of Rominger to confirm the results with uropterin was noted (47). However, a subsequent report (48) indicated that xanthopterin, 20 micrograms daily, produced a growth response and an increase in the white cell count in rats receiving a purified diet containing succinylsulfathiazole. Once again, difficulty in repeating these results was encountered (35, 49, 50, 51).

Xanthopterin was found to have a hemopoietic effect when 50 micrograms was injected into fingerling salmon with nutritional anemia (52). The salmon were made anemic by feeding "a high protein diet which contains yeast as a source of the vitamin B complex". The red cell counts per cu. mm. ranged from 416,000 to 916,000 for the control fish and from 659,000 to 1,305,000 for the injected fish 2 to 3 days after the injection.

A consistent but transitory response was obtained when monkeys with nutritional cytopenia were treated with xanthopterin 2.5 to 10 mgm. daily (53). The response consisted of a reticulocyte rise and a marked increase in both white and red cell counts, but the counts soon declined again, and three of the four animals died.

When fresh rat liver tissue was incubated with xanthopterin, a marked increase in the "folic acid" content of the preparation was obtained as compared with rat liver which was incubated alone or with leucopterin, adenine, guanine, xanthine, uracil or cytosine. The "folic acid" content was measured by assay with *L. casei* (50). This observation was confirmed (35), and it was noted that the liver tissue of monkeys, but not that of chicks, appeared to show a higher "folic acid" content when incubated with xanthopterin than when incubated alone. In another report, it was stated that the "folic acid" content of rat liver and muscle was influenced during digestion by various factors including neutral salts, cyanide, xanthopterin, the degree of dispersion of the tissue, the pH, the length of the digestion period and the addition of taka-diastase (54). A protective effect of xanthopterin against the destruction of "folic acid" by enzyme in rat liver was suggested (55). Another obvious variable is the possible effect of the various factors upon the action of "vitamin B_c conjugase" enzyme system (p. 79) present in rat liver, particularly evident in one experiment (54, fig. 4) in which rat liver, incubated alone at various pH values was found to yield a maximum amount of "folic acid" at pH 7.

Pteroylglutamic acid in poultry nutrition. A dietary deficiency of pteroylglutamic acid may readily be produced in young chicks on purified diets. Chicks appear to derive very little of their vitamin B-complex requirement from "intestinal synthesis", and they are, in contrast to rats, quite susceptible to a dietary lack of pteroylglutamic acid. The ease with which deficiencies were produced in chicks on purified diets led to the extensive use of this species as an experimental animal.

Starting in 1938 investigations were made to determine whether the chick needed B-complex vitamins in addition to the factors then known. The require-

ment of the chick for pyridoxine, nicotinic acid, choline and biotin was unexplored at that time. In one report (56), rice bran extract and a fullers' earth adsorbate of whey were added to a simplified basal diet. Growth was improved by a water-soluble factor which was present in alfalfa meal and which was precipitated by alcohol and adsorbed to some extent by fullers' earth, from which it was eluted by a mixture of water, acetone, and ammonia. It was found (6) that chicks grew slowly on a diet consisting principally of polished rice and washed fish meal, supplemented with thiamine and whey adsorbate, and with filtrate factor (pantothenic acid) preparations from rice bran or whey. Growth was greatly increased by yeast, or by alfalfa meal or a water extract of it. The factor, termed "Factor U", extracted from yeast, was adsorbed on fullers' earth and eluted by a mixture of pyridine, alcohol and water. Evidence for the presence of an unidentified factor in cereals, yeast and milk was advanced (57). The factor was needed for growth and hatchability, and was destroyed by prolonged dry heat. Biotin is destroyed by such treatment (58). In a further report on "Factor U" (59), pyridoxine was found to promote growth when added to the basal diet, but additional growth was produced by adding the yeast fullers' earth eluate. The presence of a growth-promoting factor in yeast distinct from thiamine, riboflavin, nicotinic acid, pyridoxine, pantothenic acid and choline was noted (60).

In another investigation (7), acid-hydrolyzed yeast, rice polishings extract, and alcohol extract of liver were used as supplements to a purified diet. Presumably the pteroylglutamic acid in the yeast was destroyed by the acid hydrolysis. A macrocytic hyperchromic anemia was developed by the chicks on this diet; it was stated that evidence indicated that it was not prevented or cured by any vitamin previously described. The antianemic factor from an undescribed source was adsorbed on fullers' earth at pH 1. The factor was given the name "vitamin B_c" (61). This investigation was important in that it reported the specific anti-anemic properties of the new vitamin and thus foreshadowed future developments.

Strong evidence that the norite eluate factor required by *L. casei* was essential for the chick was reported (62). A concentrate of the factor had a marked growth-promoting effect when added at a level of only 0.1 gram per kilo of diet. The "norite eluate factor" for *L. casei* is now presumed to be pteroylglutamic acid. It was found that disparities existed between the microbiological potency of certain concentrates and their activity for chicks. Apparently on this basis, the existence of two new vitamins, B_{10} and B_{11}, active for chicks, was postulated (11). "Vitamins B_{10} and B_{11}" were the subject of a number of publications (63, 64, 65) but in a subsequent report from the same laboratory (66) it was noted that "neither para-aminobenzoic acid nor vitamins $B_{10} + B_{11}$ gave a supplementary effect in the presence of synthetic folic acid". Presumably "vitamins B_{10} and B_{11}" represented a conjugate or conjugates of pteroylglutamic acid.

The biological activity of crystalline pteroylglutamic acid for the chick was first reported in 1943 (67). "Vitamin B_c" from liver was fed at a level of 2.5 parts per million of diet which prevented anemia and enabled good growth to

take place. The chicks which received the purified diet plus pteroylglutamic acid grew more rapidly than chicks on a "broiler ration". It was indicated (68) that 0.4 parts per million of diet was sufficient for normal hemoglobin, hematocrit, red cell count and thrombocyte values, but about a level of 4 parts was required for the production of normal leucocyte levels. A comparison of injected and orally administered dosage (69) indicated that subcutaneous injection was, if anything, slightly more effective than feeding by pipet. In further studies, it was reported that maximum growth, with submaximal hemoglobin response, was obtained by adding pteroylglutamic acid, either free or as the conjugate, at a level of 0.25 parts per million of diet (70).

The pteroylglutamic acid requirement of chicks was studied on a purified diet using growth, feathering and pigmentation as criteria (71). The vitamin was administered by injection 5 times weekly and it was found that a level calculated to supply 10 micrograms per day produced feathering and pigmentation equivalent to that observed in chicks receiving 10 per cent of brewers' yeast as a supplement to the basal diet but growth was somewhat more rapid on the yeast-supplemented diet. Growth and feathering were in proportion to the level of pteroylglutamic acid used.

Chicks on a purified diet were found to need at least one part of pteroylglutamic acid per million parts of diet for normal feather pigmentation and two parts per million to give growth equal to that obtained with a liver fraction (72). Pteroylglutamic acid, added at levels of one to five parts per million of purified diet, was found to be essential for the normal growth of feathers in chicks (73). The effect was not lessened by adding sulfamerazine or certain other "intestinal antiseptics".

In experiments with chicks on a purified diet, pteroylglutamic acid was found to produce maximum growth and hemoglobin values when added at a level of 0.5 to 1.0 mgm. per kilo of diet (74). Chicks were found to need 0.25 part of pteroylglutamic acid per million parts of purified diet for growth, feathering and hemoglobin formation (66). When sulfasuxidine was added to the diet the requirement was increased to between 0.5 and 1.0 part per million. In contrast, the pteroylglutamic acid requirement of chicks was found not to be increased by the addition of sulfasuxidine to a purified diet (75). Chicks were found to require 0.45 part of pteroylglutamic acid per million parts of diet for growth and hemoglobin formation to four weeks of age. The requirement for hemoglobin formation to six weeks of age was stated to be slightly less, 0.35 part per million and 0.55 part per million were required to produce normal feathering at six weeks of age. Attention was drawn (76) to earlier low estimates of the pteroylglutamic acid requirement of chicks (11, 63, 65, 77) which had resulted from feeding preparations of pteroylglutamic acid conjugates. It was pointed out that microbiological assays of such conjugates, even after treatment with taka-diastase, did not reveal the total pteroylglutamic acid content.

Perosis was observed in chicks on a purified diet without pteroylglutamic acid and the incidence was increased by adding 2 per cent of sulfasuxidine. The perosis was prevented by adding 0.2 part per million of pteroylglutamic acid

to the diet without sulfasuxidine and 0.3 part per million to the diet containing sulfasuxidine. The higher incidence of perosis on the sulfonamide-containing diet led to the suggestion that pteroylglutamic acid stimulated the intestinal flora to produce an unknown anti-perotic factor (78).

A relation was observed between pteroylglutamic acid deficiency in chicks and an absence of the normal response to the administration of diethylstilbestrol (79). Large doses of stilbestrol produced an only slight increase in the weights of oviducts of young chicks maintained on a purified diet which was deficient in pteroylglutamic acid, but stilbestrol produced a marked increase in the weight of the oviducts of control chicks receiving a supplement of 20 micrograms of pteroyltriglutamic acid daily. The failure of the deficient birds to respond was not due merely to inanition, for chicks with pantothenic acid deficiency and of comparable body weights showed substantial responses to stilbestrol as measured by increase in the weight of the oviduct.

Differentiation of pteroylglutamic acid from the erythrocyte maturation factor of concentrated liver extracts, previously indicated by studies with human subjects and with monkeys (14, 15, 16), was further emphasized by observations with chicks. In one investigation, an undescribed preparation of the anti-pernicious-anemia factor was found to be ineffective in the prevention of "vitamin B_c deficiency" (80) and subsequently (81) it was reported that pteroylglutamic acid was not liberated in appreciable quantities from concentrated liver extract by treatment with "conjugase" as supplied by dried chicken pancreas. The maturation factor as present in liver extract was ineffective for growth when injected into pteroylglutamic acid-deficient chicks at a level which would correspond, on an anti-pernicious-anemia basis, to about 0.4 mgm. of pteroylglutamic acid daily.

Reproduction in chickens, previously not reported with purified diets, was obtained on a diet which contained pure vitamin B complex factors including pteroylglutamic acid (82).

Young turkeys were found to develop marked nutritional deficiency on diets deficient in pteroylglutamic acid. A spastic type of cervical paralysis was observed on purified diets which were deficient in the vitamin (83). The paralysis was reversed by the administration of pteroylglutamic acid. These observations were confirmed (84) and the occurrence of a blood dyscrasia characterized by macrocytosis and elongation of the erythrocytes was described. The requirement for pteroylglutamic acid under the conditions of the experiment appeared to be at least twice as great for turkeys as for chickens.

Studies of the effects of pteroyltriglutamic acid in chicks. This compound was found to be "active in the nutrition of the chick" (85).

It was reported that pteroyltriglutamic acid, when added at the rate of 0.5 mgm. per kilo of purified diet, was only partially effective in promoting growth and preventing anemia (86). However, when the lactone of either 5-pyridoxic acid (2-methyl-3-hydroxy-4-hydroxymethyl-5-carboxy pyridine) or 4-pyridoxic acid (2-methyl-3-hydroxy-4-carboxy-5-hydroxymethyl pyridine) was added at a level of 0.5 mgm. per kilo to the diet together with pteroyltriglutamic acid,

termed *L. casei* factor, at the same level, a marked gain in weight was produced and anemia was completely prevented. The names alpha and beta "pyracin" were proposed for the two pyridoxic acids.

Hens on a commercial diet were made anemic by bleeding, and the rate of regeneration of hemoglobin was followed (87). The injection of either 4-pyridoxic acid or pteroyltriglutamic acid, 50 micrograms daily, hastened the rate of the hemoglobin level, and when these supplements were administered together, the rate was still further increased.

In another communication, the incubation of pteroyltriglutamic acid with fresh chick liver was found to result in an increase in "folic acid content" as measured with *S. fecalis* R. There was a further increase when 4- or 5-pyridoxic acid was added but a diminution when pyridoxic acid lactone was added rather than the acid (88). Pteroylglutamic acid was found to be effective against anemia in chicks without the addition of pyridoxic acid lactone to the diet (89, 76). Evidence was obtained with chicks (90) which tended to confirm the report of the supplementary effect of 4-pyridoxic acid on the utilization of pteroyltriglutamic acid. The experimental data were meager due to the small amount of pteroyltriglutamic acid which was available.

Other workers reported that pteroyltriglutamic acid without added pyridoxic acid was an effective as pteroylglutamic acid, on a molar basis, in promoting growth and preventing anemia in chicks (74) (91). As compared with pteroylglutamic acid, pteroyltriglutamic acid appeared to be completely utilized with or without the addition of 4-pyridoxic acid by chicks on a diet similar to that described by the Cornell group (91). It has also been found that pteroylheptaglutamic acid was utilized as efficiently as pteroylglutamic acid, without the addition of pyridoxic acid, by chicks on a purified diet (92).

Rôle of pteroylglutamic acid in microbiological nutrition. A summary of the pteroylglutamic acid requirements of microorganisms is given in table 3.

A large number of lactic acid bacteria have been shown to need pteroylglutamic acid for growth. In an investigation of the nutritive requirements of lactic acid bacteria (8) it was shown that an unidentified growth factor existed which later proved to be pteroylglutamic acid. Later it was shown at the same laboratory that this factor was required by other lactic- and propionic-acid-forming bacteria *Streptococcus fecalis* R, *Lactobacillus delbruckii*, and *Propionibacterium pentosaceum* (93).

Clostridium tetani was found to require a growth factor which could be replaced by concentrates of pteroylglutamic acid made from liver (94) or by a "folic acid" preparation obtained from spinach (95). Three "folic acid" preparations of widely varying potencies as measured with *S. fecalis* R were found to have corresponding activities for *Cl. tetani* thus indicating that pteroylglutamic acid was the effective agent.

The growth requirements of a large number of enterococci was investigated (96) and it was found that of 43 organisms investigated 9 required pteroylglutamic acid. It was also found by the same investigators that of 21 strains of *Streptococcus lactis* none required pteroylglutamic acid (97). The requirements

of a large number of lactic acid bacteria for pteroylglutamic acid and the "S. L. R factor" were investigated (99, 104) and are described in detail elsewhere in this review.

The nutrition of *Tetrahymena geleii* has been thoroughly investigated and this organism was shown to require pteroylglutamic acid (106, 110). The amount required for half maximum growth is 0.00065 microgram per ml. of culture medium which is about three times the amount required for half maximum growth by *S. fecalis* R. (10). The "S. L. R factor" which is active for *S. fecalis* R but relatively inactive for *L. casei* was found to be about 0.2 per cent as active as

TABLE 3

Activity of pteroylglutamic acid and related compounds for microorganisms

MICROORGANISMS	PTEROYLGLUTAMIC ACID	PTEROYL-TRI-GLUTAMIC ACID	PTEROYL-HEPTA-GLUTAMIC ACID	PTEROIC ACID	S. L. R. FACTOR	THYMINE
Clostridium tetani....	+(94,95,100)					
Lactobacillus casei...	+(67)	+(85)	−(70)	−(101)	−(102)	+(103)
L. helveticus.....	+(93)					
L. delbruckii.....	+(93)					
L. delbruckii LD5....	+(104)					
L. bulgaricus D5.....	+(104)					+(105)
Propionibacterium pentosaceum.....	+(93)					+(105
Streptococcus durans 98A.....	+(104)				+(104)	+(105)
S. fecalis 732.....	+(104)				+(104)	+(105)
S. fecalis F-24.....	+(104)				+(104)	+(105)
S. fecalis R.....	+(104)	±(85)	−(70)	+(101)	+(104)	+(105)
S. fecalis S-108A.....	+(104)				−(104)	+(105)
S. zymogenes 5Cl.....	+(104)				+(104)	+(105)
Tetrahymena geleii...	+(106)		+(108)	−(108)	−(107)*	
Yeast "Old process".....	+(109)					

* Approximately 0.2 per cent as active as pteroylglutamic acid.

pteroylglutamic acid for *T. geleii* (110). Later it was reported by the same workers (108) that pteroylheptaglutamic acid was active. This contrasts *T. gelcii* with *S. fecalis* and *L. casei* which are unable to utilize this conjugate. Thus the ability of *T. geleii* to utilize the conjugate is similar to that of animals, and parallels the similarity of *T. geleii* and the higher animals in amino acid requirements (111).

It seems likely that those organisms which do not require pteroylglutamic acid are able to synthesize it. *Bacillus lactis acidi, Lactobacillus arabinosus, Lactobacillus pentosus, Bacillus brassicae, Leuconostoc mesenteroides* and *Lactobacillus gayonii* are not stimulated by pteroylglutamic acid but were able to synthesize significant amounts of the factor (93).

The synthesis of pteroylglutamic acid by five microorganisms was reported (112). The assays were made after papain and takadiastase digestion of the cells and using *S. fecalis* R as the assay organism. This enzyme treatment would presumably not hydrolyze the conjugates present so the results must be interpreted to measure primarily free pteroylglutamic acid and any S. L. R factor which may be present. The organisms were grown on a purified media containing no pteroylglutamic acid and the cells and media assayed separately. The results were as follows:

ORGANISM	MICROGRAMS PTEROYLGLUTAMIC ACID FOUND PER GRAM DRY CELLS*	
	Cells	Medium
Aerobacter aerogenes (aerobic)	5	31
Aerobacter aerogenes (anaerobic)	2	7
Serratia marcesans	6	28
Pseudomonas fluorescens	3	23
Proteus vulgaris	7	7
Clostridium butylicum	1	6

* Calculated on the basis of pteroylglutamic acid having a "potency" of 137,000.

The growth-promoting activity of pteroylglutamic acid for *S. fecalis* R was reported to be reversed by the addition of a synthetic product, "methylfolic acid", which was prepared by reacting 2,4,5-triamino-6-hydroxy pyrimidine and *p*-aminobenzoyl-d(−)-glutamic acid with 2,3-dibromobutyraldehyde (113).

A relationship was established between pteroylglutamic acid synthesis and sulfanilamide in *E. coli* (114). A sulfonamide resistant and a sensitive strain of *E. coli* were grown in concentrations of sulfanilamide which permitted some growth. Both strains produced less pteroylglutamic acid when grown in the presence of sulfanilamide than in the absence of it. Biotin synthesis on the other hand was not greatly affected by the presence of the drug which demonstrated that the sulfanilamide did not exert a general depressing action on synthesis of all the vitamins.

While pteroyltriglutamic acid and pteroylglutamic acid were approximately equally active for *L. casei*, the shapes of their respective response curves were different (115). There was a lag in the response to low concentrations of pteroyltriglutamic acid which resulted in a sigmoid shaped curve. The lower part of the response curve with pteroylglutamic acid approached a straight line. A sample of "folic acid, potency 3100" obtained from spinach gave the same shape response curve as that of pteroylglutamic acid, and on this basis it was suggested that the active constituent in the "folic acid" preparation was pteroylglutamic acid. Comparative assays with *L. casei* and *S. fecalis* R showed the "folic acid" preparation to have the same relative potency as pteroylglutamic acid for these two organisms. By using a folic acid preparation with potency standardized in terms of a liver fraction the "potency" of pteroylglutamic acid was estimated to be approximately 137,000.

Para-aminobenzoic acid and its relation to pteroylglutamic acid in bacterial nutrition. There is evidence that p-aminobenzoic acid functions as a precursor of pteroylglutamic acid in the nutrition of certain bacteria. The bacteriostatic effect of the sulfonamides is due in the case of some microorganisms to inhibition of the enzyme system which synthesizes pteroylglutamic acid from p-aminobenzoic acid.

A possible indication of the relation between p-aminobenzoic acid and pteroylglutamic acid appeared before the chemical nature of pteroylglutamic acid was revealed. It was reported (116) that a yellow pigment was formed in cultures of a certain strain of *Mycobacterium tuberculosis* when it was grown in media containing high concentrations of p-aminobenzoic acid, and it was suggested that the pigment might be related to the vitamin B complex. It was also suggested that the enzyme responsible might be a specific oxidase (117). The pigment was reported to contain oxidized aromatic nitrogen groups as indicated by an increase of aromatic amino nitrogen after treatment with zinc. The pigment gave negative results for the presence of "folic acid" when tested with *S. fecalis* R. This negative result presumably would not exclude the possible presence of certain conjugates of pteroylglutamic acid which are known to be of quite low activity for *S. fecalis* R. Pteroylheptaglutamic acid (Table 1) is an example of such a conjugate. The formation of the pigment could be lessened or inhibited by sulfanilamide (118). It was noted that a similar culture of this strain of *M. tuberculosis* contained a fraction which promoted growth in chicks. This effect was attributed to considerable amounts of "B_{10} and B_{11}" rather than to pteroylglutamic acid (64); the relation between pteroylglutamic acid and "B_{10} and B_{11}" (66) has not then been evaluated. It thus appeared possible that an effect of p-aminobenzoic acid upon this strain of *M. tuberculosis* was to increase the production of conjugated pteroylglutamic acid. The nature of the yellow pigment (116) has not yet been described.

A mixed culture of organisms was obtained from the duodenum of chicks. The growth rate of the culture was increased by p-aminobenzoic acid to the medium, and simultaneously a three-fold increase in folic acid production as measured by assay with *S. fecalis* R was observed (119).

It was noted that the growth of *S. fecalis* R and *L. casei* in the presence of pteroylglutamic acid, pteroyltriglutamic acid or pteroic acid was affected very little by sulfonamides (120). Growth of these organisms did not take place if p-aminobenzoic acid or p-aminobenzoyl glutamate was substituted for pteroylglutamic acid, pteroyltriglutamic acid or pteroic acid. This indicated that these organisms cannot form pteroic acid or pteroylglutamic acid from p-aminobenzoic acid or p-aminobenzoyl glutamate and that they need preformed pteroic acid or pteroylglutamic acid. Hence there is no opportunity for a sulfonamide to compete with p-aminobenzoic acid in the nutrition of these organisms. With another organism, *S. fecalis* Ralston, which responded to p-aminobenzoic acid, p-aminobenzoyl glutamate, pteroylglutamic acid, pteroyltriglutamic acid or thymine, competitive inhibition between sulfadiazine and p-aminobenzoic acid or p-aminobenzoyl glutamate was observed. In the presence of pteroylglutamic acid,

pteroyltriglutamic acid or thymine, the organism became highly resistant to sulfadiazine, indicating that the inhibitory action of sulfadiazine was due to interference with the formation of pteroylglutamic acid from p-aminobenzoic acid and that when preformed pteroylglutamic acid was supplied, the organism did not "need" to carry out this synthesis and hence sulfadiazine was noninhibitory. The observations with thymine recall the previous observation that for certain bacteria this substance in the absence of pteroylglutamic acid is a growth promoting factor, and the suggestion that the function of pteroylglutamic for these bacteria is to catalyze the formation of thymine (p. 66). A third type of organism, represented by *E. coli*, did not require preformed pteroylglutamic acid, and the bacteriostatic effect of sulfadiazine on this type was reversed by p-aminobenzoic acid but not by pteroylglutamic acid, which observation might be presumed to indicate that these organisms could not utilize preformed pteroylglutamic acid.

Thymine and pteroylglutamic acid. A relation between the pyrimidine base, thymine (5-methyl uracil) and "folic acid" was observed in studies with *S. fecalis* R (121). This lactic acid organism was enabled to grow by the addition of both thymine and a purine base. Thymine could not be replaced by uracil, but the purine requirements were less specific, adenine and guanine being almost interchangeable.

Thymine was also found to have a growth-promoting effect on *L. casei* (103). A combination of both thymine and a purine base was necessary. Approximately 0.5 microgram of thymine and 5.0 micrograms of guanine per ml. of media were required to give the maximum response. The maximum growth obtained with thymine and purines was only half that obtained with concentrates of *L. casei* factor (pteroylglutamic acid). Thymine could not be replaced by uracil or cytosine while the purine requirements were met by guanine, xanthine, adenine or hypoxanthine.

A report on the requirements of *S. fecalis* R showed that thymine could not be replaced by 4-carboxyuracil, 4-carboxythymine, or 5-carboxyuracil (109). Nucleic acid was found to be inactive as a source of thymine for *S. fecalis* (122), indicating the inability of the organism to utilize thymine in the form of a nucleic acid. While no data has appeared on the activity of thymine nucleotide, the nucleoside thymidine has been shown to be as active as thymine on a molal basis (105). In this report the inactivity of a number of pyrimidines was also described.

The interfering effect of thymine on the assay of pteroylglutamic acid with *L. casei* received comment (123).

A possible role of thymine in serving as a substitute for "folic acid" was formulated on the basis of experiments with these two compounds in the nutrition of *S. fecalis* R and *L. casei* (105). It was found that while pteroylglutamic acid alone would give a response, addition of a purine was needed to give maximum response. In the presence of a purine, approximately 5,000 times as much thymine as pteroylglutamic acid was required. With *S. fecalis* R, thymine permitted the same maximum growth as was obtained with pteroylglutamic acid,

but with *L. casei* thymine *plus* a purine gave only half the maximum response produced by pteroylglutamic acid.

The possibility of formation of pteroylglutamic acid by *S. fecalis* R cells during growth on thymine was tested by autoclaving the cells with dilute hydrochloric acid and assaying with *L. casei*. A "plateauing" of the *L. casei* assay response at approximately half-maximum growth was characteristic of the response obtained with thymine and suggested that the material in the *S. fecalis* R cells giving the response was thymine and not pteroylglutamic acid. These observations led to the suggestion that pteroylglutamic acid functioned directly or indirectly as a coenzyme in the synthesis of thymine or related compounds by *S. fecalis* R. Three to four micrograms of thymine were taken up per mg. of *S. fecalis* R cells during growth, an amount which may be compared with the value of about 2.0 micrograms of thymine present per milligram of dried tubercle bacillus (124).

Studies of the effect of a large number of pyrimidines on the growth of *L. casei* were reported (125). Derivatives of thymine in which one or both of the oxygen atoms were replaced by an imino or thio group in many cases promoted the growth of *L. casei*. The replacement of oxygen by the imino group appeared to lower the activity to about 0.1 that of the corresponding oxy compound. Other relationships of the effect of chemical structure upon biological activity were discussed. It was found that isobarbituric acid and 5-amino uracil reversibly antagonized the growth promoting effect of thymine or pteroylglutamic acid. It was also observed that bromouracil could inhibit completely the growth of *L. casei* with thymine as the nutrient but had no effect or produced slight stimulation when pteroylglutamic acid was used as a nutrient. This observation did not support the hypothesis (105) that the function of pteroylglutamic acid in promoting the growth of lactic acid bacteria was to enable the organisms to synthesize thymine. It was further observed (125) that nitrouracil at certain concentrations could antagonize the growth-promoting action of pteroylglutamic acid but had no effect on the growth-promoting action of thymine. Thymine in large doses has been reported to cause hemopoietic remission in pernicious anemia and sprue (pp. 84, 90); the substance appears to be ineffective in preventing pteroylglutamic acid deficiency in rats and chicks (126, 127).

The "Streptococcus lactis R factor". A factor was described which was highly active for *S. fecalis* R, but relatively inactive for *L. casei* (102). One microgram of this "S. L. R factor" had an activity for *S. fecalis* R equal to 1.25 micrograms of "folic acid (potency 137,000)" but for *L. casei* it had an activity equal to less than 0.00001 microgram of the same preparation of folic acid. Pteroylglutamic acid has been shown to have the same potency as a "folic acid preparation of potency 137,000" (115).

A survey was made of the "S. L. R factor" and pteroylglutamic acid requirements of a number of lactic acid bacteria (104). The results which appear in table 4 revealed that those organisms which are able to utilize the "S. L. R factor" can also use pteroylglutamic acid. A certain number, mostly Lactobacilli, were able to utilize pteroylglutamic acid only.

These results also demonstrated that different strains of the same organism have widely differing requirements. Thus one strain of *S. fecalis* can use either the "S. L. R factor" or pteroylglutamic acid, another requires pteroylglutamic acid while a third requires neither. Synthesis of pteroylglutamic acid was stated to be established for three of these strains.

It was also reported that certain enterococci were able to convert the "S. L. R factor" into a form which is active for *L. casei*. It was found that 0.06 microgram of this factor per 10 ml. of media did not permit growth of *L. casei* but that 1 ml. of a fluid culture of "*S. fecalis*" grown with 0.006 μg per ml. of the factor would permit rapid growth. This demonstrated that *S. fecalis* R had converted the "S. L. R factor" into a form active for *L. casei*, which was presumed (104) to be pteroylglutamic acid. In the same report it was stated that incubation of the "S. L. R factor" with rat liver suspensions did not give rise to "folic acid".

TABLE 4

Streptococcus lactis R factor and pteroylglutamic acid requirement of lactic acid bacteria

Organisms requiring		
"S. L. R. FACTOR" OR PTEROYLGLUTAMIC ACID (INTERCHANGEABLE)	PTEROYLGLUTAMIC ACID	NEITHER
Streptococcus lactis R	*Lactobacillus casei*	*Lactobacillus arabinosus* 17-5
Streptococcus fecalis 732	*Lactobacillus delbruckii* LD5	*Leuconostoc mesenteroides* 6205
Streptococcus fecalis F24	*Lactobacillus bulgaricus* O5	*Streptococcus lactis* 874, 4487, 8039, 7963, 4386, L103, L104, L206
Streptococcus zymogenes 5Cl	*Streptococcus casei* 19	*Streptococcus fecalis* 10Cl
Streptococcus durans 98A	*Streptococcus fecalis* S108A	*Streptococcus zymogenes* 6054

This constituted evidence for the belief that the "S. L. R factor" is not a conjugate of pteroylglutamic acid since liver enzymes are capable of cleaving conjugates.

In a later paper from the same laboratory a detailed study of the conversion of the "S. L. R factor" to pteroylglutamic acid by various enterococci was reported (99). The ability of these organisms to effect this conversion varied widely. Resting cell suspensions of these organisms in phosphate buffer were used. A 10 ml. suspension of *S. lactis* R or *S. zymogenes* converted 5 micrograms of S. L. R factor to about 1.0 microgram of pteroylglutamic acid[1] in three hours while *S. fecalis* 232 and *S. durans* 98A formed only 0.18 microgram. The addition of carbohydrates, e.g. *d*-ribose, glucose and fructose increased the formation of pteroylglutamic acid 5- to 15-fold. Other carbohydrates tested permitted growth but did not accelerate the formation of pteroylglutamic acid. Those

[1] The data have been recalculated on the basis of pteroylglutamic acid assuming that pteroylglutamic acid has the same activity as folic acid of potency 137,000.

sugars which could act as hydrogen acceptors as evidenced by reduction of methylene blue during the incubation with resting cells, were the ones capable of increasing the rate of conversion. Sucrose was unable to stimulate this conversion by resting cells which had been grown on glucose. However, if the organism were initially grown on sucrose, then sucrose was as efficient as glucose in stimulating the conversion of the S. L. R factor, and methylene blue was reduced by the product obtained when sucrose was incubated for three hours with resting cells.

In the assay of pteroylglutamic acid formed by conversion from the "S. L. R factor" the entire cell suspensions were added directly to the assay medium without any preliminary treatment. Practically all this activity was found to be within the cells. When these cells were autoclaved with water only 12 per cent of the activity was recovered and only 28 per cent when autoclaved with phosphate buffer. However, when the cells were autoclaved in the assay medium, in 0.5 per cent sodium thioglycollate solution or in 5 per cent ascorbic acid and then assayed, complete recovery was obtained. This suggests that reducing substances exerted a protective influence during extraction from the cell. However, the residual pteroylglutamic acid in the liquid phase after autoclaving was resistant to further autoclaving. These results emphasize the difficulties that can be encountered in assaying microbial cells and the care needed to assure complete extraction.

The "S. L. R factor" ("rhizopterin") was characterized as 4(((2-amino-4-hydroxy-6-pteridyl)methyl)formamido) benzoic acid; a compound of pteroic and formic acids (306). It is inactive for hemopoiesis in sulfonamide-treated rats and pteroylglutamic acid-deficient chicks (128). Pteroic acid is similarly inactive for the chick (1). There appears to be a small difference in the relative potencies of the S. L. R factor and pteroic acid for *S. fecalis* R and *L. casei*. The activities of the "S. L. R factor" when directly compared with pteroylglutamic acid have not been reported and the only evidence available is based on recalculation of the data obtained with "folic acid" with a potency of 40,000 obtained from spinach. Using this data, the "S. L. R factor" is 125 per cent as active as pteroylglutamic acid for *S. fecalis* R and only 0.001 per cent as active for *L. casei*. Pteroic acid is 50 to 100 per cent as active as pteroylglutamic acid for *S. fecalis* R depending on the time of incubation (127) and 0.01 per cent as active by *L. casei* assay.

Pteroylglutamic acid in the nutrition of rats. On purified diets which do not contain pteroylglutamic acid, the dietary need of the rat for this factor appears to be satisfied by the production of pteroylglutamic acid by the intestinal bacteria. The addition of any of several sulfonamides to the diet depresses the growth of the intestinal bacteria and results in the appearance of certain deficiency syndromes, one of which is characterized by agranulocytosis, leukopenia, anemia and slow growth. Administration of pteroylglutamic acid or of its conjugates cures this syndrome. Some difficulties of interpretation were encountered until the fact was appreciated that certain conjugates are microbiologically inactive. A relationship may exist between the utilization of pantothenic acid and pteroylglutamic acid in rats on such diets. Lactation leucopenia

due to pteroylglutamic acid deficiency has been observed in rats on purified diets.

It was found that rats would grow and reproduce on a purified diet (129). Shortly thereafter, it was shown that when sulfaguanidine was added to a similar diet, the growth of young rats was greatly reduced and could be restored by the addition of liver extract (130). A concentrate of "folic acid" was fed at the rate of 5 milligrams daily to rats on a purified diet containing 1 per cent of sulfasuxidine (131). Definite growth responses were observed when biotin was also fed. Similar responses were obtained with a more concentrated "folic acid" preparation. In addition to promoting growth (132), "folic acid" concentrates were found to restore the color of the hair in black rats which had become grey on diets containing sulfaguanidine. The depressed growth under such conditions was found to be counteracted by feeding yeast, yeast extract or rat feces (133). Specific signs were described in rats on such diets (134), including agranulocytosis, leucopenia, anemia and hypocellularity of the bone marrow. The changes were prevented or reversed by a liver fraction which was precipitated from aqueous solution by addition of 80 per cent ethanol. A crude norite eluate containing "folic acid" was found to improve the leukocyte picture (49). These findings were confirmed with a "folic acid concentrate" (135) which was found to exert an effect similar to that of liver extract in promoting growth and preventing leucopenia in rats on diets containing sulfasuxidine. In another investigation (136) signs of pantothenic acid deficiency were observed in rats on purified diets with sulfasuxidine and the pantothenic acid content of the liver tissue of the rats was lowered, although the diet contained 40 parts of pantothenic acid per million. When biotin and a "folic acid" concentrate were added to the diet, growth was improved and the pantothenic acid content of the liver was increased. These additions were also reported to lower the prothrombin time (137). The signs of pantothenic acid deficiency, including porphyrin-caked whiskers, were found to be removed by supplementation with biotin and a "folic acid" concentrate (138), but not by additional amounts of pantothenic acid.

It was shown (50) that pteroylglutamic acid or pteroyltriglutamic acid, 20 micrograms per day for 4 days, were effective in markedly increasing the granulocytes and the total white cell count, and in relieving the anemia occurring in rats on purified diets containing sulfaguanidine or sulfasuxidine.

The anemia induced by bleeding was studied in rats which were fed a purified diet containing sulfasuxidine (139). The administration of pteroyltriglutamic acid was found to have a preventive and corrective effect on the anemia as judged by measurement of hemoglobin concentration, hematocrit readings and white counts. These observations may indicate a need for further studies of the possible role of pteroylglutamic acid in the regeneration of hemoglobin following bleeding.

Attention was drawn to the unexpectedly high activity of milk in preventing "folic acid" deficiency in rats on a purified diet containing sulfasuxidine (140). The "folic acid" content of the milk was found to be quite low as determined by assay with *L. casei* and *S. fecalis* R. When the milk was treated with "conjugase" the "folic acid" content was found to be increased more than 20-fold

(141) which indicated that the preceding observation could be explained on the basis of utilization of conjugated pteroylglutamic acid by the rat.

Storage of pteroylglutamic acid in the liver of rats was reduced by adding sulfasuxidine to a purified diet and the folic acid content of the liver was increased when a liver fraction was added to the diet (142). A series of investigations (143, 144, 145, 146) emphasized the relationships between pteroylglutamic acid and other dietary ingredients. It was shown that granulocytopenia due to pteroylglutamic acid deficiency was obtained in a small percentage of rats which were fed purified diets without sulfonamides. The dyscrasia was also produced by feeding purified diets which were low in pantothenic acid; under these conditions anemia, leucopenia, granulocytopenia, and bone marrow hypoplasia were prevented by adding pantothenic acid. It was considered that the primary deficiency producing granulocytopenia under these experimental conditions was that of pteroylglutamic acid. The administration of pantothenic acid appeared to prevent the development of pteroylglutamic acid deficiency. Riboflavin-deficient rats were found to become anemic, granulocytopenic or both. The granulocytopenia was corrected by pteroylglutamic acid and the anemia, somewhat less consistently, by riboflavin. Severe granulocytopenia and anemia were produced in rats fed protein-free diets. The condition was prevented by casein but not by pteroylglutamic acid or pteroyltriglutamic acid. Casein did not correct the granulocytopenia but when casein and pteroylglutamic acid were both added the granulocytopenia was cured.

Anemia, leucopenia, and hemorrhage and necrosis of the adrenals were observed in rats on a purified diet containing thiourea. The leucopenia and granulocytopenia were not prevented by thyroid powder or thyroxin but were corrected by treatment with pteroylglutamic acid. Pteroyltriglutamic acid, 100 micrograms daily, was effective, but treatment for 4 days with 24 micrograms did not consistently correct the dyscrasia (147). These results may be contrasted with the reported ineffectiveness of pteroylglutamic acid in preventing the onset of agranulocytosis in two patients receiving thiouracil (148).

A description of the pathology of the bone marrow of rats as affected by pteroylglutamic acid deficiency was given (149). In the deficiency the marrow was hypocellular and showed general depletion especially of the myeloid series, although the erythroid series was usually also depleted even in the absence of anemia. Administration of pteroylglutamic acid resulted in a characteristic proliferation and regeneration and the marrow returned to normal after temporary over-compensation.

Hypochromic anemia was induced by promin and promizole in young rats on a purified diet. The administration of pteroylglutamic acid, 18 micrograms daily, exerted an anti-anemic effect (150).

It was reported that concentrates of pteroylglutamic acid improved the performance of rats during lactation (151). "Lactation leucopenia" in rats on purified diets was found to be partially prevented by addition of brewers' yeast or liver extract (152) and subsequently pteroylglutamic acid was found to have

a similar effect (153) although the effect was not as complete as that of a liver fraction.

Intestinal synthesis of "folic acid" in rats on purified diets, was studied by examination of the cecal contents (154). Dextrin produced the largest amount of synthesis. A marked decrease in synthesis was caused by adding 2 per cent sulfathalidine to the diet. Increases were produced by adding niacin, lactose or milk powder to the basal diet; all these increases were prevented by adding sulfathalidine.

In experiments with rats a liver fraction was encountered which had a marked effect in relieving pteroylglutamic acid deficiency in rats which received a purified diet with sulfonamides. Its effectiveness appeared to be in excess of its pteroylglutamic acid content as measured by microbiological assay, even after treatment with acid, alkali, or a "conjugase" preparation had been used to liberate pteroylglutamic acid (155).

Isolation of pteroylglutamic acid and related compounds. Methods for the concentration of the "norite eluate factor" (pteroylglutamic acid) were described by the Wisconsin workers. In their first report (8) this factor was found to be relatively stable to acid and alkali; it could be adsorbed on activated charcoal and Lloyd's reagent; it was precipitated by phosphotungstic acid and by basic lead acetate and could be extracted from an acid solution by butanol. A later publication from the same laboratory (93) described additional methods of purification by adsorption on Superfiltrol. The factor was also concentrated from liver by adsorption and elution with norite and precipitation of the manganese salt with manganese chloride and ethanol (103).

The isolation of a highly active preparation of "folic acid" from spinach was reported by the Texas group (9) and was later described in more detail by the same group of workers (109, 156, 157). These workers employed repeated adsorption and elution from activated charcoal, precipitation with silver and lead, adsorption on Lloyd's reagent, chromatographic adsorption on alumina and precipitation of the free acid from cold acidic solutions. Since "folic acid" from spinach has never been crystallized it is not possible to establish its identity with pteroylglutamic acid. The activity of the material described in the first publication (9) had a "potency" of 40,000 as compared with a sample of a liver fraction B which was arbitrarily given a potency of 1. In a subsequent paper (156) a preparation with a "potency" of 137,000 was reported. While the activity of this preparation has not been given, a report (115) stated that synthetic pteroylglutamic acid gave a "potency" of approximately 137,000 when compared with a sample of "folic acid" of known potency obtained from spinach. Another communication (158) presented data showing that pteroylglutamic acid would have a "potency" of approximately 160,000. This data would indicate that the final product which was obtained from spinach and which had a potency of 137,000 was almost pure.

Two reports of the isolation of pure pteroylglutamic acid from liver have been made (10, 67). This compound was also isolated from a yeast concentrate which

had been enzymatically hydrolyzed (92). This yeast concentrate contained "B_c conjugate" which was active in preventing anemia in chicks but which possessed little microbiological activity until after it had been hydrolyzed enzymatically. "Vitamin B_c" isolated from a hydrolyzed yeast concentrate was identical with pteroylglutamic acid obtained from liver.

Two different methods have been described for the isolation of pteroylglutamic acid from liver. One of these starts with whole liver in which the conjugates have been converted into free pteroylglutamic acid by autolysis (67, 159). The isolation process involved essentially the following steps: extraction with boiling water, adsorption on and elution from Amberlite 4R, adsorption and elution using activated charcoal, extraction of an aqueous solution of the free acid with butanol at pH 3 to 4, formation of a barium salt, extraction of barium salts with hot water, formation of a zinc salt and crystallization of the free acid from water. The other method (10, 160) employed as the starting material an 80 per cent-alcohol-insoluble fraction of an aqueous extract of liver. The fraction contained approximately 20 micrograms of pteroylglutamic acid per gram. The pure substance was obtained by the following steps: adsorption and elution with norite, adsorption and elution with Superfiltrol, formation of a barium salt with barium chloride and methanol, esterification of the barium salt with 0.2N HCl methanol, extraction of the methyl ester from aqueous solution with butanol, chromatographic adsorption of the ester on Superfiltrol, and fractional precipitation of the ester from water and from methanol. The free acid obtained by hydrolysis of the ester was crystallized from hot aqueous solutions.

Properties of pteroylglutamic acid. Pteroylglutamic acid crystallizes as yellow spear shaped platelets. Its solubility (160) as the free acid is 10 micrograms per ml. at 0°C and more than 500 micrograms at 100°C. The sodium salt is much more soluble, having a solubility greater than 15 mgm. per ml. at 0°C. Pteroylglutamic acid has characteristic ultraviolet absorption spectra. In 0.1N NaOH it exhibits maxima at 257, 282, and 365 mμ and corresponding E (1%) 1 cm values of 585, 570, 206. Another report (161) described the absorption spectrum at pH 11. This possessed maxima at the same points but with absorption coefficients which were slightly higher; 603, 600 and 213 at wavelengths of 256, 282, 365 mμ.

The isolation of pteroyltriglutamic acid (85, 162) was accomplished by a method different from that used for the compound obtained from liver. The starting material was a cell-free filtrate obtained from aerobic fermentation of a diphtheroid-type organism. The method involved adsorption on charcoal, formation of a barium salt, esterification and extraction of the methyl ester from an aqueous solution with butanol and fractional precipitation of the ester from hot methanol. The methyl ester was dissolved in hot methanol and precipitated by cooling. In the presence of 0.05N sodium chloride the ester precipitated in a flocculent form while in the absence of electrolytes a gel was formed. After several such precipitations from methanol the ester was obtained in a microcrystalline form. The free acid was obtained in the pure state by converting the ester to the barium salt, removing extraneous pigments with Florosil and

precipitating the free acid at pH 2.8. The free acid could be crystallized from water containing electrolytes such as sodium or calcium chloride. In the absence of electrolytes gels were obtained. The pure ester was obtained by esterification of the free acid and crystallization of the ester from 0.05N sodium chloride in methanol.

While pteroylglutamic acid and its ester can be precipitated from water and methanol respectively in the absence of electrolytes, pteroyltriglutamic acid and its ester require the presence of electrolytes for their precipitation. The solubilities of pteroyltriglutamic acid in the presence of $CaCl_2$ at pH 2.8 are 3.0 mgm. per ml., at 80°C and 0.10 mgm. per ml. at 5°C.

A conjugate was isolated from yeast (70) and was later reported to contain seven glutamic acid residues (3). This compound was crystallized from 5 per cent sodium chloride. The use of electrolytes in crystallization of this compound was similar to that reported for the triglutamic acid derivative (162). The heptaglutamic acid conjugate possessed an activity for chicks which was proportional to its content of pteroylglutamic acid (70). Its microbiological activity was 0.3 to 0.6 per cent of that of pteroylglutamic acid by assay with *L. casei* and 0.2 per cent by assay with *S. fecalis* R (see table 2). The conjugate is as active on a molal basis as pteroylglutamic acid in promoting the growth of *Tetrahymena geleii* (108).

Degradation. The relationship between pteroyltriglutamic acid and pteroylglutamic acid was shown by anaerobic alkaline hydrolysis (163). The triglutamic acid derivative is active for *L. casei* but only slightly active for *S. fecalis* R (table 2). It was found that while aerobic alkaline hydrolysis produced rapid biological inactivation for both organisms, anaerobic hydrolysis produced only a slight decrease in the activity for *L. casei* and greatly increased the activity for *S. fecalis* R. The ratio of the activity for these two organisms approached that of pteroylglutamic acid isolated from liver. Two mols of alpha-amino acid nitrogen were liberated during anaerobic alkaline hydrolysis and the active compound which was formed was approximately half as active as pteroylglutamic acid by both *L. casei* and *S. fecalis* R assay. This compound was later identified as racemic pteroylglutamic acid.

Aerobic alkaline hydrolysis (163) of pteroyltriglutamic acid or racemic pteroylglutamic acid resulted in the formation of a fluorescent pigment and a diazotizable aromatic amine which could be estimated by the method of Bratton and Marshall (164). In the absence of oxygen no diazotizable amine or fluorescent pigment were produced by alkaline hydrolysis. The fluorescent pigment proved to be a dibasic acid having pKa values of 3.9 and 7.7. Elementary analysis suggested the empirical formula $C_7H_5N_3O_3$. Decarboxylation of the fluorescent dibasic pigment at 300° resulted in the liberation of approximately 1 mol of CO_2 and the formation of a fluorescent monobasic acid with a pKa of 8.0. Oxidation of the original dibasic acid with chlorine water, followed by hydrolysis with 0.1N HCl at 140°C yielded a compound which gave a positive test for guanidine. The formation of guanidine under such conditions constitutes evidence for a pyrimidine ring with an amino group in the 2-position (165). The fluorescent

dibasic acid showed characteristic absorption spectra in 0.1N NaOH with maxima at 253 and 365 mμ. The empirical formula $C_7H_5N_5O_3$, the titration data, and formation of guanidine suggested a 2-amino purine or a 2-amino pteridine. The absorption spectra, however, eliminated the possibility of a purine because purines do not have absorption maxima above 300 mμ. Thus the available evidence pointed toward a 2-amino pteridine with an enolic and a carboxy group. With this evidence available, attempts were made to synthesize pteridine having these functional groups. The compound was identified as 2-amino-4-hydroxypteridine-6-carboxylic acid by comparison with the synthetic compound.

The monobasic fluorescent pigment produced by decarboxylation was identified as 2-amino-4-hydroxypteridine.

The structure of these two pteridines was established by the following series of reactions (166). Diethylmesoxalate was condensed with 2,4,5-triamino-6-hydroxypyrimidine to yield isoxanthopterin carboxylic acid (II) (167). The structure of isoxanthopterin carboxylic acid (II) is that shown by formula II although it had not previously been definitely established whether the carboxyl group occupied the 6- or 7-position. On chlorination of isoxanthopterin carboxylic acid and subsequent reduction with hydrogen iodide one of the hydroxyl groups was removed to give compound IV which was identical with the dibasic fluorescent pigment (166). Presumably either the 4 or the 7 hydroxyl could have been removed by this procedure. The presence of the 4 hydroxyl in compound IV was shown in two ways. First decarboxylation of 2-amino-4-hydroxypteridine-6-carboxylic acid (IV) gave 2-amino-4-hydroxypteridine (V), the structure of which was established by its synthesis from glyoxal and 2,4,5-triamino-6-hydroxypyrimidine (I). Its formation by this method demands a hydroxyl group in the 4 position. Second, the synthesis of 2-amino-4-hydroxypteridine-6-carboxylic acid was accomplished by condensation of 2,4,5-triamino-6-hydroxypyrimidine (I) and ethyl-β,β-diethoxy-α-bromo propionate. This reaction also establishes the presence of a hydroxyl group in the 4 position of the pteridine.

The final proof establishing the 6 position of the carboxyl group in compound IV was obtained by degrading 2-amino-4-hydroxy-6-methyl pteridine (VI) to give a compound identical with 2-amino-5-methyl-pyrazine (VII). The corresponding 7-methyl pteridine would have yielded 2-amino-6-methyl-pyrazine instead. The structure of 2-amino-4-hydroxy-6-methyl pteridine (VI) was established by oxidation with alkaline permanganate to give the corresponding 2-amino-4-hydroxypteridine-6-carboxylic acid (IV). These reactions are outlined in Fig. 2.

By hydrolysis with 0.5N sulfurous acid at room temperature pteroyltriglutamic acid was rapidly inactivated and gave an aromatic amine and a fluorescent pigment (168). This pigment reacted rapidly with typical aldehyde reagents such as hydroxylamine, phenylhydrazine and semicarbazide, indicating the presence of an aldehyde group. This fluorescent pigment did not possess a carboxyl group as evidenced by the fact that its distribution coefficient between water and buta-

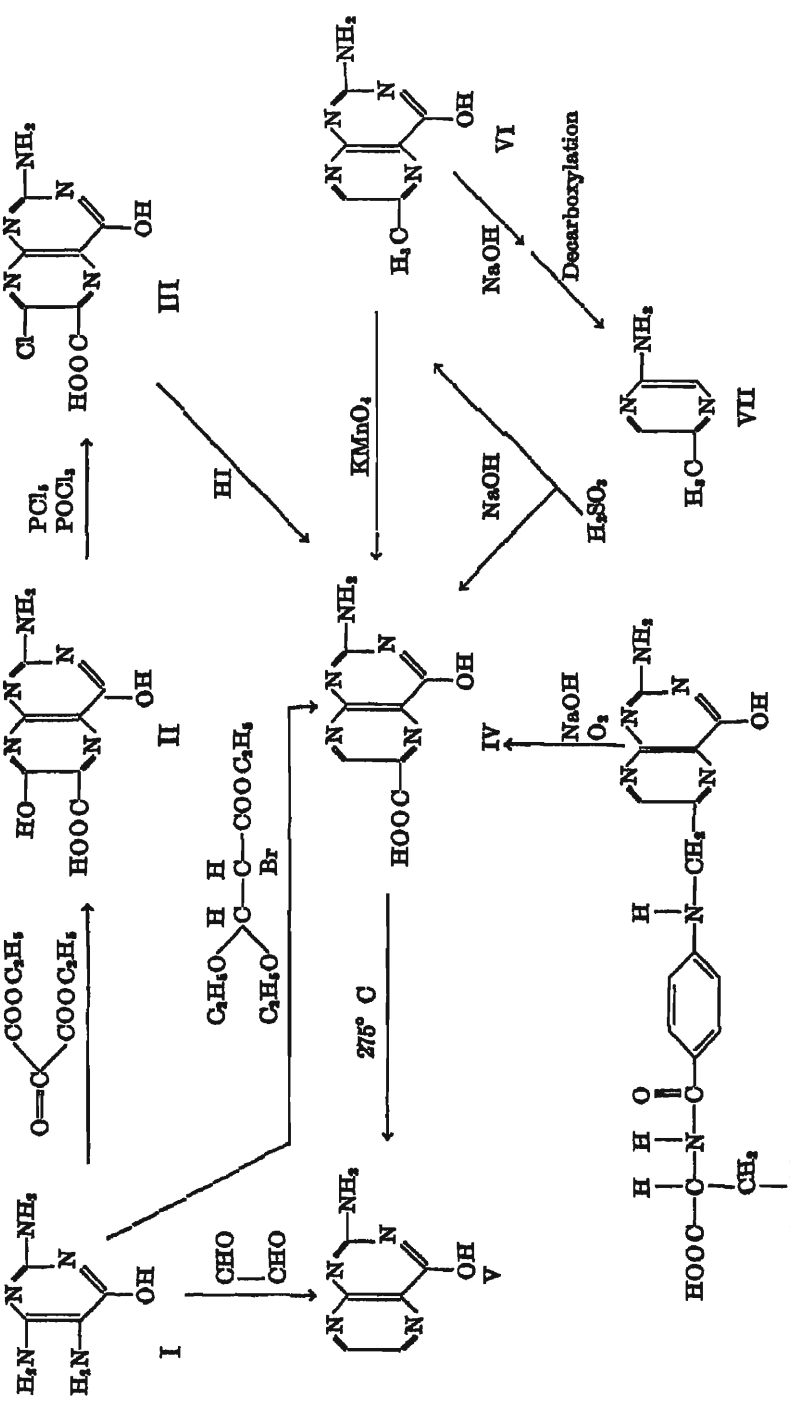


Fig. 2. Chemical reactions leading to the establishment of the structural formula of the pteridine (IV) obtained by aerobic alkaline hydrolysis of *L. casei* factor.

nol was the same at pH 3.0 as at pH 7.0. When the pigment obtained by sulfurous acid hydrolysis was treated anaerobically with dilute sodium hydroxide, approximately equal amounts of 2-amino-4-hydroxypteridine-6-carboxylic acid (IV) and a second pteridine, identified as 2-amino-4-hydroxy-6-methylpteridine (VI), were formed. The latter compound could be oxidized with alkaline potassium permanganate to yield 2-amino-4-hydroxypteridine-6-carboxylic acid (IV). The formation of approximately equal molal quantities of carboxy- and methyl-derivatives from what is apparently an aldehyde probably involves a Cannizzaro type reaction although the mechanism of this reaction is obscure.

Prolonged aqueous hydrolysis of pteroyltriglutamic acid at pH 4 produced biological inactivation and yielded a compound which was crystallized and identified as 1-pyrollidonecarboxylic acid (168). On hydrolysis with alkali this yielded 1 (+)-glutamic acid which was estimated microbiologically.

The aromatic amine which was produced during sulfurous acid hydrolysis was isolated as the barium salt (168). This compound when diazotized and coupled with N(1-naphthyl)ethylenediamine dihydrochloride yielded a red pigment (164) which indicated a primary aromatic amine with a highly negative substituent group. The aromatic amine nitrogen as measured by the method of Bratton and Marshall (164) constituted approximately 25 per cent of the total nitrogen. The remaining 75 per cent of the nitrogen could be converted into alpha-amino-acid nitrogen by alkaline hydrolysis. From such hydrolysates the aromatic amine was isolated and identified as *p*-aminobenzoic acid. Microbiological assay of the hydrolysate indicated the presence of 3 mols of glutamic acid. The peptide linkage of the glutamic acid to *p*-aminobenzoic acid must involve the carboxyl group of the latter as a primary aromatic amine is required for reaction in the Bratton and Marshall test (164).

The diazotizable aromatic amine obtained by aerobic alkaline hydrolysis of racemic pteroylglutamic acid was found to contain 2.1 atoms of nitrogen for each atom of aromatic amino nitrogen (163). The distribution coefficient of this aromatic amine was greatly different from that of p-aminobenzoic acid. After hydrolysis with 2N sulfuric acid, 45 per cent of the total nitrogen appeared as alpha-amino-acid nitrogen, and the distribution coefficient of the aromatic amine became the same as that for p-aminobenzoic acid. The latter compound was isolated from the hydrolysate.

Evidence regarding the mode of linkage is furnished by the results of alkaline hydrolysis (163). The absence of fluorescence and of the free aromatic amine in the original pteroylglutamic acid, and the simultaneous appearance of these two during aerobic alkaline hydrolysis suggested that the pteridine is linked to the aromatic amine nitrogen. As hydrolysis proceeded the liberation of pteridine and aromatic amine appeared at approximately the same rate.

Reduction in acid solution either catalytically or with zinc dust yielded the aromatic amine and a reduced pteridine (168). After reoxidation with manganese dioxide the pteridine obtained by zinc reduction was identified as 2-amino-4-hydroxy-6-methylpteridine, the structure of which has already been established.

The foregoing evidence indicated the following conclusions regarding the structure of pteroylglutamic acid.

1. Aerobic alkaline hydrolysis, sulfurous acid cleavage, and chemical or catalytic reduction each yielded a pteridine and a primary aromatic amine. This indicated the linkage of the pteridine to the nitrogen of the aromatic amine.

2. The aromatic amine formed during sulfurous acid cleavage of pteroyltriglutamic acid was a tetrapeptide, p-aminobenzoyldiglutamylglutamic acid. The aromatic amine from pteroylglutamic acid was p-aminobenzoylglutamic acid.

3. There was a single carbon atom linkage between the pteridine and the aromatic amine. This was indicated by the fact that only pteridines with a single carbon atom side chain were obtained and that no other two-carbon fragments could be detected in the two degradation reaction products. The evidence also indicated that this single carbon atom is present in a methylene link. If it were present as a CO group in an amide linkage the cleavage would be hydrolytic and would not require oxygen. The formation of 2-amino-4-hydroxy-6-methylpteridine also constituted evidence for the methylene linkage.

Synthesis. The final proof of the structure was obtained by synthesis of pteroylglutamic acid by different methods. One of these (101) involved the simultaneous condensation of 2-4-5-triamino-6-hydroxypyrimidine (I), p-aminobenzoylglutamic acid (IX) and α,β-dibromopropionaldehyde in aqueous solution. The other (169) involved the reaction of α,β-dibromopropionaldehyde with pyridine, 2,4,5-triamino-6-hydroxypyrimidine (I) and potassium iodide to yield N[(2-amino-4-hydroxy-6-pteridyl)methyl] pyridinium iodide (X). This was then treated with p-aminobenzoylglutamic acid (IX) in ethylene glycol to yield pteroylglutamic acid. The position of the methyl pyridinium group on the 6 position of the pteridine was established by oxidation with alkaline permanganate to yield 2-amino-4-hydroxypteridine-6-carboxylic acid (IV). These two methods of synthesis are schematically outlined in Fig. 3

Pteroic acid was obtained by the corresponding reaction (101) except that *p*-aminobenzoic acid was used instead of *p*-aminobenzoylglutamic acid.

Pteroylglutamic acid in the nutrition of mice. Indications that folic acid was required by mice on a purified diet containing sulfasuxidine were reported (170). The "lactation performance" of mice on purified diets as measured by the percentage and size of litters weaned was increased by adding a concentrate containing pteroylglutamic acid (171, 172).

Dogs. Observations with dogs (173) and pigs (174) indicated that an unidentified B complex factor or factors present in liver and yeast, were needed as a supplement with purified diets. It seems probable that the liver and yeast extracts used in these investigations supplied pteroylglutamic acid. Pteroylglutamic acid was reported to improve the response to niacin in dogs on purified diets deficient in niacin (175).

Guinea pigs. A series of studies with guinea pigs on purified diets indicated that three unidentified dietary factors were required, two of which were found present in linseed oil meal. It was later shown that pteroylglutamic acid could

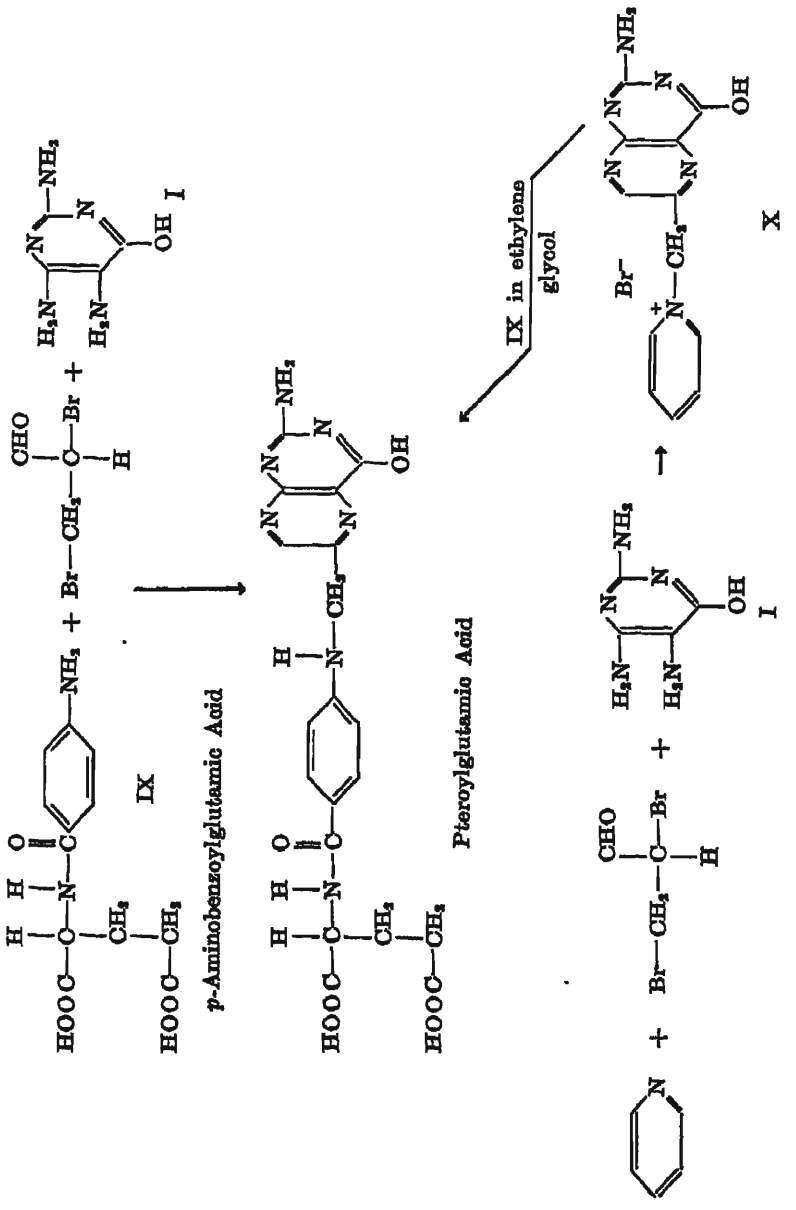


Fig. 3. Two syntheses of pteroylglutamic acid.

replace one of the factors (176). The deficiency was described as being characterized by a rapid decline in weight, lethargy, salivation, terminal convulsions and death. The daily administration of 6.5 micrograms of either pteroylglutamic acid or pteroyltriglutamic acid prevented the deficiency.

Nutrition of insects. Larvae of the mosquito *Aedes aegyptii* were found to need concentrates of pteroylglutamic acid for pupation; xanthopterin or thymine were ineffective. Pteroylglutamic acid was also found to promote growth and to increase survival rate (177). It was found that the larvae of the flour moth and the meal worm needed pteroylglutamic acid for growth (178). Growth of the larvae of a carpet beetle was increased by concentrates of pteroylglutamic acid (179).

Mink. The effects of pteroylglutamic acid deficiency in mink were found to include loss of body weight, diarrhea, irritability, general weakness, anorexia and leukopenia. The deficiency responded to administration of pteroylglutamic acid. There was some indication that an unidentified factor in liver was also needed (180).

Pigs. It was suggested that pteroylglutamic acid deficiency in pigs might be associated with the development of normocytic anemia, although no actual test with pteroylglutamic acid was carried out (181). The pigs from sows fed a supplementary "folic acid concentrate" were thriftier and more vigorous than were pigs from sows fed the basal diet of corn and soy bean meal supplemented with vitamins, not including pteroylglutamic acid, and minerals (182). It was reported that pteroylglutamic acid "may have helped slightly with hemoglobin formation" in pigs on a purified diet (183). Other observations (174) were mentioned above.

The enzymic liberation of pteroylglutamic acid from its "conjugated" forms. The effect of enzymic action in liberating folic acid from tissues was indicated by an observation that the apparent "folic acid" content of such materials could be increased several-fold by treatment with a crude enzyme-containing preparation ("taka-diastase") (184). It was found possible to prepare a dialyzable fraction from yeast which was active for the "vitamin B_c" deficient chick but which had relatively little potency in stimulating the growth of *L. casei* (92) until the concentrate was subjected to an undescribed "enzymatic digestion" which liberated "vitamin B_c" in a microbiologically active form.

Almost at the same time, it was noted that yeast was "rich in the substances which give rise to folic acid when incubated with fresh liver" (30). A crude enzymically-active preparation was made from rat liver (36) by extraction with phosphate buffer, fractional precipitation with ammonium sulfate, and dialysis. The preparation liberated the *S. lactis* R-stimulating factor at pH 7 from cell-free water-soluble fractions. These fractions had previously been extracted from natural materials with the aid of "taka-diastase" which thus had brought pteroylglutamic acid conjugates into solution without splitting them. Hence taka-diastase did not supply appreciable quantities of the "folic-acid-liberating" enzyme system. This observation indicated a degree of specificity on the part of the enzyme concentrate prepared from rat liver. The name "vitamin B_c

conjugase" was applied to the enzyme system (185). Hog kidney, liver, small intestine and beef liver were reported to be rich sources; it also occurred in sweet almonds. The optimum pH of the hog kidney preparation was 4.5, while that for almond was 7.0. Hog kidney was used as a source of the enzyme in a method for the assay of "vitamin B_c conjugate" (186). Samples were incubated for 16 hours at pH 4.5 and 45°. The values obtained after enzymatic treatment in many cases showed close agreement with the values obtained by biological assay with chicks on a purified diet (68). An exception was observed in the case of certain liver extracts and plant extracts. The presence of inhibitors in the plant extracts was suggested, or, as an alternative, the presence of compounds which were active for the chick but inactive for *L. casei* even after treatment with the enzyme.

Chicken pancreas (187) was found to be an excellent source. An increase in potency of more than one-thousand fold was reported to occur upon concentrating the enzyme from chicken pancreas extract by adsorption and precipitation techniques. The optimum pH for the action of the enzyme was between 7 and 8; a considerable loss of activity was observed on dialysis. Further studies (158) described modifications in the procedure for concentrating the enzyme. The pancreatic tissue was ground and allowed to autolyze at pH 8. A fraction was then separated which precipitated at between 40 and 80 per cent saturation of ammonium sulfate. The precipitate was dialyzed, redissolved, reprecipitated with cold alcohol, redissolved in buffer and reprecipitated with ammonium sulfate. The procedure resulted in an approximately 3000-fold concentration of activity. The concentrated preparation was activated by calcium, the optimal concentration being 0.01M. Optimum pH and temperature were found to be 7.8 and 32°.

The Michaelis constant for conjugase preparations from rat liver, potatoes and chicken pancreas was measured (188).

The production of "vitamin B_c activity" in several hundred different microorganisms was measured by *L. casei* assay. Various commercial enzymic preparations were tested for their activity in liberating pteroylglutamic acid from its conjugates but none of the preparations were very effective. Chicken pancreas was used as a source of the enzyme (189).

A study was made of the comparative potencies of various tissues in liberating pteroylglutamic acid from a concentrated preparation of pteroylheptaglutamic acid (vitamin B_c conjugate) (190). Chicken and turkey pancreas were the most potent tissues; their optimum activity was at a pH of about 7.0 while other tissues; including rat, mouse, hog and guinea pig pancreas, chicken and hog liver and hog kidney had their optimum at a pH of about 4.5. Hog kidney was used as a source of the "conjugase" enzyme system for further studies. The optimum temperature for the action of the enzyme was found to be 45° to 48°. The presence of an inhibitor in yeast extract was demonstrated. Loss of activity occurred during attempts to concentrate the conjugase; the addition of calcium failed to restore the loss. A unit of activity for the enzyme was proposed and defined.

The p-aminobenzoyl-polyglutamic-acid polypeptide isolated from yeast (191)

was found under certain circumstances to inhibit "vitamin B_c conjugase" preparations obtained from chicken pancreas and rat liver (Totter, J. R. Cited in (192)).

Clinical effects of pteroylglutamic acid. It is beyond the scope of this review to discuss the extensive literature of recent years regarding the erythrocyte maturation factor present in liver and the extrinsic and intrinsic factors which have been postulated to be concerned in its formation. Mention must be made, however, of the baffling circumstances that simultaneously relate and contrast pteroylglutamic acid and the erythrocyte maturation factor. Both are present in liver, and both will produce hematologic remission of addisonian pernicious anemia. However, the processes commercially used in the refinement of concentrated solutions of the erythrocyte maturation factor do not result in the concentration of pteroylglutamic acid, in fact the original pteroylglutamic acid content of crude liver extracts is largely diverted into side fractions during the process. Experimental animals, even when they are acutely deficient in pteroylglutamic acid, do not respond to preparations of the erythrocyte maturation factor. In the treatment of pernicious anemia, the erythrocyte maturation factor is far more effective when injected than when fed to pernicious anemia patients, while pteroylglutamic acid is approximately equally effective by either route of administration. Differentiation of pteroylglutamic acid from the "extrinsic factor" appears to be established by the activity of pteroylglutamic acid when fed or injected in producing hemopoietic remission of pernicious anemia without the addition of normal human gastric juice.

The results of various clinical experiments, most of which did not permit of definite conclusions and which were made with crude materials or natural concentrates, were published prior to the availability of pteroylglutamic acid. The investigations of Wills were mentioned on p. 54; these findings served to differentiate the erythrocyte maturation factor from an unidentified factor in yeast and liver which was effective against tropical macrocytic anemia and against a corresponding anemia in monkeys. Recently this question was reopened (193) in an article which drew attention to preceding observations (194, 195, 196, 197, 198, 199, 200) that certain macrocytic anemias usually associated with pregnancy and encountered both in the tropics and the temperate zone did not respond to the injection of liver extracts which were effective against pernicious anemia but these anemias responded to the oral administration of liver or autolyzed yeast. Three cases with histories of "striking dietary inadequacy" were found to respond to oral liver extract after no response had occurred to smaller volumes of injected liver extract.

Five patients with pernicious anemia were found to respond to comparatively large doses of brewers' yeast 1 to 2 grams per kilo of body weight daily without the addition of gastric juice (201). One may speculate that this response was probably due to pteroylglutamic acid.

A concentrate of "vitamin B_c" from yeast, standardized by chick assay, was fed to ten patients with refractory macrocytic anemias. A dosage rate corresponding to 0.6 mgm. of pteroylglutamic acid daily was used for the first week,

and it was increased to 1.5 mgm. daily for the next 3 weeks. With the exception of an increase in the hematocrit reading, no significant changes were obtained in the blood picture (202).

Negative results were obtained in the treatment of 2 cases of pernicious anemia with pteroyltriglutamic acid 3.6 or 2.3 mgm. daily by mouth for 10 days (203). This may be contrasted with the subsequent report of a single case which responded to pteroyltriglutamic acid, 3 mgm. daily, by injection (204).

As a result of the preceding and other studies (16, 193, 205, 206, 207, 208, 209, 210, 211, 212, 213, 214) it became evident by 1945 that there existed a nutritional macrocytic anemia often associated with pregnancy, which responded to the feeding of crude liver extracts, yeast or yeast extracts, but which did not respond to the injection of potent sources of the anti-pernicious-anemia factor of liver extracts. The anemia was characterized in some cases (215) by a history of marked dietary inadequacy especially with respect to meat; the presence of gastric hydrochloric acid; the absence of neural manifestations; and the presence of a megaloblastic bone marrow. Maintenance therapy with liver was not required. A similar condition was encountered which responded to the injection of concentrated liver extracts (193, 194, 216, 217, 218, 219); the differentiation of this from the preceding type of anemia appeared to be possible only on the basis of response to such extracts. The time was now ripe for the appearance of a new therapeutic substance. This substance proved to be pteroylglutamic acid.

In the summer of 1945, synthetic pteroylglutamic acid was made available in fairly large quantities for clinical experiments. Results were soon obtained and reported in the literature; it was apparent that the use of adequate dosage schedules led to the establishment of positive responses to pteroylglutamic acid in the treatment of anemias which were accompanied by megaloblastic erythropoiesis.

Addisonian pernicious anemia. Descriptions of the response of this disease to pteroylglutamic acid first appeared in December, 1945 (22, 220).

The effects of pteroylglutamic acid on 14 cases of macrocytic anemia in relapse were described (22). Five of these cases were classified as addisonian pernicious anemia, five as nutritional macrocytic anemia, and two as indeterminate. The patients received pteroylglutamic acid, 20 mgm. to 50 mgm. injected or 100 mgm. by mouth daily. One patient received 150 mgm. by mouth. A feeling of subjective improvement occurred between the third and fifth days and was accompanied by an increase in appetite, which was in some cases associated with "remarkable weight gains", in most patients. The initial red blood cell count varied from 1.64 million to 2.97 million cells per cu. mm. Except in the case of one patient who did not respond, the final red cell count at 18 to 55 days varied from 2.92 to 4.29 million cells per cu. mm. Oral administration produced greater reticulocyte responses and more rapid regeneration of blood than did parenteral administration. However, the oral dosage was usually 5 times as great as the parenteral. Increases were also noted in per cent hemoglobin and in total white cell count. Reticulocyte crises were observed in from 3 to 10 days.

The administration of pteroylglutamic acid was continued for periods up to 30 days and the red cell count and the per cent hemoglobin continued to rise after the treatment was discontinued.

In the other study (220) two patients with pernicious anemia were studied. The first patient with pernicious anemia was given 100 mgm. of pteroylglutamic acid orally each day for 10 days. Her initial red blood cell level was approximately 1.2 million cells per cu. mm. On the third day of therapy she experienced a feeling of well-being and increased appetite and on the next day her reticulocytes began to rise and a peak value of 40 per cent was reached on the seventh day. Her red blood cells began to increase in number on about the seventh day and the count rose rapidly until a level slightly over 3 million was reached. The second patient responded in a similar manner.

The use of pteroylglutamic acid in a case of untreated addisonian pernicious anemia was described (221). The patient received 2.0 mg. of pteroylglutamic acid intravenously daily for 20 consecutive days. The red cell count before treatment was 1,490,000 per cu. mm. with 5.8 grams of hemoglobin. Microscopic examination of the bone marrow showed a picture typical of a deficiency of the maturation factor. After treatment "daily bone marrow examinations demonstrated a gradual megaloblastic maturation with the marrow picture approaching normal on the tenth day of therapy." The maximum reticulocyte response (26.8 per cent) was obtained on the fifteenth day of therapy, after a total cumulative dosage of 30 mgm. to see if a secondary reticulocyte rise could be induced. This was not observed. On the fortieth day of therapy the red cells reached 4,000,000 per cu. mm. and the hemoglobin 13.0 grams. There had been a definite corresponding rise in total white cells and platelets. The same publication described the effects of pteroylglutamic acid in three patients sensitive to liver extract. In each instance, the local reaction to the injection of liver extracts was extremely marked, and in no instance was there any reaction to pteroylglutamic acid greater than to normal saline.

In another report (222) hemopoietic responses to pteroylglutamic acid in 5 cases of pernicious anemia were described after daily dosage with 20 mgm. of pteroylglutamic acid intravenously or 100 mgm. by mouth. Increases in erythrocyte counts and hemoglobin levels were observed, together with reticulocyte crises ranging from 6.4 to 14.8 per cent. Positive responses to pteroylglutamic acid in the treatment of 4 cases of pernicious anemia were described (223). Results similar to the preceding were noted (23), and it was found that one patient with pernicious anemia had neurologic changes which improved with pteroylglutamic acid therapy, although complete return to normal had not been obtained at the time of writing.

The responses of 6 patients with addisonian pernicious anemia to pteroylglutamic acid were described (224). Clinical improvement and hematological remission were noted in all cases. In one case muscular weakness and loss of vibratory sense of both lower extremities were observed; these findings were unchanged after 79 days of treatment with pteroylglutamic acid, 25 mgm. to 100 mgm. daily.

Other reports (225, 226, 227) described hematological responses to pteroylglutamic acid in pernicious anemia patients.

Studies were reported (227) on the response of various macrocytic anemias. Macrocytosis alone did not necessarily imply either megaloblastic erythropoiesis in the marrow nor responsiveness to pteroylglutamic acid. It was considered justifiable to regard megaloblastic erythropoiesis as the morphologic expression of pteroylglutamic acid deficiency. The authors studied 11 cases of macrycotic anemia treated with pteroylglutamic acid. Five of these were pernicious anemia cases which responded promptly. The remaining six cases, all of which showed normoblastic marrow patterns, did not respond; these included four cases of portal cirrhosis, one of acute infectious hepatitis (Weil's disease) and one of subacute lymphatic leukemia.

Pteroyltriglutamic acid. A patient with pernicious anemia in relapse was treated with pteroyltriglutamic acid, 3 mgm. daily, by intramuscular injection for 11 days. A submaximal hemopoietic response was observed, accompanied by subjective improvement (204). It may be presumed that a more marked response could have been obtained with a higher dosage.

Thymine. Observations of the growth-promoting effects of this substance on *L. casei* and *S. fecalis* R (121, 103, 105) were followed by studies of its effect in pernicious anemia. Large amounts were necessary to produce an effect; the administration of 1 gram or less gave no response in pernicious anemia, sprue or nutritional macrocytic anemia (228). One case of pernicious anemia responded to 6 grams daily by mouth for 14 days; three cases to doses up to 3.4 grams daily for 11 days (229) and six cases to doses of 4.5 to 10.2 grams daily (230). The necessary amounts appeared to be in the neighborhood of 1000 times as great as the adequate dosage of pteroylglutamic acid. A similar ratio of effective dosage between thymine and pteroylglutamic acid was observed with the lactic acid bacteria (see p. 65).

Combined system disease. By the summer of 1946 it had been well established that pteroylglutamic acid would consistently produce a prompt and satisfactory hemopoietic response in addisonian pernicious anemia. A long period of study was necessary to compare the effects of liver extract and pteroylglutamic acid in preventing the onset of neurological signs and symptoms in pernicious anemia.

The results of a 12-month investigation of this point were reported (231). Twenty-six patients were studied, 21 of whom had pernicious anemia which had been controlled for from 2 to 17 years by injections of liver extract. Three had pernicious anemia in relapse and two had sprue which had been poorly controlled by liver extract. Liver therapy was discontinued and the patients were treated with 70 to 105 mgm. pteroylglutamic acid per week in divided doses given orally for 10 to 12 months. Most of the patients noted an increase in appetite and weight. In two instances there were moderate increases in the hematological values after 5 to 8 months which increased until there was evidence of combined system disease. The dosage of pteroylglutamic acid was increased without signs of improvement, following which refined liver extract, 5 cc. daily,

was administered and neurological improvement was reported to be observed in 10 days.

In another study (232) the following data were obtained regarding the effect of pteroylglutamic acid on 14 cases of pernicious anemia.

	GLOSSITIS	PARESTHESIA	COMBINED SCLEROSIS
Before treatment			
	7	10	2
During treatment			
Improvement	3	4	
Temporary improvement	3	4	
No improvement	1	2	2
New manifestations		2	3

The effect of pteroylglutamic acid on pernicious anemia and combined system disease was studied in a number of patients (233). Three patients with neurological involvement received pteroylglutamic acid, 25 to 50 mgm. by mouth or 20 mgm. intramuscularly daily. The patients showed hematological responses but their neurological symptoms continued to progress. Administration of liver extract resulted in improvement in the signs of nervous system changes. Other pernicious anemia patients in relapse received 0.5 unit of liver extract with 5 to 10 mgm. of pteroylglutamic acid daily and showed reticulocyte responses which were greater than those anticipated as a result of previous experience with liver alone.

Rapidly progressive neurological relapse was observed in a patient with pernicious anemia who had been maintained on pteroylglutamic acid for 12 weeks. The diet during this period was poor. The patient subsequently improved during treatment with liver extract. The patient was one of a group of 47 patients with pernicious anemia who were maintained with pteroylglutamic acid for periods up to one year. Only two others showed neurological relapse. Both of these had a poor dietary history. They showed mild symptoms which responded readily to liver extract (234).

These preliminary studies indicated that the erythrocyte maturation factor, or some unidentified substance which accompanies it in therapeutic amounts in refined liver extract, is needed for the prevention of neurological signs and symptoms in certain cases of combined system disease and that pteroylglutamic acid is not consistently effective in this regard. It would be of interest to study the effects of pteroylglutamic acid upon the incidence of combined system disease when administered together with large amounts of the known B complex vitamins.

Possible relation of pteroylglutamic acid to the biochemical defect in pernicious anemia. A disturbance in the utilization of pteroylglutamic acid appears to

occur in pernicious anemia. This is evidenced by the following considerations:

a. In contrast to certain other anemias, pernicious anemia has never been associated with an obviously incomplete diet.

b. Dosage with pteroylglutamic acid, usually in amounts which are greater than those occurring in ordinary diets, will produce a hemopoietic response in pernicious anemia.

c. Certain cases of pernicious anemia do not respond to crude concentrates of pteroylheptaglutamic acid. This finding is discussed below.

Preliminary publications speculated upon the possibility that a basic defect in pernicious anemia is a failure to liberate pteroylglutamic acid from its conjugates (235, 236). It was observed (236) that when pteroylheptaglutamic acid (vitamin B_c conjugate) was administered to normal individuals there was increased urinary excretion of pteroylglutamic acid. However, no such increase was observed when pteroylheptaglutamic acid was administered to pernicious anemia patients (235, 236). It was therefore portulated that in these patients a pteroylglutamic acid deficiency resulted from the imperfect utilization of conjugated forms of pteroylglutamic acid and that as a result the supply of pteroylglutamic acid was insufficient for the maintenance of the hemopoietic mechanism. Three patients with pernicious anemia and one with macrocytic anemia following gastrectomy showed no evidence of a therapeutic effect from the administration of pteroylheptaglutamic acid, equivalent to 2.3 to 4 mgm. of pteroylglutamic acid daily for 8 to 12 days. When equivalent amounts of pteroylglutamic acid were substituted for the conjugate all of the patients showed significant clinical and hematologic responses (235). It was reported (236) that the daily administration of 1 mgm. of "yeast conjugate", presumably pteroylheptaglutamic acid, for 10 days to a patient with pernicious anemia in relapse produced no response. During a second period of 11 days, 100 cc. of normal human gastric juice was administered with the yeast conjugate, again without response. The patient then responded submaximally to a daily dose of 0.35 mgm. of pteroylglutamic acid. A second patient received intramuscular injections of 2.5 mgm. of yeast conjugate daily for 12 days, followed by a single injection of 30 mgm. of conjugate, without responding. After a further 12 days, administration of pteroylglutamic acid causes a theoretically maximal reticulocyte response. In two patients with pernicious anemia, injection of concentrated liver extract containing insignificant amounts of pteroylglutamic acid doubled the urinary excretion of pteroylglutamic acid. This observation fitted the interpretation that a constituent of liver extract might be concerned with the activation of a "conjugase system", or with the removal of an inhibitor of such a system (237) but it should be noted that the urinary excretion of pteroylglutamic acid on a normal diet is extremely small both in normal subjects and in pernicious anemia patients, so that doubling the urinary excretion of pteroylglutamic acid is quantitatively only a small increase.

In another report (238) earlier findings (235) were extended. The heptaglutamate was given orally in the form of a concentrate to nine cases of pernicious anemia in relapse, three in remission induced by liver extract, two with macro-

cytic anemia following gastrectomy, and six healthy subjects, without effect on the anemias, all of which subsequently responded to pteroylglutamic acid. No pteroylheptaglutamic acid was found present in the urine in any of the experiments. The patients excreted variable amounts of pteroylglutamic acid following its administration while the amounts excreted by the normal subjects were "considerably higher and more constant". After the administration of pteroylheptaglutamic acid the patients with one exception showed no significant increase in excretion of pteroylglutamic acid, but when pteroylheptaglutamic was administered to the patients in remission induced by liver extract, an excretion of pteroylglutamic acid in amounts similar to those observed with healthy subjects was found. This suggested that "the principle of liver active in pernicious anemia may be concerned with the conversion of the conjugated vitamin to the free form".

More detailed findings were subsequently reported (239) and the earlier postulations regarding the function of the erythrocyte maturation factor were modified in the light of these results. Emphasis was laid upon the role of "conjugase inhibitors", as present in certain yeast concentrates, in modifying the utilization of "hexaglutamyl conjugate" (pteroylheptaglutamic acid) by pernicious anemia patients. The urinary excretion of pteroylglutamic acid after the administration of conjugate with "large amounts of inhibitor" was much less in pernicious anemia patients than in normal subjects. Two patients, one in relapse and one in partial remission, were treated with conjugate *plus* liver extract without markedly increasing the urinary pteroylglutamic acid excretion over the levels observed when the conjugate was given alone. In contrast, three patients in remission who received the conjugate excreted pteroylglutamic acid in amounts approximately equal to those observed in normal subjects. It was concluded that the diminished ability of pernicious anemia patients to utilize the conjugate was not absolute, varied in different patients, and was partly dependent upon a conjugase inhibitor present in natural materials. The effect of the inhibitor was studied in normal persons (240), and its administration was found markedly to reduce the urinary excretion of pteroylglutamic acid following the feeding of conjugate, 4 mgm. daily. Attention was drawn to the high inhibitor content of liver, yeast and spinach. The conjugate was not found present in the urine in any of the experiments.

Others have also pointed out that certain pernicious anemia patients are able to utilize concentrates of the conjugates (192). It is difficult in the present state of knowledge to generalize regarding the biochemical defects in pernicious anemia. It appears to be evident that there is an abnormality in the utilization of pteroylglutamic acid. The variability in the findings may be due to variations in the extent and type of the biochemical lesions in the patients.

Another basis for possible speculation lies in some observations of the excretion of xanthopterin (47). It was reported that the normal daily excretion of xanthopterin in the urine was increased by approximately 50 per cent in untreated pernicious anemia. The excretion dropped to normal in 11 of 13 patients after remission had been brought about by liver extract. If pteroyl-

glutamic acid is broken down to xanthopterin and is excreted as such in the urine, these observations might imply that there is an increased rate of breakdown of pteroylglutamic acid in pernicious anemia. However, two other patients in this study had extremely low urinary xanthopterin values which increased after treatment with liver extract.

Sprue. The successful treatment of sprue with pteroylglutamic acid was first reported in November 1945 (241). Two patients were treated daily with 15 mgm. of pteroylglutamic acid by intramuscular injection. The symptoms of glossitis disappeared after 4 days treatment in the first case. After nine days the reticulocytes reached a peak of 15.3 per cent and a marked increase in thrombocytes was noted. The red cell count rose from 1.56 to 3.88 cells per cu. mm. and the per cent hemoglobin rose from 6.0 to 9.5. These changes were accompanied by marked general betterment, including regeneration of the lingual papillae, subsidence of the diarrhea and considerable gain in weight. The tolerance curves for oral glucose and vitamin A became more nearly normal. The second case received similar treatment and showed a reticulocyte crisis on the 4th day accompanied by subjective improvement.

In another report (220) 20 mgm. of pteroylglutamic acid was injected intravenously each day for 10 days to a patient with sprue. Following the initial ten-day period 40 mg. was injected every other day for an additional two weeks. A reticulocyte peak of 30.2 per cent was obtained on the seventh day although the initial red count was 2.6 million cells, so that the reticulocyte response was greater than was anticipated. The patient gained 8 pounds within 3½ weeks and the oral glucose tolerance test showed much better absorption.

Three cases of "tropical" sprue were treated with 200 mgm. of pteroylglutamic acid daily by mouth (242). Subjective improvement was noted after 3 or 4 days. The erythrocyte count was 1.15 to 2.16 million cells per cu. mm. on the 10th day. Reticulocyte crisis occurred in 6 to 7 days with peak values of 17 per cent to 22 per cent. Gains in strength, vigor and appetite were noted.

Further laboratory findings regarding patients with sprue were reported (243). Three patients were studied who fulfilled all of the criteria necessary for the diagnosis of sprue including glossitis, diarrhea with increased fat content of the stools, marked loss of weight, pigmentation of the skin, macrocytic anemia, moderate leucopenia, impairment of absorption as indicated by a flat oral glucose tolerance curve with a normal intravenous tolerance curve, a flat vitamin A tolerance curve and a very low serum carotene content, and a characteristic gastrointestinal pattern on x-ray examination. Free hydrochloric acid was present in the gastric juice of each. Sternal marrow of two of the patients was examined and found to be typical of that seen in untreated sprue and pernicious anemia. After a preliminary period of observation these patients received daily intramuscular injections of 15 mgm. of pteroylglutamic acid as the sole therapeutic agent. In all three cases prompt hematological and clinical improvement was noted including disappearance of glossitis within 3 or 4 days, regeneration of the lingual papillae, an improved sense of well-being, subsidence of diarrhea, improvement in appetite and gain in weight. One patient gained 26 pounds

within 6 weeks following the institution of treatment with pteroylglutamic acid. Reticulocyte crises were noted in 6 to 8 days together with increases in the number of platelets, the number of white blood cells, the number of red blood cells and the percentage of hemoglobin. Examination of aspirated sternal marrow after treatment showed the disappearance of the more primitive red blood cells and return of the white cell series to normal proportions.

Other reports with sprue have indicated comparable findings (23).

The effect of pteroylglutamic acid on plasma tocopherol levels in sprue was reported (244). The level gradually decreased to a very low value in one patient during a relapse following the withholding of pteroylglutamic acid therapy and increased following readministration of pteroylglutamic acid. During relapse it was observed that the patient showed a maximum rise in serum concentration of tocopherols of 0.09 mgm. per 100 cc. after an oral dose of 600 mg. of mixed tocopherols in contrast to an average peak of 0.37 mgm. in seven healthy adults after a similar dose. Attention was drawn to findings elsewhere that muscle tissue at autopsy from patients with sprue showed pigment resembling that seen in vitamin E deficient animals and that tocopherol deficiency has been observed in rats on purified diets containing sulfonamides.

Findings regarding the metabolism of pteroylglutamic acid and its conjugates were obtained in studies with two sprue patients (192). The first patient had been treated irregularly and inadequately with liver extract. He responded promptly to intramuscular injections of pteroyltriglutamic acid, 4.9 mgm. daily. After 10 days, concentrated liver extract was also administered, and the patient's recovery continued uneventfully. Microbiological assays of the urine showed that the amount of pteroylglutamic acid excreted daily before the initiation of treatment was so low as to be undetectable. Following the administration of pteroyltriglutamic acid, the urinary excretion of pteroylglutamic acid rose to a maximum of 3.66 mgm. on the sixth day, then declined to 1.34 mgm. on the tenth day. Upon starting liver extract which contained insignificant amounts of pteroylglutamic acid, the excretion rose to the remarkably high level of over 7 mgs. of pteroylglutamic acid daily for two days, in spite of the fact that the daily intake of pteroyltriglutamic acid corresponded to only 3.1 mgm. of pteroylglutamic acid. This finding indicated that some factor in liver extract had a marked effect on pteroylglutamic acid metabolism in sprue. The second patient was treated orally with a concentrate of pteroylheptaglutamic acid containing 8.4 mgm. of pteroylheptaglutamic acid and about 0.3 mgm. of free pteroylglutamic acid per daily dose. Remission promptly occurred with urinary excretion of only small amounts of pteroylglutamic acid. The excretion rose to a maximum of 0.32 mgm. on the tenth day, at which point the injection of liver extract was started, following which the urinary excretion continued to increase to a maximum of 1.5 mgm. Treatment of the urine of either patient with "conjugase" indicated the absence of conjugates. The number of cases is so small that the results until confirmed should be treated with reservation in spite of their great biochemical interest.

A patient with sprue was treated with 5 mgm. of pteroyltriglutamic acid twice

daily by intramuscular injection. A total quantity of 83 mgm. was given over a period of 9 days. A reticulocyte peak of 38 per cent occurred on the fifth day of therapy and was accompanied by a rise in erythrocyte count and in hemoglobin and by clinical improvement (245).

Thymine in sprue. Clinical and hematological improvement were reported in four patients with sprue who received 15 grams of thymine daily (246). Reticulocyte peaks were reached on the eighth or ninth day, and were followed by increase in erythrocytes and hemoglobin, subjective improvement and a return of the stools toward normal. The authors observed that the response was less dramatic than that observed with pteroylglutamic acid.

Nutritional macrocytic anemia. This condition was discussed and reviewed (17). In December 1945 (22), a consistent effect of pteroylglutamic acid in producing remission of 9 cases of nutritional macrocytic anemia was described. The responses were similar to those described for pernicious anemia in the same article (see p. 82). Additional details were given in a later publication (222). Similar results were obtained elsewhere (23). "Nutritional macrocytic anemia" occurring in a patient who had previously responded to pteroylglutamic acid and who had subsequently relapsed, was found to respond to pteroylheptaglutamic acid, 14 mg. daily for 9 days (247). The clinical improvement was rapid and was comparable to that observed with pteroylglutamic acid. A case of nutritional macrocytic anemia did not respond to pteroic acid, 5 mgm. by intramuscular injection daily for 8 days. The patient subsequently responded to dosage with pteroylglutamic acid, 5 mgm. daily.

Macrocytic anemia of pregnancy. A patient was described as having a red blood cell count of 1.1 to 1.2 million red blood cells per cu. mm. 18 days after parturition. She received 20 mgm. of pteroylglutamic acid intramuscularly for each of 10 days. Her subjective improvement was marked on the third day, and a peak value of 48 per cent reticulocytes was reached on the seventh day. The red cell count rose quite rapidly (220). Another report (24) described a response to pteroylglutamic acid in what was presumably a case of macrocytic anemia of pregnancy.

Megaloblastic anemia in infancy. A series of studies (248, 249, 250) described this condition as being characterized by:

1. Normochromic anemia, usually but not invariably macrocytic
2. A tendency toward leukopenia and neutropenia
3. A diminution of platelets, often associated with an increased bleeding tendency
4. A megaloblastic bone marrow pattern resembling or identical with the pattern seen in pernicious anemia in relapse
5. A frequent incidence of splenomegaly; an evidence of infection usually present; a histamine refractory achlorhydria present but reversible.

The treatment of 29 patients was described, 12 of these received pteroylglutamic acid either synthetic or in the form of a natural concentrate prepared by eluting a charcoal adsorbate of liver extract. The dosage rate was 5 to 20 mgm. per day for 8 days to 3 weeks. Three of the patients died with severe

infections. The remaining 9 responded, showing reticulocyte peaks, a return of the bone marrow pattern to normal, and increases in hemoglobin and red cell counts. Increases in the platelet counts were noted in blood smears at around the ninth day. The effect of pteroylglutamic acid was stated to be indistinguishable from that produced by liver extract and no relapses were observed in follow-up studies which lasted up to 10 months. The etiology of the condition, which was commonly associated with coughs and coryza, was indefinite. Of interest is the observation that one child had taken nothing but goats' milk for four months. This recalls the early observations with rats (44, 46). Another early observation (251) described the response of "goats' milk anemia" in infants to liver extract and to yeast. No response was obtained to iron.

Celiac disease. The occurrence of macrocytic anemia in celiac patients has been stated to be "exceedingly rare" (252). Results with the use of pteroylglutamic acid in the U. S. A. have not been encouraging (253). However, two reports have appeared in England; in one of these (254) the case of a 17-month old boy was described. The patient was emaciated and anemic. He had responded previously to treatment with liver extract, but a second treatment was ineffective. Pteroylglutamic acid, 25 mgm. daily, was administered orally, following which there was immediate clinical improvement and a gain in weight of 13 pounds in 16 days. In the second article (255) two cases of celiac disease (infantile sprue?) were encountered with "typical clinical features" including flat glucose tolerance curves and megaloblastic bone marrow. Both cases responded to daily dosage with 5 mgm. of pteroylglutamic acid with striking clinical improvement and reticulocyte responses of more than 25 per cent.

Glossitis of pellagra. It was reported (256) that the glossitis of pellagra started to respond within two days to the daily oral administration of 10 mgm. of pteroyl glutamic acid. By 5 days the glossitis had disappeared completely in one case and in a second case there was "evidence of a great deal of healing". The patients had previously shown glossitis which had been successfully treated with niacinamide. These observations may indicate an interesting relationship between niacinamide and pteroylglutamic acid deficiencies in pellagra.

Radiation sickness. Pteroyltriglutamic acid was administered at the rate of 5 mgm. daily for 6 days to 8 patients with leukopenia resulting from local intensive x-ray treatment of carcinoma of the cervix. Elevations of the leukocyte count were noted, but the amounts of pteroyltriglutamic acid available were so limited that the results obtained were not considered to lead to definite conclusions (257). A group of patients with lymphoblastomas received radiation therapy which resulted in an aggravation of anemia and neutropenia. When a crude preparation of pteroylglutamic acid was fed by mouth in addition to the use of radiation therapy in 69 of the patients, it seemed that the deleterious and depressant effects of radiation on the bone marrow were decreased, the frequency of necessary transfusions was lessened, and the period of hospitalization was lessened (258).

Miscellaneous observations. It was noted in India that certain cases of chronic diarrhea responded to treatment with 40 to 60 mgm. of pteroylglutamic acid

daily. Therapy restored the stools to normal or approximately so within two to five days. It was suggested that in long-standing diarrhea a nutritional factor prolonged the production of abnormal stools and that pteroylglutamic acid appeared to correct this defect (259).

The finding that edema occurs in "vitamin-M" deficient monkeys (260) is paralleled by a recent case report of the disappearance of "nutritional edema" in 7 days in a one-year-old child upon treatment with pteroylglutamic acid (261).

TABLE 5

Blood dyscrasias reported not to respond to pteroylglutamic acid

CONDITION	NO. OF CASES	REFERENCE
Anemia of premature infants	18	(248)
Hypochromic macrocytic anemia	5	(248)
Chronic hypoplastic anemia	2	(248)
Mediterranean anemia	1	(248)
Subacute myelogenous anemia	1	(248)
Acute lymphatic anemia	1	(248)
Sickle cell anemia	1	(248)
Hypoplastic marrow	6	(264)
Monocytic leukemia	5	(264)
Leukopenia in virus influenza	3	(264)
Macrocytic anemia secondary to cirrhosis	1	(264)
Anemia associated with portal cirrhosis	4	(227)
Acquired hemolytic anemia	1	(264)
Chronic myelogenous leukemia	1	(264)
Chronic lymphatic leukemia	1	(264)
Aplastic anemia	3	(222)
Iron deficiency anemia	4	(222)
Anemia associated with leukemia	3	(222)
Anemia associated with Weil's disease	1	(227)
Subacute lymphatic leukemia	1	(227)
Refractory anemia following insecticide exposure*	4	(257)
Refractory anemia, other causes*	4	(257)
Leukopenia following x-ray treatment* in Hodgkin's disease	1	(257)
Leukopenia following sulfonamide treatment*	1	(257)

* Treatment with 5 mgm. pteroyltriglutamic acid daily for 6 days.

Relief of two cases of dermatitis was observed in patients who received supplementation with a crude concentrate of pteroylglutamic acid prepared from liver (262). A synthetic pteroyldiglutamic acid was found to produce remission in two relapsed cases of pernicious anemia and one case of nutritional macrocytic anemia (263). One of the cases received 20 mg. daily by mouth for 10 days.

Blood dyscrasias not responding to pteroylglutamic acid. Information regarding these is summarized in table 5.

Discussion. An attempt to define two broad groups of megaloblastic anemias

on the basis of recent results with pteroylglutamic acid is made in table 6. No clear-cut differentiation between the two groups is implied, indeed, it seems that sprue embodies certain characteristics of both groups. A metabolic defect in sprue is indicated by its tendency to relapse and by its response to refined liver extract; in one case report (192) an effect of liver extract on pteroylglutamic acid metabolism in sprue is indicated. A dietary deficiency in sprue is implied by the effects of the accompanying diarrhea and frequently by the nutritional history of the patients.

It has been pointed out that some cases of nutritional macrocytic anemia not only fail to respond to the erythrocyte maturation factor but also do not respond

TABLE 6

Differentiation of two groups of megaloblastic anemias

CHARACTERIZATION	GROUP 1	GROUP 2
Hemopoietic response to:—		
a. Erythrocyte maturation factor of concentrated injectable liver extracts	+	−
b. Lean beef + normal human gastric juice	+	−
c. Pteroylglutamic acid	+	+
Occurrence	May occur on "normal" diet	On deficient diets, usually in tropics
Suggested cause	Metabolic defect	Dietary deficiency of pteroylglutamic acid
Analogue in experimental animals	Unknown	Pteroylglutamic acid deficiency in monkeys, chickens, rats
Examples	Addisonian pernicious anemia	Tropical macrocytic anemia

to feeding lean beef with or without normal human gastric juice, as a source of the extrinsic factor (18). It has been observed that dietary pteroylglutamic acid deficiency in chicks and rats is not alleviated by lean beef (265).

The metabolic disturbance in addisonian pernicious anemia not only involves pteroylglutamic acid but in some patients also includes neurological disturbances which in certain cases may be controlled by liver extract and not by pteroylglutamic acid.

Further light on the classification of the megaloblastic anemias may result from studies in the utilization of pteroylheptaglutamic acid (p. 86). Another possibility for biochemical investigation may lie in additional studies with thymine, which in large doses has been reported to produce remission of pernicious anemia (p. 84) and sprue (p. 90).

Excretion. Only very small amounts of "folic acid" were reported to occur in human urine (266). It was found (50) that the daily urinary excretion of

L. casei factor by 15 normal individuals averaged less than 1 per cent of the probable dietary intake.

Studies of the daily urinary and fecal "folic acid" excretion were measured by assay with *S. fecalis* R (267). Seven young men on a "normal" diet served as experimental subjects. The average daily "folic acid" excretion was about 4 micrograms in the urine and 300 micrograms in the feces. The average daily dietary intake was found to be only 62 micrograms, which seems to be very low, although the diet was not described. In a further communication (268) the effect of supplementation with various vitamins, including 90 micrograms of pteroylglutamic acid daily, was measured in the case of 2 of 7 subjects on a "restricted" diet. Urinary excretion of pteroylglutamic acid was not increased by the supplementation, but the fecal excretion was possibly increased somewhat. Again, the fecal excretion exceeded the intake. The extent to which fecal pteroylglutamic acid is nutritionally available is at present unknown. The occurrence of nutritional macrocytic anemia presumably as a result of dietary pteroylglutamic acid deficiency (4, 21, 22, 23, 24) may indicate that "intestinal synthesis" is ineffective as a source of pteroylglutamic acid under certain conditions, although the production of pteroylglutamic acid deficiency in human subjects on purified diets has not been reported.

It was reported (192) that the daily urinary excretion of pteroylglutamic acid rarely exceeds 5 micrograms and is usually 2 to 4 micrograms on ordinary diets. Oral or parenteral administration of pteroylglutamic acid led within 24 hours to an excretion of 15 to 75 per cent of the amount administered depending on the size of the dose; one individual excreted 16 per cent of one mgm. intramuscular dose, while on a dosage of about 10 mgm. daily either orally or parenterally the excretion usually ranged between 35 and 50 per cent.

Doses of 5 to 16 mgm. of pteroylglutamic acid were fed to 9 normal subjects and 9 hospital patients (269). The normal subjects before receiving pteroylglutamic acid had an average daily urinary excretion of between 2 and 3 micrograms of pteroylglutamic acid. They excreted an average of 28.5 per cent of the administered dose. Most of the excretion took place the second and eighth hours after dosage. The hospital patients excreted much lower percentages of the administered dose. Oral administration of 5 mgm. of sodium pteroylglutamate was found (270) to result in excretion of between 44 and 57 per cent of the administered dose within 6 hours. After 24 hours the excretion fell to basal levels.

Pharmacology. Studies of the pharmacology of pteroylglutamic acid indicated that the substance had a low acute and chronic toxicity and showed an almost complete absence of side reactions even when the dose was far above the therapeutic range (271). Mice, rats, guinea pigs, rabbits, cats and dogs were used in the studies. Renal damage was observed in some of the animals following intravenous injection of large doses of sodium pteroylglutamate. The substance did not affect the respiration or the blood sugar and the effects on the blood pressure and the isolated intestine were of a minor order. In chronic experiments, the daily administration of 5 mgm. per kilo intraperitoneally to rabbits and

rats for two months produced no unfavorable reactions. In a similar period, daily intraperitoneal injections of 50 mgm. per kgm. to rabbits and 75 mgm. per kgm. to rats produced some changes in the tubules of the kidney, but no deaths.

The acute toxicity was low. Rats and mice tolerated approximately 200 mgm. per kgm. intravenously, with no evidence of action.

Relation of pteroylglutamic acid to an anemia produced in dogs. An anemia was reported to occur in dogs which received choline by stomach tube or acetyl choline by injection. Acetyl-choline-like activity was detected in trichloracetic acid filtrates of the serum of blood drawn from dogs one to 1½ hours after the oral administration of 200 mgm. of choline chloride. Trichloracetate was not removed from the filtrate before the acetyl choline assay was made. The activity was diminished in the serum of dogs which had previously received liver extract or pteroylglutamic acid. Cholinesterase activity of the serum was increased by incubation with pteroylglutamic acid or liver extract at 37°C. In two normal human subjects, administration of 5 to 7.5 mgm. of pteroylglutamic acid by mouth led to increases of 33 per cent and 16 per cent in their serum cholinesterase activities within 5 hours. It was concluded that liver extract and pteroylglutamic acid acted by increasing the formation of choline esterase (272). Further experiments indicated that the rate of regeneration of the cholinesterase activity of the serum of diisopropylfluorophosphate treated-dogs was increased by the administration of liver extract or pteroylglutamic acid (273). No confirmation of these results has been reported. No anemia-producing effect of choline was observed in various experiments with human subjects (274, 275, 276).

Pteroyltriglutamic acid, pteroylglutamic acid and mammary tumors in mice. A series of studies was made of the effect of intravenous injections of yeast extract on spontaneous breast tumors in mice (277, 278, 279, 280). Spontaneous tumors were studied in the earlier experiments and later (281) tumors were developed in Rockland mice by transplantation with Sarcoma 180. The mice were then used as test animals. They were kept on a normal diet and the effect of four intravenous injections over a period of 48 hours 8 days after the transplantation was judged by comparing sizes and weights of tumors in matched treated and untreated mice. Inositol alone among crystalline factors of the B complex was reported to inhibit the rate of tumor growth over a range of 50 to 250 micrograms daily when administered by intravenous injection, but subcutaneous or oral administration were ineffective (282). Inhibition of tumor growth in this test was reported to be produced by injecting a concentrate of "folic acid" 0.16 to 5.0 micrograms, or by injecting pteroyltriglutamic acid, 0.0063 to 0.40 microgram (305). Studies with spontaneous breast tumors in mice (283) showed that intravenous injections of pteroyltriglutamic acid, 5 micrograms daily, led to complete regressions of spontaneous breast cancers in mice in the case of 43 per cent of a group of 89 animals. No tumors disappeared among the 60 controls. Mice from three strains were used. In contrast, pteroylglutamic acid had no inhibitory effect and it was reported that primary tumors in mice receiving 100-microgram doses of pteroylglutamic acid intravenously grew more rapidly than the tumors of untreated controls (284).

Further experiments with the transplanted tumors were reported (285). Xanthopterin was found to be inhibitory. It was reported that leucopterin antagonized the inhibitory effect of xanthopterin. Studies of the effects of mutually competitive substances on transplanted tumors were reported (286). Inositol was an inhibitor of tumor growth and was neutralized by p-aminobenzoic

TABLE 7

Media described for the assay of pteroylglutamic acid

INGREDIENT	AMOUNTS PER 10 ml. AND BIBLIOGRAPHIC REFERENCE				
	(8)	(287)	(288)	(98)	(289)
	mgm.	*mgm.*	*mgm.*	*mgm.*	*mgm.*
Na acetate	60	60	60	20	200*
K_2HPO_4				50	
Na citrate					250†
Acid-hydrolyzed casein	50	50	50	50	50
Glucose	100	100	100	100	200
Cystine	1.0	1.0	1.0	1.0	2.0
Tryptophane	1.0	0.5	1.0	3.0	2.0
Adenine		0.1	0.05	0.1	0.1
Guanine		0.1	0.05	0.1	0.1
Uracil		0.1	0.05		0.1
Xanthine		0.1	0.05	0.1	0.1
Asparagine			2.5		1.0
Norited peptone					Present
dl-Alanine					2.0
	μg.	*μg.*	*μg.*	*μg.*	*μg.*
Thiamine		1.0	1.0	2.0	2.0
Riboflavin	1.0	2.0	2.0	2.0	2.0
Nicotinic acid	2.0	1.0	2.0	6.0	6.0
Pyridoxine		1.0	4.0	12.0	12.0
Ca pantothenate	5.0	1.0	2.0	4.0	4.0
Biotin		0.002	0.05	0.004	0.004
p-aminobenzoic acid					0.1
	ml.	*ml.*	*ml.*	*ml.*	*ml.*
Salts A‡	0.05	0.025	0.05		
Salts B§	0.05	0.025	0.05	0.05*	0.05

* For *L. casei*.

† For *S. fecalis* R.

‡ K_2HPO_4, 5 gram; KH_2PO_4, 5 gram; H_2O, 50 ml.

§ $MgSO_4,7H_2O$, 10 gram; NaCl, 0.5 gram; $FeSO_4,7H_2O$, 0.5 gram; $MnSO_4,2H_2O$, 0.337 gram; H_2O, 250 ml.

acid or pyridoxine; desthiobiotin inhibited tumor growth and was neutralized by biotin.

Microbiological assay methods. A variety of methods has been proposed (8, 186, 287, 288, 289, 98) for the microbiological assay of pteroylglutamic acid. A number of attempts to improve the basal media have been made by increasing the amounts of vitamins, amino acids and the supplementary factors, and by increasing the amounts of glucose and buffering capacity to permit a higher maximum growth. These media are described in table 7.

The two organisms which have been used for assay purposes are *S. fecalis* R and *L. casei*. *S. fecalis* has the advantage of giving more reproducible results and of requiring shorter incubation periods than with *L. casei*, but it requires approximately 5 times as much pteroylglutamic acid as *L. casei* to give half maximum growth, thus limiting the assay of very low-potency materials.

Growth with *S. fecalis* R can be measured turbidimetrically after 16 hours or after 40 hours by titrimetric methods. *L. casei* requires an additional growth stimulant to attain maximum growth during the first 16 hours. This growth stimulant, which has been termed "strepogenin" (290) is liberated from casein by tryptic digestion. The acid-hydrolyzed casein usually used as a nitrogen source in microbiological assay media is devoid of strepogenin and thus it is not possible to conduct an assay with *L. casei* in 16 hours for pteroylglutamic acid with such media. A method has been devised (291) to permit a 16-hour assay with *L. casei* by using a tryptic hydrolyzate of casein as a source of amino acids and strepogenin. There have been insufficient published reports on this method, however, to properly assess its value.

In the assay of enzymatically hydrolyzed conjugates *L. casei* will frequently give higher results than those obtained with *S. fecalis*. This is probably due to the fact that *L. casei* will respond to partially hydrolyzed conjugates which are inactive for *S. fecalis* R.

The method of choice would seem to be that of Teply and Elvehjem using either *S. fecalis* or *L. casei*. The medium has a high concentration of buffers and glucose which permits a high maximum growth and contains added quantities of *dl* alanine which has been shown to markedly stimulate growth in the presence of suboptimal amounts of pteroylglutamic acid (292).

The importance of using a pure grade of cotton for plugs is indicated by the observation (293) that nonabsorbent cotton may contain appreciable amounts of pteroylglutamic acid.

Pteroylglutamic acid is very labile at pH 7 to direct or indirect sunlight but four hours exposure to artificial light produced only 10 per cent destruction (294).

The need for appropriate enzymatic hydrolysis of conjugates to precede microbiological assay has already been discussed. In the hydrolysis of natural materials two steps probably occur, first, the release of pteroylglutamic acid or its conjugates from the tissue to give a soluble form and second, the hydrolysis of the soluble but inactive conjugates to microbiologically active compounds. It was found that while taka-diastase cannot hydrolyze all the conjugates present (295, 189) it does aid in releasing the factor from tissues (184). Autoclaving at pH 4 or with 2N potassium hydroxide has been found effective (296) in splitting a conjugate obtained from liver. The use of alkaline hydrolysis has the disadvantage that while pteroylglutamic acid is stable to alkali under anaerobic conditions, it is quickly destroyed by alkali in the presence of oxygen (163). It was also found that (163) pteroyltriglutamic acid could be hydrolyzed anaerobically by alkali but that racemization of the pteroylglutamic acid which was released occurred. A comparison was made of a variety of chemical and enzymic methods for the liberation of pteroylglutamic acid (297). Hydrolysis by auto-

claving at pH 4, by autoclaving with 0.1N potassium hydroxide, and by digestion with taka-diastase and with conjugase from chicken pancreas were compared. With certain tissues e.g. chicken kidney, taka-diastase digestion gave higher results than digestion with conjugase from chicken pancreas. However, with other products, such as yeast extract, conjugase treatment gave the highest values. These data show that there is no digestion method which will yield the highest result with all types of products. The authors (127) have found digestion with taka-diastase, followed by hydrolysis with chicken pancreas conjugase to give higher results than treatment of either enzymic preparation alone.

Assay with chicks. The assay of "vitamin B_c" with chicks on a purified diet was described (80). The chicks were placed on the basal diet until they became anemic, following which the supplements were administered. A prophylactic method has been used more recently (186, 298, 299, 300) based on growth and examination of the blood. The chick assay appears to express the total pteroylglutamic acid content of the sample including both the free and conjugated forms (186).

The use of rats and monkeys for assay animals has been described in detail (301).

Distribution of pteroylglutamic acid. A number of studies have appeared which describe the assay of foods and other natural products for pteroylglutamic acid. The interpretation of some of the early results is complicated by several observations. Pteroylglutamic acid exists naturally to a considerable extent in the form of conjugates which are inactive in the microbiological assay until treated with a specific enzyme preparation which liberates pteroylglutamic acid (185). Even when a preparation of the enzyme is used, there is no criterion for the completion of the hydrolysis, and this consideration is brought into sharp focus by the reported existence of inhibitors of the enzyme system in natural foods (240). Attention has been drawn to the growth promoting effect of thymine as a possible complicating factor in the interpretation of the assay (123). The availability of pteroylglutamic acid conjugates in the nutrition of animals appears to be good (37, 68, 70, 92) although questions have been raised as to the utilization of conjugates by certain species (43) and by pernicious anemia patients (235, 236).

The "folic acid" content of a number of natural foods was carried out by assay with *S. fecalis* R (302). Taka-diastase was used in the preparation of the extracts. This enzyme preparation has been reported not to liberate pteroylglutamic acid completely from its conjugates (36). "Folic acid" showed the greatest losses due to cooking of all the B vitamins studied; cooking losses among meats ranged from 46 per cent in halibut to 95 per cent in pork chops, and in vegetables from 69 per cent in cauliflower to 97 per cent in carrots. The authors postulated that the vitamins may become "bound" in tissues during cooking. Muscle meats ranged from 0.7 to 2.0 micrograms of "folic acid" per gram of fresh tissue, liver 3 to 6 micrograms and vegetables 0.5 to 2 micrograms. The "folic acid" content of samples of various canned foods were assayed for "folic acid" with *S. fecalis* R and *L. casei* following incubation with taka-diastase for 24 hours at 37° (303). Considerable disparity between *S. fecalis* and *L. casei* values was

encountered, for example yellow corn was found to contain 0.017 microgram per gram by *S. fecalis* R and 0.056 by *L. casei*. Most of the values obtained with canned foods were quite low, averaging less than 0.1 micrograms per gram, except for canned spinach.

Similar assay methods were applied to a study of meats (304). Muscle meats were found to contain 0.06 to 0.33 microgram per gram of fresh tissue, liver 0.4 to 1.5 micrograms and kidney 0.3 to 0.6 microgram. Cooking resulted in destruction ranging up to 92 per cent of the original content.

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CONDITIONING FACTORS IN NUTRITIONAL DISEASE[1]

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MALNUTRITION MAY BE CAUSED by a number of factors other than inadequate dietary intake. These include conditions that interfere with the absorption or utilization of nutrients, or those that increase their requirement, destruction or excretion. These "conditioning factors", as Jolliffe terms them (237), have one point in common: they increase body requirements for specific nutrients or precipitate nutritional deficiencies on diets that would otherwise be adequate were such factors not operative. The effects of "conditioning factors" on vitamin deficiencies have been the subject of earlier reviews (236, 237, 239, 391); it is the object of the present contribution to summarize their effects on other nutrients as well. The present review makes no attempt to be a complete compendium of the literature but is designed as a critical survey of pertinent material in a few main sections of the field.

Factors Interfering with Absorption. A. *Fat soluble Vitamins.* Fat is necessary for the efficient absorption of carotene and vitamin A (26). Wilson (441) found that in men on a fat-free diet the absorption of carotene was nearly halved; while Ahmad (7) observed that in animals on a fat-free diet only ten to twenty per cent of carotene was absorbed when dissolved in ethyl laurate with the percentage rising to eighty or ninety per cent when ten per cent fat was also taken. The importance of fat for the absorption of vitamin A is largely academic, for vitamin A, particularly in foods, is almost invariably found associated with fat; the small amounts of fat in highly concentrated medical preparations of vitamin A may, however, decrease their value. Rough clinical proof that fat, in normal amounts at least, aids absorption is given by the large number of investigations which show that in all diseases where the absorption of fat is impaired, absorption of vitamin A and vitamin A-active carotenoids is also impaired. These include a number of conditions consisting of celiac disease (45, 65, 211, 292), sprue (6), fibrosis and cystic disease of the pancreas (15, 146, 292), congenital atresia of the bile ducts (14, 292), intestinal obstruction, ulcerative colitis, dysentery and diarrhea. Fever also exerts an adverse effect on the absorption of vitamin A and A-active carotenoids (211, 282), an effect most probably due to impaired digestion of fats. Among twenty-nine patients with pulmonary tuberculosis, most of them exhibiting symptoms of intestinal tuberculosis as well, the absorption of vitamin A was

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found to be less efficient than in healthy individuals (47). Absorption of vitamin A was similarly impaired in persons with lambliasis or giardiasis (247).

Evidence is available indicating that carotene requires for its absorption the presence of bile in the intestinal tract. In those conditions in which bile is excluded completely or partially from the intestinal tract or whenever there is a deficiency of bile salts, bile salts must be given as a supplement in order to assure proper absorption (162). Vitamin A appears to be absorbed, however, in the absence of bile or bile salts, though absorption of it is more efficient when one or the other is present (46, 47, 162, 163, 247, 388).

Liquid paraffin seriously interferes with the absorption of carotene by dissolving it from the food in the intestine, with the result that it is excreted with the paraffin in the feces (91, 348). Curtis and Ballmer (90) have shown by careful work on man that on a high carotene diet the carotene level in the blood only rises to one-half the normal value when 20 cc. of paraffin are taken thrice daily after meals; taking it twice daily has nearly as bad an effect, and even taking it once at night has some effect. Similar results have been obtained in work on animals (116, 227). Available evidence indicates that liquid paraffin does not materially interfere with the absorption of vitamin A (16, 116).

In general, the absorption of vitamins D, E and K is believed to be influenced by the same factors that affect vitamin A. Taylor and co-workers (410) found that dogs with biliary fistulae did not absorb vitamin D when given by mouth unless bile salts were given at the same time. Other investigators, among whom is Heymans (212) have confirmed these results by very similar experiments on rats or dogs. Brinkhous and Warner (50) report that dogs with chronic biliary fistulae develop within seven to nine months both the nutritional muscular dystrophy and testicular degeneration typical of a deficiency of vitamin E. In one dog, the muscular dystrophy was arrested and improved when bile was fed by mouth. Greaves and Schmidt (164) have obtained similar results in rats. Available evidence indicates that the bile salts are also essential for the proper absorption of natural vitamin K. Greaves and Schmidt (165) made the important observation that in bile fistula rats the clotting time was markedly increased when the prothrombin level of the blood dropped to a level of 20–30 per cent of the normal. This level of prothrombin was raised by feeding 2–3 cc. of beef bile daily or massive doses of vitamin K. The best results were obtained by combining bile and vitamin K. Similar results have been observed by Smith et al. (380) in dogs, and Brinkhous and co-workers (49) in man. Synthetic vitamin K analogues, however, particularly the water-soluble ones, do not need bile salts for their absorption if given in adequate amounts (246).

Available data indicate that liquid paraffin, or mineral oil, hinders the absorption of vitamins D, E and K. Smith and Spector (381) observed that rats on a non-rachitic diet developed rickets if as much liquid paraffin was added to their food as would correspond to the amount taken by man for constipation. Five times the normal requirements of cod liver oil were required to counter-balance the effect of this amount of liquid paraffin. Similar results were obtained for vitamins E and K (10, 25, 41, 122, 228).

Although data are lacking for hypovitaminosis D and E, hypoprothrombinemia, the chief manifestation of hypovitaminosis K, has been observed for a number of gastro-intestinal diseases and subsequent to short-circuiting surgical procedures. Vitamin K deficiency may result from excessive vomiting and loss of succus entericus from a drainage tube. In conditions associated with chronic and severe diarrhea, food is hurried through the intestine, so that absorption of vitamin K, and of other vitamins for that matter, is impaired (69). Hypoprothrombinemia has been observed in cases of ulcerative colitis (35, 326), regional enteritis, intestinal obstruction, Banti's disease, gastrocolic fistula, enteroanastomosis, ileac enterostomy, intestinal neoplasm, polyposis coli, and those diseases in which fat absorption is particularly impaired, such as tropical sprue, idiopathic steatorrhoea, celiac disease, and chronic diarrheas in general.

B. *Water soluble vitamins.* Absorption of water soluble vitamins may be impaired by factors resulting in anatomic, chemical or physiologic changes in the gastro-intestinal tract (30). These changes are mediated by reduced absorbing surfaces, altered secretions and hypermotility, and further modified by various alkalis, adsorbents and lubricants introduced by mouth. Vitamin B_1 may escape absorption from the intestine in certain digestive disturbances such as vomiting, diarrhea (100), alterations of the gastro-intestinal mucosa (ulcerative colitis) (240), and defective secretion due to atrophy, inflammation, and neoplastic disease. Operations designed to short circuit the intestines (422), internal and external fistulae and strictures will also lead to diminished absorption (20, 436). Available evidence indicates achlorhydria may impair the absorption of vitamin B_1 (143, 297). Absorption may also be impaired by various adsorbents such as aluminum hydroxide and kaolin.

In general, the factors referred to above apply to other water soluble vitamins as well. Diarrhea has been a causative factor in the production of riboflavin deficiency (31, 68, 364), pellagra (11, 31, 64, 68, 389, 436), and scurvy (3, 451). Frequent reports have appeared on the development of pellagra secondary to lesions such as carcinoma, duodenal ulcer and a malfunctioning gastro-enteric anastomosis which obstruct the stomach and duodenum, and similar reports are available for internal and external fistulae (436).

It has long been known that patients suffering from gastro-intestinal diseases excrete subnormal amounts of vitamin C in their urine. This has been attributed in the past to a low intake resulting from a restricted diet, but the work of a number of investigators suggests that defective absorption may be a more important factor in some cases. Under abnormal conditions of the gastro-intestinal tract associated with abnormal bowel motility (catharsis, diarrhea, ulcerative colitis) marked losses of vitamin C taken by mouth may occur. Thus, these conditions are frequently severe enough to decrease the blood level of vitamin C as well as the excretion in the urine, even when large amounts of the vitamin are given by mouth (301). Available evidence indicates that absorption of vitamin C may be impaired in achlorhydria (5, 451).

C. *Minerals.* Absorption of calcium is governed chiefly by three factors: (1) Vitamin D, (2) the hydrogen-ion concentration within the intestine and (3)

other substances in the diet. The exact mode of action of vitamin D is not known. However, although its chief effect may be exerted in the intermediary metabolism of phosphorus and calcium and in their disposition in the bones, there is little doubt that this factor, in physiologic dosage, plays an important rôle in favoring absorption of calcium from the intestine. Factors interfering with the absorption of vitamin D may thus indirectly contribute to an impaired absorption of calcium.

Under conditions of normal gastric acidity, compounds of calcium with weak organic acids are converted into the chloride and, if retained in the stomach for a sufficient period, even the less soluble basic phosphate may go into solution. The acidity of the duodenum is of considerable importance for the adequate absorption of calcium. Normally, it ranges from pH 2.3 to 7.0; and this factor largely determines whether calcium is primarily in the form of the acid or the basic phosphate. Since the acid phosphate is the more soluble, a high acidity tends to facilitate absorption of calcium. Calcium chloride and acid phosphate are probably absorbed from the duodenum before the gastric acidity is neutralized and, subsequently, continued absorption may be favored by the formation of organic acids (carbonic and lactic) (62, 363). Factors which tend to decrease the acidity of the duodenum will tend to impair absorption of calcium. Achlorhydria and the administration of alkalinizing agents are of particular importance in this regard.

Calcium absorption may be impaired by a number of substances commonly ingested in our diet. The oxalate content of our foods is a case in point. Spinach, New Zealand spinach, Swiss chard, beet tops, lambs quarters, poke, purslane and rhubarb leaves contain, on a dry weight basis, as much as 10 per cent oxalic acid (for the most part in the form of its salts). Traces of oxalic acid are also found in many other fruits and vegatables (257). Fincke and Sherman (144) showed that when rats were fed diets containing 0.33 per cent calcium, half of which was supplied by milk and half by spinach, only 40 per cent of this calcium was deposited in the bones of the growing animals, whereas when the spinach was replaced by kale (an oxalate-free leafy green), the deposited calcium rose to 80 per cent. Kohman (258) later reported similar findings and showed further that a diet which was satisfactory in calcium for growth could lead to injury by the addition of oxalate-containing leafy greens. However, if enough calcium were added to the defective diets they again became innocuous. There seems to be no doubt that oxalates do interfere with efficient calcium utilization. Whether this interference leads to a deficiency depends upon the oxalate and calcium contents of the diet.

Phytic acid is another substance commonly present in our diet whose ingestion may interfere with the absorption of calcium. Some twenty years ago, Mellanby (293) made the important observation that the addition of certain cereals to diets low in calcium led to severe rickets in growing animals. Subsequent work (52, 201, 221, 294, 412) has demonstrated that inositol-hexaphosphoric acid (phytic acid) was the substance present in cereals responsible for this rachitogenic effect. Sodium phytate is similarly rachitogenic, although phytin (the Ca-Mg

salt) is without effect. The rachitogenic effects of phytic acid or its sodium salt are counteracted by calcium. Apparently, phytic acid interferes with the absorption of calcium, either by precipitation or by converting it into a non-ionized form which is not absorbed from the intestine. As in the case of oxalates, whether this interference leads to a deficiency depends upon the phytic acid and calcium content of the diet

Normal nutrition depends more upon an adequate intake of both calcium and phosphorus than upon the calcium/phosphorus ratio; the latter, however, acquires more significance as the absolute values for either element approach the minimum requirement levels. An excessively high phosphorus intake tends to inhibit absorption of calcium, especially if the latter is supplied in insufficent amounts. A similar inhibiting effect is apparently exerted by an excess of Mg and of K. Diets unusually high in fat may tend to inhibit absorption of calcium from the intestine, due to the formation of insoluble calcium soaps. A similar situation has been observed in celiac diseases, sprue and so-called "idiopathic" steatorrhea due to impaired intestinal absorption of fats and the resulting formation of relatively insoluble calcium soaps. Inadequate absorption may also result from protracted diarrhea, due to the rapid passage of intestinal contents through the bowel (201).

Absortion of phosphorus is diminished by factors which favor the formation of poorly soluble salts of phosphoric acid. This can be demonstrated strikingly by experimental administration of beryllium which, through the formation of insoluble beryllium phosphate, prevents absorption of phosphoric acid and results in a characteristic form of rickets (180). Similar inhibition is produced, to a much smaller degree, by an excess of other cations, such as calcium, strontium, manganese, magnesium, barium, aluminum and thallium. Waltner (429) showed that rickets could be produced in rats by the addition of soluble iron salts to a non-rachitogenic diet, a finding confirmed by Brock and Diamond (51). Severe phosphorus depletion was produced in young chicks by Deobald and Elvehjem (104) who treated their normal ration with soluble iron and aluminum salts equivalent to 0.5 and 0.75 per cent of the theoretical amount required to combine with the dietary phosphorus as $FePO_4$ and $AlPO_4$. Normal bone ash values of 50 to 60 per cent were reduced to 25 per cent. The latter investigators warned that the clinical usage of very high doses of iron in the treatment of anemia might seriously interfere with phosphorus utilization.

A number of factors may contribute to an impaired absorption of iron (186). The relatively high pH in the duodenum facilitates the formation of insoluble basic iron compounds. The alkalinity of pancreatic juice and the relative insolubility of iron salts of bile acids probably interfere with the absorption of iron. Absorption appears to be hampered by the absence of free HCl and of bile and is also influenced unfavorably by administration of alkalis. It has been shown by Brock and Diamond (51) and others that phosphates have a deleterious effect on the absorption of iron, probably because of the precipitation of ferric phosphate. Absorption of iron is probably decreased by diarrhea and inflammation of the gastro-intestinal tract, but conclusive evidence is lacking. Absorption

may also be decreased by phytic acid (278). Hahn, Bale and Whipple (187) have recently reported still another factor that may seriously interfere with iron absorption. These investigators demonstrated that inflammation following the injection of turpentine in the subcutaneous tissue of dogs markedly reduced the absorption of iron from the gastro-intestinal tract. Subsequent to abscess healing, the rate of absorption returned to normal.

D. *Miscellaneous.* Carbohydrate, protein and fat are present in the diet largely as complex foodstuffs which must be broken down to their respective monosaccharides, amino acids, and fatty acids before adequate absorption may occur. Factors interfering with digestion will tend, therefore, to impair absorption. The diarrheal diseases are a case in point. In colitis, dysentery, intestinal parasitism, intestinal tuberculosis, sprue and pellagra, food is rushed through the intestinal tract so rapidly that little time is left for digestion, solution and absorption. The digestive secretions may be altered in amount and kind. In theory, this might be expected to be a serious cause of malnutrition; in practice, however, such is not the case. In spite of the high specificity of the digestive enzymes, the body is not entirely dependent on any single source for digestion. Thus, such radical measures as complete gastrectomy, removal of the duodenum, removal of the pancreas or of parts of the small intestine do not seriously interfere with the degree of digestion. On feeding large amounts of certain foods, some derangement of digestion may be demonstrated under the foregoing circumstances, but the degree of digestion that does occur is impressive. A more serious cause of inadequate absorption would be an impairment of the absorbing surface of the gut, present in a number of conditions, including chronic ulcerative colitis, sprue, and possibly food allergy.

Digestion and absorption of protein may be impaired by a number of factors in addition to those listed above. Recent work indicates that the pH 4.2-soluble, acetone-insoluble fraction of raw soybeans has a growth-retarding effect on both chicks (191) and rats (256). Inasmuch as Ham and Sandstedt (190) and Bowman (42) demonstrated the presence of a trypsin inhibitor in the pH 4.2-soluble fraction of unheated soybean flakes, the question of the identity or non-identity of the trypsin inhibitor with the growth inhibitor reported above is very much to the fore Since both are present in the acid-soluble fraction of raw soybean protein, the two may be identical. Evidence is available that the digestibility of milk proteins may be decreased by the administration of cocoa (273, 314).

Normal absorption of carbohydrate does not occur if there is an anterior pituitary deficiency. This probably depends for the most part upon the secondary hypofunction of the thyroid gland, for the same result may be obtained after removal of the thyroid gland when the hypophysis is intact. Furthermore, the defect in absorption accompanying hypopituitarism may be relieved by the administration of thyroid extract (351). The adrenal cortex influences carbohydrate absorption through its regulation of the sodium chloride exchange in the body. The absorption of carbohydrate from the intestine is subnormal in adrenal cortical deficiency, but can be restored to normal without the use of adrenal cortical extracts if the sodium chloride of the blood is raised to normal levels by adequate salt intake (13).

Factors Causing Destruction or Inactivation of Vitamins. Several of the water soluble vitamins are readily destroyed or inactivated in the gastrointestinal tract prior to absorption. Melnick, Robinson and Field (297) have demonstrated that thiamine was stable in gastric juice over a pH range of 1.5 to 8.0 but in a more alkaline pH the vitamin was readily destroyed. When antacids were added, destruction of thiamine was complete. In patients with achlorhydria significant destruction of thiamine may occur in the gastric juice (267) or further along the gastrointestinal tract where the vitamin comes in contact with the bile or pancreatic juices (143). Melnick and co-workers (296) have shown that when an oral dose of vitamin B_1 is taken by a normal subject, a much smaller fraction is excreted than when the same dose is ingested during or just after a meal. The difference may be due to the instability of the vitamin in a more alkaline gastrointestinal tract when gastric secretion is at a minimum. Alt, Chinn and Farmer (12) obtained a 65 per cent destruction of ascorbic acid in three hours at a pH of 7.95, representing achlorhydric gastric juice. Vitamin E may be destroyed during digestion in the presence of rancid fats (284, 285, 291). Destruction of carotene has similarly been reported (374).

Certain foodstuffs contain substances that may destroy or inactivate B vitamins. A number of workers have demonstrated that carp, pike, smelt and other raw fish when mixed in the diet of foxes precipitate a condition known as Chastek paralysis (168, 169, 217, 320). The animals develop anorexia, followed in a few days by weakness, hyperesthesia, ataxia and death. At autopsy, bilaterally symmetrical lesions are found in the grey matter of the brain, mainly in the paraventricular region and similar, pathologically, to the cerebral lesions of Wernicke's hemorrhagic polio-encephalitis of man (8, 136). The condition is preventable or curable by administration of thiamine (169) and is identical to that observed in adult silver foxes fed a diet deficient in vitamin B_1 (217). Subsequent work has demonstrated that Chastek paralysis is due to a thiamine deficiency resulting from the destruction of this vitamin by a heat labile enzyme-like substance (subsequently termed "thiaminase", (295), present mainly in the viscera, head, skin and scales of certain fish (38, 105, 169, 259, 295, 366, 367, 390). Thiamine deficiency has been observed in cats fed an exclusive diet of raw carp or herring (377); and Melnick et al. (295) report a 50 per cent destruction of dietary thiamine in persons fed diets containing raw clam. Putney (336) had recently reported the presence of an anti-thiamine factor in raw meat. Still another thiamine-destroying factor has been reported by Bhagvat and Devi in certain cereals, legumes and oil seeds (39). The properties of the factor indicate that it is not an enzyme and therefore not identical to the substance responsible for Chastek paralysis. When 50 micrograms of thiamine were added to 50 cc. of a buffered (pH 5.6) 10 per cent suspension of rice polishings, ragi, wheat germ, cottonseed, linseed, and certain other seeds, as much as 90 per cent of the thiamine was destroyed overnight. In certain cases, after only seven minutes of treatment, as much as 75 per cent of added thiamine had disappeared. Thiamine deficiency may also be induced by the feeding of live yeast (318). In the latter instance, however, the effect is believed due to the withholding of the vitamin from absorption rather than direct destruction.

Certain strains of bacteria appear to be able to decompose vitamin C in vitro, but whether they exert this effect in the intestine is open to question. Stepp (394) and Einhauser (121) have shown that strains of *E. coli* and *B. paratyphosus* were capable of destroying vitamin C. Kendall and Chinn (249) found that certain bacteria. isolated from the stomach and intestinal contents, were able to ferment the vitamin; and Young et al. (455, 456) demonstrated that numerous strains of enteric organisms, including *E. coli*, *Salmonella*, *Eberthella*, *intestinal streptococci*, *vibrios*, and *Proteus morgagni* could decompose vitamin C. While normally this may be of no significance, in some pathological conditions it might account for a clinical deficiency of vitamin C, even in cases where intake is adequate. Thus cases of clinical scurvy have been reported which failed to respond to oral ascorbic acid therapy, although recovery was rapid following parenteral administration.

Biotin deficiency is another condition that may be induced by the ingestion of certain foods. Raw egg white contains a substance capable of combining stoichiometrically with biotin and rendering it unavailable to the organism, presumably by preventing its absorption from the gastro-intestinal tract (183). The isolation and properties of this material, a protein subsequently termed avidin, has been described (448), and its crystallization and analysis effected (330). Avidin is denatured by heat and its ability to bind biotin lost. This explains why raw egg white inactivates biotin physiologically, but cooked egg white does not. Curiously enough, the avidin-biotin complex cannot be broken down by the gastro-intestinal enzymes, yet it can be utilized if injected (182). There is some evidence that biotin can also be inactivated by rancid fats (328).

Factors Interfering with Utilization. As pointed out by Kruse (260) malnutrition denotes a deficiency of essential nutrients in the tissue cells rather than in the diet. Any factor interfering, therefore, with the utilization of an essential nutrient may precipitate a nutritional deficiency despite the composition of the diet or the adequacy of digestion or absorption. The subject has been extensively reviewed by Jolliffe (237) and more recently by Hickman and Harris (213). It is obvious that before a nutrient can be employed by the body it must be transported from the intestinal wall subsequent to absorption to the functional body sites. This journey requires a carrier. For the fat-soluble vitamins, however, the carrier must be a specialized adjunct of the vitamin and necessary for its proper distribution— for instance, a suspended lipide or a phospholipide or protein complex. In the absence of sufficient lipide carrier according to Hickman and Harris, the *efficiency of conveyance* may be depressed (213). Failure of utilization may also result from an interference with the *mechanism of acceptance* (213). Certain tissues have an affinity for specific nutrients and can *accept* or concentrate them from blood or lymph prior to use. The concentration of iodine by the thyroid gland is a case in point (194, 210). Further examples may include the concentration of vitamin C by the adrenals (453), of vitamin A by the liver (426), of vitamin E by heart muscle and mammary gland (290) or of riboflavin by liver and kidney (424). If the mechanism of acceptance is faulty, the efficiency of utilization will be impaired.

Failure of utilization may also result from inadequate conversion of essential nutrients into active physiological components, in which form they participate in body metabolism. Thus carotene must be converted into vitamin A, thiamine to cocarboxylase, riboflavin to flavoprotein, and nicotinic acid to coenzymes I or II. Since the liver is considered to be the principal organ in which these conversions occur, hepatic dysfunction due to cirrhosis or other conditions may manifest itself as a nutritional deficiency of the tissue cell. The high frequency of nutritional disease in alcoholics may be due in part to hepatic dysfunction in such cases. A tissue deficiency of vitamin A has been reported in liver cirrhosis (188, 337, 338), diabetes mellitus (44) and obstructive jaundice (396), presumably due to impaired conversion of carotene to vitamin A; and similar effects have been observed in hypothyroidism (263, 442). According to Wendt (430), thyroid is necessary for the conversion of carotene to vitamin A and for the storage of vitamin A in the liver, a finding in agreement with the observations of Wohl and Feldman (442). Deficiency of protein is another factor that may contribute to poor utilization of nutrients. Trufanov (420) found that synthesis of flavin-adenine-dinucleotide failed to occur in the liver and tissues of rats fed for 50 days on a low-protein diet, although when an adequate diet was subsequently fed, a marked synthesis of dinucleotide took place both in liver and muscle tissue. Therapy with sulphonamides and other drugs may also interfere with utilization of B vitamins. West (432) has shown that sulphapyridine inhibits the activity of nicotinic acid in the dog, although similar results were not obtained in man (237). Evidence is available indicating that radiation sickness may be due to failure of coenzyme formation from thiamine (32, 226, 288, 428) or nicotinic acid (32).

A number of workers have demonstrated that calcium utilization may be adversely affected by the ingestion of large amounts of magnesium. As far back as 1905 Malcolm observed an increased calcium excretion in dogs fed magnesium chloride. Eight years later, Hart and Steenbock found that magnesium chloride and magnesium sulfate fed to swine caused an increased excretion of urinary but not fecal calcium. Soluble phosphates fed with the magnesium, however, decreased the loss of urinary calcium with scarcely any increase in the fecal fraction. Similar results were found in cattle by Palmer et al. (327). Cunningham (89) observed a reduction in bone calcium of rats fed magnesium as the sulfate or carbonate salt, a finding in agreement with that of Buckner et al. in chicks. These findings did not occur when magnesium phosphate was the salt administered (55). Evidence is available indicating that the utilization of calcium as well as phosphorus and protein may be impaired by the ingestion of certain brands of cocoa (309, 314).

Administration of inhibitory structural analogues of the various vitamins constitutes another factor that may impair the utilization of nutrients with a resultant deficiency in the tissue cell. This deficiency may occur in spite of the level of vitamins fed provided, of course, sufficient amounts of analogue are administered. The subject has been extensively reviewed by Roblin (346) and Woolley (445). The most popular hypothesis to explain why analogues exhibit

competitive antagonism with an essential metabolite is that the two compounds vie for space on a portion of an enzyme normally engaged with the metabolite. If the concentration of the inhibitor is great enough in comparison to that of the metabolite, the latter is displaced, and the biological system is thereby deprived of it. The net result is the precipitation of a deficiency characteristic of the structurally related metabolite and reversible by the administration of adequate amounts of the appropriate vitamin. To date, inhibitory structural analogues have been synthesized for thiamine (387, 450), riboflavin (125, 126, 262, 443), nicotinic acid (444, 449), pantothenic acid (261, 281, 383, 384, 446), biotin (106, 128, 272), p-aminobenzoic acid (206, 346), ascorbic acid (447) and vitamin K (298, 325).

Factors that Increase Excretion. A number of factors such as polyuria, lactation, excessive perspiration or administration of therapeutic agents may contribute toward malnutrition by increasing the elimination of essential nutrients from the body. Although such factors are unlikely to be the primary cause of tissue deficiency, they may nevertheless accentuate existing deficiencies or speed up the appearance of symptoms on an inadequate diet. Cowgill et al. (81, 83) have shown that, on a restricted thiamine intake, symptoms of deficiency appeared earlier in dogs receiving a forced fluid intake than in animals permitted to drink *ad libitum*. The forcing of fluids for prolonged periods of time in certain urinary tract infections or the loss of fluids by diuresis in anasarca may have a similar effect in man. Polyuria as present in diabetes mellitus or diabetes insipidus may also result in increased excretion of thiamine and other water soluble nutrients (237). Lactation may seriously deplete body stores of essential nutrients. Loss of calcium is of particular importance in this regard, (225, 286) although a significant loss of B vitamins (137, 398), ascorbic acid (28, 150) and protein (283) also occurs. Data are available indicating that excessive perspiration may result in appreciable losses of thiamine from the body, particularly under tropical conditions or in subjects doing heavy manual work in a hot environment (78). Other investigators, however, have found that the thiamine, riboflavin, pantothenic acid, nicotinic acid and ascorbic acid content of sweat was virtually negligible (197, 233, 302, 360, 413, 452). A significant excretion of sodium chloride may occur as a result of excessive perspiration (406).

Various drugs commonly employed as therapeutic agents may increase the excretion of vitamin C. Salicylates, atropine, aspirin, cincophen, barbiturates, amidopyrine, antipyrine, adrenaline, chloroform, chloretone, paraldehyde, the commoner anesthetics, stilbestrol, estradiol, and sulfonamides all increase excretion of vitamin C (37, 138, 218, 242, 248, 275, 303, 304, 341, 353, 370, 402). Certain foodstuffs have a similar effect. Basu and Ray (27) report that ingestion of cabbage extract resulted in a vitamin C excretion temporarily greater than the intake; and Musulin et al. (317) found that oats and certain fractions of halibut liver oil exerted a similar effect. According to Selkurt et al. (370) the increased excretion of vitamin C, at least that following administration of estrogens, was due to changes in renal tubular absorption. Increased urinary excretion of other vitamins has also been reported. The excretion of thiamine is consider-

ably increased by the administration of mercurial diuretics such as mercurophylline (439), an increase that is not due entirely to increased diuresis, since the concentration of thiamine in the urine is several times greater than normal. Over short periods salicylates may also increase urinary thiamine excretion, although output is subsequently reduced (71). Sure (399) has reported an increased urinary excretion of riboflavin in the thiamine deficient rat. An increase of urinary riboflavin has also been observed in experimental hyperthyroidism (400). Significant losses of vitamin A may also occur. Vitamin A is never excreted by the kidneys of man in good health, except possibly as a breakdown product (415), but it may appear in the urine during illness, being found frequently and in the highest concentrations in pneumonia. A daily output of 3,200 I.U. has been recorded, which ceased abruptly with the crisis (265). In chronic nephritis vitamin A is common in the urine, though in smaller amounts than in pneumonia. Still smaller amounts have been reported in chronic infections, rheumatic fever, skin diseases, pernicious anemia, asthma, cancer and normal pregnancy (265). The effects of magnesium salts on calcium excretion have already been reviewed. A significant loss of calcium may occur in hyperthyroidism and hyperparathyroidism. An increase in the urinary excretion of calcium and phosphorus has been reported following fracture (222).

Excessive loss of protein may occur in a variety of conditions: for example, after hemorrhage (56, 127, 311), operations and injuries (53, 93, 94, 223, 224, 315), gastro-intestinal obstruction (73, 184, 282) or burns (74, 87, 408, 409). Diseases characterized by chronic bleeding (ulcers, intestinal carcinomas, uterine fibroids, etc.) may precipitate hypoproteinemia by depletion of plasma proteins through prolonged blood loss. Excessive protein loss through proteinuria resulting from kidney disease (139, 250, 268, 332) or other causes (63) exacts a severe toll upon plasma proteins and may deplete body protein reserves. Injury to blood vessels, through mechanical or chemical means, or as a result of severe burns, may so alter cell permeability as to permit loss of blood proteins through exudation (79, 216, 276). In the same manner continuous drainage of pus (as in peritonitis, osteomyelitis, etc.) represents an additional pathway for the loss of protein material (333). Damage resulting from surgery may cause not only alterations in cell permeability, permitting leakage of proteins (80), but actual breakdown of tissue protein, as well as severe hemorrhage, thereby contributing significantly to protein loss. Loss of nitrogen resulting from breakdown of body protein is particularly marked in patients with fractures (93, 94, 223, 224), meningococcus meningitis (178), or during the febrile stage of most acute infections. Interestingly enough, in such cases negative nitrogen balances are not appreciably modified by increasing dietary protein nor by supplementing diets with intravenous injections of protein hydrolysates (333). Amino acids may be lost to the body in the form of proteoses or closely related substances which are frequently found in the urine in certain febrile disorders, particularly pneumonia, diphtheria and pulmonary tuberculosis, as well as in peptic ulcer, carcinoma and osteomalacia (63). An increase in the amino acid content of the urine may occur in conditions in which hepatic function is impaired or those characterized by extensive tissue

autolysis. High figures are obtained in acute yellow atrophy of the liver, eclampsia, and in chloroform, phosphorus, cincophen, arsenic and carbon tetrachloride poisoning. Under such circumstances, leucine, tyrosine, glycine, arginine, phenylalanine and other amino acids may appear in the urine in large quantities. Urinary amino acids are also increased in conditions associated with excessive tissue wasting as in protracted fevers (typhoid) (63). In addition to the general loss of amino acids reported above, specific amino acids, or products of their metabolism, may appear in the urine in certain pathologic conditions. Tyrosine, for example, may be excreted in the urine in amounts of 0.9 to 2.0 grams daily in cases of acute hepatic necrosis (271). Tyrosinuria may occur in the presence of extrahepatic foci of autolysis, as in degenerating lung tumors or extensive sloughing of the skin. To a lesser extent it may occur in subacute atrophy of the liver, toxic hepatitis, and, on occasion, in prolonged calculous obstructive jaundice (63). Tyrosinuria may occur as an "inborn error of metabolism" (151). Other "inborn errors of metabolism" include cystinuria, in which 0.4 to 1.0 gram daily of cystine may be lost in the urine and alkaptonuria, characterized by the urinary excretion of homogentisic acid, due to an abnormality in the intermediary metabolism of phenylalanine and tyrosine. Homogentisic acid has also been found in the urine of normal subjects given tyrosine and a diet deficient in vitamin C, disappearing after administration of the vitamin. The latter, however, has no effect upon clinical alkaptonuria (365, 368). Phenylpyruvic acid has been found in the urine of certain patients with mental deficiency and manifestations of extrapyramidal system disturbance (147, 229). The condition is apparently due to impairment of the metabolism of phenylalanine. P-hydroxyphenyllactic acid and p-hydroxyphenylpyruvic acids have been found in the urine of premature infants fed diets of vitamin C-free cow's milk containing 5 grams or more of protein per kilogram. These substances disappeared after administration of vitamin C (269). Histidinuria has been observed both in males and females, in health and disease (421). The condition is particularly pronounced during pregnancy, where it appears about the fifth week of gestation, coincident with the appearance of urinary prolan, and disappears rapidly following parturition (244, 245, 425).

Factors Increasing Body Requirements (Stress Factors). Body requirements for essential nutrients may be increased by physical exertion, fever, drugs, toxins, abnormal environmental conditions, pregnancy, lactation, hyperthyroidism, and related conditions. These constitute what are referred to as "stress factors" in the present review and may be defined as any condition resulting in an increased metabolic requirement for an essential nutrient on the part of a tissue cell. Such nutrients may be employed for purposes of detoxification or they may be used in the increased metabolism of specific tissues. The net result, however, is that nutritional requirements are increased beyond the usual or average range.

A. *Increased metabolism.* Evidence is available indicating that the requirement for many nutritive essentials is proportional to the metabolic rate. Accordingly, body requirements may be elevated in conditions with an increased B.M.R. such as hyperthyroidism, toxic adenomata and malignancy of the thyroid gland,

diabetes insipidus, fever, cardiorenal disease with dyspnea, severe anemias, diabetic pseudodwarfism, leukemia and polycythemia. Considerable work has been reported on the increased requirements of pyrexia, particularly for vitamin C. Clinically diminished urinary excretion and reduction in the blood level of vitamin C occurs in a number of infections, including pneumonia (339), diphtheria (264, 323), osteomyelitis (1), rheumatic fever (2, 345), rheumatoid arthritis (189, 343, 344), tuberculosis (230, 403) and others (140, 141, 198). Faulkner and Taylor (140, 141) found that whereas the average vitamin C content of the blood serum of healthy persons averaged 1.31 mgm. per 100 cc.; patients with infectious diseases had values as a rule fifty per cent lower or about 0.65 mgm. per 100 cc. Examination of the vitamin C reserves of the body (blood, urine, saliva, cerebrospinal fluid) revealed that in pulmonary tuberculosis, pneumonia, diphtheria, and febrile diseases generally the concentration of vitamin C was low as compared with normal standards (22, 23, 57, 58, 203, 205). Similarly, a comparison of the vitamin C content of the organs of experimental animals suffering from infections such as diphtheria and tuberculosis with those of healthy controls revealed that vitamin C stores were significantly reduced in the infected animals (156, 198, 417). The question naturally arises as to whether the increased utilization of vitamin C in febrile diseases is due to elevation of body temperature or to the infectious process *per se*. Reductions in the serum level of vitamin C in artificial pyrexia suggest that elevation of body temperature was the responsible agent (19, 102, 107, 204, 457), presumably by increasing metabolism (fever increases basal metabolism by approximately 7.2 per cent for each degree F.) (112). Osborne and Farmer (324), however, failed to confirm these findings. They found no significant change in the vitamin C blood level before or during pyrexia in patients submitting to temperatures of 102 degrees F. in fever cabinets. Abt and co-workers (4) also concluded from studies on scarlet fever, rheumatic infections and diphtheria that pyrexia alone does not affect the vitamin C reserves or utilization. Reduction of the serum level of vitamin A in febrile states has been observed during infection and artificially produced pyrexia (48, 70, 388, 414). Available data indicate, however, that such effects are more likely due to impaired liberation of vitamin A from the liver to the blood than increased utilization. May et al. (292) report a spontaneous rise in the vitamin A level of the blood after fever abatement without any vitamin A being taken; and similar results have been observed for typhus fever (142), rheumatic fever (371), and pneumonia (241). The effects of fever in preventing absorption of vitamin A and A-active carotenoids have previously been reviewed (70, 211). Schaefer (361) has reported an increased iron requirement during the febrile stage of infection in children, but whether a similar effect would occur in artificial pyrexia has not been determined.

Evidence is available that strenuous physical exertion, particularly if continued for prolonged periods of time, will increase body requirements for various nutrients. The primary need is for more calories, a point recognized since antiquity but placed on an exact basis by the work of Atwater and Benedict (21) and others (34, 112, 379). Christensen et al. (67) have found that during caloric restriction

(fasting) pronounced physical exertion may result in considerable deterioration in a period as short as two days. Under such circumstances even a single meal containing only a fraction of the day's requirements will result in significant improvement (148). The marked increase of metabolism during strenuous physical exertion (112) and the consequent ingestion of larger amounts of food both serve to increase body requirements for vitamin B_1 (81). In this regard, Cowgill et al. (84) have pointed out that the length of time required for dogs to develop anorexia on a vitamin B deficient diet was one-third to one-half less in dogs permitted to exercise each day than in those who were not; and Guerrant and Dutcher (179) have similarly observed an increased thiamine requirement subsequent to exercise in the rat. Circumstantial evidence indicates that a similar relationship pertains to man. Thus Egan (119) reports that in an epidemic of beriberi on shipboard, the stokers were affected more seriously than the sailors in the ratio of 34 to 3, an effect that may have been due to the greater physical exertion of the former and the resulting higher food consumption. Similarly in the literature of beriberi, epidemics in Japan, Burma, Siam and elsewhere a great preponderance of beriberi is noted in males. This, again, may be due in part to the harder physical work to which men are customarily subjected. The high incidence of thiamine deficiency observed in the past in mental hospitals among patients with delirium or mania may have been due in part to a greater requirement, resulting from increased psychomotor activity and a corresponding elevated metabolic rate (437, 438). Recently, the subject has been attacked experimentally in man. Egana et al. (120), Johnson et al. (234) and others (18, 24, 438) report that severe physical exertion on a diet deficient in the B vitamins greatly hastened the onset of symptoms. Physical deterioration, according to Johnson et al. (234) occurred within one week and could be prevented or cured by the entire B complex containing 0.6 mgm. of thiamine daily, but not by 2.0 mgm. of thiamine hydrochloride alone, suggesting an increased requirement not only for thiamine hydrochloride but other B complex factors as well. Diametrically opposite results were obtained, however, by Keys et al. (251, 252, 253) who, in the most careful study of the subject so far reported, observed no evidence of physical or psychologic deterioration after fourteen days on a diet containing as little as 0.16 mgm. of thiamine, 0.15 mgm. of riboflavin, and 1.8 mgm. of niacin per 1000 calories. These results, according to Keys (251), "completely negate the claims that important effects on work performance occur in a few days or weeks when normal young men do very hard work while subsisting on a diet severely restricted in the vitamins of the B complex." Similar experiments extending for longer periods of time are necessary, however, to establish the effects of physical activity on thiamine requirements and to determine to what extent strenuous physical exertion will hasten the onset of symptoms on a diet deficient in the B vitamins. Available data indicate that physical exertion does not significantly increase body requirements for vitamin C. Johnson et al. (235) observed no adverse effects in subjects engaged in manual labor on a vitamin C free diet during a period of eight weeks. Similar results were obtained by Crandon et al. (85, 86) and others (342) for even longer periods, in spite of a blood plasma virtually free of ascorbic acid

after the sixth week of feeding. Recent experiments by Darling et al. (101) and others (335) confirm the earlier findings of Chittenden (66) that physical exertion does not increase the protein requirement of man.

Requirements for essential nutrients may be significantly increased during hyperthyroidism both in experimental animals and man. This may occur in exophthalmic goiter, toxic adenoma or malignancy of the thyroid gland or from the administration of thyroxin, desiccated thyroid or iodinated casein. These requirements are due, in part, to an increased metabolism and food consumption; but they also reflect a specific response to the thyroid hormone *per se*. The subject has been extensively reviewed, with particular emphasis on vitamin requirements, in a most thorough manner by Drill (109). An increased requirement for calcium, protein, and vitamins A and C was observed during hyperthyroidism, both in experimental animals and man; but the B vitamins apparently were most affected (109). As early as 1932 Himwich et al. (215) found that the "vitamin B" requirement was increased in the hyperthyroid dog, and similar results were soon obtained for other species (82, 108, 401). In 1938 Drill and co-workers initiated a series of experiments on the effects of vitamin B_1 and yeast on adult hyperthyroid rats that had lost weight. They found that further loss in weight could be prevented by vitamin B_1 alone, but that rats did not regain their initial weight unless a rich source of the vitamin B complex was also supplied (108, 111). Subsequent work has demonstrated that pyridoxine and calcium pantothenate could effectively replace the vitamin B complex in the diet of the hyperthyroid rat (110). Thus, in addition to vitamin B_1 both pyridoxine and pantothenic acid are required in increased amounts during experimental hyperthyroidism in the rat.

B. *Pregnancy and lactation.* Pregnancy and lactation constitute two additional stress factors which may significantly increase body requirements for essential nutrients. In part, these requirements may be due to increased metabolism; they also reflect, however, an increased utilization in the formation of fetal or placental tissue and increased secretion in the form of milk. During the early period of pregnancy the basal metabolic rate is maintained within normal limits. During the later months, however, the basal metabolism rises and at term may reach an average value of plus 30 (280). Some observers believe this increase can be entirely accounted for by the metabolism of the fetus; others maintain, however, it is due in part to factors resident in the maternal organism in the nature perhaps of increased thyroid or pituitary activity (92, 129). During lactation, even higher values are obtained, due most likely to the increased activity of the secreting mammary glands. The increased metabolic rate observed above is correlated with a corresponding increase in thiamine requirement. Toverud (418) found that 46 per cent of a group of 114 pregnant women excreted practically no vitamin B_1 in their urine, and that a daily dose of 4 to 5 mgm. of thiamine was necessary to obtain an excretion corresponding to normal nonpregnant controls. Lockhart et al. (274) observed that three times as much vitamin B_1 were required to produce a urinary excretion peak in pregnant and nursing women as were required in normal controls. In agreement with the

above, numerous reports are available on the increased incidence and severity of polyneuritis during pregnancy and lactation in man and its successful treatment with vitamin B_1 (9, 319, 440). Evans and Burr (137) found that the nursing rat required five times its usual amount of vitamin B. Available data indicate that riboflavin requirements are also increased during pregnancy in man (43, 114).

All the evidence points to an increased requirement for vitamin C in pregnancy and lactation. Blood levels of vitamin C fall progressively throughout pregnancy (61, 123, 385, 407, 411). Elmby and Becker-Christensen (123, 124) found that the serum ascorbic acid level in non-pregnant women was double that of pregnant women of the same social class. Bucher (339) asserts that pregnancy causes a four to six-fold increase in requirements for vitamin C. Other workers are more conservative but agree requirements are increased by at least 100 per cent (149, 322, 418). During lactation, vitamin C is required not only for the needs of the mother but for those of the child, as well. Baumann and Rappolt (28, 29) have calculated that a nursing mother secreting 800 cc. of milk a day, containing at least 5 mgm. of vitamin C per 100 cc., requires a minimum of 50 mgm. of vitamin C beyond the normal daily requirements. Other workers have arrived at substantially the same figure (123, 418, 435). Protein and calcium requirements are also increased during pregnancy and lactation in man (117, 152).

C. *Detoxification.* In recent years considerable work has been published concerning the rôle of amino acids and other nutrients in detoxification. By feeding substances which are "detoxified", deficiencies of glycine, methionine and other nutrients may be produced, correctable by the administration of increased amounts of the appropriate nutrient. If benzoic acid or one of its salts is administered, either parenterally or by mouth, only a part of it is excreted as benzoate, the major portion being converted to hippuric acid, in which form it may be recovered from the urine. Since glycine is considered a dispensable amino acid and can readily be synthesized from other components of the diet, it was not regarded as a nutritive essential and hence a glycine deficiency was not considered probable, at least for the rat. Marked inhibition of growth has been observed, however, in rats fed benzoic acid or its sodium salt on a low protein diet, suggesting that glycine requirements for detoxification were not being met and that animals were suffering from a "glycine deficiency". Removal of benzoic acid or sodium benzoate from the experimental ration or the administration of increased amounts of glycine restored normal growth (175, 433). Inhibition of growth has been observed following administration of bromobenzene to rats, presumably due to a cystine deficiency resulting from the excretion of mercapturic acid (392, 434). Cysteine, methionine and glutathione were all effective in restoring normal growth (392). Growth was similarly retarded following the feeding of biphenyl or chrysene and restored to normal on the addition of methionine or cysteine (431). A deficiency of labile methyl groups has been reported in rats fed diets containing 1 per cent nicotinic acid or its amide. The attendant growth retardation was alleviated by methionine, choline plus homocystine or choline plus cystine, but not by choline, cystine or homocystine alone (195, 393).

Requirements for methionine and other nutrients may be significantly increased following exposure to various drugs, chemicals and other toxic compounds. High protein diets have been shown to exert a beneficial influence when administered to experimental animals exposed to chloroform, carbon tetrachloride or benzene, or when administered to animals given toxic amounts of selenium, trinitrotoluene, arsphenamine or mapharsen (154, 181, 299, 362, 382, 386). Following the demonstration of the protective action of protein, there was an intensive search for the fraction or fractions chiefly responsible for it. Methionine, and to some degree cystine, proved to be most responsible for the above effects. Miller et al. (303) found that methionine, and to a lesser extent, cystine, protected against chloroform poisoning in the protein depleted dog. The beneficial effect of methionine has similarly been observed following administration of mapharsen or exposure to carbon tetrachloride in dogs (155, 307), 1,2-dichloroethane in rats and mice (208, 209) and trinitrotoluene and carbon tetrachloride in man (33, 118). The available data indicate that requirements for methionine and other amino acids may be significantly increased following exposure to various noxious agents. The increased requirements are due to the use of these amino acids for detoxification. On a low protein diet, insufficient amounts of such amino acids may be present to meet the requirements for maintenance plus detoxification with the result that an amino acid deficiency may develop. The effects of such a deficiency will be particularly marked in the liver cell, since the liver is the organ where detoxification primarily occurs. The hepatotoxic effects of chloroform, carbon tetrachloride and other toxic substances may be explained therefore, at least in part, on the basis of a methionine deficiency in the liver cell. Particularly pertinent in this regard is the similarity of symptoms resulting from a dietary deficiency of methionine with that obtained on the administration of noxious substances requiring methionine for detoxification. It appears further that the body will employ nutrients preferentially for detoxification, even at the expense of sacrificing its own tissues to obtain the required material. A recent report by Croft and Peters (87) is pertinent in this regard. It has been demonstrated that the excessive urinary loss of nitrogen following severe burns could be checked both in the human (409) and the rat (87) by the ingestion of large amounts of protein. Croft and Peters found that methionine was equally effective in the rat (87). Such data suggest that following a severe burn, methionine is required in increased amounts, at least by the rat; that insufficient amounts of this amino acid may be available from dietary sources, and that body tissue will be broken down to meet the increased demand. Increased urinary nitrogen is explained on the basis of deaminization of excess amino acids and the resulting rise in urinary nitrogen excretion.

Detoxification may similarly increase body requirements for ascorbic acid. An increased requirement for this vitamin has been observed following the administration of toxic doses of l-tyrosine (368), gold (96, 354, 369), lead (219, 220, 287) and arsenic compounds (59, 75, 76, 77, 95, 96, 397, 423), benzene (185, 270, 300), phosphorus (352), trichlorethylene, T.N.T. (99), barbiturates (167), anesthetics (115, 266), sulphonamides (40, 97, 98), hydrazine (36) and various other

compounds. It appears that ascorbic acid combines with at least some of these substances and is excreted in such combination in the urine. Similar detoxification effects are observed toward diphtheria toxins (254, 375, 376), tuberculosis (231, 349), and many other infectious diseases (331). Guinea pigs which died from diphtheria intoxications showed reduced vitamin C content of the adrenals (196, 243), whereas no significant differences were found between the vitamin C content of the adrenals of animals injected with a sublethal dose of diphtheria toxins and those of normal animals (417). Bacterial toxins can cause a decrease of as much as 50 to 85 per cent of the normal vitamin C content of the adrenals (198, 277, 416).

Requirements for other nutrients may similarly be increased following the administration of various drugs, toxins, poisons and related substances. Higgins (214) found that the toxic effects of orally administered promin (sodium p,p′ -diaminodiphenyl sulfone -N,N′ -didextrose sulfonate) were counteracted by increased amounts of thiamine, riboflavin and pyridoxine. Fishman and Artom (145) observed that the toxic effects of dl-serine were modified by ingestion of pyridoxine. Sandground (355, 357) and Sandground and Hamilton (358, 359) showed that p-aminobenzoic acid was highly effective as a detoxicant for lethal doses of phenyl arsenates such as "Tryparsamide", arsanilic acid, carbarsone and acetarsone; and virtually the same results have been obtained with the pentavalent antimonial compound "Stibosan" (sodium-m-chloro-p-acetylaminophenyl stibonate) (199, 356). P-aminobenzoic acid was found to alleviate the symptoms of hydroquinone poisoning (289) and to reduce, at least in rats, the toxicity of neoarsphenamine (279). More recently, p-aminobenzoic acid has been shown to be of value in the treatment of experimental tsutsugamushi disease (scrub typhus) (316, 386), murine (170, 171, 193) and epidemic typhus (193) and Rocky Mountain spotted fever (17, 192). Clinical trial of p-aminobenzoic acid has demonstrated it to be of value in the treatment of louse-bourne typhus fever (454) and apparently Rocky Mountain spotted fever as well (347). Vitamin K therapy will raise the lowered prothrombin level due to sulphonamide (72), aspirin and salicylate therapy (372), dicumarol (88, 103, 373) or quinine sulfate (334). Iodide will protect against poisoning by thiourea (172) or ANTU (alpha-napthylthiourea (60)). Selenium poisoning was prevented by the administration of arsenic salts (113, 313).

Data are lacking regarding the specific mechanisms through which the above nutrients exert their effects. Some results were apparently due to detoxification, but the *modus operandi* remains obscure; others may reflect a pharmacologic, as distinguished from a nutritional, response. The net result, however, remains the same, namely, that a toxic or deleterious effect was alleviated or prevented by increased amounts of a specific nutrient. It may be added further that these effects occurred in animals that were normal at the start of the experiment and were fed diets that were qualitatively and quantitatively adequate in the absence of the various stress factors employed; and further, that animals responded to doses of nutrients that could be supplied by diet alone.

D. *Anoxia.* Available data suggest an increased vitamin requirement in the

therapy of shock and anoxia. Govier and Greer (160) found that thiamine would prolong the life of dogs in hemorrhage-induced shock, although the animals were in excellent health at the start of the experiment and were fed a diet apparently adequate in all respects. Blood pyruvic acid was determined in a number of animals during shock, and levels as high as 4 to 5 mgm. per 100 cc. were observed in contrast to the normal range of 1 to 2 mgm. per cent (159). This level is actually higher than that seen in most cases of beriberi, clinically. Thus, it would appear either that these animals became thiamine deficient as shock was induced or that their thiamine became incapable of functioning in a normal manner. It is well known that for thiamine to be effective as a coenzyme in pyruvate metabolism, it must be phosphorylated to diphosphothiamine or cocarboxylase. Under normal conditions most of the thiamine is in the phosphorylated form, but under abnormal conditions, such as shock, Govier (157) suggests that breakdown of cocarboxylase may occur, thus reducing the amount of metabolically "active" thiamine. Ochoa (321) has shown in vitro that under anaerobic conditions such breakdown of cocarboxylase does occur, probably by means of a phosphatase. Dephosphorylation of cocarboxylase was similarly observed by Govier and Greig (161) in the tissues of dogs after shock. Govier points out, therefore, that although shocked animals may be well supplied with thiamine, they may nevertheless become in a sense thiamine deficient, since their thiamine becomes converted to a form which is useless in tissue metabolism (157). Under such circumstances large doses of thiamine are required to raise the intracellular concentration of thiamine so that resynthesis of cocarboxylase may occur. A similar breakdown of cocarboxylase has been observed in dogs allowed to breathe oxygen-nitrogen mixtures containing 10 per cent oxygen (172). It appears, therefore, that anemic anoxiaand anoxic anoxia both produce a tissue anaerobiosis which results in a breakdown of cocarboxylase. Cocarboxylase, however, is not the only coenzyme which may be broken down in anoxic conditions nor are shock and anoxic anoxia the only conditions in which coenzymes are broken down. Greig (173) found that in shock a pronounced breakdown of coenzyme I and alloxazine adenine dinucleotide (flavine adenine dinucleotide) may occur and that increased amounts of nicotinic acid and riboflavin are required respectively for re-synthesis to take place. Destruction of coenzyme I has also been observed following experimental coronary ligation (158).

E. *Miscellaneous factors.* A group of factors not readily classified under one heading may also raise body requirements for various nutrients or hasten the onset of deficiency states. Prolonged parenteral administration of glucose, particularly to patients in a borderline state of nutrition, increases body requirements for thiamine and may precipitate deficiency disease (238, 404). Excessive consumption of carbohydrate acts in the same way; beriberi due to this cause has, in fact, been reported (153, 395). Exposure to sunlight or other forms of irritation such as radiant energy, dirt, filth, chemical trauma, tight clothing or repeated friction may precipitate skin lesions in nicotinic acid deficient persons (200, 350, 378). Johnson and Eckhart (232) found that sunlight hastened the onset of corneal vascularization in riboflavin deficient rats, and Sydenstricker

et al. (405) have suggested that exposure to bright light might cause destruction of riboflavin in the cornea with resulting ocular signs in the absence of lesions in other tissues. Mills et al. (308) observed that rats on a vitamin K deficient diet had four times as much internal hemorrhage when kept at 90 to 91 degrees F. as rats kept at 68 degrees F., an effect presumably due to an increased requirement for vitamin K at the higher temperature. For the water-soluble vitamins, however, requirements not only were not increased at higher environmental temperatures but in some cases were actually lower, due to decreased activity and reduced food intake (207, 255). Riboflavin requirements may be increased at high altitudes and other conditions where the oxygen supply is decreased (310). Administration of growth hormone has accentuated deficiency symptoms in the vitamin A deficient rat (133). An increased requirement for folic acid was reported for rats fed reduced caloric intakes (132). A pyridoxine deficiency was observed in the suckling young of rats fed increased amounts of thiamine during pregnancy and lactation (340).

Evidence is accumulating that in adition to the major nutrients, substances are present in our diet which may be required in increased quantities during conditions of stress. Such factors are apparently dispensable under normal conditions or their requirements are so small that they may readily be met by amounts present in the diet or through the synthetic activity of the intestinal flora or the animals' own tissue. Certain drugs or other stress factors may, however, increase requirements for these substances to the extent that deficiencies occur, manifest by retarded growth or tissue pathology, and preventable by the administration in appropriate amounts of the missing nutrient. Many effects commonly attributed to drug toxicity or endocrine hyperfunction may in reality reflect a tissue deficiency due to an increased requirement for one of these "minor vitamins". A recent report by Ershoff and Hershberg (135) is a case in point. It has been demonstrated that requirements for thiamine, pyridoxine and pantothenic acid are increased in the hyperthyroid rat (110). The above authors found that still another factor, present in yeast, is required in increased amounts by the hyperthyroid rat. Length of survival was significantly longer in thyroid-fed rats on yeast containing rations than those on similar diets containing synthetic B vitamins (135). Furthermore, neither wheat germ, pancreas, nor individual supplements of thiamine, riboflavin, pyridoxine, calcium pantothenate, nicotinic acid, p-aminobenzoic acid, inositol, folic acid nor biotin prolonged the life of thyroid-fed rats on synthetic rations; liver, however, was as effective as yeast in this regard (131). Moreover, it contains still another factor required in increased amounts by the hyperthyroid rat. It is well known that excessive thyroid feeding retards the growth of young rats. Futhermore, if feeding is begun at weaning, ovaries remain infantile, both in weight and microscopic appearance (130). Both effects, i.e., retardation of growth and failure of ovarian development, were counteracted by liver, although yeast, wheat germ, pancreas, thiamine, riboflavin, pyridoxine, calcium pantothenate, nicotinic acid, p-aminobenzoic acid, inositol, folic acid and biotin were ineffective in this regard (131). Similar experiments have been reported by Ershoff and Deuel (134) in which

alpha-estradiol served as the stress factor. Male and female rats were raised to maturity on purified rations containing excessive amounts of alpha-estradiol. Two basal rations were employed: in one, the B vitamins were administered as synthetic factors; in the other, they were present as yeast. After sixty days of feeding, both body and gonadal weight were significantly greater in the yeast series. The list of minor vitamins may be further extended to include substances effective as anti-hypertensive agents. Harrison et al. (202) prepared kidney extracts which were effective orally or parenterally in lowering the blood pressure of rats rendered hypertensive by surgery or renin, although without effect in normal animals. Similar results were obtained with marine oil concentrates, both in experimental animals (177, 427) and man (176, 329). At first, such results were attributed to the vitamin A content of the preparations employed; subsequent work has demonstrated, however, that the blood pressure-reducing factor was some unrecognized substance and that vitamin A was neither the antipressor substance nor its precursor (177, 312). More recently, Grollman (176) obtained the active principle in aqueous solution and suggested it might be identical or of related chemical structure to the active substance in renal extracts.

SUMMARY

Malnutrition may be caused by conditions other than a primary dietary deficiency. These include factors that interfere with the absorption and utilization of nutrients or those that increase their destruction and excretion. Body requirements for essential nutrients may furthermore be significantly increased for purposes of detoxification or by factors such as physical exertion, fever, drugs, toxins, abnormal environmental conditions, pregnancy, lactation, hyperthyroidism and related conditions which result in an increased metabolic requirement on the part of the tissue cell. The net result of such factors is an increased body requirement beyond the usual or average range and the precipitation of nutritional deficiencies on diets that would otherwise be adequate were such factors not operative. In the present review a critical survey has been made of current literature in respect to these factors and their effect on the vitamin, mineral and amino acid requirements of experimental animals and man.

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NEURAL CONTROL OF THE PITUITARY GLAND

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THE SECRETORY ACTIVITY of most endocrine glands appears to be outside the direct control of the nervous system. Recent work casts doubt on any nerve supply to the adrenal cortex (274, 392, 396) though experimentally it may be stimulated by the secretion of the adrenal medulla (419). This latter undoubtedly receives a rich innervation from the sympathetic system via the splanchnic nerves and lumbar sympathetic chain (392, 444) and possibly some fibers from the vagus (395). The innervation of the gonads by (in man the tenth thoracic segment) the spinal cord through the spermatic and ovarian plexuses is well known, though the exact site of nerve fiber termination in the gonads is open to dispute (257, 258, 320). However, experimental evidence clearly indicates gonadal activity to be controlled hormonally rather than by a direct nerve supply. The thyroid gland receives nerve fibers from the cervical sympathetic system and vagus (313, 357). Nerve nets around individual follicular cells have been described (391) and their presence denied (314), but the evidence that thyroid activity is under the control of secreto-motor fibers is doubtful. Removal of or stimulation of the cervical sympathetic system (45, 148, 188, 272) may possibly affect thyroid secretion indirectly by a vasomotor influence over the thyroid (357) or pituitary (406). The vagal innervation of the parathyroids (214) appears to be inessential for normal function as demonstrated by transplantation experiments (264). Similarly the pancreas, which receives vagal and sympathetic fibers (351), can still regulate the blood sugar level after vagal section or after transplantation in a depancreatized dog (227). Regarding the nerve supply and control of the pituitary gland, see below.

Thus, of the endocrine glands only the adrenal medulla and neurohypophysis possess a rich nerve supply and are directly influenced in their activity through this supply. (It is of interest to note that developmentally, the neurohypophysis may be classified as the central nervous system and the cells of the adrenal medulla as post-ganglionic sympathetic neurones.) The more slowly acting endocrines are apparently regulated by humoral means. Some activities of the adenohypophysis are clearly influenced by the nervous system, and it may be that nervous control of the endocrine system as a whole is enacted through the mediation of this gland. This surmise would place the hypothalamico-adenohypophysial unit as a key link in the

chain of neural endocrine regulation, and would clarify the position regarding the apparent lack of secreto-motor nerves to the other endocrine organs.

This review is concerned with the evidence relating to neural excitation and control of both the neurohypophysis and adenohypophysis. The terminology used for the various subdivisions of the gland will be that proposed by Rioch, Wislocki and O'Leary (354). The term 'adenohypophysis' (or glandular lobe) includes the pars distalis, the pars tuberalis and the pars intermedia. The neurohypophysis includes the infundibular process (neural lobe) and the infundibulum (neural stalk). The infundibulum may in turn be subdivided into the infundibular stem and the median eminence of the tuber cinereum. The term 'posterior lobe' will be retained to refer to the infundibular process and pars intermedia, and 'hypophysial or pituitary stalk' to include the neural stalk together with its sheath of portions of the glandular lobe.

NEUROHYPOPHYSIS

1. *Nerve Supply of Neurohypophysis*

It is now recognized that the neurohypophysis consists of three parts which function as a unit: the median eminence of the tuber cinereum, the infundibular stem and the infundibular process. That the median eminence is part of the neurohypophysis is shown by its reactions on vital staining (442), vascular supply (171, 437, 438), embryology (401) and cytology (164, 422). Tilney (401) believes the pars tuberalis to be coextensive with the median eminence, but this is denied by Weaver and Bucy (422), who state that the upper boundary of the median eminence is as yet inexactly known. However, the fact remains that the infundibular stem and the lower part of the tuber cinereum probably function as part of the pituitary gland rather than the hypothalamus. Fisher, Ingram and Ranson (140) showed that the median eminence and infundibular stem atrophy (as does the infundibular process) after lesions in the supraoptico-hypophysial tract, and that after simple removal of the infundibular lobe, the presence of the infundibular stem and median eminence are sufficient to prevent the onset of diabetes insipidus. Magoun, Fisher and Ranson (277) found that the median eminence and upper part of the infundibular stem of monkeys constituted 12 to 16 per cent of neurohypophysial tissue and were sufficient to preserve a normal urine output.

The nerve supply of the neurohypophysis is derived from two sources, a scanty sympathetic supply from the carotid plexus running with the posterior median hypophysial artery into the posterior pole of the gland (98) and a hypothalamic supply which reaches the infundibular lobe through the neural stalk. Regarding the sympathetic supply little is known as to the cells of origin, termination or function, whereas the hypothalamic supply, which would appear to be of greater functional significance, has been the subject of much anatomical and physiological study. A comprehensive list of references to work dealing with the anatomy of the hypothalamico-hypophysial tract in all types of vertebrates may be found in the monograph by Fisher, Ingram and Ranson (140).

The hypothalamico-hypophysial tract has been subdivided into the supraoptico-hypophysial tract running in the ventral or more rostral wall of the neural stalk and the tubero-hypophysial tract in the dorsal or more caudal wall of this structure (138, 358).

(*a*) NUCLEI OF ORIGIN OF THE HYPOTHALAMICO-HYPOPHYSIAL TRACT. Ramon y Cajal, 1894 (63) described a tract of fibers running from a nucleus situated behind the optic chiasma into the pituitary stalk and 'superior' lobe in young mice. Pines (335) placed the source of some of these fibers as the supraoptic nucleus (nucleus hypophyseus of Pines). This was confirmed by Greving (176) who described also a tractus paraventricularis-cinereus, arising in the paraventricular nucleus and running either to the supraoptic nucleus or to join the fibers in the supraoptico-hypophysial tract. Laruelle (259) described two groups of paraventriculo-hypophysial fibers, an external and internal set. The similar connection of the supraoptic and paraventricular nuclei with the hypophysis is not surprising in view of their common phylogenetic origin (33) and similar vascular and cytological architecture. In the rabbit some fibers entering the ventral wall of the stalk are said to arise from a group of cells situated behind the chiasma in the midline, termed by Young (445) the nucleus hypothalamicus ventralis pars centralis. Fisher *et al.* (138, 139) describe the origins of fibers in the supraoptico-hypophysial tract as the supraoptic nucleus mainly but also the paraventricular nucleus (filiform), anterior hypothalamic, ventromedial hypothalamic and ventral periventricular hypothalamic nuclei. The tubero-hypophysial tract is said by these authors to arise in the central and posterior parts of the hypothalamus and possibly from the nucleus periventricularis ventralis (343). Scattered cells and nuclei in the tuberal region are also suggested as giving origin to fibers in the lateral and posterior regions of the stalk (358), and there is some evidence that fibers from the pars mammillaris of the hypothalamus enter the tubero-hypophysial component (415).

More recent work on the origin of the hypothalamico-hypophysial fibers has been concerned with the retrograde degeneration that occurs in some hypothalamic nuclei following lesions in the tuber cinereum and infundibular stem. Such lesions are reported to cause clear-cut degeneration in only the supraoptic and paraventricular nuclei, but it is possible that less well-defined nuclei associated with the tubero-hypophysial tract also undergo at least partial degeneration. Kary (248), and Lewy (265) first described retrograde degeneration in the supraoptic nuclei. The normal number of cells in each supraoptic nucleus has been estimated at about 60,000 (man, 305, 346), 30,000 to 40,000 (monkey, 278), 35,000 to 40,000 (dog, 305, 316, 334, 345) and 7,000 (rat, 305, 345). After lesions in the neurohypophysis these cells are considerably reduced in number, the figures recorded varying from 70 per cent loss of cells on removal of the infundibular process to 90 per cent (or more) loss on section of the supraoptico-hypophysial tract above the median eminence (190, 211, 278, 316, 334, 345, 346). Less information is available for the paraventricular nuclei. Many workers have described an apparent loss of cells in this nucleus after partial or complete denervation of the neurohypophysis [Rasmussen (345) rat; Fisher, Ingram and Ranson (139) cat; Biggart and Alexander (21) and Heinbecker and White (211) dog; Rasmussen and Gardner (346) man]. Estimates have been given that about 20 per cent of the cells in the paraventricular nuclei degenerate after pituitary stalk section in the monkey (278), about 90 per cent degenerate after sectioning the supraoptico-hypophysial tract in the dog (316, 334) and 35 per cent degenerate after hypophysectomy in the rat (154). The difference in these estimates is probably due, in part, to the indefinite demarcation of the nucleus. The loss of paraventricular

cells would appear to be greater after denervation or destruction of the median eminence than after pituitary stalk section (211, 316). The loss of supraoptic and paraventricular cells after different lesions is not always proportional, and it has been suggested that some other factor such as interference with the vascular supply of the nuclei may account for this fact (154). O'Connor (316) found that the number of cells remaining in the supraoptic nuclei after various lesions in the supraoptico-hypophysial tract could not be related to the magnitude of the final polyuria.

(*b*) FIBERS IN, AND COURSE OF, THE HYPOTHALAMICO-HYPOPHYSIAL TRACT. The fibers of the hypothalamico-hypophysial tract are usually described as being unmyelinated. Vidal (415) describes these fibers in the rat as losing their myelin sheath at the median eminence. Trocello (402) saw myelinated fibers in the stalk and neural lobe in various forms, and Vasquez-Lopez (412) states that some fibers acquire a myelin sheath as they run to supply what he terms the 'meningeal terminal corpuscles'. Some authors ascribe sensory functions to these fibers (84, 412). It is felt that both the presence of myelinated fibers and the sensory nature suggested for them need confirmation.

As the supraoptico-hypophysial tract enters the median eminence it occupies a very superficial position where it is liable to be affected by basal infections of the brain (139). The fibers here have been described as pursuing a sinuous course (412) and of undergoing partial decussation (259, 359, 360). This decussation is probably not of any magnitude, for unilateral injury of the median eminence (211), or neural stalk (240), causes appreciable retrograde degeneration only in the supraoptic nucleus of the same side. The tubero-hypophysial tract in the dorso-caudal region of the tuber cinereum and infundibular stem is smaller and consists of finer fibers (139). In the neural stalk the fibers are collected into densely aggregated bundles lying in a central position, leaving a peripheral zone in contact with the pars tuberalis, relatively free of nerve elements (412). In the neural lobe the compact arrangement of the nerve fibers is broken up, although two main hypophysial fasciculi have been described in the rat (403). The general picture of fibers in this lobe is that of a tangled, gnarled mesh. Actual counts of nerve fibers in the neural stalk have given figures of about 50,000 for man (344). This is probably an underestimate, for the total number of cells in the two supraoptic nuclei alone exceeds this figure. A closer approximation would probably be 10,000 in the rat, about 60,000 in the dog and monkey and 100,000 in man (345).

(*c*) TERMINATION OF THE HYPOTHALAMICO-HYPOPHYSIAL TRACT. The fibers of the hypothalamico-hypophysial tract end mainly in the neurohypophysis, a few penetrating the pars tuberalis, pars intermedia and possibly the pars distalis. (The fibers that may enter the adeno-hypophysis are discussed below.) Although it is now generally held that the potent extracts obtained from the neuro-hypophysis are formed in situ and not by the pars intermedia (105, 140, 159, 160, 321, 322), it is doubtful which are the 'secretory' elements. The evidence of Gersh (164) that his 'parenchymatous' glandular cells are secretory has been questioned by Hickey, Hare and Hare (218), and it would seem impossible as yet to describe a secreto-motor ending in the neurohypophysis. Histologically the mode of termination of the nerve fibers in the neural lobe is obscure. The lack of a specific stain for nerve fibers (184) and the heterogenous group of structures among which the nerve endings are situated

make it hardly surprising that the fiber terminals are far from clear. [The structures comprising the neurohypophysis may be enumerated as follows, see (343): nerve fibers; nerve cells (377); neuroglia, at least in the form of microglia (411); various cells such as pituicytes (56, 178, 179, 180), mast-like cells (169), wandering cells (57), ependymal lining cells in some forms such as the cat, and cellular cords invading posteriorly from the pars intermedia; a rich vascular supply (442); and the connective tissue framework (56).]

From the evidence given above, that more cells in the supraoptic and paraventricular nuclei degenerate following lesions above or in the median eminence than in the hypophysial stalk, it seems probable that the hypothalamico-hypophysial tract ends in all three parts of the neurohypophysis. Cajal (64) described nerve fibers ending near the surface of the stalk in varicose extremities. Perivascular terminations to the fibers have been described by many authors (89, 184, 397, 412), Tello (397) and Vasquez-Lopez (412) both claiming that this type of ending is particularly numerous and dense in the infundibular stem as compared with the infundibular process. (This point will be referred to again when considering possible modes of transmission of stimuli to the pars distalis.) Other methods of termination appear to be by means of terminal enlargements, bulbs, menisci and similar structures (56, 184, 397) in relationship to cells in the neural lobe (53, 56), in the connective tissue capsule (56), in end organs described as sensory (84, 412) and in the neurohypophysial ependyma (85).

2. *Neural Control of Neurohypophysis*

During the last 50 years various effects produced by administration of extracts of the neurohypophysis have been investigated, the original studies of Oliver and Schafer (323), Howell (231) on the pressor activity, Magnus and Schafer (275), Schafer and Herring (366), Von den Velden (413) on the diuretic-antidiuretic and chloruretic activity, Dale (96) on the oxytocic activity, Borchardt (34) on the hyperglycaemic activity of such extracts, leading the way to the accumulation of much valuable data. Posterior pituitary extracts are now known to influence water and salt metabolism, the cardiovascular system, carbohydrate metabolism, the smooth muscle of the uterus, intestine and other viscera, water balance of amphibians and possibly blood coagulation, fat metabolism and other functions (409). Only recently, however, has evidence accumulated as to the physiological rôle of the neurohypophysis, and it is still not possible to accredit with certainty more than one hormonal function to the organ. Much of the evidence has been derived from studies of experimental or clinical lesions of the organ or its nerve supply. Direct stimulation of the gland has been a less productive field of enquiry owing to the complications associated with anesthesia. Indirect stimulation by means of emotional reactions, peripheral sensory stimuli and other means has yielded valuable information regarding the liberation of the antidiuretic hormone.

The more probable endocrine actions of the neurohypophysis will now be considered with special reference to their loss on damage to, or production on stimulation of, the gland or its nerve supply. Stimulation of the gland will, for convenience, be described as indirect or direct.

(*a*) NEUROHYPOPHYSIS AND THE ANTIDIURETIC HORMONE. The recognition that

the antidiuretic activity shown by extracts of the neurohypophysis represents a true hormonal action is due particularly to the work of Ranson and Verney and their respective colleagues. A detailed account of this work may be found in reviews by Pickford (333), and O'Connor (317).

Lesions. Ranson and his co-workers have demonstrated very clearly the effects of damage to the neurohypophysis or its nerve supply. The disturbance to the water balance of the body (139, 240, 241, 277) (and restoration of normal balance by posterior pituitary extracts), the degenerative changes which occur in the neurohypophysis and associated hypothalamic nuclei (138, 240, 277, 278) and the hormonal content of the atrophic neurohypophysis (136, 137) and other points have been investigated following experimental trauma to the hypothalamus or pituitary. Their work has been made the subject of a monograph (140). The main conclusions of this school are as follows: The supraoptico-hypophysial tract regulates the secretion of the antidiuretic hormone from the neurohypophysis, the term neurohypophysis being taken to include the median eminence, infundibular stem and infundibular process. Under normal conditions in the intact animal the activity of the antidiuretic hormone is balanced against that of the diuretic processes of the body, which appear to be influenced by the adenohypophysis. If the supraoptico-hypophysial tract is interrupted above the level of the median eminence, secretory activity ceases, degeneration and atrophy of the neurohypophysis and major parts of the supraoptic nuclei occur, and a state of diabetes insipidus supervenes. Extracts of the atrophic neurohypophysis show loss of antidiuretic, oxytocic and pressor activity, though the melanophore activity is retained. Major dissentients from these views have little sound evidence in their support (307, 308, 309, 310, 412). Some points still require clarification, such as the part played by the adenohypophysis in the development of a maximum diabetes insipidus (211), and the possibility of extra-hypophysial formation of an antidiuretic principle (251, 301, 302). Keller (251) believes the hypothalamic formation of an antidiuretic substance is a possible explanation of his results, in which complete separation of the hypophysis from the hypothalamus did not give rise to diabetes insipidus. It is generally accepted now that denervation of the neurohypophysis leads to atrophy of the gland and cessation of secretory activity. In this respect, Gellhorn (161) believes the neurohypophysis to be unique among the endocrine organs.

Indirect stimulation. Indirect stimulation of the neurohypophysis either by reflex nervous pathways or humorally has been the explanation put forward to account for a change in rate of urine flow on the application of many different types of stimuli.

Muscular exercise and emotion. For many years it has been known that muscular exercise causes inhibition of a water diuresis. Rydin and Verney (362) produced evidence that the primary inhibiting factor is the emotional accompaniment of exercise. They found that denervation of the kidney (see also 254, 255, 400) did not alter the response, and after excluding the participation of hemodynamic factors suggested that the antidiuresis is due to some agent humorally conducted to the kidney. Further experiments showed that the adrenal glands were not concerned, that the responses to emotional stress and injection of posterior pituitary extracts were closely similar as regards both the urine flow and chloride and nitrogen excretion by the kidney and that removal of the posterior pituitary (318) reduces the inhibition

to about 5 per cent of its original magnitude. O'Connor (315) has also shown that section of the supraoptico-hypophysial tracts in dogs, besides producing the changes described in cats and monkeys by Fisher, Ingram and Ranson (140), very largely abolished the inhibition of a water diuresis produced by emotional stress. Much of the above work was performed on dogs in which the kidneys and adrenals had been denervated. In further work (319) in which this denervation had not been performed, it was found that many dogs respond to emotional stress by a sharp, short inhibition of a water diuresis. After section of the splanchnic nerves, removal of the second, third and fourth lumbar sympathetic ganglia and denervation of the kidneys, a long slow inhibition of pituitary origin was apparent in all cases. This latter could be abolished, however, by injection of adrenaline just before the application of the emotional stimulus, but since similar injections of adrenaline did not abolish the inhibition produced by injection of posterior pituitary extracts, it was concluded that adrenaline acted by preventing the release of the antidiuretic hormone from the pituitary. Tyramine in equipressor doses is as effective as adrenaline in this respect, so it would seem that an increased cerebral blood flow is the common factor in preventing the release of antidiuretic substance (414). This beautiful sequence of experiments shows very clearly that the neurohypophysis may be aroused to secretion by emotional stress, which is a component of the psychological pattern produced by pain, exercise and other natural stimuli. The neural pathway by which this excitation occurs is almost certainly the supraoptico-hypophysial tract. [It is possible that emotional factors may have been responsible for the changes in urine flow described in man (5, 355).]

Hypertonic solutions. The presence of osmoreceptors in nervous connection with the pituitary was postulated by Klisiecki *et al.* (254). The discovery that administration of hypertonic saline inhibits a water diuresis (73, 165, 194, 414) appears to support this view. Chambers reports that the neurohypophysis of the rat, following prolonged administration of hypertonic saline, shows histological changes (71) and a loss of assayable antidiuretic hormone (72). Verney (414) found that the intracarotid injection of hypertonic saline was followed by a definite antidiuresis, which was largely abolished by removal of the posterior lobe of the pituitary. The intravenous injection of the same amount of hypertonic saline had no effect on a water diuresis. It was also shown that the effect is not due specifically to sodium chloride but to the rise in osmotic pressure of the blood, that the osmoreceptors are freely permeable to urea, less freely permeable to dextrose and relatively impermeable to sodium chloride. It seems likely that the antidiuresis evoked by hypertonic saline is in the nature of a physiological response, especially since it has been calculated that the change in blood chloride necessary to elicit the response is well within the range of the falls reported in water diuresis in man (414). The nature and identity of the osmoreceptors is uncertain. The supraoptic nuclei have been suggested as the effective site, largely on the grounds of their highly vascular nature. Also Hare (192) has shown that the antidiuretic response to injection of hypertonic saline is abolished by cutting the pituitary stalk, but if the diencephalon is isolated from peripheral receptors by cutting the midbrain, the cervical sympathetic trunks and the first three cranial nerves, the response is still retained.

Cranial and sensory nerve stimulation. Stimulation of the rat by flashes of light

has been held responsible for indirect stimulation of the neurohypophysis, with a resultant diuresis (36). Similar results had been obtained in the frog which responds with a loss of body water (37). Electrical stimulation of the vagus nerve in the isolated head of the anesthetised dog produced a diuresis and the appearance of an antidiuretic substance in the urine. Hypophysectomy abolished this response, which was attributed to reflex secretion of the antidiuretic hormone (75). Sensory stimulation of the lumbar area has been shown to inhibit a water diuresis (398), the inhibition remaining after renal denervation (400) but not after cauterization of the pituitary stalk (204). Hare (191) however states that inhibition of a water diuresis still occurs after hypophysectomy or pituitary stalk lesions in response to restraint or painful stimuli. Under these circumstances renal clearance tests show the antidiuresis to be due mainly to reduction in the glomerular filtration rate.

Administration of various drugs. Injection of morphine (103), barbiturates (104), nicotine (58), yohimbine (155), atropine (386) or acetyl-choline (332) has been shown to inhibit a water diuresis, and there is evidence that this effect is produced by mediation of the neurohypophysis.

Direct Stimulation. The demonstration that stimulation of the supraoptico-hypophysial tract causes liberation of the antidiuretic hormone is rendered difficult by the fact that administration of anesthetic inhibits a water diuresis. Haterius (204) found that of several anesthetics investigated, a chloralose-urethane mixture injected intravenously had least depressant action on a water diuresis. Under this narcosis, electrodes were inserted into the region of the pituitary stalk of rabbits by means of a stereotaxic instrument in which the animals' heads were clamped and orientated. This manipulation invariably caused abrupt diminution in urine flow, and subsequent gavage frequently resulted in death. However, good diureses were eventually obtained in eight experimental and six control animals. Of the eight experimental animals, five showed a definite antidiuresis on stimulation of the pituitary stalk and three only a transient depression. In one of these three the electrodes were found at post-mortem to be 1 to 2 mm. lateral to the stalk. In the six control rabbits that had had electrolytic lesions placed in the region of the stalk two to four days previously, four failed to show an antidiuresis on stimulation. In the two animals in which stimulation checked the diuresis, autopsy revealed intact pituitary stalks.

In a recent communication (199) electrical stimulation of the neurohypophysis and hypothalamus has been investigated with regard to the secretion of the antidiuretic hormone in the conscious rabbit. Unipolar stimulation was applied by the remote control method, a small coil being buried between the scalp and skull, and an insulated electrode carried down through the skull, corpus callosum, hippocampal commissure and other median structures into some part of the hypothalamus or hypophysis. Rabbits have been shown to live normal healthy lives for periods of at least three years with this unit in situ. After full recovery from the initial operation and anesthetic, stimulation may be performed by placing the animal's head in an electromagnetic field. Repeated experiments may thus be performed on the same animal. With the electrode in, or in contact with some part of the supraoptico-hypophysial tract, short or long-lived antidiuresis could be produced at will, in the

conscious animal, by varying the intensity of stimulation These effects were constant, easily repeatable, and in every way similar to the antidiureses produced by injection of appropriate doses of posterior pituitary extract. Control experiments showed that so far as the nerve fibers in the neurohypophysis were concerned, the effective current spread was ½ mm. or less, indicating a very localized stimulus. If during a water diuresis a given stimulus was repeated the antidiuretic responses tended to increase. The explanation of this phenomenon was not clear for it could not be correlated entirely with a diminishing water load. A given stimulus repeated under identical conditions from day to day gave remarkably constant responses. Sedative doses of a chloralose-urethane mixture tended to depress the response. The antidiuresis following stimulation was unchanged by removal of the left adrenal gland and denervation of the right. A chloruresis was found to occur concurrently with the inhibition of urine flow, the intensity and duration of the two effects running parallel. The chloride concentrating reaction could also be duplicated exactly by intravenous injection of posterior lobe extracts.

(*b*) NEUROHYPOPHYSIS AND THE UTERUS. *Lesions.* Since Dale (96) first reported the oxytocic action of posterior pituitary extracts, many workers have found that normal labor may occur after hypophysectomy or lesions of the neurohypophysis (121, 252, dog; 4, 299, cat; 328, guinea-pig; 298, ferret; 135, rabbit; 371, 384, rat; 372, mouse), and the idea became prevalent that the oxytocic factor was of pharmocological interest only. Some workers suggested the foetal pituitary may play an important part during parturition (15).

Fisher, Magoun and Ranson (141) noticed that pregnant cats which had developed diabetes insipidus following interruption of the supraoptico-hypophysial tract exhibited disturbances in parturition. Of seven animals, four died during labor, two survived for some days and only one remained alive and in good condition. The dystocia took the form either of total inability to deliver the young or a very prolonged labor. The conclusion was drawn that the supraoptico-hypophysial tract and neurohypophysis probably form an integral part of the labor mechanism, and previous work showing normal labor may occur after hypophysectomy was criticized on the grounds that most workers had failed to control the following points: *a*) complete removal of the neurohypopnysis (including median eminence) demonstrated by serial sections after death, *b*) the presence of at least some secretory tissue of the glandular lobe, and *c*) the duration and normality of the various stages of labor. The statement that delivery occurred at term was held to be insufficient. In a further report Dey, Fisher and Ranson (117) confirmed their previous findings. They state that with hypothalamic lesions in the supraoptico-hypophysial tract slightly less than one third of their pregnant guinea-pigs were able to deliver their young normally, and that about one half of them had a definitely prolonged labor or delivered their young dead. Confirmatory results to the above are given by Pencharz and Long (327) from a study of hypophysectomized rats and by Smith (385) for hypophysectomized monkeys. Smith found that of 13 hypophysectomized pregnant rhesus monkeys, 6 completed pregnancy and had normal babies although labor was prolonged, caesarean operations being performed on 3. From this evidence then, it would seem likely that the maternal neurohypophysis is involved in

the process of parturition, but the fact that some animals with diabetes insipidus may deliver their young normally (guinea-pig, 117; dog, 252; and man, 99) makes the evidence not wholly convincing. Further work on the effect of hypothalamic lesions on parturition in different species is needed.

Stimulation. It is claimed (77) that stimulation of the central end of the vagus in the isolated head preparation of the dog causes reflex liberation of the oxytocic factor. This was found to have no effect on the dog's own uterus, but could be detected in the jugular blood by its action on a guinea-pig uterus. Control sera and sera obtained after stimulation in the hypophysectomized dog had no effect on a guinea-pig uterus. Stimulation of the cervical sympathetic in rats has been reported as increasing the amount of oxytocic factor in the pituitary gland (325).

Haterius and Ferguson (206) were the first to investigate the effects of faradic stimulation of the infundibular stem on uterine motility in anesthetized rabbits. They found the response in virgin rabbits to be variable. In the postpartum animal, stimulation produced an increase in uterine activity as shown by increased frequency and amplitude of contractions. This response was simulated closely by an intravenous injection of 'Pitocin,' and from a comparison of the responses they inferred stimulation could cause the liberation of as much as 0.5 U of oxytocic substance at one time. Spinal transection, section of the splanchnic nerves or vagotomy did not abolish the increased uterine motility on stimulation, though an electrolytic lesion placed in the pituitary stalk rendered subsequent stimulation of the structure ineffective. In a further study by Ferguson (133) on postpartum cats and rabbits, it was shown that in animals in which the neck had been crushed (except the carotid arteries, jugular veins and a flap of skin), stimulation of the infundibular stem still evoked an increase in uterine activity equivalent to the injection of 0.5 U 'Pitocin'. Various observations were adduced showing that the increased activity of the uterus was not dependent on an associated rise in blood pressure. In one experiment it was noted that the uterine response to stalk stimulation was more closely simulated by an injection of 'Pitocin' than 'Pituitrin', and the suggestion was made that on stimulation the pituitary liberates a hormone with relatively little pressor activity.

The effects of stimulation of various parts of the hypophysis and hypothalamus have been studied (199) in the unanesthetized rabbit, using the remote-control method of stimulation and a modification of Reynold's (349, 350) uterine fistula for recording uterine activity. In animals retaining their ovaries, uterine activity shortly after operation was usually that of the oestrous state and a marked increase in activity would occur in response to stimulation of the neurohypophysis. These reactions tended to disappear in a few days, possibly because the stimulation also caused a release of luteinizing hormone from the adenohypophysis. Good standard preparations were obtained, however, following ovariectomy and the subcutaneous implantation of a tablet of stilboestrol di-n-butyrate. Stimulation of the median eminence or infundibular stem in the oestrous or oestrogenized rabbit was shown to cause a very marked increase in uterine activity. The response to a given stimulus in the oestrogenized rabbits was very constant from day to day. The magnitude of the effect could be graded at will by varying the intensity of the stimulus and could be duplicated exactly by the intravenous injection of posterior lobe extracts in doses up to 200–500 mU. Similar stimuli applied in control rabbits

with the electrode tip in the pars distalis below the infundibular stem, in the posterior part of the tuber cinereum above the infundibular stem, or in the region of the paraventricular nucleus were ineffective in altering uterine motility providing stimuli were applied at a distance greater than ½ mm. from the supraoptico-hypophysial tract. A further group of control animals in which stimuli were applied not directly to, but within ½ mm. of the ventral, lateral or dorsal sides of the infundibular stem showed submaximal increases in uterine activity. (The magnitude of the antidiuretic responses in these same animals could be correlated in a similar manner with the site of stimulation.) The uterine responses to stimulation of the neurohypophysis and to intravenous injection of posterior lobe extract were also observed through periods of anoestrus, pseudo-pregnancy and progesterone treatment and found to be qualitatively similar. Deep ether anesthesia inhibited the uterine response to stimulation markedly, but had much less effect on the response to injection of posterior lobe extract; light anesthesia (intravenous chloralose-urethane) had little inhibitory effect on the uterine reactions. Removal of the left adrenal gland and denervation of the right did not alter the uterine response to stimulation of the median eminence. Administration of adrenaline had an effect on the uterus qualitatively dissimilar to that following stimulation of the neurohypophysis or injection of posterior lobe extracts.

The data given above indicate that, in the rabbit and cat at least, the neurohypophysis has the potentiality of secreting an oxytocic factor into the blood stream which can exert a marked effect on the uterine musculature. The proof that this oxytocic principle is a true hormone rests on the demonstration that such a release and action occur in response to 'natural' stimuli. It has been suggested (199) that the act of coitus, known to stimulate the adenohypophysis of the rabbit, stimulates also the neurohypophysis, and thus may increase uterine motility and aid sperm transport. The fact that stimulation of the vulva of the rabbit increases uterine motility (207) and the rate of transmission of fluids up the uterus (256) could be adduced as evidence in favor of this view. Ferguson (133) has described a reflex release of oxytocic substance from the neurohypophysis following the stimulus of distension applied to various parts of the birth canal and has suggested that reflexes of this type may play a part in the mechanism of parturition.

(*c*) NEUROHYPOPHYSIS AND BLOOD SUGAR. Borchardt (34) first showed that injection of extracts of the posterior lobe of the pituitary produces a hyperglycemia and glycosuria. This observation has been confirmed and extended, the general conclusions (409) being that posterior lobe extracts on injection will produce a moderate hyperglycemia persisting for a few hours and often followed by a fall in blood sugar. Most work indicates the vasopressor principle to be the active constituent of such extracts, although some (130, 225, 230) believe the oxytocic principle to be more potent in this respect. The doses of extract necessary to produce hyperglycemia are large. In the rabbit the following doses have been used: 2–4 U/kgm. body weight, intravenous injection of 'Post-Lobin V' (129); 7½ U posterior lobe extract injected intravenously (66); 20–60 U 'Pituitrin' injected subcutaneously (436). Since the pituitary of the rabbit has been estimated as containing only 1.3–2.5 U of the pressor principle (381), these doses appear to be outside the physiological range.

Injuries to the hypothalamus have been stated by different authors to cause

both hyperglycemia or hypoglycemia. If these changes in blood sugar are mediated via the pituitary gland, it would seem likely that they are related to the activity of the adenohypophysis rather than the neurohypophysis and will be discussed further below. Keller (250), however, suggests that the decreased tolerance to insulin found in cats with large hypothalamic lesions is due to lack of secretion by the neurohypophysis.

Electrical stimulation of the cervical sympathetic system, or hypophysis itself, has been described by many workers as causing a glycosuria or hyperglycemia. Weed, Cushing and Jacobson (423) claimed stimulation of the hypophysis produced a glycosuria in cats and dogs, even after preliminary transection of the spinal cord and cervical sympathetic trunk, and that stimulation of the superior cervical ganglion in cats, rabbits or dogs by faradization or by the manipulation necessary for its exposure likewise resulted in a glycosuria. This latter reaction remained after section of the cervical sympathetic trunk, spinal cord and vagi but was abolished by removal of the posterior lobe of the pituitary. They suggested the infundibular process is innervated by the superior cervical ganglion, and that stimulation of any part of this mechanism liberates posterior lobe hormone so producing a glycogenolysis. Direct stimulation of the pituitary gland has been described by Keeton and Becht (249) as causing a glycosuria, abolished by previous section of the splanchnic nerves, and by Ingram and Barris (238) as causing a glycosuria often accompanied by a diuresis. Harris (199) found that stimulation of various regions of both the neurohypophysis and adenohypophysis of the unanesthetized rabbit by the remote-control method failed to produce a hyperglycemia or diuresis that could be ascribed to pituitary activation, and suggested that the results of previous workers had been complicated by the administration of an anesthetic. That stimulation of the cervical sympathetic system causes a glycosuria or hyperglycemia has been confirmed (100, 233, 376) and denied (219, 342). These experiments were all performed under anesthesia, except those of Rabens and Lifschitz (342) who criticize the results of previous workers as being due to prolonged anesthesia. Stimulation of the central end of the cut vagus nerve has also been reported to cause hyperglycemia due to reflex excitation of the neurohypophysis (233).

There is little evidence that the neurohypophysis plays a physiological rôle in the regulation of blood sugar.

(*d*) NEUROHYPOPHYSIS AND BLOOD PRESSURE. In 1895 Oliver and Schafer (323) reported that injection of pituitary extracts into anesthetized animals raised the blood pressure. Howell (231) showed the active principle was contained in extracts prepared from the posterior lobe and not from the anterior lobe of the pituitary gland. As is well known, these observations have been amply confirmed, and a transient depression of blood pressure, which often precedes the more prolonged rise following injection, has been ascribed to a constriction of the coronary arteries with impairment of the heart's action and diminution in cardiac output (303 and others). Injection of whole or fractionated posterior lobe extracts in unanesthetized animals or man is commonly stated to lower the systolic blood pressure and cardiac output, effects which are attributed to coronary constriction and reflex nervous activity (181, 182, 183), but pressor responses are also described (198, 367).

Total hypophysectomy results in a fall of blood pressure in the rat (260, 443) and dog (39), but removal of the pars neuralis alone (39, 443) does not produce hypotension. In view of these results it might be expected that lesions in the nerve supply of the neurohypophysis would affect the blood pressure little, if at all. Section of the pituitary stalk in a case of malignant hypertension produced a marked reduction in blood pressure (346), but the significance of this case is doubtful, the result possibly being secondary to vascular damage to the pars distalis. In dogs showing experimental hypertension of the Goldblatt type, lesions in the supraoptico-hypophysial system produced a fall in mean blood pressure (364), and one normal dog in which the median eminence was sectioned showed a fall of blood pressure for one month followed by a return to normal levels, though a marked polyuria was maintained. The evaluation of these results is difficult.

Stimulation of the neurohypophysis by reflex pathways has been held responsible for the pressor response in the trunk produced by stimulation of the central end of the vagus nerve in the isolated head of the dog (74). This rise was abolished by cutting the pituitary stalk or by hypophysectomy and was duplicated by the intravenous injection of 100 mU 'Pitressin' (see also 363). An attempt to trace the nervous pathway involved has been made (234). Similar reactions have also been obtained in the toad, turtle, chicken, duck, rabbit and cat, but not the snakefish (273). Stimulation of other cranial nerves (olfactory, optic, lingual, vestibular, glossopharyngeal and hypoglossal) also produces, in the dog preparation, slight pressor responses abolished by hypophysectomy (235). Repeated stimulation of the central vagus causes exhaustion of the pressor response which returns with rest. These changes have been correlated with the exhaustion and reappearance of granules in pituicytes (76), but in view of the work of Hickey, Hare and Hare (218), the significance of granules is open to doubt. That the arterial hypertension due to stimulation of the central vagus is abolished by hypophysectomy has been denied (293) and partially confirmed (363). It has been suggested that thermal trauma, which elicits a pressor response, may activate the neurohypophysis (324).

Direct electrical stimulation of the pituitary gland or its hypothalamic nerve supply has been investigated with regard to a possible pressor response. Cyon (93, 94, 95) one of the first workers to stimulate the pituitary gland directly reported a bradycardia and rise in blood pressure as resulting from mechanical and electrical stimulation of the gland. Stimulation of the supraoptic region of the anterior hypothalamus of the anesthetized spinal cat was reported by Clark and Wang (79) to give a pressor response. The characters of this response were a long latent period, a slow smooth rise of about 30 mm. Hg, and a duration of approximately two to four minutes, indicating the possibility that the response was activated humorally through the supraoptico-hypophysial tract and the neurohypophysis. These results have been confirmed, striking elevations in blood pressure being obtained on bipolar stimulation of the infundibulum in animals with crushed spinal cords (Sattler, 363). Ferguson (133) records small rises in blood pressure on stimulating the pituitary stalk in rabbits and cats anesthetized with chloralose-urethane and in which vascular connections only remained between the head and trunk. In some cases the pressor response was very slight (see his fig. 2), although the oxytocic effect of the stimulus

was well marked. In view of the different effects recorded following injection of pituitary extracts into anesthetized and unanesthetized animals, observations were made (201) on the response of the conscious rabbit to injection of posterior pituitary extract and remote control stimulation of the pituitary stalk, using Grant's capsule (167) for recording the blood pressure. The unanesthetized rabbit exhibits a pressor response on intravenous injection of 'Pituitrin', and on stimulation of the infundibular stem slight pressor responses (less than those evoked by 100 mU of 'Pituitrin' injected intravenously) of a similar type were obtained.

(*e*) NEUROHYPOPHYSIS AND INTESTINAL PERISTALSIS. There are few subjects in the literature on which more discordant results have been recorded than the effects of posterior pituitary extract on intestinal motility, and there would seem little justification at the present time for correlating results obtained by in vitro experiments on isolated intestinal segments, and in vivo experiments performed with and without the use of anesthetics on a variety of animal species and with a wide variation in dosage of extracts used.

There is some evidence available, however, concerning the effects of stimulation of the neurohypophysis or its nerve supply on intestinal motility. Wang, Clark, Dey and Ranson (421) found that electrical stimulation of the region anterior to the infundibulum of the anesthetized cat produced an excitation of gastro-intestinal motility. This response had a long latent period, persisted for several minutes after cessation of the stimulus, was not abolished by sectioning the vagi, but was seen in slight form in only 4 out of 11 cats after sectioning the spinal cord. In view of this last observation the authors were unwilling to emphasize the participation of the neurohypophysis in the reaction. Vagal effects on intestinal motility, observed when the hypothalamus, at or behind the infundibular level, was stimulated, were more immediate in onset and completion. Relative to these results are those of Steggerda, Gianturco and Essex (387), who found the threshold dose to activate the colon of the conscious cat was two to three pressor units injected intravenously and that no response was obtained after injection of 'Pitocin' or 'Pituitrin'. Wang *et al.* (421) found intestinal motility increased in three out of five cats after injection of four pressor units. Assays of the pressor content of the cat's neurohypophysis have been estimated at about 6 pressor units (140) and 5.6–13.1 pressor units (381). It would seem possible then for the gland of this animal to secrete a sufficient amount of pressor factor to activate the intestines, but the evidence that this occurs is incomplete, since Ferguson (133) found the response to stimulation of the pituitary stalk in cats was about equivalent to that obtained by injection of ½ U. 'Pitocin' and that the secretion appeared to have relatively less pressor than oxytocic activity.

In the case of the rabbit, it has been shown that the colon is the most sensitive part of the alimentary canal to the action of posterior lobe extracts, and that the pressor rather than the oxytocic is the active fraction (158, 304). The effect of stimulation of the infundibular stem and median eminence on intestinal peristalsis has been studied in the unanesthetized rabbit (201). It was found that the peristaltic waves as observed through the intact abdominal wall of the unanesthetized rabbit tied in the supine position are small in amplitude and infrequent or, more usually, absent. On intravenous injection of posterior lobe extract (50–500 mU), a

marked increase in the visible waves occurs in the right lumbar and umbilical areas, that is, in the regions overlying the colon and cecum. This increase occurs after a latent period of about thirty seconds and persists for many minutes. On stimulation of the median eminence or infundibular stem a similar increase in peristalsis was observed. This response had a somewhat longer latent period (30–90 seconds), though in other respects it was similar to that following injection of posterior lobe extracts in doses less than 100 mU. In these experiments the stimulus spread, with regard to the supraoptico-hypophysial tract, was less than ½ mm. It is possible then, that colonic activity in the rabbit may be excited humorally by the neurohypophysis, which is in turn under the neural control of the hypothalamus.

3. *Nature of the Substance Liberated from the Neurohypophysis on Electrical Stimulation of the Supraoptico-hypophysial Tract.*

The nature of the secretory product of the neurohypophysis has been discussed for many years. Early views have been summarized (247). The view that the gland secretes more than 1 active principle (multiple hormone theory) has been strongly contested by Abel and his co-workers (1, 2), who support the 'unitary theory' that the gland secretes a single specific substance with multiple actions. Two closely related problems are involved; first the nature of the substance(s) elaborated and stored by the gland and second the nature of the substance(s) liberated from the gland into the blood stream. Van Dyke, *et al.* (410) have isolated a protein from the dried posterior lobe of frozen gland material which behaves as a homogenous substance to solubility, electrophoresis and ultra-centrifugation tests, and which possesses oxytocic, pressor and anti-diuretic activities in the same proportion as those of the U. S. P. reference standard. These authors suggest that even if the pars neuralis elaborates a single protein with multiple activities, it is possible that specific enzymes liberate active fragments of the parent molecule into the blood stream, depending upon the requirements of the organism. As is well known posterior lobe extracts may be fractionated into preparations showing a high degree of separation of pressor and oxytocic activities (242, 247, 388). The pressor principle is usually considered responsible for the renal action of extracts, but according to Heller (212) some separation of these activities is also possible. Fraser (146) maintains that the hydrolysis, as used by Heller, involves no real change in the ratio of antidiuretic and pressor activity.

A few observations indicate that the substance(s) liberated from the neurohypophysis on stimulation of its nerve supply exert relatively less pressor (or antidiuretic) activity as compared with oxytocic, than do whole posterior lobe extracts. Ferguson (133) noted in one experiment that stimulation of the pituitary stalk was more comparable in its proportionate effects on the uterus and blood pressure of the anesthetized animal to one U. 'Pitocin' than one U. 'Pituitrin'. In other experiments he noted the uterine contraction elicited by stimulation of the stalk was qualitatively similar to that produced by injection of 'Pitocin' rather than 'Pituitrin'. He concluded that stimulation of the pituitary stalk liberates a hormone from the neurohypophysis with little pressor activity. Harris (199) has found that stimulation of the median eminence or infundibular stem in rabbits causes a substance to be

liberated from the neurohypophysis which possesses less antidiuretic in proportion to oxytocic activity than various standard unfractionated extracts. Stimulation was performed in the unanesthetized state, the antidiuretic and oxytocic activities being measured simultaneously and compared with similar responses evoked by intravenous injection of posterior lobe extracts into the same animals over a series of days. Also the closer correspondence between the uterine contraction produced by stalk stimulation and injection of 'Pitocin' rather than 'Pituitrin' was confirmed. Injection of 'Pituitrin' caused a greater degree of inhibition of uterine activity following the initial contraction than either of the other two procedures, this probably being due to the higher pressor content producing spasm of the uterine vessels (311). It has also been found (201) that, whereas stimulation of the infundibular stem or median eminence produces an oxytocic action equivalent to about 250 mU. of posterior lobe extract, similar stimulation produces an effect on the blood pressure and intestinal peristalsis less than that evoked by 100 mU. of such extracts. On the assumption that the pressor and antidiuretic activities run parallel, and that the action on intestinal motility is due to the pressor principle, there would seem to be substantial evidence for the belief that the secretion of the neurohypophysis produced by stimulation of the supraoptico-hypophysial tract in the rabbit possesses relatively low pressor activity.

Recently it has been shown (201) that the reactions of the oestrogenized uterus, following intravenous injection of a solution containing 'Pitocin' and 'Pitressin', gives a roughly quantitative assay of the 'Pitocin' : 'Pitressin' content of such mixtures. The 'Pitocin' content is related to the initial uterine contraction and the 'Pitressin' content to the subsequent inhibitory phase. One minute stimulation of the infundibular stem by the remote control method in the rabbit produces a secretion equivalent (in action on the uterus) to a mixture of 250 mU. 'Pitocin' and 50–100 mU. 'Pitressin.' One hour after intravenous administration of hypertonic saline, a similar stimulus produces a secretion equivalent to about the same amount of 'Pitocin' but less 'Pitressin.' After various control procedures the most likely explanation appears to be that the injection of hypertonic saline has depleted the neurohypophysis of its pressor content, but not the oxytocic.

ADENOHYPOPHYSIS

1. *Evidence That the Adenohypophysis Is under Neural Control*

There is much evidence that the adenohypophysis is under neural control but only conflicting evidence regarding the presence of a direct secreto-motor nerve supply to the gland.

The evidence that the nervous system affects the secretory activity of the pars distalis of the hypophysis may be conveniently summarized as follows.

(*a*) INDIRECT EVIDENCE. EXTEROCEPTIVE FACTORS WHICH ACT UPON THE NERVOUS SYSTEM AFFECT ORGANS IN WHICH DIRECT NERVOUS CONTROL IS UNLIKELY, BUT WHICH ARE KNOWN TO BE INFLUENCED BY PITUITARY HORMONES. (i) *Gonads.* The effects of exteroceptive stimuli on the reproductive processes of vertebrates have been the subject of several recent reviews (25, 286, 288, 361 and especially 289). The intrinsic sexual rhythm is in many vertebrates adjusted to external seasonal changes,

so that variation in the seasonal environment produces a corresponding adaptation in the reproductive processes. One example of this phenomenon is the change-over that occurs in the oestrous cycle of some mammals after transference across the equator (287). The active factors of the general environmental pattern are in many cases doubtful. Light has been shown to be a potent stimulus to the sexual processes in some fishes, amphibians, reptiles and many birds and mammals (289). Among the common mammals the reproductive rhythm of the mouse (9, 175), rat (55, 109, 142, 337, 404), ferret (23), hedgehog (3), cotton-tailed rabbit (28), cat (101), raccoon (27), goat (26) and sheep (470) is sensitive to changes in light exposure, while that of the adult cotton rat (306), squirrel (246, 426), guinea-pig (107) and rabbit (382) is not. Other environmental factors which may alter the reproductive processes in mammals and birds are temperature and humidity (10, 32, 261, 287, 427), visual stimuli (295) and tactile or proprioceptive stimuli (393). The relative constancy of the number of eggs in a clutch for any particular species of bird, and the repeated laying of the bird if by removal of eggs from its nest it is prevented from attaining its usual quota (289), again point to an influence of visual or tactile stimuli on the adenohypophysis. Desclin (112) believes that the act of suckling exerts a direct influence on the basophil cells of the maternal pituitary and consequently on its gonadotropic activity.

The presence of a sterile mate or companion of the same sex may exert a marked effect on the sexual rhythm. Exteroceptive stimuli of various kinds would appear to be involved. Olfactory and auditory stimuli probably play a part in some forms (59), though little is known about these. Brooks (49) showed that removal of the olfactory lobes and neocortex or destruction of the labyrinths and cochleae in the female rabbit does not prevent mating and subsequent ovulation, but that ablation of the neocortex and olfactory bulbs in the male rabbit permanently abolishes mating behavior. Tactile or proprioceptive stimuli appear significant in many forms, but especially in those animals which ovulate only after sexual excitement or mating [viz. rabbit (208), ferret (187, 285), cat (102, 271), ground-squirrel (145), short-tailed shrew (326) and mink (189)], though the work of Brooks (49) on the rabbit indicates that the stage of sexual excitement necessary to elicit discharge of the pituitary secretion may be produced by stimuli from a variety of sensory end organs. For a review of the factors underlying sexual behaviour in mammals see Beach (14). The sight of another animal is occasionally sufficient to produce ovulation in the rabbit, and more particularly, in the pigeon. In the latter case even the reflected image from a mirror may produce the same result in the isolated bird (295). The sex display of many birds probably affords another example of this type. Marshall (286, 288) has suggested that the main function of visual stimuli in the form of sex display and courtship phenomena among various classes of animals is to promote an effective synchronization of the male and female sexual processes, thus favoring successful procreation. He suggests this synchronization is mainly effected by pituitary stimulation through the intermediation of the hypothalamus.

(ii) *Breasts, thyroid, adrenals.* The maintenance of milk secretion by the mammary glands is largely dependent on the exteroceptive stimulus of vigorous suckling (373, 374). Since there is evidence that afferent nervous pathways from the nipples are involved (see below) and that the breast lacks a secreto-motor nerve supply, the

most likely hypothesis would appear to involve a reflex stimulus to the secretion of the lactogenic hormone by the pituitary gland.

Similarly the changes which occur in the thyroid and adrenal cortex under the influence of seasonal variations or experimental changes in the environmental temperature (19, 109, 448, 449) may be due to reflex alteration in the activity of the adenohypophysis, since the thyroid gland appears to receive only a vasomotor innervation, and the adrenal cortex lacks any nerve supply. Brolin (46) gives a detailed discussion of the effect of environmental temperature on the thyroid and adrenal glands.

(iii) *Clinical data.* Evidence of a similar nature to that presented above may be adduced for man. It is well known that the usual menstrual rhythm may be interrupted by environmental change [viz., the women of Patagonia and Eskimo women during the long winter nights (268)], by suckling and lactation and by worry or fright (269, 399). The process of lactation is also greatly affected by the emotional state. Cases of exophthalmic goiter (20, 267) frequently present a history of psychic trauma preceding the onset of the disease. A possible mechanism underlying these reactions is a nervous, reflex activation or inhibition of the adenohypophysis resulting in a hormonal effect on the ovaries, breasts or thyroid.

(*b*) DIRECT EVIDENCE. DAMAGE OR STIMULATION OF POSSIBLE NERVE PATHWAYS TO THE ADENOHYPOPHYSIS. The large amount of evidence derived from experimental studies of this type will be discussed below when the individual nervous pathways to the pars distalis are under consideration. Clinical observations, in which lesions of the hypothalamus and other parts of the brain have been found to interfere with sexual development and function, are numerous (see 51). Hypothalamic lesions have been held responsible for hypogonadism, either alone (177) or in association with Frohlich's adiposogenital syndrome (122, 353, 389) or the Laurence–Moon–Biedl syndrome (347). Hecker and Warren (209) concluded that many changes seen in the Laurence–Moon–Biedl and Frohlich's syndromes are due to involvement of the hypothalamus and, indirectly, the pituitary. Pineal tumours may also be associated with genital dystrophy, but it seems likely that this disturbance is due to secondary involvement of the hypothalamus (81, 353). Since hypogonadism may be a non-specific change, due perhaps to a state of malnutrition associated with the primary lesion, better evidence concerning a possible hypothalamico-hypophysial relationship is offered by a study of cases showing precocious puberty in association with neural lesions. That hypothalamic lesions may cause pubertas praecox is well known (47, 120, 122, 144, 341). In these cases the lesion is usually situated at the level of, or caudal to, the tuber cinereum, and it has been suggested (425) that the precocity is due to destruction of a posterior hypothalamic mechanism with release of an anterior hypothalamic innervation of the pars distalis. Weinberger and Grant (425) review the literature on this subject. Changes in thyroid activity associated with a cyst near the infundibulum (312) and other lesions of the brain (267, 336) have also been reported. Three cases of thyrotrophic exophthalmos, possibly of pituitary-diencephalic origin, have been described by Zondek and Ticho (450). In view of the known actions of the diabetogenic hormone the fact that hypothalamic lesions are occasionally found associated with diabetes mellitus (420) may indicate that a similar mecha-

nism underlies these cases, as suggested above to explain pubertas praecox with hypothalamic lesions.

(*c*) EVIDENCE DERIVED FROM THE STUDY OF HYPOPHYSIAL TRANSPLANTS. In studying the replacement capacity of hypophysial grafts in hypophysectomized animals, attention should be paid to the following points. First, the grafted tissue should be situated at a distance from the normal site of the gland thus obviating the danger of vascular or nervous repair; second, the studies should be extended over a period sufficient to ensure that any effects observed are due to functional activity of the transplant, and not to absorption of an implant; and third, serial sections through both the transplant and sella turcica with surrounding tissue are necessary to control the activity of the graft and the complete removal of the pituitary.

The male reproductive organs in hypophysectomized animals are reported to be maintained or restored to function by ocular grafts of the pituitary in rats (296) and (incompletely hypophysectomized) mice (221) by transplants in the sella turcica in rats (91, 174) and by ocular transplants in rats (91) and guinea-pigs (369). Cutuly (91) obtained fertile matings from two male rats 200 and 209 days after hypophysectomy and transplantation, in spite of the fact that the testicular weights of his experimental animals were all below the average.

In hypophysectomized female rats, pituitary transplants have produced inconstant effects on the reproductive organs. Martins (294) found only 1 out of 4 animals exhibited oestrous changes, and these cycles were irregular. May (297) obtained normal oestrous cycles with ocular transplants, but these cycles were not entirely due to the transplants, for removal of the eyeball bearing the grafted tissue did not immediately result in their loss. Greep (174) has reported normal reproductive processes (oestrous cycles, pregnancy, parturition and lactation) in rats bearing transplants in the sella turcica. Loss of oestrous cycles in hypophysectomized rats bearing intramuscular (330) or ocular (92, 431) transplants has been reported more recently. Hypophysectomized female guinea-pigs bearing ocular pituitary transplants show follicular development, hypertrophy of the uterus and proliferated mammary glands, though cyclical activity is lacking and a state of constant oestrus finally intervenes (368). It is perhaps significant that the most complete replacement of pituitary function was that obtained by Greep (174) with grafts in the sella turcica, a position in which regeneration of nervous or vascular connections may possibly occur.

An increase in body weight following transplants in hypophysectomized animals has been reported by several observers (91, 174, 296, 368, 369, 431), though in most cases the increase is slight or less than in controls (91, 174). Maintenance by transplants of the thyroid gland has been observed by Schweizer, Charipper and Haterius (368) though not by Cutuly (91). Differing accounts have been given regarding maintenance of the adrenals (91, 221, 368, 369, 431).

2. *Possible Nerve Pathways to the Adenohypophysis*

From the above data it would appear that there is strong presumptive evidence in favor of some degree of neural control over the adenohypophysis. The mechanism whereby this neural control is exerted is uncertain. The nervous system may affect the secretory activity of the pars distalis either by a direct nerve supply through the

mediation of the cervical sympathetic system, the parasympathetic system via the spheno-palatine ganglion or petrosal nerves, or the hypothalamico-hypophysial tract; indirectly through a neurovascular pathway involving the hypophysial portal vessels; or by some other unknown route. At the present time the balance of evidence is in favor of the view that nervous stimuli affect the pars distalis by means of humoral transmission through the hypophysial portal vessels. This hypothesis will be discussed in detail after consideration of the anatomical and experimental evidence.

(*a*) CERVICAL SYMPATHETIC SYSTEM. The first account of any nerves passing to the pituitary gland was brief mention of a sympathetic supply by Bourgery in 1845 (35). Henle (213) described small nerve twigs passing from the carotid plexus medially to the hypophysis, which, he said, had been previously described by Fontana, Ribes and Bock. Berkley (18) stated that only sympathetic fibers were to be found in the anterior hypophysis of the dog. The first detailed description of the sympathetic pathway to the gland was given by Dandy (98) who used the intravitam methylene blue method of staining. He described the nerve supply of the glandular hypophysis as passing with the arterial supply from the circle of Willis down the stalk to the gland. These nerve fibers came mainly from the plexus around the posterior communicating artery, and as many as three or four small nerve filaments may accompany an arterial branch to the glandular lobe. Pines, (335) using methylene blue and silver staining methods, also demonstrated a sympathetic innervation of the pars glandularis. Hair (184) found a rich innervation of the pars glandularis running with two sets of vessels, first with vessels from the circle of Willis running directly to the pars distalis and second with vessels passing via the pars tuberalis, where an extensive vascular network was found. Rasmussen (344), in a comprehensive study of the nerve fibers to the human pituitary supplemented by observations on the rat, guinea-pig, rabbit, cat, dog and monkey, found sympathetic fibers from the cavernous plexus entering the pars distalis. Large areas of the gland were found to be free of nerve fibers, however, the conclusion being drawn that the fibers present were probably vasomotor in nature. Truscott (403) estimated that out of 2,000 nerve fibers entering the glandular lobe of the rat's pituitary, 20 per cent are carried in on the walls of blood vessels. Drager (123, 124) states that the pars tuberalis of the porpoise and bird receives both autonomic and hypothalamic nerve fibers, but that none pass on to the pars distalis. In the armadillo (125) this author describes sympathetic fibers from the carotid plexus (as prominent nerve fiber bundles) which end in contact with, or between, the glandular cells of the pars distalis.

Maiman (281) found chromatolytic changes in the superior cervical ganglion after destruction of the anterior lobe in dogs. This result was not confirmed by Mahoney (279) in the chimpanzee. Nerve fibers are still present in the pars distalis of the cat (184), rat and rabbit (53) after superior cervical ganglionectomy. In the rabbit they are said to be less than normal in number (53). It should be remembered that sympathetic fibers with cell stations in the scattered ganglia along the internal carotid artery may reach the gland, or alternatively, that fibers from the vertebral plexus may be concerned. Phillips (331) recorded action potentials from the pars distalis during stimulation of the cervical sympathetic trunk. Collin and Hennequin (86, 87, 88) and Popjak (340) have observed histological changes in the pituitary and hypothalamus, respectively, after removal of the superior cervical ganglion.

Many fragmentary claims have been made that stimulation or removal of the cervical sympathetic system affects the secretion of the gonadotropic hormone by the pars glandularis. Several workers have shown (147, 202, 416) that cervical sympathectomy abolishes or reduces the pseudopregnancy response that normally follows artificial stimulation in the rat. (Pseudo pregnancy in the rat, and ovulation with consequent pseudopregnancy in the rabbit, may be induced by sterile mating or some form of artificial stimulation. There is much evidence that in these reactions, the anterior lobe of the pituitary gland is activated by a nervous reflex.) Ball (11) thinks a quantitative variation in the stimulus may be, at least partially, responsible for this result. Friedgood and Bevin (149) also report that removal of the superior cervical ganglion, cervical sympathectomy or blank neck operations may induce an immediate pseudopregnancy, while Britt (41) found psychic stimuli, which previously caused an oestrogenic response in rats, were ineffective after sympathectomy. In a few rabbits, Friedgood and Pincus (152) produced ovulation and maturation of ova by stimulation of the cervical sympathetic trunk, but more recently Friedgood and Cannon (150) failed to obtain ovulation following sympathetic stimulation, though maturation of ova occurred. These results are unconvincing as a demonstration of a secretory nerve supply to the adenohypophysis, for the specificity of the pseudopregnancy response in the rat would appear in doubt, and the slight ovarian changes in the rabbit may have been due to vasomotor effects on the hypophysial vessels. The large mass of negative evidence forces the conclusion that the sympathetic system plays little part in the control of secretion of the gonadotrophic hormones. Pseudopregnancy still follows sterile coitus in the partially sympathectomized rat (147, 202, 416), ovulation still follows sterile coitus in the partially or completely sympathectomized rabbit (48, 203, 224, 417) and cat (69) and may follow stimulation of the tuber cinereum after cervical sympathectomy in the rabbit (196). Stimulation of the cervical sympathetic nerves has failed to produce ovulation in the rabbit in the hands of most workers (150, 193, 203, 284). Perry (329) obtained not a stimulation but a regression of the reproductive system following administration of adrenalin to female rats.

It has been claimed that the cervical sympathetic system may affect the output of the thyrotropic hormone from the adenohypophysis (143, 406). Stimulation of the cervical sympathetic either by anastamosis with the phrenic nerve (151) or electrically (188) has been found to increase the metabolic rate. Contrary results have, however, been reported (45, 148, 283). More significant evidence has been obtained by Brock, Doty, Krasno and Ivy (45), who obtained a marked fall in metabolic rate in seven out of nine rabbits and two out two cats following cervical sympathectomy, and by Uotila (406) who noted a temporary and mild hypoactivity of the thyroid in rats following a similar operation. It should not be forgotten, however, that removal of the sympathetic system in cats (69) and rats (262) may be performed without any very significant change in the metabolic rate, and further that the cervical sympathetic system may well exert a vasomotor control over the hypophysial or thyroid vessels.

(*b*) OTHER AUTONOMIC NERVES. Cobb and Finesinger (83) and Chorobski and Penfield (78) described parasympathetic fibers in the cat and monkey which run from the greater superficial petrosal nerve to join the carotid plexus. These were shown

to carry vasodilator fibers for the pial vessels. Hinsey and Markee (224) suggested this pathway might be involved also in transmitting secretomotor impulses to the adenohypophysis. In the rat, Zacharias (446) reported that a branch from the Vidian ganglion at the junction of the superficial and deep petrosal nerves runs to at least the capsule of the pituitary gland.

Experimental support for a parasympathetic innervation of the adenohypophysis appeared to be forthcoming when it was found that anesthetization of the nasal mucous membrane (378) or extirpation of the spheno-palatine ganglion (356) was followed by pseudopregnancy in approximately 50 per cent of rats. It was further found (447) that removal of the Vidian ganglion produced a pseudopregnancy response in 100 per cent of the cases, and an increased insulin sensitivity in 66 per cent, although section of the greater superficial petrosal nerve which caused pseudopregnancy in a high percentage of cases had no effect on insulin sensitivity. This possible innervation of the adenohypophysis cannot be of more than subsidiary importance, for female rats deprived of the sphenopalatine ganglion show normal sex functions as far as the date of the vaginal opening, reproductive capacity and pseudopregnancy response to stimulation of the uterine cervices are concerned (379). In the rabbit also, bilateral avulsion of the facial nerve and geniculate ganglion (185) and bilateral section of the greater superficial petrosal nerve (418) did not prevent ovulation following coitus. The immediate pseudopregnancy response of rats following operative trauma would appear to be a non-specific reaction, for Friedgood and Bevin (149) report pseudopregnancy occurs in 64 per cent of animals following bilateral removal of the superior cervical ganglion, in 39 per cent following bilateral cervical sympathectomy and in 23 per cent following blank neck operations. The fact that in the rabbit ovulation follows coitus even after the intravenous injection of large doses of atropine (282) again suggests the parasympathetic system plays little part in this response.

(*c*) HYPOTHALAMUS AND HYPOPHYSIAL STALK. Cajal (63), who gave the first clear description of the nerve fibers in the pituitary stalk, mentioned that some of these fibers penetrate between the epithelial cells of the pars intermedia. That nerve fibers enter this part of the adenohypophysis has been confirmed by many workers (31, 53, 56, 89, 162, 163, 171, 184, 335, 343, 344, 358, 397, 403, 412). Some authors indicate these nerve fibers to be relatively numerous (184, 397, 403), though most recent accounts describe them as being scanty (53, 171, 343, 344). Tello (397) described intercellular and intracellular endings for these fibers, but most workers have found only simple intercellular nerve terminals in the pars intermedia (184, 344).

The pars tuberalis appears to receive some nerve fibers from the hypothalamico-hypophysial tract as it lies in the median eminence and infundibular stem (65, 123, 171, 184, 344, 358). No very close relationship of nerve terminals to cells has been seen here, though a close neuro-vascular relationship has been noted (171). Vasquez-Lopez (412) described a close relationship between the nerve fibers and blood vessels in the narrow cortical zone of the median eminence and stalk, that is, the tissue adjacent to the pars tuberalis.

The innervation of the pars distalis is a much debated subject. An absolute (57, 163, 216) or relative (18, 53, 89, 171, 344, 397) lack of nerve fibers in this structure

has been reported many times, though a few workers describe the presence of numerous nerve fibers (184, 403). Particular attention is drawn to the careful work of Rasmussen (334), who gives a summary of the literature. Pericellular nerve nets have been described around the glandular cells of the pars distalis (53, 335), though as pointed out by Green (170), the published illustration of these nets (232) resemble the reticular nets previously described by Tello (397). Truscott (403) describes a well marked innervation to the pars distalis of the rat, passing forward from the neurohypophysis. This finding is not in accordance with previous observations on the same animal (53). Attempts have been made in the rat and rabbit to determine the origin of the nerve fibers in the pars distalis, by studying the glands of animals after section of the pituitary stalk and after cervical sympathectomy (53). Stalk section was found to eliminate most of the nerve fibers to all parts of the pituitary, while sympathectomy caused a partial reduction of nerve fibers in the pars distalis of the rabbit and no detectable reduction in the rat. Of much interest are the studies by Drager on the nerves to the pars distalis of the porpoise, bird (123, 124) and armadillo (125). In these forms a connective tissue septum intervenes between the infundibular process and pars distalis, thus limiting the route for transference of nerve fibers from the neurohypophysis to adenohypophysis. No hypothalamico-hypophysial nerve fibers were found in the pars distalis of the bird or porpoise, and only a few in the armadillo. These observations on the bird's pituitary are of especial significance, since gonadal activity in the bird appears to be particularly responsive to environmental change.

In summary, it may be said that the hypothalamic innervation of the adenohypophysis appears scanty if it constitutes a true secreto-motor nerve supply to the organ.

There is some experimental evidence that interference with the hypothalamus or pituitary stalk, by damage or stimulation, affects the activity of the adenohypophysis. This work will now be considered with respect to the actions this gland exerts through the gonadotrophic, lactogenic, thyrotrophic, adrenotrophic and diabetogenic hormones.

(i) *Hypothalamus and gonadotrophic hormones.* That damage of the hypothalamus may lead to genital atrophy is well known from clinical observations (quoted above) and from the early observations of Camus and Roussy (67), Bailey and Bremer (8) on dogs and Smith (383) on rats. Cushing (90) suggested Smith's results were due to interference with hypophysial blood supply or of interrupted nerve supply. More recent observations indicate that hypothalamic injuries may interfere with sexual functions through damage to the nervous structure underlying the integration of reaction patterns of sexual behavior, or through damage to some neural mechanism controlling the output of gonadotropic hormones from the pituitary. In the normal animal these two mechanisms are doubtless closely inter-related, but in experimental work they are frequently separated, studies of the first type often being performed on gonadectomized animals under controlled hormonal administration (12, 108).

Cahane and Cahane (60, 61) noted that hypothalamic lesions may produce genital atrophy in the rat. Following the observation that female cats with diabetes insipidus (produced by lesions in the hypothalamico-hypophysial tract) never bred in

the laboratory (141), Dey, Fisher, Berry and Ranson (116) and Dey (113, 115), found that lesions in guinea-pigs situated between the optic chiasma and pituitary stalk resulted in sexual disturbances. Some animals with lesions anterior to the mammillary bodies in variable sites showed normal oestrous cycles though many would not mate; others with lesions between the chiasma and median eminence showed a constantly open vagina, hypertrophied external genitalia and a lack of corpora lutea in the ovaries; while a third group with lesions at the junction of the hypothalamus and pituitary stalk showed genital atrophy with a constantly closed vagina. Loss of cyclical phenomena and genital atrophy were found more commonly after lesions in the median eminence than after lesions in other sites in the hypothalamus or in the pituitary stalk (114) or after hypophysial lesions (115, 118). In rats Hetherington and Ranson (217) describe obesity and sexual dystrophy following lesions in the tuber cinereum, as do Biggart and Alexander in dogs (21), and it has been stated (434) that an uninterrupted connection of hypothalamus, pars tuberalis and pars distalis is needed to ensure a normal production of gonadotrophic hormone. In spite of the claim of Diaz (119) that interference with the oestrous rhythm may be consequent to nonspecific trauma, such as nephrectomy, it would seem probable that lesions in the tuber cinereum which interrupt the oestrous cycle exert their effects by altering the hormonal output of the pituitary gland. The most direct route by which this effect could be mediated is the pituitary stalk, but data derived from experiments in which this structure has been sectioned are far from harmonious.

Section of the pituitary stalk in rats has been claimed to produce lengthened oestrous cycles (352), gonadal atrophy in male and female animals (46, 429, 430) and normal, prolonged or absent cycles (in the rats showing normal cycles, pseudopregnancy occurred following sterile coitus, 109, 110). Westman and Jacobsohn (429), who noted gonadal atrophy in the rat following stalk section, inserted a small silver plate between the cut ends of the stalk. In the rabbit stalk section has been reported to produce gonadal atrophy (196, 223, 432). Oestrous rabbits have been obtained following this operation (50, 54), however, though the normal ovulatory response to mating was absent in these animals. Brooks (50) investigated the blood vessels of the stalks in some of his operated animals and stated that they either had not been completely destroyed by the operation or had been reestablished in part. Westman and Jacobsohn (432) found that transection of the pituitary stalk in rabbits, within forty minutes of copulation, did not prevent subsequent ovulation, but injection of novocaine solution through the foramen opticum of unanesthetized female rabbits (433), which were then mated, did prevent the ovulatory response. Transection of the stalk in guinea-pigs may cause little interference with the reproductive system (106) or may be followed by normal, irregular or absent cycles (263). In dogs, silver clips applied to the pituitary stalk (280), or division of the stalk (121), caused sexual atrophy, while other workers (252) reported normal sex functions in the bitch following stalk section. Breckenridge (40) removed the hypophysial stalk and upper part of the pars anterior in nine dogs and found two bred normally, four showed anatomically normal reproductive tracts and three showed atrophic sex organs. There was no correlation between the amount of pars anterior tissue remaining and the state of the reproductive system. Brooks (51) makes brief mention of similar experiments on

monkeys. Dandy (99) describes a case of stalk section in a young adult woman that was followed by normal menstrual cycles, pregnancy, labor and lactation.

From this evidence it seems that lesions of the median eminence produce a greater disturbance of sex function than section of the pituitary stalk. It appears unlikely that all the cases of sexual dysfunction reported to follow lesions of the stalk can be explained in terms of concurrent damage to the median eminence or hypophysis. A more likely hypothesis is that lesions of the median eminence irreparably damage a neural mechanism which normally exerts a controlling influence over the activity of the adenohypophysis by vascular transmission down the pituitary stalk. These vessels might undergo regeneration following stalk section.

Stimulation has been performed by electrical and chemical means. The electrical method was first used by Marshall and Verney (291), who found strong diffuse stimulation of the brain of the oestrous rabbit was followed by ovulation and pseudopregnancy. Harris (195) showed pseudopregnancy followed similar stimuli applied to the rat. Attempts were made to localize the active focus, and it was shown that stimulation of the pituitary gland directly (196), the tuber cinereum (196 and see 51), the preoptic region (205) or posterior hypothalamus (196) produced ovulation in the rabbit. Stimulation of the tuber cinereum was still effective after cervical sympathectomy (196), though previous cauterization of the pituitary stalk prevents ovulation following brain stimulation (428). More recent attempts to delimit the focus (284) indicate that direct electrical stimulation of the pituitary gland is not followed by the follicular rupture which occurs after similar stimulation of the hypothalamus. The most reliable technique for investigating this problem is the remote-control method of stimulation, which renders unnecessary any anesthesia or concurrent trauma. With this procedure stimulation of the tuber cinereum is more effective in eliciting ovulation than stimulation of the infundibular stem or adenohypophysis (201). The fact that repeated electronarcosis of guinea-pigs (128) causes increased ovarian weight may be a further example of neural excitation of the adenohypophysis.

Intravenous administration of copper (134) and cadmium (131) salts, picrotoxin (292), metrazol (52) or various plant juices (38) is followed by ovulation in the rabbit. Other drugs and salts [pilocarpine, eserine, acetylcholine and adrenaline (291); strychnine, apomorphine, β-tetrahydro-naphthylamine, ergometrine, doryl, coriamyrtin (292); strychnine, insulin, benzedrine and ephedrine (52); salts of barium, cobalt, gold, iron, manganese, nickel, silver and zinc (131)] have been tried without success. It was first believed that the active substances might produce their effects by augmenting the action of the circulating pituitary hormones. Bischoff (22) then suggested that copper salts act on some nervous mechanism, and this idea is now supported by the evidence that transection of the hypophysial stalk, before or shortly after the intravenous injection of copper acetate, picrotoxin or metrazol inhibits the usual ovulatory response (52), and further, that the dose of copper acetate necessary to produce ovulation on injection into the region of the third ventricle is approximately $\frac{1}{200} - \frac{1}{300}$ of the intravenous dose (197). Intravenous administration of copper salts to oestrous rats is followed by pseudopregnancy (126). Pretreatment with oestrone makes it possible to induce pseudopregnancy or ovulation and pseudopregnancy, in met- or di-oestrous rats or anoestrous rabbits, respectively, by intravenous

injection of copper acetate (127). On the other hand, administration of progesterone to oestrous (post-partum) rabbits blocks the ovulation induced by copper salts (153). Injection of yohimbine is also reported to upset the normal sex rhythm in rats, producing a state of constant oestrus of three to nine days' duration (156).

Although intravenous injection of acetylcholine is not followed by ovulation in the rabbit (291), Taubenhaus and Soskin (394) claim that direct application of an acetylcholine-prostigmine mixture to the exposed pituitary glands of rats results in pseudopregnancy, and that similarly applied atropine prevents the pseudopregnancy which normally follows electrical stimulation of the uterine cervix. This evidence is taken by the authors to support the view that the adenohypophysis is normally excited by a humoral transmission from the hypothalamus via the hypophysial portal vessels in the pituitary stalk.

A further method whereby the adenohypophysis may be excited is by increased illumination. It is well known that increased illumination in midwinter causes female ferrets to come into full oestrus earlier than control animals (23). Ultraviolet light is more active in this respect than luminous rays (290). Ferrets in which the pituitary has been removed are non-responsive to extra illumination (220), and similarly, if the optic nerves are severed (24, 80), the cycles are freed from seasonal or photic influences or do not occur at all. It seems likely from this and other evidence that in the ferret, light stimulates the adenohypophysis by a neural, or neuro-humoral pathway from the retina. There is evidence that the nervous impulses pass from the optic tract either to the ventral nucleus of the lateral geniculate body or to the subthalamus by way of the accessory optic tracts (80). However, it is claimed (244) that the ferret lacks any accessory optic tracts. The final pathway serving this reflex activation of the pituitary remains obscure. It has been shown in the drake that light which has a marked effect in stimulating the testis of the normal bird is also effective after enucleation of the eyeballs. Gonadal stimulation was also obtained if the light was directed, by means of a quartz tube, on to the region of the pituitary gland (16, 17). These latter results are difficult to evaluate.

(ii) *Hypothalamus and lactogenic hormone.* It seems probable that nervous activity plays a part in the removal of milk from the actively secreting breast [the 'letting down' of milk from cattle (186)], but it is unlikely that it is directly concerned with the actual secretion of milk, which would appear to be under hormonal control by the pituitary gland. Stricker (390) showed that a mammary gland grafted into a rabbit's ear continues to secrete providing normal glands are being suckled. Also, lactation may occur normally in the completely sympathectomized cat (69), though some reports indicate that occasional impairment of lactation follows sympathectomy (7, 68, 380).

The idea was advanced by Selye and his co-workers (370, 373, 374, 375) that lactation is maintained in rats and mice by the act of suckling, which stimulates the adenohypophysis by some nervous pathway to liberate the lactogenic hormone. It was found that the stimulus of suckling prevents involution of the mammary glands, even those from which the nipple had been excised and no milk was removed. Weichert (424) found that the posterior breasts tend to be neglected in rats with small litters, but that regression of these is delayed by suckling of the more anterior glands.

Ingelbrecht (236) showed that severance of the spinal cord, in lactating rats, between the last dorsal and first lumbar segments resulted in failure of milk secretion and death of the young if only the posterior, anesthetic nipples were suckled, whereas if suckling of the anterior nipples was allowed milk secretion was maintained in all glands. The observations of Hooker and Williams (226) that mammary involution (after removal of the young) is retarded in all breasts by application of spirits of turpentine twice daily to all or some of the nipples also support Selye's theory. The influence of suckling on the lactogen content of the pituitary gland in rats and rabbits has been studied (300, 348).

The nervous pathway by which the stimulus of suckling excites the adenohypophysis is unknown. That lactation can occur normally in sympathectomized animals eliminates the cervical sympathetic system. The data concerning the hypophysial stalk are inconclusive. Desclin (111) and Herold (215) claim that transection of the stalk in rats causes failure of lactation in spite of functioning anterior pituitary tissue and continued suckling. Jacobsohn and Westman (243) found that the mammary involution which occurs in lactating rats following transection of the pituitary stalk is not so complete as that which occurs following hypophysectomy and state that their results do not uphold Selye's views. According to Dempsey and Uotila (110), however, stalk-transected rats may lactate normally. These latter results would indicate that the nerve fibers in the stalk are not essential to the mechanism.

(iii) *Hypothalamus, thyrotrophic and adenotrophic hormones.* Lichtwitz (267) suggested that the hypothalamus reacts to variations in thyroxine concentration, thus regulating and controlling the secretion of the thyrotrophic hormone from the pituitary. The evidence concerning this hypothesis is controversial.

Hypothalamic lesions have been stated to affect metabolic rate and thyroid histology. With lesions in the central hypothalamus, a fall in metabolism was observed in cats (29). Cahane and Cahane (62) noted that infundibular lesions may produce a histological picture of increased or decreased activity in the thyroid gland. From this and other evidence they postulated the presence of two centers in the infundibular region, one situated between the optic chiasma and stalk which excites the secretion of the thyrotropic hormone and the other in the group of tubero-mammillary nuclei which inhibits the secretion of this hormone. Grafe and Grunthal (166) noted a fall in metabolism of 35 per cent on destruction of the tuberal centers in a dog.

Section of the hypophysial stalk has been shown by most workers to have little effect on the metabolic rate or thyroid histology. Uotila working on stalk-sectioned rats found that the thyroid remained histologically normal (405), that it reacts with the usual atrophy to injections of thyroxine or with the usual compensatory hypertrophy after subtotal thyroidectomy (408), but that the normal hypertrophy evoked by a cold environment is lacking (405, 407). He suggests that the basic secretion of the thyrotrophic hormone is humorally controlled and independent of the pituitary stalk, but under certain environmental conditions this basic secretion can be modified by hypothalamic impulses which reach the hypophysis via the pituitary stalk. Brolin (46) records a decrease of thyroid activity following stalk section in rats and agrees with Uotila that cold exposure of such animals does not lead to the usual hypertrophy. Uotila (407) also found that the adrenal cortex was normal in weight

and histological appearance after stalk section, and that unlike the thyroid, some hypertrophy was elicited by the stimulus of cold. Brooks (50) remarks that the thyroid and adrenal glands of stalk-cut rabbits were generally of normal weight and showed no obvious histological abnormalities. Mahoney and Sheehan (280) described metabolic changes in puppies following stalk section as evinced by retardation of growth, sexual infantilism, adiposity, refinement of hair and sluggish general behavior. Since the pituitaries of these animals showed marked degenerative changes, they feel the condition may be explained by interference with the major blood supply to these glands. This work confirms earlier work on dogs, such as that of Dott (121), who divided the stalk and inserted a small platinum plate between the pituitary gland and tuber cinereum. He reported among other signs a lowered body temperature and degenerative changes in the thyroid gland. In stalk-sectioned monkeys, Mahoney and Sheehan (280) found a normal metabolic rate. Westman, *et al.* (435) divided the pituitary stalk in 14 rabbits and found that in 11 cases the thyroid gland exhibited the histological picture of diminished function. All their rabbits showed marked genital atrophy. These latter findings stand in contrast to those of Brooks (50).

The effect of stimulation of the hypothalamus or hypophysis upon thyroid or adrenal function has not been the subject of much study. Saxton and Greene (365) report that the stimulus of coitus causes the liberation of thyrotrophic and perhaps corticotrophic hormone from the hypophysis of female rabbits as well as the gonadotrophic hormone(s). Ellis and Wiersma (128) found that repeated electronarcosis of dogs and guinea-pigs caused hypertrophy of the thyroid and an increase in thyrotrophic hormone in the blood. Preliminary experiments by Green and Harris (172) have failed to demonstrate any increase in oxygen consumption in rabbits following electrical stimulation of various regions of the hypothalamus and hypophysis. Stimulation was applied by the remote control method for periods up to three hours daily for one week. It is possible that further work, involving longer periods of stimulation of various regions, may produce different results.

There is some evidence then, that the secretion of the thyrotrophic hormone is under hypothalamic control, mediated by the pituitary stalk.

(iv) *Hypothalamus and the diabetogenic hormone.* Hypophysectomy produces an increase in insulin sensitivity (229), a tendency to hypoglycemia and amelioration of a pre-existing pancreatic diabetes (228). Injection of anterior pituitary diabetogenic extracts may produce the reverse effects. It is of interest, therefore, with regard to hypothalamic control of the adenohypophysis, to consider how far these states may be duplicated by lesions or stimulation of the hypothalamus or hypophysial stalk. For a previous review of this subject see Long (270).

It has long been known that injury or puncture of the tuber cinereum may be followed by glycosuria (6, 67, 423). In many cases the glycosuria was of an acute nature, and it seems likely that it was induced by excitation of the sympathetic system rather than the pituitary gland.

An increased sensitivity to the action of insulin has been reported to follow hypothalamic lesions. Ingram and Barris (239) showed that lesions in the anterior, suprachiasmatic portion of the hypothalamus in cats produce an increase in insulin

sensitivity and a lowered sensitivity to adrenaline. Cleveland and Davis (82) found comparable changes in the reactions to insulin and adrenaline followed lesions in the medial hypothalamus, which included the paraventricular and ventromedial hypothalamic nuclei. Keller (250) similarly reports a markedly increased sensitivity to insulin in the majority of cats with large hypothalamic lesions, though there was little correlation between the extent and site of the lesion and the decrease in insulin tolerance. After hypothalamic lesions in many cats Brobeck (42) found insulin hypersensitivity in a very small proportion of cases. Barris and Ingram (13) found hypoglycemia followed lesions in the anterior hypothalamus, especially if the paraventricular nuclei were damaged. Similar hypoglycemic effects have been reported by Keller and his associates (97, 250, 253) and by Cleveland and Davis (82). Bloch (30) reports only a wider range of blood sugar than normal after destruction of the medial hypothalamic area in cats. Amelioration of a pancreatic diabetes by previous hypothalamic injury has been observed in cats by Davis, Cleveland and Ingram (100) and Cleveland and Davis (82). However, this finding was not confirmed by Brobeck and Long (43) in partially depancreatized rats. Ingram (237) in a recent report states that hypothalamic lesions greatly reduce the insulin requirments of pancreatectomized cats (9 out of 13), though there was no specificity as regards localization of the lesion. It would appear then that the cardinal signs of interference with carbohydrate metabolism which follow hypophysectomy may also be found with lesions in the hypothalamus. The possibility that the neural lesions exert their effects by pituitary mediation may explain the frequent association by different workers between their lesions and the paraventricular nuclei. Paraventriculohypophysial connections have now been proven to exist, and there is no evidence associating these fibers with the functions of the neurohypophysis.

It is generally agreed that section of the hypophysial stalk produces slight or no derangement of carbohydrate metabolism. Brooks (50) found that stalk-cut rabbits showed a normal blood sugar level and sugar tolerance, and there was no increased sensitivity to insulin. However, Westman and Jacobsohn (432) state that rabbits with transected stalks, in spite of normal blood sugar values, show the same extreme sensitivity to insulin after 24 hours' starvation as shown by hypophysectomized rabbits. Mahoney and Sheehan (280) noted no significant variation of blood sugar to follow stalk section in monkeys. These results have been confirmed and extended by Brobeck, Magoun and Ranson (44).

Stimulation of the lateral hypothalamus was shown by Himwich and Keller (222), and Magoun, Barris and Ranson (276) to produce hyperglycemia. As pointed out by Fulton (157) this acute phenomenon is probably brought about by activation of the sympatho-adrenal medullary system at the hypothalamic level. Lewy and Gassmann (266) obtained a more prolonged hyperglycemia lasting a few days following stimulation of the (perifornical) periventricular nucleus. In preliminary experiments Harris (201) has obtained a marked decrease in insulin sensitivity following prolonged stimulation (one to three hours daily for one week) of the tuberal region in rabbits. This glycotrophic effect is maximum after one week and slowly decreases after ceasing stimulation. These time relations may indicate a slow hypertrophy of the adrenal cortex as suggested by the work of Jensen and Grattan (168, 245) and

Heinbecker and Rolf (210). A possible explanation for the onset of diabetes mellitus during a course of metrazol therapy (173) may be hypothalamico-hypophysial excitation by this drug.

3. *Difficulties in Accepting a Direct Secreto-motor Innervation*

The outstanding features at the present time are the large bulk of evidence relating the activity of the adenohypophysis to neural control in general and hypothalamic control in particular; the conflicting evidence concerning interference with adenohypophysial function that follows pituitary stalk section; and the scarcity of nerve fibers in the pars distalis. There is no convincing evidence implicating the peripheral autonomic system with pituitary activity.

The pars distalis of the pituitary may, in general terms, be described as a gland under nervous control but lacking a nerve supply. This statement would appear to be most literally true for the bird where exteroceptive factors play a large part in regulating gonadal activity in spite of the apparent lack of nerve fibers in the pars distalis (124).

There is much experimental and clinical evidence that the hypothalamus is functionally linked with anterior pituitary activity. The most direct route by which this influence might be exerted is by means of the nerve fibers or blood vessels of the hypophysial stalk. It seems possible to eliminate the nerve fibers of the stalk from participation, since section of the hypophysial stalk may not interfere very markedly with the functions of the gland. However the portal vessels of the stalk, unlike the nerve fibers, may undergo repair following stalk section, and so reestablish humoral transmission of stimuli to the pars distalis.

4. *Evidence Concerning Neuro-vascular Transmission of Stimuli to the Adenohypophysis*

Popa and Fielding (338, 339) were the first to describe a true portal system of blood vessels in the pituitary stalk. From a study of human material they described a system of vessels collecting blood from all parts of the pituitary gland and uniting into larger trunks which could be traced up the stalk into the hypothalamus, where they were seen to break up into the 'secondary net' of capillaries. Wislocki and King (442) and Wislocki (437, 439) confirmed the presence of these vessels, but differed from Popa and Fielding in describing the upper limit of the portal vessels as situated in the expanded lower end of the tuber cinereum (known as the median eminence), the direction of blood flow as being downward and the blood draining entirely into the pars distalis. Since the median eminence is part of the neurohypophysis, the term 'hypophysial portal system' was substituted for 'hypothalamico-hypophysial portal system.' Green and Harris (171) reinvestigated the region in a variety of mammals (rat, guinea-pig, rabbit, dog and man) and largely confirmed the findings of Wislocki. They described small arterial twigs running from the internal carotid and posterior communicating arteries to supply a large vascular plexus situated between the pars tuberalis and median eminence. From this plexus very characteristic sinusoidal capillary loops penetrate the median eminence where they are surrounded by the wealth of nerve fibers of the hypothalamico-hypophysial tract. These capil-

laries then pass into large trunks which run down the hypophysial stalk, draining into the pars distalis where the blood is redistributed by the large sinusoids of the gland. The pattern differs in detail in different animals; in the human the upper system of capillary loops and tufts being present in the infundibular stem as well as the median eminence. Nerve fibers of the hypothalamico-hypophysial tract, besides being intimately related to the sinusoidal loops in the median eminence, pass in moderate numbers into the pars tuberalis, where they may be seen associated with the vascular plexus.

Harris (196), Hinsey (223), Brooks (50), Taubenhaus and Soskin (394) and others, have mentioned the possibility that neural control of the adenohypophysis is humorally transmitted from the neurohypophysis to the pars distalis. From the anatomical studies mentioned above, it seems possible that nervous stimuli might cause the liberation of some substance into the capillary sinusoids of the median eminence, this substance then being transported via the hypophysial portal vessels to excite or inhibit the pars distalis. Evidence concerning this view has been summarized by Harris (198) and Green and Harris (171).

(i) Ovulation in the rabbit normally occurs 10 hours after coitus. If hypophysectomy is performed within one hour of coitus, ovulation does not occur (132). It appears that the pituitary must remain intact for one hour following coitus for the secretion of sufficient gonadotropic hormone to produce follicular rupture. Since the nervous stimulus of mating takes only a few minutes, this may indicate that one link in the chain of events is a slow, humoral excitation of the adenohypophysis. Westman and Jacobsohn (432) claim that transection of the stalk 40 minutes after coitus does not prevent the subsequent ovulation, so it would seem that the delay occurs in the pituitary gland itself.

(ii) Markee, Sawyer and Hollinshead (284) have shown that ovulation is more easily elicited by electrical stimulation of the hypothalamus than of the hypophysis. At the time of their report the same result had been obtained from experiments in which the hypothalamus or pituitary gland had been stimulated in rabbits by the remote control method (Harris, 201). Direct stimulation of the adenohypophysis or the hypothalamico-hypophysial tract in the infundibular stem for 15 hours, in hourly periods distributed over 5 days, failed to elicit ovulation, whereas similar stimulation of the tuber cinereum for three minutes (a time period approaching that of the normal stimulus of coitus) was followed by an ovulatory response. If the adenohypophysis is devoid of a direct secreto-motor nerve supply, it is possibly not excitable by direct electrical stimulation (cf. electrical stimulation of the denervated adrenal medulla, 70).

(iii) The different effects recorded after section of the hypophysial stalk (40, 50, 54, 106, 109, 110, 111, 121, 196, 215, 223, 252, 263, 280, 352, 405, 434, 435) may be explained by variations in degree of regeneration of the hypophysial portal vessels. The observation of Brooks (50) that the hypophysial portal vessels of his stalk-cut rabbits either had not been completely destroyed by the operation or had become reestablished in part is of interest in this connection. Those workers who have taken the precaution of placing small metallic plates between the divided ends of the stalk (121, 429) have described gonadal atrophy following the operation. India ink

injections of the vascular tree of rats with divided stalks (201) indicate that regeneration of the hypophysial portal vessels may occur. The observation of Westman, Jacobsohn and Hillarp (434) that it is the connection of the pars tuberalis with the hypothalamus and not that of the infundibular process which is of significance in preserving a normal cytology of the pars distalis and a normal output of gonadotrophic hormone indicates the importance of the structure bearing the portal vessels.

(iv) The results of Dempsey (106), Dey (114) and Leininger and Ranson (263) indicate that a greater disturbance of the oestrous cycle in the guinea-pig may follow a lesion in the median eminence rather than section of the hypophysial stalk. This might be due to the former producing a complete, irreparable denervation of the median eminence and sinusoidal capillaries, while stalk section allows the possibility of vascular repair.

(v) In some animals, such as the whale (Wislocki and Geiling, 441), porpoise (Geiling, Vos and Oldham 160), sea-cow (Oldham, McCleery and Geiling, 322), armadillo (Oldham, 321) and Indian elephant(Wislocki, 440) the infundibular lobe is separated from the pars intermedia, or pars distalis if the intermedia is lacking, by a connective tissue septum derived from the dural capsule of the gland. This septum eliminates the main pathway for nerve fibers between the infundibular process and pars distalis. However, from the literature it seems these animals have a pars tuberalis in contact with the median eminence and probably a portal system of vessels. The pars distalis of the porpoise is said to be free of any nerve fibers (123), but this animal shows a very well marked hypophysial portal system originating in the median eminence, passing ventrally in front of the connective tissue septum separating the two lobes and ending in the pars distalis (200).

(vi) Taubenhaus and Soskin (394) state the pars distalis of the rat may be stimulated by local application of a prostigmine-acetylcholine mixture. They adduce this fact, among others, as evidence for the theory of humoral control of the adenohypophysis.

At the moment there is insufficient evidence available to estimate the functional importance of the few nerve fibers which enter the adenohypophysis or of the hypophysial portal vessels. Further information regarding this basic problem of endocrine physiology is eagerly awaited.

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FERTILIZATION AND IMMUNITY[1]

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IN THE PRESENT ARTICLE an attempt is made to review the work on fertilization which, for the most part, correlates the reactions involved with those of immunological processes. Such correlation has been indicated by many earlier writers. Thus Loeb (124) pointed to the similarity of fertilization to phagocytosis, although he did not go much beyond the superficial resemblance of these two processes. A closer correlation is derived from the presence in eggs and sperm of specific substances that interact in the manner of antigens and antibodies. F. R. Lillie (107–118), who provided the first substantial demonstration of one of these substances, which he termed fertilizin, also attempted a description of the fertilization process in immunological terms. The correlation becomes even stronger as a result of recent work showing that an enzymatic lytic agent termed hyaluronidase may be involved in both fertilization and infection.

In many respects present-day knowledge of immunological reactions is further advanced than is that of the reactions of fertilization. So, to the extent to which the two may be correlated, immunological principles may help explain various features of fertilization. However, it can be anticipated that the relation will not remain one-sided and there are, in fact, already indications of the directions from which studies on fertilization may contribute to our knowledge of immunity

There are four specific interacting substances, or groups of substances, of eggs and sperm which will be considered in this review. These have been designated fertilizins of eggs, antifertilizins of sperm, antifertilizins of eggs and lytic agents of sperm. Hartmann (64) has introduced the term gamone to designate the substance derived from eggs (gynogamones) and sperm (androgamones). However, the hormonal analogies of that terminology seem at least as objectionable as the implication of Lillie's terms. The term ovulin, suggested by Dalcq (26), and a corresponding spermulin might be preferable, but since the fertilizin terminology is so widely known it would be best to adhere to it for the present. When the various substances become better characterized, designation can, of course, be more readily made on the basis of specific chemical action and properties. Thus one of the lytic agents obtained from sperm is now termed hyaluronidase because of its enzymatic action on a polysaccharide known as hyaluronic acid (134).

FERTILIZIN

A. EFFECTS. Several early embryologists in studies on fertilization in echinoderms had noted effects of egg extracts on spermatozoa of the same or foreign species. In

[1] Work of the author reviewed here has been aided by grants from The Rockefeller Foundation.

particular, agglutinative and other effects had been observed by von Dungern (231), Buller (6), Schücking (192) and DeMeyer (29) but little attention was paid to these until F. R. Lillie (107–116) initiated an extensive series of investigations of the subject. Lillie showed that the supernatant sea water (termed egg water) from a suspension of ripe, uninjured, sea urchin eggs would, when added to a suspension of sperm of the same species, cause an agglutination of the spermatozoa. Another effect of the egg water that he noted consisted in an increase in the activity of the spermatozoa. A third effect, namely chemotaxis of spermatozoa by egg water, has also been claimed by various workers (see 144, for critical review) but this still requires adequate demonstration. A fourth effect of egg water consists in inducing spawning of ripe males of certain species of animals. While the term fertilizin has been used to designate the substance or substances in egg water having all of these as well as other related effects, the agglutinative action is the one most generally employed in the tests.

1. *Agglutination.* The agglutinating action of egg water upon spermatozoa of the same species has been observed in a large number of species of animals, particularly among the echinoderms A fairly complete list of species in which such agglutination has been reported is given in table 1. Certain of these require some qualification. Thus, the report of agglutination in the fish Fundulus and the frog by Glaser (52) is contained simply in a parenthetic sentence, so Montalenti and Schartau's (141, 191) account in the Lamprey is the first real description of the phenomenon in vertebrates. In Chiton tuberculatus, Southwick (195) reports negative results with egg water but confirms Crozier's (25) finding of agglutination of spermatozoa that have passed through the mantle cavity of a male or immature female. In the starfish several workers (190, 91, 204, 133) find no agglutinative effects of ordinary egg water. Negative results are, of course, not frequently reported in the literature since failure can often be attributed to lack of sufficient trial under proper conditions. However, in addition to the cases mentioned above, it seems reasonably certain that agglutination of sperm does not occur with ordinary egg water in several species of chitons, the abalone and Cumingia (190) nor in Urechis campo (204). Even among the echinoids the phenomenon is evidently not universal since Vasseur and Hagström (230) report failure of isoagglutination in Brissopsis lyrifera.

Lillie (114) was aware of the fact that in many species of animals the agglutinative action of egg water was lacking. Nevertheless, he assumed that an analogous fertilizin was present in such species but in insufficient concentrations or in altered conditions, so that the readily visible agglutination was not apparent. Support for this view will be presented below in a discussion of 'univalent' fertilizin.

In the agglutination reaction in the sea urchins the sperm ordinarily unite mainly by their heads (114, 36). However, tail to tail and head to tail as well as head to head unions have been reported in other groups of animals such as the chiton Katharina tunicata (190) and the keyhole limpet Megathura crenulata (202). They may occur in sea urchins, too if the sperm suspensions are allowed to age (190). The types of reaction obtained with egg water are quite similar in appearance to those observed in the agglutination of sperm in antisera, such as figured by Henle, and Chambers (78) for bull spermatozoa. The latter authors have demonstrated separate antibodies for

head and tail antigens in such antisera produced in rabbits. For the action of the egg waters the evidence (202) indicates that only one kind of agglutinin is involved. However, the essential absorption tests with separate head and tail fragments of spermatozoa have not as yet been performed.

TABLE 1. SPECIES OF ANIMALS IN WHICH AGGLUTINATION OF SPERM BY EGG WATER HAS BEEN DESCRIBED

Echinoids
- Arbacia punctulata (Lillie, 1912, 1913)
- Arbacia pustulosa (Elster, 1935; Hartmann *et al.*, 1940)
- Strongylocentrotus purpuratus (Loeb, 1914; Lillie, 1921; Tyler, 1940)
- Strongylocentrotus franciscanus (Loeb, 1914; Lillie, 1921)
- Paracentrotus lividus (Just, 1929; Elster, 1935)
- Echinus microtuberculatus (Just, 1929; Elster, 1935)
- Echinus esculentus (Carter, 1932)
- Psammechinus miliaris (Vasseur and Hagstrom, 1946; Runnstrom and Lindvall, 1946)
- Echinometra subangularis (Southwick, 1939)
- Sphaerechinus granularis (Elster, 1935)
- Echinocardium cordatum (Elster, 1935; Vasseur and Hagstrom, 1946; Runnström and Lindvall, 1946)
- Echinarachnius parma (Just, 1919)

Asteroids
- Asterias forbesii (Glaser, 1914)
- Asterina pectinifera (Nomura, 1924)

Amphineuran mollusks
- Chiton tuberculatus (Crozier, 1922)
- Katharina tunicata (Sampson, 1922)

Gastropod mollusks
- Megathura crenulata (Tyler, 1940)

Pelecypod mollusks
- Ostrea virginica (Glaser, 1921)
- Ostrea circumpicta (Terao, 1926)
- Solen ensis (von Medem, 1942)
- Pecten varius (von Medem, 1942)

Polychaet annelids
- Nereis limbata (Lillie, 1912, 1913)
- Platynereis megalops (Just, 1915, 1930)

Cyclostome
- Lampetra fluviatilis (Montalenti and Schartau, 1941)

Fish
- Fundulus heteroclitus (Glaser, 1921)
- Salmo irideus (Hartmann, 1944, 1947)

Amphibia
- Rana pipiens (Glaser, 1921)

An interesting feature of the agglutination reaction, as it occurs in most species of sea urchins, is its spontaneous reversibility. Lillie (114) and Just (91) considered this feature to be characteristic of isoagglutination reactions as contrasted with the more permanent union they observed in the heteroagglutination of sperm by egg water or body fluids of a foreign species. However, Lillie (109, p. 552) noted that in Nereis "the agglutinations are essentially permanent" and irreversible isoagglutination has

also been described in Katharina (190), in Megathura (202) and even in a sea urchin, Echinocardium cordatum (230, 183). Spontaneous reversal cannot, then, be regarded as a criterion for typical isoagglutination. It is a phenomenon that is not ordinarily encountered in serological reactions and, therefore, requires further consideration. This will be given below in the section of 'univalent' fertilizin. Apart from the occurrence of spontaneous reversal in sea urchins, the agglutination of spermatozoa by egg water is essentially a serological type of reaction as Lillie (114) indicated. Even such features as the zone phenomena are manifested (202, 196) and other evidence, to be presented below, points to basic similarity to antigen-antibody reactions.

2. *Activation.* The stimulating action of egg water on the motility of spermatozoa has been noted in most of the species listed above, including many of those not exhibiting agglutination. Most of the observations on increased activity of sperm are highly subjective, so that it would be difficult to make a very useful list. When the spermatozoa are normally highly active, as is the case with freshly shed dilute sperm from fully ripe males of most species of sea urchins, an activating effect of egg water is not readily discernable, as Lillie (114) noted. However, when they are in a relatively quiescent condition, as a result of aging or special treatment, tests of activation by egg water may be made and several workers have reported such action in sea urchins as well as in other animals. Accompanying such increased activity increase in rate of oxygen uptake of the spermatozoa under the influence of egg water has also been noted in certain species. Thus Gray (59) presents data on the initial respiratory rate of Echinus esculentus sperm in egg water that ranged in different tests from 210 to 425 per cent of the control values. In another species, Echinus miliaris, no such increases are noted (59, 60), but rather a retardation in the normal decline in respiratory rate that occurs with time. The difference in response correlates with difference in activity of the sperm suspensions in the two species, those of E. miliaris being ordinarily much more active than those of E. esculentus. In Arbacia, Hayashi (72) notes a decrease in the rate of oxygen uptake under the influence of egg water. He attributes this to the agglutinating action of the egg water. However, it has been shown (e.g., Haurowitz and Schwerin, 70) that cells may be agglutinated by antibodies without reduction of their respiratory rate. In another species of sea urchin, Strongylocentrotus purpuratus, tests in this laboratory (196) have shown no increase in respiratory rate upon the addition of egg water to the suspensions. On the other hand, in the keyhole limpet, Megathura crenulata, the rate increases to between 300 and 400 per cent (221).

3. *Chemotaxis.* Various early investigators have presented evidence both for and against the existence of an attractive influence of eggs or egg water for spermatozoa. In an extensive review of the subject Morgan (144) concluded that the evidence presented in favor of such chemotaxis does not actually support it. The principal difficulty consists in distinguishing between chemotaxis and the trap action type of behavior described by Jennings (84). Although there has been a number of more recent reports concerning chemotaxis (21, 66–69, 230, 232), in none of these does this difficulty appear to be resolved.

4. *Spawning.* In marine animals the shedding of gametes by animals of one sex in

response to the presence of gametes of the opposite sex is quite commonly observed. Such stimulation of spawning has been reported in chitons (73), in nereids (117, 85), in sea urchins (43), in oysters (46–49, 158) and in mussels (250). In the case of the nereids and the oysters it has been shown that egg water alone will induce spawning in males of the same species. Properties of the spawning-inducing agent and its relation to the agglutinin present in egg water will be considered below.

B. CHEMICAL PROPERTIES. 1. *Agglutinin.* The source of the agglutinative agent of the egg water has been the subject of some controversy. Lillie (110, 116) showed that the gelatinous coat of the sea urchin egg contains the agglutinin in high titer but considered it to be continuously produced by the ripe unfertilized eggs as they remained in sea water. Loeb (122) on the other hand considered the agglutinin to be simply particles of the gelatinous coat. While Lillie's experimental data have been confirmed, recent evidence of Tyler and Fox (222, 223), Tyler (202, 204), Evans *et al.* (38), Hartmann *et al.* (69), Vasseur and Hagstrom (230) and Runnström and Lindvall (183) identifies the agglutinin with material of the gelatinous coat that goes into solution as the eggs stand in sea water, and rules out any continuous production or secretion by the eggs after they are shed from the ovary.

Lillie (110) showed that the sperm agglutinin of Arbacia egg water, by its failure to dialyze through a collodion membrane, is of large molecular size. Glaser (50) and Woodward (247) failed to obtain, with Arbacia egg water, the common protein tests except for a weak xanthoproteic test. More recently Tyler and Fox (222, 223) obtained positive xanthoproteic, biuret and Millon's reactions with agglutinin preparations of Strongylocentrotus and of Megathura prepared by dialysis and ammonium sulfate precipitation of 'uncontaminated' egg water, while Kuhn and Wallenfels (95) obtained positive ninhydrin reaction with an agglutinin (termed by them Hilfsträger) preparation of Arbacia. From various other chemical properties, including especially inactivation by crystalline preparations of the proteinases trypsin and chymotrypsin, it has been concluded by Tyler and Fox (222, 223) that the agglutinins of the sea urchin and the keyhole limpet are of the nature of proteins. However, the nitrogen content is considerably lower than the values usually obtained with simple proteins. Values around five per cent have been obtained in Megathura and Strongylocentrotus (223) and 8.0 to 8.5 per cent in Arbacia (95). With agglutinin preparations of Strongylocentrotus that show a single electrophoretic boundary and from which the organic material can be practically completely absorbed by the sperm, nitrogen values of 5.6 to 5.7 per cent have been obtained (221).

It has been suggested by Carter (11) that the agglutinin is a substance related to thyroxine which, he finds, causes an irreversible agglutination of sperm of sea urchins and starfish. Possibly some such substance may be liberated from egg water but this remains to be adequately demonstrated.

For the preparation of the agglutinin of Strongylocentrotus purpuratus the following procedure has been found (221) to be most effective. The washed egg suspension is acidified to pH 3.5 which dissolves the gelatinous coat of the egg. A 20 per cent suspension of eggs thus yields an egg water having a titer (maximum dilution giving agglutination when tested with an equal volume of one per cent sperm suspension) of about 1000. Addition of 4 cc. 1 N NaOH per 100 cc. of the egg water completely

precipitates the agglutinin along with certain sea water salts. The precipitate is taken up in 3.3 per cent NaCl. The agglutinin is again practically quantitatively precipitated by the addition of five volumes of 95 per cent alcohol to four volumes of the saline solution in the cold. Larger amounts of alcohol give no increase in the amount of precipitate or agglutinin. The agglutinin can be reprecipitated from saline solution with alcohol or with ammonium sulphate, which Woodward (247), Tyler and Fox (222, 223) and Kuhn and Wallenfels (95) showed to be an effective salting-out agent. However, electrophoretically homogeneous preparations are obtained without the use of ammonium sulphate. An egg water with an agglutinative titer of about 4000 yields approximately one mgm. of alcohol-precipitable material per cc.

Runnström, Tiselius and Vasseur (189) have shown that the material of the gelatinous coat of Psammechinus eggs is of pronounced acidic character migrating electrophoretically to the anode at pH 6.0 to pH 3.8. Ultracentrifugation of a solution containing 0.26 mgm. N per cc. showed a main component with a sedimentation constant of 2.9×10^{-13} varying with concentration in the manner characteristic of elongated, gel-forming molecules. In the absence of measurements of double refraction of flow, estimates of the molecular weight cannot readily be made. They also report a positive carbohydrate reaction of the material by a carbazole method. Their solutions did not exhibit agglutinating action, which is weak and often lacking in Psammechinus (cf. 230). This may, however, be due to condition of the sperm or some modification of the material, so it is not unreasonable to assume that their data may apply qualitatively to agglutinins of other species.

With hydrolyzed (three hours boiling in one N HCl) agglutinin preparations of Strongylocentrotus carbohydrate reactions have been obtained (221) with Molisch's and related reagents. With phenylhydrazine an osazone having the crystal form of galactosazone has been obtained. The hydrolyzed agglutinin yields reducing sugars amounting to about 15 per cent (calculated as glucose) of the original material present. Using Kunitz's (99) method, glucosamine has also been found in the hydrolysate by Krauss (93) in amounts equivalent to about four per cent of the original material. In Arbacia, Kuhn and Wallenfels (95) find about two thirds of the nitrogen in the supernatant of a vigorously hydrolyzed agglutinin preparation to be amino nitrogen. It is evident that the agglutinin of sea urchins contains both sugars and amino acids and may, then, be considered a glycoprotein.

The agglutinins have been found to be heat labile (114). The inactivation time is a function of pH and differs considerably in different species (223). Both Strongylocentrotus purpuratus and Megathura crenulata agglutinins are most stable around pH 4 in sea water but, whereas the former is half inactivated in 15 minutes at 100°C., the latter requires over 24 hours. A similar difference is found in their rate of inactivation by trypsin and chymotrypsin. No coagulation occurs when the fertilizins are inactivated by boiling and the initial loss of agglutinating activity does not involve destruction of ability to combine with sperm.

2. *Activating agent.* In 1939 Hartmann, Kuhn, Schartau and Wallenfels reported that the sperm-stimulating agent of Arbacia pustulosa egg water is identical with a pigment known as echinochrome, which Ball (4) and Lederer and Glaser (102, 56) had

been able to isolate in crystalline form from sea urchins. This pigment occurs in the test and elaeocytes of sea urchins, but only in the genus Arbacia is it also found in the eggs. It is not surprising, then, that no activating influence of echinochrome, nor of the related spinochrome, was found (201) in experiments with Strongylocentrotus purpuratus. However, in Arbacia punctulata negative results have also been reported (21) and it has been suggested that the original stimulating action may have been caused by alkalinity of the solutions. Kuhn and Wallenfels (94–97, 233–238) have determined the structure of echinochrome and related pigments and find them to be substituted naphthoquinones, the first examples of this class of pigments to be found in animals. Although it is closely related and may be readily converted into compounds having vitamin K activity, echinochrome A (3,5,6,7,8-hydroxy, 2-ethyl, 1:4-naphthoquinone) itself was found (98) to have no antihemorrhagic action in chickens. Possibly it may act in such manner in sea urchins. According to Shapiro (193) it is partially released into sea water from fertilized but not unfertilized eggs.

Kuhn and Wallenfels (95) showed that, when eggs of Arbacia pustulosa are deproved of their gelatinous coat, frozen, thawed and extracted with sea water, the echinochrome is obtained in combination with a nondialyzable carrier. According to Hartmann *et al.* (69) this 'binary complex' has no stimulating or agglutinating action on spermatozoa but active echinochrome can be split off by means of dilute acid. If the eggs are not deprived of their gelatinous coat the extract contains the latter material bound to the binary complex as a cocarrier (Hilfsträger) to form a tertiary complex which, they find, has both activating and agglutinating action. Upon dialysis against distilled water the binary complex precipitates out leaving the cocarrier in solution. The latter shows agglutinating but no stimulating action on the sperm. Cornman (21) has confirmed the stimulating action of a similarly prepared tertiary complex in Arbacia punctulata but attributes its activity to the protein portion. However, he and also Vasseur and Hagström (230) report that the activating agent is at least partially dialyzable. On the other hand Kuhn and Wallenfels (95) report that the activating agent in egg water fails to pass through cellophane even after several days dialysis against sea water. In Strongylocentrotus and Megathura, Tyler and Fox (222, 223) also find the activating agent to remain associated with the agglutinin upon precipitation with ammonium sulphate and dialysis. Clowes and Bachman (19) had noted that a sperm-stimulating agent can be distilled from egg water by boiling. Cornman (21) confirms this and adds that the distillate becomes inactive very quickly at pH 6. Possibly some nonspecific agent such as ammonia may be involved. Thyroxine has been shown by Carter (9–12) to activate unripe sperm and he suggests that a related substance in egg water is the effective agent.

On the whole the evidence indicates that the activating agent is normally bound to the agglutinin and may perhaps be split off as a smaller molecular constituent under certain conditions, but its chemical identity does not appear to be unequivocally established.

3. *Spawning-inducing agent.* There were indications in the early work of Lillie and Just (117) that the agent in Nereis egg water that induces spawning of the males might be the same as the sperm-agglutinin which it resembled in being nondialyzable,

similarly inactivated by heat, species specific and bound by sperm. Townsend (199) has suggested that the agent may be glutathione but her evidence does not appear very convincing.

Galtsoff (46–49) has presented some data concerning the nature of the spawning-inducing agent in the oyster egg water without making any direct comparison with the sperm agglutinin. He finds it to be relatively heat stable and dialyzable. Eggs of the pelecypod mollusks and of the starfish, but not the sea urchin, will induce spawning of male oysters which will respond occasionally also to oyster sperm. Female oysters will respond to the presence of species-sperm or a thermolabile constituent that is insoluble in sea water and soluble in alcohol and benzene, but not to sperm of other pelecypods. The male oysters also respond to a variety of chemical substances including thyroxin and glutathione, but Galtsoff (49) does not conclude that either of these are the active agents in egg water.

Nelson and Allison (158) have shown that the receptors, for the stimulation of spawning of the male oyster by eggs, are located on the surface of the demibranchs and that the shedding of sperm results from a relaxation of the sphincter of the distended sperm duct. A new hormone-like substance, termed diantlin, was discovered earlier by Nelson (1935) in oyster sperm. When added to the incurrent water this agent causes a great increase in the rate of water pumpage through the oyster's gills. Nelson and Allison show this effect to involve relaxation of the adductor muscle, enlargement of the gill openings and increased ciliary activity. This effect is not given by sperm of other mollusks or annelids, nor by various substances that induce spawning of the male oyster. By means of various tests, including inactivation by trypsin, they show diantlin to be of a protein nature. Upon acidification (0.1 N HCl) they obtain separate basic protein and nucleoprotein (nucleic acid ?) fractions which, by themselves, are inactive but which can be recombined to give the active agent again. Diantlin is evidently quite different from the alcohol soluble substance of sperm which, according to Galtsoff, induces spawning of the females. It could, however, be related to the antifertilizin of sperm which is discussed below (p. 193).

C. 'UNIVALENT' FERTILIZIN. 1. *Spontaneous reversal of agglutination.* In the usual serological reactions with immune sera or specific normal sera, agglutination ordinarily persists as long as the cells remain intact. While this is the case, too, for the action of egg water on sperm in certain species of animals, spontaneous reversal occurs in various species of sea urchins. If interpretations of the mechanism of agglutination reactions are to apply to these reactions, too, the reversal must be taken into account. There is now fairly general acceptance of the mutual multivalence theory of Heidelberger (74, 75) and Marrack (128) which recent evidence of Pauling *et al.*, (see 167, 169) strongly supports. Briefly, the theory postulates that antigen and antibody are both multivalent with respect to their structurally complementary combining groups. Agglutination or precipitation, then, results from the building up of large aggregates by combination of each molecule of antigen or antibody with more than one molecule of the other. 'Univalent' antigen (e.g., a single hapten) or 'univalent' antibody, although still capable of reacting specifically, would be incapable of forming such aggregates and would inhibit agglutination or precipitation of the 'multivalent' reagents.

After spontaneous reversal in the sea urchin following agglutination by excess egg water the spermatozoa cannot be reagglutinated (114), although they remain intact and active. It has also been shown (223) that the agglutinin is considerably more stable in species like Megathura in which the agglutinates persist. Several interpretations of the reversal, consistent with the mutual multivalence theory, have been suggested by Tyler (204), including the view that the fertilizin molecules are split by action of the sperm leaving essentially univalent fragments combined with the antifertilizin of the sperm. This view is supported by experiments in which material having the properties expected of 'univalents' is obtained.

2. *Production of 'univalent' fertilizin.* When sea urchin egg water is exposed to the action of heat, proteolytic enzymes, ultraviolet or X-radiation to the point where it no longer agglutinates sperm, it still is found to contain material capable of combining specifically with the sperm (204, 206, 132). Such solutions inhibit agglutination of the sperm by untreated egg water. The altered agglutinin is still nondialyzable and is absorbed by species sperm. It also impairs the fertilizing power of sperm as does untreated agglutinin (109, 204). By analogy with inhibiting antibodies the altered fertilizin is termed 'univalent'. Sea urchin fertilizin does not require very drastic treatment (compared, for example, with the hydrolysis of proteins and polysaccharides) to effect conversion into the 'univalent' form. Thus 20 minutes boiling at pH 8 is effective. Evidently it is not necessary to rupture strong bonds for this purpose. In the spontaneous reversal of agglutination it is, presumably, these same bonds that are broken. It is not inconceivable that the sperm accomplish this mechanically, but it seems more reasonable to suppose that they do it enzymatically. The fact that relatively inactive or aged sperm do not exhibit spontaneous reversal can be interpreted to support either interpretation, depending on additional evidence. The possible presence of a depolymerase in sea urchin sperm will be discussed below.

3. *Species lacking sperm-agglutinin.* Egg waters of the starfish, Patiria miniata, and the gephyrean worm, Urechis caupo, which do not ordinarily cause agglutination of homologous sperm, were found by Tyler (204) to reduce the fertilizing power of the latter. That such egg waters actually contain material that may be termed fertilizin has been shown by the work of Metz (133). He found that the addition of some nonspecific adjuvant (from hen's egg white or the serum of lobster, fish, rabbit or chicken) resulted in agglutination of starfish sperm with homologous egg water. In tests with egg waters of three species of starfish no cross agglutination was obtained. The adjuvant of hen's egg white was found to be a heat stable, nondialyzable substance, insoluble in acetone or alcohol. Without egg water it activates but does not agglutinate the sperm. From various tests, including the obtaining of an agglutination-inhibiting substance by ultraviolet irradiation of the egg water, Metz concludes that it is the sperm which is effectively 'univalent' and that the adjuvant exposes more combining groups on it. Apart from the question as to whether it is the sperm or egg water that is 'univalent', this work supplies strong support for Lillie's (114) view that fertilizin is present in species in which sperm-agglutination by egg water is not ordinarily obtained. In addition it provides an important technical means of investigating the specific interacting substances of such species.

4. *'Univalent' antibodies.* The work on 'univalent' fertilizin has its counterpart in the field of immunology. Analogous conversion of agglutinins or precipitins of immune sera into 'univalents' has been obtained by various workers (see 212 for references) by means of such agents as heat, diazo compounds, formaldehyde and photo-oxidation. In these cases the effect is also interpreted (209–213) as due to a splitting of the antibody molecules and, in addition, a reassociation of the 'univalent' fragments with similarly split nonantibody protein of the serum. The 'univalent' antibodies retain their original specificity and, in the case of an antitoxin (229), may still function as a protective agent. Such treated antisera are found to have, in general, reduced antigenicity and may thus be of some practical value in connection with serum sickness, etc. Analogous to the occurrence of nonagglutinating fertilizin in some species of animals, immune antibodies have also been found to be formed as 'univalents' in certain cases (20, 163, 77). Of recent interest are the blocking ('univalent') antibodies found in Rh antisera (242, 177, 40, 106). In the presence of serum albumin these antibodies are found (243, 30, 27) to be capable of agglutinating Rh positive cells. This again resembles the situation in the starfish where an adjuvant is needed to enable the egg water to agglutinate homologous sperm (133).

D. Specificity. The sperm-agglutinin of egg water is fairly specific for homologous sperm. Cross-reactions may occur between related species and, while these are generally weaker than the homologous reactions, occasionally they may be even stronger (see 36, 230). In addition there are instances of cross-reactivity between remotely related species, such as the agglutination of Nereis sperm by Arbacia egg water (109). The Nereis sperm are also agglutinated by Arbacia body fluid which has no agglutinating action on homologous sperm. Lillie (110) showed that the heteroagglutinating activity of Arbacia egg water could be removed by absorption with Nereis sperm, or even sperm of the teleost fish Ctenolabrus, leaving the isoagglutinin intact. Just (87) has also presented evidence for a separate heteroagglutinin to account for the action of Arbacia egg water on Echinarachinus sperm. However, Elster (36) finds in cross-tests among echinoids, no indication of distinct heteroagglutinins.

Heteroagglutinins are of quite widespread occurrence in the normal serum and body body fluids of vertebrates (see 241, 101) and invertebrates (82, 225, 215). It is not surprising then that cross-reactions are found (e.g., 109, 110, 87, 91, 190, 57, 69, 184) to occur between spermatozoa and foreign egg waters, body fluids and spermatozoa or their extracts. A recent investigation by Tyler (215) of the body fluid and seminal fluid of 12 species of invertebrates showed these fluids to have agglutinating action on the spermatozoa or blood cells of some or most of 34 species of animals. In lobster serum, which normally acts on the sperm or blood cells of a wide variety of animals throughout the animal kingdom, at least ten distinct, relatively class-specific, agglutinins have been demonstrated, by Tyler and Metz (224, 225) by means of absorption tests. Cells (blood cells or spermatozoa) of one species remove the agglutinins for other species of the same group, but not those for species belonging to other groups. The ten different heteroagglutinins of lobster serum are evidently (227) represented by a single protein component that is electrophoretically homogenous and of large molecular size. In starfish body fluid at least four distinct heteroagglutinins are

found (215). In work on various immune or normal sera it has been established (see 1, for references) that antibodies with different specificities may be represented by a single, electrophoretically homogenous, protein component. The serological differences evidently do not involve changes in such physicochemical properties. The differences have come to be regarded (see Pauling, 167, 169) as differences in spatial configuration rather than of composition, the specificity of interaction being dependent upon the degree of structural complementariness of antibody and antigen. In the case of heteroagglutinins of such wide reactivity as described here, it has been suggested (225, 215) that the reactants are not complementary in detail but in the overall structure of large regions. Substances of large molecular size would presumably have greater possibilities as to the variety of configuration they may assume, and it has been suggested (225) that the agglutinating action of viruses (80) on the red blood cells of a variety of species of animals (18) may be based on this rather than on the presence of any special constituents.

The heteroagglutination reactions obtained with the body fluids and cells of the invertebrates discussed above do not, in general, occur within a taxonomic class of animals (215). On the other hand the cross-reactions of egg waters occur with spermatozoa of closely related species. Where spermatozoa of a remotely related species are agglutinated (e.g., Nereis sperm in Arbacia egg water), the reaction is attributed (110) to a separate substance in the egg water. Whether this will be found to hold in general needs to be determined. It is quite possible that the same molecule may possess both isoagglutinating and heteroagglutinating activity. If the situation is analogous to that found for the body fluids of the lobster and the starfish, then one may expect to find a number of heteroagglutinins present in egg water. If this activity is carried by the same substance that represents the isoagglutinin then only a fraction (e.g., one-tenth if there are ten equally distributed heteroagglutinins) would possess any particular heteroagglutinating activity. Absorption of the egg water by sperm of any one remotely related reactive species would then remove only that fraction and reduce the isoagglutinating potency of the egg water relatively little.

In defining the specificity of fertilizins the above considerations should, then, be taken into account. On the basis of present evidence it appears that the isoagglutinins are dominantly species-specific.

E. ROLE IN FERTILIZATION. 1. *Removal of fertilizin.* Various features of fertilizin (defined in particular as the isoagglutinin) led Lillie (114), Just (91) and others to consider it of essential importance in fertilization. The critical experiments concern the fertilizability of eggs that are deprived of this material. Lillie (110) reported that prolonged washing (1 to 3 days) to remove fertilizin rendered Arbacia eggs nonfertilizable. Loeb (122, 123) objected that this was due to death of the eggs during the washing period and, in Strongylocentrotus, obtained fertilization of fresh eggs deprived of detectable fertilizin by means of acid treatment. Using this method with Arbacia, Lillie (111) obtained reduction in fertilizability and noted the presence of fertilizin in suspensions of acid-treated eggs. In Strongylocentrotus, however, he (115) obtained no detectable fertilizin after such treatment and found the eggs to be fertilizable. In more recent work, following identification of fertilizin with the gelat-

inous coat of the egg, removal of this material has been found (69, 204) to impair the capacity of the eggs for fertilization. Although fertilization occurs in eggs that yield no detectable fertilizin, the jellyless eggs must be inseminated with much higher concentrations of sperm than are necessary for fertilization of the control eggs (204). It appears, then, that fertilizin serves as an aid to fertilization, its presence as the gelatinous coat enabling fertilization to be effected with smaller amounts of sperm than in its absence. While sea urchin eggs that have been deprived of their jelly layer by acid treatment, by shaking or by proteolytic enzymes, give off no further detectable fertilizin into the medium, they still react with solutions of antifertilizin from the sperm (see p. 198). This means that a layer of fertilizin remains intimately bound to the surface. The fact that jellyless eggs are still fertilizable does not, then, exclude the possibility that the presence of fertilizin may actually be essential for fertilization. This would be consistent, too, with Chamber's (15) and Just's (89) demonstration that egg fragments devoid of original surface material (cortex) are unfertilizable.

Runnström *et al.* (189) have reported experiments on so-called underripe eggs of Psammechinus miliaris in which fertilizability is evidently improved by removal of the gelatinous coat. In these 'underripe' eggs they note that the coat is greatly contracted and more solid than in the ripe eggs, due presumably to lack of water in the material of the coat. The spermatozoa cannot penetrate such a coat. Upon standing in water for some time the coat generally softens and swells and fertilization can then be effected. Removal of the contracted coat by means of acid also permits fertilization. There is, then, no direct contradiction between these experiments and those discussed above. Presumably in the contracted hydrophobic condition few, if any, combining groups of the fertilizin of the coat are available for interaction with the antifertilizin (see below) of the sperm.

In many species of marine animals the unfertilized eggs show no distinct gelatinous coat. Presumably, the surface of such eggs contains a fertilizin which would function as in the jellyless eggs of sea urchins. Aside from the case of the polychaet annelid Nereis, such eggs have not been particularly investigated in this regard. Nereis presents an interesting situation in that a gelatinous coat forms on the surface of the egg shortly after attachment of the sperm and continues to enlarge during and for a while following the slow penetration of the fertilizing spermatozoön. There is a distinct, rather tough, vitelline membrane at the surface of the unfertilized egg and the jelly exudes through this as it is converted into the fertilization membrane. Lillie (109) found that the unfertilized eggs charge the surrounding sea water with small amounts of fertilizin and that as the jelly exudes, following fertilization, large amounts are obtained. It appears likely that, in Nereis, the jelly is also composed of fertilizin which, either because of the small quantities exuded before fertilization or special properties, cannot form a gelatinous coat on the unfertilized egg. Novikoff (160) and Costello (22, 23) have discovered that treatment of unfertilized Nereis eggs with alkaline NaCl causes the jelly-precursor to swell without exuding through the vitelline membrane, thereupon rupturing the latter and going into solution itself. Costello (22) finds that such jellyless, vitelline membraneless eggs are incapable of fertilization, but if the jelly and membrane is similarly removed shortly after insemination,

normal development may ensue. These results may again be interpreted as indicating a necessity of fertilizin for fertilization, it being assumed that the fertilizin is present as the jelly precursor and as part of the vitelline membrane of the unfertilized egg. The vitelline membrane of the Nereis egg would, then, correspond to the vitelline membrane of the sea urchin egg, its location with respect to jelly material being reversed.

2. *Addition of fertilizin.* Reports that the addition of fertilizin improves the fertilizability of washed or unripe eggs (247, 53) were disputed by Lillie and Just (118). In more recent experiments by Tyler (204) the addition of solutions of fertilizin to jellyless and normal eggs of Strongylocentrotus was found to interfere with fertilization. Since agglutination is temporary in this species, the sperm being intact and active after reversal, the inhibition is not attributable simply to unavailability of free spermatozoa. Further tests confirm Lillie's (109) observation that the fertilizing power of sperm that have agglutinated and reversed is considerably reduced, the amounts of treated sperm required for fertilization being 40 to 200 times the minimum amounts of control sperm. These results do not actually conflict with those described in the preceding section which indicate a helpful action of fertilizin. It should be recalled here that fully agglutinated and reversed sperm cannot be reagglutinated. Having reacted with all the fertilizin that they are capable of binding, they would not be expected to react further with fertilizin present on the eggs. The inhibiting action of fertilizin in solution tends then to support the view that this material is important for fertilization. However, to participate in the fertilizin process it must be present on the surface of the egg. In solution it acts as a barrier to fertilization, the reaction with the sperm being completed at a distance from the egg. These considerations help explain why sea urchin eggs that have stood for a short time in unchanged sea water require more sperm for fertilization than if they are washed immediately prior to insemination. Fertilizin goes into solution as the eggs stand in sea water and effectively incapacitates some or all of the sperm depending upon the relative amounts present. As Rothschild (178) has aptly stated it, in a recent article on fertilization, a false fertilization takes place and the sperm are 'muzzled' by reaction with fertilizin in solution.

3. *Cross-fertilization.* Several investigators (109, 110, 115, 122, 123, 87, 36) have examined the question of the relation of cross-fertilization to cross-agglutination. While there are differences in detail and interpretation of their results, all agree that there is no correspondence between the degree of cross-agglutination and of cross-fertilization obtained with various species of sea urchins. Both cross-agglutination and cross-fertilization occur to a greater or less extent among most of the species of echinoids examined. As an index of cross-agglutination the relative titers of the egg waters on the various sperm is employed. For cross-fertilization it is the concentration of sperm required for the foreign eggs, in comparison with the minimum needed for the homologous eggs, that serves as a measure, it being known (115, 36) that the per cent of cross-fertilization increases with amount of sperm employed and that, in general, much more is needed for cross-fertilization than for fertilization within the species. Absence of correlation has been interpreted (123, 36) as implying lack of significance of fertilizin in fertilization. However, the reaction of the sperm with

fertilizin is undoubtedly only one of a series of reactions that may be involved in the fertilization process and its specificity would not necessarily be expected to determine the overall specificity of the process.

4. *Parthenogenesis.* Recent work on the subject of artificial activation of the egg has been reviewed by Tyler (205, 219) and R. S. Lillie (119). For the present purpose the possible role of fertilizin in the process must be considered. Moore (142, 143), Just (88) and Lillie (116) showed, in contradiction to Loeb's (123) results, that sea urchin eggs that have been optimally activated by means of butyric acid are incapable of fertilization, even after removal of their membranes. Since, after butyric acid treatment, fertilizin can no longer be obtained from the eggs Lillie and Just considered lack of its further production to be responsible for the failure of superposition of fertilization on parthenogenesis. However, since fertilizin has now been identified as a component of the gelatinous coat of the egg and since this coat is dissolved by butyric acid treatment (110, p. 559), their interpretation no longer appears warranted. A similar interpretation of the block to polyspermy, or failure of fertilized eggs to refertilize, was based on the view that fertilizin was continuously produced by the unfertilized egg, but production ceased after fertilization. However, Lillie (110, p. 553) noted that demonstration of cessation of production depended upon removal of the gelatinous coat of the egg which he found to contain fertilizin in high concentration. This feature of the experiment is evidently responsible for what now appears to be a misinterpretation of the facts. In this connection it may be mentioned that refertilization of fertilized eggs has been obtained under special conditions (228). There does not, then, appear to be much justification, as yet, for assigning to fertilizin a rôle in artificial activation or in the establishment of the block to polyspermy. Further investigation along this line would be desirable.

ANTIFERTILIZIN FROM SPERM

The substance of the sperm with which fertilizin combines has been termed antifertilizin (110). It was first extracted from sperm by Frank (44) and Tyler (200) by different methods and is reported to have various effects on eggs and on spermatozoa.

A. Effects. 1. *Neutralization of fertilizin.* Extracts of sperm of sea urchins and mollusks prepared by brief heating in sea water (44) or by freezing and thawing (200) are found to be capable of neutralizing the agglutinating action of fertilizin on homologous sperm. In the supernatant obtained by centrifugation of 'dry' or concentrated sperm suspensions this agglutinin-neutralizing property has been reported to be present by Southwick (194) and Hartmann *et al.* (69), absent by Frank (44) and Hayashi (71) and weak by Tyler and O'Melveny (226). Hartmann *et al.* (69) also reported that both the supernatant and the extracts neutralize the sperm-activating component of egg water in Arbacia pustulosa, while Frank (44) found no such action of sperm extracts in Arbacia punctulata. Runnström *et al.* (189, 184, 185) and Vasseur and Hagström (230) likewise obtained negative results with the supernatant of sperm of Psammechinus miliaris and Echinocardium cordatum.

The antifertilizin is evidently rather firmly bound to the sperm; however, it does, in our experience, slowly dissolve as the sperm age in sea water, and slight acidification (pH 4–6) of fresh suspensions yields substantial amounts without killing the

sperm. Extracts prepared by disruption of the cells neutralize more fertilizin than is absorbed by an equivalent amount of live sperm. Evidently, not all of the antifertilizin carried by the sperm is available for immediate action.

2. *Agglutination of eggs.* Another effect of sea urchin, sperm-extracts consists in agglutination of homologous eggs (246, 44, 203, 226, 189). Microscopic examination shows that a precipitate forms on the surface of the gelatinous coat, outlining the latter sharply. The reaction resembles the so-called Neufeld reaction obtained with encapsulated micro-organisms, such as pneumococci, in the presence of specific antisera. Agglutination of the eggs and formation of the precipitation membrane are quite evidently two aspects of the same reaction. It is also clear that the agglutinating substance in the sperm-extract reacts with the gelatinous coat of the egg, and various tests (44, 203, 226) identify the egg-agglutinin with the agent (antifertilizin) that neutralizes the sperm-agglutinin (fertilizin). The term antifertilizin may then be used for the substance having these two effects. Another manifestation of the same reaction is the formation of a precipitate when solutions of fertilizin and antifertilizin are mixed under appropriate conditions (44, 203). Eggs that have been deprived of their gelatinous coat by shaking or by acid treatment are also agglutinated by solutions of antifertilizin and the latter is absorbable by such eggs. There is, then, present on the surface of such eggs a layer of fertilizin that is more intimately bound than is the rest of the gelatinous coat.

3. *Dissolution of the gelatinous coat.* According to Hartmann *et al.* (69), sperm-extracts of Arbacia pustulosa also have the property of dissolving the gelatinous coat of the egg. Tyler and O'Melveny (226) have noted a similar disappearance of the gelatinous coat of eggs of Strongylocentrotus purpuratus and Lytechinus anamesus but do not interpret this as dissolution. If the action of a strong sperm-extract is followed under the microscope a precipitation membrane is first observed to form on the surface of the gelatinous coat. The latter then contracts and the precipitation membrane becomes increasingly more distinct until it reaches the surface of the egg itself. It is not then readily distinguished from the surface of the egg. However, in the process some of the eggs may be freed of their gelatinous coat, particularly if they are shaken. Long after the precipitation membrane has shrunk to the surface and apparently disappeared on the intact egg it can be seen on the isolated jelly hulls as a distinct heavy membrane forming a hollow sphere that contracts to less than one-half of the diameter of the egg proper. It appears, then, that the material of the gelatinous coat becomes incorporated in the precipitation membrane, where it occupies considerably less volume than in the original gel form. There is, as yet, no evident necessity for the assumption of a jelly-dissolving agent in the sperm-extracts unless the recent findings of Monroy and Ruffo (140, 180), discussed in connection with hyaluronidase, refer to something other than the above sequence of events, and receive confirmation.

4. *Paralysis of the spermatozoa.* In several species of sea urchins the supernatant fluid (seminal fluid) obtained by centrifugation of 'dry' or concentrated sperm-suspension has been found to have an immobilizing action on the homologous sperm. Positive results in such experiments have been presented by Southwick (194) on Echinometra subangularis, by Hartmann, Schartau and Wallenfels (69) on Arbacia pustulosa

and by Vasseur and Hagström (230) on Psammechinus miliaris. On the other hand, in Echinocardium cordatum, Runnström, Tiselius and Lindvall (188) find no immobilizing action of seminal fluid and, in Arbacia punctulata, Hayashi (71, 72) finds that it actually maintains the motility and respiratory activity of the sperm. Hartmann *et al.* (69) found their sperm-immobilizing agent to be soluble in methanol. In partial accord with this, Runnström and Lindvall (183) obtain inhibition of motility with a methanol extract of E. cordatum, but not of P. miliaris, sperm. This methanol extract is also found to have a certain lytic action on the egg surface which will be discussed below. Hayashi's (71, 72) seminal fluid factor is found to be nondialyzable and precipitable by ammonium sulphate.

In the work of Runnström *et al.* (183, 188), Vasseur and Hagström (230) and Hayashi (71, 72) possible effects of CO_2 tension and pH were taken into account. It would appear then, that different species differ considerably in regard to the action of seminal fluid and sperm extracts on sperm. No general conclusions can, therefore, be made as yet concerning the relation of such action to antifertilizin activity.

B. CHEMICAL PROPERTIES. Various workers have examined the chemical properties of antifertilizin (as defined by agglutinating action on eggs, formation of precipitation membrane or neutralization of the sperm-agglutinin). Frank (44) found the active agent in Arbacia punctulata to be nondialyzable, relatively heat stable, insoluble in alcohol and ether and nonprecipitable by ammonium sulphate. His preparations gave negative results in the common tests for proteins and also failed to reduce Benedict's solution or Nylander's reagent. With solutions of Strongylocentrotus and Megathura antifertilizin, that were evidently stronger, Tyler (200, 203) obtained positive protein reactions, precipitation with ammonium sulphate and inactivation by proteolytic enzymes. In Arbacia pustulosa (69) and in E. cordatum (189) the active agent is also found to be insoluble in methanol, nondialyzable and relatively heat stable. Upon ultracentrifugation of a dialyzed sperm extract Runnström, Tiselius and Vasseur (189) find a main component with a molecular weight under 10,000 and no appreciable amounts of any larger molecules. Upon electrophoresis they find only one component and this behaves as an acid down to pH 4. Tyler and O'Melveny (226, 216) showed that antifertilizin of Strongylocentrotus and Lytechinus is extractable in acidified sea water (ca. pH 3.5). Dialysis, ammonium sulphate precipitation and isoelectric precipitation (pH 3.0 in the absence of salts) yields an electrophoretically homogenous preparation (216) whose single component shows mobilities that correspond closely with those reported by Runnström *et al.* Purified antifertilizin preparations of Lytechinus are found to contain about 16 per cent nitrogen. Solutions containing 0.2 to 0.3 mgm. N/ml. give titers (maximum dilution giving visible precipitation membrane when tested with an equal volume of a suspension of ca. 10,000 eggs per ml.) of 128 to 256.

The antifertilizin preparations are found (226, 216, 183) to be antigenic in rabbits, the antibodies agglutinating the intact sperm as well as precipitating with original solutions. This is consistent with both its protein nature and location on the surface of the spermatozoa. Electron microscope examination of spermatozoa from which antifertilizin has been extracted (pH 3.5 to 2.8) show (216) no effect on the acrosome, midpiece or tail, while the intervening region of the head (which is normally elongate)

becomes spherical, increasingly swollen and less dense. The antifertilizin is evidently present on this region of the surface of the head.

C. Specificity. Since antifertilizin reacts with fertilizin its specificity may be expected to parallel that of fertilizin. However, different tests, corresponding to different manifestations of the interaction, may give different results. Thus, if one of these agents is effectively 'univalent' with respect to the other in homologous or cross-reactions, the results obtained by direct agglutination tests would differ from those obtained by neutralization tests. Comparisons on this basis have not as yet been made, but several workers have obtained data on cross-reactions of antifertilizin. Frank (44) showed that the egg-agglutinin was not obtainable from tissues or fluids of Arbacia other than the sperm (see below for an antifertilizin from eggs) and that it acted only on the eggs. Reciprocal cross-reactions were obtained with another echinoid, Echinarachinus. Cross-reactions among the echinoids, tested by egg-agglutination, or in some cases by inhibition of sperm-agglutination, have also been reported by Tyler (203), Hartmann *et al.* (69) and Runnström *et al.* (189). In addition, however, reactions with material of remotely related species have been reported. Thus Frank (44) obtained agglutination of Arbacia eggs with sperm extract of the polychaet annelid Nereis, but not the reciprocal. Runnström *et al.* (189, 184) obtained agglutination of sea urchin eggs with extracts or salmon and bull sperm and with serum of a shark and a bony fish. These evidently represent heteroagglutination reactions of the type discussed above (see p. 189). It may be recalled that egg water and blood of Arbacia agglutinates Nereis sperm, and that the seminal fluid of various species of animals has agglutinating action on sperm and blood cells (and probably eggs) of different assemblages of whole groups of animals (224, 225, 227, 215). To what extent such heteroagglutinating action may be the property of substances distinct from the isoagglutinin or the property of the same molecules involved in isoagglutination needs to be determined. It is clear, however, that the heteroagglutinating action of the materials obtained from sperm is a property that such material has in common with substances of the body fluid (and probably other tissues) of the body. Thus in defining the specificity of antifertilizin (or of fertilizin) cross-reactions that are also given by body fluids or extracts of other tissues may be ruled out. On this basis it appears that the fertilizin-antifertilizin reaction is dominantly species-specific.

D. Role in fertilization. 1. *Removal or blocking of antifertilizin.* To test the significance of antifertilizin in fertilization it would be desirable to deprive the spermatozoön of this agent. Complete removal without destruction of the sperm has not proved feasible. However, when antifertilizin of Strongylocentrotus and Lytechinus is partially extracted by means of slightly acidified sea water or warm sea water, the spermatozoa are found to remain viable and motile (Tyler and O'Melveny, 226). Tested on eggs, such extracted spermatozoa showed a very much reduced capacity (in terms of amount required) for fertilization, roughly in proportion to the amount of antifertilizin removed. At the same time their rate of oxygen consumption was found to be considerably less affected. Thus, in different tests, sperm having 80 per cent of the control respiratory activity showed between 3 and 25 per cent of the control fertilizing capacity and when respiratory rate was reduced to 25 per cent, fertilizing

capacity was found to be less than one percent. While other effects of the treatment are not ruled out, the experiments indicate that deficiency in antifertilizin impairs fertilizing capacity.

Another method of eliminating antifertilizin without physical damage to the sperm consists in neutralizing it by means of a specifically reacting agent. This has been done by reaction with fertilizin, as described above (p. 000), with the result that the sperm become relatively nonfertilizing. It has also been attempted by means of antibodies produced in rabbits by injection of purified antifertilizin (Tyler and O'Melveny, 226; Tyler, 216). Since the spermatozoa are agglutinated by the antiserum any interference with fertilization could be attributed to the locking up of the sperm and the experiment would supply no information concerning the possible effect of neutralizing the antifertilizin. To overcome this difficulty, use was made of 'univalent' antibodies prepared by photo-oxidation of the ordinary antiserum. After treatment with such 'univalent' antibodies the spermatozoa were found to be of normal appearance and motile. When tested on homologous eggs in experiments with Lytechinus and with the gephyrean worm Urechis, the treated sperm showed a considerable reduction in fertilizing power, roughly in proportion to the titer of antibodies employed for treatment. The heterologous antibodies were found to be ineffective. This experiment further supports the view that antifertilizin is involved in the fertilization process. It does not, however, necessarily mean that the antibodies bodies combine with the same specific groups on the antifertilizin molecule that are involved in the interaction of the latter with fertilizin. The antibodies might very well be directed against other specific groups, and, in combining, physically block those concerned in the reaction with fertilizin.

2. *Some immunological analogies.* In the field of immunology analogous experiments are employed in attempting to identify the factor involved in virulence of pathogenic organisms. Thus, for example, it has been shown that antibodies directed against the polysaccharide coat of pneumococcus will reduce the infective power of the organism. A further analogy is provided by experiments on bacteriophage treated with a specifically reacting substance of the susceptible bacterium or with antisera. Thus Levine and Frisch (105) and Gough and Burnet (58) showed that phage can be specifically inactivated by a substance derived from the coat of the susceptible organism. The infective power of phage on the bacteria can also be impaired by means of antiphage immune sera, the inactivation following a percentage law (30, 8). According to Burnet (7, 8) the antibodies combine with specific groups other than those by which the phage particles react with the surface of the bacterium but may spatially block some of the latter and thus interfere with adsorption of phage to the bacterial surface. The action of specific bacterial substance and of specific antibody on phage resembles then the action of fertilizin and of specific antibody on sperm. There is also evidence (see Delbrück, 28) that only a single phage particle (perhaps the first to attach to the surface) actually enters the bacterial cell and multiplies. This would imply a further analogy with the phenomenon of monospermy or establishment of the block to polyspermy in fertilization. Of course, the outcome of phage action in producing lysis of the bacterial cell is quite different from the development that normally ensues following fertilization, but it may be mentioned in passing

that Lindahl (120) has reported cytolysis of specially treated eggs as a result of fertilization. Some bacteriophage particles seem to be provided with a tail (see 126), but this slight structural resemblance may be taken less seriously than the above analogies.

3. *Sperm-immunity and sterility in mammals.* Numerous investigators have examined the possibility of inducing sterility in various animals, including humans, by means of antibodies against homologous or heterologous sperm or their extracts. A brief review of the subject has been previously presented (206). Although there have been many conflicting reports, the evidence as a whole is to the effect that sterility is not induced by such immunization of females even when antibodies are found to be present in the serum of the animals at the time of mating. In one type of experiment a temporary sterility is evidently obtained. This consists in the introduction of the antispermatozoal serum into the vaginas of rabbits followed by matings within one to 30 hours (Parsons and Hyde, 165). The experiment is essentially a direct treatment of the sperm with antibodies such as reported above with invertebrates and it is not surprising that the results are similar. The negative results in other types of experiments involving active or passive immunization of the female may be attributed to unavailability of the antibodies, insufficient concentration relative to the sperm or other factors.

4. *Addition of antifertilizin.* Treatment of sea urchin eggs with antifertilizin or insemination in the presence of antifertilizin has been found (44, 69, 203, 226) to inhibit fertilization. It has also been reported by Frank (44) that treatment of the sperm with antifertilizin impairs their fertilizing capacity, but this has been denied by Hartmann *et al.* (69) and by Tyler and O'Melveny (226). Reaction of antifertilizin with the egg results in the formation of a precipitation membrane as described above (p. 194). In dilute solutions this membrane may be incomplete and it has been noted, in another connection (203), that such eggs may be fertilized. More complete interaction with antifertilizin renders the eggs nonfertilizable. Eggs deprived of their gelatinous coat are also found to lose their fertilizability upon treatment with antifertilizin (44). This may be interpreted as support for the view mentioned above (p. 191) that a layer of fertilizin remains on the surface of the egg after removal of the coat by the usual methods. In any event it appears that antifertilizin in solution acts as a block to fertilization, its interaction with fertilizin of the egg evidently preventing interaction of the latter with the antifertilizin present on the sperm. As in the case of the fertilizin discussed above (p. 191), the substance must remain on the surface of the cell in order to function in the fertilization process.

5. *Alexin (complement) and the fertilizin-antifertilizin reaction.* The interaction of fertilizin and antifertilizin has been considered to be analogous to that of antigen and antibody. An interesting feature of antigen-antibody reactions is their ability to bind alexin (usually called complement—a heat labile, relatively nonspecific material present in the serum of vertebrate animals, the action of which is ordinarily manifested by the lysis of erythrocytes, or other cells, when reacting with specific antibody in its presence). Ability to bind alexin is not, by any means, characteristic of all antigen-antibody reactions. There are a great many exceptions, such as in toxin-antitoxin reactions (see Osborn, 161), reaction of pneumococcal capsular polysac-

charide with its specific antibody produced in horses (251) or in humans (197), and most cases of the reactions of the normal blood-group isoantibodies (see 241). In many cases, too, antigen or antibody alone may combine with alexin (termed anticomplementary action) to such an extent that no decision can be made as to whether or not alexin is fixed by their interaction. It is of interest, then, to learn how the fertilizin-antifertilizin reaction behaves in this regard. This was examined by Tyler (208) with fertilizin and antifertilizin of Strongylocentrotus and alexin of guinea-pig serum. It was found that the fertilizin alone bound alexin. Upon reaction with antifertilizin a rather novel situation was discovered. The antifertilizin destroyed the ability of fertilizin to combine with alexin, or, if the alexin was first bound by fertilizin, addition of antifertilizin released it. This alexin-release reaction could in fact serve as a measure of the fertilizin-antifertilizin reaction, just as, conversely, alexin-fixation is used to follow antigen-antibody reactions. 'Univalent' fertilizin by itself was also found to bind alexin which could be released by antifertilizin. Alexin is known to be composed of two heat-labile and two relatively heat-stable components (see 34, 76). It is with the relatively heat-stable fourth component ($C'4$), which is found in a muco-euglobulin fraction of serum, that the fertilizin was found to combine. Since antifertilizin and $C'4$ compete in the interaction with fertilizin, it might be expected that antifertilizin could substitute for $C'4$ in the hemolytic action of alexin. Tests of this possibility gave negative results. This means that the antifertilizin is quite different from $C'4$ in those properties that enable the latter to act with the other three components and the sensitized cells in the hemolytic system. The binding of alexin by fertilizin is also manifested by agglutination of the sea urchin eggs and formation of the precipitation membrane in guinea pig serum. This may, then, be simply another aspect of the heteroagglutination type of reaction above (p. 190) and it seems reasonable to conclude that the specific groups by which fertilizin combines with antifertilizin are different from those involved in union with $C'4$, the release phenomenon being the result of spatial interference due to proximity of the different groups.

6. *A hypothetical scheme for attachment of the sperm.* On the basis of the present evidence fertilizin and antifertilizin appear to be concerned primarily with attachment of the spermatozoön to the egg. The sperm-activating agent associated with fertilizin may be helpful in speeding up encounter of the gametes and in stimulating fatigued sperm to an extra burst of energy for entrance into the gelatinous coat, but there is little to substantiate this. Since the gelatinous coat is composed primarily of fertilizin, the question may be raised as to whether the spermatozoön reaches the egg surface proper (vitelline membrane) before it has reacted completely. Experimental evidence concerning this is lacking, but to provide a basis for discussion the following picture of the mechanism of attachment of the sperm may be suggested. The fertilizin comprising the gel micelles of the coat of the egg would have relatively few combining groups available for reaction with the sperm. Additional combining groups would be available on the egg surface proper. A small amount of fertilizin present in solution in the interstices of the gel would react with the sperm or with any antifertilizin that may dissolve off it. The effective initial reaction would, however, be between the active groups available on the micelles of the gel and on the surface of the sperm. These groups are probably absent from the acrosome and midpiece of

the sperm (see p. 195). If the complementary fertilizin groups are radially arranged in the gel and absent from its outermost surface, then on contact with the latter the spermatozoön would be held in a position normal to the egg surface. Since interaction of fertilizin and antifertilizin results in precipitation (presumably because the specific combining groups are also the ones that render these substances hydrophilic and the interaction destroys their affinity for water) the micelles would also tend to precipitate. Since it is anchored to the surface of the cell, precipitation of a micelle would be manifested by its contraction. The spermatozoön would thus be brought into contact with more deeply located combining groups and cause further contraction of micelles until the surface of the egg is reached. Radial arrangement of the available groups in the gel would keep the spermatozoön oriented normal to the egg surface, where further interaction of the fertilizin on the vitelline membrane with the laterally disposed antifertilizin on the head of the sperm would result in more intimate contact and could even account for the depression of the membrane that is seen at the point of attachment. To speculate further, reaction with the fertilizin of the vitelline membrane might involve a weakening of the latter, so that the egg substance tends to protrude in the form of the entrance cone which engulfs the sperm and is then partially (e.g., extrusion cones) or completely retracted into the egg by purse-string closure of the plasma membrane, as the vitelline membrane is converted into the fertilization membrane. To account for the block to polyspermy one might assume a rapid, chain-like reorientation of the molecules comprising the vitelline membrane in response to the first sperm to attach so that the supernumeraries cannot attach as firmly. One might also assume that a lytic agent such as that described below (p. 206) is involved in penetration and block to polyspermy.

Detailed support for the above kind of picture of approach and attachment of the spermatozoön will not be attempted here but a few observations pertaining thereto may be presented. The sea urchin spermatozoön does not swim very actively as it passes through the coat of the egg; rather the tail becomes progressively motionless as it enters the jelly. The intimate attachment to, and depression of, the vitelline membrane may not, then, be attributed to mechanical activity of the sperm. The first observations of fertilization, made by Fol (41, 42) on the starfish egg, showed this very strikingly along with the formation of a curious filament which Chambers (16, 17) and Hörstadius (81) have carefully studied. When the starfish spermatozoön strikes the surface of the coat of the egg it becomes motionless and a filament appears extending from the head of the sperm directly to the vitelline membrane. The filament then contracts dragging the sperm with it until it reaches the surface of the egg. A single egg may show a large number of such filaments extending to many of the spermatozoa on the surface of the jelly coat and their contraction brings many of the supernumerary sperm into contact with vitelline membrane where their entry is evidently blocked. According to Hörstadius the filament has the form of a hollow cylinder. I should interpret the formation and contraction of the filament to be due to precipitation of the fertilizin micelles of the gel by interaction with antifertilizin of the sperm as described above. Runnström (182) has examined starfish sperm extracts for the presence of antifertilizin and obtained no precipitation membranes on the surface of the coat of the egg. However, since the presence of fertilizin in solu-

tions of starfish egg jelly has been convincingly demonstrated (133), antifertilizin must evidently be present on the sperm. Runnström's failure to obtain precipitation membranes with sperm extracts may, then, be attributed to other factors, such as occurrence of antifertilizin in 'univalent' (nonprecipitating) form in the extracts or condition of the egg coat (e.g., few combining groups available on the surface) at the time of testing. The above account also implies that the spermatozoön must approach radially. This is consistent with most observations (see Morgan, 144) showing failure of obliquely approaching sperm to attach. In connection with the postulated radial structure of the gelatinous coat of the sea urchin egg, Runnström *et al.* (187) and Monné (139) observed no birefringence under normal conditions. A strong birefringence appeared, however, upon addition of antifertilizin or upon staining with acridine-orange. This was found to be negative in the radial direction with the former agent and positive with the latter. Monné points out that the isotropy of the normal coat may be attributed to irregular or to regular structure, the latter being obscured by compensating positive and negative birefringencies of its constituents or by hydration of a birefringent substance present in small amount. The ready appearance of double refraction upon treatment may be taken to favor the latter view, but further work will be necessary to decide the question.

In attempting at the present time to present a picture, such as the above, of the manner in which fertilizin and antifertilizin may act in effecting attachment of the sperm, one runs the risk of having the evident weaknesses of a particular scheme confused with the evidence that such substances are definitely concerned in the processes under consideration. However, I feel sure that most workers in the field will not fail to make the distinction. The great extent to which the present picture is obscure and hypothetical is a measure of the lack of specific information. It may be hoped that this attempt to visualize a mechanism will help stimulate experimentation designed to discover the true mode of action of the substances involved in fertilization. The problem of sperm attachment may seem of rather minor, special importance to some biologists. It should, then, be remarked that it is part of the general problem of the adherence of the cells of the various tissues and organs to form a differentiated multicellular organism and that it has seemed reasonable to assume (see Tyler, 220; Weiss, 239) that the same kind of factors are in operation.

ANTIFERTILIZIN FROM EGGS

A. Effects. Lillie (110) presented evidence to show that the interior of the egg of Arbacia contained a substance (that he also termed antifertilizin) capable of inactivating fertilizin. Tyler (203) confirmed this with extracts of frozen and thawed Strongylocentrotus eggs that had been deprived of their gelatinous coat and showed that the extracts would agglutinate intact eggs and cause the formation of a precipitation membrane on the surface of the gelatinous coat. [For the record it may be mentioned that Runnström (181) had earlier observed precipitation of the gelatinous coat of Arbacia eggs by means of an extract of the eggs but did not attempt to relate this to fertilizin-antifertilizin reactions.] No particular action of the extracts on the sperm has been noted. The active agent in the egg extract behaves, then, in a manner similar to the antifertilizin obtained from sperm and it is perhaps justifiable to

use the same term for both. As in the case of the antifertilizin from sperm, absorption of the extracts with eggs or treatment with solutions of fertilizin rapidly neutralizes the antifertilizin. When intact fresh eggs are extracted by freezing and thawing in sea water, no antifertilizin is generally obtained in the solution. This is evidently due to its neutralization by the fertilizin of the gelatinous coat. From freshly shed eggs, that are provided with a large coat, the relative amounts of fertilizin and antifertilizin extractable is of the order of ten to one, as tested by reciprocal neutralization. The occurrence of antifertilizin within the cell along with fertilizin on the suiface has interesting implications that will be discussed further below.

B. CHEMICAL AND ANTIGENIC PROPERTIES. Some information concerning the chemical nature of the antifertilizin of eggs has been obtained by Tyler (203, 221). For extraction it is of importance first to remove the coat of the egg, since this would otherwise neutralize much or all of the antifertilizin. Freezing and thawing of the jellyless eggs of Strongylocentrotus yields a coagulum of insoluble egg material along with a yellowish colloidal solution containing the antifertilizin. The active agent is nondialyzable and precipitates in ammonium sulphate or, in the absence of salts, at pH 4 to 4.5. It is insoluble in alcohol and inactivated by heat and by proteolytic enzymes. While there is resemblance to the antifertilizin of sperm in these few properties, much further information will be needed before any definite conclusions can be drawn concerning the extent of similarity or dissimilarity. The present evidence indicates that the material is a protein.

The solutions of antifertilizin from eggs are found to be active antigens. The antiserum, obtained by immunization of rabbits with such solutions, neutralizes and precipitates the antifertilizin but does not agglutinate the intact eggs. The latter effect is understandable since the antifertilizin is a subsurface antigen. On the other hand, the antiserum does agglutinate homologous sperm. This means then that the antifertilizin from eggs is antigenically similar to that from sperm. It does not, however, necessarily imply any overall chemical similarity.

C. ROLE IN FERTILIZATION. Direct tests of the function of the antifertilizin of eggs by removal or neutralization experiments are not readily feasible. Treatment of eggs with the solutions is found (203) to interfere with fertilization, as in the case of treatment with the antifertilizin of sperm.

Lillie (114) proposed that the antifertilizin of eggs is involved in the establishment of the block to polyspermy. He pictured it as reacting with fertilizin immediately upon fertilization, thereby rendering the latter unavailable for reaction with additional spermatozoa. Since apparent secretion of fertilizin by unfertilized eggs and its cessation upon fertilization is now attributable to dissolution of the gelatinous coat, the proposal in its original form seems no longer tenable. It is conceivable that the antifertilizin might act in some such manner by interaction with a layer of fertilizin on the vitelline membrane of the egg, but experimental evidence concerning this is completely lacking.

Concerning the specificity of the antifertilizin from eggs, present evidence shows cross-reaction between the related, cross-fertilizing species of echinoids, S. purpuratus, S. franciscanus, Lytechinus pictus and Dendraster excentricus.

D. NATURAL AUTO-ANTIBODIES. 1. *Terminology.* In considering the fertilizin-antifertilizin reaction to be analogous to that of antigen with antibody, one may ask which represents antigen and which antibody. With the usual immune bodies the answer is relatively simple. The antibody is the substance that is produced as a result of injection of the antigen and is ordinarily found in a protein component of the serum of the immunized animal. The antigen is the inducing agent, which may be a protein or polysaccharide in solution or as part of a cell and which will react with the antibody to give precipitation, agglutination, alexin fixation, etc., depending upon the conditions of the reaction. The term is also applied to substances (simple and complete haptens) which by themselves are incapable of inducing antibody-formation but can react with the specific antibodies. When normally occurring substances, such as those involved in the blood group reactions, are considered, the definitions must be broadened somewhat. In this case the agglutinins are present in the non-immunized animal, having been formed as a result of gene-controlled reactions. They are generally termed antibodies by immunologists because of their presence in the serum, but such designation immediately conflicts with that part of the definition of antibody as something produced as a result of the introduction of a foreign substance into the organism. Likewise, designation of the so-called agglutinogens of the erythrocytes as antigens broadens the definition of that term. There are also cases where two substances occurring normally in the sera of different animals interact, as for example in the inhibition of blood group reactions (241). The designations in such cases would depend upon the direction in which the test is set up. This is the type of situation involved in attempting to use these terms for fertilizin and antifertilizin. Thus fertilizin can act as an agglutinin for sperm and might therefore be termed an antibody. But antifertilizin can act as an agglutinin for the eggs and should likewise be termed an antibody. Rather than abuse the terminology to that extent it is preferable to refer to such substances as complementary substances (complementary proteins, etc., further designation depending upon their chemical nature) of which the more familiar antigens and antibodies form a class whose manner of interaction is fundamentally the same. Professor Sterling Emerson, of this institution, has suggested (not too seriously) alleloplastology in place of immunology to designate the study of such reactions which are involved in problems of biological specificity. The reactants might then be termed alleloplasts. Some such general terminology would have definite use. The purpose of mentioning Emerson's suggestion here is to encourage consideration of the terminology. In the meantime substances like fertilizin and antifertilizin may be designated mutually complementary substances whose interaction occurs in the manner of antigen and antibody.

2. *Mutually complementary substances in cells.* The finding of antifertilizin within the egg means, then, that a single cell contains a pair of components, capable of interacting with one another in antigen-antibody manner. The implications of this and related findings in immunological and general biological problems have been presented in previous publications (Tyler, 203, 206, 217, 220) and only a brief summary of some of the points will be given here.

It is of importance first to know whether or not the situation is general for all kinds of cells. In immunological literature there are a number of reports of agglutination

or lysis of cells by means of extracts of the same cells. The present author, too, has been able to extract such auto-agglutinins from various bacteria and red blood cells, but not consistently. One of the difficulties evidently involved in such attempts is the probable occurrence of interaction and precipitation of the complementary substances in processes of extraction that involved destruction of the whole cell. If surface substance be present in excess it would bind the complementary subsurface substance and the latter would not be obtained in the ordinary extracts. Since the complementary substances may combine in multiple proportions and since one or both may be 'univalent' there is further difficulty in extraction and identification. In attempting to extract a complementary subsurface material it is evidently essential first to remove the surface substance. While this is readily feasible in the case of the sea-urchin egg, it is more more difficult in other kinds of cells. These factors may account for the above-mentioned lack of consistent results and more significance may, then, be attached to cases of active extracts.

There is no reason to expect complementary substances to occur simply in pairs, so in the present state of our ignorance we may conceive of cells as constructed of a number of complementary substances. For purpose of emphasis I have termed this the auto-antibody concept. Since the complementary substances are capable of combination, it is pertinent to ask how they may exist together in the cell. The tentative answer is that they are actually in combination in the regions in which they adjoin and by their union form the various membranes on and within cells. The manner of formation of the complementary structures will be considered below.

In a multicellular organism it may be expected that some of the surface material of the cells of various tissues would also be present in the body fluids. On this basis the demonstration by Kidd and Friedewald (92) that normal rabbit serum reacts, in antibody manner, with a sedimentable constituent of extracts of various organs of the same animal is consistent with the auto-antibody concept. A more familiar auto-antibody is the so-called Wassermann reagin found in syphilitic serum. Similar examples are known in other diseases, such as infectious mononucleosis, yellow fever, acute hepatitis, malaria and virus pneumonia. The reagin reacts with a lipoid substance that is extractable from the tissues of the same animal. Since it appears after infection it is assumed to be an immunologically induced antibody. The usual interpretations assume either that the lipoid tissue constituent combines with some protein of the infecting organism to form a complete antigen capable of inducing the formation of antibodies directed against the lipoid or that the pathogen possesses an antigen that is serologically similar to the tissue lipoid. A third interpretation is provided by the auto-antibody concept. It assumes that, in the tissue destruction occasioned by the pathogen, lipoid and complementary protein are liberated and that the smaller lipoid molecules are more rapidly eliminated from the body, leaving the complementary protein present in excess in the circulation and capable of reacting in vitro with added lipoid. Agents that tend to increase the concentration of lipoid in the blood would presumably render the serum nonreactive and this may explain why alcohol imbibition results in false negative Wassermann reactions. It should, on this basis, also be possible to cause false positive reactions without infection, and in this connection it may be noted that donors for blood banks have often been found

to give false positive reactions after repeated bleedings. Presumably the drain on the tissue cells in replacing the serum proteins results in liberation of some protein that is complementary to the tissue lipoid. While real evidence is as yet lacking, these examples perhaps suffice to show that the question is subject to experimental attack.

3. *An auto-antivenin.* A practically, as well as theoretically, important property of immune antibodies induced against pathogenic organisms, toxins or venoms, is their ability to act as protective agents. It is of interest, then, to learn whether the so-called auto-antibodies might serve in that manner. Experiments by the author with extracts of pneumococci have not, as yet, yielded definite results one way or the other. However, in other experiments with a venomous reptile called the Gila monster, material capable of neutralizing the venom of the same animal as well as of other individuals has been obtained (217). This material was found in the serum and in extracts of the liver of the animal, but not in extracts of the venom glands where it was first sought. The latter point would be difficult to reconcile with the auto-antibody concept if the venom gland is the site of synthesis of the venom. However, this is not known to be the case and the evidence (see 125) indicates that it may be produced elsewhere and transported in bound form to the gland where it is released. The antivenin in the blood may, then, represent complementary substance left behind when the venom is taken up by the gland, both substances presumably coming from the liver. Aside from the precise interpretation the finding of antivenin in the animal is favorable to the view that naturally occurring complementary substances may act as protective agents.

There are many examples, that may be cited from the immunological literature, of the destruction of cells by means of substances derived from the same kind of cell. Thus certain antibacterial proteins such as lysozyme and diplococcin are derivable from the same species of organism on which they act (135, 162). Certain autolytic enzymes perhaps belong in this category, although in most instances they do not attack the intact cell (see 31). Bacteriophage may be regarded as an example of this type since it attacks the cell from which it is produced and is known to react in antibody-like manner with a surface constituent of the cell (see 24). In fact it seems just as reasonable to regard the virus as being originally a gene-like cellular component as to consider it a distinct parasitic living entity. The facts of spontaneous lysogenicity would perhaps be better understood on that basis than on the basis of phage carriers.

4. *Antibody formation.* The antigen-antibody analogy leads to the view that the complementary substances of cells are formed by the same kind of processes as are involved in antibody formation. The exact mechanism of antibody formation is not known, but there is now wide acceptance of the general theory proposed by Breinl and Haurowitz (5), Alexander (2) and Mudd (157) which has been extended and experimentally supported by Pauling and Campbell (166–169). Briefly, this theory involves incorporation of the foreign antigen into the site of synthesis of serum globulin, where it so influences the construction of the globulin that various regions of the latter become structurally complementary to specific regions of the antigen. In this general theory no statement is made as to the structure of the globulin in absence of foreign antigen. On the basis of the auto-antibody concept I have proposed that the

normal globulin is similarly complementary to the substance comprising the normal site of synthesis. The formation of serum globulin may be regarded as an expression of the normal process by which the cells form various macromolecular constituents including those involved in its own growth. These constituents, then, would be complementary to the substance or substances comprising the site of synthesis, i.e., synthesis of cellular constituents would occur in the same manner as antibodies. Many pairs of such mutually complementary substances should be extractable from cells plus the surrounding fluids. For the formation of self-duplicating bodies (e.g., genes) of cells, it would be necessary to assume either direct mirror-imaging of identical materials as Pauling and Delbrück (170) have done or formation of an intermediate template which Emerson (37) lists as an alternative.

The specific structure of many constituents of cells is now known to be genetically determined. This is particularly well exemplified in the work on the antigens of the blood cells (see 83). It seems reasonable to conclude, then, that the configuration of various specific structures of the macromolecular constituents of cells are the same or the complement of those on the genes.

The bearing of the auto-antibody concept on problems of differentiation involves further speculation which has previously been sufficiently indulged in (203, 206, 217, 220) and which has received some possible experimental support (Weiss, 239).

LYTIC AGENTS FROM SPERM

A. Effects. 1. *Egg-membrane lysis.* The unfertilized eggs of many species of animals among the mollusks, fish and amphibia normally possess viscous coats and a rather tough membrane which elevates from the surface when the eggs are shed into their aqueous medium. To effect fertilization the sperm must penetrate these barriers. Several early workers have postulated the presence of a lytic agent on the spermatozoön to effect penetration. Hibbard (79) and Wintrebert (244, 245) observed that extracts or macerated suspensions of spermatozoa of the amphibian Discoglossus would break down one of the coats of eggs of that species. Tyler (200) found that sea-water extracts of frozen and thawed sperm of two mollusks, the keyhole limpet Megathura crenulata and the abalone Haliotis cracherodii, were able to dissolve the egg membrane in the respective species. Von Medem (232) confirmed this with other species of keyhole limpet and abalone. Cross-reactions were not obtained with these mollusks, but more closely related forms have not been tested.

The process of dissolution of the membrane can be readily followed in the eggs of mollusks. The membrane becomes thin, increases in diameter, may indent in one or two regions and finally, if the extract is sufficiently strong, vanishes completely. With strong extracts, or with concentrated sperm suspensions, disappearance of the membrane will occur in about three minutes if the gelatinous coat of the egg is present or in less than half a minute if the coat is first removed.

2. *Follicle cell dispersal by hyaluronidase.* The unfertilized tubal egg of mammals (with some exceptions such as the sheep, opossum and monotremes) is surrounded by a layer of follicle cells, the cumulus oöphorus, that persists for a considerable length of time. The cells are held together by a viscous material derived from the follicular fluid and the egg is thus covered by a tough coat through which the sperm must pass

to effect fertilization. It has been shown by Yamane (248, 249) and Pincus (171, 172) that when unfertilized tubal eggs of the rabbit are exposed to dense sperm suspensions or to saline extracts of the sperm, the follicle cells are dispersed within a few minutes. This effect could also be brought about by sperm of other mammals. At about the same time, and quite apart from the above-mentioned experiments, Duran-Reynals (32) and McClean (129) discovered what they called a spreading or diffusing factor in extracts of mammalian testes. This factor, when injected into the skin along with some indicator (dyes, India ink, hemoglobin, toxins, viruses) permitted widespread diffusion of the material which would otherwise be restricted to a small region. Used with a vaccinia virus a considerable enhancement of vaccinal infection occurred. Extensive investigations by these workers and others showed that the spreading factor was obtainable from various bacteria, snake venoms, poisonous insects, leech tissues and other sources besides mammalian testes and sperm (see reviews by Duran-Reynals, 33 and Meyer, 136), and have emphasized the importance of this factor in infection. Working along somewhat different lines, Meyer *et al.* (137) found an enzyme (called hyaluronidase) in autolysates of pneumococci, and later in extracts of rabbit iris, ciliary body and spleen, that hydrolyzed a polysaccharide (called hyaluronic acid) which he obtained from vitreous humor, umbilical cord and other sources. The latter two independent lines of work were correlated by Chain and Duthie's (13, 14) demonstration that testis extract also hydrolyzed hyaluronic acid, and subsequent work showed hyaluronidase activity in various other preparations containing spreading factor. The relation to fertilization came about through the discovery by McClean and Rowlands (131) and Fekete and Duran-Reynals (39) that the dispersal of the follicle cells of rat and mouse ova could be brought about by preparations of hyaluronidase from various sources besides mammalian testes, and that the active agent roughly paralleled the hyaluronidase in enzymatic and spreading action and in physicochemical properties.

The dispersal of the follicle cells results from a dissolution of the intercellular cementing material which is evidently hyaluronic acid, or at least similar to it. Considering simply the sperm extracts, the effect is relatively nonspecies-specific, cross-reactions being obtained with various species of mammals (e.g., bull and sheep sperm extracts on mouse ova). It is, however, rather tissue specific in that mammalian tissues other than the sperm yield little if any of the active agent.

3. *Dissolution of the gelatinous coat of sea-urchin eggs by hyaluronidase.* It was mentioned above in connection with antifertilizin (p. 194) that Hartmann, Schartau and Wallenfels (69) observed a disappearance of the gelatinous coat of the eggs of Arbacia under the influence of extracts of homologous sperm. In other species of sea urchins, Tyler and O'Melveny (226) accounted for a similar apparent disappearance by incorporation of the material of the coat in the precipitation membrane that is formed by interaction of the fertilizin of the coat with antifertilizin of the sperm extract and the rapid shrinkage of this membrane to the surface of the egg. In recent experiments, Monroy and Ruffo (140, 180) again report a dissolution of the coat of eggs of several species of sea urchins found at Naples (Psammechinus micr., Arbacia lixula and Spharechinus gran.) under the influence of a homologous or heterologous sperm extract prepared in the manner employed for extraction of hyaluronidase

from bull testis. They also find that bull testis extract causes a similar initial swelling of the coat but no final dissolution, and that the sea urchin sperm extract has viscosity-lowering action on hyaluronic acid preparations but is much less active in that regard than is the bull hyaluronidase. They conclude that sea-urchin sperm contain a hyaluronidase-like substance and the difference from bull testis-hyaluronidase is due to difference in relative amounts of two components of hyaluronidase. While there is evidence for the presence of two or more enzymes, or enzymatic activities, that may differ in relative concentration in preparations from different sources, the situation is not as yet well enough understood (see Meyer, 136) to permit ready application to the present case. Monroy and Ruffo also report no inhibiting action of egg water on the dissolution of the jelly coat by sperm extracts.

Runnström *et al.* (187) reported that hyaluronidase from bull testis did not attack the coat of eggs of the sea urchins P. miliaris and E. cordatum. They do not mention whether or not there was any swelling such as Monroy and Ruffo report. In this laboratory bull testis-hyaluronidase exhibited no dissolving or swelling action on the coat of eggs of L. pictus (221, 93) nor was such action obtained with the sea-urchin sperm extracts, prepared according to the methods used for hyaluronidase. When tested by the mucin-clot method these sea-urchin extracts showed no hyaluronidase activity. They also showed no spreading activity in guinea-pig skin and similarly negative results were obtained with a Megathura sperm extract containing the egg-membrane lysin. Another finding that is of interest in this connection is that concentrated preparations of sea-urchin fertilizin give a mucin-clot reaction (93). This reaction consists in the formation of a fibrous clot or a flocculent precipitate, depending upon the degree of polymerization or aggregation of the material, when protein is added to it in acid solution. Hyaluronic acid preparations give this reaction (138) but not after they are treated with hyaluronidase, which can then be assayed by this method (see Meyer, 136). That fertilizin, too, gives this precipitation reaction means only a very general resemblance to hyaluronic acid, since many mucins of acidic nature behave in this manner. As yet no inhibition of this reaction of fertilizin has been obtained with bull testis-hyaluronidase, and sea-urchin sperm extracts tend to enhance rather than to prevent it.

It is evident that further work will be required to ascertain definitely the presence or absence of hyaluronidase in sea urchins.

4. *Egg surface (vitelline-membrane) lysis.* Runnström *et al.* (183–189) have discovered an interesting new effect of methanol extracts on sea-urchin sperm. This is interpreted as a liquefaction of the egg surface and is manifested in the following ways: *a*) when placed in hypertonic solution the treated eggs shrink with a much smoother surface and cytolyze more readily than do the controls; *b*) cytolysis in hypotonic solution occurs in less dilute solution than is needed for the control eggs; *c*) the constriction into two fragments that occurs upon high speed centrifugation takes place more readily in the treated eggs; *d*) eggs of P. miliaris and E. cordatum, which are often quite elongate when shed, become spherical very soon after addition of the sperm extract. In the intact, unfertilized sea urchin egg the vitelline membrane is not readily discernible, but various observations of these authors strongly indicate that it is this structure that is attacked by their sperm extracts. Other effects of this

material include inhibition of fertilization in P. miliaris, inhibition of motility of sperm of E. cordatum, but not of P. miliaris, inhibition or retardation of agglutination of E. cordatum sperm by homologous egg water and by antiserum and sphering of mammalian erythrocytes. The authors find that most of these effects can be imitated by certain detergents (Duponol and Dupont QB), and by honey bee venom.

B. CHEMICAL AND ANTIGENIC PROPERTIES. 1. *Egg-membrane lysin.* Some of the properties of the lytic agent in extracts of Megathura sperm have been examined by Tyler (200). It is nondialyzable, salts out in saturated ammonium sulphate, is not extractable by alcohol and is readily inactivated by crystalline trypsin or chymotrypsin. It seems fairly safe to conclude that the lysin is of protein nature. It is extremely heat labile; at 50° C., for example, its half life is about one minute. Solutions prepared by ammonium sulphate precipitation and dialysis possess antifertilizin activity and the latter remains unaltered when the lysin is heat-inactivated. This does not necessarily mean that there are two distinct, separate-acting substances present. Considerably more information will be required before definite conclusions may be drawn in this regard.

Antiserum prepared in rabbits against active or inactivated preparations of the lysin precipitate with the solutions and render them inactive for membrane lysis (221). Whether or not they also neutralize the antifertilizin is not readily determinable, since the antiserum agglutinates the sperm. Cross reactions with abalone sperm were not obtained. The results show that the specific structures causing lysis are not really antigenic. This is consistent with the results of similar, more extensive investigations with hyaluronidase (see below).

2. *Hyaluronidase.* Work on the chemical properties of this agent has been reviewed by Meyer (136). In the present connection it should be emphasized that heat labile proteins are again involved. This is shown primarily by their inactivation by pepsin and trypsin, but not carboxypolypeptidase, nondialyzability and precipitation by many reagents commonly employed for proteins. At 60°C. solutions of bull testis-hyaluronidase at pH 7 lose 99 per cent of their activity in five minutes, 99.9 per cent in 15 minutes, and at room temperature activity is very rapidly lost below pH 4 and above pH 10 (127). Chemically purified preparations were found to be electrophoretically and ultracentrifugally inhomogeneous, the active component being isoelectric at about pH 5.7 and having a sedimentation constant (S_{20}) of 4.3 (62). Detectable hydrolytic or spreading reactions are obtained with as little as 10^{-4} mgm. of purified material.

There is evidence that hyaluronidase from different sources may differ enzymatically (see Meyer, 136). While all cause an initial depolymerization as shown by viscosity reduction (and presumably also by spreading and mucin clot tests), they differ in the extent to which they complete the hydrolysis of the hyaluronic acid into its acetyl glucosamine and glucuronic acid components. Testicular and leech hyaluronidase give only about half the theoretical yield (measured as reducing sugars), while pneumococcal hyaluronidase gives almost complete hydrolysis and can supplement the action of the testicular and leech materials. Apparently there are different enzymes (or different active groups on the same molecule) involved in the depolymerization and in the hydrolysis of the aldobionic acid. Hahn (63) has re-

ported separating two such enzymes from testicular extracts, as well as an acetylglucosaminidase which is not related to hyaluronidase activity.

Numerous investigators have shown that the activities of hyaluronidase can be blocked by means of specific antisera (see Duran-Reynals, 33; Meyer, 136). In contrast to the lack of species-specific action of hyaluronidase, the antisera are highly specific. Thus, antisera against the hyaluronidases of Clostridium welchi and Cl. septicum do not cross-react (130). In tests of dispersal of follicle cells of rat ova, antisera prepared in rats against bull testis-hyaluronidase did not cross-react with the rat testis enzyme (103). It may be concluded that the enzymatically active groups of the hyaluronidase are nonantigenic. Neutralization by homologous antibody may then be attributed to precipitation of the enzyme (which might also be linked to some antigenic protein) or blocking of the enzymatically active regions of the molecule by the presence of antibody combined with adjacent antigenic groups, or both.

3. *Egg-surface lysin.* Preparation of this substance according to the latest method described by Runnström and Lindvall (183) involves methanol extraction of frozen-dried sperm, solution in chloroform, absorption on zinc carbonate column which separates it from accompanying yellow coloring matter, elution in ethyl alcohol and repeated solution in distilled water and evaporation at 80°C. The substance is heat stable and dialyzes through cellophane.

The chemical properties of the egg surface lysin are quite different from those of antifertilizin. Its ability to neutralize fertilizin might imply some similarity in constitution or it might even represent the determinant groups of the antifertilizin molecules. Against this is the observation that it is not in turn neutralized by fertilizin and that it is relatively nonspecific. It seems more likely that it inhibits the agglutinating action of fertilizin by some nonspecific action such as tying up calcium, the absence of which interferes with the agglutination of sea urchin sperm. Its other effects seem to be more direct and lack of calcium, for example, has an effect opposite to that of the lysin in the hypertonicity test (186).

C. Role in fertilization. 1. *Egg-membrane lysin.* The manifest action of the lysin on the membrane barriers in molluskan and amphibian eggs implies that it is instrumental in enabling the individual spermatozoön to penetrate to the surface of the egg proper. For this purpose the membrane need not be completely dissolved. The membrane of eggs of this type remains visibly intact when penetrated by one or a few spermatozoa which evidently cause a small, hardly discernable lesion. Loeb (121) developed a theory of fertilization that postulated the presence of a lysin in sperm along with a corrective factor. The lysin was supposed to act on the surface of the egg and would lead to its destruction but, being checked by the corrective factor, results in activation. The membrane lysin cannot be considered a lytic agent of this sort. It has no effect on the surface of the egg proper, does not cause artificial activation nor affect the development of fertilized eggs (200). There have been reports (35, 164) of artificial activation of amphibian eggs by microinjection of sperm extracts but, since such eggs can be activated by simple puncture and by chemical means (see 205), it cannot be concluded that the extracts contained the particular activating agent of the sperm.

Since the egg membrane lysin is carried by the spermatozoön and probably enters

the egg with it, there is certainly the possibility that it participates in other activities, but there is as yet no evidence of further action beyond that described.

2. *Hyaluronidase.* The follicle cell mass of the mammalian egg is of tough consistency and evidently constitutes a barrier which would be difficult for the spermatozoön to traverse by mechanical means alone. The demonstration that the cementing material (probably hyaluronic acid) is dissolved by hyaluronidase, which the spermatozoön carries, provides a ready explanation of its ability to penetrate this barrier. Since the hyaluronidase that the sperm carries evidently functions in this manner it has seemed reasonable to deduce that addition of solutions of hyaluronidase to nonfertilizing quantities of sperm would effect fertilization. Experiments along this line have been reported by Rowlands (179) and by Kurzrok, Leonard and Conrad (100). According to the latter workers, fertilization was obtained in six human females, with a history of sterile marriage, when bull testis-hyaluronidase was added to the semen specimen used for artificial insemination or placed in the genital tract prior to coitus. Since appropriate controls are not readily feasible in experiments of this type with humans, the effect of hyaluronidase on the results is difficult to assess. Rowlands reports a series of seven experiments with rabbits. In two experiments the number of spermatozoa required for fertilization was checked and found to range from 4.5×10^5 to 1.8×10^6. In the other five experiments hyaluronidase (filtrate from 2×10^7 sperm) was added to the inseminates and increased fertility was obtained in four of them. The median effective concentration of sperm in these range from 5.4×10^4 to 1.5×10^5, and the average increase in fertility is such that one-sixth the normal amount of sperm is needed when the enzyme is present, the range being from one-thirtieth to equal concentration in different tests.

There is, then, evidence that hyaluronidase enhances fertilization in mammals. Since it is added in solution it would appear that it acts by contributing to the dispersal of the follicle cells. This would support the previous explanation, offered by McClean and Rowlands (131), of the large number of sperm required for fertilization in mammals. The additional sperm are presumably required to break down the follicle coat of the egg. This implies that a single spermatozoön does not carry enough enzyme for this purpose, not even enough to make a little pathway for itself. Since enzymes are not ordinarily used up in the reactions that they catalyze, it might be supposed that in time a single spermatozoön would manage to break down the coat sufficiently to get through. But this might involve a longer time than its life span would allow. There are, of course, other possible explanations for the apparent failure of a single spermatozoön to get through the coat unaided. Thus there may be loss of hyaluronidase from the spermatozoa in transit and inactivation of dissolved hyaluronidase with time at body temperature. It has been shown that the hyaluronidase content of semen is proportional, within limits, to the sperm concentration (240, 100). The hyaluronidase is evidently given off continuously by the spermatozoa and this is apparently aided by secretions of the seminal vesicles, since the latter tissue, when incubated with testis homogenate, gives an increased yield of hyaluronidase (104). In the genital tract of female rabbits the ratio of hyaluronidase to spermatozoa was found to decrease with time (61) and this has been interpreted to mean that 'secretion' of the enzyme ceases there. However, to establish that

point it would be desirable to know how the activity of hyaluronidase solutions, introduced into the genital tract, varies with time. In artificial insemination experiments the added hyaluronidase must evidently reach the eggs and it is, then, of importance to learn how its transportation up the genital tract is accomplished. It is also of interest to know to what extent the follicle cell mass must be dispersed in order to effect successful fertilization. By way of comparison, a striking example of fertilization without loss of follicle cells may be mentioned. The eggs of ascidians are surrounded by a layer of so-called test cells, covered by a continuous membrane on which is another layer of follicle cells. The spermatozoön must penetrate through these cells, or the intercellular cement, and through the membrane (which has no pores large enough for the sperm). This is accomplished without apparent effect on these coats which persist until the developing embryo is ready for hatching.

It should be emphasized that it is not only in mammals but in practically all animals that large numbers of spermatozoa must be present in order to effect fertilization. One reason for this is quite evidently the necessity of providing sufficient opportunity for sperm and egg to encounter within their respective life span. Another factor is the proportionate incapacitation of sperm by their reaction with specific egg substance in solution in the medium (see p. 000). Another factor may be the occurrence of large numbers of physiologically defective sperm, but there is as yet no specific evidence concerning this. In any event, such factors may also be expected to be involved in fertilization in mammals. The great interest that has been aroused by hyaluronidase will undoubtedly result in experiments designed to answer these and other questions relating to the precise manner in which it is involved in the fertilization process.[2]

3. *Egg-surface lysin.* The lytic agent postulated by Loeb (121) might appear to be represented by the surface-liquefying agent that Runnström *et al.* (183–189) extracted from sperm. Runnström has mentioned (personal communication) that the substance is an unsaturated fatty acid. It would thus resemble agents known to initiate activation and also cause lysis in sea urchin eggs. Runnström *et al.* (188) consider their results to offer some support for Loeb's lysin theory of activation.

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[2] While the present article was in press papers dealing further with these questions have been published by G. I. M. Swyer (Biochem. J. **41**: 409, 1947 and **41**: 413, 1947), by M. C. Chang (Proc. Soc. Exp. Biol. Med. **66**: 51, 1947) and by S. L. Leonard, P. L. Perlman and R. Kurzook (Proc. Soc. Exp. Biol. Med. **66**: 517, 1947). Swyer finds the hyaluronidase content of semen of various mammals to be directly correlated with sperm count. He also demonstrates that hyaluronidase is not actively manufactured by the ripe spermatozoa, but simply dissolves off at various rates depending upon conditions. The rate of liberation of the enzyme is found to increase with dilution of the suspension and to decrease upon addition of hyaluronidase to the suspension.

The other two papers further justify the doubts expressed above as to the necessity for dispersal of the follicle cells. Chang finds no enhancement of fertilization due to addition of hyaluronidase in rabbits. He interprets Rowland's positive results as due to the action of the seminal fluid present in his hyaluronidase preparations since that fluid, free of hyaluronidase, is found to be capable of maintaining the fertilizing capacity of the sperm. The observations of Leonard *et al.* on rats show that fertilization normally takes place before there is any distinct dispersal of the follicle cells and that introduction of hyaluronidase into the uterine horns of rats in heat results in no denudation of tubal eggs. These papers support, then, the view that the individual spermatozoon carries enough hyaluronidase to effect its penetration through the follicle coat of the egg.

However, there is no evidence that their lytic agent induces membrane elevation or causes general cytolysis upon prolonged treatment of the eggs except perhaps in very high concentration. Hypertonic sea water, which is a corrective agent according to Loeb, has the reverse action in connection with Runnström's lytic agent. Runnström *et al.* (188, 183) have also expressed the view that the lytic agent may be responsible for the establishment of the block to polyspermy. They note that it is obtainable from eggs as well as sperm and so could be released in the egg surface upon attachment of a single spermatozoön. Since the agent inhibits agglutination they consider it to be effective in inhibiting the attachment (agglutination to the egg surface) of supernumerary spermatozoa. It should be remarked, however, that supernumerary sperm attach themselves to the surface of eggs and remain (attached?) there after fertilization. That part of the hypothesis appears then unnecessary and they could appeal simply to the demonstrated fertilization-inhibiting action of their lytic agent to support the view that it is instrumental in preventing polyspermy, provided that there is evidence of sufficiently rapid release and action on the egg surface.

That materials with detergent-like action can be extracted from sea urchin sperm and eggs is of decided interest, but before speculating on their possible role in fertilization, it is desirable to know whether they are available under physiological conditions. Runnström *et al.* (188) report preliminary tests showing that the agent is present in the supernatant obtained by centrifugation of a heavy suspension of live sperm or frozen and thawed sperm. They conclude that it is rather loosely bound to the spermatozoa. Presumably, too, it is not given off by living spermatozoa in sufficient amount to render the egg nonfertilizable before the spermatozoa reach it.

4. *Self-sterility in Ciona.* In connection with egg-membrane lysins, as well as the other specific substances of eggs and sperm, the phenomenon of self-sterility in the hermaphroditic ascidians should be discussed. In recent years this has been the subject of extensive investigations by T. H. Morgan (145–156), who is also responsible for most of the early work on this topic, and by H. Plough (173–175). Only a brief review of such features as pertain to sperm and egg extracts will be attempted here. In many hermaphroditic animals including the ascidians the eggs are more or less refractory to sperm of the same animal. This is strikingly exhibited in the ascidian Ciona in which a sperm suspension that gives 100 per cent cross-fertilization (with eggs of another individual) will, even when over 200 times more concentrated, generally yield little if any self-fertilization. The absolute amounts of sperm required varies in different individuals and, in all cases, increasing the concentration of sperm increases the percentage of self-fertilization, but only rarely can 100 per cent selfing be obtained. It is evident that self-sterility is a relative affair. For brevity, however, we shall use the term without qualifying adjectives. Cross-insemination tests with wild individuals show occasional cases of cross-sterility and these occur more frequently among offspring of a self-fertilized individual or of a single pair of individuals. Morgan showed that the situation has a genetic basis, although the exact factor-analysis has not as yet been accomplished. He also demonstrated by various ingenious experiments that the block to self-fertilization resides in the membrane (between test and follicle cells) that surrounds the egg. This membrane

and the adhering cells can be removed mechanically (with some difficulty) or by means of proteolytic enzymes, and the eggs are then self-fertilizable. It is not, however, necessary to remove the membrane. Treatment of the eggs for five minutes with pH 2.7 sea water has no visible effect on it, but renders the eggs self-fertilizing.

The block to self-fertilization is evidently of highly specific nature since the gametes of almost any pair of individuals selected at random fertilize readily. The materials on which it depends must, then, be of a highly specific nature. In some early experiments by Fuchs (45) egg water and egg extracts were found to enhance the cross-fertilizing power of Ciona sperm. Morgan extended the experiments to self-fertilization with tests of various kinds of egg and sperm extracts. For the most part no marked effects were obtained. The presence of, or treatment with, self-egg water was found (145, 146) to reduce the self-fertilizing, but not the cross-fertilizing capacity of the sperm. However, in the cross-fertilization tests an excess of sperm was generally present and occasional experiments did show inhibition. Foreign egg water gave no increase in self-fertilization. Sperm extracts would cause a slow (24 to 48 hours) breakdown of the membrane in many cases and this seemed to occur more readily with foreign eggs than with eggs of the same individual. No significant facilitation of selfing nor inhibition of crossing was obtained by use of the sperm extracts.

There is, then, some indication that lytic and specific interacting substances may be extracted from Ciona sperm and eggs. The relative weakness of the extracts may be attributed to various factors, such as methods of extraction, instability, etc. The facts of self-sterility preclude the possibility that the lytic agent is simply a proteolytic enzyme, although it is possible that some such enzyme may be released by the interaction of the specific substances of the egg and sperm. These substances must evidently possess a degree of specificity of interaction that is as great or greater than that exhibited in the human blood groups.

GENERAL CONCLUSIONS

From the work reviewed here it is evident that fertilization involves the interaction of a number of substances on and within the eggs and sperm. Certain of these substances interact in the manner of antigen and antibody. Others have enzymatic activities and others even may have detergent-like action. The general chemical nature of certain of these agents has been established. Their precise role in fertilization has not as yet been determined, but the evidence shows them to be concerned in the initial steps, including the penetration of coats and membranes surrounding the egg and attachment to the surface. The action of these and other substances in the further steps, involving incorporation of the spermatozoön into the egg and activation of the latter, is for the most part unknown. The specificity of interaction of these substances provide a basis for understanding the tissue and species-specificity of fertilization, although none of the specificities so far examined show precise correspondence. Various properties of the interacting substances provide analogies with phenomena in the fields of immunology and virology. These include such features as blocking antibodies, heteroagglutination, zone phenomena, phage neutralization, bacterial invasiveness, etc. Concepts employed in these fields find application in

studies of fertilization and these in turn suggest new general views, such as the auto-antibody concept of cell structure that is briefly discussed.

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CHEMORECEPTION IN INSECTS

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HIGHLY SPECIALIZED SENSORY RECEPTORS normally capable of being excited by direct contact with chemicals attain their most spectacular development in two groups of animals, the insects and the vertebrates (cf. von Frisch, 59). Despite radical histological and chemical differences in the superficial structure of the end organs within the two groups, there appear to be striking physiological similarities in the behavior of each toward numerous chemical compounds and in the manner in which stimulation is presumed to be mediated. This apparent paradox has not only stimulated interest in the study of insect chemoreceptors but has also promoted a wealth of confusion by reason of anthropomorphic interpretation. Nevertheless, it is becoming increasingly apparent that chemoreception throughout the animal kingdom is basically similar and that much is to be gained by the study of forms, such as insects, which frequently lend themselves more advantageously to experimentation than does man. The present review, therefore, considers chemoreception in insects, not as an isolated physiological phenomenon, but as one facet of the intricate relation between animal chemoreceptors and the compounds which excite them.

DISTINCTIONS BETWEEN TASTE AND SMELL

It is now generally accepted that the chemical senses of insects may be separated into three categories which correspond roughly to smell, taste and the common chemical sense of vertebrates (39, 59, 103, 104, 145). However, the criteria upon which this division is based are at least as tenuous and unsatisfactory as those applied to vertebrates (73, 96, 111). The following well-known points advanced to permit of distinction between the gustatory and olfactory senses of vertebrates are here re-examined in the light of newly acquired data on the physiology of invertebrate chemoreceptors (59, 73, 103):

1) the two types of end organs differ anatomically and with respect to their distribution in the body; 2) olfaction is subserved by primary neurones and gustation by secondary neurones; 3) the two senses differ with respect to their threshold concentrations; and 4) compounds which stimulate one set of receptors usually do not stimulate the other set.

1) The adequacy of anatomical and topographical factors as valid criteria in insects suffers on two counts. Although the distribution of chemoreceptors has been the subject of heated controversy since 1798, the great body of accumulated experimental evidence is inadmissible by reason of questionable techniques (cf. Mar-

shall, 96). For this reason and because new loci of chemical stimulation are still being discovered (40), it is not yet advisable to relegate each chemical sense to specific areas of the body. A second difficulty is imposed by the fact that the end organs subserving both senses frequently occupy the same segments of the same appendage (e.g., the antennae of honeybees and the labellum of muscoid flies). It follows, therefore, that nerve fibers from both types of receptors are contained in the same nerve wherefore they enter the same lobe of the brain. In the case of the antennal nerve of honeybees this would be the deutocerebrum. Here the fibers even follow the same tracts inasmuch as all terminate in glomeruli situated principally in the deutocerebral neuropile. Sensory fibers from the labellum are gathered into the labial nerve, which enters the suboesophageal ganglion. Hence no analogy can be drawn with vertebrates where olfactory receptors are innervated by the olfactory nerve and taste receptors by the chorda tympani, glossopharyngeal and vagus (7, 81, 115, 120, 154).

2) As Hanström has pointed out, the histological criteria applied to vertebrates are inadequate here because all receptors in insects are primary sense cells (cf. Snodgrass, 128). Moreover, histological information sufficient to permit a comparison on the basis of any other points of finer structure is still incomplete, since considerable doubt surrounds the identity of the chemoreceptors in many insects.

3) Quantitative differences based upon threshold values constitute the most promising point of comparison. Parker and Stabler (112) have shown that the minimum concentrations of ethanol perceptible by each of the three chemical senses in man are: for smell 1.25×10^{-4} M, for taste 3 M, for the common chemical sense 5 to 10 M. Comparable values measured as rejection thresholds have been determined for insects. Ethanol acting as a gas on the antennal and labial end organs of female houseflies is rejected at 5×10^{-3} M (144). Acting as an aqueous solution in 0.1 M sucrose on the tarsal receptors of blowflies it is rejected at a concentration of 3.2 M (42). Both values should, of course, be obtained for the same species; however, the similarities between the sensitivities of the tarsal receptors of unrelated species indicate a strong likelihood that this comparison between two closely related flies may be a valid one.

The relative sensitivities of taste and smell are more strikingly illustrated when the limits in each category are examined. For man, one of the most effective taste substances is quinine hydrochloride. It is perceived in concentrations which are approximately 1.5×10^{-7} M (108). Aquatic beetles may be conditioned to it at dilutions of 1.25×10^{-6} (9). One of the most effective olfactory stimuli for man, a mercaptan (probably methyl), is perceived in concentrations which are approximately 9×10^{-13} M. Values for this or similar compounds have not, however, been obtained with insects. Values which approximate the limits of sensitivity for the two categories of receptors have been obtained with a few other compounds. The tarsal receptors of some butterflies (e.g., *Danaus menippe*) can be stimulated by solutions of sucrose as dilute as 9.8×10^{-6} M (5); those of some flies (*Calliphora vomitoria* and *Cynomyia*) by 3.9×10^{-5} M sucrose (27, 105). Carefully conducted experiments designed to test the sensitivity of olfactory receptors have been few. Warnke (140) has found that dung beetles of the genus *Geotrupes* respond to the odor of a 2.3

$\times 10^{-8}$ M skatol gas. The tent caterpillar, *Malacosoma americana*, is repelled by a gaseous mixture of benzaldehyde equivalent to 4.1×10^{-7} M (39). In neither case, however, do these values represent the limit of sensitivity for it is known that insects can be stimulated by incredibly low concentrations of substances in the gas phase. By way of illustration, odors, probably lipoid, protein or ester (23, 72) emanating from female gypsy moths attract males from distances as great as $2\frac{5}{16}$ miles.

That a strict distinction between taste and smell cannot be drawn even on a quantitative basis is illustrated by a comparison of thresholds of the most stimulating taste and smell substances for man (63). When these are compared in terms of molecules per cubic millimeter, a continuous spectrum and overlapping of stimulating effectiveness is observed from the most effective olfactory substance (trinitrobutyltoluene at $1.03–2.06 \times 10^4$ molecules per cu. mm.) through one of the least effective (nitrobenzene, 2.006×10^{11}) to the most effective taste substance (quinine sulfate, 2.42×10^{11}). Nevertheless, the fact is inescapable that taste is on the whole less sensitive than olfaction.

4) The proposition that that which is smelled is usually not tasted and vice versa (111) is even less applicable to insects than it is to vertebrates. While it is true that sugars and many inorganic compounds (e.g., salts) which stimulate insect gustatory receptors apparently fail to stimulate olfactory receptors, many organic compounds (viz. alcohols, aldehydes, fatty acids, etc.) as solutions, stimulate oral and tarsal receptors (22, 42) and as gases stimulate olfactory receptors (24, 144). Two crucial experiments which would test this proposition have not been undertaken with insects and with vertebrates have yielded controversial results. It is not known for insects whether solutions which stimulate gustatory receptors can, as solutions, stimulate olfactory receptors nor whether compounds which in the gas phase stimulate olfactory receptors can do so equally well in the liquid phase.

Kunze (90) and Marshall (97) have shown that the antennae of the honeybee can be stimulated both by 'mild odors' and by solutions of sucrose or certain other taste substances. The antennae bear, however, innumerable sensilla of several different types, and there is no proof that the same receptors are responding in each case. Thus this cannot be construed as evidence to support a hypothesis that there is no rigid barrier between the concepts of gustation and olfaction.

Despite the inadequacy of the foregoing criteria it must be concluded that there is as much ground for separating taste and smell in insects as in vertebrates. In terrestrial insects and those aquatic species which are adventitiously terrestrial there are clearly two categories of receptors involved. The olfactory receptors react to very low concentrations of compounds which are volatile at ordinary temperatures; the gustatory receptors react to higher concentrations of liquids or solutions which may or may not be volatile at room temperature.

With regard to aquatic species the situation stands in need of clarification by additional experimental inquiry. While it appears that two categories of receptors are involved here also, the experiments of different workers are contradictory in several important respects. In each study, water beetles were conditioned to one of several compounds arbitrarily designated as odor substances (artificial musk, coumarin,

heliotropin and skatol) or to one of several taste-substances (sucrose, NaCl, HCl and quinine hydrochloride). According to Schaller (125) *Dytiscus* perceives odors under water by means of the nine funicular segments of the antennae and by the tips of the maxillary palpi. Out of water the same compounds stimulate as vapors. Taste substances, on the other hand, are perceived by the submerged beetle through the maxillary palpi, the labial palpi and the inside of the mouth. According to Ritter (122) *Hydrous piceus* bears olfactory receptors on the apices of the maxillary palpi only. Sucrose, NaCl and quinine salts stimulate organs on the tips of the maxillary palpi while stimulation by HCl is restricted to the labial palpi. Bauer's (9) results are in disagreement in that he failed to prevent responses to sugars, salts and bitter substances by removing the maxillary palpi of *H. piceus*.

Localization of the sour taste on a different appendage from that bearing the other taste organs, if indeed true, casts further doubt on the wisdom of attempting to distinguish taste from smell solely on anatomical grounds. Moreover, the conditions of all of the experiments cited above precluded the accurate control of concentrations; hence, the criterion of sensitivity cannot yet be applied to the separation of taste and smell in aquatic species. Furthermore, at least two of the 'odors' tested, heliotropin and coumarin, stimulate the gustatory as well as the olfactory sense of man and in the case of the aquatic beetles may conceivably have acted upon receptors other than olfactory.

Experiments with fishes have indicated that water solutions of compounds which are taste substances for terrestrial vertebrates act as taste substances for aquatic vertebrates also, while compounds which in aqueous solution are olfactory for fishes are olfactory for terrestrial animals (58, 59). If the results of Schaller, Ritter and Bauer can be reconciled one with the other, they constitute grounds for extending this parallel to insects.

With wireworm larvae (*Agriotes* spp.), which burrow in moist soil, the response to chemical stimulation is essentially like that of aquatic organisms. There are clearly two categories of stimulating compounds. Low concentrations of aqueous solutions of several dibasic acids, amino acids and amides elicit an orientation response. Solutions of sugars, triolein and animal proteins in high concentration elicit both an orientation response and a biting response. Neither group stimulates in the gas phase (136). The concentrations necessary to evoke a biting response are said to be comparable to those usually adequate for organs of taste while effective concentrations in the case of those compounds which elicit only an orientation response are of the order characteristic of olfactory stimulation. For example, the activity (negative logarithm of concentration) of aspartic acid is 11; that of triolein, 2. Recent experiments (26) place the receptors on the antennae and galeae of the maxillae. The former are large sensilla basiconica; the latter, small sensilla basiconica. Stimulation of the maxillary organs by compounds of the second above-mentioned category (e.g., two per cent glucose) provokes both orientation and biting responses while stimulation by compounds of the first category (e.g., asparagine at 10^{-8}–10^{-9} mgm./l.) provokes only an orientation response. On the other hand, stimulation of the antennal organs either by two per cent glucose or 10^{-8}–10^{-9} mgm./l. asparagine provokes only orientation. It is difficult to interpret these results in terms of taste and

smell, and the authors of the experiments scrupulously avoid doing so. At best it can be pointed out that both categories of compounds stimulate both types of receptors, and that the animal may be able to distinguish between compounds of the two categories when they stimulate the maxillae but not when they stimulate the antennae.

Solely for convenience of discussion, therefore, we shall consider the gustatory sense of terrestrial insects as that which is mediated by chemical stimuli acting as liquids or solutions at relatively high concentrations on contact and the olfactory sense as that mediated by chemical stimuli in a gaseous state at relatively low concentrations.

COMMON CHEMICAL SENSE

It can no longer be doubted that insects possess a third category of chemoreceptors in addition to and distinct from olfactory and gustatory receptors. Adequate stimuli are high concentrations of numerous so-called irritating compounds of which ammonia, chlorine and essential oils are examples (74). Early failure to recognize the existence of a common chemical sense was largely responsible for the contradictory results which for a century and a half stemmed from experiments designed to locate the olfactory organs (cf., e.g., 93, 94).

It appears that several classes of compounds can stimulate both the olfactory and common chemical senses. Insects which have been conditioned to weak odors of essential oils lose their ability to recognize these odors after the olfactory receptors are extirpated (51) but continue to exhibit violent avoiding reactions if the concentration is increased to maximal. Mealworm beetles fail to respond to odors from food or the opposite sex if the antennae are amputated, but responses to the vapors of essential oils persist. Unilateral amputation results in circus movements toward the intact side if normal odors are presented. When the vapors of essential oils are used as stimuli, circus movements are away from the intact side (137). In this connection the observations of Weyrauch (143) concerning the earwig *Forficula auricularia* L. are interesting. Food odors directed to the antennae of this insect elicited no response, but strong artificial odors did. On the other hand, food odors evoked responses when directed to the maxillary palpi. Following amputation of the palpi these responses ceased. Weyrauch concluded that the maxillary palpi mediated positive chemotaxis; the antennae, negative chemotaxis.

Few quantitative experiments dealing with the common chemical sense have been recorded. Dethier (38) demonstrated with *Pieris* larvae that the threshold of response to the odor of benzaldehyde is increased 17 times following removal of the olfactory receptors; however, no attempt was made to localize the sensitive region.

This much can be said with certainty regarding the common chemical sense. For excitation, a high concentration of stimulus is required, and the response is always in the nature of an avoiding reaction. The receptors have not yet been located.

OLFACTORY SENSE

Adequate stimuli for olfaction in terrestrial insects are the vapors of compounds which volatilize at physiological temperatures. Literally thousands have been in-

vestigated (cf. Dethier, 39), albeit under pressure of economic needs. The behavioristic aspects of olfaction have been adequately treated by Fraenkel and Gunn (50) and Dethier (39), but the physiology of olfaction has been less extensively studied.

1. *Receptors*

Olfactory receptors in insects are located chiefly on the antennae (96, 145). They are, in addition, commonly found on the maxillary and labial palpi or their homologues. These sites of action have been discovered through observations of the ability of insects from which one or more appendages have been extirpated to orient to food or sex odors or odors to which they have been conditioned. In evaluating localization experiments of this sort it has been necessary to take into consideration possible deleterious effects attendant upon destruction of stimulatory organs. Some insects bear in the antennae such stimulatory organs (153) whose somewhat secondary proprioreceptive function is to assist in the maintenance of muscular tone and in the initiation of muscle kinetics. Following antennal amputation these insects fall into a state of akinesis from which they are aroused only with difficulty (149). For many species there are no untoward effects following destruction of the various appendages.

In a few species the identity of the actual receptive surfaces involved has been determined experimentally. Previously function was ascribed on morphological grounds entirely. Three basic types of receptors have now been identified: pore plates (sensilla placodea) (57), thin-walled cones and pegs (sensilla basiconica, 11, 19, 38, 41, 140, 141, 146), and thin-walled pegs or cones sunken in pits (sensilla coeloconica, 11).

In his classic experiments von Frisch (57) trained honeybees to associate food with the odor of an essential oil. Bilateral amputation of the flagellar segments of the antennae did not impair this response as long as the proximal three-fourths of the fourth flagellar segment of one antenna remained intact A histological survey of the antennal sensilla revealed that the pore plates alone coincided in distribution with the physiological results. Warnke (140) was able to demonstrate with dung beetles (*Geotrupes sylvaticus* and *G. vernalis*) that the areas of the antennae and palpi which were necessary for olfaction bore a profusion of sensilla basiconica peculiar to those areas. Dethier (41) showed that species of *Necrophorus* and *Silpha* were able to orient from distances to decomposing flesh as long as a small fraction of one antennal lamella remained intact. Again this bore a type of sensillum basiconicum not found elsewhere. With lepidopterous larvae he demonstrated that the antennae bore olfactory receptors and that of the four types of sensilla present the sensilla campaniformia were proprioceptive, the sensilla trichodea tactile and the sensilla basiconica olfactory. Wigglesworth (146) proved that the louse *Pediculus humanus corporis* de Geer loses its ability to respond to odor when the sensilla basiconica of the antennae are covered with cellulose paint, but that removal of the paint restores olfactory perception. Begg and Hogben (11) compared the olfactory responses of wild type *Drosophila melanogaster* with those of the mutants *antennaless* (A_0) and *aristapedia* (ss_a). *Antennaless* fails to respond to odors which normally lead the

flies to food, and *aristapedia* responds less strongly than do normal flies. A comparison of the antennae of the two forms (wild type and ss_a) indicated that either the sensilla basiconica or sensilla coeloconica or both are olfactory receptors. Bolwig (19) has proved that amputation of the dorsal papillae of the cephalic lobes of housefly larvae completely destroys the olfactory sense. These papillae are forms of sensilla basiconica.

All of these olfactory receptors possess in common a cuticular covering which is completely or partially thin-walled, innervation by a *group* of bipolar sense cells, and minute refringent bodies (Riechstäbchen) situated on the distal processes of the neurones (128). Nothing is known concerning the chemical or physical properties of the cuticle surmounting these receptors.

2. *Acuity*

Attempts have been made to assay the acuity of the olfactory sense and its powers of discrimination by determining threshold values. Such values are empirical inasmuch as it has been impossible thus far to determine the absolute threshold. This may be defined as the lowest concentration necessary to excite the most sensitive receptor element. As molecular concentration exceeds this amount a point is attained which may be termed the threshold of response. There is little doubt that this includes an expression of central nervous system phenomena. Whether it expresses itself initially as acceptance or rejection is predetermined by the genetic constitution of the animal as well as by its physiological state. It may be altered experimentally by such factors as preimaginal conditioning (134). If the threshold of response is manifested as a true rejection, the compound is rejected throughout the entire supraliminal range. Since the response frequently becomes more violent as the concentration is increased, it suggests that a different group of receptors, those of the common chemical sense, for example, may become involved. If a compound is initially accepted, it may become more attractive as the concentration is increased until an optimum is attained. Beyond this point attractiveness decreases with increased concentration till the nature of the response is completely reversed. Again it is probable that either a different set of receptors becomes involved or there is an increase in the frequency of impulses discharged by the receptors initially stimulated. For every attractant thus far tested there has been found a concentration in excess of which the substance is rejected. This has been illustrated by Dethier's analysis of data from Reed (121) for *Drosophila*, Folsom (48) for the Mexican boll weevil and Wieting and Hoskins (144) for the housefly.

Thresholds of response have been determined for a few odorous compounds with a small group of insect species. These values are given in the accompanying table 1. It is obvious that the olfactory acuity of insects and vertebrates cannot be compared on the basis of available data. On the strength of an insect's ability to orient to food, host, mate or nest by means of odors imperceptible to man, it is generally conceded that the insect sense has a vastly lower threshold. On the other hand, von Frisch (56) has demonstrated that the minimum perceptible odor of many essential oils is of the same order of magnitude for the honeybee and man. His experiments were based upon observations of the responses of conditioned bees to the odor of

various dilutions (in paraffin oil) of a test compound (essential oils). They were thus semiquantitative at most inasmuch as the concentration in terms of weight per unit volume of gas was unknown. Conditioning as a technique appears to be legitimate in that thresholds so determined depend in no wise upon the concentration to which the insect is conditioned (56).

All of the olfactory organs of insects are not equally acute. In general the thresholds for antennal organs are probably lower because many species can respond to distant sources of odor only so long as the antennae are intact. At the same time the ability to respond to stronger odors or those near at hand is not impaired as long as the palpi are intact (41, 51, 64, 140). Measurements of the threshold of response of larvae of the cabbage butterfly (*Pieris rapae*) for benzaldehyde before and after amputation of either the antennae or maxillae also suggest dual sensitivity (38).

TABLE 1. COMPARISON OF OLFACTORY THRESHOLDS OF CERTAIN INSECTS AND MAN

COMPOUND	MAN (RECOGNITION THRESHOLD, MGM./L.)	SPECIES OF INSECT (THRESHOLD OF RESPONSE, MGM./L.)		AUTHORITY
skatol	0.000,000,4	*Geotrupes sylvaticus* *G. vernalis*	0.003–0.009	Warnke (140)
indol		" "	0.003	Warnke (140)
benzene	0.0053	*Habrobracon juglandis*	0.5–3.0	Wirth (151)
cyclohexane	0.25	" "	3–5	" (151)
ethanol	5.76	" "	5–20	" (151)
"		*Musca domestica*	230	Wieting and Hoskins (144)
ammonia	0.035	" "	0.04	" "
"		" "	1 (rejection)	" "
benzaldehyde		*Pieris rapae*	0.058	Dethier (38)

Before antennectomy or maxillectomy the threshold is 0.058 mgm./l. No change follows maxillectomy; following antennectomy the threshold is 0.077 mgm./l. Following combined antennectomy and maxillectomy the threshold rises to one mgm./l. This may represent a threshold of the common chemical sense.

3. *Factors Influencing Threshold of Response*

Any values obtained by an experimental method which utilizes some component of behavior to signal stimulation are subject to at least the same factors of variation as is the behavior itself. Such variations in behavior are discussed at some length by Fraenkel and Gunn (50). It is to be expected, then, that olfactory thresholds of response may be profoundly influenced by various environmental and internal physiological factors. Among the former are temperature and humidity.

Nearly all investigators working with olfactometers have found temperature and humidity control necessary. In most of the apparatuses response depended upon locomotion and orientation. Alterations of response were then traceable to the effects of temperature and humidity on the overall activity of the animal. This condition may also apply to feeding responses for which optimum temperatures and

humidities have been observed (2). Because of the relation between food and odors it is to be expected that responses to the latter may be altered in the same manner by the same variables. No evidence has been forthcoming to indicate that absolute thresholds in insects are affected by these factors, although for man it has been reported (152) that odor perception is more acute at low humidities than at high.

Internal physiological states which may affect thresholds of response are age, sex, state of nutrition and conditioning.

Records of age effects are few. Schwarz (127) has suggested, but not proved, that olfactory acuity decreases with the onset of senility. He offers this as an explanation of the fact that old individuals of certain moths frequently oviposit on plants unacceptable to the larvae. Bolwig (19) cites experiments to indicate that fly larvae in the first instar are less responsive to olfactory stimulation by normal food odors than are older larvae. He warns that the lack of response may be an artifact arising from the technique employed. He did prove, however, that housefly larvae suddenly change their behavior toward ammonia and other normally attractive substances three and one-half days after the second molt. At this time the larvae cease feeding, and the lateral portions of the ring gland undergo accelerated growth. If the ring gland is extirpated, feeding does not cease and pupation never occurs. Experiments in which the ring gland was removed and which were designed to indicate whether or not there existed a relation between the hormone and olfactory response were, unfortunately, inconclusive.

Effects which are associated in time with gonad development are observed also in the behavior of the parasite (*Pimpla ruficollis*) of the pine shoot moth (*Rhyacionia buoliana Schiff.*) toward essential oils found in its normal habitat (135). Emerging from its host long before the next generation of host larvae is available for oviposition, the parasite forsakes pine trees for Umbelliferae upon which it then commences to feed. At this time its ovaries are small, and oil of *Pinus sylvestris* is repellent. Later in the third or fourth week of life the ovaries enlarge, oil of pine becomes attractive and the females return to pine where hosts are now available.

The influence of sex upon thresholds of response is most evident in species where feeding habits are markedly different, e.g., species of mosquitoes of which the males feed on plant juices and the females on blood, but exact measurements of differences have been made only with *Drosophila* and houseflies. For the former, Reed (121) found that females exhibited a maximum response to ethanol and acetic acid at concentrations of 10 to 15 per cent and 0.4 per cent, respectively, while the maximum responses of males occurred at five per cent and 0.2 per cent, respectively. It was demonstrated in addition that these values represented differences in response to odor and not merely differences in activity. Wieting and Hoskins (144) found that female houseflies were more strongly attracted than males to ammonia, while the reverse was true with respect to ethanol.

Marked effects of nutritional states upon olfactory thresholds have not yet been demonstrated.

Conditioning, natural or experimental, may alter the threshold even to the point of reversing the sign of the response. Thus in some species, substances which normally elicit a rejection may be made to provoke acceptance. By preimaginal con-

ditioning Thorpe (134) has produced *Drosophila* adults which respond positively to the odor of peppermint. Crombie (25) has changed the response of blowflies to menthol from negative to positive. In every case, however, the change is of a transient nature lasting for a few days only. Moreover, there are limits to the changes which can be evoked. Though an insect may be conditioned to accept a normally repellent material, this conditioning does not survive if the concentration is too greatly increased. In the case of certain compounds conditioning is impossible. Von Frisch (56) could not condition bees to lysol, skatol and other compounds of of this type.

Although there are no very conclusive experiments proving adaptation to odors, a few suggestive observations have been recorded. Strebel (131) stated that Collembola exposed to stimulating odors became much less excited, as indicated by movements of the antennae and by general behavior, after being exposed to the same odor for some period of time. Wigglesworth (146) reported that the human louse shows adaptation in a constant field of stimulation. This conclusion is based upon observations of orientation in an odor gradient. If an animal should continue to proceed in a straight line in a favorable zone and followed a sharply convoluted course upon entering an adverse zone, it would become trapped in the unfavorable region. Actually, as a result of gradual adaptation, it makes increasingly long excursions before turning and finally goes straight. On reaching a favorable zone it continues to go straight. On the other hand, Wieting and Hoskins reported that the response of houseflies to an attractive concentration of NH_3 did not vary over a 20-minute period of constant exposure.

Finally thresholds of response may be altered if the animal is placed in a compound field of excitation involving antagonistic stimuli (25, 66). *Lucilia* exposed to light and the repellent odor of menthol illustrate this point. Crombie (25) placed flies in an olfactometer so constructed as to offer only two alternatives. The insects could travel toward light to which they are negatively phototactic in order to escape from the odor of menthol or toward the menthol in order to escape from the light. At 0.095 meter-candles 79 per cent of the flies were repelled by an air stream saturated with menthol; at 0.64 meter-candles, 74 per cent; at 2.58 meter-candles, 73 per cent; at 9.15 meter-candles, 69 per cent.

4. *Relation Between Physiological Action and Chemical Structure*

Most inquiries into the relation between stimulating effectiveness and chemical structure have assumed the form of field tests designed primarily to study attractance and repellence. These procedures introduced necessarily large experimental error in that many variables were beyond control. Not the least of these is the frequent occurrence of highly specific attractants. However, where field data can be translated into terms of acceptance and rejection thresholds, some interpretation of chemical and physiological interrelationships is possible. Such is true of the work of Cook (24).

In a study designed to ascertain the relation of physical properties of paraffin derivatives to the dilution most attractive to flies, Cook tested aliphatic alcohols from methyl to amyl and esters from acetates to valerates. To each of several small

flytraps he added a basic bait consisting of 25 ml. of 10 per cent molasses plus an aliquot of the alcohol or ester solution to be tested. The number of flies caught in each trap was taken as a measure of the attractiveness of the particular concentration contained therein. In this manner the optimum concentration of each compound was determined. It is a rough measure of the modal acceptance threshold.

Within each series of homologous compounds tested it was found that the acceptance threshold decreases with increased boiling point. A remarkably close correlation was observed A logarithmic plot of concentration and boiling point gives a straight line for which the approximate equation is: log. opt. conc. = 13.78 − 7.1 log. B. P. A comparable relationship exists between concentration and any property of the compounds which can be related thermodynamically to boiling point.

The relation between stimulating effectiveness and chemical properties can be examined in still another fashion. Instead of comparing the relative efficiencies of a homologous series by determining separately for each the most attractive concentration, the various compounds may be tested simultaneously in competition at optimum concentration. Since most of the compounds exhibit some repellence, the number of flies attracted is also a measure of the repellency. When the stimulative efficiency of each compound is expressed as the reciprocal of the number of flies trapped, the same general correspondence with boiling point as expressed above is observed. Increasing the chain length in a homologous series of aliphatic alcohols or esters brings about increased stimulative efficiency. This was first stated by Speyer (129). The addition of successive CH_2 groups to the acid side of the ester molecule results in a more pronounced and a more uniform increase in stimulative efficiency than when the additions are made to the alcohol portion of the molecule. This observation is borne out to a degree by similar studies of the codling moth by Eyer and Medler (46). Although in this case the addition of CH_2 groups to the acid portion of the molecule results in greater changes in physiological activity than similar additions to the alcohol side, the curve for the latter shows markedly fewer irregularities. It must be concluded that more exact experiments with the esters are required before any generalizations can be extended beyond the simple statement that stimulative efficiency is proportional to chain length, molecular weight and boiling point and inversely proportional to water solubility.

Several sets of the esters studied are isomeric. Cook stated that generally the isomer with the lowest acid radical attracts the greatest number of flies. In this series of experiments, however, there is extreme variation, and further experimentation is clearly indicated before data can be correlated. Especially is this true of those series where boiling points and solubilities of compounds with the same empirical formulae are very nearly identical. Differences between normal and iso compounds are more consistent. The normal compound possesses the greater stimulative efficiency as would be expected from its higher boiling point.

Many field experiments do not lend themselves to analyses such as the foregoing. The relative effectiveness of different members of homologous series can be determined only when the compounds are tested at comparable concentrations. Peterson's data (114), which indicate that saturated alcohols are attractive to onion maggot flies in the order iso-propyl, ethyl, butyl, amyl and methyl cannot be evaluated because the compounds were compared at concentrations which were not optimum.

Bunker and Hirschfelder (20), using mosquitoes, attempted to relate repellency to chemical structure but realized that the field technique employed introduced enormously high experimental error. Certain tendencies were suggested by the data. Ketones (e.g., acetophenone) appeared more stimulating than phenol esters (anisol) and than alcohols (camphor vs. borneol).

Studies of fly repellents (109) indicate that as a rule esters are more stimulating than their alcohols. This is to be expected in terms of boiling point, solubility, chain length and other physical properties.

5. *Humidity*

Whether or not responses to water vapor may be correctly characterized as chemoreception rests in the final analysis upon a fuller understanding of humidity reactions than is now enjoyed. Many aspects of hygrostimulation are so strikingly reminiscent of olfaction that water vapor cannot be denied the rôle of a chemical stimulus solely on the basis of its omnipresence in the environment.

The responses of insects to humidity may take the form of orientation to water vapor from a distance (80, 86, 87), proboscis responses to water vapor near at hand (86, 90, 138) and avoidance of regions of high or low humidities with consequent aggregation in a zone of preferred humidity. The response is frequently determined by the state of water balance of the insect. For example, the roach *Blatta orientalis* normally exhibits a preference for drier regions, but desiccation may reverse the reaction so that the animals become hygropositive (68). The behavior of the beetle *Ptinus tectus* Boie is dependent in a large measure on the degree to which H_2O is lost. Wet insects aggregate in a dry region until they become desiccated, whereupon the response is reversed (13). This is especially true of mosquitoes (133).

Only aggregation responses have been studied in great detail. Experiments usually involved observations of the reactions of insects placed in some type of ring gradient or alternative humidity chamber (69). In these arenas insects may show an intense response. Wireworm larvae, for example, entering a dry area, stop, move the head, manipulate the appendages, then retreat. Blood-fed female mosquitoes (*Culex fatigans*) show an equally decisive avoiding reaction when flying into an area of high humidity. In general, orientation involves klino-kinesis, klino-taxis, and ortho-kinesis (50, 69). No tropo-taxis or circus movements occur (50, 70).

With the African migratory locust (*Locusta migratoria migratorioides* R. and F.), which 'prefers' dry microclimates, the intensity of reaction to a humidity gradient as measured by the excess percentage ratio is related to the steepness of the gradient (85). With the mosquito *Culex fatigans*, which avoids high humidities (133), the mealworm beetle *Tenebrio molitor*, which avoids high humidities (117) and the wireworms *Agriotes obscurus* L. and *A. lineatus* L., which avoid low humidities (91), the intensity of reaction is related to the value of the highest humidity. The reaction of *Ptinus tectus*, which avoids high humidities, is most intense at low humidities. The discrimination factor varies in the same way. *Culex* distinguishes differences of one per cent RH near saturation while between RH 30 and 85 a difference of 50 per cent is necessary before it is appreciated (133). In general, the louse also shows greater sensitivity within the higher ranges of humidity (146). Wireworms can detect the difference between 100 per cent and 95.5 per cent RH. Lees (91) sug-

gested that the failure of reaction is due to an elimination of an integral part of the orientation mechanism under unfavorable physiological conditions rather than any real 'indifference' to humidity as such.

With all species studied the intensity of reaction to a given RH at different temperatures has been investigated. In this manner it has been found that the reactions of *Culex* and *Tenebrio* are more in accord with relative humidity than with saturation deficiencies. With wireworms, on the contrary, the intensity of reaction is more in accord with saturation deficiencies.

The behavior of species in the first category suggests that the sense organs are reacting as hygrometers to relative humidity. The reactions of wireworms suggest receptors of an evaporimeter type in that the intensity of reaction is dependent upon evaporation from some site on the body (91). This appears to be true also with the terrestrial isopod *Oniscus asellus* (139). In neither case, however, is the mode of action definitely understood. If receptors sensitive to RH are to act as hygrometers, the hygroscopic properties of the receptors determine their recording characteristics. Pielou found that curves relating RH to the hygroscopic properties of *Tenebrio* cuticle closely parallel curves relating RH to intensity of reaction. As he questioned, however, how can a receptor of this sort be affected more by the RH of the external environment than by that of the body fluids bathing its internal surface?

Various hypotheses have been advanced in an attempt to explain the mode of action of the evaporimeters. Pielou (116) postulated that evaporation could act by *a*) producing quantitative changes of chemical concentration, changes in osmotic pressure or mechanical stress, or *b*) by lowering the temperature, thus exciting heat receptors. Waloff (139) also suggested a concentration of body fluids to initiate reaction and as an alternative, secondary proprioception. Lees believed that the rapidity of reaction suggested local changes in osmotic pressure which might stimulate nerves directly. But none of these hypotheses is wholly satisfactory. Nor has positive identification of the receptors in some species illuminated the problem.

Humidity responses by *Tenebrio* are completely abolished when the antennae are amputated or covered with an impervious material (116). Two types of receptors are involved, pegs and pits. The former are confined to the distal seven segments. The latter are located on all segments except the terminal one. Removal of the terminal segments is followed by a decrease in the intensity of response, hence the peg organs are concerned in hygrostimulation. That they are not the sole receptors is proved when all seven segments bearing pegs are extirpated. The animals still respond. If eight pairs of segments are removed, response is abolished although the remaining segments bear pit sensilla. It has been shown by asymmetrical amputation that a threshold number of pit sensilla is necessary. An animal with the basal three pairs of segments (bearing pits only) intact does not respond to humidity changes. When a single segment on one antenna and five (two of which bear pegs) on the other are left intact, there is an 11 per cent response. This indicates that the pegs are humidity receptors but does not eliminate the pits since an animal thus treated bears both pits and pegs. When three segments are left intact on one antenna and five on the other, there is a 20 per cent response. The only difference between the two preparations is the greater number of pit sensilla, hence the con-

clusion that a greater number of pit sensilla is required. The peg receptors are also suspected of subserving the olfactory sense (137).

The humidity responses of wireworm larvae are abolished by the combined removal of antennae, maxillary palpi and labial palpi. No particular receptors have been incriminated and it has been concluded that responses are mediated by evaporation through these areas (91).

Wigglesworth (146) proved that the humidity receptors of the human louse are distinct from the olfactory receptors. Both are borne on the antennae. The former are tuft organs each of which consists of a minute cone bearing at its apex four tiny delicate hairs. The sensillum is innervated, like typical chemoreceptors, by a group of bipolar sense cells.

6. *Modalities and Discrimination*

Efforts to reduce the olfactory sensations of man to some system analogous to conceptions of taste modalities have been notably unsuccessful. It is hardly surprising, therefore, that few inquiries have been directed toward this aspect of chemoreception in insects.

Under the circumstances little more can be done than to study the reactions of insects to those odors which provoke distinctive sensations in man. This approach has been followed in a study of the reactions of honeybees to the odors of 47 essential oils and component compounds (56). Bees were trained to one odor against several empty odorless containers serving as controls. This odor was then exposed in the company of others and the frequency of visits to each recorded.

Of the 47 substances tested initially, three, in addition to the training odor were highly attractive. Each was an essential oil distilled from a citrus fruit and as such contained a motley array of chemicals. Common to all was limonene so that each emanated a similar odor. A tabulation of the number of visits to each indicated that this similarity of odor exists for bees also. Tests in which the four were exposed simultaneously indicated, however, that they were not indistinguishable. Comparisons of pairs of pure compounds showed that for the bee as well as man compounds with similar odors but different chemical structure were confused as one, while compounds with nearly identical structure but quite distinctive odors were easily distinguished. Of the first type nitrobenzene was tested with benzaldehyde and methyl ester of anthranilic acid with beta naphthol methyl ether. Counts of bees indicated that while the odors may have been very similar they were not completely indistinguishable. Man experiences like sensations.

If the olfactory sense of honeybees reflects similarities with that of man, it is to be expected that compounds with similar structure even to the point of stereoisomerism would evoke different sensations. This has been one of the obstacles to all attempts to relate smell to chemical structure. Thus within each of the following pairs the two members are clearly distinguishable by bee and man alike: amyl acetate and methyl heptenone, bromstyrol and phenylacetaldehyde, isobutyl benzoate and salicylic acid amyl ester, para-cresol methyl ether and meta-cresol methyl ether. It appears very likely, therefore, that whatever the mechanism of olfactory stimulation in bees, it resembles remarkably closely that of man.

Numerous theories which attempt to explain the nature of olfactory stimulation have been proposed and are reviewed briefly by Moncrieff (108). Recently Beck and Miles (10A, 98A) have advanced an infrared theory of olfaction, but complete details of their experiments have not yet been published.

CONTACT CHEMORECEPTION

1. *End Organs of Contact Chemoreception*

Contact chemoreception is treated below as a sense distinct from olfaction and the common chemical sense although in the present state of our knowledge, as pointed out above, we lack wholly satisfactory criteria for separating it from the former in the case of certain aquatic and subterranean insects. Localization of receptors and greater sensitivity afford a reasonable basis for distinguishing contact chemoreception from general reactions to irritants. Functionally, contact chemoreception as here limited is concerned principally and immediately with the acts of feeding and oviposition, and doubtless plays a rôle, often largely negative, in the choice of resting places.

Demonstrated loci of the receptors include: *a*) the *antennae* of bees (52, 84, 90, 97, 107) and of ants (126); *b*) the *mouthparts* or adjacent surfaces of the preoral cavity in many species (9, 32–37, 47, 49, 52–55, 60–63, 76, 86, 90, 98, 102, 106, 122, 125, 131, 138, 150); *c*) the distal segments of the *legs* in bees (52, 90, 97, 107); in flies (1, 8, 22, 27, 31–35, 42, 51, 55, 76, 79, 86, 102, 105); and in adult Lepidoptera (5, 99–101, 142); and *d*) the *ovipositor* of certain ichneumonids and braconids (40); and of *Gryllus*.

Verlaine (138) contended that the tarsal response of adult Lepidoptera is a reaction to humidity, viscosity and mechanical stimulation, dependent partly on associative memory; while McIndoo (95) ascribed reactions mediated by the tarsi of calliphorid flies to a combination of olfactory and mechanical stimuli: these views do not accord with the findings of the great majority of workers who have studied the tarsal chemical sense.

Phylogenetically it is of interest that ambulatory appendages have been identified as the seat of contact chemoreceptors in several other classes of aquatic and terrestrial arthropods (6, 12, 18, 78, 83, 92, 113); and in this connection it may also be pointed out that of the other loci demonstrated in insects, at least the mouthparts and the gonopods are presumed to have been derived from ancestral walking legs (128).

In most cases the nature of the actual receptors in insects is uncertain. Either the appendages, such as the antennae of bees, have a variety of end organs and subserve a variety of functions (tactile, olfactory, gustatory, hygroreceptive, thermoreceptive), so that attempts to match structure and specific function have been unsuccessful, or the regions concerned in contact chemoreception lack easily recognized end organs to which a chemoreceptive function could confidently be assigned. However, Minnich (103, 106) reported that he had succeeded in stimulating individually some of the long curved hairs on the aboral surface of the oral sucker of *Phormia* and *Calliphora* by direct contact with 1 M sucrose. In the earlier paper he said that "stimulation of the tip of a single hair may be sufficient for the response." This statement has been questioned by Frings and O'Neal (55), who quote Minnich's

later assertion that he moistened with sugar solution "all the hairs, marginal as well as central," and who therefore conclude that his experiment could not differentiate between the labellar setae and the numerous shorter hairs which lie among them. This later statement was not intended by Minnich to refer to experiments on the localization of receptors (personal communication). In their own examination of *Tabanus*, Frings and O'Neal were unable to obtain a response when only the longer marginal setae were moistened but found that contact of sugar solution with the tips of the thin-walled medium-sized hairs clothing the labella and lying among the longer hairs caused extension of the proboscis. Whether other types of sensilla present on the labella also may act as organs of taste could not be determined. Similarly, Frings and O'Neal showed that chemoreception by the legs of *Tabanus* is mediated either by medium-sized thin-walled hairs scattered among the long hairs which clothe the tarsus thickly, or by undiscovered organs on the integument beneath these hairs. The sensitive areas were the ventral surface of the distal half or two-thirds of the proximal tarsal segment and of the four more distal segments; the longer setae on these regions were insensitive. Dethier (36) observed that longer and shorter thin-walled spines are the only types of end organ common to the epipharyngeal and hypopharyngeal surfaces of the preoral cavity in larval Lepidoptera, and since these were the only areas which yielded a response to contact chemical stimulation he concluded that one or both types are among the specific receptors involved.

The above instances are the only ones known to the reviewers in which the effort to verify by direct experiment the gustatory nature of presumptive end organs has been even partially successful, and it is clear, as others have pointed out, that assignment of function on a morphological basis only, without experimental support, tends merely to obscure the situation. For this reason we are making no attempt at this time to reinterpret that rather extensive literature descriptive of the varied types of sensilla that are to be found on regions thought or known to be chemoreceptive on contact. The 'olfactory pores' (sensilla campaniformia) of McIndoo are now generally recognized as organs of proprioception (118, 119, 123, 124, 145), so that their presence on the tarsal segments of *Lucilia* and *Calliphora* (95) is probably beside the point. Eltringham (45) found on the tarsus of *Pyramcis* thin-walled hairs with multiple innervation; presumably these are the end organs of the function first investigated by Minnich (99), but the critical experiments which would prove that they rather than other undiscovered organs are the ones which respond to solutions have not been done. Hayes and Liu (77) have described from the tarsi of the housefly long, thin-walled setae, with a distinct joint at the base and with an innervation consisting of groups of spindle-shaped bipolar sensory cells; their inference that these are the end organs of contact chemoreception, though probably correct, is unsupported by experimental evidence other than observations (31–35) that *Musca* possesses a well-defined tarsal chemical sense.

Thus a major shortcoming of much of the work to date on contact chemoreception in insects has been the failure to identify positively the specific receptors involved. Since it is obvious that any attempt to comprehend the mechanisms concerned must take into account not only the characteristics of the stimulatory substances but also those of the receptors with which they interact, it is clear that progress in this field

will be limited until more definite knowledge of the structure and properties of the receptor elements is available.

2. *Methods of Investigation and Their Results*

Most of the physiological studies of contact chemoreception in insects have centered around the feeding response, which has been utilized in various ways by different workers as an indicator of chemical stimulation. By this means the location of the receptors has been roughly mapped out, many chemical compounds have been classified as acceptable or unacceptable and a considerable body of quantitative data has been developed in reference to the relationship between concentration and stimulatory effect. Further analysis of the categories acceptable and unacceptable has also been attempted with some success, and progress has begun toward a correlation of the physical and chemical properties of compounds with their potency in stimulation. A brief discussion of the more important methods that have been used in these studies will serve to indicate their limitations and provide a basis for a critical evaluation of the data which have been obtained.

a. FEEDING EXPERIMENTS; ACCEPTANCE AND REJECTION. The type of experiment most commonly employed leads to the determination of acceptance or rejection thresholds. For the former, an insect which has been allowed to drink until it no longer responds to plain water is presented with a series of dilutions of the test chemical, and the lowest concentration which induces an attempt to feed is noted. In the measurement of rejection thresholds, the test compounds may be given to thirsty insects in water, but since it is difficult to regulate the degree of thirst it is often more convenient first to let the specimens satisfy their desire for water and then to offer dilutions of the test compound in a standard solution of some acceptable substance, such as sucrose. In either case, the least concentration which prevents acceptance or causes an avoiding reaction is taken as the threshold of rejection. Obviously such measurements can have only a relative value, and the results are dependent in part on the strength of the attractive stimulus.

b. FACTORS WHICH AFFECT THE RESPONSE. Responses of acceptance or rejection, separated as they are from the initial process of sensory excitation by a complex chain of physiological events and subject therefore to modification at various levels by the physiological state of the animal, are open to influence by a variety of factors which must be suitably controlled if consistent results are to be obtained. Among these have been noted: previous association (conditioning), preceding chemical stimulation, availability of natural sources of nectar or other food, age, preparation for experiment and especially the condition of the animal in regard to nutrition and thirst.

Conditioned responses. Several workers have succeeded in conditioning insects to respond positively to one of a chosen pair of chemicals and to reject the other (9, 84, 122, 125, 126). In repeated trials the animal learns to associate one stimulus with the presence of food, with which it is rewarded after a positive response, and the other with some disagreeable experience, such as the taste of food heavily contaminated with quinine. The ability of aquatic beetles to distinguish between NaCl and HCl, NaCl and sucrose, HCl and sucrose, etc., was demonstrated in this way; and ants

were trained to respond positively to 0.5 M sucrose and negatively to water. Theoretically it should be possible with this technique to determine specific thresholds for the test substances, but the few studies of contact chemoreception in which the method was used were made under conditions which usually did not permit a very accurate estimate of the strength of the chemical stimuli employed. Associations thus formed seldom persisted more than a few hours without reinforcement, yet the fact that they were established readily on repeated occasions suggests that care should be taken in routine measurements of acceptance and rejection thresholds to avoid complicating the results by introducing factors of this sort.

Preceding chemical stimulation; adaptation. Chemical stimulation which precedes a given test by a sufficiently short interval also may influence the results, but in a manner somewhat different from the learning process just described. Probably several distinct mechanisms are involved. Frings (54), for example, noted what he termed a 'shock reaction' in roaches exposed to supraliminal concentrations of acid: after contact with such a solution, they often refused everything, even sucrose, for a time. Similar behavior was seen in houseflies after exposure of the tarsi to five per cent $HgCl_2$ in 1.0 M sucrose (31, 33). One hundred and twenty flies were tested, and immediately after exposure only one fly would respond to sucrose. Recovery was gradual, with 15.8 per cent response after one hour, 46.4 per cent after three hours and 68.0 per cent after five hours. It is possible that the receptors were injured temporarily in these experiments. Another type of reaction was demonstrated by Minnich (105). When testing was begun below threshold and flies were transferred in immediate succession from one sugar solution to another in increasing order of concentration, individuals whose threshold for sucrose was ordinarily 1/1600 M did not respond until a concentration of 2/100, 4/100 or even 8/100 M was reached. This result was ascribed to fatigue or adaptation. Others (90, 138, 142) have recorded similar observations, which they also interpreted as evidence of fatigue or adaptation of the receptors, but in these cases the differences measured are hardly significant statistically. Von Frisch (63) noted that weaker solutions of sucrose were taken hesitantly or refused by bees for the first 5 or 10 minutes after stronger ones had been fed, though he states also that the acceptance threshold (0.25 to 0.125 M) was not changed even after 0.5 M sucrose had been fed for hours nor when the threshold was approached gradually from above. Other workers (44, 97) could find no difference in the threshold of acceptance determined when sugar solutions were offered in ascending or descending order of concentration. Thus, while it seems probable that sensory adaptation does occur, the evidence which has been gathered is for the most part weak or contradictory, and all of it is based on responses contributed to by an extrasensory element which it is difficult to assess. The observations suggest that, if adaptation does take place, recovery is fairly rapid; failure to demonstrate it more conclusively may have resulted in some cases from too long an interval between tests. Further study of the phenomenon, in experiments designed to separate central and peripheral factors, would be desirable.

Availability of natural food. Both von Frisch (60, 63) and Kunze (89, 90) have remarked that the threshold of bees for sucrose and other sugars is dependent to some extent on the availability of nectar and the sugar concentration of the latter. At

seasons when nectar with a high concentration of sugar is in good supply, bees will refuse sugar water in dilutions which are accepted readily when natural sources are less favorable, even though under the latter conditions they are kept equally well fed and have a plentiful store of sugar or honey in the hive. The mechanism whereby the response is modified is obscure but does not seem to involve either sensory adaptation or the state of nutrition.

Age. No thorough investigations have been made of the effect of age on the contact chemoreceptive response. In the extensive observations of von Frisch (63) little influence of age on threshold was seen until the workers had been gathering nectar for about a month. These elderly bees had an elevated acceptance threshold for sucrose. Eger (44) found that fifth instar larvae of *Acidalia* had higher thresholds of rejection for NaCl, HCl and quinine than younger specimens, but Dethier (36) noted little change in thresholds with age among other caterpillars, except for a general disinclination to feed immediately before pupation. In wireworms, orientation to chemical stimuli shows a biennial rhythm, of which the biting response is independent (136). Probably age is of minor significance in most investigations of contact chemoreception apart from cases where marked changes in dietary behavior are associated with the process of development.

Preparation of specimens. The practice of mounting insects for experiment, introduced by Minnich (99) and since used by many other workers, often renders the specimens erratic or unresponsive for the first 24 hours or so after preparation. Later this inconsistency disappears, and with suitable treatment the insects may be maintained in a responsive state for weeks or months.

Nutrition. By far the most important factor affecting acceptance thresholds is the nutritive state of the animal, and it is a common observation that acceptance thresholds fall when insects are maintained continuously on a water diet or are otherwise subjected to complete or partial starvation (5, 27, 34, 35, 60–63, 76, 79, 89, 101, 102, 105, 136, etc.) Hunger may reverse the effects of training and cause an insect to accept quinine-flavored meat which he has been taught to avoid (125). However, a marked decrease in threshold with starvation does not invariably occur (5, 37, 55, 63, 106, 126), and the time course and extent of the decrease may be quite different for different compounds (76, 105) or for oral and tarsal receptors (76). Where rejection thresholds are determined against the background of a contrasting acceptable stimulus, an increase in the rejection threshold may be expected with starvation if the latter results in increased acceptability of the contrasting stimulus (150). Thus, lack of water has been shown frequently to be a potent force in improving the acceptance of otherwise repugnant solutions, and it is known that, even apart from starvation or thirst, variations in the strength of the attractive stimulus do produce differences in the rejection thresholds determined (33, 34, 44, 63, 138). Haslinger (76), in recognition of this effect, measured the rejection threshold for HCl of blowflies during starvation by presenting the acid in a fructose solution the concentration of which was varied so as to be just three times the threshold for fructose on each day of the test. Under these conditions, no change in the rejection threshold for acid was observed, and similar results were obtained with unacceptable sugar alcohols, salts and quinine. However, von Frisch (63), who presented NaCl, HCl and quinine in 1.0 M sucrose to bees at all stages of starvation, found no change in

the rejection threshold for salt and an increase with HCl and quinine. Bees succumb to starvation so rapidly that they are not the animal of choice for experiments of this sort (15–17, 63). Acceptance of $HgCl_2$ in 1.0 M sucrose by *Phormia regina* was better after 48 hours than after only 24 hours on a water diet (34). Frings and O'Neal (55) observed somewhat variable rejection thresholds in *Tabanus* during starvation, but no consistent trend appeared in their data. In summary: there is usually but not always a decrease in acceptance thresholds during starvation; the data in respect to rejection thresholds are scantier and to some extent conflicting so that more study is needed.

The contention of various authors, that the lowest threshold of acceptance reached during the penultimate stages of starvation represents a threshold of receptivity or a specific threshold, requires examination. The state of starvation is conceived as eliminating a central inhibition which under normal circumstances prevents a visible response to low, though stimulating, concentrations of the acceptable compound (105, 106, 136). With bees, a rather constant value for the acceptance threshold of sucrose is always obtained during starvation, irrespective of initial differences, and it is considered unlikely that a starving insect would refuse an acceptable substance that it was capable of perceiving (63). A weakness of these arguments, apart from their speculative nature, lies in the fact that many variables other than hunger may be operating to condition the central nervous state: although starvation may have lowered the resistance, so to speak, between excitation and acceptance, there is no guarantee that the resistance has been reduced to zero. Granted, however, that the premise is correct, the threshold measured can be only a specific threshold and not an absolute threshold. From experiments with man (e.g., 82) it has long been known that the specific threshold for a given compound need not be the absolute threshold of excitation, since it is often possible for human subjects to distinguish dilute solutions from distilled water at concentrations so low that the solute cannot be identified nor even classified in one of the usual taste categories. Whether a similar situation exists for insects is uncertain, but that the sensory process may be at least as complex as in the vertebrates is shown, for example, by the following experiment of Frings (54) with the American roach: on adding small amounts of acid to 0.1 M sucrose, a concentration was presently found which caused hesitation and rejection. A further increase of acid brought the solution into a range in which it was accepted more readily than sucrose solution, while a still further increase disclosed a threshold above which all concentrations were rejected vigorously. This level was interpreted as the rejection threshold. In this experiment it is clear that the acid was in some manner stimulating the roach at a concentration below either the acceptance or rejection threshold, and it is possible that the reaction to very low concentrations represents a process similar to the phenomenon of a general or difference threshold in man.

c. VARIABLES WITH LESS PRONOUNCED EFFECTS. Somewhat surprising are the observations that the factors of light intensity, temperature, humidity and sex, in contrast with their effects on olfaction, and the presence or absence of odors, appear in general to have little effect upon the sensitivity of insects to chemical contact with solutions in laboratory investigations, although it is well known that these may exert profound effects upon the feeding behavior of insects in nature.

Light intensity. Only casual observations of variations in light intensity during

tests have been recorded, and any effect which they may have had has been overshadowed by other variables. Kunze (90) states that bees whose eyes were covered with opaque shellac responded no differently than normals.

Temperature and humidity. Weis (142) obtained essentially identical distributions of acceptance thresholds for sucrose in tests run with butterflies at 25.5° C., 56.7 per cent RH and at 35° C., 100 per cent RH. Nor was the threshold altered when the butterflies were kept at 21° C. and the sugar solutions offered at 15° C., 21°C. and 35° C. For bees, von Frisch (63) found temperature changes within wide limits to be without significant effect on the acceptance ratio, both in the field where the temperature of the bees followed the ambient temperature and in laboratory tests where the temperature of the solutions was varied independently over the range from 10° to 35° C. Yet the amount of sugar solution drunk by bees is greater at higher temperatures. In another group of studies with bees at air temperature (20° to 25° C.), von Frisch observed no change in acceptance of 1.0 M sucrose within the range from 8° to 50° C., but the majority of specimens became hesitant when the solutions were at 3° or 5° and at 55°, while general refusal occurred at 1° and 60° C. These experiments give incidental added proof that viscosity is not a significant factor in insect chemoreception. The apparent absence of a temperature effect in insect gustation is in contrast with results which have been obtained with man, but it may be remarked that there has been little agreement among the numerous investigators who have studied human taste from this point of view. References to many of the studies on man may be found in the papers of Goudriaan (65) and Hahn and Günther (71).

Sex. No difference in the sensitivity of male and female specimens appeared in the studies of Abbott (1) with *Cochliomyia macellaria*, of Deonier (34) with *C. americana* and *Phormia regina* nor in our own tests with the latter, of Minnich (105) with *Calliphora vomitoria*, of Anderson (5) with various butterflies, nor of Frings (54) with the American roach. Drone bees responded more readily to 1.0 M sucrose than workers, while no difference was detected in the response of young queens and workers of the same age (90).

Odors. The acceptance of sucrose by bees was not affected by the presence or absence of the odors of essential oils or honey, and removal of the olfactory organs by antennectomy left the taste threshold unaltered (63). Amputation of antennae, labial palpi and the forelegs of nymphalid butterflies did not significantly affect the tarsal response to solutions (99). The average tarsal acceptance threshold of 181 specimens of *Phormia* for sucrose was 0.019 M ± 0.005 (standard deviation); for 122 of these same flies which survived combined antennectomy and labellectomy it was 0.017 M ± 0.013. After operation the flies no longer responded to odors (42). Ritter (122) found that amputation of the antennae was without effect on the gustatory response of *Hydrous;* however, this beetle has olfactory receptors on the maxillary palpi. Bauer (9), who worked with the same species and with *Dytiscus*, states that removal of both the antennae and the maxillary palpi, which abolished the olfactory response, did not prevent a response to taste substances, but that the threshold was raised and the animals were generally more sluggish. The increase in threshold in this case may be ascribed to loss of part of the contact chemoreceptors, some

of which are located on the maxillary palpi, to a deficient central excitatory state, or to both factors.

d. EXTENSIONS OF THE FEEDING TECHNIQUE. *Method of mixtures.* While many of the facts already cited have come from work with conditioned insects or from straightforward feeding experiments, certain variations of the latter technique have been made to yield additional information not otherwise obtainable. One such refinement was developed by von Frisch (62, 63), who noted that the stimulative effect of various sugars for the bee is additive. For example, a mixture of sucrose and fructose, each present in a concentration which would have been refused if offered alone, was accepted readily. It occurred to von Frisch that this fact could be utilized in retesting the acceptability of sugars which had appeared to be without stimulative power even in saturated solution when presented singly. Such sugars were now mixed with a concentration of sucrose to which some but not all of the bees would respond, and it was found in fact that the acceptance ratio was improved in several instances. By comparing the concentration of the second sugar with the concentration of sucrose which had to be added to produce an equal effect, a quantitative estimate of the relative 'sweetness' of the second sugar was obtained. In similar experiments, von Frisch determined the stimulating power of various compounds repugnant to the bee by adding them in known quantities to an acceptable solution of sucrose and observing the decrease in acceptability. This latter procedure illustrates the usual approach in the measurement of rejection thresholds.

Measurement of 'crop loads'. Another variation of the feeding technique, also due to von Frisch (63), is based on the observation that the quantity of sugar solution taken by a bee at one feeding is a function of the concentration. At 0.5 M, the average bee drank 44.9 cu. mm. per trial, but when 1.0 M or 2.0 M sucrose was offered the amount taken rose to 50.5 and 55.2 cu. mm., respectively. Thus a difference in the degree of response was revealed beyond that indicated by a simple count of acceptances and rejections. Having provided a concentration of sucrose just sufficient to ensure acceptance by all the bees in the test group, von Frisch then showed that addition of NaCl in concentrations too low to reduce the percentage accepting still produced a measurable decrease in the average amount of solution drunk per trial per bee. This was interpreted as an indication that the bees perceived the presence of the salt even though they drank the solution containing it; in other words, that the specific threshold for NaCl must lie well below the rejection threshold. If this conclusion is acceptable, the technique offers an additional means of approximating specific thresholds, and of testing intensity discrimination.

The same procedure has also been used by von Frisch to draw a distinction between various types of compounds unacceptable to the bee. When concentrations of quinine hydrochloride below the rejection threshold were added to sucrose solution, the outcome differed from that obtained with NaCl in that no decrease occurred in the quantity of solution taken; and this, von Frisch reasons, shows that there is a difference in the quality of the perception of the bee for these substances. HCl was found to behave in this respect like NaCl, but by no means all of the bitter substances tested gave results similar to quinine. Von Frisch concludes that what is bitter for man may not constitute a homogeneous grouping for the bee.

Duration of feeding as an index of acceptability. A somewhat similar modification of the acceptance technique was used in conjunction with the usual procedure by Weis (142), who recorded the length of time that butterflies, stimulated tarsally, would leave the proboscis in an acceptable solution. With *Pyrameis atalanta* Linn. as the test animal, she observed that the proboscis remained in distilled water for 25 seconds on the average, while the average drink of 0.5 M sucrose required 4 minutes 19 seconds. Dulcin (*p*-phenetyl urea), a synthetic sweetening agent, caused withdrawal of the proboscis after only eight seconds, and was therefore judged to be somewhat distasteful to the insects. Without further investigation, too much reliance should not be placed on the use of this method with Lepidoptera in view of our ignorance of the process of chemoreceptive adaptation in insects and of Stober's (130) finding, incidental to a study of digestive function, that several species drank indiscriminately the following: various concentrations of sugar solution, with or without added dyestuffs; water; litmus; congo red; neutral red; brom cresol purple; brom thymol blue; milk; frog or rabbit blood, with or without Na-oxalate or hirudin; alcohol; a variety of salts and acids; starch; water colors; Flemming's fixative; and Schaudinn's solution. It is true, of course, that in Stober's experiments the tarsi were not in contact with the fluids as they were in the experiments of Weis, but Weis herself points out that duration of feeding is not a reliable criterion of acceptability if the animals are starved and thirsty. Kunze (89) likewise used duration of feeding as an index of acceptability of solutions for the bee.

Tarsal stimulation. One of the most fruitful modifications of the food acceptance technique was introduced by Minnich (99) consequent upon his discovery that contact chemoreceptors are present upon the tarsi of certain nymphalid butterflies and that stimulation by an acceptable substance results in extension of the proboscis. The location of these sense organs makes it a simple matter to investigate their function without giving the animal an opportunity to ingest the test substances, a fact which facilitates control of the important variable of nutrition and which permits one to avoid some of the complications which arise when toxic compounds are investigated. Further observations by Minnich (102, 107) demonstrated similar sensitivity to chemical stimulation in the legs of flies and bees. Since then both he and others have taken advantage of this convenient arrangement on numerous occasions (see references under section on location of receptors above). The present writers recently combined the technique with extirpation of the olfactory receptors in flies, a procedure which permits testing of water-soluble organic compounds whose odors might otherwise cause confusion in the results (42). A method which would allow similar tests with substances which are insoluble in water would be highly desirable as this would extend greatly the range of compounds whose effects on contact chemoreceptors could be investigated. The principal problem presented here is the choice of a suitable diluent, which must combine the following characteristics:

1. It must be a solvent for the compounds to be tested and must not react chemically with them.

2. Preferably it should be nonstimulating; if stimulating, then preferably attractive; if inert or repellent, then it must dissolve some standard attractant, such as sucrose, in sufficient concentration to ensure a positive response.

3. It must lack high contact toxicity.

An attempt in this direction, using ethylene glycol as a carrier for the attractant (sucrose) and for the test substances, has been only moderately successful.

Ovipositor preparations. One further technique which offers considerable promise developed from the observation that the ovipositors of certain insects respond to contact chemical stimuli (40). It has been found that the isolated abdomen of some parasitic hymenoptera makes characteristic avoiding movements if the valvulae are placed in solutions which are rejected when presented to the antennae, mouthparts or tarsi of other insects. Rejection thresholds for such compounds can therefore be determined with this preparation, which not only eliminates the nutritional variable of other techniques, but also renders unnecessary the addition of an attractant to the test solution. Abdomens protected from desiccation by coating the cut surface of the petiole with paraffin remain responsive for several days if kept in a moist atmosphere at room temperature. Similar preparations may be made from the cricket *Gryllus*, and probably from some other insects.

Electrical recording from chemoreceptors. To have some means of studying receptor function more directly than via the initiation or suppression of reflex responses would have many advantages. Recording of sensory action potentials offers a possibility, but so far all efforts along these lines with the chemoreceptors of insects have been fruitless. Dethier and Prosser tried in vain to record chemically stimulated action potentials from the antennal nerve of caterpillars, while Dethier and Roeder obtained only negative results with preparations of the nerves which supply the ovipositor of parasitic wasps and the cricket, and the mouthparts of the roach (unpublished experiments). The individual sensory fibers are of small diameter; the presumably small potentials are apparently shunted out by adjacent tissue and fail to rise above the noise level in the amplifying system. In these unsuccessful trials all the preparations responded with visible movements to chemical stimulation, so that there can be no doubt that normal receptor function was maintained; and tactile stimulation of the regions concerned produced typical spikes in the oscillograph records (38). Additional attempts of this sort should be made whenever favorable preparations are encountered, in spite of the technical difficulties imposed by the small diameter and short length of the nerves, for they offer the best prospect of obtaining information uncomplicated by central nervous factors about the chemical response in insects. The fact that some success has attended similar efforts in vertebrates (3, 7, 75, 81, 115, 120, 154) should encourage further endeavor.

3. *Sensitivity in Taste*

a. SPECIFIC DIFFERENCES. Conspicuous differences in threshold are found even among closely related insect species (cf., e.g., 5, 44, 126), and consequently it is hardly possible to generalize in comparing the acuity of the insect gustatory sense with that of man and other animals. The known range of concentrations adequate for stimulation lies approximately between 12 M and 1×10^{-6} M, depending on the species, the individual, the organ, and the substance concerned. Limitations of space prevent the citation of more than the following representative figures for commonly tested compounds (see table 2, page 244).

b. INDIVIDUAL DIFFERENCES. As nearly all workers have noted, different individuals of the same species show a wide range of thresholds for a given compound, in spite of all precautions taken by the experimenter. Von Frisch (63) even gives evidence that the sensitivity of the same bee may vary from day to day. These variations pose difficulties in the comparison of data and in the presentation of results, which lose much of their significance unless accompanied by some measure of their reliability. In this connection it is useful to know that the scattering of thresholds in a population of *Phormia* reared under standard conditions is not significantly different from a normal distribution when plotted against the logarithm of concentration (42), and that a similar relationship may be demonstrated for both acceptance and rejection thresholds of other insects in most cases where sufficient data have been reported (e.g., 44, 54, 63, 142). It is therefore possible to characterize the reaction

TABLE 2. COMPARISON OF TASTE THRESHOLDS

COMPOUND	THRESHOLD CONCENTRATIONS[1]
sucrose	man, 0.02 M; bee, 0.06–0.125 M; butterfly (*Pyrameis*), average ca. 0.01 M, in starvation as little as 8×10^{-3} M, or for *Danaus*, 9.8×10^{-4} M; horsefly (*Tabanus*), 0.005–0.11 M.
NaCl	man, 0.009 M; bee, rejects ca. 0.24 M in 0.5 M sucrose; various caterpillars reject at 0.2 M, while others accept over the full range up to and including 5.0 M.
HCl	man, 0.00125 M; bee, rejects 0.001 M in 1.0 M sucrose; various caterpillars reject at 0.01–0.2 M.
quinine	man, 1.5×10^{-7} M; bee, rejects at 8×10^{-4} M in 1.0 M sucrose; various caterpillars reject at 0.002–0.033 M; aquatic beetles were conditioned to respond to 1.25×10^{-6} M.

[1] Data on man are for specific thresholds, adapted from (108); for the bee, from (63); for *Pyrameis*, from (101); for *Danaus*, from (5); for *Tabanus*, from (55); for caterpillars, from (44); for aquatic beetles, from (9). The figures for insects are thresholds of response

of a population to a given chemical mathematically in terms of the regression of responses, expressed in probability units, on log concentration, and to calculate within stated limits of accuracy the most probable value of the concentration required to produce a chosen degree of response. While one might be tempted to regard the logarithmic nature of this distribution as evidence for the operation of Fechner's rule in insect chemoreception, it should be kept in mind that the relationship describes the distribution of thresholds in a population of individuals, each of whom bears a population of receptors, and that there is at present no information as to the manner in which the number of receptors responding or the response of a single receptor element depends on the intensity of the stimulus.

However, information obtained with other animals suggests that the phenomenon is probably more widespread in chemoreception than is generally realized. Rather similar to the results with insects are the data of Krinner (88), who trained the min-

now, *Phoxinus*, to distinguish sucrose or salt from water and from each other, and who noted that the intensity of reaction (number of responses) was roughly proportional to the logarithm of concentration. Crozier (29, 30) demonstrated a direct proportionality between the logarithms of speed of reaction and concentration of the stimulating compounds with worms exposed to acid and alkaline solutions. In the rabbit, Hasama (75) found an approximately logarithmic relationship between the strength of the negative electrical potential developed on the hippocampal lobe and the intensity of the odor of quinoline, while Hahn and Günther (71), in experiments on the temperature dependence of human taste thresholds, remarked that the range of variation was approximately proportional to the concentration. Lemberger (91A) demonstrated that the discrimination factor in human taste is constant for sucrose and Na-saccharin, except at the extremes of concentration. It is thus apparent that the factor of stimulus intensity enters logarithmically into a number of the phenomena associated with chemoreception although the relationship has been investigated less thoroughly here than with some of the other senses.

c. *Sensitivity differences of different organs.* Even in a single individual the threshold for a given substance may be different if different organs are stimulated (32, 34, 55, 76, 97, 102, 105, 106, 122). Thus, in *Tabanus*, the average labellar threshold for sucrose was 0.021 M; for the tarsi, it was 0.060 M (55). With most of the compounds tested on *Calliphora erythrocephala* by Haslinger (76), the labella were more sensitive than the tarsi; but in *C. vomitoria*, according to Minnich (106), the oral lobes are only one-sixteenth as sensitive to sucrose as the legs although the fly reacts to lactose applied orally but not when it is touched to the tarsi (105). Houseflies would extend the proboscis after contact of the tarsi with 1.0 M sucrose which contained a toxicant in suitable dilution but avoided the fluid or refused to drink it when it touched the mouthparts (32); and the same was observed with *Cochliomyia americana* and *Phormia regina* (34). Rather similar observations were reported by Verlaine (138) for *Pieris*. The antennae of the bee are more sensitive to solutions than are the legs (52, 97, 107), and the proboscis more sensitive than the antennae (90). According to Ritter (122) the beetle, *Hydrous*, can detect HCl at a concentration of 0.007 M with the tips of the labial palpi; these are said to be insensitive to NaCl and sugar, which are perceived via the maxillary palpi. An interesting problem which should repay future investigation is the extent to which threshold differences of different organs in the same insect are dependent on receptor sensitivity, number of receptor units activated and central nervous factors. As yet there has been no productive research on the possibilities of temporal and spatial summation, nor is there any information on latency or other temporal aspects of receptor function.

d. *Intensity discrimination.* Only an approximate estimate is available of the ability of the individual insect to discriminate between slightly different concentrations of the same chemical. Von Frisch (63) showed that there were measurable differences in the response of a group of bees to 1/8 M and 5/32 M, or to 1/4 M and 5/16 M sucrose, while no changes in the response were detectable by his methods when the concentration intervals were half as great. In other cases where geometric series have been used, the least ratio between successive concentrations appears also to have been 1:1.25 (54, 55); thus we know that, like the bee, the American roach and

the horsefly, *Tabanus*, can distinguish at threshold between solutions which differ by no more than this factor, but the lower limit of their discriminatory ability has not been explored. Somewhat greater sensitivity was claimed by Verlaine (138) for the oral receptors of *Pieris* on the basis of tests with two specimens. No technique has been devised which would measure the discrimination factor in insects at concentrations above threshold. According to Moncrieff (108) the discrimination factor for the chemical senses of man is about 0.3, i.e., nearly the same as for the insects mentioned above, but Lemberger's data for human taste indicate values of about 0.15 for sucrose and 0.11 for Na-saccharin, except at threshold and at very high concentrations (91A).

4. *The Modalities of Taste in Insects*

It is generally believed that man distinguishes four types of taste sensation, viz., sweet, salt, sour and bitter, and that most complex tastes can be resolved into some combination of these four primary qualities (14, 108). In view of the work of Allen and Weinberg (see also the study by Pfaffmann, 115), this interpretation, criticized vigorously by Henning (77A), probably represents an oversimplification of the actual state of affairs, although their measurements of the critical frequency of electrical stimulation did reveal four distinct sets of receptors, each of which it was possible to identify chiefly with one of the primary sensations (4).

In insects all that has been observed directly is that some substances are accepted and others rejected, and it has been necessary to resort to less straightforward methods in the attempt to ascertain whether the categories acceptable and unacceptable are homogeneous or whether insects also are capable of a finer degree of discrimination. Recent evidence has been derived principally from five sources: *a*) observations of the behavior of specimens conditioned to respond to a given chemical when confronted with other compounds; *b*) determinations of the additive or non-additive capacity of diverse chemical stimuli; *c*) measurement of the amount of sucrose solution drunk by bees after contamination with subliminal concentrations of different chemicals: *d*) comparison of the threshold changes for different compounds during starvation; and *e*) localization of the receptors for certain types of substances on definite organs.

Bauer (9) trained *Dytiscus marginalis* and *Hydrous piccus* to respond positively to sucrose; these animals could not be trained simultaneously to avoid glucose, and also reacted positively in the majority of cases to some 15 or 20 other sugars and sugar derivatives. As in the experiments of Schaller (125) and Ritter (122) with the same or related species, the beetles learned readily to distinguish between pairs chosen from sucrose, NaCl, acids (Hcl and HAc) and quinine, and could even be trained to accept quinine and avoid NaCl. But specimens which had learned to avoid HCl also avoided HAc and could not be taught to respond differently to the two; the same result was obtained when quinine was matched against salicin or aloin. Bauer concluded that the sweet substances (with the possible exception of mannose, which was avoided by some individuals) constitute a single homogeneous grouping; salt, acids and bitter substances, normally avoided, were distinguished from sweet and from each other, so that taste substances could be classified into the same four qualities for these beetles as for man.

As mentioned above in the discussion of methods, von Frisch (63) found additive the stimulatory effects of all sugars which are acceptable to the bee. Summation was noted also between NaCl and LiBr, NH_4Br or HCl, but the repellent effect of quinine was lessened rather than enhanced by the addition of acids (hydrochloric, acetic, sulfuric, citric, lactic). When sucrose solutions, one containing NaCl and another quinine hydrochloride, were prepared which were accepted by equal proportions of the bees, they were drunk in different amounts. Almost as much of the quinine was taken as of the control, but considerably less of the solution containing salt. Thus far, the results would indicate three taste qualities: sweet, acid-salt and bitter. But in other experiments von Frisch found that starved bees show no better acceptance of NaCl than when fully fed, although the threshold for rejection of quinine rises eight times and that for HCl by a factor of five. Therefore, he concluded that salt represents a quality different from either acid or bitter. Furthermore, although there is no summation of repellency between quinine and acids, other bitter substances, such as aloin, arbutin, colocynthin and salicin, are rendered more repugnant by the addition of HCl, so that the category bitter is not homogeneous for the bee. It is possible that investigations of this kind would reveal similarly complex relationships in man.

Other evidence as to the separation of the taste qualities in insects was provided by Ritter (122), who found that after amputation of the maxillary palpi specimens of *Hydrous piceus* would still react to 0.007 M HCl but not to salt, sugar or quinine. Removal of the tips of the labial palpi then abolished the response to acid although animals lacking both sets of palpi and the antennae still reacted positively to meat juice, presumably via receptors in the mouth. These observations would seem to set the acids apart from other taste substances for *Hydrous* but require reinvestigation in view of Bauer's (9) finding with the same and related species that amputation of the maxillary palpi left the gustatory response to sucrose, etc., unaltered except for an increase in threshold. It is unfortunate that Bauer failed to comment on this discrepancy between his results and those of Ritter.

Several of the observations above are rather difficult to reconcile with the hypothesis put forward tentatively by Frings (54) that all specific tastes may be merely points in a continuous spectrum of taste sensation, the critical dimension of which is envisioned as penetrating power or some colligative property. While it is true that in general sweet substances are those which penetrate cells slowly, that some salts and alkalis at low concentrations are acceptable to insects and sweet for man, that there is a gradual transition from typical salty to bitter in the series of inorganic salts as their ionic mobilities increase, and that the most mobile cation, H^+, is the principal factor in the sour taste, such facts as summation of certain types of chemical stimuli but not of others, the apparent grouping by conditioned insects of chemically similar compounds or of those which taste alike to man and the sharp localization of acid reception alleged to occur in *Hydrous* do not fit easily into the scheme proposed. On the other hand, the experiments of Allen and Weinberg (4) reveal a high degree of interdependence among the four receptor types in man and thus raise a question as to whether the concept of a pure primary taste sensation may not be an abstraction (cf. Henning, 77A). There are also anomalies in human taste, such as the sensations aroused by alkalis, borax, hydrogen peroxide, ginger, menthol and some

metallic salts, not all of which are readily assigned to one or another combination of the recognized primary qualities (Moncrieff, 108). The invocation of thermoreceptors, tactile receptors and the common chemical sense to explain such aberrant phenomena is not wholly convincing in the absence of experimental evidence that they are actually concerned. In the cat, Pfaffmann (115) was able to distinguish only three types of taste fibers. Individual fibers of one type responded to acid stimuli (and to KCl); of another to acids, NaCl, NaAc, KCl, and $CaCl_2$; and of a third to acids and quinine. Among the several students of vertebrate gustation who have used electrical methods, only Zotterman (154) succeeded in obtaining a single fiber response to stimulation with sugar solutions, and this in only one instance. This suggests that most of the nerves which mediate the sensation 'sweet' in the vertebrates, like the chemoreceptive fibers of insects, may give rise to potentials too small for recording with present methods. The implication of the other observations above is that the entire question of the physiological basis for the modalities of taste stands in need of much fuller investigation than it has yet received, in man as in the insects, so that an hypothesis such as that of Frings may yet have value, even if it is only that it will stimulate work along these lines. Among the insects, further experiments with conditioned responses and more studies on the interaction of diverse stimuli should be productive pending the development of methods for a more direct attack on receptor function.

5. *Stimulation in Relation to Chemical and Physical Properties*

According to available data about 310[1] compounds have been presented experimentally to the contact chemoreceptors of insects. Of these, some 46 are sugars or sugar derivatives, 31 of which have been found acceptable to one or another species. With few exceptions the remainder, which include unacceptable sugars and related compounds, alcohols, aldehydes, alkaloids, amides, amines, amino acids, bromo- and chlorohydrins, carbitols, cellosolves, esters, ethers, fats and oils, fatty and mineral acids and their salts, glycols, glycosides, ketones and proteins, plus a few miscellaneous cyclic compounds and synthetic sweetening agents, are all rejected actively if presented in sufficient concentration. Of this array, the acids and salts, at low concentration, are sometimes taken in preference to plain water or dilute solutions of sucrose (cf., e.g., Butler, 21; Frings, 54), so that they alone exhibit a double modality, being acceptable in one range of dilutions and rejected at higher concentrations. Several fats, oils and proteins release a biting response in wireworms (136) and would doubtless be acceptable to various other insects. Exceptional behavior toward certain compounds is occasionally encountered. Thus, saturated solutions of NaCl were accepted by some butterflies (142) and by *Calliphora erythrocephala* (76), as by certain species of caterpillars (44). *Cecropia* larvae took strychnine freely when it was placed on the leaves of their food plant (53), and there are of course many similar examples of the ingestion of toxicants, not considered here, to be found in the literature on insect control.

[1] This estimate excludes the several thousand organic substances which were screened as potential insecticides and repellents by various agencies, principally the U. S. Department of Agriculture, during the war. A full report on these tests has not yet been made public.

Concerning the manner in which chemoreception in insects is related to the physical and chemical characteristics of the stimulating compounds little more is known at present than in the case of the vertebrates. However, insects offer certain advantages in studies of this sort, such as the accessibility of the sensory organs, the possibility of avoiding the complications of salivary and nasal secretions and the availability of statistically useful numbers of animals, which invite more attention than the group has been accorded in the past. The fact that the basic features of gustation and olfaction in insects now appear to be not too different from those of the analogous processes in man should also encourage greater use of this advantageous material for fundamental studies of chemosensory function, the more so since the results may be expected to find immediate application in the fields of preventive medicine and agriculture through the development of improved insect attractants and repellents. Recent experiments on contact chemoreception in insects which bear on the problem of the relationship between chemical and physical properties, on the one hand, and stimulatory effect, on the other, are summarized below.

Acceptable compounds. Since von Frisch (63) reviewed the subject, work on the sugars has been amplified principally by the studies of Haslinger (76) with blowflies, Bauer (9) with water beetles, Schmidt (126) with ants and Thorpe *et al.* (136) with wireworms. It is now clear, as Weis (142) had already demonstrated for butterflies, that many insects accept a wider variety of sugars than bees, and that there is no hard and fast correlation between what is acceptable and what is nutritious. Equally clear, unfortunately, is the fact that no information has been obtained which would permit a revision of von Frisch's conclusion: that the factors involved in stimulation by sugars, in insects as in other animals, simply do not reach expression in our structural formulae.

Unacceptable compounds. Somewhat more encouraging progress has been made in the field of the unacceptable. For several series of salts with a common anion (acetates, bromides, chlorides, iodides, nitrates and sulfates), Frings (54) has shown that the efficiency in stimulation may be correlated directly with the mobilities of the cations, including H^+. These studies, conducted on the mouthparts of the American roach, were an outgrowth of earlier work on oral reception in *Cecropia* larvae (53), in which similar results were obtained, and have been extended by Frings and O'Neal (55) to the legs of the horsefly, *Tabanus sulcifrons*, and by Dethier (40) to the ovipositors of parasitic Hymenoptera. The relationship thus appears to be of rather general validity among insects and, according to Frings, is in substantial agreement with most of the data reported in the literature for chemical stimulation of other animals by the series of cations employed ($Li^+ < Na^+ < Mg^{++} < Ca^{++} = Sr^{++} < K^+ \leqq Cs^+ = Rb^+ < NH_4^+ << H^+$), including their gustatory effect in man. As pointed out by Frings, the order of effectiveness of the salts is also that of their partition coefficients, which parallels that of ionic mobilities (110). The exact modus operandi of stimulation by cations thus remains in doubt, since either penetration or adsorption at a lipoprotein interface would fit the facts (and as yet it is not known that either occurs); however, it is a step forward to have related their stimulatory effects quantitatively to some definite physical property. In regard to the anions the situation is more dubious. The work of Frings (54) establishes the following

empirical order of effectiveness: $PO_4^{---} < Ac^- < SO_4^{--} = Cl^- \leqq Br^- < I^- < NO_3^- << OH^-$, for which there is at present no obvious physical basis; it is to be hoped that further studies will shed light on this question, and on possible summation or antagonism between ions of the same and of opposite sign.

Progress has been reported also in work with organic compounds. Using flies which had been desensitized to odors by removal of the antennae and labella, Dethier and Chadwick (42) measured rejection thresholds for a series of aliphatic alcohols by presenting the compounds to the tarsi in 0.1 M sucrose. Exceptionally high correlations were found between the stimulating power of the compounds and such properties as boiling point, molecular area, oil-water distribution coefficients, molecular moments, vapor pressures and activity coefficients. Molecular weight, number of carbon atoms and osmotic pressure were eliminated from consideration in part by the use of isomers, which proved usually to have different thresholds, while the correlation with vapor pressures was inverse. It was concluded that surface energy relationships must be involved in stimulation. Similar studies now under way with aldehydes, ketones, glycols, bromo- and chloro-hydrins, cellosolves, carbitols, amines and amides have so far given concordant results, but the data are still incomplete. Tests with a number of fatty acids indicated that while the H ion is the principal factor in stimulation with compounds of this type, a contribution is made also by the anion(s) or undissociated acid and that this component is effective in inverse proportion to the hydrophile nature of the molecule (22). The relationship is thus very similar to that found with the same class of compounds for human taste (cf., e.g., Taylor *et al.*, 132) and for their penetration into other organisms (e.g., Crozier, 28).

Though all evidence at present available points to the importance of free energy relationships in the limiting process in contact chemical stimulation, a more exact definition of the mechanism is impeded by gaps in information relative to the physical characteristics of the compounds tested, by the lack of knowledge as to the nature of the receptor surface and by our complete ignorance of the essential features of the stimulatory event. Another obstacle is that the techniques at hand limit the investigations largely if not entirely to compounds soluble in water or in dilute sucrose, so that in most homologous series only a few members can be examined although it is recognized that many organic liquids, nearly insoluble in water, are highly stimulating when applied in the undiluted form. As indicated in a study of the glycols (43), in which it proved feasible to measure rejection thresholds for compounds with an average molecular weight as great as 1540, the possibility must be reckoned with that curves which appear linear when based on only the first few members of an homologous series may prove to have a different order of complexity when extended to include molecules of greater size.

At present, the one relationship which appears to reconcile all the known facts of contact chemoreception in insects, including those derived from studies of inorganic electrolytes, is between stimulating efficiency and lipid solubility or oil-water distribution coefficients. Unfortunately it is just in this area that reliable physical data are lacking for many of the compounds tested, and the existence of a lipoid layer or lipo-protein mosaic at the surface of or within the receptors is still wholly

hypothetical, plausible though it may be in view of recent work on the general surface of the insect cuticle (10, 147, 148). Exact studies of the constituents and structure of the receptor surface are urgently needed.

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USE OF MICROORGANISMS FOR ASSAY OF VITAMINS

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THE PRESENT WIDESPREAD USE of microorganisms for the determination of th water-soluble vitamins represents a practical application of an essentially academic study—the nutritive requirements of microorganisms. Of necessity, microbiologists were, until recent years, more interested in the development of practical media, concocted from crude materials, which would permit continued growth under laboratory conditions of the vast numbers of different organisms found in nature, than they were in defining the exact nutritive requirements of these cultures. Isolated and sporadic investigations of the latter subject were indeed made, especially with yeast, but serious investigation of the subject has been undertaken only within the past fifteen years. This period of intensive investigation thus coincided with that during which rapid advances in knowledge of animal nutrition were being made. As has been pointed out elsewhere (1–3) the resulting cross fertilization between the two fields has immeasurably speeded development of knowledge in both fields. It was found, in brief, that the vitamins required by animals and the growth factors required by certain microorganisms were, in many cases, identical. Demonstration of the essential nature of some of these substances for growth (e. g., thiamine, riboflavin, pyridoxine, choline) was made primarily with animals, of others (e.g., inositol, biotin, pantothenic acid, pyridoxal, pyridoxamine, p-aminobenzoic acid) primarily with microorganisms. Both animals and microorganisms are inextricably involved in the early history of nicotinic acid and folic acid.

For each of the B-vitamins known to be required for growth of animals, there are thus known several different microorganisms which also require that vitamin for growth. In many cases (e.g., biotin, 4; pantothenic acid, 5–7; folic acid, 8–11; inositol, 12), the growth responses of such organisms were adapted to provide a quantitative or semiquantitative measure of the amount of the growth factor present even before the responsible factor was identified, and this measure was used to guide the course of concentration from natural materials in the initial isolation of these substances. It is from such procedures, used to identify the growth factors essential for the various test organisms, that our present microbiological assay methods have developed.

In theory, any microorganism which, under a specified set of conditions, requires addition of a vitamin to the culture medium for growth, might be used for the estimation of that vitamin in natural materials. Examination of known nutritional requirements of microorganisms (reviews: 3, 13, 14, 15) reveal large numbers of different organisms which might be thus used for almost any of the known vitamins. In practice, however, comparatively few groups of organisms have been thus used. Various microbiological assay methods which have been proposed for individual vitamins are listed, together with incidental information about the methods, in tables 1, 2 and 3. It will be observed that the majority of methods utilize lactic acid bac-

TABLE 1. VITAMIN ASSAY METHODS USING LACTIC ACID BACTERIA

TEST ORGANISM	CONCENTRATION FOR STANDARD CURVE	INCUBATION TIME	MEASUREMENT OF RESPONSE	AUTHOR, DATE AND REFERENCE
A. Biotin				
	γ per 10 cc.	*hrs.*		
Lactobacillus arabinosus	0–0.001	72	acidimetric	Snell and Wright, 1941 (16)
Lactobacillus arabinosus	0–0.002	72	acidimetric	Wright and Skeggs, 1944, (17)
Lactobacillus casei	0–0.002	72	acidimetric	Landy and Dicken, 1942 (18)
Lactobacillus casei	0–0.001	72	acidimetric	Shull *et al.*, 1942–3 (19, 20)
Lactobacillus casei	0–0.0012	16–72	turbidimetric or acidimetric	Roberts and Snell, 1946 (21)
Streptococcus faecalis	0–0.0015	16–20	turbidimetric	Luckey *et al.*, 1946 (22)
Streptococcus faecalis	0–0.001	20	turbidimetric	Rabinowitz and Snell, 1947 (23)
B. Folic acid (pteroylglutamic acid)				
Lactobacillus casei		72	acidimetric	Snell and Peterson, 1940 (24)
Lactobacillus casei	0–0.0015	72	acidimetric	Landy and Dicken, 1942 (18)
Lactobacillus casei	0–0.0015	72	acidimetric	Bird *et al.*, 1945 (25)
Lactobacillus casei	0–0.001	30–72	acidimetric	Teply and Elvehjem, 1945 (26)
Lactobacillus casei	0–0.0012	16–72	turbidimetric or acidimetric	Roberts and Snell, 1946 (21)
Streptococcus faecalis	0–0.0015	16	turbidimetric	Mitchell and Snell, 1941 (27)
Streptococcus faecalis	0–0.002	16	turbidimetric	Luckey *et al.*, 1944 (28)
Streptococcus faecalis	0–0.008	30–72	acidimetric	Teply and Elvehjem, 1947 (26)
Streptococcus faecalis	0–0.008	20	turbidimetric	Rabinowitz and Snell, 1947 (23)
C. Nicotinic acid				
Lactobacillus arabinosus	0–0.5	72	acidimetric	Snell and Wright, 1941 (16)
Lactobacillus arabinosus	0–0.5	72	acidimetric	Krehl *et al.*, 1943 (29)
Lactobacillus arabinosus	0–0.5	72	acidimetric	Barton-Wright, 1944 (30)
Lactobacillus arabinosus	0–0.5	40–72	acidimetric	Sarett *et al.*, 1945 (31)
Lactobacillus arabinosus	0–0.5	72	acidimetric	(Collaborative, 1945 (32) (USP-AOAC))
Lactobacillus arabinosus	0–0.5	72	acidimetric	Lawrence *et al.* 1946 (33)
Lactobacillus casei	0–0.8	72	acidimetric	Landy and Dicken, 1942 (18)
Lactobacillus casei	0–0.2	16–72	turbidimetric or acidimetric	Roberts and Snell, 1946 (21)
Leuconostoc mesenteroides	0–2.0	72	acidimetric	Johnson, 1945 (34)
Streptococcus faecalis	0–0.5	20	turbidimetric	Rabinowitz and Snell, 1947 (23)

D. Pantothenic acid				
Lactobacillus casei	0–0.2	72	acidimetric	Snell *et al.*, 1937 (6, 35)
Lactobacillus casei	0–0.2	72	turbidimetric or acidimetric	Pennington, *et al.*, 1940 (36)
Lactobacillus casei	0–0.15	72	acidimetric	Strong *et al.*, 1941 (37)
Lactobacillus casei	0–0.20	72	acidimetric	Landy and Dicken, 1942 (18)
Lactobacillus casei	0–0.10	72	acidimetric	Neal and Strong, 1943 (38)
Lactobacillus casei	0–0.10	72	acidimetric	Ives *et al.*, 1945 (39)
Lactobacillus casei	0–0.20	16–72	turbidimetric or acidimetric	Roberts and Snell, 1946 (21)
Lactobacillus arabinosus	0–0.2	72	acidimetric	Snell and Wright, 1941 (16)
Lactobacillus arabinosus	0–0.2	18–72	turbidimetric or acidimetric	Skeggs and Wright, 1944 (40)
Lactobacillus arabinosus	0–0.2	14–72	turbidimetric or acidimetric	Hoag *et al.*, 1945 (41)
Streptococcus faecalis	0–0.4	20	turbidimetric	Rabinowitz and Snell, 1947 (23)
E. Riboflavin				
Lactobacillus casei	0–0.3	72	acidimetric	Snell and Strong, 1939 (42)
Lactobacillus casei	0–0.4	72	acidimetric	Landy and Dicken, 1942 (18)
Lactobacillus casei	0–0.15	72	acidimetric	Greene and Black, 1943 (43)
Lactobacillus casei		72	acidimetric	Barton-Wright and Boothe, 1943 (44)
Lactobacillus casei	0–0.2	16–72	turbidimetric or acidimetric	Roberts and Snell, 1946 (21)
F. Thiamine				
Streptococcus salivarius	0–0.002	24	turbidimetric or acidimetric	Niven and Smiley, 1943 (45)
Lactobacillus fermenti	0–0.050	16–18	turbidimetric	Sarett and Cheldelin, 1944 (46, 47)
G. Vitamin B_6				
1. Pyridoxal				
Lactobacillus casei	0–0.10	24–36	turbidimetric	Snell and Rannefeld, 1945 (48)
Lactobacillus casei	0–0.04	24	turbidimetric	Rabinowitz *et al.*, 1947 (49)
2. Pyridoxal plus pyridoxamine				
Streptococcus faecalis	0–0.004	16	turbidimetric	Snell and Rannefeld, 1945 (48)
Streptococcus faecalis	0–0.006	22–24	turbidimetric	Rabinowitz and Snell, 1947 (23)
H. p-Aminobenzoic acid				
Lactobacillus arabinosus	0–0.0005	72	acidimetric	Lewis, 1942 (50)
Leuconostoc mesenteroides	0–0.001	15	turbidimetric	Pennington, 1946 (51)

TABLE 2. VITAMIN ASSAY METHODS USING YEASTS

TEST ORGANISM	CONCENTRATIONS FOR STANDARD CURVE	INCUBATION TIME	MEASUREMENT OF RESPONSE	AUTHOR, DATE AND REFERENCE
A. Biotin				
	γ per 10 cc.	*hrs.*		
Saccaromyces cerevisiae	0–0.0002	16	turbidimetric	Snell *et al.*, 1940 (52)
Saccaromyces cerevisiae	0–0.0004	18–22	turbidimetric	Hertz, 1943 (53)
Saccaromyces cerevisiae	0–0.001	16–18	turbidimetric	Atkin *et al.*, 1944 (54)
B. Nicotinic acid				
Zygosaccharomyces marxianus	0–0.2	72	turbidimetric	Leonian and Lilly, 1945 (56)
Torula cremoris	0–0.2	12–40	turbidimetric	Williams, 1946, (57)
C. Pantothenic acid				
Saccharomyces carlsbergensis	0–0.4	16–18	turbidimetric	Atkin *et al.*, 1944 (58)
D. Thiamine				
Saccharomyces cerevisiae	0–0.01	15	turbidimetric	Williams *et al.*, 1941 (59)
Saccharomyces cerevisiae	0.5–2.0	3	CO_2 evolution	Schultz *et al.*, 1942 (60)
Saccharomyces cerevisiae	0.01–0.02	3	CO_2 evolution	Atkin *et al.*, 1939 (61); Josephson and Harris, 1942 (62)
Endomyces vernalis	0–0.1	24	turbidimetric	Woolley, 1941 (63, 64)
E. Vitamin B_6 (pyridoxal plus pyridoxamine plus pyridoxine)				
Saccharomyces cerevisiae	0–0.002	15	turbidimetric	Williams *et al.*, 1941 (65)
Saccharomyces cerevisiae	0–0.004	16–18	turbidimetric	Siegel *et al.*, 1943 (66)
Saccharomyces carlsbergensis	0–0.040	16–18	turbidimetric	Atkin, *et al.*, 1943 (67) (55)
Saccharomyces oviformis	0–0.008	20	turbidimetric	Burkholder, 1943 (68)
F. Inositol				
Saccharomyces cerevisiae	0–20	16	turbidimetric	Woolley, 1941 (69)
Saccharomyces cerevisiae	0–5	15	turbidimetric	Williams *et al.*, 1941 (70)
Saccharomyces carlsbergensis	0–10	16–18	turbidimetric	Atkin *et al.*, 1944 (55)
G. General method for all six of the above vitamins				
Kloeckera brevis			turbidimetric	Burkholder *et al.*, 1944 (71)

teria as test organisms. Yeasts and *Neurospora* mutants are also widely used. The extensive use of lactic acid bacteria for such assays is due in part to the historical accident that knowledge of their nutritional requirements was sufficiently advanced to permit application to bioassay at a time when need for such procedures became most acute. Consequently, they were among the first organisms to be thus applied. The specific and complex nature of their nutritional requirements, their rugged nature, and the fact that their growth could be easily followed either by turbidimetric means or by titration of the lactic acid produced during growth, have served to keep this group of organisms foremost among those used for assay both of vitamins (3, 13) and of amino acids (reviews: 96, 97, 98). Yeasts and *Neurospora* mutants have provided not only excellent alternative methods for some of the vitamins required by lactic acid bacteria but are applicable to the determination of certain vitamins not required for growth of the latter organisms.

In addition to the original descriptions of the various proposed microbiological methods referred to in tables 1 to 3, many of these procedures have been critically discussed and described in detail or in summary form in recent monographic treatments (99–101). Except for illustrative purposes, the present review will avoid these technical details and will attempt, rather, to present the basic information necessary to the development, understanding and evaluation of various assay procedures. Before this can be done effectively, it will be necessary to have in mind the general procedure used in carrying through a microbiological assay.

In general, the procedure used in each of the assay methods is similar. The essential steps, common to all methods, but modified in detail according to the test organism being used and the vitamin to be determined, can best be illustrated by a brief description of an actual example. For this purpose, the microbiological method of Snell and Strong (42) for riboflavin will serve. This procedure was the first microbiological method for the routine determination of a vitamin in natural materials to achieve widespread use and general acceptance. The use of lactic acid bacteria for such assays, and many of the general techniques so widely followed today, were introduced with this method, itself an adaptation of earlier procedures used in studying growth factor requirements of these organisms (3, 6, 95). The history, procedural details, range of applicability and evidence for the specificity and validity of this method have been considered in detail elsewhere (100–102).

The test organism, *Lactobacillus casei*, is carried as stab cultures by monthly transfer in a yeast dextrose agar. Such cultures are incubated at 37°C. for 24 to 48 hours, or until good growth along the line of the stab is visible, and are then held in the refrigerator for the remainder of the interval between transfers. About 24 hours before an assay is to be made, a transfer is made from this culture to a tube of sterile inoculum medium. This consists of the riboflavin-deficient basal medium supplemented with riboflavin (1 γ per 10 cc.). This inoculum culture is then incubated at 37° C. until used.

The assay is conveniently carried out in 16 x 180 mm. lipless test tubes, held in one or more metal racks which are easily autoclaved. To one series of tubes, increasing amounts of a standard solution of riboflavin (from 0 to 0.25 γ in increments of about 0.05 γ) are added. To other similar series, increasing amounts of extracts

TABLE 3. VITAMIN ASSAY METHODS USING MOLDS AND MISCELLANEOUS MICROORGANISMS

TEST ORGANISM	CONCENTRATION FOR STANDARD CURVE	INCUBATION TIME	MEASUREMENT OF RESPONSE	AUTHOR, DATE AND REFERENCE
A. Biotin				
	γ per 10 cc.	*hrs.*		
Neurospora crassa	0–0.002	72	weigh mycelium	Hodson, 1945 (72)
Neurospora crassa	0–0.002	72	weigh mycelium	Tatum *et al.*, 1946 (73)
Clostridium butylicum	0–0.01	72	turbidimetric	Lampen *et al.*, 1942 (74)
Rhizobium trifolii	0–0.003	120	turbidimetric	West and Woglom, 1942 (75)
B. Nicotinic acid				
Proteus HX 19	0–0.12	36	turbidimetric	Lwoff and Querido, 1939 (76); Morel, 1943 (77)
Proteus HX 19	0–0.10	40	turbidimetric	Grossowicz and Sherstinsky, 1947 (78)
Shigella paradysenteriae	0–0.25	16–22	turbidimetric	Isbell *et al.*, 1941 (79)
Shigella paradysenteriae	0–0.01	96	acidimetric	Dorfman *et al.*, 1940 (80, 81)
C. Pantothenic acid				
Proteus morganii	0–0.005	24	turbidimetric	Pelczar and Porter, 1941 (82)
D. Thiamine				
Phycomyces blakesleeanus	0–0.2	144–480	weigh mycelium	Schopfer, 1935–1945 (83, 84)
Phycomyces blakesleeanus	0–0.2	336	weigh mycelium	Hammer *et al.*, 1943 (85)
Staphylococcus aureus	0–0.005	36	turbidimetric	West and Wilson, 1938 (86)
E. Vitamin B_6 (pyridoxal plus pyridoxamine plus pyridoxine)				
Neurospora sitophila (mutant)	0–1.0	120	weigh mycelium	Stokes *et al.*, 1943 (87)
Neurospora sitophila (mutant)	0–0.07	72	weigh mycelium	Tatum *et al.*, 1946 (73)

F. Aminobenzoic acid				
Actobacter suboxydans	0–0.01	48	turbidimetric	Landy *et al.*, 1942–3 (88, 89)
Acetobacter suboxydans	0–0.01	60–70	turbidimetric	Cheldelin and Bennett, 1945 (90)
Clostridium acetobutylicum	0–0.002	20–24	turbidimetric	Lampen and Peterson, 1944 (91)
Neurospora crassa (mutant)	0–0.03	20	diameter of mycelial growth	Thompson *et al.*, 1943 (92)
Neurospora crassa (mutant)	0–0.05	72	weigh mycelium	Tatum *et al.*, 1946 (73)
G. Choline				
Neurospora crassa (mutant)	0–8	72	weigh mycelium	Horowitz and Beadle, 1943 (93)
Neurospora crassa (mutant)	0–8	120	weigh mycelium	Hodson, 1945 (72)
Neurospora crassa (mutant)	0–10	72	weigh mycelium	Tatum *et al.*, 1946 (73)
I. Inositol				
Neurospora crassa (mutant)	0–15	72	weigh mycelium	Beadle, 1942 (94)
Neurospora crassa (mutant)	0–10	72	weigh mycelium	Tatum *et al.*, 1946 (73)
J. General method for p-aminobenzoic acid, biotin, vitamin B_6, choline and inositol				
Neurospora (mutants)		72	weigh mycelium	Tatum *et al.*, 1946 (73)

of the various samples are added in volumes (<5.0 cc.) considered by approximation to furnish amounts of the vitamin between 0.05 and 0.25 γ. All tubes are diluted with distilled water to 5 cc., then 5 cc. of the double strength, riboflavin-deficient medium are added. The tubes are plugged with cotton or otherwise appropriately covered and sterilized by autoclaving at 15 pounds pressure for 15 minutes. After cooling to body heat or below, each tube is inoculated with one drop of the turbid inoculum culture. The racks are then incubated at 37° C. The response to riboflavin may be measured turbidimetrically after 24 to 30 hours incubation, but is more commonly determined by titrating the lactic acid produced after 72 hours incubation.

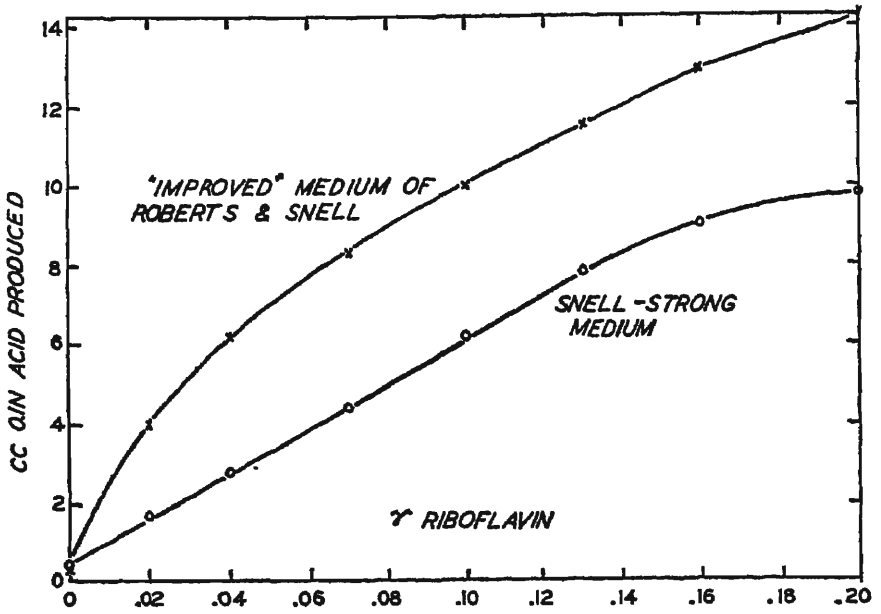


FIG 1. RESPONSE OF LACTOBACILLUS CASEI to riboflavin in two different media

To obtain the assay results, a standard curve is commonly plotted relating the response of the test organism (in turbidity units or cubic centimeters of alkali used in titration) to the concentration of riboflavin. The lower curve of figure 1 is an example of such a standard curve. The riboflavin content of the various aliquots of sample used may be obtained by interpolating the response obtained in tubes containing these aliquots onto the standard curve. Results falling outside the assay range (0.05–0.25 γ of riboflavin per tube) are discarded. At least three different sized aliquots of the sample should contain amounts of riboflavin within this range. The riboflavin content of the sample is then calculated from that of each aliquot. The figures obtained should agree within ±10 per cent and should show little or no tendency to increase or decrease regularly with increasing size of the aliquot ('drift'—see further discussion below). If the values meet these conditions, they are averaged to obtain the final result.

From this example we may now outline the essential steps involved in this or any

similar assay. These are: *a*) preparation of media for carrying stock cultures and maintenance of these cultures; *b*) preparation of the vitamin-deficient basal medium; *c*) preparation of inoculum medium and of the inoculum culture; *d*) extraction of the vitamin from the sample preparatory to assay; *e*) setting up the assay; *f*) sterilization of the assay tubes and media; *g*) inoculation with the test organism; *h*) incubation; *i*) determination of response to the vitamin and the vitamin-containing extracts; and *j*) calculation of the results.

The complexity of this series of steps is such that the end result may be influenced by the manner in which almost every operation of the procedure is carried out. In many (perhaps most) cases, the effect of variation in procedure at any of the various steps has not been studied. Conditions have been selected which appear optimum and which give consistent results. The fundamental assumption is made that any uncontrolled variations in procedural details which occur under conditions of normal practice will affect the response of the test organism alike to standard and sample, and hence will not materially affect the accuracy of the assay result. Certainly, in most cases, this assumption appears to be borne out in practice. It will, however, be useful to point out instances in which variation at the various stages of an assay are known to affect the results obtained.

PHYSICOCHEMICAL REQUIREMENTS FOR GROWTH OF THE VARIOUS ASSAY ORGANISMS. Steps (*a*) through (*c*) of the above generalized procedure involve knowledge of the nutritional requirements and general cultural conditions necessary for growth of the various test organisms. Of the three widely used groups, *Neurospora* has the simplest nutritional requirements. Butler, Robbins and Dodge (104) and Beadle and Tatum (105) showed that wild type strains of this organism required biotin as the sole growth factor of vitamin-like nature, and that if this were supplied, these organisms grew well on a medium containing a carbon and energy source (e.g., sucrose), an inorganic source of nitrogen (ammonium salts) and several inorganic salts. Such wild types could, of course, be used only for assay of biotin. The present use of these organisms for assay stems from the work of Beadle and Tatum (105) who showed that, by irradiation with X-rays or ultraviolet light, mutant strains of the organism could be derived which were unable to synthesize other vitamins or amino acids and therefore required addition of these substances to the minimal basal medium for growth. In a recent tabulation, Beadle and Tatum (106) list individual mutants which require, in addition to biotin, one of the following vitamins: thiamine, riboflavin, vitamin B_6, nicotinic acid, pantothenic acid, p-aminobenzoic acid, inositol and choline. Thus all of the presently characterized B-vitamins, folic acid excepted, could be determined with these organisms, if desired. As is apparent from table 3, several such applications have been made.

Somewhat more complex nutritionally are the various yeasts. The nutritional requirements of these organisms have been extensively investigated. Inositol (12), thiamine (107), biotin (4), pantothenic acid (5, 108) or its cleavage product, β-alanine (109), vitamin B_6 (110, 111, 48) and nicotinic acid (71, 112, 113) have been identified as necessary or stimulatory for various yeasts. Different species or strains of yeast require different combinations of these six vitamins for growth. As an example, the work of Burkholder *et al.* (71) may be cited. Of 163 strains of yeasts belonging in

110 named species and varieties, 86 required one or more of the six vitamins mentioned above, as follows: biotin, 78; thiamine or its split products, 33; pantothenic acid (or β-alanine), 30; inositol, 15; nicotinic acid, 13; vitamin B_6, 13. Some species required no vitamins at all (e.g., *Hansenula lambica*); others required all of the six vitamins (e.g., *Kloeckera brevis*. Between these two extremes, other species required from one to five vitamins in 18 different combinations. Only a single yeast, *Rhodotorula aurantiaca*, has been found which requires p-aminobenzoic acid for growth (114), and none are yet known which require riboflavin or folic acid.

In the presence of a complete assortment of vitamins, yeast, like *Neurospora*, will grow when furnished with the appropriate inorganic salts and a fermentable carbohydrate (e.g., glucose or sucrose). Ammonium salts are a commonly used nitrogen source. Although good growth can be obtained on such minimal media, and no yeasts have been reported to require preformed amino acids, it is found quite generally that addition of amino acids often improves growth considerably (71, 53, 115). For this reason, protein hydrolysates are often added to assay media employing yeasts as the test organisms.

By far the most complex nutritionally of the microorganisms used for bioassay are the lactic acid bacteria. The nutritional requirements of those members of the group which are used for assay are fairly well known and have been summarized elsewhere (3, 13, 96, 101). Vitamins required for growth include biotin, folic acid, nicotinic acid, pantothenic acid, riboflavin, thiamine, vitamin B_6 and p-aminobenzoic acid. No known culture requires all of these. Those with simpler requirements, such as *L. arabinosus*, require only biotin, nicotinic acid and pantothenic acid; others with more complex requirements (e.g., *L. casei*) may require in addition folic acid, riboflavin and vitamin B_6. Other combinations of requirements also exist. No organisms of this group are known which require inositol or choline for growth.

In addition to various vitamins, these organisms require a variable assortment of amino acids, certain purine and pyrimidine bases, a fermentable carbohydrate and certain mineral salts for growth. Under defined conditions, many cultures are stimulated by substances which are still unidentified.

To obtain good growth of any of these organisms, it is necessary to satisfy other physiological requirements. Thus, yeasts and *Neurospora* grow best under aerobic conditions. Stock cultures of these organisms are therefore carried on agar slants, exposed to air. In assay work, similarly, these organisms are cultured in vessels where the ratio of surface to volume of the culture medium is high, to permit ready access of air. Alternatively, with yeasts, where the procedure does not interfere with subsequent determination of growth, aeration may be effected by active shaking during the course of incubation.

Lactic acid bacteria, on the other hand, grow essentially anaerobically, although small amounts of air are not toxic, that is, these organisms are microaerophilic or facultatively aerobic (103). Aeration by shaking or exposure of large surfaces to air is generally deleterious. For this reason, stock cultures are carried as stabs in agar, and for assay purposes, ordinary test tubes serve well as containers since the organisms grow well throughout a deep tube of medium. These organisms produce large amounts of lactic acid during growth, and to prevent the pH of the medium from

rapidly dropping to levels which would inhibit growth (varying with different organisms from pH 3.0 to about 4.3 (116)), buffers must be added to the assay medium. Some of the organisms (e.g., *Streptococcus lactis*, *Leuconostoc mesenteroides*) are inhibited by much smaller hydrogen ion concentrations than are others (e.g., *Lactobacillus arabinosus*, *L. casei*, *L. delbrueckii*), so that to achieve high acid production by the former group it is necessary to pay careful attention to the type and amount of buffer present. In a nutritionally adequate medium, the amount of acid produced by a given organism can often be increased by increasing the amount of sugar, or of buffer, or both.

Other important factors to be controlled in use of organisms for quantitative assay work are the temperature of growth and the initial pH of the medium. It has been general practice to incubate assays at the assumed 'optimum temperature' of the test organism, although this temperature has never been accurately determined under assay conditions, and may well vary with the same organism when used in different basal media with different limiting nutrients. Such comparisons as have been made (e.g., 117, 118) indicate that the exact temperature selected for incubation is not of crucial importance, so long as it lies reasonably near to the optimum temperature, in a range which permits good growth of the test organism. Since the rate of growth of an organism is a function of temperature, it is important that the temperature selected for incubation be maintained constant throughout the area occupied by the assay vessels. Temperatures most extensively used with various lactic acid bacteria are from 30 to 37°C., varying somewhat with the organisms. Yeasts and *Neurospora* are most commonly incubated at 30°C., although lower temperatures have sometimes been used, especially with the former organisms.

The optimum pH for growth of most lactic acid bacteria is stated to lie between 5.5 and 6.5 (119, 120). Since some acid production occurs during autoclaving, the initial pH of the medium should be somewhat higher than this, 6.8 to 7.0 (119), and this is common practice. Like other fungi, yeasts and *Neurospora* grow over a wide range of hydrogen ion concentrations. Ordinarily, the initial pH of the media used with these organisms is about 5.0.

With this basic information about the nutritive requirements of cultures in mind, it is possible to proceed to consideration of the individual steps involved in microbiological assay, enumerated earlier.

A. *Carrying stock cultures of test organisms.* In all cases, it is essential that pure cultures of the test organism be maintained. If, after a period during which successful assays have been obtained, sudden transition to irregular behavior is noted, the purity of the stock culture may be suspected. For carrying cultures, the customary media and procedures long followed by bacteriologists suffice. Although, in tracing the history of the use of an individual organism for assay, it will often be found that modifications in the composition of the medium for carrying stock cultures have been introduced, these have usually been made arbitrarily, on *a priori* grounds, and have not been shown to result in improved assays. In a recent study (121), however, it was found that the magnitude of response of two commonly used assay organisms, *Lactobacillus arabinosus* and *Lactobacillus casei*, to limiting amounts of nicotinic acid or of riboflavin, decreased after long periods of time if these organisms were trans-

ferred monthly in a yeast extract-dextrose-agar, as is common practice. Such cultures could be 'rejuvenated' by a series of transplants in an enriched medium containing peptones and a liver extract.

In an independent investigation (46) dealing with thiamine assay with *Lactobacillus fermenti*, the customary yeast-glucose-agar was recommended for carrying this culture. In subsequent assays conducted much later, it was noted (47) that excessive growth occurred in assay tubes containing no added thiamine. This was attributed to development of a limited capacity of the test organism to synthesize thiamine when carried in a thiamine-low medium. This tendency was eliminated by enriching the medium used with an excess of thiamine (47).

Such experiments illustrate that the medium in which stock cultures are carried may affect assays subsequently carried out with the assay organisms, and point to the possibility of improving some methods by systematic investigation of this neglected aspect of the overall procedure. It might be similarly expected that frequency of transfer, incubation temperatures, etc., might similarly affect subsequent growth behavior. Again, these factors have not been investigated.

Recently, it was found possible to use lyophilized cultures of microorganisms as inoculum for certain assays (121). If generally successful, such procedures would relieve laboratories conducting occasional assays of the burden of carrying stock cultures and would make possible use of an inoculum of constant physiological activity from place to place and from time to time.

B. *Preparation of the vitamin-deficient basal medium.* The nature of the vitamin-free basal medium is certainly one of the most important single factors which determine the accuracy of an assay method. It is not surprising, therefore, that successive modifications of a method, which appear after its introduction, are most frequently concerned with the nature of the basal medium. What, then, are the essential characteristics of a satisfactory basal medium? It must, of course, be free of the substance to be determined. It must also be sufficiently complete, in a nutritional sense, so that addition of the sample, in the amounts necessary for assay, will not supply substances other than the essential vitamin which increase or decrease the amount of growth (or other measured quantity) obtained at the end of the recommended incubation period. Finally, the amount of growth (or other product) produced by additions of graded amounts of the vitamin must be sufficient for accurate measurement by the method adopted.

The adequacy of a basal medium for assay thus cannot be judged apart from other experimental details. A basal medium, which, when supplemented with the missing essential nutrient, contains all of the substances *essential* for growth, but is lacking in one or more substances which markedly stimulate rate of growth, may give very satisfactory assays if the period of incubation is sufficiently long to eliminate the effect of nonessential growth stimulants, so that the essential substance being assayed is the only substance limiting growth. Such a medium will also be satisfactory for assaying samples which are rich in the vitamin to be determined and hence are added in amounts too small to supply interfering materials. Satisfactory assays can also be obtained by removing the interfering materials from the extract being assayed. Conversely, such a medium will tend to give erroneous results if used with a short

incubation period, and with samples low in the substance being determined or high in other growth stimulants not present in the medium. Where possible, of course, the most satisfactory way to eliminate the effects of growth stimulants present in samples for assay is to add these to the basal medium, either as pure compounds or as crude extracts suitably freed from the vitamin to be determined.

Examples in support of the above general statements may now be cited. In assay of vitamin B_6 in yeast extract with *Streptococcus faecalis*, Rabinowitz and Snell (23) noted high results following incubation periods of 12 to 20 hours. If the incubation period was extended to 22 hours or longer, constant assay values of the proper magnitude were obtained. The effect of stimulating substances present in the extract but not in the medium, and which contributed to the value found for vitamin B_6 after short incubation periods, was eliminated by extending the incubation period. It is well known that fatty materials markedly interfere with the determination of riboflavin and pantothenic acid with lactic acid bacteria (122–125). These effects are prevented by removing them from the extracts, either by filtration at pH 4.5, by extraction with ether, or both (122, 125). Skeggs and Wright (40) have shown, however, that it is also possible to avoid these effects (in pantothenic acid assay with *Lactobacillus arabinosus*) by adding oleic acid to the assay medium. An additional instance of the same nature is afforded by observations of Stokes *et al.* (87) that the presence of thiamine in extracts stimulated response of *Neurospora sitophila* 'pyridoxineless' to vitamin B_6, with consequent high results. These authors avoided this source of error by destroying the thiamine in each sample by sulfite treatment, a procedure which left the vitamin B_6 undamaged. Tatum *et al.* (73) showed that interference from this source could also be eliminated by the simple expedient of adding an excess of thiamine to the basal medium.

In attempting to improve previously devised basal media, it has often been assumed by investigators that any modification which will permit increased growth (or acid production, etc.) of the test organism in the presence of an excess of the vitamin to be determined will increase the specificity or accuracy of an assay method; mere obtention of such increased growth has been deemed sufficient to prove the modified medium to be an 'improved' medium for assay. Although, in some cases, the assumption appears logical, in no case have the necessary data to prove it been published. In an investigation bearing on this point, Roberts and Snell (21) devised a medium which permitted more rapid growth and higher acid production by *Lactobacillus casei* in response to added riboflavin than did the widely used medium of Snell and Strong (42). The dose response curves obtained in parallel trials on the two media are shown in figure 1. Despite the more luxuriant growth obtained in the modified medium, a series of parallel assays on the two media gave identical results, within experimental error of either method. The modified medium did permit assays to be obtained in less time, however. A similar result was obtained by Sarett *et al.* (31) in comparative assays for nicotinic acid with three different assay media. In a third instance, it was found (53) that supplementation of a previously devised assay medium for biotin (52) with hydrolyzed casein considerably increased the range of concentration over which growth of yeast depended on the biotin concentration. In the modified medium, the amount of growth obtained and the assay

range for biotin were both increased; the modification was therefore valuable since it increased the convenience of use. The conclusion (53) that the modified method has increased specificity over the original method due to elimination of nonspecific stimulation which might result from the amino acid content of samples would appear invalid, however, in view of comparative curves presented with the modified method, which showed that addition of excess hydrolyzed casein did not influence the response to biotin over the assay range used in the original method.

Once a medium and technique have been devised which afford assays of proven accuracy over a convenient concentration range with gradations of growth in an easily measurable range, it would appear that other data besides a demonstrated superiority in the maximum levels of growth (or acid production, etc.) permitted by the medium should be required to prove the superiority of a modified procedure. This is not to deny that an adequate assay medium should promote excellent growth when supplemented with the missing vitamin, but merely to emphasize that the prime objective is not obtention of maximum growth, but rather of accurate and convenient assays, and that the two goals are not *necessarily* identical.

Finally, it should be re-emphasized that in general, the complexity of basal media required by various organisms reflects the complexity of their nutritional requirements. This is well illustrated by the composition of four assay media for biotin, given in table 4; that used with *Lactobacillus arabinosus* is most complex, those used with yeast occupy an intermediate position and that used with *Neurospora* is simplest. Simplicity in minimal nutritive requirement is no assurance, however, that simple media will suffice for accurate assay of an essential nutrient in complex natural mixtures. Thus, Thompson *et al.* (92) found a fairly complex medium necessary to permit accurate assay for p-aminobenzoic acid with *Neurospora crassa* p-aminobenzoicless, and simple minimal media have not proved generally adequate for assay of various amino acids required by certain *Neurospora* mutants (126). Similarly, the early yeast growth method for pantothenic acid (5), by means of which pantothenic acid was originally discovered, and which served admirably as a guide for purification and near isolation of this substance, is completely unsatisfactory for its routine determination in natural materials (36) because of the presence in these materials of substances other than pantothenic acid which affect growth of yeast in the minimal media originally used. Very satisfactory, though more complex, media for determination of pantothenic acid in natural materials with yeast have since been developed (58).

C. *Preparation of the inoculum medium and inoculum culture.* With lactic acid bacteria, which are carried as stab cultures, it is necessary to grow an inoculum culture in liquid medium for use in inoculating the assay tubes. Most commonly, the vitamin-deficient basal medium, supplemented with adequate amounts of the missing vitamin to support growth, is used for this purpose, although sometimes a special medium is used. Almost no information is available concerning the effect of variation in composition of this medium on success of the subsequent assay. Nebulous evidence suggests, however, that growing the inoculum in an 'enriched' inoculum medium may produce improved growth in the subsequent assay, due presumably to carry over of stimulating factors not supplied by the basal medium. In some cases

(particularly pantothenic acid, biotin and folic acid), the cells may store sufficient amounts of the vitamin so that subsequent use of heavy inocula results in considerable growth in unsupplemented assay tubes. This can be prevented by using a

TABLE 4. COMPARATIVE ASSAY MEDIA USED FOR DETERMINATION OF BIOTIN WITH DIFFERENT TEST ORGANISMS

CONSTITUENT	*Lactobacillus arabinosus* (17)	*Saccharomyces cerevisiae* (53)	*Saccharomyces cerevisiae* (52)	*Neurospora crassa* (73)
	Amount per 100 cc. of double strength medium			
Glucose	4.0 gm.			
Sucrose		3.4 gm.	3.4 gm.	4.0 gm.
Acid-hydrolyzed casein (charcoal-treated)	1.0 gm.	0.1 gm.		
Ammonium sulfate		0.5 gm.	0.5 gm.	
Ammonium tartrate				1.0 gm.
Sodium acetate	1.2 gm.			
Aspartic acid		17 mg.	17 mg.	
Cystine and tryptophane	20 mg. ea.			
Adenine, guanine, xanthine, uracil	1.0 mg. ea.			
p-Aminobenzoic acid	20 μg.			
Calcium pantothenate	200 μg.	50 μg.		
β-Alanine			50 μg.	
Inositol		900 μg.	900 μg.	
Pyridoxine	400 μg.	100 μg.	3 μg.	
Nicotinic acid, riboflavin	200 μg.			
Thiamine	200 μg.	100 μg.	3 μg.	
KH_2PO_4	100 mg.	340 mg.	340 mg.	200 mg.
K_2HPO_4	100 mg.			
$MgSO_4 \cdot 7H_2O$	40 mg.	45 mg.	45 mg.	100 mg.
$CaCl_2 \cdot 2H_2O$		45 mg.	45 mg.	20 mg.
NH_4NO_3				200 mg.
Trace elements	[1]	[2]	[2]	[3]

[1] $MnSO_4 \cdot 4H_2O$, NaCl, $FeSO_4 \cdot 7H_2O$, 2.0 mg. each.
[2] $MnCl_2$, H_3BO_3, $ZnSO_4$, $TlCl_3$, 180 μg. each; $FeCl_3$, 90 μg; $CuSO_4 \cdot 5H_2O$, KI, 18 μg. each.
[3] NaCl, 20 mg.; B, 2 μg.; Mo, Mn, 4 μg. each; Fe, 40 μg.; Cu, 20 μg.; Zn, 400 μg.

diluted, washed inoculum (e.g., 21, 40) or by growing the inoculum in a medium containing barely enough of the vitamin to be assayed to permit good growth. In other assays (e.g., riboflavin, nicotinic acid), large inocula may be used without danger of 'carry over.' It is generally agreed that least 'lag' in growth of subsequent assays

occurs if inoculation is made from inoculum cultures which are in the actively growing, logarithmic phase (cf. 41).

With yeasts and *Neurospora* cultures, inocula are usually prepared directly from stock cultures by removing a portion of growth (cells or spores, respectively) from a slant and suspending in water. With the former organisms, it is now most common to use freshly prepared (i.e., 24 to 36 hours old) slant cultures for this purpose (e.g., 54, 58, 65), although older cultures will also serve (52). Further work on this phase of microbiological assay, to establish the preferable procedures, would likely be worthwhile.

D. *Extraction of the vitamin from the sample preparatory to assay.* It is now well known that the majority of the water-soluble vitamins occur in tissues in combined or 'bound' forms, which are often unavailable to microorganisms used for assay. An essential phase of any microbiological assay method, therefore, is development of a procedure for liberating the free vitamin from such combined forms. Solution of this problem is also necessary, of course, for valid application of chemical procedures for the estimation of vitamins. Properly speaking, it should be considered as an independent problem, apart from the method subsequently used to estimate the vitamin; and in comparing the merits of two assay media, the same extraction procedures should be used for preparation of samples in both cases. It is apparent that, if inadequate extraction procedures are employed, no assay medium, however good, can yield reliable values.

The object, then, of a good extraction procedure, is obtention of all of the vitamin of a sample in a form utilizable by the test organism, with the elimination so far as possible, of known interfering materials. The state of combination of a given vitamin, however, apparently is not the same in different tissues. Pantothenic acid, for example, is fully extracted from wheat in a form utilizable by yeast by water alone, whereas a preliminary enzymatic digestion is required to permit its complete extraction from corn (58). Similarly, different assay organisms differ in their ability to utilize naturally occurring, combined forms of a vitamin. Biotin in some yeast extracts, for example, is fully utilized by *Lactobacillus casei*, but the same extract requires hydrolysis with strong acid to permit full utilization of its biotin by *Lactobacillus arabinosus* (17). Many other similar examples might be cited. Thus an extraction procedure which is adequate for one set of samples and one test organism may be quite inadequate with different samples or with a different test organism. In the absence of special information, one should apply the most thoroughly tested and widely applicable extraction procedure known. These are listed in table 5 and should be considered tentative procedures, to be modified where necessary with special products (cf. also 99, 100, 101 for further discussion of this problem).

E. *Setting up the assay.* This is merely a matter of mechanical manipulation and need not be further discussed. With large assays, it may be a time-consuming operation; and with highly light-labile vitamins (riboflavin and vitamin B_6), should be conducted, together with step (D) above, in subdued light or with low actinic glassware. Cannon (139) has described an automatic dispenser which is very useful in decreasing the amount of time required for pipettings in large or routinely conducted assays.

F. *Sterilization of assay tubes and media.* The necessity for maintaining pure cultures of the test organism being employed has been mentioned previously, and it is for this purpose that sterilization of test media is required. This has always been effected by autoclaving. The amount of autoclaving, and the extent of other precautions required to prevent contamination, vary widely from one type of procedure to another. In a yeast method for biotin (52), for example, sterilization was

TABLE 5. TENTATIVELY RECOMMENDED EXTRACTION PROCEDURES FOR VARIOUS VITAMINS

VITAMIN	KNOWN COMBINED FORMS	RECOMMENDED EXTRACTION PROCEDURE AND REFERENCES
Biotin		Variable with product. Hydrolysis with 6N H_2SO_4 at 120° (15 lbs. pressure, autoclave) for 1 to 2 hours appears most generally applicable (17, 53, 127). In some instances, some destruction is reported by this treatment, especially if prolonged (127).
Choline	Phospholipids, acetylcholine	Autoclaving (15–17 lbs.) with 10 cc. of 3N H_2SO_4 or HCl per 100 mg. of dry, ground sample (93, 128).
Folic acid	Pteroyldiglutamylglutamic acid Pteroylhexaglutamylglutamic acid	Autoclave finely divided sample with water, cool, then incubate with a specially prepared enzyme from hog kidney (25) at pH 4.5 for 16 hours at 45°. Other conjugate-splitting enzymes from almond (23) or chicken pancreas (129, 130, 131) have also been used and may give superior results with some samples.
Inositol	Phytic acid, lipositol	Reflux sample with excess 18% HCl for 6 hours. Remove HCl by distillation *in vacuo*. Dissolve residue in water and adjust to appropriate pH (69, 55, 132).
Nicotinic acid	Nicotinamide, coramine, nicotinuric acid, coenzymes I and II	Suspend finely ground sample in sufficient N/1 H_2SO_4 to give estimated nicotinic acid content of 1 μg. per cc. of mixture. Autoclave at 15 lbs. pressure for 15 minutes, cool, neutralize with NaOH (32, 29, 133).
Panthothenic acid	Coenzyme A	Autoclave finely ground sample at pH 6.8–7.0 for 15 minutes at 15 lbs. pressure with sufficient water to give 3–10 μg. of vitamin per 50 cc. Cool, add "Mylase P" in an amount equal to $\frac{1}{10}$ the estimated dry weight of sample, and 2 cc. of 2.5 M sodium acetate. Adjust pH to 4.8 with HCl, incubate under toluene for 12–24 hours at 50°. Dilute to 100 cc., filter, repeatedly if necessary, until a clear filtrate is obtained (134).
Riboflavin	Flavin monophosphate, flavin adenine dinucleotide	The finely divided sample is suspended in not less than 10 times its weight of N/10 HCl, autoclaved 15–20 minutes at 15 lbs. pressure, cooled, adjusted to pH 4.5 by addition of 2.5 M sodium acetate solution, and filtered to remove interfering fatty acids (102, 122). An enzymatic digestion procedure (135) was found (135, 136) also to give excellent results (cf. discussion in 101).

TABLE 5—*Continued*

VITAMIN	KNOWN COMBINED FORMS	RECOMMEND EDEXTRACTION PROCEDURE AND REFERENCES
Thiamine	Thiamine pyrophosphate	The finely divided material is suspended in at least 15 times its weight of 0.1 N H_2SO_4 and digested on a steam bath for 30 minutes. If the pH becomes greater than 1.5, additional acid is added. After cooling, sodium acetate solution is added to a pH of 4–4.5. For each 10 μg. of estimated thiamine, 1 cc. of a 10% solution of taka diastase, clarase, mylase P, or other similar enzyme is added. The mixture is incubated at 45–50° for 3 hours, or overnight (under toluene) at 37° (137). A simpler, enzymatic digestion procedure with papain and clarase (135) has also proved effective (46).
Vitamin B_6	Pyridoxal phosphate Pyridoxamine phosphate	A finely divided sample estimated to contain about 2 μg. of vitamin B_6 is suspended in 180 cc. of 0.055 N HCl. The mixture is autoclaved at 20 lbs. pressure for 5 hours, cooled, and adjusted to proper pH for assay (138).
p-Aminobenzoic acid	Pteroyl glutamic acid and higher conjugates p-Aminobenzoylpolyglutamylglutamic acid	No recommendation possible. Procedures may break down variable amounts of folic acid, etc. cf. (50, 51, 91, 92, 101).

effected by merely steaming at 100°C. for five minutes, and inoculated assay vessels were incubated without covers for the incubation period of 16 hours. No trouble from contamination was encountered, perhaps because the simple medium employed, the low initial pH of the medium, the short incubation period and the heavy inoculation with the test organism made contamination of a sufficient magnitude to be observed highly improbable. When complex media are used, suitable for growth of many organisms, together with long incubation periods and small inocula, the need for aseptic precautions becomes correspondingly greater. For such assays, it is customary to autoclave at 15 pounds pressure for from 5 to 15 minutes.

It should be recognized that during the autoclaving process, deep-seated chemical changes can and do take place in the medium, only some of which have been delineated. Many lactic acid bacteria fail to grow in unautoclaved media (119, 140, 141, 23, 142) and the nature of the substances produced which permit growth is imperfectly understood. In some cases, (e.g., for *Streptococcus faecalis*, 23, 140), these appear to be reducing substances, replaceable by thioglycollate or cysteine. In other cases (e.g., *Streptococcus salivarius*, 142), addition of compounds such as pyruvate or acetaldehyde permits growth in unautoclaved media. In all cases investigated, the effective substances appear to be derived from partial breakdown of the carbohydrate of the medium during autoclaving. Such decomposition of sugars during heating has long been known to occur, especially at neutrality or above, and in the presence of phosphates and oxygen (143). Moderate autoclaving can also render a medium

incapable of supporting growth. Thus, autoclaving a medium of conventional composition (48) for as long as 10 minutes rendered it unsuitable for growth of *Streptococcus faecalis*, apparently due to destruction of cystine (49), an essential amino acid for this organism, through interaction with glucose. Again, the interaction of glucose and amino acids at high temperatures is to be expected from their chemical constitution and has been recognized to occur for some time, (cf. 144).

Other recognized effects of autoclaving with the medium which may disturb certain microbiological assays include *a*) the production from pyridoxine, itself inactive in promoting growth of lactic acid bacteria, of small amounts of pyridoxamine and pyridoxal (48), *b*) the conversion of pyridoxal, by transamination reactions, into pyridoxamine (145), *c*) the destruction of thiamine which can be prevented, however, by appropriately modifying conditions of sterilization (46).

These results indicate that more attention needs to be given to the possible disturbing effects of variable autoclaving in microbiological assays, for both vitamins and amino acids. Ordinarily, any minor effect of autoclaving would escape notice because both standards and sample would be equally subject to it. Positional differences in an autoclave, however, could result in different tubes receiving different heat treatments and thus contribute to variability in an assay.

G. *Inoculation with the test organism.* Most commonly, inoculum is added dropwise from a pipette to each assay tube. In most cases, the final amount of growth obtained is dependent almost solely on the concentration of limiting vitamin present and is not materially influenced by fluctuations in drop size. In some cases (e.g., in biotin assay with *L. casei*, 20), inadvertent variation in the size of inoculum will materially affect the result, especially if a heavy inoculum is used. Such variation was successfully eliminated either by reducing the amount of the inoculum (by diluting the inoculum suspension) or by carefully standardizing the amount added. A syringe arrangement said to be useful for the latter purpose has been described by Black and Arnold (146). Another useful device for insuring equal inocula for different assay receptacles is to sterilize the basal medium separately, before its addition to the assay tubes, cool, inoculate, then dispense aseptically to the sterile assay vessels. This procedure has been widely and successfully used in assays with yeast (cf. 52, 53). Alternatively, the inoculum used can be diluted, and a comparatively large volume (e.g., 1 cc.) used to inoculate each tube.

H. *Incubation.* The effect of variations in temperature of incubation on assay results and the manner in which length of incubation is related to the specificity of an assay have been considered previously. For accurate assay, it is essential that all tubes be incubated at the same temperature. Small cabinet-type incubators do not generally maintain sufficiently uniform temperatures throughout the cabinet to permit the best results (cf. 147). Circulating air incubators or well-stirred water baths are much superior. It has also been found (148) that where water baths are used, care should be taken that circulating water does not impart variable vibration to the assay tubes, since variations in growth response may result.

I. *Determination of response to the vitamin.* The response of lactic acid bacteria to additions of the test substance is most commonly followed by titrating the acid produced during growth. In complete media containing one per cent of glucose, many

of these organisms (e.g., *Lactobacillus arabinosus, Lactobacillus casei*) will convert this almost quantitatively to lactic acid, producing from 10 to 11 cc. of 0.1 N acid per 10 cc. of medium. Larger quantities of acid (17 to 20 cc.) will be produced if two per cent of glucose is added and the buffer capacity of the medium is sufficiently high. Less acid-tolerant organisms (e.g., *Streptococcus faecalis, Leuconostoc mesenteroides*) will produce as much as 12 to 15 cc. of 0.1 N acid under similar conditions. This range is a very convenient one for titration. By using microburettes, with more dilute alkali for titration, the assays can readily be adapted to a 'micro' scale, in which the total volume of medium is 0.2 cc. or even smaller (149, 150), and such procedures have proved useful for some purposes.

Despite the fact that turbidimetric assays have been less widely made, comparative assays (117, 21, 151, 152, 153) indicate that this procedure gives results as reliable as those obtained by titration. When turbidity measurements are made samples assayed must be optically clear, and turbidity not due to cells must not develop on incubation. The organisms must be uniformly suspended by shaking before measurement. A moment or two is allowed after shaking to permit any air bubbles to rise and break. Any stable photoelectric colorimeter may be used. By proper choice of the wave length at which measurements are taken, interference from the slight color imparted to the medium by some samples is usually readily avoided. The procedure is somewhat faster than titration.

Turbidity measurements are used exclusively in yeast growth assays (table 2) and for almost all assays using miscellaneous bacteria (table 3). In no case has difficulty been reported to arise from this method of measurement, except where extremely dark-colored samples were assayed.

In assays with *Neurospora* and other filamentous fungi, the mycelium is harvested with a needle or filtered from the medium, washed, dried and weighed. Although more laborious than titration or turbidity measurements, accuracy of assays conducted in this manner is very satisfactory.

In all cases, it is possible to make the comparison of responses to standard and sample either before or after growth has stopped for lack of the essential vitamin. In the former case, one measures comparative growth rates; in the latter, the extent of growth. Reasons for believing that the latter procedure is the preferable of the two (with any one organism) have been considered earlier.

J. *Calculation of the results.* The response to various concentrations of the standard vitamin is plotted against the concentrations to produce a *standard curve.* Most commonly, the amount of the vitamin present in any given sample tube is determined by interpolation onto this standard curve. From simple dilution factors the value for the original sample is readily calculated. If properly set up, several increasing concentrations of the sample yield responses which fall in the assay range of the standard curve. Values for the vitamin content of the sample calculated from each of these concentrations should check within the limits of error of the assay (usually considered to be about ± 10 per cent for the better microbiological assays). If they do so, the values are averaged to obtain the final result. Sometimes it is found that the assay values calculated from several such levels of the sample will show a steady rise (or fall) with increasing concentration. Such a 'drift' in values indicates that

the test organism is not responding to the sample in the same way it does to the pure standard and is evidence against the validity of the assay. Some known reasons for such 'drifts' will be considered below.

Recently, a number of investigators (154-157) have attempted to devise mathematically sound procedures to supplement, and in some cases replace, the empirical method described above for calculating results of assays. Where the dose-response curve to the test substance is a straight line, or where it can be converted into a straight line by a suitable transformation of response, such procedures are already available (155, 157). When combined with a statistically valid design for setting up the assay, they permit use of the microbiological data themselves to determine the variance and standard error of the assay result. For this reason they promise to be most valuable for some purposes. This subject has been fully discussed by Bliss (158). Application of certain findings made in connection with these studies may change the mechanics of assay considerably. Customarily, for example, each concentration level of standard and sample is prepared in duplicate, and increasing concentration levels are prepared, inoculated, incubated and the response measured in consecutive, nonrandom order. Such a procedure is statistically unsound, and investigation has shown (159) that under these conditions, excessively good agreement, which does not reflect the true degree of variation encountered in the assay as a whole, is obtained between duplicate tubes. Replicate points prepared in this manner, therefore, may be largely valueless. If, however, duplicate tubes are prepared, inoculated, etc., in random order, agreement between them is not so good, and their variance is a true reflection of the precision of the method. Similarly, current practice usually calls for several more points for the standard curve than for the sample curve. Where the standard curve is regular and its nature well established, however, this procedure is valueless, for the final estimate of potency cannot be more accurate than the least accurate observation made in obtaining it. Logically, the number of points determined for the standard need be no more than that determined for each sample.

CRITERIA FOR ESTABLISHING RELIABILITY IN AN ASSAY. The general procedures available for establishing whether or not an assay method is yielding reliable results have been fully discussed elsewhere (133, 160) and need only be listed here. They include *a*) agreement with results of other reliable methods, *b*) obtention of theoretical recoveries on addition of known amounts of vitamin to a sample, *c*) obtention of consistent values on repeated assay, *d*) agreement in assay values obtained with different assay organisms having different nutritive requirements, and *e*) agreement of results calculated from different assay levels, and the absence of 'drift.'

Unfortunately, (*a*) is applicable in only a few instances, since the reliability of other methods for determination of a vitamin is usually established no better than that of proposed microbiological methods. If methods as widely different as chemical and microbiological assays do give the same values on a given extract, it is, however, strong presumptive evidence for the validity of both procedures; (*b*) and (*c*) are obvious requirements of any analytical method; although nonfulfillment is certainly sufficient to condemn an assay, neither good recoveries nor consistent values on repeated assay suffice to prove a method reliable. Points (*d*) and (*e*) are more con-

clusive. If different assay organisms having different nutritive requirements give values which check, it is excellent evidence for the reliability of the values, since it is improbable that the different organisms would respond to interfering materials in exactly the same manner and to exactly the same extent. The absence of 'drift' in assays is evidence that the assay organism is responding to the vitamin and the sample in exactly the same way and is thus excellent evidence for the absence of interfering materials in the sample, and hence for specificity of the assay.

'DRIFT' AND ITS POSSIBLE CAUSES. It has been emphasized above that occurrence of a regular shift or 'drift' in assay values, either increasing or decreasing with concentration of the sample, is evidence for the presence of interfering materials in the sample, and that where such drift occurs, complete reliance cannot be placed on the assay result. Such drifts are quite frequently found in assay work, and consideration of some cases in which their cause is known may suggest procedures for eliminating them in other cases as well.

In several instances 'drifts' in assay values can be produced by assay of a known substance or of mixtures of known substances. Three general cases have been recognized, as follows.

(A) A substance chemically unrelated to the vitamin may stimulate or inhibit response of the test organism to suboptimal levels of the vitamin. The effect of free fatty acids on the response of *L. arabinosus* and *L. casei* to pantothenic acid, and of *L. casei* to riboflavin, is an example (122–125, 44). Depending upon the concentration, the fatty acid may stimulate or inhibit the growth response to riboflavin. At levels ordinarily encountered in samples, stimulation occurs, but the per cent stimulation varies with the amount and type of fatty acid, so that a drift in assay values is obtained at increasing levels of sample. In both the riboflavin and pantothenic acid assays, this effect is eliminated by filtration of the sample at pH 4.5 (122), which removes the interfering lipids. In the latter assay, it can also be avoided by addition of an excess of oleic acid to the basal medium (40).

(B) Substances chemically related to the vitamin may replace the vitamin in the nutrition of the test organism, but the dose-response curve to the vitamin and the related compound may be entirely dissimilar. In such a case, the ratio of growth-promoting activity of analogue to that of vitamin is not constant at various concentration levels, and if both substances are present in a sample, drift results. For example, Bird and Robbins (161) showed that the activity for *L. casei* of partial enzymatic hydrolysates of vitamin B_c conjugate (pteroylhexaglutamylglutamic acid) in terms of free vitamin B_c (folic acid, pteroylglutamic acid) varied greatly, increasing as the dosage level was increased. This conjugate occurs naturally, and when incompletely hydrolyzed samples are assayed with *L. casei* against a standard of pteroylglutamic acid, upward drift at increasing levels of sample is encountered. A similar example is provided by work of Rabinowitz and Snell (162), who found that pyridoxamine phosphate, which occurs naturally, was active in place of pyridoxamine for *Streptococcus faecalis*. Its activity varied from 0.5 to 2.0 times that of pyridoxamine when tested at various levels. Incompletely hydrolyzed samples which contained this substance showed marked drift when assayed. In both of the above instances, drift could be eliminated by appropriate treatment of the sample to completely hydrolyze active combined forms of the vitamin to the free compounds.

(C) Substances physiologically related to the vitamin, in that they may replace it for growth, may occur naturally, and interfere in the same way as described under (B) above. Thymine, for example, replaces folic acid under the testing conditions used with lactic acid bacteria, and if present in sufficient quantity in natural extracts, will interfere with assays for folic acid (163). In a similar fashion, oleic or linoleic acids replace biotin for lactic acid bacteria so far suggested for its assay (164, 165, 166), and D-alanine may replace vitamin B_6 (167). Where interferingly large amounts of these substances were present in samples for assay, the only remedy would be adoption of an extraction procedure which would eliminate them from the sample, adoption of a different test organism unaffected by the interfering material or development of assay conditions under which interference no longer occurred.

SPECIFICITY OF TEST ORGANISMS. A mistaken impression of the specificity of most test organisms might be gained from examination of the instances in (C) above. As a general rule, microorganisms show marked specificity in their requirement for individual vitamins. For example, animals can satisfy their nicotinic acid requirements from dietary nicotinic acid or nicotinamide, or by synthesis from tryptophane (168, 169). Microorganisms so far used for assay are unable to utilize tryptophane in place of nicotinic acid. While the most widely useful assay organisms utilize nicotinamide, nicotinic acid and coenzymes I and II with equal facility (as do animals), other microorganisms are available which require either free nicotinic acid, nicotinamide or the intact coenzymes I and II (133). Similarly for thiamine, microorganisms are known which require only the thiazole portion of the molecule, only the pyrimidine portion, both thiazole and pyrimidine or intact thiamine (170). By using such microorganisms as assay agents, useful information concerning the metabolism of vitamins can be obtained (e.g., 171, 172). Such procedures lie outside the scope of this review and have not been listed in tables 1 to 3. Knowledge of the specificity of response of an organism to a vitamin and its naturally occurring derivatives or degradation products is very helpful in devising valid procedures for assay. The pertinent information so far available for most widely used assay organisms has been summarized elsewhere (99, 101) and will not be further considered here.

PREFERRED METHODS OF ASSAY. The number of publications dealing to some extent with the problem of microbiological assay of vitamins and the number of alternative methods for a single vitamin have become so large that it is useful to list what may be called 'preferred' methods of assay. These are given, together with certain substances which may interfere in their accurate use, in table 6. Any such list of methods will vary to some extent with the experience and personal preferences of its author. The tentative and temporary nature of any such list should also be clearly apparent. In compiling the present list the following points have been given most emphasis:

a) Widely tested procedures which have proved generally satisfactory are preferred over others which, though they may possibly be superior, have not been tested by widespread use.

b) Since the most widely used assay procedures employ lactic acid bacteria, and since all methods which employ these organisms are very similar, assay methods using these organisms are preferred in the absence of clearly superior methods.

c) From the standpoint of simplicity and speed, assays employing yeast are usually preferable to those using lactic acid bacteria, and these in turn to those using *Neurospora.*

In each case, the method listed should be used with the most suitable extraction procedure known (table 5), which may or may not be that described with the method. In some cases, where there appears little choice between two or more methods on the basis of the above criteria, more than a single method is given.

TABLE 6. PREFERRED METHODS FOR MICROBIOLOGICAL DETERMINATION OF THE VITAMINS

VITAMIN	TEST ORGANISM	REFERENCE	KNOWN INTERFERING MATERIALS
p-Aminobenzoic acid	*Neurospora crassa* (mutant)	(73)	
Biotin	*Lactobacillus arabinosus*	(17)	Oleic and linoleic acids
	Saccharomyces cerevisiae	(53, 54)	Desthiobiotin
Choline	*Neurospora crassa* (mutant)	(73, 93)	Methionine
Folic acid	*Lactobacillus casei*	(21, 26)	Folic acid conjugates, thymine
	Streptococcus faecalis	(26)	Thymine
Inositol	*Saccharomyces carlsbergensis*	(55, 58, 67)	
Nicotinic acid	*Lactobacillus arabinosus*	(16, 29, 32)	
Pantothenic acid	*Lactobacillus arabinosus*	(40)	Fatty acids
	Saccharomyces carlsbergensis	(58)	
Riboflavin	*Lactobacillus casei*	(42, 137)	Fatty acids
Thiamine	*Lactobacillus fermenti*	(46, 47)	Cocarboxylase
	Saccharomyces cerevisiae	(60)	
Vitamin B_6			
Total (pyridoxal + pyridoxamine + pyridoxine)	*Saccharomyces carlsbergensis*	(67)	
Pyridoxamine + pyridoxal	*Streptococcus faecalis*	(23)	Pyridoxamine phosphate, D-alanine
Pyridoxal	*Lactobacillus casei*	(49)	D-alanine

CONCLUDING REMARKS

The attempt has been made above to summarize, in general terms but with specific examples, certain basic information helpful in understanding and using microbiological assay methods. No attempt at an exhaustive treatment of the literature has been made. All of these assay procedures are based on the controlled growth of living organisms. In discussing procedures based on such a complex and imperfectly understood phenomenon, it is difficult to avoid making statements which appear logical but which may be based on untested assumptions. Some such assumptions have been discussed in the body of this review. An additional example is the statement, often heard, that microbiological assays cannot be more precise than ±10 per cent because of the 'biological variability' of the test organisms employed. As indicated elsewhere (96, 98), the large numbers of organisms

involved in microbiological assays should free the result of variation attributable to 'biological variation,' and actual experimental results conducted with pure vitamins (150) indicate that much more precise results than this can be obtained under favorable circumstances. Present procedures are rarely more precise than this when applied to natural materials, but this should be ascribed to our improper understanding and control of variables in the assay procedure, and not to the assay organisms. The future improvement of these methods would appear to lie, then, in an improved understanding of processes involved in growth and metabolism of the test organisms, which would permit more precise control of those factors at present leading to variation.

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SOME THERMODYNAMIC AND KINETIC ASPECTS OF METABOLIC PHOSPHORYLATION

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DURING THE LAST FIFTEEN YEARS, the part played by phosphate in cellular processes has been made clear: phosphate is the means by which a large part at least of the energy of catabolic processes is made available for adaptive processes in the cell. The immediate source of energy for these adaptive processes is believed to be unstable ('energy rich') phosphate compounds e.g., creatine phosphate and adenosine triphosphate). These are formed from inorganic phosphate at the expense of the free energy of degradation of carbohydrate and probably of other intermediates.

The chemistry and some thermodynamic aspects of reactions involving phosphorylation have been reviewed by Barron (4, 5), Kalckar (26, 27), Meyerhof (46) and Lipmann (40–42). We have attempted a rather more thorough use of the thermodynamic approach under the following heads:

I. A general examination of the use that can be made of thermodynamic data and a consideration of what additional information, especially kinetic, is needed for a full understanding of cellular processes.

II. An examination of the thermodynamic information concerning phosphorylation brought about by anaerobic glycolysis.

III. An examination of the thermodynamic information concerning phosphorylation brought about by oxidation of carbohydrate, together with a critical study of some of the experimental data on the amount of phosphorylation that is produced.

IV. An examination of the possible functions of intermediary carriers in the transport of hydrogen and of phosphate.

V. An examination of some problems of kinetics that arise from these considerations.

It must be pointed out that the thermodynamic argument applied in sections II and III is unlikely to lead to incorrect results provided that the assumptions made and the numerical values used are accurate. The assumptions made have been clearly indicated in the text, and the experimental figures are unlikely to be far wrong; for this reason the figures given in these sections should be of value for comparison with experimental results. On the other hand much of the argument used in the last two sections is based on kinetic considerations of a fairly simple nature. Since almost nothing is known of the actual kinetic conditions within the cell, it is uncertain how far the results obtained can be rigidly applied to the cell. For this reason, and in view of the much greater un-

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certainty in the assumptions made and the numerical values used, the figures deduced in the last two sections are to be taken more as illustrative of the type of calculation which must eventually be made than as of immediate use for comparison with experiment.

Finally we have suggested what sort of experimental evidence is needed to solve the problems that have been raised.

I. GENERAL

Only in the case of the anaerobic breakdown of carbohydrate is the exact nature of the process known by which inorganic phosphate is transferred to phosphate acceptors; however, the mechanism can reasonably be supposed to be general and no evidence opposes this supposition.

The type of changes involved may be expressed as follows: *a*) An intermediate R forms a compound with inorganic phosphate whose free energy of hydrolysis is relatively low, $R + P \rightarrow RP$; *b*) the compound RP then undergoes a reaction (such as dehydrogenation or decarboxylation), $RP \rightarrow R' P$, with the formation of a product which is relatively unstable thermodynamically, that is, of high free energy of hydrolysis; and *c*) R′ P reacts with an acceptor A (such as creatine or adenylic acid), $R' P + A \rightarrow R' + AP$.

In order that such a series of changes may occur in any given state of concentrations of reactants and products, it is a necessary condition that the total change of free energy shall not be positive. The total change of free energy may be regarded as the sum of the changes of free energy involved in the processes $R \rightarrow R'$ (which will be negative) and $A + P \rightarrow AP$ (which will be positive).

It is convenient to classify reactions according to their *standard free energy*, ΔF_0. This is usually defined as the change of free energy when the activity of each reactant and product is unity. (To simplify further argument we shall take the activity as being measured by the molar concentration.) ΔF_0 is related to the conventional equilibrium constant of the reaction by $\Delta F_0 = -RT \ln K$ (where ln represents $\log_e$). However, where the reactants and products have concentrations other than 1 molar, the actual free energy change is given by

$$\Delta F = \Delta F_0 + RT \ln \frac{\text{Product of concentrations of products}}{\text{Product of concentrations of reactants}}.$$

Thus, in order to predict whether a given reaction $R \rightarrow R'$ can lead to the phosphorylation of a given acceptor, it is necessary to know not only the respective values of ΔF_0 but also the concentrations of all the substances concerned. A conclusion based on the values of ΔF_0 alone might be seriously wrong.

Usually the change $R \rightarrow R'$ occurs not as an isolated reaction but as one stage of a sequence of reactions such as

$$Q \xrightarrow[\Delta F_{01}]{} R \xrightarrow[\Delta F_{02}]{} R' \xrightarrow[\Delta F_{03}]{} S$$

and the concentrations of R and R′ will then be partly determined by the preceding and succeeding reactions. Suppose that only the reaction $R \rightarrow R'$ leads to phosphorylation of A and cannot occur unless A accepts phosphate, that the free energy of $A + P \rightarrow AP$, under the conditions chosen, is $\Delta F'$, and that the concentrations of

R and R′ are not such that ΔF_2 is negative enough for phosphorylation of A to occur (i.e., $\Delta F_2 + \Delta F' > 0$). Then R may accumulate as a result of conversion of Q to R, and R′ may become depleted by conversion to S. ΔF_2 thus becomes more negative and, if this can go far enough, a point is reached at which phosphorylation can occur. However, ΔF_2 can reach a sufficiently negative value only if the overall free energy change of Q → S is large enough and negative. This depends on the overall change of standard free energy and on the concentrations of Q and S. Thus for phosphorylation to occur

$$\Delta F_{01} + \Delta F_{02} + \Delta F_{03} + RT \ln [S]/[Q] + \Delta F' < 0 \qquad 1$$

must be satisfied. This condition may also be stated in the form

$$\Delta F_{02} + RT \ln [R']/[R] + \Delta F' < 0. \qquad 2$$

The whole of the free energy of the reaction Q → S will be available for phosphorylation (at the stage R → R′) when the reactions Q → R and R′ → S are at equilibrium (and therefore involve no changes of free energy). In this situation the maximum amount of energy is made available at a single stage.

If the reaction R → R′ can occur without phosphorylation of A, then the concentrations of R and R′ reached, when a steady state is attained, will depend on the rates of this and of the other reactions. In this case, a purely thermodynamic argument is not enough to answer the question whether the reaction Q → S can lead to the phosphorylation of A, since the ratio [R′]/[R] might never reach a low enough value even if *equation 1* were satisfied. Therefore, kinetic data are also required.

It will not be found in practice that any system can transfer energy with an efficiency of 1. This could be so only in the absence of any side reaction, and if all stages were at equilibrium, when the rate of reaction would be infinitely slow. The rate may also be insufficient, even if the total free energy is sufficiently negative, if the concentration of any intermediate should fall too low. Thus, in the example given, if ΔF_{02} is too positive, the value of [R′]/[R] needed to satisfy *equation 2* may be very small; consequently, the low concentration of R′ may become a rate-limiting factor.

In general, knowledge of cellular processes must be based, in the first place, on information about the chemical nature of the substances and reactions concerned. Thermodynamic information next enables limits to be set to the possible development of free energy by reactions and thence to the possibilities of coupling between them. But knowledge of the standard free energies of reactions is not enough for this purpose; the actual free energy changes are needed and this involves knowledge of the concentrations of all the substances concerned. This introduces the necessity for kinetic information, to set limits to the concentrations of reacting substances consistent with the rates at which individual and overall reactions occur. Ultimately, the aim is to be able to account for cellular reactions, both in the steady state and when the steady state is disturbed in any particular way.

At present, naturally, chemical information is most complete; thermodynamic information is nearly complete in some fields but incomplete in others; kinetic information which is relevant to conditions within the cell is largely lacking.

II. PHOSPHORYLATION ASSOCIATED WITH ANAEROBIC GLYCOLYSIS

Lundsgaard (45) showed that phosphorylation of creatine occurs in whole muscle following a short tetanus. In his most favored experiments (his table 7, experiments 7–11) the molar ratio of creatine phosphate (CrP) formed to lactate (HL) formed varied between 1.6 and 1.9. Meyerhof and Lohmann (48) and Lehnartz (39) further showed that phosphorylation of creatine accompanies anaerobic glycolysis in muscle brei and press juice, but they did not show an exact proportionality between the two processes. Lehmann (38) showed a quantitative phosphorylation of adenylic acid by 2-phosphopyruvate (PyP) and of creatine by adenosine triphosphate (ATP).

This and later work has made it clear that adenylic acid or adenosine diphosphate (ADP) accepts phosphate (P) at two stages of the glycolytic process: *1*) from 1:3 diphosphoglyceric acid (diPG acid; Needham and Pillai, 51; Bücher, 11; Meyerhof *et al.*, 49); *2*) from 2-phosphopyruvic acid (PyP) (Lehmann, 38, Lardy and Ziegler, 36). In turn, Banga (2) confirmed that *3*) adenosine diphosphate and adenosine triphosphate can transfer phosphate to creatine. Phosphate is acquired by the glycolytic system at three stages: *4a*) glycogen, through phosphorolysis by inorganic phosphate, gives glucose-1-phosphate (G-1P) or *4b*) glucose, with phosphate from ATP, gives glucose-6-phosphate (G-6-P); *5*) fructose-6-phosphate (F-6-P), with ATP, gives fructose 1-6-diphosphate (HDP); *6*) 3-phosphoglyceraldehyde (PGA), with inorganic phosphate, after dehydrogenation forms 1-3-diphosphoglyceric acid.

The stoichiometry of these stages does not seem to be in doubt. It appears, therefore, that the maximum ratio of moles of phosphate combined to moles of lactate formed is 1.5 in the case of glycogen and 1 in the case of glucose. This appears to be in conflict with the results of Lundsgaard quoted above, but close examination of his data shows that the discrepancy can be accounted for. Thus, while after a one-minute recovery the inorganic phosphate had returned nearly to its value in resting muscle (13 to 15 millimolar), the creatine phosphate (at 14 to 17 millimolar) was still considerably below its resting concentration (17 to 24 millimolar). Furthermore, the decrease of inorganic phosphate (4.5 to 8.5 millimolar) is greater than the increase of creatine phosphate in the same experiments (4.5 to 5.5 millimolar). These results can be explained if there is a fall in the concentration of hexose diphosphate during the recovery, leading to a ratio of CrP/HL greater than 1.5, with a simultaneous increase in the concentration of hexose monophosphates; this would cause a decrease of inorganic phosphate greater than the increase of creatine phosphate. These considerations illustrate the difficulties of dealing quantitatively with changes in a complex system.

It is interesting to consider the stoichiometrical ratio of 1.5 in the light of the thermodynamic data, now very nearly complete, which are set out in table 1. Column 1 gives the index number of the reaction; column 2 gives the reaction (in certain cases main reactions are dissected into simpler reactions which are indexed by letters); column 3 gives the reference to the literature; columns 4 and 5 give the experimental values of K or of E'_0, whichever has been measured; column 6a gives the values of ΔF_0, per $\frac{1}{2}$ mole of glucose for those reactions which sum to glycogen → lactate; and column 6b gives the values of ΔF_0 for the other reactions. All values

are for pH 7 to 7.5 and for 37°C. At the bottom of the table are the summed values of ΔF_0 for the two main overall reactions.

The following points require special comment:

Reaction 4a. This is the only reaction in the series for which no exact data are available. It is known that fructose-6-phosphate is not phosphorylated by inorganic

TABLE 1

1	2		3	4	5	6a	6b
			REF. NO.	K	E'_0	ΔF_0 cal/½ mol. of glucose	
1	Glycogen + P	→ G-1-P	15	0.42		+270	
2	G-1-P	→ G-6-P	69	15.7		−840	
3	G-6-P	→ F-6-P	69	0.43		+260	
4	F-6-P + ATP	→ HDP + ADP					−5,100
a	F-6-P + P	→ HDP	[1]	10^{-2}		+1,400	
b	ATP	→ ADP + P	42				−6,500
5	HDP	→ PDHA + PGA	22	1.2×10^{-4}		+2,800	
6	PDHA	→ PGA	47	2.8×10^{-2}		+1,100	
7	PGA + P + Co	→ diPGAcid + CoH_2	74	0.9		+70	
a	PGA	→ PGAcid + H_2	[2]		−0.67		−18,100
b	H_2 + Co	→ CoH_2	8		−0.28		
c	PGAcid + P	→ diPGAcid	[2]				+18,200
8	diPGAcid + ADP	→ 3-PGAcid + ATP	[2]	5.1×10^{2}			−5,200
a	diPGAcid	→ 3-PGAcid + P	[2]			−18,200	
b	P + ADP	→ ATP	42				+13,000
9	3-PGAcid	→ 2-PGAcid	50	0.11		+1,340	
10	2-PGAcid	→ PyP	44	3.6		−790	
11	PyP + ADP	→ Py + ATP	36				−3,000
a	PyP	→ Py + P				−16,000	
b	P + ADP	→ ATP	42				+13,000
12	Py + CoH_2	→ HL + Co				−4,600	
a	Py + H_2	→ HL	6		−0.18		
b	CoH_2	→ Co + H_2	8		−0.28		
13	ATP + Cr	→ ADP + CrP	2	2.3×10^{-3}			+3,700
a	ATP	→ ADP + P	42				−13,000
b	Cr + P	→ CrP					+16,700
	½ glucose (glycogen)	→ HL				−33,200	
	2 Cr + 2P	→ 2 CrP				+33,400	

[1] Value assumed; see text. [2] Values deduced; see text.

phosphate to a detectable extent, whereas ATP leaves no detectable amount of fructose-6-phosphate unphosphorylated. Taking the limit of detectability as 1 per cent, the value of the standard free energy of phosphorylation of fructose-6-phosphate must lie between $-2.303 \log_{10} 10^{-2}$, that is +2800 calories/mole, and +13,000 minus 2800, that is +10,200 calories/mole. We have taken the lower limit (see below), which agrees with Lipmann's (40) estimate for a 'low-energy' phosphate ester.

Reaction 8. Bücher (11) appears to have made a direct study of this equilibrium, but we have been unable to find any publication of the quantitative results. Fortunately, it has been possible to obtain information about this equilibrium indirectly through the measurement of *reaction 7* by Warburg and Christian (74) of the reaction $\frac{1}{2}$ HDP + CoI + P + ADP = PGAcid + $CoIH_2$ + ATP by Meyerhof *et al.* (49) and from *reactions 5* and *6*.

For *reaction 7* Warburg and Christian (74) obtained the values of K given in table 2.[2] From the data of Meyerhof *et al.* (their table 5), using the equilibrium constants for *reactions 5* and *6*, it is possible to calculate values of K for *reaction 8* (table 3). Using Lipmann's (42) value of −13,000 calories for ΔF_0 of hydrolysis of ATP, a value of −18,200 calories for ΔF_0 of hydrolysis of diphosphoglyceric acid is obtained (*reaction 8a*). Using this value and −0.28 volt for E'_0 of CoI, the value of E'_0 for phosphoglyceraldehyde-3-phosphoglyceric acid (*reaction 7a*) is −0.67 volt.

TABLE 2

$$K = \frac{[\text{diPGAcid}]\,[CoH_2]}{[\text{PGA}]\,[P]\,[Co]}$$

[P] millimolar	K
0.87	0.99
16.8	0.87
33.6	0.88

log. mean K = 0.91

TABLE 3

$$K = \frac{[\text{PGAcid}]\,[\text{ADP}]}{[\text{diPGAcid}]\,[\text{ADP}]}$$

EXPERIMENT[1]	K × 10^{-3}	EXPERIMENT[1]	K × 10^{-3}
1a	2.5	2	3.3
1b	9.7	3	8.2

log. mean K = 5.1 × 10^3

[1] Meyerhof *et al* (49), table V.

Reaction 13. Banga (2) measured the equilibrium constant of *reaction 13*. ΔF_0 for *reaction 13b* is thence obtained using Lipmann's value of ΔF_0 for the hydrolysis of ATP.

Discussion. Table 1 shows that, under standard conditions, the conversion of glycogen to lactate supplies just insufficient energy to phosphorylate 2 moles of creatine per mole of lactate; alternatively, that at equilibrium there will be less creatine phosphate than creatine, if the concentrations of glycogen, lactate and phosphate are all unit. Correction for the actual stationary concentrations of initial and end products is applied, taking the concentrations (in resting muscle)

[2] In a paper which appeared after the manuscript of this review was complete, Meyerhof and Oesper, *J. Biol. Chem.* 170: 1, 1947, describe extensive measurements on this and related equilibria; their value of K for reaction 7 at pH 7 is close to that of Warburg and Christian. We cannot agree with Meyerhof and Oesper that Warburg and Christian did not regard this reaction as an equilibrium; indeed, they specifically stated that it is so and their quantitative results (table 2) support this assertion.

of phosphate and of lactate as 14 and 2 millimolar, respectively. We neglect the effect of concentration of glycogen on the free energy, for the effect is small *per residue of glucose* since glycogen is a high polymer. Since at equilibrium the total free energy change of the reaction

$$\tfrac{1}{2} \text{ glycogen} + 2\ \text{Cr} + 2\ \text{P} = \text{HL} + 2\ \text{CrP}$$

is zero, then

$$-33{,}200 + 33{,}400 + 2.303\ \text{RT}\ (\log [\text{HL}] - \log [\text{P}] + 2 \log [\text{CrP}]/[\text{Cr}]) = 0$$

whence

$$[\text{CrP}]/[\text{Cr}] = 2.3.$$

This agrees reasonably with the values found for resting frog muscle (18) under anaerobic conditions.

In this calculation we have assumed that fructose-6-phosphate is phosphorylated by inorganic phosphate at 14 millimolar; in fact, it receives its phosphate only from ATP. This corresponds to a net phosphorylation of 1.5 moles of creatine instead of 2 and would lead to a much higher predicted value for [CrP]/[Cr]. This discrepancy would, however, largely disappear if it were assumed that the free energy of phosphorylation of fructose-6-phosphate had the higher value (see above). On the other hand, if its value is about +2800 calories/mole (the value assumed above), the reaction with ATP must be far from equilibrium; otherwise, in resting muscle at least one of the following would have to hold: very low ATP, very low phosphate, very high HDP or *reactions 1*, *2* and *3* very far from equilibrium, none of which appears to be true. Our assumption of the lower value is consistent with that made in explaining why Lundsgaard obtained ratios CrP/HL greater than 1.5. This assumption of the lower value implies a 'loss' of most of the free energy of ATP breakdown at this stage and an overall efficiency of about 0.75.

It is to be noticed also that while ΔF_0 for *reaction 8a* is sufficient to phosphorylate creatine to a [CrP]/[Cr] ratio greater than 1, ΔF_0 for *reaction 11a* is insufficient. This must mean that the stationary ratio of [PyP]/[Py] must be considerably greater than 1. The high thermal efficiency estimated in the whole muscle (45) means that there is little margin of energy and that all reactions, except *4*, *8a* and *11a*, must be near their equilibria.

III. PHOSPHORYLATION ASSOCIATED WITH THE OXIDATION OF CARBOHYDRATE

The information available here is in every way less complete than in the anaerobic case. There is no doubt that phosphorylation of creatine does occur during the aerobic recovery of whole muscle, associated with the oxidation of glycogen, but there seem to be no reliable estimates of the ratio of phosphorylation to oxidation. For example, different methods, thermal and chemical, give a wide range of values for the Meyerhof Quotient; Lipmann (40) accepts the value of 6, but though this is near the mean value, no great reliance is to be placed upon it. Closer study of phosphorylation in tissue preparations has necessarily awaited the chemical definition of the path of oxidation of glycogen; this can now be taken as established, at

least in its main features (31). Little work has been done on the case corresponding to glycolysis, namely the oxidation of glycogen to pyruvate, and no clear evidence has been obtained on the degree of phosphorylation connected with this process.

However, various measurements of oxidative phosphorylation accompanying the oxidation of pyruvate and its intermediates have been made, chiefly by Cori and collaborators, by Ochoa and by Tzibakowa and Belitzer.

If we take the Meyerhof Quotient as being not less than 5, then the number of moles of phosphate combined, per ½ mole of glucose oxidized, must be at least 5 × 1.5; allowing 1.5 for the contribution of the process glycogen → pyruvate (as in the anaerobic reaction) this leaves six to be accounted for by the oxidation of pyruvate. This is a minimum estimate and evidently the P:O ratio (56) must be greater than 1 in its average value and probably considerably higher in some of the individual stages of the oxidation. Since in none of the work of Cori and collaborators is a P:O ratio as great as 1 found, we shall confine attention to the work of Ochoa. (The work of Belitzer and Tzibakowa (7) is not directly available to us.)

Free Energy of Oxidation of Pyruvate. We shall first calculate the total free energy derivable from the oxidation of pyruvate, in order to find the limit of possible values for the P:O ratio. The heat of combustion of dry glycogen is given as 4238 calories/gram by Kharasch (29) or 686,600 calories/glucoside residue. The entropy of glycogen per glucoside residue may be estimated as 35.4 cals/°C. from the value of 50.7 for glucose less 15.3, the average decrease found for several reactions in which water is eliminated (75). Then for the complete oxidation $(C_6H_{10}O_5) + 6\ O_2 = 6\ CO_2 + 5\ H_2O$, $\Delta S = +62.7$ calories/°C. (using the values for O_2, CO_2 and H_2O given by Parks and Huffman, 61) and $\Delta F = \Delta H - T\Delta S = -686{,}600 - 298 \times 62.7 = -705{,}300$ calories per glucoside residue at 25°C. (O_2 and CO_2 at one atmosphere and liquid H_2O). Now

$$\left\{\frac{\partial\left(\frac{\Delta F}{T}\right)}{\partial T}\right\}_p = \frac{-\Delta H}{T^2}.$$

Therefore, $\Delta F_0(37°) = -704{,}900$ calories/glucoside residue.

The standard free energies of formation of lactate from glycogen and of the oxidation of lactate to pyruvate (at 37°C. and pH 7) are already known (table 1), and the standard free energy of formation of H_2O is obtained from Parks and Huffman (61). By difference, the standard free energy of oxidation of pyruvate is obtained (table 4). This value is for molar pyruvate at 37°C. and pH 7, O_2 and CO_2 at one atmosphere and liquid H_2O.

It is convenient to correct this value to the conditions used in experiments, namely pyruvate 0.025 M and CO_2 at 40 mm. Hg (these being of the order of magnitude of those in the cell). Furthermore, the pressure of O_2 may be less than one atmosphere. Applying these corrections we obtain:

$$\Delta F/\text{atom of oxygen} = -55{,}500 - 705 \log_{10} p(O_2) \text{ calories} \qquad 3$$

where $p(O_2)$ is the partial pressure of the O_2 in atmospheres.

The standard free energy calculated here is for the oxidation of glycogen in the

dry state, but the free energy of solution of glycogen, per glucose residue, is certain to be relatively small and may be neglected. The value of ΔF for the oxidation of pyruvate is also affected by the value assumed for ΔF_0 of *reaction 4a* of table 1; however, at the worst, the value given for ΔF per atom of oxygen would be 700 calories too positive. This difference also is unimportant.

Several interesting conclusions can be drawn at once from these results:

a) The value of the free energy depends on the partial pressure of oxygen at which the oxidation effectively occurs. Now a given pressure of oxygen is related to a potential on the redox scale by the equation for an oxygen electrode

$$E'_h = 0.82 + \frac{2.303RT}{4F} \log p(O_2) = 0.82 + 0.0153 \log p(O_2) \qquad 4$$

where F is the electrochemical equivalent and where E'_h is measured against a hydrogen electrode at pH 7. We can then, by means of *equations 3* and *4*, calculate the free energy available from the oxidation of pyruvate at any value of E'_h. If we take the standard free energy of phosphorylation of creatine as +16,700 calories/mole, and assume that phosphorylation occurs at a [CrP]/[Cr] ratio of unity, we can calculate from these free energies the maximum P:O ratio obtainable from the oxidation of pyruvate at any E'_h, that is, at any effective pressure of oxygen that we choose. Some examples are given in table 5.

Evidently a P:O ratio of about 3 is the greatest possible, even if atmospheric oxygen exerts its full oxidizing power. It seems unlikely, however, that it can do so in the cellular system (see below). The next lower known redox system is cytochrome C, whose E'_0 is +0.26 volt (67); if oxidation occurred effectively at this potential, a P:O ratio of only about 1.8 could be obtained. Both these values assume an efficiency of 1.

b) The standard free energy (37°C., solid glycogen, pyruvate 1 M, pH 7, $p(O_2) = p(CO_2) = 1$ atmos.) available from the aerobic conversion of glycogen to pyruvate is obtained from table 4. Per atom of oxygen it is $\frac{1}{2}(-704{,}900 + 548{,}600) = -78{,}300$ calories. This is far in excess of the minimum needed to form 1.5 or even 2 moles of creatine phosphate at a [CrP]/[Cr] ratio of unity.

c) Calculation of the free energy of formation of glycogen from its elements, from the heat of combustion and entropy of glycogen and from the known free energies of formation of CO_2 and H_2O, gives −142,200 calories per glucose residue. Thence for the reaction glycogen + H_2O = glucose, $\Delta F_0 = -18{,}500$ calories per mole of glucose. (The values of the free energies of formation of water and of glucose were taken from Parks and Huffman, 61). This value may be wrong by several thousand calories, but it makes it clear why glucose must be phosphorylated by ATP, in contrast with glycogen which can react with inorganic phosphate.

P:O Ratio. In view of the conclusions reached in section *a*) above, a close examination of Ochoa's results becomes necessary.

a) *Brain dispersions* (56), to which were added pyruvate, fumarate, phosphate, magnesium, coenzyme I and adenylic acid, with creatine, glucose or hexosemonophosphate as phosphate acceptor and fluoride to hinder the hydrolysis of ATP, showed some phosphorylation of adenylic acid or of creatine; the phosphorylation

was greater with glucose or hexosemonophosphate as acceptors. The P:O ratio fell rapidly with time from initial values near 2. Lipmann (42) has suggested the extrapolation of Ochoa's data to a P:O ratio greater than 2 at zero time, but this seems to be very risky.

b) Heart muscle preparations were studied under similar conditions (57). With these, the P:O ratio did not fall with time, and the same P:O ratio was found whether glucose or creatine was the phosphate acceptor. The values averaged 1.91 with glucose and 1.88 with creatine, with considerable variation about these values in different experiments. Fluoride was again included.

It was shown in separate experiments that fluoride retarded the liberation of phosphate from added ATP and increased the extent of phosphorylation of glucose by added ATP, though considerable liberation of inorganic phosphate still occurred. Ochoa argued that adenosine triphosphatase (ATP-ase) was still partly active, even in the presence of 0.025 M fluoride (at which concentration its maximum effect is obtained), and that this lowers the P:O ratio. He attempted, therefore, to allow

TABLE 4

REACTION	ΔF_3 (37°) *cal/mol. of glucose*
1. Glycogen + $6O_2$ → $6CO_2 + 5H_2O$	−704,940
2. Glycogen + H_2O → 2 HL	−66,400
3. 2 HL → 2 Py + $2H_2$	+23,000
4. $2H_2 + O_2$ → $2H_2O$	−113,120
5. (1 − 2 − 3 − 4) 2 Py + $5O_2$ → $6CO_2 + 4H_2O$	−548,620

TABLE 5

E'h *volt*	−ΔF *cal/atom of oxygen*	MAXIMUM P:O	E'h *volt*	−ΔF *cal/atom of oxygen*	MAXIMUM P:O
+0.82	55,500	3.3	+0.26	29,700	1.8
+0.70	50,100	3.0	−0.02	16,700	1.0
+0.34	33,400	2.0			

for this assumed lowering by measuring the P:O ratio obtained in the same system during the anaerobic reaction hexosediphosphate to phosphoglyceric acid. Now Needham and Pillai (51) and Meyerhof *et al.* (49) had demonstrated in similar preparations, which did not contain ATP-ase, a P:O ratio of about 1 for this reaction; Ochoa obtained P:O ratios of about 0.6. He therefore applied a 40 per cent proportionate correction to the aerobic values, obtaining corrected values for the P:O ratio of about 3.

We do not accept the validity of this corrected value for the following reasons. Ochoa's results for the liberation of phosphate from ATP added to the preparation, in the absence of fluoride and in the presence of 0.025 M fluoride, are plotted in figure 1. Although he does not quote an analysis of his sample of ATP, it is clear from his data that the content of labile phosphate is near to the value required and that all the labile phosphate is removed by enzymes in the preparation. Hydrolysis in the presence of fluoride follows a first-order course closely (full line in fig. 1). Hydrolysis in the absence of fluoride also follows approximately a first-order course

with the same velocity constant as in the presence of fluoride (full line in fig. 1), but a closer inspection of the data shows that the rate of liberation of phosphate is nearly the same in the two cases after five minutes and that the uninhibited reaction is therefore a compound one. The rapid component of this compound reaction (complete within five minutes) is inhibited by fluoride, while the slower component is unaffected. Moreover, the end point of the reaction in the presence of fluoride is at 60 per cent of that of the uninhibited process.

These facts exclude both the possibility that fluoride produces a *partial* inhibition of adenosine triphosphatase, since this could not give a first-order process going to a 60 per cent end point, and also the possibility that fluoride inhibits the breakdown of adenosine diphosphate, since this could explain neither the inhibition of the faster component reaction nor the 60 per cent end point. On the other hand, ATP-ase

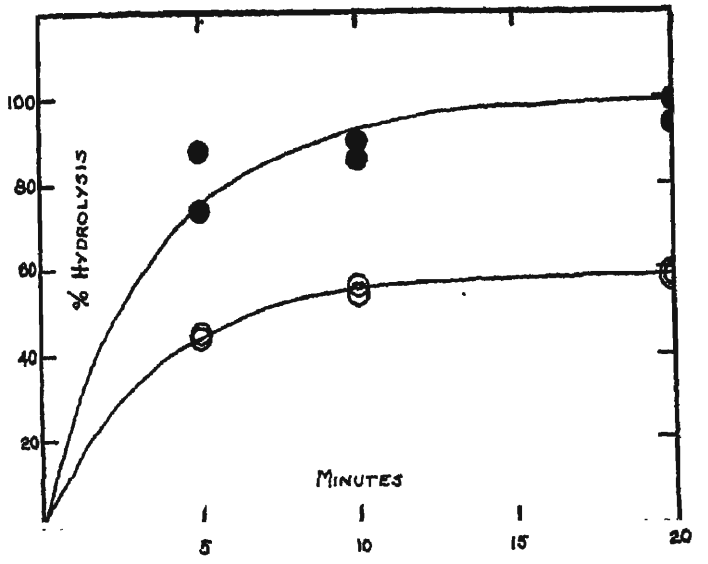


Fig. 1. FILLED CIRCLES: without NaF; open circles: 0.025 M NaF

must be inhibited by fluoride because fluoride is shown to bring about an absolute increase in the amount of phosphate transferred from added ATP to glucose and a great increase in the aerobic phosphorylation of glucose; inhibition of the breakdown of ADP would not account for either of these facts since ADP does not phosphorylate glucose (14). We are forced to conclude, therefore, that 0.025 M fluoride produces a complete inhibition of adenosine triphosphatase and that the phosphate which is liberated from added 'ATP' in the presence of fluoride is not derived from ATP.

This implies that Ochoa's sample of ATP, though it contained the theoretical amount of labile phosphate, consisted of only 40 per cent of true ATP, the residual 60 per cent being another material of identical composition. Although this conclusion is a surprising one, it is supported by Bailey's (1) finding that solid barium-ATP may break down to a considerable extent on long storage, mostly into adenylic

acid and pyrophosphate; while Lohmann (43) showed that frog muscle containing a pyrophosphatase is not inhibited by fluoride at 38°. The actions of ATP-ase on ATP (40 per cent inhibited by fluoride) and of pyrophosphatase on pyrophosphate (60 per cent, not inhibited by fluoride) would account for Ochoa's results.

This interpretation of Ochoa's results also explains the fact that the same P:O ratio was obtained for the phosphorylation of both glucose and creatine. It would be difficult to understand this finding if adenosine triphosphatase were still residually active in the presence of 0.025 M fluoride, since the great difference of the free energies of phosphorylation of the two acceptors would demand very different ratios of [ATP]/[ADP] to effect their phosphorylation; one would have expected appreciably lower values of P:O for creatine than for glucose. The single experimental objection to our interpretation is Ochoa's value of only 0.6 for the P:O ratio of the anaerobic reaction in the presence of fluoride. We are unable to explain this.

We consider, in spite of this, that the balance of evidence favors the conclusion that 0.025 M fluoride completely inhibits ATP-ase, and we conclude that Ochoa's observed P:O ratios for the aerobic reaction do not require correction but measure directly the uncomplicated oxidative transfer of phosphate.

c) Ochoa (58) observed the phosphorylation which accompanies the oxidation of α-ketoglutarate in the heart muscle preparation in the presence of malonate and fluoride. He obtained similar P:O ratios with α-ketoglutarate and pyruvate in parallel experiments, though all the values were rather lower than in the previous work. There is no reason why the conclusions given above should not apply to this work also, and the P:O ratios should again be taken at their face values.

These results, as we interpret them, suggest that, per .5 mole of glucose oxidized, the number of moles of phosphate combined is about 11 ($1.5 + 5 \times 1.9$), assuming a contribution of 1.5 moles of phosphate from the aerobic stage glycogen $\rightarrow$ pyruvate. Assuming the same yield from the anaerobic reaction glycogen $\rightarrow$ lactate, this corresponds to a value for the Meyerhof Quotient of 7.3. This value is in the upper range of direct estimates of this quotient, whereas any considerably higher value for the P:O ratio in the oxidation of pyruvate would give a value outside the range.

Mechanisms for Phosphorylation. The essential nature of any reaction which leads to phosphorylation has been indicated. The intermediate with which the phosphate first combines must be capable of undergoing a reaction whose change of free energy is negative and which leads to a phosphate compound of lower stability. Such a reaction could be a dehydrogenation, a dehydration, a hydration or a decarboxylation. Some light may be thrown on oxidative phosphorylation by an examination of the supposed stages of oxidation of pyruvate from this viewpoint; at the same time we shall examine the thermodynamic data. The data at present available are given in table 6 (columns as in table 1).

The whole series of reactions involves 5 dehydrogenation, 3 decarboxylation and 3 hydration or dehydration stages. If it is supposed that each of these could be mechanistically concerned in the transfer of 1 phosphate, the total is 11; if the hydration and dehydration stages are excluded (no experiment suggests otherwise), the total is 8. For comparison with this, if we assume oxidation at +0.26 volt, the total free energy change during the oxidation of molar pyruvate would allow for

phosphorylation of 8.8 moles of creatine per mole of pyruvate, at a [Cr]/[CrP] ratio of unity. It is interesting to note that the data available on individual stages show that the sum of the free energies of reactions 7, 8, 9 and 11 is −32,600 calories and of reactions 3, 4, 5 and 10 is −35,600 calories, each being approximately sufficient for the synthesis of 2 moles of CrP. Experimentally it appears that the P:O ratios for *reactions 7* and for *9* plus *11* are not greater than 1 (12, 13, 25), and Ochoa (57) has

TABLE 6

	2	3	4	5	6	
					ΔF_3 = cal/mol. CO_2 = 40 mm.; other substances molar	
		REF. NO.	K	E'₀	*a.* O_2 = 1 atmos. E_h' = +0.82 volt	*b.* E_h' = +0.26 volt
1	Pyruvate + oxaloacetate → condensation product + H_2O		—	—		
2	Condensation product + O → cis-aconitate + CO_2		—	—		
3	Cis-aconitate + H_2O → iso-citrate	24	4		−850	−850
4	Iso-citrate + CoII → oxalosuccinate + H_2CoII (pH7: 22°C)	59, 60	3.3		−730	−730
5	oxalosuccinate → α-ketoglutarate + CO_2 (pH7: 22°C)	59, 60	2.3×10^3 (CO_2 molar)		−8970	−8970
6	α-ketoglutarate + O → succinate + CO_2 + H_2O		—	—		
7	Succinate + O → fumarate + H_2O	37, 9		0	−37,900	−12,000
8	Fumarate + H_2O → malate	33	3.2		−720	−720
9	Malate + CoI → oxaloacetate + H_2CoII	35		−0.17	+5,080	+5,080
10	H_2CoII + O → CoII + H_2O	[1]		−0.28	−50,800	−25,000
11	H_2CoI + O → CoI + H_2O	8		−0.28	−50,800	−25,000
	Accounted for				−145,690	−68,190
	Balance (reactions 1, 2, 6)				−131,810	−79,910
	Total				−277,500	−148,100

[1] Value assumed.

shown that the oxidation of H_2CoI (*reaction 11*), added to a heart muscle preparation, does not cause phosphorylation.

Now, if the phosphate transferred is more than 8 moles per mole of pyruvate (a P:O ratio greater than 1.6), then, excluding the hydration and dehydration reactions, one or more single stages must lead to the transfer of more than one phosphate each. This could happen in two ways: the substrate molecule could combine with two or more molecules of phosphate, all of which are made less stable by a subsequent reaction (though no experimental evidence supports the existence

of such compounds) or (as suggested by Lipmann, 41) an intermediate carrier of hydrogen might be involved, itself undergoing an oxidation-reduction and phosphorylation-dephosphorylation cycle. Such a carrier mechanism raises certain specific problems which will be discussed in the next section. A more general kinetic problem, however, is raised by both mechanisms and this will be discussed in section 5.

IV. CARRIERS

General. Carriers have hitherto been considered almost exclusively with respect to their function in the transport of hydrogen. A carrier is a substance which can accept hydrogen from a reducing system and pass it on to an oxidizing system; it may form an essential or a permissive link in the process of hydrogen transport. Generally, the carrier will have a different stationary ratio of oxidized to reduced forms at these two sites, maintained by the oxidative and reductive processes at them, and by diffusion between them. This difference of ratios corresponds to a difference of E'_h and could be the means by which a part of the energy of the oxidative process is made available. The maximum energy obtainable from such a process is $\Delta F = -2F\Delta E'_h = -RT\Delta(\ln[Ox]/[Red])$ calories per mole of H_2 transferred, where $\Delta E'_h$ is the difference between the values of E'_h at the two sites. (For a value of ΔF of $-16{,}700$ calories, $\Delta(\log_{10}(Ox)/(Red))$ is about 12.)

Transfer of phosphate could result from reactions of a carrier molecule C by the following cycle of processes:

$CH_2 + P \rightarrow CH_2P$	'low energy phosphate'
$CH_2P - 2H \rightarrow CP$	'high energy phosphate'
$CP + A \rightarrow C + AP$	phosphorylation of acceptor
$C + 2H \rightarrow CH_2$	reduction of unphosphorylated carrier

This differs from the general mechanism of phosphorylation by a substrate in being cyclic. Thus, while it is the unphosphorylated C which is hydrogenated, it is the phosphorylated CH_2P which is dehydrogenated, implying a difference in the enzymatic processes of reduction and oxidation of the carrier. It must be assumed either that there are two different enzymes or, equally possible, that there is a single enzyme having a double specificity. Furthermore, since there is no net change in the amounts of oxidized and reduced carrier, the energy is not derived from oxidation of the carrier, but comes, indirectly, from the oxidation of the ultimate reductant (substrate) by the ultimate oxidant (effectively oxygen, cytochrome, flavoprotein etc., according to the conditions).

In principle, any carrier system could perform a transfer of phosphate when working over a sufficient interval of E'_h. Such a system need not involve an intermediate metabolite nor need the oxido-reduction involve the transfer of hydrogen. Thus coenzymes I and II flavoproteins or cytochromes could act as phosphorylating carriers, if a mechanism existed for the transfer of phosphate.

A necessary but not sufficient proof that a given substance can act as a phosphorylating carrier is that its oxidation can be shown to lead to phosphorylation. Such evidence is entirely lacking except for the carrier system postulated by Szt.

Györgyi (73), consisting of the succinate-fumarate or malate-oxaloacetate pairs; phosphorylation has been shown to accompany the oxidation, as substrates, of both malate (25) and succinate (12, 13), and it is therefore possible that it might accompany their oxidation as carriers.

Succinate and Malate as Carriers. It may be said at the outset that the evidence for the participation of succinate and malate in hydrogen transport is far from conclusive, but there is perhaps too much favorable evidence for the Szt. Györgyi scheme to be completely neglected. There is no experimental evidence that the dicarboxylic acids lead to phosphorylation when acting as carriers, but no one appears to have looked carefully for this or for any effect which their presence might have on the P:O ratio. (The system in which this should be most clearly detectable, if it occurs, is that in which Szt. Györgyi first claimed to have shown their activity in the transport of hydrogen, namely in the oxidation of glycogen, glucose or an intermediate to pyruvate.)

We shall therefore reexamine briefly the evidence that succinate or malate can be active in hydrogen transport. Szt. Györgyi's original claim was based on the activity of the enzymes malic dehydrogenase, fumarase and succinic dehydrogenase, the ability of the last to react with cytochrome C and the now well-known effects of the dicarboxylic acids in increasing and stabilizing the rate of oxidation of glucose by tissues which have been ground, dialyzed or otherwise damaged. These facts are not in doubt and have been widely confirmed by later workers. The probability that the succinate-fumarate reaction is significant in the transfer of hydrogen from substrates which reduce CoI was lessened by the failure to demonstrate a rapid reaction of fumarate with H_2CoI (16) and by the independence of succinic dehydrogenase of CoI. Furthermore, Potter (63) demonstrated that a reconstructed enzyme system could oxidize triose phosphate rapidly in the presence of enough malonate to prevent completely the oxidation of added succinate. Straub (68) has also shown that washed muscle does not need succinate to dehydrogenate malate. It seems to be agreed that succinate is not a carrier; but, as we have seen, a difference of enzymatic specificity is needed anyway if a hydrogen carrier is also to phosphorylate, and it is not necessary to assume that a carrier forms an essential link. The question of the participation of succinate in hydrogen and phosphate transport should not therefore be regarded as quite closed.

Other work bears on the activity of the malate-oxaloacetate system. (Although it is often fumarate that is added, the presence of fumarase makes this equivalent to malate.) Several workers have shown that, on the addition of these acids, true catalytic effects occur, that is, an increase of oxygen uptake which cannot be accounted for by the oxidation of the added dicarboxylic acid as a substrate. Thus Stare (65) and Stare and Baumann (66) showed clearly the catalytic effect of small quantities of fumarate in muscle preparations, this increasing with the damage to the tissue. Citrate had much smaller effects. Colowick *et al.* (13) showed that succinate is catalytic in the oxidation of glucose and pyruvate by homogenized kidney; Banga *et al.* (3) showed that citrate and α-ketoglutarate are much less active than are the dicarboxylic acids in dispersions of brain. The interpretation of these catalytic effects is made doubtful, however, by the existence of an alternative

explanation, namely, that oxaloacetate acts by condensing with pyruvate or a product of pyruvate in the 'modified citric acid cycle' (31). None of the experiments quoted can distinguish between these two explanations. In either case, it is oxaloacetate which is believed to cause the catalytic increase in respiration, either by acting as a hydrogen carrier or by condensing with 'pyruvate,' and other intermediates are catalytic only insofar as they give rise to oxaloacetate. Krebs' (31) demonstration that various members of the cycle are equivalent as substrates (at much above the concentrations needed for a catalytic effect) does not affect this conclusion, because of the possibility of saturation of an enzyme mediating a later stage.

Another line of evidence is the demonstration that oxaloacetate can accept hydrogen from various substrates, although this evidence is permissive rather than cogent. Szt. Gyorgyi (72) and Parnas and Szankowski (62) showed that during 'carbohydrate' oxidation the reduction of oxaloacetate to malate occurs. (Negelein and Brömel, 53, 54, have shown that this oxidative stage involves phosphoglyceraldehyde.) Breusch (10) demonstrated the anaerobic reduction of oxaloacetate, and Krebs *et al.* (30–34) showed that oxaloacetate is reduced to malate by hydrogen from pyruvate plus oxaloacetate and by citrate. However, the demonstration of the anaerobic reduction of oxaloacetate does not prove that this is of importance under aerobic conditions and again another explanation of the aerobic formation of malate from oxaloacetate is possible. Thus, the 'modified citric acid cycle' allows malate to be generated aerobically through an oxidative process, so that the demonstration of its formation does not prove that it was formed reductively from oxaloacetate. (This possibility does not exist in the anaerobic experiments.) We conclude that, on the experimental evidence, the case for succinate or malate as hydrogen carriers is neither proved nor disproved.

One general objection to the idea of the malate-oxaloacetate system will be discussed further. This has been forcibly stated by Ball (1), that since, as far as is known, oxaloacetate must receive hydrogen from and malate give up hydrogen to CoI, "we are right where we started" and the scheme seems "pointless".

Diffusion in Hydrogen Transport. Unless all the enzymes concerned with the oxidation of a substrate are arranged in the cell with their active groups closely adjacent, diffusion through space of carrier molecules must occur. What evidence there is suggests that they are not so arranged. The proteins concerned (dehydrogenases, flavoproteins, cytochromes) could diffuse relatively slowly, if at all, and transport from one to another must be performed by smaller molecules. We may suppose that the coenzymes perform such a function. The rate of oxidation might then depend on the rate of their diffusion as well as upon the rates of their reactions with substrate (at the surface of a dehydrogenase molecule) and with flavoprotein. In order to investigate this possibility theoretically, we choose the case where the molecules of protein occur randomly in the solution. A molecule of coenzyme I or II is considered to react immediately on collision with an enzyme molecule so that the rate of diffusion determines the rate of hydrogen transport.

Let the *number of molecules* per ml. of dehydrogenase be n_1 and of flavoprotein n_2; let the *concentrations* of unbound Co be c_1 and of unbound H_2Co be c_2 moles per liter; let the effective collision areas of Co with dehydrogenase and of H_2Co with

flavoprotein be A_1 and A_2 cm.^{2}, respectively, and the diffusion constant of Co and H_2Co be D cm.^{2} sec.$^{-1}$

Consider the diffusion of a molecule of H_2Co, which has just been hydrogenated. Irrespective of the direction of its diffusion, the probability that its first effective collision with a molecule of flavoprotein will occur at a distance between x and $x + dx$ from its position at zero time is $n_2A_2 \cdot e^{-n_2A_2x}\,dx$. According to Einstein (17) the time taken for a particle to diffuse a distance x is $t = x^2/2D$. The *mean time* taken by all molecules of H_2Co, from the moment of hydrogenation to that of the next dehydrogenation, is therefore:

$$\bar{t}_2 = \frac{1}{2D}\int_0^\infty n_2A_2 \cdot e^{-n_2A_2x} \cdot x^2 \cdot dx \Big/ \int_0^\infty n_2A_2 \cdot e^{-n_2A_2x} \cdot dx = 1/Dn_2^2A_2^2$$

Similarly the mean time for the reverse process is $\bar{t}_1 = 1/Dn_1^2A_1^2$; for a steady state, $c_1\bar{t}_2 = c_2\bar{t}_1$; and the rate of hydrogen transport is given by $c_1/\bar{t}_1 = c_2/\bar{t}_2$ moles/liter/second.

This result is of no interest unless it can be given a quantitative value, and in order to do this, we shall have to guess fairly boldly. Schlenk (64) indicates that the total concentration of all dehydrogenases is 1 to 2 grams per 100 ml. in muscle, and, if an average molecular weight of 35,000 is assumed, this makes n_1 about $N \times 4 \times 10^{-7}$ molecules/ml. (where N is the Avogadro number 6.06×10^{23}). Ball (1) gives a value for the concentration of flavoproteins of 8×10^{-5} molar, whence n_2 is about $N \times 8 \times 10^{-8}$ molecules/ml. He also gives 25×10^{-6} molar for the total concentration of CoI.

However, a fraction of the Co is bound by dehydrogenases and by flavoproteins; the values of K_m for the combination of CoI with dehydrogenases seem to lie between 10^{-4} and 10^{-5} molar (e.g., Negelein and Wulff, 55; Green, 19; Green and Brosteaux, 20; Needham *et al.*, 52), and Haas (21) gives 10^{-5} for the K_m of combination of H_2CoII with cytochrome reductase. Thus, taking a mean value for all K_ms of 3×10^{-5}, the concentration of *free* coenzymes ($c_1 + c_2$) is about 3×10^{-5} molar.

The radius of a protein molecule is about 2×10^{-7} cm. so that its spherical area is 5×10^{-13} cm.^{2} However, the *effective* collision area is likely to be much less than this, since only a part of the enzyme surface will be active and since the Co and enzyme probably must collide within a certain range of relative orientation for reaction to occur. We assume, therefore, an effective collision area 50 times less, that is of 10^{-14} cm.^{2} (= 100 Å^2). D is likely to be about 5×10^{-6} cm.^{2} sec.$^{-1}$

Using these values

$$\bar{t}_1 = 0.034 \text{ seconds and } \bar{t}_2 = 0.85 \text{ seconds}$$
$$c_1 = 0.11 \times 10^{-5} \text{ molar and } c_2 = 2.9 \times 10^{-5} \text{ molar.}$$

Thus the rate of transport of hydrogen by diffusion becomes 3.3×10^{-5} moles/liter/second. This value may be compared with observed values of the $Q(O_2)$ of whole muscle, a reasonable value for which is -10. Such a $Q(O_2)$ corresponds to about 10^{-4} moles/liter/second for the whole volume of the muscle.

This result indicates that the diffusion of coenzyme could be a limiting factor in the transport of hydrogen and thence in the total process of oxidation. It would

be easy, by modifying the quantitative assumptions, to obtain much higher or much lower predicted values for the rate of the diffusion process, but we regard the assumptions which we have made as reasonable. Moreover, arrangements in space of the proteins other than random would also make a great difference to the demands on diffusion. If the dehydrogenases were closely grouped with the flavoproteins the demand would be less, but if the flavoproteins and dehydrogenases were separately grouped, at a distance from each other, the importance of diffusion would be still greater.

One can now see what might be the importance of the dicarboxylic acids in hydrogen transport; they could relieve the diffusion 'bottleneck' by acting as more rapid carriers between the two enzyme systems—oxaloacetate receiving hydrogen from H_2CoI at one site and the malate, after diffusion, passing hydrogen to CoI and thence to flavoprotein at another site. This facilitating effect could be considerable because of the relatively high concentration of unbound dicarboxylic acids, though their presence would not be essential to the transport of hydrogen. At the same time, energy could become available for phosphorylation, provided that the conditions discussed earlier were fulfilled.

It is to be noted that this scheme implies the close spatial apposition of the enzyme which dehydrogenates malate or phosphomalate (whichever is concerned) to the flavoprotein and of that which hydrogenates oxaloacetate to the substrate dehydrogenases.

V. KINETIC PROBLEMS

We have seen that the amount of energy available from the oxidative process is dependent on the E'_h at which oxidation of the substrate effectively takes place. There is no evidence that phosphorylation accompanies the oxidation of any substances other than intermediary metabolites, and Ochoa (57) found no phosphorylation during the oxidation of H_2CoI. If we assume, in accordance with this evidence, that all oxidative phosphorylation is associated with the oxidation of metabolites by CoI or CoII, then the E'_h at which the coenzymes oxidize the substrate determines the energy available and therefore limits the amount of phosphorylation. Table 5 shows that E'_h must be at least +0.26 volt to give a P:O ratio of 1.8 in the oxidation of pyruvate, whether a carrier is involved or not.

Since the E'_0 of uncombined CoI is −0.28 volt (8), at E'_h+0.26 volt the ratio of uncombined $[H_2Co]/[Co]$ is 10^{-18}. The total concentration of uncombined Co is of the order of 10^{-4} molar, so that the concentration of uncombined H_2Co is 10^{-22} molar. (This conclusion holds whether it is considered that dehydrogenation of the substrate takes place directly or that the malate-oxaloacetate system intervenes.) At such a concentration the rate of reaction would be effectively zero since *a*) the rate of collision between H_2Co and flavoprotein is effectively zero; and *b*) the rate of reaction of the H_2Co-flavoprotein complex, in equilibrium with 10^{-22} molar uncombined H_2Co, would be effectively zero. (Assuming that Hogness' (23) values for K_m and K_r, for the complex between H_2CoII and cytochrome reductase, apply here, the concentration of this complex would be 10^{-21} molar and its rate of reaction 3×10^{-20} moles/liter/second.)

Clearly someting is wrong. In order to account for the observed phosphorylation and an adequate rate of oxidation, we must assume one or both of the following:

1 *a*) that the rate of collision between *uncombined* H_2Co and flavoprotein does not limit the rate, because hydrogen is transferred from the substrate or a carrier, directly to Co which is already in combination with flavoprotein. This necessarily implies that the dehydrogenase of the substrate or carrier forms a part of a complex with flavoprotein and *b*) that there is a very much larger concentration of the H_2Co-flavoprotein complex in equilibrium with 10^{-22} molar uncombined H_2Co than Hogness' figures would suggest, this being necessary in order to give an adequate rate of reaction of the complex. This would mean that K_m must have a very low value, of the order of 10^{-20}, compared with the value 10^{-5} found by Hogness.

2) that the initial assumption is wrong, though it was suggested by the experimental data, and that further phosphorylation can accompany the oxidation of some carrier system or systems of E'_0 higher than that of the coenzymes, such as succinate, flavoprotein or cytochrome. No mechanism of phosphate transfer by the last two has been proposed.

Even with *assumption 2*), it is necessary that the E'_h at which coenzyme is oxidized should be, at the lowest, −0.02 volt if phosphorylation of creatine is to accompany the oxidation of pyruvate intermediates by coenzyme. This would imply a concentration of about 10^{-13} molar for uncombined H_2Co, which is still very low. Furthermore, there is some difficulty in understanding the kinetics of any carrier system which is to produce 16,700 calories per mole of hydrogen transferred since it must work over an interval of E'_h of at least 0.36 volt. Optimally, its E'_0 would lie in the middle of this interval, when its upper and lower values of E'_h would then correspond to ratios of oxidized to reduced forms of 10^6 and 10^{-6}.

More experimental evidence bearing on these problems is needed for further discussion of them to be profitable.

VI. CONCLUSION

We have surveyed the evidence which bears on the relationship between oxidation and phosphorylation mainly from the viewpoints of thermodynamics and kinetics. The result has been to set limits and to emphasize the existence of problems rather than to make a positive contribution towards the understanding of these processes, but we hope that our criticism may be a help to future work. In particular:

1) We doubt whether the present evidence justifies the assumption of a P:O ratio greater than 2 in the oxidation of pyruvate.

2) According to our present knowledge it is difficult to understand on kinetic grounds how a P:O ratio even as low as 2 can be achieved at an appreciable rate of reaction. Light should be thrown on this problem as information accumulates about the spatial distribution of enzyme systems in cells and about the possibility of phosphorylation accompanying any oxidative processes other than those of substrates by pyridine nucleotide coenzymes.

3) It has been shown that catalytic effects of intermediates on oxidation might be due to their assisting in the diffusional transport of hydrogen. In this connection, the question of the spatial distribution of enzymes is again of importance. Such an

action of intermediates could be combined with that of phosphate transport. Information on these two aspects might be best obtained from the study of some simple oxidative process, such as that of phosphoglyceraldehyde to phosphoglyceric acid.

In general, we have tried to indicate how chemical, thermodynamic and kinetic types of evidence may supplement each other in leading to the understanding of cellular processes.

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ALLOXAN DIABETES

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EXPERIMENTAL DIABETES was first produced by total or partial pancreatectomy. The second method of inducing diabetes was by means of anterior pituitary extract. The third form of experimental diabetes was produced in 1943 by the administration of alloxan after the discovery by Dunn, Sheehan and McLetchie (59) that this substance caused necrosis of the pancreatic islets. These persistent or permanent forms of experimental diabetes are to be distinguished from the various temporary glycosurias or hyperglycemias. The literature on alloxan diabetes will be reviewed under the following headings: I. History and general description of alloxan diabetes; II. The mechanism of action of alloxan on susceptible cells, especially those of the islands.

HISTORY, NATURE AND COURSE OF ALLOXAN DIABETES

Early Bio-Chemistry of Alloxan. Alloxan was first made by Woehler (172) in 1838 by the oxidation of uric acid. Since then there have been only a few references to its biological significance. In 1862 Liebig (133) found alloxan in the intestinal mucus of a patient and in 1866 Lang (124) reported a patient who excreted alloxan or a precursor thereof in the urine. The tests were probably valid by modern appraisal (3). Early studies (4, 149) on the reversible reaction, uric acid $\rightleftarrows$ dialuric acid, indicate that alloxan may be present in traces in blood (3). The alloxan-dialuric acid oxidation-reduction system has been described (150, 171). The reactions of alloxan with sulfur compounds (132) and its action as a capillary poison have been studied (122). Its excretion was investigated by Cerecedo (34) and little has been added by later investigators. Cerecedo (34) cites older references indicating that alloxan is to a large extent destroyed in the body and describing the red color of the urine after its ingestion in dogs and mice. Although a fraction of the alloxan may be converted into alloxantin and excreted in the urine as murexide most of it was thought to be eliminated in the bile, perhaps as ethereal sulfate. There was a slight increase in the total nitrogen output but the urea figures showed that alloxan was not broken down to urea. In every case there was a drop in the inorganic sulfates on the day after its administration which was by ingestion with food. The fall in inorganic sulfates was not compensated by a rise in any other sulfur fraction of the urine, hence the assumption that it was possibly excreted by the bile. He also concluded that alloxan was not a physiological breakdown product of uric acid. Dilute concentrations of alloxan increased the oxygen uptake of liver suspensions and greatly accelerated their oxidation of ethyl alcohol (18). Alloxan inhibited the conversion of the Cori ester to the Robison ester (129). The acute hypoglycemic action of alloxan was first described by Jacobs (104).

Determination of Alloxan. Since the discovery of alloxan diabetes numerous methods for the quantitative determination of alloxan have been developed. The first to appear was that of Leech and Bailey (128) which, though of limited specificity, demonstrated the instability of alloxan in blood, a finding which has been fully confirmed (3, 22, 84, 115). In his article describing six methods for the determination of alloxan, Archibald (3) gives a detailed review of the chemistry of alloxan and of the problems of specificity, accuracy and choice of these methods. The fluorometric methods (3, 115) which are the most sensitive have shown that if alloxan is present in normal blood there is less than 10–20 gamma per 100 cc. These results appear to disagree with the reports (152, 167) that, by qualitative tests, alloxan or its reduction products may be a normal constituent of certain tissues. A microbiological and another fluorometric method (15) as well as another colorimetric method applied to blood and tissues (22) provide the investigator with a large choice of procedures. In addition to other chemical considerations the extent to which related compounds may be converted *in vivo* to alloxan or to its reactive derivatives must be taken into account in the interpretation of results obtained by these procedures.

Production and Course of Alloxan Diabetes. This has been previously reviewed (51, 73, 77, 112, 113). The background of the original observation in rabbits is cited in the authors' words (59).

"Experiments were being conducted recently by one of us (J. S. D.) with the object of elucidating the pathogenesis of the renal lesion of the crush syndrome and of the somewhat similar condition which has been observed to follow the accident of a mismatched blood transfusion. In the course of these experiments trial was made by intravenous injection of a number of substances which might influence particularly the lower segments of the renal nephrons. Among other lines investigated in this way uric acid and related products were exhibited, as it had been found by Dunn and Polson (1926) that uric acid, suitably applied experimentally, could produce a selective lesion in the renal second convoluted tubules and collecting tubules. Among the substances in this series alloxan was found initially to give very promising results which may be recorded at another time. A difficulty which arose, however, in the successful establishment of a tubular nephritis by use of alloxan was that with the order of dosage required for this purpose many animals died during the first day or so with distinctive symptoms which were not referable to renal disease. It was in the investigations of these early deaths that a lesion was discovered in the islets of Langerhans."

These authors found the initial rise and subsequent fall in blood sugar, and they proposed the search for animals that would survive with diabetes. This was soon achieved by them (58) and by others (5, 74).

Species. Following the original announcement that alloxan caused necrosis of the islets, permanent diabetes was soon produced in rabbits (5, 39, 52, 54, 117, 118), rats (53, 58, 80, 86, 102, 116, 126) and dogs (33, 74, 93, 111, 159, 166). In the successful experiments, diabetes was apparent in 24 hours and was permanent. In certain species with few beta cells, such as the sheep, pigeon and frog, it has been difficult to define islet lesions. Although minor lesions of the islands are reported in the toad, diabetes does not occur, in contrast to the response of this species to pancreatectomy. Before discussing the general range of dosage given in table 1, certain general comments may be made. The use of alloxan has enlarged our knowledge of diabetes in different species because alloxan diabetes can be produced in those animals with a diffuse pancreas. The ease with which the rat, in particular, is made available

for the study of diabetes is a boon to investigators. In general the use of alloxan has confirmed previous knowledge of the response of different animals to diabetes. Thus, the duck does not have the sustained hyperglycemia after pancreatectomy or after alloxan. Any type of diabetes in ruminants is relatively mild; in carnivora, it is usually severe. Like the toad, the carnivorous birds (owls) do not develop diabetes after alloxan, presumably because the beta cells are resistant to the drug. When a new species is used three variables must be kept in mind: species difference in response to the lack of insulin; the variable susceptibility of different animals to alloxan; and the variable severity of the diabetes produced by alloxan in susceptible animals. The uratic deposits in birds will be discussed under complications.

TABLE 1. STUDIES ON ALLOXAN IN VARIOUS SPECIES

SPECIES	LESIONS OF ISLETS	HYPERGLYCEMIA (DIABETES)	DOSAGE	URATIC DEPOSITS	REFERENCES AND NOTES
			mgm/kgm.		
Rabbit	+	+	25–300		see text
Rat	+	+	100–200		see text
Hamster	+	+	40–200		(87)
Dog	+	+	50–200		see text
Cat	+	+	150		(148, 154)
Sheep	+	+	90		(60, 109, 110)
Monkey	+	+	300		(12)
Man	+	+	100–600		see later section
Frog	+	o	50–1000		(158)
Toad	o	o	200		(21, 96)
Turtle	–	+	400		(69)
Pigeon	+	+	75–200	+	(78, 157)
Duck	+	o	50–750	o	(143, 157)
	o	o	200–300	o	(157)
Chicken	o	o	75–400	+	(143, 157)
Barn owls	o	o	50–200	+	(157)
Horned owls	o	o	100–250	o	(157)

+ = present; o = absent; – = no data.

Dosage, Toxicity and Methods of Administration. Table 1 surveys the range of dosage used. The susceptibility to both toxic and diabetogenic doses varies widely not only in different species but also among animals of the same species. In the common laboratory animals (rat, rabbit, dog) the picture described by Goldner and Gomori (74) has been confirmed. They defined the response of the dog to single intravenous doses of alloxan as follows:

125–200 mgm/kgm.: Sudden death with convulsions.
75–100 mgm/kgm.: Diabetic-uremic syndrome.
50–75 mgm/kgm.: Diabetes
25 mgm/kgm.: No observed effect.

Thus for each species the lethal, the nephropathic and the diabetogenic doses must be considered.

The symptoms and findings after toxic doses of alloxan were first described by Jacobs (104) and by Dunn *et al.* (57–59). The latter noted early death from hypo-

glycemia or from uremia of varying severity at one- to five-day intervals. In the light of subsequent work the following events may occur after a single intravenous dose. *a*) Death may occur in a few hours in convulsions without hypoglycemia or nitrogen retention and without anatomical lesions. This is unexplained in the dog (33, 74) and has been attributed to toxic action on the nervous system in toads (21, 96), although no lesions of the nervous system have been reported. *b*) Acute pulmonary edema has been seen in dogs (93) and cats (148). The resultant anoxia might be a cause of convulsions. *c*) Death may occur at 6–18 hours from hypoglycemia. *d*) Death often occurs in one to five days from uremia with oliguria, albuminuria, anuria or hematuria, usually associated with diabetes (45, 57, 58, 59, 74, 82). Table 2 gives the data on the minimum lethal dose. The toxicity is greatest after intravenous and least after subcutaneous administration. The coincidental agreement between the LD_{50} (95) and ED_{50}, the minimum effective or diabetogenic dose (25), does not alter the practical ease of preparing diabetic rats. The subcu-

TABLE 2. MINIMUM LETHAL DOSE (LD_{50}) OF ALLOXAN

ANIMAL	ROUTE OF ADMINISTRATION	LD_{50}	REFERENCES	NOTES
		mgm/kgm.		
Rat	intravenous	49	(95)	
	intravenous	45[a]	(25)	(a) ED_{50}—see text
	intraperitoneal	152	(95)	
	intraperitoneal	360–140	(72)	5 to 200 grams body weight
	subcutaneous	175[b]	(116)	(b) 90% diabetogenic; see text
Hamster	intravenous	65.4 ± 9.8[c]	(87)	(c) S.E.
Pigeon	intravenous	118.8 ± 11.8	(157)	
Rooster	intravenous	199.8 ± 19.4	(157)	

taneous dose of 175 mgm. per kgm. (116) is not a calculated LD_{50} but is included because of its usefulness.

The production of diabetes without nephritis or other demonstrable injury is dependent on a practical difference between the nephrotoxic and diabetogenic doses. In rabbits and dogs this difference is such that a fair proportion of animals will have uncomplicated diabetes if a suitable dose is used. The margin is narrower in rats, for diabetes could not be obtained without an elevation of blood non-protein nitrogen (16). However, as the islet injury is usually permanent and the renal damage is usually reversible, an essentially uncomplicated diabetes may be secured. A few examples should be cited of the irregularity with which diabetes is produced. "Of 43 dogs injected only 7 developed a high degree of glycosuria without serious complications" (166). In 30 dogs given 75 mgm. per kgm. by Houssay *et al.* (93) only 8 survived more than a week with diabetes; with the dose of 50 mgm. per kgm., 20 of 70 dogs were permanently diabetic. Data on other doses are given (93). In rabbits 10 out of 13 (54), 12 out of 27 (118), and 53 out of 56 (52) were made diabetic, all on

the dose of 200 mgm. per kgm intravenously. The response of rabbits to various doses has been described (86). Subsequent knowledge of the influence of diet and other factors accounts in part for the variations of different workers (see below).

Alloxan is given in freshly prepared solutions of 1 to 5 per cent concentration in water or saline. From what has been said intravenous administration is the route of choice in most animals. The subcutaneous or intraperitoneal route is quite satisfactory in rats although in rabbits doses up to 700 mgm. per kgm. intraperitoneally were entirely ineffective (118). Diabetes has been produced in rats (153) and rabbits (155) by the injection of alloxan into the alimentary canal and in cats (154) if alloxan was rapidly ingested with food by fasted animals. However, oral administration is usually unsatisfactory.

The repetition of small doses of alloxan which are ineffective singly may lead to diabetes. In rabbits, doses of 40 and 50 mgm. per kgm. daily for 4 to 13 days have produced permanent diabetes (6, 118). Nephritis is largely avoided by this procedure. More accurate studies in the rat have shown a progressive diminution in glucose tolerance on as little as 25 mgm. per kgm. per day intravenously for 4 weeks (161). Dogs likewise respond to repeated small doses (93). Thus, a minimal effective concentration of alloxan causes progressive damage to the islands. This explanation is chosen because the injury is histologically apparent after small doses (56) and because the rapid disappearance of alloxan from the blood (3, 22, 115, 128) makes a cumulative action unlikely.

Diet and Other Conditions Influencing the Response to Alloxan. Martinez (136) found that rats were more sensitive to a constant dose of alloxan after a foreperiod on a high fat diet (90–100 per cent mortality) and less sensitive after a high carbohydrate and protein diet (33–40 per cent mortality). In their paper on the production of alloxan diabetes, Kass and Wasibren (116) studied the influence of fasting on the response to alloxan. Withdrawal of all food from adult rats for 48 to 60 hours rendered them almost uniformly susceptible to the subsequent administration of 175 mgm. per kgm. subcutaneously. Ninety to 95 per cent became diabetic where as only 25 per cent of animals which had not been fasted responded to a similar dose. Older and heavier rats required a longer period of starvation. Several authors have observed that animals which have not responded to one injection may be refractory to the reinjection of a similar dose of alloxan (74, 86, 93, 116, 118). These results have been partly explained by Kass and Wasibren (116) who found that in their animals this occurred in the group which had not been fasted. After fasting for 60 hours the refractory animals became diabetic on the standard dose. The value of these observations is apparent. When the intravenous route is used, the rate of injection is important (93), for the slow injection of an otherwise effective dose produced no diabetes. Rats of both sexes were equally sensitive but wide differences in response were associated with variations in body weight (23, 72). In the detailed analysis of Gitter and Prieto Diaz (72 and table 2) the toxicity of alloxan changed in linear relation to the weight of the animal raised to the 0.73 power which suggested a direct relation between toxicity and this metabolic unit. They also applied this formula to an estimation of the LD_{50} for each rat. Barbiturate anesthesia renders animals more sensitive to the toxic effects of alloxan (118, 159), and this may also be true of chloralose

(98). If anesthesia is needed at the time alloxan is given, light ether anesthesia may be the safest (118). Other agents which enhance or prevent the action of alloxan will be discussed in relation to its mechanism of action.

Response of the Blood Sugar to Alloxan. Jacobs (104) first described the initial hyper- and hypo-glycemic phases following intravenous alloxan in rabbits, and observed the symptomatic relief of the hypoglycemia by the administration of glucose. Later, the phase of permanent hyperglycemia was added to the picture. Many reports have established the tri-phasic blood sugar curve associated with a diabetogenic dose of alloxan (5, 39, 54–58, 86, 104). This curve is composed of a rise in blood sugar at 2 to 4 hours after the administration of alloxan, a marked hypoglycemia at 6 to 12 hours, and a permanent hyperglycemia at 18 to 24 hours. The initial hyperglycemic phase does not occur in every case, whereas the hypoglycemic and diabetic phases are consistent except for minor variations in timing in different animals. Shipley and Beyer (159) observed a slight initial fall in blood glucose at 15 to 30 minutes and so refer to the curve as tetra-phasic. The knowledge that hypoglycemia caused early death in many instances led to the prophylactic administration of glucose (5, 54, 58). When animals were thus sustained during the period of hypoglycemia they survived and became permanently diabetic. Additional studies on the response of the blood sugar will be discussed under the mechanism of action of alloxan.

Duration and Constancy of the Diabetes. Alloxan diabetes may be permanent. This statement is true if one recognizes the experimental conditions to which it is applied and the exceptions that may be encountered. Diabetes has persisted for 5 to 11 months in rabbits (6, 52, 54, 118), 2 to 8 months in rats (28, 41, 58), 8 months in dogs (93) and 2 months in sheep (60, 109). As a rule the degree or severity of the diabetes remains constant but certain changes in its intensity have been seen. Thus, when less than the optimal dose was used, Houssay *et al.* (93) observed 3 dogs in which transitory diabetes lasted 5, 5 and 8 days. A similar example of spontaneous recovery in a dog after a few days of hyperglycemia had been previously reported (33). Slight but definite improvement in diabetes has followed the dietary treatment of diabetes in rats (28). Kass and Wasibren (116) state that 4 of 6 rats from the group which had not been starved before the injection of alloxan had normal blood sugars when tested three months after they had been shown to be hyperglycemic. Janes (104a) found that rats which had severe diabetes remained diabetic but that rats with mild initial diabetes tended to improve. When partial pancreatectomy is performed in young rats diabetes develops about three months later, i.e., after they have grown, and a similar increase in the manifestations of diabetes might well occur after alloxan under appropriate circumstances. Although this increase of severity with growth was not mentioned in the report on the dwarfing of young rats by diabetes (36), it has been suggested that diabetes may become more severe in the rat (27). A gradual increase in the severity of diabetes during the first three months has been noted in dogs (93) and in rabbits (5). In short, although alloxan diabetes may be permanent and constant, appropriate measurements of these characteristics must be made as required.

Severity of Diabetes. Alloxan diabetes should be regarded as partial pancrea-

tectomy produced by a chemical agent. This means that the subsequent diabetes may vary from the mildest to the most severe in type. There are many criteria for appraising the severity of diabetes, no one of which is entirely satisfactory for all situations. The glucose tolerance test reveals the earliest abnormalities in glucose metabolism but does not measure severity in a severely diabetic animal. Likewise the quantitative excretion of glucose and nitrogen during fasting, which are quite normal in mild diabetes, are valuable measurements of severe diabetes. Therefore the various measurements which have been made in alloxan diabetes must be evaluated with particular reference to the degree of 'chemical pancreatectomy' that has been achieved.

Hyperglycemia is an index of the presence of diabetes but is inadequate as a measure of its severity in the presence of glycosuria. In the dog, hyperglycemia has been of the degree usually seen in other types of experimental diabetes (e.g., 200–350 mgm. per 100 ml.). Rabbits and rats tend to have higher blood sugar levels (300–600 mgm. per 100 ml.) even when there is no apparent renal lesion and when the diabetes is mild as estimated by glycosuria (118, 127). Renal insufficiency may be responsible for some of the very high levels reported in alloxan diabetes. Diabetes with marked glycosuria and relatively lower blood sugar levels has been seen in sheep (60, 110). The glucose tolerance test varies from the slight impairment after small doses of alloxan to frankly diabetic curves (54, 65, 93, 97).

Diabetic animals respond readily to insulin and insulin resistance has not been encountered. Insulin tolerance or sensitivity tests resemble those seen in depancreatized animals (54, 74, 76, 93, 118). The diabetes may be controlled by the regular use of insulin, the dose of insulin varying with the severity of diabetes and the species used. The control of diabetes in rabbits is stated without details (54) and no data were found on the maintainance of diabetic rats, a species relatively resistant to insulin. In dogs, Houssay *et al.* (93) used 1 to 3 units per kgm. per day. In 3 dogs insulin treatment was followed by recovery but this cannot be distinguished from the spontaneous recoveries noted above. On the other hand 4 dogs treated for 25 to 45 days did not recover and this has been the experience of others with well established alloxan diabetes (166). The insulin requirement was comparable to that of pancreatic diabetes but they note (93) that the glycosuria of their animals was in general less than occurs after total pancreatectomy. Hence their results may not be comparable to those of Thorogood and Zimmerman (166) and Candela (29). In dogs with apparently severe alloxan diabetes the insulin requirement of fed animals was 3.3 to 3.8 units per kgm., calculated from the data of Thorogood and Zimmerman (166). After total pancreatectomy 1.1 to 1.4 units per kgm. were needed (166). Incidentally, the limitation of insulin requirement as a criterion of the severity of diabetes is shown in their experiments. Thus, a dog with severe glycosuria and moderate ketonuria from alloxan diabetes lived 91 days without insulin in contrast to 4 depancreatized dogs which died in coma 5–7 days after insulin was withdrawn. Although their insulin requirement was less, the depancreatized dogs had more severe diabetes by the criteria of survival, rate of loss of weight and acidosis. It would be interesting to know the insulin requirement during fasting in both types of diabetes. In any case, alloxan is a new aid in attacking such problems.

The quantitative excretion of glucose, nitrogen and acetone bodies has received little attention. In the dog, Houssay *et al.* (93) give values which in table 3 have been compared with their earlier figures for depancreatized dogs. One may assume that in alloxan diabetes as in pituitary diabetes occasional animals will be met which have 'total urinary diabetes', i.e., they are the equivalent of depancreatized animals in terms of these urinary metabolic measurements. In the dog the glycosuria and nitrogen excretion of pancreatic diabetes and phlorizin glycosuria are of the same order. Hence phlorizin glycosuria has been used as a basis for comparison in the case of the rabbit (118) in which there is little data on total pancreatectomy. If one uses an approximate estimate of the 'available glucose' of the diet (all the carbohydrate plus 50 per cent of the protein), the alloxan diabetic rats of Janes *et al.* (106) excreted about 80 per cent of the available glucose when fed. When fasted for one day the glycosuria was about 0.8 gram per kgm. per day, i.e., they had mild diabetes by comparison with the depancreatized dog. The proportion of the available glucose excreted by fed animals is a useful index of severity in moderately severe diabetes. Some such criteria are required if the degree of insulin deficiency is to be even approxi-

TABLE 3. EXCRETION OF GLUCOSE, NITROGEN AND ACETONE BODIES DURING FASTING[1]

DOGS	URINE				MAXIMUM WT. LOSS	SURVIVAL
	Glucose	Nitrogen	Ketones	D:N		
	g/k/d[2]	*g/k/d*	*mgm./k/d*		%	*days*
Depancreatized.....	2–4	0.7–1.8	—	2.8	50	5–30
Alloxan diab.	1.2–2.8	0.5–1.3	10–50	2–2.7	35	90–240

[1] From Houssay *et al.* (91, 93).

[2] Grams per kilogram per day.

mated in alloxan diabetes. Ketonuria has been measured in fed dogs in which quantities up to 200 mgm. per day contrast with 1400 mgm. per day after pancreatectomy (166). In dogs and cats ketonuria usually appears when 80 per cent or more of the available glucose of the diet is excreted in the urine, so that it indicates relatively severe diabetes. Ketonuria has occurred in rabbits (5, 131) and rats (53, 58, 80) but its relation to quantitative glycosuria in these species is uncertain. The quantitative ketonuria of rats was increased by adding nicotinic acid to the diet (108). The mechanism of this is not understood. The description of alloxan diabetic rats as generally free from excessive ketone bodies but always on the borderline of ketosis, when they are severely diabetic, aptly summarizes the situation (108). Blood ketones have been elevated in some animals (74, 123) as would be expected from the presence of ketonuria.

Liver glycogen has been determined. Histologically it is diminished (80). On chemical determination, seven rabbits examined 3 hours to 25 days after alloxan had an average of 3.46 per cent of liver glycogen, a normal value (86). In diabetic rats (not fasted?) the average value of 0.83 per cent was obtained instead of 3.05 per cent in controls (123). In fasted diabetic rats, Weber (169) found liver glycogen higher than in the controls and a slightly higher level of glycogen in alloxan diabetes was

noted by Houssay *et al.* (97). Janes *et al.* (106) give an average of 0.88 per cent for diabetic rats in agreement with Weber (169). In diabetic acidosis the average liver glycogen was 0.06 per cent (114). In diabetic rats, muscle glycogen falls slightly (97, 123, 169) and cardiac glycogen increases strikingly in agreement with the results in other types of diabetes (123). The levels of liver and muscle glycogen were increased by the ingestion of glucose but the increment was much smaller in diabetic than in normal rats (97). The variation in the liver glycogen of the normal rat with different periods of fasting must be taken into account in reading these papers. The behavior of muscle and liver glycogen follows the pattern that would be expected in partial insulin deficiency of varying degree.

Complications. Death in acidosis and coma has been observed (6, 114). This occurs in a few days when large doses of alloxan cause the combination of severe diabetes and renal insufficiency. The carbon dioxide combining power of the blood was slightly decreased when dogs became drowsy although the NPN remained normal (74). In acute acidosis rats were semiconscious or comatose with hyperventilation, severe glycosuria and ketonuria (114). In this condition the changes in the blood and liver phosphate fractions resembled those recently described in diabetes mellitus.

The most striking complication of alloxan diabetes in rats and rabbits has been the development of cataract (6, 11, 36, 54, 131, 156, 160). This occurs as early as the sixth week in rats and at eight weeks in rabbits (131). The incidence of cataract is greater and the appearance time shorter when diabetes is severe, facts which explain the irregularity of this complication in different laboratories. Insulin treatment seemed to delay the development of cataract (11). Diabetic cataract resembles galactose cataract (144), cataract after partial pancreatectomy (40, 64) and the usual cataract in man (11). It is distinct from that produced by certain dietary deficiencies. In addition to cataract one group of investigators (131, 156) has reported the occurrence of retinal lesions, consisting of capillary hemorrhages seen by ophthalmoscopic and histologic examination. Exudates were not observed. Of their 21 diabetic rabbits 18 showed progressive hemorrhages. The retinal lesions were not related to the severity of diabetes and appeared as early as one month but usually three to four months after alloxan. No effect of insulin treatment is claimed. These investigators (131, 156) state that the plasma proteins were altered in all cases. Plasma albumin was decreased and beta globulin increased in severe diabetes and in all rabbits with hemorrhages. When plasma proteins were depleted by plasmapheresis before the production of diabetes retinal changes developed more rapidly.

Immature rats with alloxan diabetes were strikingly dwarfed, some remained sexually infantile and the intestinal tract was enlarged in proportion to body weight (36). The effect of alloxan diabetes on pregnancy and on the fetus has been observed in the dog (93, 142), rabbit (142) and rat (43, 44, 67, 142). In the 4 dogs reported there was one abnormal pregnancy, in another (93) the fetuses had normal blood sugars and the islands were normal 48 hours after birth. In rabbits there were many abortions, premature deliveries and stillbirths, only about half of the pregnancies going to term. There was no increased birth weight of the young rabbits. All of the pregnancies in diabetic rats were full term and the fetuses were of normal weight. In this connection, Miller (142) cites unpublished work of Long who observed that more severely

diabetic depancreatized rats had a high incidence of fetal reabsorption. Reabsorption of the fetus and its prevention by insulin treatment have been described by others (43, 44). In diabetic rats maternal hyperglycemia, fetal hyperglycemia and normal fetal blood sugar after delivery have been found (67). The fetal islands were normal despite the demonstration of alloxan in fetal blood. Bailey (113) found hyperplasia of the fetal islands but more information is needed on this point. In spite of poorly understood fluctuations in diabetes during pregnancy there is no definite or consistent relief of maternal diabetes of any type by the fetal islands. In contrast alloxan diabetes in the rat is alleviated by parabiotic union with a normal rat (160). The most interesting conclusion of all of these reports is that there may be differences in function between fetal and adult islands of Langerhans.

Lowry and Hegsted (134) showed that rats with alloxan diabetes had no increased tendency to develop thiamine deficiency, and that their thiamine requirement was less than that of normal controls. Neuritis has not been reported in alloxan diabetes.

The deposition of sodium urate on the serous surfaces in certain birds (table 1) is apparently a non-diabetic complication which makes it possible that alloxan may be useful in the experimental study of gout. Blood uric acid reached extremely high levels 24 hours after the injection of alloxan, and death occurred in 48 hours when this response was severe. In birds which survived the initial rise in blood uric acid, the normal level was restored in two to three days and at autopsy little or no uratic deposition was found (55). Marked muscular atrophy occurred. The rôle of dosage and the time factor have been discussed in connection with the differences in various birds (157). It is noteworthy that Dalmatian dogs, which are unable to oxidize uric acid, respond like other dogs by the development of diabetes and no abnormalities of uric acid metabolism have been reported in this breed (77). An increase in blood uric acid 48 hours after alloxan and the prevention of this phenomenon by colchicine has been reported (42).

Pathology of the Pancreas. Ordinarily there are no gross changes in the pancreas although congestion and hemorrhage is occasionally seen. The histological alterations have been fully described by the first observers (54–59) and other detailed accounts (11, 48) and reviews (51, 77, 101, 113) have confirmed their results. The review by Duff (51) is particularly recommended. The lesions are similar in the rabbit (6, 11, 26, 54, 56, 57, 86, 164), rat (7, 53, 58, 102, 127) and dog (26, 33, 48, 74, 166) so that a single description will suffice. In other species the changes have not been as fully reported and the reader is referred to the individual accounts (table 1). The resistance of some species to anatomical damage and the difficulty in defining the islet injury in others have already been noted.

Slight diminution of the granules in the nuclei and cytoplasm of the beta cells is detectable as early as 5 minutes after the injection of a diabetogenic dose of alloxan (11, 102). At 10 to 15 minutes this is definite and seems to affect first the beta cells at the centers of the larger islands. By one hour shrinkage of the injured cells is apparent and by two hours definite pyknosis of the nuclei is evident (11, 26, 53, 56). Nuclear pyknosis progresses and by three hours there may be some separation of the cells with homogeneous, eosinophilic cytoplasm. Thereafter there may be either

shrinking or swelling of the cells which coalesce as the necrosis advances. Pyknotic nuclei may remain for as long as 24 hours or more but evidence of karyolysis and complete disintegration and disappearance of cells may be observed from 5 hours onward (11). At 24 hours the centers of the islands are composed of pale staining debris with only shadows of nuclei or cell outlines recognizable. The time of final disappearance of the granules of the beta cells has ranged from 15 minutes (102) to 3 days (80). While these changes are proceeding in the beta cells, the alpha cells are often undamaged although they may show slight degenerative change (56). Descriptions of necrosis of occasional alpha cells (53, 56, 57, 102) and of the destruction of both alpha and beta cells in almost all of the islands (5, 7, 11) indicate the variable extent of the damage. Sometimes after prolonged diabetes it has been impossible to find any islet tissue at all (7, 58, 102), but usually a few islands composed largely or entirely of alpha cells remain. The final picture after diabetes has existed for a few weeks or more is that of striking atrophy of the islands. Thus, the late stages of alloxan diabetes are indistinguishable from the final appearance of the pancreas in pituitary diabetes in dogs.

The rapid necrosis of the islands after alloxan, like the slower destruction in partial pancreatectomy (1) or pituitary diabetes (135), is remarkable for the complete absence of cellular inflammatory reaction.

There is a wide variation in the injury of the islands produced by any one dose of alloxan. In addition, the anatomical response to varied doses has been studied. After small single doses the changes are of the same type but less extensive and severe (56). After massive doses necrosis is accelerated, the condition of the islands at 10 hours being comparable to islands at 24 hours after the usual diabetogenic dose (37). The response to repeated small doses has also been studied (7, 11, 51, 101). As acute changes are thus produced at different times the picture is complex, all stages of necrosis and atrophy appearing together with normal cells which have escaped injury (11). With daily doses of 25 mgm. per kgm. or less for two months there were abnormalities of the granules of the beta cells. The animals did not develop glycosuria but this agrees with the alterations in the glucose tolerance test under similar conditions (161). From the effects produced by small doses of alloxan, Hughes and Hughes (101) postulated a cycle of maturation in the islands. According to them alloxan in low dosage destroys the oldest beta cells which are normally in the centers of the largest islands. In normal rats the smallest beta cells were in the largest islands; after repeated doses of alloxan, the largest cells were in the largest islands. In the alloxan-resistant strain of hooded rats (51) repeated subdiabetogenic doses produced transitory diabetes with minimal lesions in the larger islands. Thereafter, the animals were resistant to the continued administration of alloxan.

Alloxan has not produced hyalinization or fibrosis of the islands, the lesions most commonly seen in human diabetes. Hydropic degeneration of the beta cells has been noted occasionally (6, 11, 48, 52, 54, 118). This is seen rarely in man and commonly in other types of experimental diabetes. Hydropic change has not occurred in rats, but in dogs (48) and rabbits (11, 52) it has been found after diabetes had been present for several weeks. This has been attributed to the effect of hyperglycemia on beta cells that had escaped destruction by alloxan (118). Mitotic figures have been only rarely observed in the surviving cells (7, 56, 58, 80) but it has been suggested that the

alpha cells may increase in number (11, 53, 80). The disintegration of the Golgi apparatus in the beta cells has been described (42). The timing of the histological changes in the first 24 hours may be related to the alterations in the blood sugar (11, 56). Pathologists agree that the injury from alloxan takes place within a few minutes of its injection and that the subsequent events are the consequence of this initial injury. Although there was "no alteration in the character of the lesion which could be correlated with the fall in blood sugar" (11), the changes which began immediately after the injection of alloxan had progressed to the complete disintegration of many cells by 6 hours, i.e., at the onset of the hypoglycemia phase.

The acinar tissue is commonly described as normal. Duff and Starr (53) observed numerous mitoses in the acinar cells at about 17 hours, but not thereafter. In the small intralobular ducts the hydropic change noted by Goldner and Gomori (74) has been confirmed (47, 52, 58, 93). Small foci of fat necrosis with the usual inflammatory reaction were found in 23 per cent of dogs by one group (47, 48, 93) who suggest that this was the result of more advanced injury of the small ducts with leakage of the external secretion. Grossman and Ivy (85) found a diminished threshold response to secretin 18 to 30 days after alloxan. The amylase activity of the secretion was normal. They suggested that this was due to the injury of the ducts which they confirmed and that therefore duct cells as well as acinar cells might participate in the formation of pancreatic juice.

Kidneys. The remarkable toxicity of alloxan for the beta cells of the islands of Langerhans is accompanied by damage of the renal tubules which may be due to the sensitivity of this tissue to the drug or to the increased concentration of the drug during excretion. Occasionally gross pallor of the cortex has been mentioned. The glomeruli are normal. The convoluted tubules are often the site of vacuolation or hydropic change, necrosis and desquamation of varying degree (7, 11, 26, 48, 53, 57, 58, 74, 93, 127, 154). Albuminuria and occasional casts may be present. The lesions have been compared to those of mercurial poisoning (93). Renal lesions are most conspicuous in the first four days after the administration of alloxan. Thereafter they tend to disappear (11, 80, 93, 127, 154) so that in late diabetes the kidney is usually normal. This corresponds to the changes in the nonprotein nitrogen of the blood which rises after the injection of alloxan and returns to normal in a few days if the animals survive (16, 127). Infiltration of leucocytes about the affected tubules is seldom seen (127). The relation of dosage to renal injury has been studied in the dog (74, 93) and rat (16, 127). As an example, Lazarow and Palay (127) found that after 40 mgm. per kgm. intravenously, 16 of 18 rats had no kidney injury whereas half of the animals given 200 mgm. per kgm. intraperitoneally showed moderate to severe changes. In diabetes of some duration glycogen deposition in the loops of Henle has been found (41, 80, 93). Curtis *et al.* (41) found that this occurred only if the terminal blood sugar was 350 mgm. per 100 ml. or higher. Glycogen deposition in the kidneys is a manifestation of diabetes and not of alloxan itself. Intercapillary glomerulosclerosis has not been reported. In summary, an acute necrosis of the renal tubules may follow appropriate doses of alloxan. If death from uremia does not occur these lesions are reversible, a fact in which the kidneys differ from the islands of Langerhans.

Liver. Although many writers report little or no abnormality of the liver in

alloxan diabetes (7, 11, 53, 80, 86), definite lesions have been observed by others. In an attempt to differentiate lesions caused by alloxan from those due to the ensuing diabetes, particular attention will be paid to the time after alloxan when the lesions were found. Thus, Goldner and Gomori (74) saw no hepatic lesions in dogs dying acutely from large doses of alloxan. Fatty infiltration, slight necrosis and increased phosphatase (by stain) were present in dogs diabetic for two weeks. In contrast, in dogs given large doses, Houssay *et al.* (93) described marked jaundice, positive cephalin-cholesterol flocculation tests and other signs of severe hepatic damage. They distinguish this from jaundice occurring late in 2 animals which they regarded as secondary to severe diabetic changes. They frequently found fatty infiltration and central lobular necrosis at three days or longer after alloxan. As fatty livers can develop in 48 hours in depancreatized dogs, the primary rôle of alloxan is not clear in these animals but the emphasis on necrosis may indicate an effect of the drug. In cats (154) the occurrence of small and large areas of hepatic necrosis and fatty degeneration, beginning at the third day, was not related to the severity or duration of diabetes. Focal necrosis has been seen with varying frequency in rats (80, 127, 146). The typical lesion is a small focus of necrosis usually located in the midzonal or peripheral region of the lobule. It is 45 to 200 micra in diameter, uniformly distributed and inflammatory reaction is slight or absent (127). After intraperitoneal alloxan, subcapsular lesions instead of diffuse foci were noted. The incidence and severity of hepatic involvement could not be related to the dosage of alloxan or to the occurrence of renal lesions. Some of the highest intravenous doses did not produce hepatic necrosis whereas some of the lower doses were accompanied by severe damage. Occasionally slight liver damage was encountered after nondiabetogenic doses (127). When cysteine was given intravenously with alloxan to protect the islets, the incidence of hepatic necrosis increased from 22 to 85 per cent. As cysteine alone was harmless this was attributed to the conversion of cysteine to cystine which is known to cause hepatic necrosis. Herbut *et al.* (89) describe portal cirrhosis with iron deposition suggestive of hemochromatosis after alloxan in rabbits.

Other Organs. The adrenal glands increase in weight in alloxan (17) as in pancreatic (63) diabetes. The adrenal glands are usually described as normal or as having minor changes not regarded as significant (7, 11, 53, 57, 58, 74, 93). Congestion and hemorrhage have been seen (59). Hard and Carr (86) found necrosis of the medulla in animals given acutely toxic doses of alloxan. Lesions were slight or absent in diabetic animals examined one to three weeks after alloxan. Focal necrosis of the adrenal cortex, especially of the fascicular zone, has occurred with varying frequency (54, 117, 154, 164). In one report (117) cellular inflammatory reaction was noted. One gains the impression that injury of the adrenal gland, particularly of the cortex, may occur but that it is an unimportant factor in the routine use of animals with alloxan diabetes. The thyroid is atrophic (17) as in pancreatic diabetes (63), and the Golgi apparatus is reduced in size (2), changes which are attributed to the diabetes rather than to a direct effect of alloxan. The thyroid, parathyroid and pituitary glands are usually described as normal (11, 53, 57, 58). With large doses degenerative changes in the basophils of the pituitary have been seen (154, 164) and the intestinal absorption of glucose is greater than normal (147). Arteriosclerosis

has been sought but not found even in rabbits which are known to be sensitive to arterial injury (11, 51). The hypercholesterolemia of rabbits after alloxan is transitory unless the diabetes is severe (117). Three rabbits showed only a slight elevation at 50 to 60 days after alloxan so that the sustained diabetic hypercholesterolemia differs in degree from the extreme value produced by the forced feeding of cholesterol.

Alloxan in Man. Alloxan was first given to man by Brunschwig *et al.* (27) for the purpose of relieving hypoglycemia due to island cell carcinoma. They also gave it to 4 patients with severe malignant disease of other types. In the 4 persons with normal blood sugar there was a negligible hyperglycemic phase and no evidence of diabetes. The doses ranged from 50 to 500 mgm. per kgm., increasing amounts being given over periods up to six days. Because of this it is frequently asserted that man is resistant to alloxan. This may be so if one discounts the slower administration of the drug in 500 ml. of saline. In one patient they mention that the injection was completed in an hour and as Houssay *et al.* (93) point out slowing the injection of an otherwise effective dose to 10 minutes abolishes its diabetogenic action. One patient who received 600 mgm. per kgm. subsequently came to necropsy and minor lesions of the islands were found. The kidneys were entirely normal. One patient in this group had a chill, nausea and cyanosis for several hours after one of the large doses.

Five patients with hyperinsulinism have received alloxan. The first (27), who was known to have metastatic carcinoma of islet tissue, received several courses of alloxan which was given daily for one to four weeks. The dose was gradually increased to a maximum of 39 gm. in this 112-kgm. patient (348 mgm. per kgm.). After each course the patient had relief of hypoglycemic symptoms for periods as long as three weeks. At necropsy, the cells of the metastatic islet tumor showed no injury, the islets of the pancreatic tissue were described as normal and there was no tubular necrosis of the kidney. Another patient is that of Talbot, reported by Bailey (9, 113). In this 9-month-old girl with hypoglycemia, no tumor had been found at laparotomy and frequent convulsions continued to occur. Beginning at 20 mgm. per kgm. the dose was increased to 100 mgm. per kgm. over a period of seven days. Symptoms were relieved for three weeks and then recurred. A second series of 8 injections of 100 mgm. per kgm. was followed by freedom from symptoms for the eight months of observation reported. Although spontaneous remissions of hypoglycemia may occur this is a striking result. A third patient with hypoglycemia is briefly reported by Conn, Hinerman and Buxton (38). She was given 50 mgm. per kgm. on each of the first two days and 100 mgm. per kgm. daily for the next seven days, with temporary relief. However, after symptoms recurred an islet tumor was removed and normal pancreas was also secured for study. Histologically there was no effect of alloxan on the neoplastic islet cells, but marked destruction of the islet cell of the pancreas itself was observed. The patient was diabetic after the tumor was removed (37a). Finally, Wilder (171a) has mentioned a patient with hypoglycemia complicated by infection who died after alloxan with extensive fatty degeneration of the liver, and Flinn *et al.* (62) gave it to a patient with islet cell carcinoma.

Most of the injections of alloxan in man have caused no untoward effects. However, in addition to the reaction noted above, one of the hypoglycemic patients

(27) had five severe reactions on scattered occasions. The symptoms consisted of chill, nausea and vomiting sometimes followed by fever. On several occasions there was jaundice and severe anemia, the erythrocyte count falling as low as 1.7 million per cmm. Whether icterus was due to the hemolysis caused by alloxan or to direct injury of the liver is uncertain. Transfusions were used to aid the recovery of this anemia. A few casts, erythrocytes and a trace of albumin in the urine were transitory findings after a few injections.

Thus, two instances (27, 38) of the resistance of tumor cells and the susceptibility of normal human islets to alloxan are on record and another case of uncertain pathology may have been benefited. The reviewer agrees with Conn (37a) that "insufficient observations have been made to warrant the repeatedly expressed opinion that human islet tissue is exceedingly resistant to the degenerative effects of alloxan".

MECHANISM OF ACTION OF ALLOXAN

The conclusion that alloxan diabetes is pancreatic diabetes of varying degree seems so apparent from the correlation of both pathological and physiological data that this concept of *alloxan diabetes* will be accepted without further discussion. On the other hand, the fundamental action of alloxan on the islet cells is unknown and a summary of the investigations on this subject will be made. In the living animal alloxan necrosis of the beta cells results from the immediate action of an evanescent substance. The immediate action of alloxan means that it acts during the first five minutes or so after its rapid intravenous injection. This is supported by the findings that the earliest lesions appear in five minutes (11, 102), and that after this time alloxan is no longer demonstrable in the blood (3, 22, 115, 128). Goldner and Gomori (81) demonstrated this immediate action by clamping the circulation to the tail of the pancreas for five minutes during and after the administration of alloxan. In the clamped portion of the pancreas the islands were normal, whereas in the remainder of the organ the usual island necrosis occurred. Finally the chemical agents which protect the islands from alloxan (see below) must be given within a few minutes of the alloxan to be effective. After reviewing the chemical properties of alloxan, Archibald (3) came to the conclusion that there are at least three types of reaction which neutralize alloxan in blood: *a*) conversion to alloxanic acid by alkali, *b*) conversion to alloxantin and dialuric acid by the thiol groups in plasma and *c*) combination with urea.

Present evidence indicates that the action of alloxan on the islands is exerted directly. It is generally agreed that the pathological changes are the consequences of an immediate toxic injury of the beta cells. In addition, the mediation of certain other agents has been excluded. The initial hyperglycemic phase has been prevented by means of insulin (75, 76), phlorizin (75, 76), adrenalectomy (103, 119), hypophysectomy (10, 103, 119) and hepatectomy or evisceration (98, 99). In these experiments the occurrence of island injury has been reported often enough to make it clear that this initial period of hyperglycemia is not essential to the toxic action of alloxan on the islets. This deserves mention even though hyperglycemia of a few hours' duration would have no demonstrable effect on the islands, as days or weeks rather than hours are required to produce marked histological lesions in dogs or cats after

partial pancreatectomy (1), pituitary diabetes (135) or glucose administration (49). Moreover, hyperglycemia has not been accompanied by similar island lesions in the rat (46, 68), yet the rat is susceptible to alloxan. Alloxan necrosis and some degree of diabetes have been produced in the absence of the pituitary (7, 10, 51, 103) or adrenal glands (51) so that the organs which are, in varying degree, involved in other types of experimental diabetes play no part in the effect of alloxan. The sustained diabetes is not produced by inhibition of insulin because insulin is not inactivated by alloxan *in vitro* (75, 118) and because animals with alloxan diabetes respond normally to insulin (54, 74, 76, 93, 118). The action of alloxan on other tissues is apparently an acute injury of the parenchymal cells followed by necrosis or recovery depending on the conditions.

There are responses to alloxan which occur after its disappearance from the blood but which are relevant to its action on the islands and on the organism as a whole. Among these are the initial hyperglycemic and subsequent hypoglycemic phases of the blood sugar during the first 18 to 20 hours.

Hyperglycemic Phase. As noted above this may be prevented by various means without interfering with the necrosis of the islands. On the other hand, the mechanism by which this hyperglycemia is produced is still debatable. Dunn, Sheehan and McLetchie (59) suggested that this might be due to excessive mobilization of glucose through the adrenosympathetic system. This hypothesis has received support from subsequent studies. In adrenalectomized rabbits (75) and rats (51, 75, 119) and in rabbits with the adrenal medullae destroyed (75), the hyperglycemic phase was abolished. Corkill *et al.* (39) found that blocking the sympathetic nervous system by ergotoxine abolished the hyperglycemia, and Hughes, Ware and Young (102) duplicated both phases of the blood sugar response to alloxan by the combined administration of epinephrine and insulin. All of these results support the concept that the hyperglycemic phase is a non-specific response mediated by the sympathetic system. However there are other observations which show that the question is not settled. Houssay *et al.* (98, 99) found hyperglycemia in dogs (98, 99) and toads (96) after adrenalectomy. The reviewer would conclude from their data (98) that normal dogs under chloralose had a small hyperglycemic response to alloxan and that this was absent after adrenalectomy or denervation. The hyperglycemic phase is less in dogs than in rabbits (26). Houssay's interpretation is supported by others who have found that the hyperglycemic phase was not prevented by ablation of the adrenal medullae or sympathectomy (103, 119, 148, 159, 162). When alloxan was injected via the portal vein (98, 99) the hyperglycemia was higher than in the control series so that the possibility of a direct influence of the liver on this phase of the blood sugar curve must be kept in mind. The results of Goldner and Gomori (75) have been criticized by Iverson (103) who concluded that the cortex, rather than the medulla, is responsible for the effects of adrenalectomy. A final understanding of the hyperglycemic phase must await further study.

Hypoglycemic Phase. This has been attributed to *a*) an insulin-like action of alloxan (104), *b*) a transitory stimulation of the beta cells (59), *c*) the release of preformed pancreatic insulin (102) and *d*) an effect of alloxan directly on the liver (99). There is now ample evidence that alloxan has no insulin-like activity as Jacobs (104)

assumed, nor has it any effect on the utilization of glucose (39). The histologic changes in the pancreas are not consistent with the concept of a temporary overactivity of the beta cells. At the time of the hypoglycemia, 6–12 hours after alloxan, the beta cells are in a fairly advanced stage or necrosis. Incidentally it may be that there is an element of stimulation of the islands at a much earlier time if one recalls the initial fall in blood sugar at 15 minutes described by Shipley and Beyer (159), and the occasional mitoses observed after small doses (56) and discussed by Berthoud (19). Most reviewers agree that stimulation of the islands is not the explanation of the hypoglycemic phase.

The most generally accepted hypothesis is that preformed insulin is liberated from the damaged islands. This is supported by the general course of the histological lesions, the knowledge that alloxan does not neutralize insulin *in vitro* (75, 118), and the reproduction of the hypoglycemia with a dose of insulin corresponding to the insulin content of the animal's pancreas (102). Moreover there has been no fall in the blood sugar when alloxan is administered to alloxan-diabetic animals (77, 118) or to depancreatized animals (13, 79) and the insulin content of the pancreas does not begin to diminish until the necrosis of the beta cells is well advanced (7–8 hours), this diminution of insulin content coinciding approximately with the development of hypoglycemia (151).

There has been some disagreement with portions of the evidence cited above. In contrast to Banerjee (13) who used rabbits, Foglia, Orias and Sara (65) using rats reported the usual hypoglycemia after partial pancreatectomy. Although Goldner and Gomori (77, 79) found no hypoglycemia in depancreatized dogs, Houssay, Orias and Sara (99) observed a fall in blood sugar. This was found in dogs depancreatized one-half hour before the alloxan was given, but if 24 hours or more elapsed between pancreatectomy and the administration of alloxan they found, like others, no effect of alloxan on the blood sugar. They interpret this to mean that the liver becomes insensitive to alloxan. Another and most important explanation of these differences is made by Best and his associates (20). They have repeated the experiments with alloxan in depancreatized dogs using two preparations of alloxan. With the alloxan used by Goldner and Gomori (79) they obtained results in agreement with the Chicago investigators, and their results agreed with Houssay's (99) when Houssay's alloxan was employed. Pending further clarification, it seems that the hypoglycemic phase of the blood sugar after the administration of alloxan is due to the liberation of insulin from the necrotic island cells to which an effect of alloxan on the liver may be added when certain preparations of alloxan are used.

Partially depancreatized rats (145) are more resistant than normal controls to the diabetogenic effect of single doses of alloxan. Partially depancreatized dogs (93) also require the full dose. However, the depancreatized rats are more susceptible than normal rats to repeated low doses of alloxan. This indicates that it is the concentration of alloxan in the blood, rather than the mass of islet tissue, which determines the effect of a single dose. On the other hand, the gradual injury of islet tissue by repeated doses is more readily revealed when the insular reserve is reduced. When diabetes is established 24 hours or more after an effective dose of alloxan, the insulin content of the pancreas falls to a very low level (75, 151). At this stage the secretion

of insulin, measured by grafting the diabetic pancreas in a depancreatized dog, was usually greatly reduced (99).

Houssay *et al.* (96) found that the diabetogenic action of the pituitaries of alloxan-treated toads seemed slightly diminished. In rats (94) there was no such diminution and all pituitary hormones appeared normal except for a reduction in gonadotrophic activity. They relate this result to the minor lesions described by Thomas and Emerson (164) in the basophilic cells which are thought to elaborate the gonadotrophic hormone. In established alloxan diabetes, where the mechanism of alloxan itself is no longer an issue, hypophysectomy in both rats (10) and rabbits (103) leads to a somewhat milder and unstable type of diabetes reminiscent of the results seen in the 'Houssay' dog. Adrenalectomized rats are more sensitive to the hypoglycemic effect of alloxan as would be expected (140). Adrenalectomy caused an amelioration of alloxan diabetes in rats (106, 107) and a fall in liver glycogen to 15 per cent of that in control diabetic animals. In both hypophysectomy and adrenalectomy the decline in food intake is an important variable which must be controlled (103, 106). Pretreatment with estrogens does not alter the blood sugar curve after the injection of alloxan (103). Established diabetes is not notably influenced by estrogenic therapy (103, 105) when the alterations in food intake are considered. When food intake is maintained by tube feeding estrogens made the diabetes more severe (102a). Estrogens caused some increase in glycogen levels of diabetic rat (97).

Protection from Alloxan Diabetes. This has been accomplished by a variety of agents *provided the protective substance is given almost simultaneously with the alloxan.* Benerjee (14) found that the following substances given immediately before alloxan prevented the development of diabetes in rabbits: 1,2-dimethyl-4-amino-5 (d-l-ribityl-amino)-benzene; nicotinic acid; pyridine-dicarboxylic acid and 2-phenyl-quinoline-4-carboxylic acid. He noted that these compounds did not prevent the initial hyperglycemia although they abolished the hypoglycemia and ultimate diabetes. He found (14) that the following substances did not prevent diabetes: calcium pantothenate, glycine, benzoic acid, p-amino-benzoic acid, ascorbic acid and pyridoxine hydrochloride. The 1,2-dimethyl-4-amino-5 (d-l- ribityl-amino)-benzene was the reagent used to react with alloxan to form riboflavin (15) and this was thought to be the mechanism of its protection *in vivo.* Nicotinic acid was studied accidentally and the other compounds were tried in an effort to explain its action. He concluded that neither the carboxyl group nor the pyridine ring was responsible and that the entire nicotinic acid molecule was involved. The first reported attempt to prevent alloxan diabetes was that of Leech and Bailey (128) who used glutathione. This followed their observation (128) that about five minutes after the intravenous injection of alloxan the blood glutathione falls, often to zero, and a fall in blood thioneine was also found. The fall in glutathione has been confirmed (25). However, glutathione in doses of 200 mgm. per kgm. failed to protect rabbits. Lazarow (126) using 2500 mgm. per kgm. of glutathione and 912 mgm. per kgm. of cysteine found that both prevented diabetes in rats. Alanine, glycine, phosphate buffer, ascorbic acid and succinic acid did not protect. If the sulfhydryl compounds were given three minutes after the alloxan injection instead of two minutes before it, there was no pro-

tection. In additional studies on the prevention of diabetes by cysteine, a marked increase in the incidence of necrosis of the liver occurred after cysteine even though the islands were protected (146). Weinglass, Frame and Williams (170) protected rabbits from the hypoglycemic and diabetogenic effects of alloxan by immediate pretreatment with 3,4-diamotoluene, orthophenylenediamine and sodium bisulfite. They found the following ineffective: sodium bicarbonate, sodium phosphates (pH 7.0), sodium pyrophosphate, sodium cyanide, phenylhydrazine, hydroxylamine hydrochloride, cysteine hydrochloride, paraphenylenediamene, sodium pyrogallol and lysine hydrochloride. Colchicine inhibits the second hyperglycemic phase and prevents the increase in blood uric acid which occurs about 48 hours in rats given alloxan (42). The prophylactic influence of ribonucleotides has been reported (61). Ascorbic acid, already referred to as having no preventive action, was regarded as increasing the diabetogenic action of alloxan (130). This was not observed after d-isoascorbic acid. British Anti-Lewisite (BAL) gave complete protection in rats (37, 126a).

The prevention of alloxan diabetes by certain physiological procedures has been the subject of several studies. The rôle of diet and the protection of a portion of the pancreas by clamping its circulation for a short time have been noted. Walpole and Innes (168) ligated the pancreatic ducts and 30 to 60 days later, when there was pancreatic sclerosis with persistence of normal islet tissue, injected alloxan. Under their conditions alloxan had little or no pathological effect on the islets and did not produce diabetes. In constrast, Goldner and Gomori (79) saw no protection against the degenerative action of alloxan after fibrosis of the pancreas had been produced in this manner. The discrepancy between these investigators is still unexplained except for the fact that Goldner and Gomori used dogs instead of rabbits. Jimenez-Diaz *et al.* (111) described complete protection of dogs from the diabetogenic and nephrotoxic effects of alloxan if the circulation to the kidneys was clamped during the time necessary for the inactivation of alloxan in the blood. They interpret this as indicating some rôle of the kidneys in the diabetogenic effect of alloxan. Both the clamped and control animals had the initial hyperglycemic and the hypoglycemic response but the clamped ones did not develop diabetes. Confirmation of this work has not yet appeared. Thymectomy increased the susceptibility of rats to alloxan although splenectomy or castration had no such effect (61). Houssay *et al.* (92, 95, 100) and Martinez (137, 138, 139) have studied the influence of the thyroid on both alloxan and pancreatic diabetes in the rat. Thyroidectomized animals were more resistant and thyroid-treated animals were more sensitive to intravenous alloxan, LD_{50} being 74 and 25 mgm. per kgm., respectively. Thiouracil caused even more resistance to alloxan than thyroidectomy. In subtotally depancreatized rats thyroid treatment caused an earlier appearance of diabetes but led to the subsequent disappearance of diabetes, a result attributed to the hyperplasia of the islets due to the thyroid treatment. They point out the differences between the dog and the rat in respect to this response of the islands (95). In summary, protection from the diabetogenic action of alloxan has been demonstrated by: *a*) substances which combine directly with alloxan, i.e., they do so *in vitro* and presumably do so on injection in animals; *b*) physiological conditions under which the inactivation of alloxan is facili-

tated by means not yet understood. These experiments tell one little about the fundamental action of alloxan although the results with most of the chemical antagonists emphasize the rapid and transitory nature of its action.

Action of Alloxan Homologues and Related Compounds. Another approach to the question of the mechanism by which alloxan destroys the beta cells is the determination of the chemical specificity of its effect. This has received considerable attention and tables 4 and 5 indicate the results. Table 6 supplements these listings with a few structural formulae. In table 4 it will be seen that some compounds have been found to be diabetogenic with a high degree of uniformity. Initial experiments (e.g., 75) which were negative may be attributed to technical difficulties and it is easy to reach the conclusion that under appropriate conditions all of the compounds listed, except barbituric acid and violuric acid, are diabetogenic. None is as readily effective as alloxan itself. In the case of barbituric and violuric acids it is possible that a positive result is more significant than the failures to produce island lesions. Further

TABLE 4. OTHER COMPOUNDS REPORTED AS DIABETOGENIC

SUBSTANCE	DIABETES REPORTED	DIABETES NOT REPORTED
Monomethyl-alloxan	(24, 25, 71, 90)	
Monoethyl-alloxan	(25)	
Monopropyl-alloxan	(25)	
Alloxantin	(7, 24, 25, 83, 88, 91)	(75)
Dimethyl-alloxantin	(24, 25)	
Diethyl-alloxantin	(24, 25)	
Dialuric acid	(8, 24, 25, 83, 121)	(75)
Monomethyl-dialuric acid	(24, 25)	
Barbituric acid	(71)	(25, 75, 83, 121)
Violuric acid	(71)	(24, 25, 75, 83, 165)

study of these substances is needed. The long list of compounds without diabetogenic action (table 5) requires only the comment that this list is neither complete nor final. That it is not necessarily the last word is illustrated by the report of Stoll (163) that ninhydrin was diabetogenic. The hypoglycemic effect of sulfanilamide-cyclopropylthiazole was not accompanied by diabetes (35).

The most recent discussion of the data in tables 4 and 5 is that of Brückmann and Wertheimer (25) which is the basis of the following remarks. An intact pyrimidine nucleus appears essential for diabetogenic activity. Some inactive substances like dimethylalloxan still produce kidney injury which may mean that the kidney damage is not due to the same mechanism as the island damage. These authors suggest the following possible types of action to account for the direct effect on the islet cells. 1) There may be selective accumulation of the drug in the beta cells. Because of the small amount of islet tissue this is possible in spite of their finding that after its injection there is less alloxan in the pancreas than in the liver and kidney.

2) Alloxan may compete with some structurally similar compound for an enzyme with resulting injury to the cells. This is entirely speculative at present. 3) A specific reaction of alloxan may occur in the islet cells with or without a specific accumulation. Lazarow (126) has suggested that the inactivation of SH groups of protein enzymes might account for such an effect. In view of the large reserve of glutathione

TABLE 5. COMPOUNDS WITHOUT DIABETOGENIC ACTION

COMPOUND	REFERENCES	COMPOUND	REFERENCES
Allantoin	(71)	Oxaluric acid	(104)
		Formyl-oxaluric acid	(104)
Alloxanic acid	(24)		
Dimethyl-alloxanic acid	(24)	Parabanic acid	(71, 165)
Benzal-barbituric acid	(165)	Pipe azine	(71)
Benzoyleneurea	(90)		
		Rhodizonic acid	(25)
Benzyl-alloxan	(25)		
Butyl-alloxan	(25)	Senecionene	(87)
Isobutyl-alloxan	(25)		
Phenyl-alloxan	(25)	Sulfadiazine	(87)
Dimethyl-alloxan	(25, 90)	Tartronic acid	(24)
Methyl-ethyl-alloxan	(25)		
Methyl-propyl-alloxan	(25)	Thiouracil	(64, 165)
Dimethyl-dialuric acid	(25)	Uracil	(71)
Isodialuric acid	(104)		
		Uramil	(71, 165)
Formyl urea	(104)	N-methyl-uramil	(25)
Guanidin	(57)	Uric acid	(57, 71, 165)
Isatin	(24, 25, 90)	Other oxidizing substances	(75)
		Ceric sulfate	(75)
Isobarbituric acid	(104)	Persulfate	(75)
N-dodecyl barbituric acid	(75)	Quinone	(75)
		Sodium molybdate	(75)
Mesoxalic acid	(104)		
Sodium mesoxalate	(90)	Related to styryl quinoline	(55)
Ethyl mesoxalate	(90)	1,2-naphthoquinone-4-sulfonic acid	(25)
Mesoxalamide	(90)	Naphthoquinone	(75)
		1,8,mesoxalylnaphthalene mesoxalyl	(25)
Murexide	(24)	Quinoline	(75)
		Cincophen	(75)
Ninhydrin	(24, 25, 90)		
Oxalic acid	(57)		

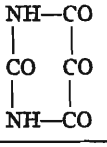
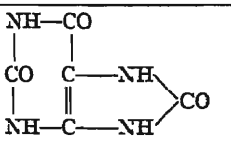
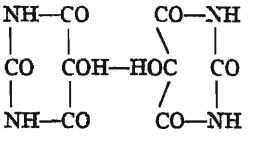
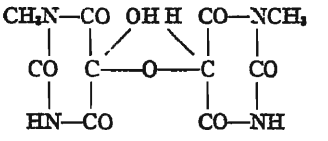
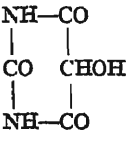
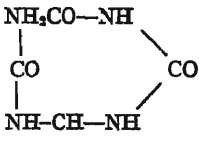
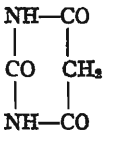
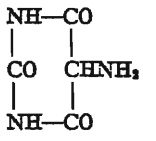
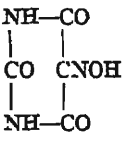
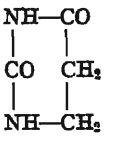
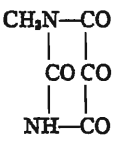
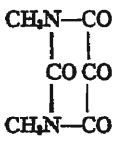
in the pancreas after alloxan (25) and the relatively unspecific nature of SH oxidation this concept is doubted. Brückmann and Wertheimer point out that lack of knowledge of the biochemical processes in the islands of Langerhans is the major barrier to any final understanding of the action of alloxan.

Possible Relation of Alloxan to Enzymatic Reactions. In reviewing the few

references in this field it is necessary to distinguish between the direct effects of alloxan and studies in established alloxan diabetes. The latter give no information about the primary action of alloxan and are included only for the sake of completeness.

Reference has already been made to the physical chemistry of the alloxan dialuric acid oxidation: reduction system (150, 171) and to the reversible reaction: uric acid-dialuric acid in the perfused liver (4). In appropriate concentrations alloxan in-

TABLE 6. CHEMICAL STRUCTURE OF ALLOXAN AND RELATED COMPOUNDS

Alloxan		Uric acid	
Alloxantin		Dimethyl-alloxantin	
Dialuric acid		Allantoin	
Barbituric acid		Uramil	
Violuric acid		Uracil	
Methyl alloxan		Dimethyl alloxan	

creased the oxygen uptake of liver suspensions and greatly accelerated their oxidation of ethyl alcohol (18). Alloxan had no direct effect on hepatic glycogenolysis (31) and it inhibited the glycolysis of glycogen to lactate in frog muscle extract and this inhibition was reversed by cysteine (70). This agrees with previous work on the inhibition of hexosephosphate formation from glycogen by alloxan (129). In experiments timed from 6 to 48 hours after alloxan, there was a diminution in the alkaline phosphatase of the kidney (141), which was also demonstrated histochemically.

Serum diastase was temporarily increased 6 hours after alloxan but returned to the normal level by the time permanent hyperglycemia was established (66).

Rats with established alloxan diabetes have been used to study the oxidation of various substrates by brain, liver and kidney *in vitro* (32). There were no differences between the normal and diabetic tissues in these studies and in contrast to earlier work (18) alloxan did not increase the oxygen uptake of liver. Increased glycogen phosphorylysis in muscle (125) and an increase in liver (50, 74) and serum (30, 125) phosphatase all point to the splitting of phosphate esters at certain stages in alloxan diabetes. As noted, studies made after the initial effects of alloxan have disappeared are not apparently related to its mode of action. For this reason major contributions to carbohydrate metabolism in which alloxan diabetic rats have been used are not discussed.

SUMMARY

Alloxan diabetes is the diabetes produced by the administration of alloxan in various ways to many species of animals. It is the result of the immediate action of alloxan on the islet cells, especially the beta cells, which results in their necrosis with subsequent atrophy of the islands. The permanent diabetes is therefore a form of pancreatic diabetes, i.e., it is partial pancreatectomy of varying degree which has been chemically induced. The acinar tissue is uninjured. Alloxan as such disappears from blood *in vitro* and *in vivo* in 10 minutes or less and has not been found in the blood in human diabetes. From its pathology and physiology alloxan diabetes is due to the destruction of islet tissue but the mechanism by which it injures the cells is unexplained. Alloxan diabetes differs strikingly from the other types of experimental diabetes in the course and nature of the lesions which are developed. It is a valuable aid in the study of diabetes and it may open the way to increased knowledge of the function of the cells of the islands of Langerhans. As yet there is no evidence that alloxan is related to human diabetes mellitus.

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RENAL FUNCTION IN EARLY LIFE

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IT IS NOW GENERALLY RECOGNIZED that the function of an organ may alter as development proceeds. Physiologists were slow to appreciate this fact, but the work of the last 25 years has demonstrated its importance so clearly that several books have been written on the subject. In these Windle (128) and Barcroft (9) have dealt mainly with function during fetal life while Smith (110) has confined himself largely to the physiology of infancy. The time now seems to have come, however, to review certain aspects of these new developments in more detail. The infant kidney has been selected in the first instance because our knowledge of it has reached a stage at which a detailed and critical résumé of the established facts has become profitable and, in consequence, advisable. This article has been written, therefore, to compile knowledge which has already been acquired, to point out the gaps in that knowledge and finally to suggest various theories and lines of work which might profitably be pursued. The functional abnormalities of the kidney, which give rise to the Fanconi and other syndromes (113, 114), will not be discussed since it is felt that they are pathological entities and as such require rather different treatment. Since the basis of this review will be a comparison of the infant with the adult, some reference to adult function will be inevitable, but in general it has been assumed that the reader will possess a working knowledge of adult renal physiology.

GENERAL CONSIDERATIONS

Work of the Kidney and the Internal Environment of the Newborn

The function of the kidney may be defined in the most general terms as the regulation of the constancy of the internal environment. In utero this regulation is largely carried by the placenta but within a few minutes of birth the kidney must take up the task. From that time onward it must excrete all the nitrogenous end-products of the body. Under the partial direction of the post-pituitary body and the cortex of the suprarenal gland, moreover, the kidney must stabilize the osmotic pressure and the chemical composition of the circulating medium. For the rest of the animal's life the kidney must play a leading part in regulating the volume of the extracellular fluids. This is a matter of great concern to pediatricians and infant physiologists but the mechanism by which it is brought about is still very obscure. By excreting non-volatile acids, moreover, the kidney from birth will share with the lungs the task of maintaining the pH of the body within the narrow limits of normality. The efficiency with which all these operations are carried out by young animals forms the subject matter of this review. Any study of their renal function, therefore, should begin with an examination of the volume and composition of the internal environ-

ment and of the circulating fluid which bathes the renal cells. It is not possible in an article of this length to do so in any detail, but it is necessary to point out various ways in which these fluids in infants differ from those in adults. *a*) The serum of an infant contains less albumin and globulin that that of a healthy adult (53). Hence the colloidal osmotic pressure of the serum will be lower and this in turn will tend to favor a more rapid glomerular filtration rate and a more rapid outpouring of fluid into the tissue spaces. *b*) It is generally conceded that the osmotic pressure of the cells at all ages is the same as that of the extracellular fluids, i.e., of the internal environment; but whereas that of the former is mainly due to K salts, that of the latter is due to the Na salts of Cl and HCO_3. All published analyses have shown that newborn animals and fetuses contain more Na and Cl/kgm. of body weight than do adults of the same species and since newborn animals have the same concentration of Na in their sera as adults (18, 84) this has generally been taken to indicate that they contain more extracellular fluid/kgm. of body weight. This has been confirmed by a comparison of the volume of the body occupied by thiocyanate and radio-sodium in infants and adults. It is to be noted that the thiocyanate space was appreciably smaller than the sodium space at all ages (39). In other words, per unit of cell mass infants have a much larger volume of internal environment than adults have. Premature babies may exhibit this peculiarity in an extreme degree for they may be frankly edematous at birth or become so shortly afterwards. *c*) A number of authors have reported on the acid base balance of the serum of newborn infants. Seham (108) found plasma bicarbonate within normal limits, but Branning (16), Hoag and Kiser (54), Lippard and Marples (72), Lucas *et al* (73), Marples and Lippard (76, 77) and Ylppö (130, 131) have all reported values which were below those usually found in adults, and their work has been interpreted as meaning that many babies are in a state of acidosis for some little time after birth due to an accumulation of fixed acids. *d*) The concentration of inorganic P in the plasma of a baby tends to lie between 5 and 8 mgm/100 cc. (7, 34, 120). In adults the range for this ion is generally given as 2.5 to 4.5 mgm/100 cc. *e*) The serum K was found to average 28.1 mgm/100 cc. by Kotikoff (68) during the first month of human life, and 30.6 mgm/100 cc. by McCance and Young (84) in babies under 14 days old. An average figure of 21.9 was obtained by the latter authors for healthy adults. No one can set out to explain renal function in early life without taking cognizance of these differences between infants and adults.

Methods of Investigation and of Comparative Expression

The methods which have yielded so much information about the adult kidney are almost all available for the study of the infant kidney but some of them require adaptation to a more micro scale. Workers have not always checked their modifications for infant work against adult material, but any one who sets out to study renal function in young animals should be prepared to make similar investigations on adults by the same methods, because the basis of every study must be comparative. Some measurements, e.g., those of osmotic pressure, may be directly compared and sometimes the principle of an internal standard may be employed. Thus the NH_3/total N or the P/total N ratio in the urine of adults and infants may be com-

pared. Sometimes, however, a more arbitrary standard must be used. This is outstandingly so when clearances are to be compared. Surface area, body weight and kidney weight have all been suggested. It makes a large difference which basis or standard is employed, since the surface area of an adult is 8.25 times that of an infant at birth whereas the kidneys weigh about 13 times and the body 21.5 times as much. These and other possible standards of comparison, such as the basal metabolic requirements, and the sources of information were discussed by McCance and Young (84) and further data about weights of infants and their organs may be found in an article by Cruickshank and Miller (26). Of these standards, surface area would appear to be the best because per unit of surface area clearances of children over the age of 2 are of the same magnitude as those of adults (55, 86). Some investigators, however, have been dissatisfied with this basis of comparison for infants because by it clearances in infancy appear to be so low. This, however, is one of the important discoveries about the renal function of infants and to establish another basis for newborn children would only confuse the issue. All investigators, however, should state clearly the 'correction' factors which they have employed or give the surface area of their infant subjects. A much more subtle difficulty has been encountered by those people who have been administering substances to newborn and adult animals and comparing the effects on renal function. If the renal functions are to be compared on a basis of surface area, should these doses be calculated and equalized on a basis of surface area or of body weight? It is suggested that if renal functions are to be compared, the object of dosage should be to raise or to lower serum levels to the same degree at both ages and that the doses should be calculated on those terms. In practice this means dosing in terms of body weight or of extracellular fluid volume.

Species Differences

One of the contributions of Homer Smith and his associates to renal physiology was the attention which they gave to species differences. There is every reason to believe that these differences will be exaggerated in very young animals for no two species are born at the same stage of development. Investigators should be on the look out for these differences and should always state clearly both the age and the type of animal used.

RENAL FUNCTION BEFORE BIRTH

Until birth the regulation of the internal environment can be carried out unaided by the placenta because babies, normally developed in almost every way, may be born with functionless urinary tracts. The kidney, however, normally begins to function before birth, if only to a limited extent (52, 91). Even as early as the second or third month of fetal life, tubule cells of man growing in tissue culture have been observed to form small cysts and to secrete dyes into their lumina (21), and an analysis of liquor amnii at different stages of development has indicated that while the osmotic pressure of this fluid gradually falls, the concentration of urea and uric acid in it steadily rise (44). It is unfortunate that studies of the maternal and fetal bloods were not combined with those of the liquor, but the findings suggest that long before birth the kidney begins to excrete a urine of low specific gravity containing

urea and uric acid in appreciable amounts. They also suggest that the amniotic sac must be osmotically isolated from both mother and fetus for a large part of its existence. Butler (19) has recently shown that inulin injected into the mother may be detected in the urine of babies delivered within two hours, so that this substance must not only pass the placental membrane but be excreted by the fetal kidney. Wells (122, 123) delivered fetal rats into the abdominal cavity of the mother about 16 hours before term, and blocked the outlet of their bladders by cautery. He found that urine was formed and distended the urinary tracts above the obstruction, and that phenosulphonephthalein was excreted by these fetuses. The urine secreted by a group of 11 control animals averaged 2.25 mgm/grams fetus/hr. Into 6 animals he injected 75 mgm. of urea in a volume of 0.15 cc. and from these he collected an average of 4.5 mgm. of urine/grams of fetus/hr. He collected an average of 5.1 mgm. of urine/grams of fetus from 4 animals to whom he had given 0.15 cc. of distilled water and he concluded that fetal rats secreted urine more rapidly than was generally supposed during the last two days of uterine life. By employing a suitably sized animal and the appropriate technique it ought to be possible to work out the division of labor between the placenta and the kidney throughout a large part of fetal life.

Tausch (117) catheterized a large number of babies at birth and concluded that the urine of the last few hours or days of fetal life was generally acid and had a lower specific gravity than that found in adults. It contained creatinine and sometimes albumin, and the average non protein N amounted in that series to 0.155 per cent. Work recently carried out by McCance and von Finck (79) is in substantial agreement with these conclusions. Fetal urine characteristically contains very low concentrations of urea and other nitrogenous end-products and has a low osmotic pressure, whereas that of the mother passed at the same time may be highly concentrated. Since mother and fetus must be regarded as being in osmotic equilibrium, there is a problem to be solved here which might give us a clue to much of the renal physiology of the infant.

RENAL FUNCTION AFTER BIRTH

Urine Volumes and Osmotic Pressure

It may be assumed that a child is fully hydrated at birth, but in a state of nature it gets little fluid from the breast for at least 24 or often for 36 or even 48 hours. An article by Thomson (118) may be consulted for data about the fluid intake of newborn infants and an account of the volumes and the characteristics of the urine passed by them. In some hospitals newborn babies are given water, but in many they are not and in a few the infants are deliberately given no fluid for 36 hours or more even if they are to be reared from a bottle. Dehydration for this length of time would leave an adult in a state of considerable hydropenia and, although they do not sweat freely at this age, there is reason to believe that newborn babies may lose a greater proportion of their body water in the same time, for they have proportionally a larger surface area from which to evaporate moisture (124). Against this must be set Heller's (48) observations on newborn rats which showed that these animals had a surprisingly small extrarenal loss of water and the explanation of this fact probably lies in the findings of Levine and Marples (71). These workers demonstrated that the in-

sensible loss of water varied directly at all ages with the heat production—and this is well known to be low in newborn infants. Bailey and Murlin (6) found the metabolism of the newborn infant under basal conditions to be 25 Cals/sq.m/hr., whereas young adults have a basal heat production of about 40 Cals/sq.m/hour (35). It may be assumed with confidence, however, that babies are as a rule short of water from about the 4th to the 36th hour of their lives. Very little is known about the effect of this physiological hydropenia upon the chemistry of the internal environment and not much more about the way in which the kidney reacts to it, but much of this information should be easy to obtain. Our knowledge at present may be summarized as follows:

1) The blood urea rises between birth and the third day of life and with it in all probability the uric acid, NPN and other nitrogenous end-products (8, 73, 106, 107, 109). The rise in the blood urea may not be great and averaged 10.2 mg/100 cc. in a small series of 12 infants. Much higher figures have been observed in some premature babies (133). The concentration of urea in the blood begins to fall about the third day of life (for references, recent data and discussion see McCance and Widdow-

TABLE 1. CHANGES IN THE CONCENTRATION OF N AND P IN THE URINE DURING THE FIRST 10 DAYS OF LIFE

	AGE					
	0	5–24 hr.	24 hr –4½ days	4½–6 days	6–7 days	7 days and over
Total N mgm/cc. of urine	0.70	5.45	5.60	1.92	3.22	2.11
Inorganic P μg/cc. of urine	6.16	116.0	106.0	41.1	79.0	65.0

son, 82). The blood urea also rises for some days after birth in newborn rats (126). 2) The urine becomes more concentrated soon after birth and urates are frequently deposited in it as it cools. The output goes up later as the intake of milk increases. The figures in the table were obtained by McCance and von Finck (79) and show the general trend. It must be emphasized that these figures are averages and there was considerable variation from one child to another. All who have studied the urine at this age are in agreement about the reality and magnitude of these variations (118). Miller (87) has made similar observations on gastric juice, and it is evident that a number of points about the physiology of infancy can only be settled by a statistical approach. 3) No statement can be made about the volume of urine passed per minute before birth but the volumes of urine passed on the second and third days of life are small. In Thomson's series (118) they averaged only 20.6 cc/24 hrs., which is probably less than 100 cc/sq.m/24 hours, and one of the two babies with dehydration fever (119) passed less than 50 cc/sq.m/24 hr. for three consecutive days. Smith (111) made observations on premature and full-term babies who were given no water for nearly 48 hours and found that they were passing about 200 cc/sq.m/24 hrs. 4) The specific gravity of the urines passed at this time is of the order of 1012 to 1015 (118), and the osmotic pressure 450 m. osmols/l. (46).

Smith (111) found values of 400 to 600 m. osmols/l., and Thomson (119) one for *baby B* with dehydration fever which was probably not much higher. If the serum be taken to have an osmotic pressure of 300 m. osmols/l., it will be seen that the infant urines are generally more concentrated than the plasma on the second day of life, but after shorter periods of water deprivation healthy adults have been shown to produce urines with osmotic pressures of 1000 to 1300 m. osmols/l. so that it is clear that the human infant does not normally produce such hypertonic urines as an adult. When McCance and Young (84) first discussed this, their data had been derived from full-term babies who had been born for at least a week and who had never been deprived of water for very long. Their conclusions were based upon chemical analyses which probably gave results somewhat lower than the true U/P osmotic ratios. They concluded that the kidneys of infants consistently produced "hypotonic urines from plasmas from which adults would certainly have produced hypertonic urines at comparable urine volumes". The recent data do not conflict with their conclusions in any way. In discussing a series of premature babies, moreover, aged 2 to 7 days, Young, Hallum and McCance (133) pointed out that the U/P osmotic ratios as measured by them were one or more in a number of the children (the ratios would have been higher had they been based upon the actual freezing points). They thought that in some instances these high ratios were due to the high blood urea found in these babies and Aldridge (3) noted in older children that when the serum chlorides were within normal limits, little NaCl was excreted in the urine which was certainly hypotonic. After NaCl or normal saline had been given to these babies, however, the serum values became quite abnormal and the concentration of NaCl in the urine went up to 1.39 per cent. These urines must have had osmotic pressures of at least 600 or 700 m. osmols/l. It is possible that many babies do not begin to produce hypertonic urines until the urea or sodium salts in their plasmas have risen to abnormal heights and it is to be regretted that Thomson's (119) study of two babies with dehydration fever should not have included investigations of the serum chemistry. It may be concluded that babies aged 2 to 4 days are hydropenic, yet they produce urines with osmotic pressures which are sometimes no greater than that of the serum and never as great as that of the urine passed by a healthy adult after being deprived of water for a similar length of time. This work on man followed some very excellent studies on puppies which were made on the Continent of Europe between 1925 and 1936 by Kramár (66, 67), Schiff, Bayer and Choremis (104), Schiff (103), and particularly by Kerpel-Fronius (27, 59, 60, 61, 62, 63). These investigators were fully aware of the fact that young puppies could not concentrate their urine as could adult dogs and Kerpel-Fronius actually stated in 1932 that the same was true of all suckling mammals (89). A knowledge of this fact and of the well-known French dictum about the 'urine obligatoire' (4, 23) enabled these men to develop a method of dehydrating puppies by feeding them upon partially evaporated milk. They showed that *a*) the same diet did not dehydrate adult dogs and *b*) it was only possible to dehydrate the pups in this way if the diet contained a certain amount of protein. It was assumed that the inability of the kidney to concentrate led to a relative polyuria and this in turn led to the dehydration which was obvious clinically and measurable osmotically after four days. High praise must be given to these pioneer investigators.

It will be recalled that the volumes of urine passed by babies during the stage of physiological hydropenia were between 80 and 200 cc/sq.m/24 hrs. These volumes are surprisingly small. The two men dehydrated by Black *et al* (14) passed about 350 cc/sq.m/24 hrs., but their diets contained between 9 and 10 grams of NaCl/day and 10.4 to 13.8 grams of N. This quantity of urine, however, is little more than that excreted by Nadal, Pedersen and Maddock's *subject E.B.*, who excreted about 330 cc/sq.m/24 hrs. on a salt poor 'dry' diet. Gamble's (41) subject passed 290 cc/sq.m/24 hrs. and Ladell's men (69) somewhat less (about 230 cc/sq.m/24 hrs.), while the person who was given nothing to eat or drink by Nadal, Pedersen and Maddock (90) passed 440 cc. in the 24 hours which would be about 250 cc/sq.m. A similar figure was obtained by Winkler, Danowski, Elkinton and Peters (129). How are these findings to be reconciled with the views of Kerpel-Fronius? The human infants do not produce concentrated urines, yet they do not appear to have any polyuria. There are several possible explanations. The volume of the 'urine obligatoire' varies inversely as the concentrating capacity of the distal tubules and directly as the quantity of the osmotically active material reaching the distal tubules per minute. The latter can only be large if the glomerular filtration rates are high, and although nothing is known about the glomerular filtration rate of Kerpel-Fronius' pups, they were presumably relatively high. If the glomerular filtration rates of infants aged 2 days were sufficiently low, then the infants might have low minute volumes in spite of a poor concentrating power. The glomerular filtration rates of infants 2 days old are already known to be low (see later). They averaged 28 cc/sq.m/min. after giving fluids (31), but this is not low enough to explain the volumes of urine passed at this age by babies who are hydropenic, for if the volume of urine passed by a 2-day old infant be taken to be 150 cc/sq.m/24 hrs., this would be reduced to about 50 cc/sq.m/24 hrs. if the baby had the concentrating power of an adult. A glomerular filtration rate of 30 cc/sq.m/min., however, is little less than one half of the rate found in dehydrated adults by Black *et al* (14) and would lead one to expect urine volumes of the order of 450 cc/sq.m/24 hrs. in the infants. The volumes actually passed suggest that the glomerular filtration rates would have to have been of the order of 8 cc/sq.m/min. to explain the findings under discussion. The glomerular filtration rates, however, may well be as low as that in infants who have never received water or food. From all that is known about glomerular filtration rates this may be so, but no measurements have yet been made and, therefore, the relationship cannot be made quantitative. There is no information at present about the relative heights of the glomerular filtration rates in infancy and the reabsorptive capacity of the proximal tubules.

The Excretion of Water

After some controversial work in the two previous years (92, 101, 116, 124) it was shown by Lasch (70) that babies under 3 months of age excreted water less freely than babies over 3 months. This confirmed the findings of Aschenheim (5). This investigator had given babies 200 cc. of water at 6 a.m. after an overnight fast and collected their urine over the next 5 hours. In the second half year of life the infants excreted 200 cc. of water in this time, but less than this in the first half year and in the first three months only some 100 cc. Aschenheim considered that these results were

due to the fact that the tissues of the younger infants had a very high affinity for water. She did not attribute them to functional underdevelopment of the kidney. Lasch gave the babies under 3 months of age 100 cc. and those between 3 and 6 months 150 cc. of water, so that he employed more comparable tests. Unfortunately, Ohlmann (92) and Rominger (101) did not arrange their experiments in a similar way and little or no further work of a similar kind seems to have been done with human subjects. There is little doubt, however, that these results were correct, although they must now be extended and confirmed, for Adolph (1) has found that young puppies excrete water at a much slower rate than adult dogs and Heller (48) and McCance and Wilkinson (83) have found the same to be true of newborn rats. In fact, the administration of 5 per cent or even more of the body weight of water produces little if any diuresis in the newborn of this species.

The excretion of water cannot be discussed without reference to the post pituitary antidiuretic hormone. Heller (46) has published some important papers on this subject. He has shown that the administration of post pituitary hormone to infants does not lead to much concentration of the urine. The tests were made on babies over a week old who were passing the large volumes of dilute urine usual at that age. It is perhaps unfortunate that water was not given to the infants, since the action of the post pituitary hormone can be demonstrated with most certainty when the animal is carrying a considerable water load. Be that as it may, Heller (49) has also found that the pituitaries of young animals can only be made to yield a fraction of the active material per 100 grams of body weight obtainable from the pars nervosa of adult glands. It is certainly an interesting adaptation of nature that for the time after birth during which the kidney appears to produce little or no diuresis after the administration of water and to be at the same time unresponsive to post pituitary hormone, the gland should contain and presumably manufacture so little.

Glomerular Filtration Rate

It is recognized that the glomerular filtration rate in adults averages about 70 cc/sq.m/min. and that it is relatively unaffected by the hydration of the subject (14), at any rate, so long as he continues to eat. Barnett (10), who employed an indirect method to estimate the inulin clearances of some newborn infants, found that on the basis of surface area the inulin clearances at birth were less than 50 per cent of those in adult life. Young and McCance (134) showed in a few babies that the inulin clearances, i.e., the glomerular filtration rates, were only of the order of 30 cc/sq.m/min. and the recent work has in general gone to confirm these findings and to show that, while the rates may be very variable from one baby to another, the average figures rise with age to approach the adult levels by the end of the first year or 18 months of life. Thus Dean and McCance (31) found the glomerular filtration rates averaged 28 cc/sq.m/min. in babies a few days old. West, Smith and Chasis (125) and Rubin, Bruck and Rapoport (102) have found the mannitol clearances in very young infants to be much lower than those of adults. The former authors considered that adult levels were reached as early as the second month but the latter not till the second or third year. Somewhere between the two will probably turn out to be true for the *average* baby, but large individual variations must be expected.

After examining a small series of infants McCance and Young (84) considered

that the glomerular filtration rates varied with the hydration of the baby. Very similar results were obtained quite independently by Barnett (10) who made an elegant study of a baby with extravertion of the bladder (12). Both ureters were catheterized and the kidneys compared. This dependence of the glomerular filtration rate upon the hydration of the animal is not peculiar to the human infant. It is shown by the adult rabbit (56, 127) and by other species. It may (40) or may not (47) be true of the rat. Recent work in the United States, however, has not demonstrated any close relationship between the volume of the urine and the glomerular filtration rate (11), but some of this work was done with mannitol as the index of glomerular filtration and this substance acts as an osmotic diuretic, so that few really low minute volumes were at first obtained. Barnett *et al* (13) have now reverted to inulin as the index of glomerular filtration rate and shown that by varying the water intake of premature infants the inulin U/P ratios could be made to vary from 3.9 to 127. The latter figure indicates that the glomerular filtration rates must have remained high even when the urine volumes had fallen to low levels. Babies have still to be tested by the new techniques under conditions of real dehydration and this should be done from the clinical point of view for, if it were to be established that the glomerular filtration rates fall to low levels in really dehydrated infants, then it would be easy to explain the grossly abnormal serum chemistry often found in them.

Urea Clearances

Urea clearances have been the standard tests of an adult's renal efficiency for a number of years, and a great deal is known about them. At urine volumes over 2 cc/min. the clearances are at a maximum value which is about 72 cc/min. As the urine volumes fall the clearances also fall and at 1 cc/min. average 54 cc. At urine volumes of 0.3 cc/min. the clearances are of the order of 34 cc. McCance and Young (84) determined the urea clearances of healthy babies of various ages and found that in the first 14 days of life they were far below those of adults when the two were compared on the basis of surface area. These findings might possibly have been predicted from some work of Stransky and Bálint (115) on Ambard's coefficient in infancy, or from what has already been said about the glomerular filtration rates and been confirmed by all subsequent investigators (102, 125).

McCance and Young (84) found, as might have been expected, that on the whole the urea clearances in infants varied as they did in adults with the degree of hydration of the subject, but Gordon, Harrison and McNamara (42), who have also determined the urea clearances in the early days of life and agree about their being so much lower than those of adults, did not find that they varied with the minute volumes of the urine. The original findings have, however, been confirmed by Barnett *et al* (13) and by Dean and McCance (unpublished data). Young and McCance (134) and Gordon *et al* (42) found that the urea clearances rose towards the adult levels as the babies grew, and Young and McCance (134) showed that they were approaching adult levels towards the end of the first year of life (102).

TUBULAR FUNCTION

Creatinine Clearances

In animals other than man and the higher apes the exogenous creatinine clearances are equal to the inulin clearances (112). In adult human beings the exogenous

creatinine clearances are greater than the inulin clearances. The ratio of the one to the other is of the order of 1.3 and some creatinine is generally considered to be excreted by the tubules. The endogenous creatinine clearances are said to be equal to the inulin clearances but, since some of the chromogenic material is most probably not creatinine, it is impossible to say what scientific value should be assigned to these endogenous creatinine clearances. Brod (17) has examined the endogenous creatinine/mannitol clearance ratios in infancy and found them less than unity and Dean and McCance (31) studied the exogenous creatinine/inulin clearance ratios in babies a few days old and found them to average 1. Both these results suggests that at birth the tubule cells have not yet developed the property of excreting creatinine which they will acquire in later life.

Diodone (B.P.) and p-Aminohippuric Acid Clearances

These substances are excreted to some extent by glomerular filtration but they are also actively excreted by the tubules. Their clearances have been used to measure the blood flow through the kidney by virtue of the fact that if the quantity of one or the other of them in the plasma is small, the whole of it is removed in one passage through the kidney. The maximum amount which the tubules can excrete from high plasma levels (Diodone or p-aminohippuric acid Tm) has been used as a measure of the active tubular mass. Dean and McCance (31) measured the diodone clearances from low plasma concentrations in the first four days of life and found them to be low by adult standards on the usual surface area basis. They obtained an average value of 66 cc/1.73 sq.m/min. against one of 600 cc/min. for adults. They also found the diodone/inulin clearance ratios to be low, which suggested to these authors that the diodone was being excreted much less readily by the tubules in infancy than in adult life, and that its clearance could not be used to measure the blood flow through the kidney at this age. The inulin/diodone clearance ratios, or the so called filtration fractions of Dean and McCance, averaged 0.42 and those of Barnett *et al* (13) 0.34 in 4 premature infants under 14 days of life. The average for adults is much lower. Workers in Homer Smith's Department (125) and also Rubin, Bruck and Rapoport (102) have studied the clearances and the Tm values of p-aminohippuric acid in older babies and communicated their results to the Fifth International Congress of Pediatrics. At the earlier ages they also found low clearances and Tm values for p-aminohippuric acid; as the infants grew the clearances and Tm values rose and approached adult levels towards the end of the first year.

Mineral Clearances

Very much less is known about mineral than about urea, inulin or creatinine clearances and no adult standards have been laid down for potassium, sodium or chloride. The clearance of a true non-threshold substance such as inulin should only be affected by the glomerular filtration rate and perhaps by the minute volume, but the clearance of a threshold body such as glucose or chloride depends not only upon the glomerular filtration rate and the minute volume, but also upon the concentration in the serum and the reabsorptive activity of the tubules. This last is partly controlled by the suprarenal cortex—at any rate so far as sodium, chloride and potassium are concerned—and it is not possible to make any measurement of the extent of this.

Variations in suprarenal activity, however, are probably responsible for some of the puzzling facts about mineral clearances. The rate of excretion of NaCl, for instance, often appears to depend upon intake rather than serum level, and Borst (15) has recorded variations in chloride output which are the very reverse of those which a knowledge of serum values would have led one to expect. McCance and Young (84) determined the sodium, chloride and potassium clearances in a number of newborn full-term babies and they showed that the clearances were much lower than those of adults. On the usual basis of surface area the figures for infants tended to be about one-fifth of those of the adults tested at the same time. It has already been pointed out that the concentrations of these elements in the sera of infants are fully as high as or higher than in those of adults, so that the cause of the low clearances must lie in the kidney itself or in the ductless glands which control the excretion of these ions by the kidney. The low clearances indeed may be the reason for the high values found in the blood and the large volume of extracellular fluids at this age. They may also be responsible for much of the edema which affects premature infants. Young, Hallum and McCance (133) found that the Cl clearances were much lower in premature than in full-term infants. At all ages the Cl clearances tended to fall as the volumes of urine passed per minute decreased. Young and McCance (134) found that the K clearances also varied with the minute volume. It is still not known whether the low glomerular filtration rates will fully explain these low clearances or whether the tubules reabsorb more of the minerals from the glomerular filtrate in infancy than they do in adult life. A further analysis can only be made when the glomerular filtration rate and the sodium and chloride clearances have been determined simultaneously.

Some small amount of attention has been given to the rate of excretion of P and its clearances in adults. Phosphate clearances have been found by several people (33, 93) to be of the order of 10 to 11 cc/sq.m/min., but there are many curious features about them which are so far unexplained. They have been found to be lower in the forenoon than at other times of the day and night (38, 65). Dean and McCance (33) found the phosphate clearances of infants less than 14 days old to be greater when the minute volumes were large than when they were small and to range from 0.12 to 3.7 cc/sq.m/min. This range is far below the average for adults and it is evident that these clearances resemble all others so far investigated in being very much lower in infancy than they are later in life. It is not known at what age the phosphate clearances approach the adult levels, but one hydrocephalic child aged 5 months investigated by Dean and McCance had a phosphate clearance of 7.5 cc/sq.m/min., which is within the adult range. It is to be noted that these low clearances may be the explanation of the high serum phosphorus characteristic of infants and young children, but until simultaneous glomerular filtration rates and phosphate clearances have been carried out on young infants it will not be possible to state whether the glomerular filtration rates are low enough to account for the clearances, or whether exaggerated tubular reabsorption also plays a part.

Osmotic Diuresis

If a strongly hypertonic solution of urea or sodium chloride is administered to a hydropenic adult or to a full-grown rat which has been deprived of water for 12 hours,

a very characteristic response is evoked. This response has been investigated by McCance and Young (85), Chambers *et al* (22); McCance (78); Hervey, McCance and Tayler (50, 51), and by McCance and Wilkinson (83). It consists of *a*) a diuresis which may be very intense for a time but always subsides before all the NaCl has been excreted and *b*) a fall in the osmotic pressure of the urine in spite of the hypertonic state of the animal's body fluids. Hervey, McCance and Tayler attributed this fall in osmotic pressure to the fact that the distal tubules were only capable of carrying out a limited amount of osmotic work per minute so that when excessive quantities of osmotically active material were passed on to them by the proximal tubules as an isosmotic solution, they were unable to remove enough water to raise the osmotic pressure of the large volume of fluid to the height usual in dehydration. The response of a newborn animal to similar treatment differs considerably from that of an adult of the same species and newborn rats which were investigated by McCance and Wilkinson show these differences to a much greater degree than human infants. When 5 per cent of the body weight of 10 per cent NaCl or 20 per cent urea was administered to newborn rats by stomach tube, the young animals passed very little additional urine, although the changes in the serum chemistry were as great as or greater than in the adults. The urine of these newborn animals, moreover, which has always got a much lower osmotic pressure than the urine of the hydropenic adult, did not become more dilute but tended to become more concentrated. The absence of any appreciable diuresis means that the newborn animal is incapable of doing much if anything to restore the internal environment to its normal composition. Dean and McCance (32) have investigated the responses of newborn infants to similar treatment. In them a diuresis of small magnitude is obtained and the osmotic pressure of the urine may rise slightly or remain unchanged. The percentage of the dose of NaCl which is excreted is always much less than that excreted by an adult. There is no doubt that these effects and phenomena must be considered in connection with the low sodium and chloride clearances which have already been described and with the work of Aldridge (2, 3), Maizels and McArthur (75), Cooper (25) and others who have so often emphasized the rapidity with which an infant's serum chemistry may become highly abnormal. There is a great deal of quantitative work to be done before the relationships of all these phenomena to each other can be satisfactorily explained.

Acid-Base Regulation

This is a most important function of the kidney but it is a very difficult one to investigate quantitatively. The evidence that babies may remain in a state of acidosis for some time after birth has already been given. A change from breast milk to cow's milk has been said to alter materially the acid-base balance of the serum towards the acid side (72). The reactions of infant urines, however, have not been found to be highly acid. The average pH was close to that of a large group of adults. There are, however, interesting differences between the manner and the facility with which adults and infants excrete acid (79). Infants tend to have higher ratios of ammonia/acidity titrated to the phenolphthalein end point and also higher ammonia coefficients than adults. Both these findings suggest either that their kidneys are

coping with an acidosis or that there are few of the normal adult buffer substances in an infant's urine. It has been known for a very long time (105) that the urine of an infant may contain extraordinarily little inorganic phosphate. This was fully confirmed by McCance and von Finck, who showed that the P/N ratio in infancy was far lower than in adults and that in infancy much less of the titratable acidity was due to acid phosphates, and much more of it to organic acids than later in life. This small excretion of phosphates by newborn infants clearly makes it difficult for them to cope with an acidosis should one arise (96, 97, 98) and may be the explanation of the findings in the serum. The low phosphate clearances are also a part of this story. No one has yet compared the capacity of the tubules to produce ammonia in infancy and in adult life by creating an internal acidosis. This would be an instructive experiment especially in the light of the data which has just been discussed.

Cellular Metabolism

Differences in cellular metabolism must underlie the changes in renal function which accompany development, but so far little work has been carried out on this subject and there is no immediate prospect of being able to correlate metabolism with function. Kay (57, 58) reported that the phosphatase activity of embryonic rabbits' kidneys was low and that it remained below the adult levels for some days after birth. Epps (37) found that the cortex and medulla of the kidney of the newborn child were deficient in amine oxidase, as compared with the same parts of an adult's kidney. The activity of the oxidase had reached the adult level by the time the infant had reached the age of 3 months. Cutting and McCance (28, 29) showed that the oxygen consumption of the kidney cells of newborn rats was a little lower than that of adult cells when the slices were first set up. The oxygen consumption of the kidney cells of newborn pigs, cats and humans was higher than that of adults, but in all species the oxygen consumption of the newborn cells was better maintained. The addition of lactate or succinate to the slices of newborn kidneys raised the oxygen consumption much less than a corresponding addition to slices from adult kidneys. Another difference between the behavior of these slices at the two ages was that the adult slices disintegrated much more rapidly so that far more of the tissue N escaped into the surrounding medium. It was shown in rats that this disintegration could be prevented to a large extent by adding calcium to the medium and this addition made the adult slices behave in other ways more like those of newborn animals. The addition of calcium was without effect upon the slices of newborn animals (30).

Anatomy

Parts of the human kidney are still undeveloped at birth. Many years ago Eckhardt (36) stated that the glomeruli at birth were smaller and much more close together than they were in adults, but he considered that no new glomeruli were formed after full term. Potter and Thierstein (99) found that developing glomeruli could no longer be found after an infant had reached a weight of 2100 to 2500 grams. Thus many premature infants must possess this functional handicap whereas full-term infants should not. Evidence has already been quoted to show that premature infants have lower clearances of urea and salt than full-term babies, but to what

extent this is due to a numerical deficiency of glomeruli (and presumably therefore of nephrons) has not yet been made clear. According to Peter (95) the glomeruli are all formed, but the nephrons attached to those near the capsule are still very primitive and the loops of Henle are very short. The most central nephrons are in a much more advanced stage of development, but even they have many primitive features (88). If the loops of Henle are really the parts of the nephron responsible for the production of a hypertonic urine, these observations might be held to explain the inability of an infant to produce a concentrated urine, but unfortunately any theories about function based upon measurements of the loops of Henle are complicated by the fact that even in adults the loops are of very different length (94).

In some young animals the kidneys seem to be less well developed at birth than they are in man, for Riedel (100) found that in mice and in other animals which were born blind new glomeruli continued to be formed for some time after birth. Yoffey (132) has studied the elimination of trypan blue by the cells in the proximal tubules of growing rats. In newborn animals the nephrons in the peripheral part of the cortex were still undeveloped and the cells took up no dye. The proximal tubules in the deeper layers of the kidney took up dye and the nearer they lay to the medulla the more actively they did so. The ability of the cells to excrete dye was accompanied by the development of a brush border. Some attempt should be made to correlate these histological findings with any changes in enzyme activity which are known to take place at about the same time. The bearing of the histological data upon the function of urine formation, as revealed by studies of the whole animals, will be more difficult to interpret.

Trueta *et al* (121) have recently made some interesting discoveries about the renal circulation. They have confirmed that in the neonatal child the cortex makes up a much smaller proportion of the kidney than it does in later life, and consequently that the medullary and juxtamedullary glomeruli must have a particular significance at that time. They believe that these glomeruli have a blood supply which differs in important respects from those in the cortex. It is possible that the function of these inner nephrons differs from those lying nearer the cortex of the kidney and that something of this kind may underlie the functional differences between the kidneys of newborn and adult animals, but this is a matter of conjecture at present. Trueta *et al* have also demonstrated the development in certain circumstances of large arterio-venous shunts which may exclude most of the cortex from active circulation. These observations may go far towards explaining some of the functional abnormalities of the kidney with which physicians have been familiar for many years (20, 74, 80, 81), but it is difficult to see at present how their discoveries further the study of function in the newborn kidney. Of more importance would seem to be the histological data about the state of the glomerular epithelium. In adult life the glomerular tuft is covered with the thinnest of pavement epithelia so that there is a minimum barrier to filtration. In fetal life, however, the tuft is covered with a layer of tall columnar cells, which must be an effective barrier to ultrafiltration. These cells were described and illustrated long ago by Klein *et al* (64), but they have been redescribed quite recently by Grunewald and Popper (43). At full term the cells are much less conspicuous and are cubical rather than columnar and may not completely

cover the tuft (24). Coincidental with this developmental change in the shape of the glomerular cells there appears also to be one in their staining reactions with silver salts, for as the cells flatten they acquire a fine reticulum of argyrophil fibrils (45). There is thus an anatomical basis for the low glomerular filtration rates and low clearances of early life. In the first place the cortical glomeruli and nephrons may not yet have commenced to function. In the second place the glomeruli, even if functioning, are still partially covered by a layer of cells thick enough to make filtration a relatively slow process.

CONCLUSIONS

In this review a short description has been given of the anatomy, cellular chemistry and physiology of the infant's kidney. It has been shown to differ in all these respects from the adult organ, but it is not yet possible to correlate these differences to any extent or to discuss the functional immaturity of the newborn organ in terms of development, anatomy and cellular metabolism. In the future no doubt it will be possible to do this.

If it is permissible to generalize about the functional differences, it may be said that the kidneys of animals begin to form and to secrete a fluid which resembles urine before birth. They continue to do so after birth and there is probably no great functional development at that time. In the early days or weeks of life the newborn kidney is, however, a less effective organ than it will later become. Thus the glomerular filtration rates are lower and the clearances of all those substances which are excreted mainly by glomerular filtration are necessarily also lower. The tubules have not yet acquired to the full their capacity to excrete creatinine, diodone or p-aminohippuric acid, but their ability to reabsorb sodium, chloride and phosphate ions may develop more quickly than the glomerular filtration rate so that a very high proportion of these ions may be reabsorbed from the glomerular filtrates. Functionally, moreover, the kidney of the newborn animal has little flexibility. It excretes water, sodium chloride and urea, but if the mutual relationships of these in the internal environment are disturbed, the kidney makes a relatively feeble attempt to restore the status quo and this is true even when the disturbance has been very great. There is some slight evidence also that the same may be true of pH.

In the light of these results a study of the capacity and limitations of the kidney in the early days of life seems to be an important subject for research. If all is normal, the kidney of a baby will do the work required of it—Nature has seen to that—but if the child has become dehydrated, acidosed, or otherwise abnormal, or has to undergo some surgical operation, then the duty of the pediatrician must be to assist the kidney to restore and maintain the normality of the internal environment and he requires for this purpose all the knowledge with which the investigator can supply him.

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THE CHEMICAL, PHYSICAL AND MORPHOLOGICAL PROPERTIES OF ANIMAL VIRUSES

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THE DIVERSITY in the biological activities and effects of viruses is well known. The range of pathological lesions and host-cell reactions extends from rapid and total necrosis of affected tissues to the stimulation to uncontrolled proliferation of parasitized cells in virus tumors (96). This versatility of viruses as a group is not cause for wonder, for variety in effects is characteristic of the action of other types of infectious agents. Considering their size, however, there might appear to be less basis for conceiving a comparable diversity in the physical, chemical and morphological attributes of the viral agents. Indeed, from the beginning, there has been a tendency, still continued, toward simplification of concepts of these characters as well as of the biological nature of viruses. The increasing accumulation of data in recent years, however, is rapidly dispelling the illusions of simplicity implied in theories of the enzymic, genic or molecular character of the agents. As one virus after another has been examined, there is added the evidence of further differences and complexity.

Since 1930, several viruses affecting man, animals, plants and bacteria have been isolated and studied in purified preparations. The plant viruses, the findings with which have been discussed on many occasions (126, 7, 129), consist of protein and ribopentose nucleic acid in varying amounts, the latter constituting as much as 40 per cent of the tobacco ring spot virus (127). Only two shapes have been observed, those of the rod and the sphere, and some of the agents are of such uniformity in size and shape that crystals are readily formed. In contrast, the few human, animal and bacterial viruses studied are of greater complexity (121, 41, 9, 91, 10) in constitution than plant viruses and much more variable in external morphology. In addition there is evident an internal structure which likewise differs from one agent to another. It is with the findings obtained with the latter group of agents represented by the viruses of vaccinia, rabbit papillomatosis (119), equine encephalomyelitis (Eastern (140) and Western (77) strains), influenza (human types A (124) and B (33, 71, 34) and the swine type (118, 120)) and Newcastle disease (61, 27, 14) and with bacteriophages that the present paper is concerned.

CONSTITUTION

From the beginning the size range of viruses has been an obstacle not only to direct experimentation but to thoughts of the possible characters of these agents. The status of viruses in this respect has been, and still is in the minds of some, no different from that of organisms beyond or at the threshold of vision in earlier times (93).

It was hard to conceive of entities so small being able to contain the substances of life. Soon after tobacco mosaic virus was first filtered (51), Beijerinck (17) referred to the infectious agent as a contagious fluid. Many have regarded viruses as enzymes incapable of self-determination and formed only by the activity of the stimulated host cell. The experiments and arguments purporting to substantiate these views have been numerous even in recent years. Northrop (85) regarded a bacteriophage of a staphylococcus as an enzyme of sizes, determined by diffusion, varying from 10 to 100 mμ. Kalmanson and Bronfenbrenner (53) considered the T_2 bacteriophage of E. coli to consist of protein associated with only traces of phosphorus and to be enzyme-like in nature. Stanley (125), reporting for the first time the chemical purification of tobacco mosaic virus, called this agent a globulin of the nature of an autocatalytic enzyme.

With the results of more direct examination, some of these findings have not been confirmed and some of the views have languished. Wyckoff (145) could not demonstrate in the analytical ultracentrifuge the variation in the size of phage particles predicted by Northrop; Bawden *et al.* (8) found nucleic acid in the tobacco mosaic virus; and Hook *et al.* (49, 108) and Taylor (134) demonstrated the complexity in the constitution of the T_2 bacteriophage of E. coli, corroborating Schlesinger's (100–103) findings years before with a bacteriophage of E. coli.

The animal viruses exhibit constitutions lacking in but few, though important, respects the chemical constitution of organisms, consisting, in addition to protein and nucleic acid, of lipid components and one, the influenza virus, carbohydrate in excess to that found in nuclei acid. Differences in the proportions of the components among the various agents are profound. For example, the T_2 bacteriophage of E. coli contains 45 per cent of nucleic acid and the influenza virus only about 2 per cent; and differences in proportions are nearly equalled by differences in kinds of constituents. The component constitution of the animal viruses studied and that of the T_2 bacteriophage are summarized in table 1.

The rabbit papilloma virus, the smallest animal agent thus far examined, appears to be the simplest in constitution (11, 135). The nitrogen content, 15 per cent, is indicative of a large proportion of protein, estimated on fractionation as 90 per cent of the whole complex. Whether or not lipid is an integral constituent of the virus is subject to question; 1.5 per cent has been the largest amount extracted from preparations of the agent. The nucleic acid, difficult (135) to free from the whole complex, is relatively large in amount, about 8.7 per cent, and appears to consist wholly of the desoxypentose type.

Vaccinal elementary bodies, one of the largest and most intensively studied animal viruses, differ in some respects but possibly not fundamentally from the papilloma virus. The amount of protein, 89 per cent, is comparable to that in the papilloma virus; the remainder of the complex is almost equally divided between nucleic acid and lipid. Many workers have contributed to the knowledge of the constitution of vaccinal elementary bodies. Protein, lipid and carbodhydrate were demonstrated by Hughes, Parker and Rivers (50); phosphorus was determined and nucleic acid of the desoxypentose type was recognized by McFarlane and Macfarlane (74) who also obtained tests for glucosamine. McFarlane *et al.* (75) found 8 per cent carbohydrate,

indicating the presence of more carbohydrate than that bound in nucleic acid. Hoagland and his associates (42–48) in studies of carefully purified virus confirmed these findings qualitatively. In contrast, however, the value representing the carbohydrate content of thoroughly washed bodies (43) was reduced to 2.8 per cent; and the amount of nucleic acid (42) was 5.6 per cent, apparently all of the desoxypentose type.

The lipid fraction, 5.7 per cent, was fractionated (43) into phospholipid, neutral fat and cholesterol. While the former two were considered to be actual constituents of the virus, questions were raised regarding cholesterol. This material could be

TABLE 1

	WHOLE COMPLEX				LIPID				NONLIPID					REFERENCES
	C	N	P	Carbohydrate	Total	Phospholipid	Cholesterol	Neutral fat	Total	Protein	Carbohydrate	Nucleic Acid		
												INA	RNA	
Broth bacteriophage	42.0	13.5	4.84	13.6	2.6	0	0	2.6	97.4	50.6	13.1	40.3	6.6	134
Synthetic medium bacteriophage	42.3	13.3	5.22	11.7	1.8	0	0	1.8	98.2	52.4	11.2	44.6	1.3	134
Vaccinia	33.7	15.3	0.57	2.8	5.7	2.2	1.4	2.2	94.0	89.0	2.8	5.6		42, 43
Papilloma	49.6	15.0	0.94	6.5	1.5				98.5	90.0		8.7		11, 135
Equine encephalomyelitis (Eastern St.)	62.2	7.7	2.2	4.0	54.1	35.0	13.8	9.6	53.0	49.1	7.2		4.4	237
Influenza A (PR8 Strain)	53.2	10.0	0.97	12.5	23.4	11.3	7.0	5.1	77.5	65.0	7.3	1.5	?	133
Influenza B (Lee Strain)	52.7	9.7	0.94	13.1	22.4	11.2	3.7	7.2	76.4	63.6	9.4	1.2	?	133
Swine Influenza	51.4	9.0	0.87	10.0	24.0	10.7	5.7	7.7	77.6	67.6	10.0	+	?	133
Broth medium bacterium	49.1	13.2	2.72	12.5	7.75	7.75	0	0	92.3	67.9	12.5	5.2	19.1	134
Synthetic medium bacterium	49.0	13.2	2.66	11.6	9.11	9.11	0	0	90.9	67.7	11.6	2.4	20.9	134

All values are percentage dry weight of the whole complex.

extracted with ether from lyophilized bodies further dried over phosphorus pentoxide without loss of infectivity. Inasmuch as approximately 98.5 per cent of the bodies were already inactive (10) due to the drying treatment, the findings of the authors are inadmissable as evidence that cholesterol is not an integral constituent of vaccinia virus. The phospholipid was of the lecithin type containing phosphorus and nitrogen in a 1:1 ratio.

Further examination of the vaccinal elementary bodies showed the presence of 0.05 per cent copper (46) which acted as the catalyst in the oxidation of cysteine. Biotin (45) and flavin-adenin-dinucleotide (47) were present. In the search for enzyme constituents, McFarlane and Salaman (70) demonstrated phosphatase and

catalase, and in addition to these, Hoagland *et al.* (43) found lipase. The latter authors also found that the 3 enzymes could be adsorbed in large amounts on the bodies, and, consequently, the place of these enzymes in the constitution of the virus was equivocal.

The two strains, Eastern and Western, of equine encephelomyelitis virus exhibit unusual and extreme properties (136, 132). Protein and lipid are approximately equally divided, and the nucleic acid, only 4.4 per cent, is entirely of the ribopentose type in contrast to the desoxypentose type of the vaccinia and papilloma agents. The lipid component consists chiefly of phospholipid, 35 per cent, of the diamino-monophosphatide type. Both cholesterol and neutral fat were found. There were no constitutional characters differentiating the two strains.

Among the animal viruses thus far studied, the agent of equine encephelomyelitis is unique in its content of only ribopentose nucleic acid. Both desoxypentose and ribopentose types have been reported to contribute to the structure of the influenza viruses and to that of the T_2 bacteriophage of E. coli. All of the plant viruses examined thus far contain only ribopentose nucleic acid.

The influenza viruses A and B have been studied by several investigators (137, 116, 133, 130, 131, 55, 56, 58) with closely similar results. In constitution the swine influenza virus (139) does not differ greatly from the two types affecting man. The three types of influenza viruses contain about 25 per cent lipid, relatively very small amounts of nucleic acid and carbohydrate in excess of that found in the nucleic acid.

Difficulties have been experienced in the analysis of the small amount of the nucleic acid component. In the earlier studies (137, 116, 139, 133), only the desoxypentose type was reported; later, Knight (55, 56, 58), taking exception to Taylor's (133) methods, reported positive tests for the other type, ribopentose, of nucleic acid. While the desoxypentose type is undeniably there, further confirmation is needed in the case of the ribopentose type.

Though discrepancies were apparent in the amounts of carbohydrate reported by Taylor (133) and Knight (58), the findings uniformly revealed the presence of far more than could have been bound in the minute quantities of nucleic acid. With the carbazole method, Taylor (133) demonstrated mannose or glucose-galactose or both in complex form. These findings were corroborated by Knight (58) who also identified glucosamine. Knight (59) has recently studied the amino acid composition of influenza viruses A and B, reporting differences between the two agents believed to be significant. On the basis of the results of precipitin studies, Knight has suggested (57) that influenza virus may contain components antigenically identical with normal components from chorio-allantoic fluid or mouse lung, depending on the host.

Schlesinger (101–103) was the first investigator to concentrate and purify a virus for chemical analysis. The agent was a bacteriophage of E. coli which he found to contain protein and fat. Phosphorus was also found in a large amount in the initial studies but related only later to the presence of nucleic acid. Northrop (85) found a high content of phosphorus in his bacteriophage of the staphylococcus, but Kalmanson and Bronfenbrenner (53) could demonstrate only traces of the element in the T_2 bacteriophage of E. coli concentrated by ultrafiltration.

The T_2 bacteriophage (25) of E. coli, like the animal viruses, consists (134) of

protein, nucleic acid and lipid, and the amount of carbohydrate present is compatible with the quantity of nucleic acid. One major difference from the animal viruses is the amount of nucleic acid, about 45 per cent. Both desoxypentose and ribopentose types have been reported, the former greatly in preponderance, but Cohen and Anderson (23) have denied the presence of the ribopentose type. To some extent, possibly, the differences may have been related to differences in techniques. By the method of Schmidt and Thannhauser (104), Taylor (134) found 40.3 per cent desoxypentose nucleic acid and 6.6 per cent of the ribopentose type in the phage cultivated on the host grown in broth medium. Only 1.5 per cent of the latter and 44.6 of the former were found in the phage obtained from lysates of the host grown in synthetic medium. The tryptophane method of Cohen (22) did not give a positive test for ribopentose.

A fundamental difference between this bacteriophage and the animal viruses is in the qualitative content of lipid. Whereas phospholipid, cholesterol and neutral fat are found associated with all of the animal viruses except the agent of rabbit papillomatosis, which has not been examined in this respect, the bacteriophage contains only neutral fat, neither cholesterol nor phospholipid being present. The total amount of lipid is small, only about 2 per cent.

Experiments with bacteriophage afford singular opportunities for the study of the relation of virus constitution to that of the host. In microchemical analyses similar to those made on the bacteriophage, fractionations (134) were carried out on the host cell, E. coli, cultured in broth medium and synthetic medium. Though in certain respects the structure of the parasite resembles that of the host, in others there are striking differences. While both consist of protein, nucleic acid and lipid, the lipid differed vastly from that of the virus, consisting wholly of phospholipid of which the virus contained none. Neither cholesterol nor neutral fat, the latter comprising the lipid of the virus, could be demonstrated. Both types of nucleic acid were found but the proportions were the reverse of those in the virus, ribopentose nucleic acid being present in the larger amounts in the bacterium. These findings constitute evidence of physiological independence on the part of the bacteriophage in the processes of reproduction; for the bacterium is able to synthesize phospholipid, none of which is found in the phage and is either unable to or does not form neutral fat which is a constituent of the bacteriophage.

Concentrates of the virus of Newcastle disease reveal (24) a constitution not greatly different from that of the influenza viruses. While the results obtained thus far have been with obviously impure material, no unusual characters of virus constitution were noted.

MORPHOLOGY

Some of the viruses are of a size within the limits of visualization with the light microscope. For many years before viruses were separated into a special category, specific structures, the inclusion bodies, had been seen in the cells of certain diseased tissues. Later, much smaller coccoid bodies were observed in some instances. Borrel (18) in 1904 discovered in fowl pox lesions the barely visible bodies which Woodruff and Goodpasture (142, 143) subsequently found were contained within the inclusion or Bollinger bodies. These workers provided evidence of the identity of the Borrel

bodies with the virus of fowl pox. Similar entities found in tissues affected with other diseases—vaccinia, molluscum contagiosum and psittacosis—have been considered to be the respective viruses and regarded as microorganisms.

Though much was learned by these methods, the various elementary bodies were just visible in the light microscope, and the data were extended but little by use of the ultraviolet light microscope. The enormously greater magnifications of the electron microscope, affording a useful range up to nearly 100,000 diameters, including photographic enlargement, have revealed shape, size and internal virus structure. Dependent on differential absorption of electrons by the various portions of the substance of the virus, electron micrography leaves much to be desired with respect to contrast at the periphery of the particle and internal regions of segregated material. Thus far no way has been found to increase differentiation by staining, though calcium chloride (111, 137) is useful in increasing contrast at the surface. Of extreme value has been the shadow technique (83) applied to the study of viruses (141, 148). The three dimensional effect thus given affords a good idea of the shape of the virus.

Some viruses are essentially spherical. This is particularly true of certain plant viruses, those of bushy stunt of tomato (92) and bean mosaic disease (92, 147). These agents appear extraordinarily uniform in shape and size, properties associated with their capacity to form crystals.

Among the animal viruses, the agents of rabbit papillomatosis (109, 113) and equine encephalomyelitis (111) appear to be spherical or nearly so. Images of these two agents are circular in conventional electron micrographs and seem highly uniform in shape and size. However, the boundaries of the images are indistinct and small variations in size could not be determined if they existed. In shadowed preparations of the papilloma virus, the shadows cast are not as long as those expected of spheres. The shadows cast by the bushy stunt virus (92) indicate a more nearly spherical shape, a possible indication that either the plant virus is more nearly spherical under natural conditions or the changes occurring during drying differ with the two agents. Shadowed preparations of the equine encephelomyelitis virus have not been obtained.

The influenza viruses, A (137, 26), B (116) and the swine agent (139), differ greatly from the papilloma and equine encephalomyelitis viruses. The three types of influenza virus, indistinguishable in electron micrographs, are neither spherical nor uniform in size. In conventional micrographs the images vary in shape from circular to oval and elliptical. Many seem bean-shaped. The evidence of considerable variation in size is corroborated by the diffuse sedimentation boundaries in the analytical ultracentrifuge. Shadowed preparations show forms varying as the images in conventional micrographs. Some flattening of the virus was evident. Mosley and Wyckoff (82) reported filamentous forms, but there is no evidence that they are significantly related to the virus.

A still greater departure from the spherical shape is seen with vaccinia virus. Conventional micrographs (39, 113) show rectangular images of fairly uniform widths but of definite variation in length. Green, Anderson, and Smadel (39) construed their pictures as indicating a brick shape. There is reason, however, to consider the particles as short rods with rounded ends. In some micrographs there occur round images of a diameter equal to that of the width of the rectangular images. Such

images are of high contrast and may indicate particles standing on end. Micrographs of shadowed preparations (113) show again rectangular or biscuit-shaped images. The relatively short shadows seen indicate a marked flattening of the bodies, due, certainly in large part, to much shrinkage on drying. The superior surface of the shadowed particles is not smooth. Instead, there are rounded, mound-like elevations which look like protrusions of internal structure elevating the surface. Substantiating the evidence of rod-forms seen in conventional micrographs, are round images with relatively very long shadows, indications of bodies standing on end. In some conventional micrographs, there are seen outline or 'ghost' forms (39) which can be produced by treatment with alkali. These forms look like the empty shells of the bodies from which the internal contents have escaped.

The elementary bodies of fowl pox (40, 19), canary pox (99, 40), molluscum contagiosum (99, 19), ectromelia (99, 19) and myxoma (19) appear in electron micrographs to be forms resembling those of vaccinia virus. Shadowed preparations of fowl pox virus showed the unevenness of surface contour like that seen with vaccinal elementary bodies (113).

Some of the bacteriophages are tadpole-shaped (98, 68, 69, 113, 49). To one end there is attached broadly a tail structure which, in the T_2 bacteriophage of E. coli (49), terminates in a brush- or disc-like expansion. In most cases the tail has the appearance of rigidity, lying in a line straight with the long axis of the head. In preparations which have been sedimented repeatedly, some of the tails lie at a sharp angle with the head, and many tails are separated from the heads. A most interesting phenomenon is the occurrence of ghost forms with the outline of the whole particles but without evidence of the high electron-absorbing material of the head. Such forms can be produced (4) in large numbers by sonic vibration and are probably analogous to the ghosts seen in preparations of vaccinia virus treated with alkali. These findings provide evidence of a limiting structure containing the internal substance of the phage.

In shadowed preparations, the headpiece appears to be a short rod with conical ends and there is evidence of flattening possibly occurring during preparation of the mount. An interesting appearance is seen (40) in some shadow micrographs of particles washed with distilled water and lightly coated with metal. The images under these conditions reveal a roughened, hourglass surface contour caused, evidently, by protrusion into the surface of the double internal structure described above. Other bacteriophages appear to be essentially spherical, as judged (98, 68) from the circular images in conventional electron micrographs. Such forms do not show the presence of tails.

At the extreme of forms removed from the sphere is the shape of the virus of Newcastle disease (24, 5). This agent is a relatively very long—about 700 mμ—slender, sperm-shaped entity. The head, about one fifth the length of the whole is narrow, oblong or nearly ellipitcal, and one end is continuous in a slender, frequently curved tail which tapers sharply at the distal end. The various curves of the tail-piece give the impression that it might be motile. In some micrographs, confusing forms are evident. There are ring shapes, shapes open in the center and great balloon forms of low contrast. These bizarre shapes are seen especially under conditions

which might result in damage to the particles, such as that caused by repeated centrifugation or suspension in distilled water. The tails are frequently torn from the heads, leaving a frayed region or only a very short remnant of the tail at one end of the head. The ring and doughnut forms have been construed as tails curved upon themselves and the balloon shapes as heads which have taken up water and become swollen. Such swollen forms of low contrast have been seen frequently in influenza virus preparations in distilled water. Pleomorphism of the Newcastle disease virus has been suggested (5), but too little is known to subscribe to this concept.

INTERNAL STRUCTURES

Some of the viruses are very difficult to picture in electron micrographs because of the low electron-absorbing power of their constituents. For this reason, though internal structure is visible in all of the agents causing disease in animals, clarity has not been obtained. The use of calcium chloride in dilute solutions is valuable in enhancing the contrast of the external particle outline in some instances, but internal structure is proportionately obscured. All save external contours is obscured in shadowed preparations.

In images of the papilloma virus (109), there is present a single, circular region of contrast higher than that of the remainder of the particle. The boundary of the region is not well limited but fades into the surrounding substance of the particle. The papilloma virus image has the appearance of a cell with nucleus and cytoplasm somewhat resembling a small lymphocyte. Similar internal structures are present (111) in the images of freshly prepared equine encephalomyelitis virus. In images of particles that have stood in Ringer solution for several days, the internal structure shows up better; here, the substance may be well outlined, circular or oblong, and two regions of segregation may be seen. Thus, under these circumstances, internal structure is not only present but may be varied in shape, size and number of regions in a single particle. Vaccinia virus reveals (39, 113) the presence of one to five regions of high contrast. These are round in conventional micrographs, are scattered through the particle and constitute a relatively small part of the substance of the particle. The mound-like protrusions in the surface seen in shadowed preparations may well represent these structures which may shrink less on drying than the remaining substance of the particle.

The internal structure of the influenza viruses (137, 116, 139, 26) appears to be a single structure of indefinite outlines, more often than not, eccentrically placed. In the bean-shaped particle, the internal material lies near the hilum.

Some of the bacteriophages (98, 68, 69, 113, 49), notably T_2 of E. coli, show unusually clearly the presence of structure within the headpiece. This material, absorbing electrons with high degree, is bow-tie or dumbbell shaped and occupies a large part of the head, there being but little surrounding substance. It was noted above (49) that the surface of phage particles coated with metal under certain conditions is roughened, giving evidence of the internal structures protruding, mound-like, into the surface contour.

The particles of Newcastle disease virus contain (24) in the headpiece internal structure of variable characters. In some the material seems single, in others double

with considerable space between masses. These masses, or portions of them, appear to reach in some instances into the proximal region of the tailpiece.

Though the findings of virus morphology are fragmentary, it is obvious that the particles have the appearance of organisms. It is supposed that the high electron absorbing material contains the nucleic acid present, for nucleic acid carries the atoms of the highest atomic numbers such as phosphorus. The internal materials look like cell nuclei surrounded by protoplasm. In vaccinia virus, which contains a small amount of nucleic acid, the internal structure is small in comparison with the rest of the particle. The opposite is true for the T_2 bacteriophage, for the particle is nearly full of the inner material.

ULTRACENTRIFUGAL CHARACTERS

Those properties of particles determining their rate and manner of sedimentation are size, shape and density. Knowledge of the size or density, provided the shape is not too complex, permits calculation of the other from the sedimentation constant. The sedimentation constant, corrected for the viscosity, temperature and other factors concerned with the suspending medium, is characteristic of the particles examined. Sedimentation diagrams provide a basis for judging the uniformity of particle characters and certain aspects of the purity of the preparations. In the period of a few years before the introduction of the electron microscope, most of the judgment of the physical characters of viruses in this respect was, and frequently still is, given a significance far beyond the limits of justification. Conservatively interpreted, however, sedimentation diagrams provide relatively accurate estimates of many properties of viruses.

If all of the particles of a given preparation are of exactly the same size, shape and density, all will sediment at the same rate, and the descending boundary will be very sharp and clear. Variation in these characters will lead to proportionate diffuseness of the boundary. Two or more different groups of particles in the same preparation, if close enough in sedimentation properties, will give rise to two or more boundaries. It should be noted at the outset that the characters of the sedimentation diagram have no significance whatever with respect to the ultimate nature of the sedimenting particles.

Sharp sedimenting boundaries are seen with several of the animal viruses. The bacteriophage T_2 of E. coli gives an exceedingly sharp boundary (49, 108), indicating a high degree of uniformity in the size, shape and density of the particles. A bacteriophage of staphylococcus concentrated by Northrop (85) and examined by Wyckoff (145) likewise showed very sharp boundaries. With some preparations, slightly less sharp boundaries are seen with the papilloma (13, 11, 84) and both Eastern and Western strains of equine encephalomyelitis viruses (111). In both instances, though relatively high degrees of particle uniformity are evident, there is slight diffuseness.

Vaccinia (12, 3, 89) and influenza viruses (35, 36, 114, 65) give diffuse boundaries indicating a lack of uniformity of sedimentation characters. It is likely that the diffuseness observed is due mainly to differences in particle size, since variations in this character of both viruses are seen in electron micrographs. In the instance of the influenza viruses, the spread of sedimenting boundaries obtained (114) with the

Lamm scale method is closely similar to the spread in size measured in electron micrographs. The whole significance of the very diffuse boundary given by the Newcastle disease virus (24) cannot be judged since it was known that preparations of the agent were not pure. In all instances there appears to be a close correlation between sharpness or diffuseness of sedimenting boundaries and the uniformity, or lack of it, in the sizes of virus particles observed in electron micrographs.

The sedimentation constants of the animal viruses are given in table 2. There is seen a variation from S = 265 × 10^{-13} for the equine encephalomyelitis virus (111) to S = 4910 × 10^{-13} for the vaccinia virus (89). The sedimentation constant for a given preparation of a virus is reproducible (66) with high accuracy even in different laboratories. Nevertheless, different preparations of an individual agent may yield values of rather wide variation. For the papilloma virus variations (84) have been seen between the limits of about S = 265 to S = 290 × 10^{-13}. It seems evident that

TABLE 2

	SEDIMENTATION CONSTANT	DENSITY IN AQUEOUS SUSPENSION	SIZE SEDIMENTATION VELOCITY DATA	SIZE ELECTRON MICROGRAPHS	PARTIAL SPECIFIC VOLUME	WATER CONTENT	REFERENCES
	× 10^{13}		mμ	mμ		% by volume	
Vaccinia	4910	1.16	236–252	222 x 284	0.793		89, 122, 106, 70
Papilloma	278	1.133	60	44	0.669	58	112
Equine encephalomyelitis (Eastern strain)	265				0.839		136
Influenza A (PR8 strain)	742	1.104	116	101	0.822	52.0	115
Influenza B (Lee strain)	840	1.104	124	123	0.863	34.5	115
Swine Influenza	727	1 100	117	96.5	0.850	43.3	115
T_2 bacteriophage (broth)	1022 695			80 x 100	0.655		108, 134

differences of this magnitude are not due to experimental error and that, while the particles of one preparation may be highly uniform, variation must occur either in the sizes or the densities of particles from different sources. Like differences have been observed (12, 3, 89) with other viruses such as vaccinal elementary bodies of different strains and preparations.

A most interesting behavior of the T_2 bacteriophage of E. coli is the occurrence (108) of two separate boundaries depending on the conditions of the experiment. This virus suspended in saline media of pH above 5.8 gives a very sharp boundary of S = about 700 × 10^{-13}. In media of pH below 5.8, there is seen a similarly sharp boundary of S = about 1000 × 10^{-13}. With proper balance of pH near 5.8, both boundaries may be seen at the same time. Both boundaries are associated with full phage activity, and the transition from the one state to the other occurs without change in infectious capacity. The boundary of S = about 1000 × 10^{-13}, occurring in the acid range, can be produced in the alkaline region by the presence of calcium. These phenomena have been interpreted (108) as related to the state of aggregation or dispersion of particles of the tadpole shape.

DENSITY

The density of virus particles as they exist in aqueous media under natural conditions has been a most difficult property to investigate. Theoretically, one of the simplest methods would be the study of change in sedimentation rate with change in density of the suspending medium. A number of such studies has been made from time to time with both bacteria (97) and viruses—bacteriophage (100, 30, 76), vaccinia (15, 16, 31, 122, 75), influenza virus A (31, 67) and the swine influenza virus (115)—but the results have been difficult or impossible to interpret. In all of the earlier studies, materials such as sodium chloride and sucrose were employed for attaining the desired changes in the density of the suspending media. Under these conditions, the density of the virus increased (122, 67, 115) with increase in the concentration of the solute Such a behavior was considered to be due, possibly, to osmotic effects and loss of water from the particles. A material offering possibilities for circumventing such an effect is bovine serum albumin, which has a molecular weight presumably in the region of 70,000 and, consequently, a negligible osmotic pressure in the range of concentrations desirable for the experiments.

Studies with the three types of the influenza viruses and on the papilloma virus have revealed linear relationships between the sedimentation rate and the density of the suspending medium. There was thus no evidence of change in density of these viruses due to the effect of bovine serum albumin. The values obtained in this way, table 2, were 1.104, 1.104, and 1.100 for the influenza viruses A and B and the swine virus (115), respectively, and 1.133 for the papilloma virus (112). These values are much lower than those for vaccinia, 1.16 (122), and influenza virus A, 1.148, obtained with sucrose. The value 1.148 was derived (115) mathematically from the curve of Lauffer and Stanley (67) from which they inferred the value 1.1. It is notable that the density range involved in the second and third decimal places, that is, from 1.100 to 1.199, represents a range of approximately 100 per cent in the sedimenting properties of the virus based on density. From these experiments it is evident that values obtained by materials such as sucrose are of use only in the estimation of possible upper limits. Bovine serum albumin is, unfortunately, unsuitable for density work with some viruses; neither the bacteriophage T_2 (106) nor the tobacco mosaic virus (64) remain in suspension in solutions of the material in the density range required.

Vaccinia virus suspended in concentrated sucrose solutions increases in density for a time and then, on continued exposure to the solution, decreases in density (122). A similar behavior has been observed for the influenza virus A (115). This property suggests that these agents are limited by a semipermeable structure or membrane. In the instance of vaccinia virus there is evidence of such a structure in electron micrographs, and it will be recalled that the headpiece of the T_2 bacteriophage is enveloped in a structure from which the contents of the head may spill to leave ghost forms.

The partial specific volumes determined in the pycnometric studies of the animal viruses thus far investigated, given in table 2, vary from 0.669 for the papilloma virus to 0.863 for influenza virus B. With these values and a knowledge of the density of the particles in aqueous suspension, the water contents, table 2, for the papilloma (112) and influenza viruses A and B and the swine influenza virus (115) are 58 and 52.0, 34.5 and 43.3 per cent, respectively, by volume. Lauffer and Stanley (67) assumed a water content of 60 per cent for influenza virus A.

SIZES

The general size range of viruses is now well known. The ultrafiltration studies by means of graded collodion membranes (29) have given close approximations with viruses which are spherical or nearly so. Serious errors are encountered, as for example with the Newcastle disease virus, where the particles are long. In this case, though, the values 80 to 120 mμ (21) were close to the widths of the heads of the sperm-shaped particles observed (24) in electron micrographs of this virus. A discussion of sizes and methods of study was given by Markham, Smith and Lea in 1942 (72). The more recent values obtained from ultracentrifugal and electron micrographic measurements are in farily close agreement from one laboratory to another. While such values are undoubtedly not precise, they represent approximations close enough for practical uses. In most of the instances, the values can represent only an average for the particles of those viruses which vary in size.

The sizes of the viruses under discussion here are given in table 2. Average values obtained from electron micrographs have been reported for the viruses of molluscum contagiosum, 255 × 178 mμ (99) and 302 × 266 mμ (19); fowl pox, 332 × 264 mμ (99); myxoma of rabbits, 287 × 233 mμ (99); canary pox, 311 × 263 mμ (99); and ectromelia, 232 × 172 mμ (99) and 300 × 210 mμ (19).

ELECTROPHORESIS

Another criterion of virus character and behavior is the manner of migration of the particles in an electrical field. Electrophoresis experiments (1) in the earlier work with various types of instruments gave little quantitative information, in part, because extraneous material in the preparations far outweighed the amount of virus. Significant experiments with some viruses became possible with development of the Tiseleus apparatus and access to purified preparations. Not all viruses are adaptable to the conditions of study with the instrument because of instability, turbidity of the preparation or other reasons. There has been some interest in possibilities of virus purification by the method but little has been done in this respect.

Several investigators have worked with vaccinal elementary bodies. McFarlane (73) observed boundaries which were not sharp and which exhibited a streaming effect that was thought to be due to endosmosis. Other workers (117, 123), however, demonstrated that the phenomenon was due to density of gradient effect that decreased when the virus concentration was raised to 0.5 per cent and disappeared when serum protein was present. Under these conditions sharp boundaries were obtained. With the Northrop-Kunitz (86) microcataphoresis method, the isoelectric point appeared (12) to be in the region of pH 4.36 to 4.6. At pH 7.9 the mobility in the Tiselius electrophoresis apparatus was about 10×10^{-5} cm/sec. per volt/cm. The virus is inactivated relatively rapidly below pH 5.5, which is still well above the isoelectric point.

The clearest and sharpest boundaries for an animal agent were those obtained (110, 107) with the virus of rabbit papillomatosis. The virus is stable both above and below the isoelectric point, which was observed (13) to be about pH 5.0 in microcataphoresis experiments. In the pH regions adjacent to 5.0 the virus agglutinates, but studies could be made on active virus at pH 3.77 to 4.0 and 6.54 to 7.77. In the

acid region there was rapid cathodic movement of an exceedingly sharp single boundary. Seemingly more diffuse boundaries moved much more slowly toward the anode in the alkaline region. Analysis of the Svensson curves in this region, however, showed that the heterogeneity values (107), H, were similar in the two regions. The findings were considered to indicate a high electrophoretic homogeneity of the virus in the acid region and but little less homogeneity in the alkaline range. The mobility was from 3.85×10^{-5} cm/sec. per volt/cm. at pH 3.78 to -1.03×10^{-5} cm/sec. per volt/cm. at pH 7.77, and the points were compatible with a smooth curve including the isoelectric point at pH 5.0.

Sharp single boundaries are obtained with influenza virus A after special efforts at purification (79). Otherwise, a second boundary related to contaminating extraneous particles presumably derived from chorio-allantoic fluid is seen. The isoelectric point, in microcataphoresis experiments, was at pH 5.0. In the alkaline region, where the virus was stable with respect to infectivity, the mobilities were from -0.67 to -0.66×10^{-4} cm/sec. per volt/cm. between pH 9.3 to 7.4. At pH 3.0 and 2.8, the virus, which must have been inactive, gave mobilities of 0.87 and 0.97×10^{-4} cm/sec. per volt/cm. All of the points, nevertheless, fell on a smooth curve drawn through the isoelectric point. Influenza virus obtained (56) from mouse lung exhibited a more variable behavior.

STABILITY

A relatively uninvestigated field is that related to the stability of animal viruses under various physical and chemical conditions. This property has been most studied in relation to the effects of hydrogen ion concentration, principally in order to know how to protect and preserve the agents during and after the purification procedures. The results of such studies have been reported for vaccinal elementary bodies (12), the papilloma (13), equine encephelomyelitis (32, 138) and influenza viruses (78, 79) and the T_2 bacteriophage (108) of E. coli. In general the animal viruses are unstable below about pH 5.5 to 6.0 and above pH 9.5. The papilloma virus is a notable exception for, like some of the plant viruses, it is stable below the isoelectric point. Indeed, the greatest stability is seen in the acid region from about pH 3.5 to neutrality. It is a general finding that particle integrity enjoys a wider range of stability than the quality of infectivity.

A field but little studied yet is concerned with the effects of salts, both of kind and concentration, on animal virus stability. The papilloma virus agglutinates at salt concentrations much below 0.05 M but disperses readily and is stable in 2 per cent NaCl solution. Vaccinal elementary bodies agglutinate (87, 12) and are damaged (12) in 0.9 per cent NaCl solution and in distilled water. Buffers—phosphate, citrate (87) and others (12)—of 0.005 M concentration appear optimum. Equine encephalomyelitis virus is more stable (136) in Ringer solution than in NaCl solution (144) and, perhaps, more so in the presence of cysteine (6). The virus agglutinates in distilled water. The influenza viruses are stable in Ringer solution (137, 139, 116) or 0.1 M phosphate buffer (54). Damage occurs in phosphate buffer of 1.0 M or 0.001 M concentration or in distilled water, in which the virus is not dispersed (133). A rather detailed study (60) showed that this virus was not

damaged by several reducing agents, except ascorbic acid; was inactivated quickly by phenol, 0.5 N; and was weakly affected by sulfathiazole sodium.

COMMENT

There have been many discussions (2, 38, 52, 37, 93, 94, 7, 128, 80, 146) in recent years concerned with the nature of viruses, most of which were written before much progress had been made in the study of the animal agents. None of the present day views is new in principle though some variation has occurred in the details. These concepts, familiar to all who have followed the literature describing the physico-chemical studies on plant viruses, have been reviewed in detail by Rivers (94) and were discussed critically by Bawden (7). In brief, there are those who believe that viruses are molecular entities and others who consider them as organisms. Recently Burnet (20) has discussed the biological reasons for regarding viruses as organisms. Stanley has been the outstanding proponent of the molecular theory. Precisely what the term molecule is intended to mean, aside from the strictly chemical implications, is difficult (94) to judge from Stanley's discussions. From time to time there have been reiterated (128) the views of many writers extending through the gamut of autocatalytic enzymes and molecular nucleoproteins to comparisons of viruses with genes. Regardless of what has been meant, many have inferred that the molecular view has been synonymous with the concept that viruses may be inanimate 'chemical agents' (95) though some authors have spoken directly of 'living molecules' (62).

Belief that some of the plant viruses are molecular is based on interpretations (62, 63) with respect to homogeneity judged on a number of criteria, uniformity of chemical findings, sharp sedimentation boundaries, sharp electrophoretic boundaries and the capacity to form crystals. It is very clear that, although high degrees of uniformity are evident within the limits of the methods employed, the criteria are not of sufficient precision to permit the conclusions drawn. There is no doubt that many of the smallest viruses must be of relative quantitative simplicity in structure because of the limitations of size, but that any virus consists of a single compound "made up of the same number of atoms arranged in the same way" (62) not only has not been established but is incompatible with the known variability and adaptability of viruses with changing conditions (20). Of course, homogeneity is not an essential criterion of molecular structure, but it is the basis which has been employed for judging the plant viruses to be molecules. It is obvious, however, that on the basis of homogeneity, the animal viruses are not molecular. If a molecule is just any unit specifically organized to perform a given function or which constitutes a stable aggregate (88), then viruses, as well as bacteria and all cells, might be so designated.

Actually, whether some of the smallest viruses may approach molecular proportions is not of fundamental importance with respect to the principal questions of the biological nature of these agents—whether they are to be classed as a special category of living organisms, their modes or reproduction and the relations of their physiological activities to those of the host cell. If the term molecule is not meant to imply an inanimate nature of viruses, the concept immediately loses significance and meaning with respect to the place of viruses in the biological scale.

It would seem that the findings with the animal viruses and the bacteriophage

should give little reason for confusion. The data obtained present a series or system continuous with the findings of other known entities, organisms and cells. The size range of animal viruses studied in some detail extends from about 250 mμ—vaccinal elementary bodies—to about 60 mμ—the papilloma and equine encephelomyelitis viruses. Between these limits of size there is no evidence of a break or gap in fundamental constitution, and in no instance, except for the size of the agents and the physical behavior of particles of this size, have the data diverged from the pattern of the qualitative character of organisms and cells. A specific comparison is that between the T_2 bacteriophage and its own host, E. coli. In this particular case qualitative component differences between the virus and the host cell indicate autonomy of the former in the process of reproduction. The nucleic acid content of the animal viruses varies from 8.7 per cent in the papilloma virus to about 2 per cent in the influenza viruses. In certain streptococci (105) the amount of nucleic acid is in the range of about 20 per cent, a value similar to that for E. coli. The content, about 45 per cent, of nucleic acid in the T_2 bacteriophage is considerably higher than that of its host and of these streptococci but is comparable with the amount in sperm and thymus gland cells (81, 80). It is possible that bacteriophages in general may be characterized by high contents of nucleic acid since the agents of this group from several sources contain large amounts of phosphorus. It is apparent that the physico-chemical findings with these viruses have closed no gaps between the animate and the inanimate; instead a known field has been broadened, and the problem of the biological nature of infectious agents of this size remains as great as ever.

The results of the analyses present no evidence confirming the belief that viruses may represent or be similar to 'genes' (28, 2). On the contrary the proportions of nucleic acid, and consequently 'genic' material, in the animal viruses are in general smaller and in no instance greater than those of organisms or cells (80). The internal structure, presumably representing condensations of nucleoprotein and a nuclear apparatus, is small in comparison with the whole particle; and there are clear regions corresponding to the cytoplasmic structures of organisms and cells. In the bacteriophage, which contains much nucleic acid, the internal material occupies a large part of the particle. Evidence of some sort of limiting peripheral structure having the appearance of a wall and the behavior of a semipermeable membrane in some instances is reason to doubt 'nakedness' of the particles. Rather than such a concept, it appears likely that viruses contain a proportionate amount of determinative material set aside within the virus cell in a unit not differing fundamentally from the analogous units of other organisms.

The group of animal and bacterial viruses thus far studied, though small in number, exhibits a diversity in chemical constitution which may well approach the limits to be expected for all of the agents of these two groups. There is the possibility that some of the agents of animal and bacterial disease, like the plant viruses, will be devoid of lipid. In neither the T_2 bacteriophage nor the papilloma virus is fat a large component, but if the fat is regarded as a vital part of these two viruses, all of the agents studied in both groups are complexes consisting of protein in association with nucleic acid and lipid and, in one instance, with carbohydrate in excess of the amount bound in nucleic acid. Reference to these agents as nucleoproteins, as frequently

practiced, is confusing and misleading, for the lipid is unlikely to be a constituent of nucleoprotein, and, further, there is no evidence that all of the protein and nucleic acid are bound in a single compound. With the information now at hand, such a usage of the term nucleoprotein is no more warranted with respect to viruses than it would be to refer to a bacterium, a liver or an oyster as a nucleoprotein.

Illusive reasonings are no longer provocative or helpful in the field for nearly everything has been said. The tools for further study are at hand, and numerous directions for new investigations are obvious. Many viruses must yet be purified, if no method is devised for their culture in simple media, before enough will be known to permit classification on a physico-chemical basis. Work in the field of the immunochemistry of viruses (57), which may tell much of virus structure and relationships, has barely begun. The usefulness of the electron microscope in physiological and morphological studies of viruses as well as bacteria is only beginning to be explored (148). Only a few investigations have been made of the physiological relations of virus and host-cell (25). Beginnings have already been made in the developments of techniques of the electron micrographic study of some aspects of virus growth and reproduction (149). As Burnet (20) has suggested, there is but one way to settle the questions, and that, undoubtedly will be the hard way of gathering the experimental data.

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THE RELATION OF NUTRITIONAL DEFICIENCIES TO GRAYING

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THE LITERATURE on the physiology, morphology and pigmentation of hair has been reviewed by Bloch (1), Danforth (2) and Urbach (3). Although considerable work remains to be done to clarify these fields of knowledge the following points appear to be fairly well established.

The color of hair is normally caused by the deposition of pigment (melanin) into the hair shaft from the follicle during growth of the hair. Different colors and shades are caused by differences in the color of the pigment deposits and also in the way in which the pigment is diffused or concentrated in granules. White hair contains no pigment. Graying of normally pigmented hair is apparently caused by a failure of the hair follicles to deposit pigment in the hair at the time the hair is being formed.

Pigmentation of feathers has been extensively studied by Willier (4). The chick embryo provides a rather ideal medium for study of the relation of genetic factors to pigmentation. Willier has concluded that the ability to form pigment exists only in certain cells, the so-called melanophore cells. These cells arise from the neural crest and migrate into the skin and its derivatives—the hair and feather follicles. In the absence of melanophores the hair and feather structure is otherwise normal but without pigment. When melanophore cells were removed from the neural crest of embryos of Barred Plymouth Rock chicks and grafted to wing buds of White Leghorn embryos, the White Leghorn chicks developed the pigmented wing feathering characteristic of Barred Plymouth Rocks.

According to Willier the specific color or pattern produced in feathers depends on the genetic constitution of the melanophores and partly on the physiology of the feather follicle, i.e., its growth rate etc. Experiments were cited which indicate that the melanophore cells which arise from the neural crest migrate laterally and invade the skin, the hair and feather follicles and furnish melanin to these structures. This is true both in mammals and in birds and is thought to account for the characteristic coloring of animals in general.

Graying, depigmentation or failure of pigmentation of hair has been variously referred to as 'achromotrichia' (5), 'canities' (6) and 'achromachia' (7). The word achromotrichia, or simply graying, will be used throughout this review to describe the condition of 'absence of pigment from hair or feathers'. These terms have received widespread use in recent scientific literature. There appears to be general agreement among nutritionists that graying of fur or feathers may be brought about in many species of animal, usually during the stage of growth, by imposing deficiencies of certain specific mineral elements or factors of the vitamin B-complex. Various names have been used in referring to these pigmentogenic nutritional factors normally

present in foods, such as trichochromogenic (8), anti-gray hair, anti-achromotrichia and chromotrichia (9) factors. For purposes of this review the various nutritional factors which appear to be needed for normal pigmentation processes are referred to in general as pigmentation factors, or as factors necessary for pigmentation of hair, feathers or skin.

Sufficient experimental evidence is not at hand to evaluate all of the various possible causes of graying in man. Barahal (10) has cited instances to indicate that extreme psychotic and psychoneurotic states or cerebral trauma may predispose to rapid and premature graying. He lists as possible causes of gradual premature graying the following: heredity, emotional strain, endocrine disturbance, dietary deficiency and the chronic diseases—tuberculosis, syphilis, malaria, diabetes, Addison's disease and Simmond's disease. He further reviews the possibilities advanced by others that rapid graying of hair may in some instances be associated with physical changes of the hair, such as thickening and opacity of the outer layer and the presence of air bubbles in the hair itself. It should be noted that as early as 1910 Stieda (11) discredited many of the famous instances of literature and history which tell of sudden graying. The American Medical Association (12) likewise early endorsed the viewpoint that sudden graying of hair is a virtual impossibility.

The finding that hair and feather color can be greatly influenced by nutritional controls in growing experimental animals has led to much speculation with regard to the possibility of arresting or correcting graying in humans. On purely theoretical grounds Hrdlicka (13) repudiated the idea that the influence of essential nutrients or drugs can have more than a temporary stimulating effect on the normal pigmentation processes. He stated empirically that graying is a normal symptom of the aging process and is an outward symptom of declining metabolism. Gibbs (9), however, pointed out that seasonal changes in the color of a variety of animals is a normal process not related to age and that there are many reasons to assume that the pigmentation processes are subject to drug action like most other body functions. The two viewpoints are not in strict opposition and the interesting question whether graying can be delayed in the aging human by nutritional or other means has yet to be answered.

Although this review deals chiefly with reports concerning nutritional studies, there is included a brief discussion of the chemistry of pigment formation, and an attempt is made to correlate some of the facts in a general way. Brief reviews on various phases of the problem have appeared from time to time in *Nutrition Reviews* (14).

EARLY EXPERIMENTAL OBSERVATIONS

Although Stieda (11) predicted it in 1910 the observation that deficiency in diet may predispose to graying was not made until 1922 when Niemes and Wacker (15) reported graying in black rats fed milk and starch. The graying was cured in a few weeks when the rats were returned to a stock diet. At about the same time Hartwell (16) in England observed fawning in rats on summer milk but not on winter milk when they grew less rapidly. She suspected a protein deficiency, particularly deficiency of tyrosine and tryptophan and reported the curative effect of adding 'food

casein' to the bread and milk diet. Hartwell made the interesting observation that there must be a small store at birth of pigment-forming essentials since only one suckling rat out of about a thousand litters developed a gray coat on first growth of fur.

Bakke, Aschehoug and Zbinden (17) in 1930 described graying of rats on synthetic diets containing wheat germ as a source of the vitamin B-complex. Since the use of whole wheat in place of wheat germ did not cause graying, these authors concluded that wheat germ probably contains a toxic factor which predisposes to unnatural graying. They apparently let the matter drop at that.

In 1931 Keil and Nelson (18) in studying the effects of copper deficiency produced by feeding milk diets reported the interesting fact that copper deficient rats become depigmented. When copper was supplied there was a gradual return to normal hair color. In 1934 Gorter (19) at Delft, Holland, produced graying in cats, rabbits and rats on a multiplicity of diets. From his extensive experiments Gorter concluded that copper was the chief, if not the only, active principle in the curative substances he examined. Gorter's work is difficult to assess in light of recent findings because the diets he used were varied and ill-defined. Sjollema (20) in Denmark reported in 1938 the concomitant occurrence of anemia and depigmentation in cattle on copper deficient pasture.

Zinc, also, appears to be concerned in the mechanism of pigmentation. Stirn, Elvehjem and Hart (21) found that acute zinc deficiency caused retardation of growth and depigmentation in young albino rats. Both deficiency symptoms were rapidly alleviated by administration of minute quantities of soluble zinc salts. The fact that zinc has been found in considerable amount in hair (22) is interesting in this regard, although little is known of the mechanism of its action.

FACTORS OF THE VITAMIN B-COMPLEX

That organic as well as inorganic nutrients are involved in graying has been shown by a large number of workers but was specifically referred to by Free (23) who demonstrated the inefficacy of B-complex factors to cure copper deficiency achromotrichia in rats and the equal inefficacy of copper to cure achromotrichia induced by B-complex deficiency.

Widespread interest in the problem from the nutritional side did not come, however, until Morgan, Cook and Davison (24) and Lunde and Kringstad (25) in 1938 reported graying in rats maintained on synthetic diets deficient only in the filtrate factors of the vitamin B-complex, i.e., factors other than thiamin, riboflavin and pyridoxine, and not adsorbed on Fuller's earth. Synthetic diets with casein, sucrose, salts, corn oil or other unsaturated fat and cod liver oil plus thiamin, riboflavin and pyridoxine induced graying in black and piebald rats in six to seven weeks. Animals on such diets, however, showed various gross deficiency symptoms, all of which were curable by addition of crude filtrate fractions. Both groups (26, 27) made the pertinent observation that yeast and yeast extracts always promoted good growth, but did not always cure graying. Likewise wheat germ and cane molasses induced rapid growth, but only late and incomplete cure of grayness, while rice bran and liver filtrates had less effect on growth but caused prompt darkening of the fur. Morgan

and Simms (27) produced nutritional achromotrichia in rats, dogs and guinea pigs on similar diets deficient in the 'filtrate fraction' of the vitamin B-complex. Both of the above groups (28, 29) also reported achromotrichia in the silver fox on diets designed to be specifically deficient in the so-called 'anti-gray hair vitamins'. Lunde and Kringstad (30) stated that they could distinguish the anti-graying factor from the growth factor by the slightly greater instability to heat of the former.

Oleson, Elvehjem and Hart (5) reported in 1939 that pantothenic acid concentrates were ineffective in preventing nutritional achromotrichia. Early in 1940, however, Gyorgy, Poling and Subbarow (32) indicated the curative effect of pantothenic acid in concentrates of that vitamin, but concluded that it is not the only factor involved. Later György and Poling (33) reported practically complete cure of graying in 5 to 7 weeks with daily doses of 75–100 μg. of synthetic d(+)calcium pantothenate. Still later (34) they showed some effect for biotin as well, but stated that even with biotin plus pantothenic acid, the pigmentation was still not entirely normal.

Nielsen, Oleson and Elvehjem (35) announced in 1940 that they had succeeded in isolating a highly active crystalline chromotrichial substance of unknown nature. In the same year Unna and Sampson (36) claimed clear-cut cure of graying in rats on synthetic diet with calcium pantothenate at 100 μg. per day. Considerable evidence was forthcoming, however, that pantothenic acid is only one, although it is probably the most important, of the filtrate factors needed for normal pigmentation. Dimick and Lepp (37) indicated the presence of additional chromotrichial factors in rice bran extracts and Williams (38) reported the inadequacy of pantothenic acid alone to cure graying. Frost, Dann and Moore (39) showed that the curative capacity of liver and yeast extracts is not entirely dependent on their pantothenic acid content, but that liver fractions containing relatively small amounts of pantothenic acid may be curative. They postulated the presence of an additional factor in yeast and liver which appeared to potentiate the action of pure pantothenic acid.

Early in 1941, Ansbacher (40) announced that p-aminobenzoic acid is a vitamin, and an achromotrichia factor as well. He reported that rats became gray on his ration supplying a high level of all of the known B-complex factors and that blackening occurred in one month following administration of 3 mg. daily of p-aminobenzoic acid. Later Martin and Ansbacher (41) reported cure of achromotrichia induced by hydroquinone toxicity in mice. In these experiments 0.8 mg. daily of p-aminobenzoic acid was reported to cure the graying more effectively than rice bran concentrates. Emerson (42) and Unna, Richards and Sampson (43) soon reported negative results with p-aminobenzoic acid alone or in combination with pantothenic acid and were unable to produce graying in rats with a diet similar to the one Ansbacher had described. Experiments at Wisconsin (44) and in this laboratory (45) also were entirely negative with p-aminobenzoic acid. The literature on p-aminobenzoic acid has been reviewed by Ansbacher (46).

The complexity of the graying and pigmentation mechanisms assumed even greater proportions in 1941 with the findings that dietary adequacy of cystine, choline and sodium chloride may be involved together with the fore-mentioned compounds. Insufficiency of cystine supply in the ordinary synthetic diet containing 18 per cent

of washed casein was indicated by Pavcek and Baum (47) who reported that administration of 70 mg. daily of cystine to grayed rats augmented the curative effect of calcium pantothenate and stimulated growth above that on pantothenate alone. Owens, Trautman and Woods (48) reported that choline deficiency results in the rusting of fur in albino rats, as does deficiency of pantothenic acid, and suggested that this may have some bearing on the achromotrichia problem. In this regard the effect of inositol on hair growth in mice (49) and in rats (50) should not be overlooked, although no evidence has yet been forthcoming that this factor has to do with pigmentation. Ralli, Clarke and Kennedy (51) reported that the amount of salt added to the filtrate factor-deficient diet influenced the incidence, extent and degree of graying in rats. Rats on low salt intake grayed 55 days sooner than those on high salt intake. Changes were also seen in the adrenals of these animals. This finding added to the already strong evidence that adrenal function is concerned with pigmentation.

Following the more or less general agreement among nutritionists that p-aminobenzoic acid was not concerned to a prominent degree in the normal pigmentation of experimental animals (42–44) came the report of McGinnis, Norris and Heuser (52) that an unidentified factor in brewer's yeast, other than pantothenic acid, is necessary for the normal feathering and pigmentation of Rhode Island Red chicks. Martin (53) and Wright and Welch (54) produced some achromotrichia in rats fed sulfadiazine or succinylsulfathiazole and reversed the condition by feeding concentrates known to contain folic acid. Frost and Dann (55) were able to produce a high incidence of graying in dogs raised on highly purified diets with adequate calcium pantothenate, copper, zinc, biotin, inositol, choline, cystine and p-aminobenzoic acid. The achromotrichia was associated with anemia, and both conditions regressed with liver extract or yeast feeding. Early in 1946 Frost, Dann and McIntire (56) reported the prevention of graying in Black-Leghorn chicks which received 10 μg. per day of synthetic pteroylglutamic acid (folic acid) in addition to a diet containing all other known dietary factors. Pigmentation was comparable to that seen in chicks which received a 10 per cent yeast supplement. Graying was pronounced in chicks which received only 5 μg. pteroylglutamic acid by injection per day. Below this level feathering and growth were very poor. Lillie and Briggs (57) confirmed the prevention of depigmentation in the chick by the addition of synthetic pteroylglutamic acid to purified diets adequate in all other known factors.

A possible interrelationship between pantothenic acid and pteroylglutamic acid in metabolism is indicated by the work of Wright and Welch (54). Their work showed that liver storage of pantothenic acid was favorably influenced by the simultaneous administration of folic acid-rich concentrates and biotin to succinylsulfathiazole fed rats. From this one might deduce that utilization of pantothenic acid is in some way dependent on the presence of pteroylglutamic acid. Other work (37, 39) had previously indicated that low levels of pantothenic acid as supplied in yeast and liver were more effective in the prevention of achromotrichia in growing rats than was a four-fold level of pure d(+)calcium pantothenate. The inference was that unknown factors in the natural materials were acting synergistically with pantothenic acid in the prevention of graying.

In the early work on the effect of pantothenate on graying considerable discrepancy was encountered between results in different laboratories. Complete prevention or cure (43, 44), incomplete prevention or cure (34, 37, 39, 40, 31, 58, 59), and no prevention or cure (38) of achromotrichia in rats or chicks by pantothenate were reported in 1940–41. It is probable that the different diets used contained different levels of folic acid. There is the further likelihood that intestinal synthesis of folic acid may proceed at different rates under differing conditions. That the rat requires folic acid when sulfaguanidine or succinylsulfathiazole is fed and that this artificial deficiency state is accompanied by achromotrichia has been demonstrated (53, 54). The rat can dispense with pteroylglutamic acid even on highly synthetic diets; however, the needs for growth and all phases of metabolism may not always be completely met by intestinal synthesis of this factor. Under these conditions transitory graying may occur.

Spontaneous cure of graying in rats approaching maturity has been reported by Unna and Richards (60) and Schwarz (61) in rats receiving minimal levels of pantothenate. Unna and Richards (60) reported a decrease in the pantothenate requirement of rats from 100 μg. per day at 3 weeks to 25 μg. per day at 10 weeks of age. Schwarz (61) made a quantitative study of the effect of low levels of pantothenic acid with regard to pigmentation in relation to growth. He reported that weanling piebald rats fed 29 micrograms of d(+)pantothenic acid per day gained an average of 11 grams per week and only 7 per cent of them showed graying. A similar group which received 5 micrograms of d(+)pantothenic acid per day gained 9.5 grams weight and 100 per cent of the animals became gray. Schwarz concluded that there is competition within the animal for pantothenic acid for the unrelated functions of growth and melanin formation and that the needs for growth are generally first satisfied. On low prophylactic doses graying appeared before growth decreased. In studying the effect of pantothenic acid on growth Schwarz (62) noted that weanling rats made pantothenate deficient by a standardized method showed a weight response in close proportion to the size of the dose. Thus each microgram of d(+)pantothenic acid gave about 62.5 milligrams increase in body weight over a wide dosage range. The effect of sub-optimal levels of pantothenic acid to satisfy certain physiological functions without meeting the needs for growth is further seen in the work of Mills, Shaw, Elvehjem and Phillips (63). These workers indicated that only 4 to 25 micrograms of pantothenic acid per day was sufficient to prevent the appearance of adrenal necrosis from developing in weanling rats.

The possible effects of intestinal synthesis of pantothenic acid and folic acid on achromotrichia are difficult to define. Although there has been no general verification of the importance of p-aminobenzoic acid in animal nutrition, there have been some indications (53, 64) that p-aminobenzoic acid acts indirectly by stimulating microbial synthesis of other factors which may then become available to the animal. The chromotrichial effects reported for biotin (34, 65) may be related to some extent to a reduced synthesis of this factor in the intestinal tract (66, 67) under certain conditions. The clearcut graying produced by Emerson and Keresztesy (65) in rats fed 15 per cent dried eggwhite in the diet is, however, more clearly related to formation of the unavailable avidin-biotin complex than to a failure of intestinal synthesis.

A still further deficiency effect related to abnormal feather pigmentation in bronze poults has been described by Fritz *et al.* (68). The syndrome was produced on diets containing a high percentage of vegetable proteins and was prevented by adding lysine. The indication was that 1.1 to 1.2 per cent lysine in the diet is needed for normal feather pigmentation and optimum growth in poults.

The interesting point has been advanced by Morgan and Simms (27) that abundance of nicotinic acid and pyridoxine in the diet predisposes to rapid graying in animals on synthetic diet. This type of diet leads also to adrenal and thyroid damage and to unnatural thymus survival. According to Morgan the changes which occur in the glands of animals deficient in pigmentation factors are typical of senescence. Further studies relative to this type of observation and the possible import in human nutrition are needed.

TABLE 1 DIETARY FACTORS REPORTED TO BE NECESSARY FOR NORMAL PIGMENTATION IN ANIMALS AND BIRDS

DIETARY FACTOR	REPORTED BY	YEAR FIRST REPORTED	SPECIES AND REFERENCE NO.
——	Niemes and Wacker	1922	Rat (15)
——	Hartwell	1923	Rat (16)
Copper	Keil and Nelson	1931	Rat (18, 23); cats and rabbits (19); cattle (20)
Zinc	Stirn, Elvehjem and Hart	1935	Rat (21)
Filtrate factor	Morgan, Cook and Davison	1938	Rat (24–30); dog (27, 55); fox (28), guinea pig (27)
Pantothenic acid	Gyorgy and Poling	1940	Rat (32–39, 58–59); chick (31, 52); mouse (34)
P-aminobenzoic acid	Ansbacher	1941	Rat (40), mouse (41)
Folic acid (concentrate)	Martin	1942	Rat (53, 54)
Pteroylglutamic acid	Frost, Dann and McIntire	1946	Chick (56)
Lysine	Fritz, Hoopei, Halpin and Moore	1946	Turkey (68)

In table 1 is presented a brief summary of the dietary factors thus far reported to be necessary for normal pigmentation of various animals and birds, together with the initial investigators, the year reported, the species studied and some of the key references.

OBSERVATIONS IN HUMAN BEINGS

A few attempts to cure graying in humans have been scientifically reported, and several reports have appeared in the popular press and in trade journals. Some of the latter have been referred to by Brandaleone, Main and Steele (69). Only those papers which have appeared in recognized scientific journals will be reviewed here.

In 1941 after p-aminobenzoic acid had been reported to be an achromotrichia factor in rats (40), Sieve reported (70, 71) changes in hair color after three to eight weeks in humans given large doses of p-aminobenzoic acid, i.e., about 100 mg. twice daily by mouth. Experimental details and observations were not clearly described in these reports.

In 1943 Kerlan and Herwick (72) reported a clinical study in 27 white men and women with graying hair given 20 mg. of calcium pantothenate daily. No significant change in hair color was noted and it was concluded that calcium pantothenate alone is of no value in the restoration of color to gray hair in humans. Similar negative results were reported the same year by Vorhaus, Gompertz, and Feder (73). Brandaleone, Main and Steele (74) also in 1943 reported that 2 out of 19 elderly grayed individuals treated daily for eight months with 100 mg. calcium pantothenate, 200 mg. para-aminobenzoic acid and about 50 grams brewer's yeast showed a significant change toward a return of normal hair color. In 1944 these same authors (69) reported their previous studies in greater detail and extended the study to include 14 younger individuals. None of the younger individuals showed a decrease in grayness with different combinations of calcium pantothenate, para-aminobenzoic acid, and brewer's yeast or yeast concentrate.

The authors point out that the hair of the two individuals who showed a positive effect gradually became grayer again after medication was stopped. The fact that both of these individuals received brewer's yeast as well as calcium pantothenate and para-aminobenzoic acid may be of some significance, especially in view of the more recent animal work indicating that folic acid, and possibly other factors in yeast and liver extract, play a part in normal pigmentation in chicks and dogs.

The appearance of graying in severely avitaminotic children has been observed in various parts of the world. Nicholls (75) reported that children in Malaya who live largely on manioc root exhibit symptoms of multiple nutritional deficiencies, one of the symptoms being graying of the hair. When the children were fed a good diet the proximal parts of the hair grew in normally pigmented, while the distal part remained gray. Graying was also seen in infants weaned on cereal gruels. A considerable loss of pigment was rarely seen and no change in the texture of the hair was noted.

Recently, nutritional deficiencies in children of a severity uncommon in the United States have been extensively observed also by Chavarria *et al.* (76) in Costa Rica. These authors reported that depigmentation of the hair is a rather generalized symptom in children severely deprived of various nutritional factors, where the diagnostic symptoms are those of pellagra, beri-beri, riboflavin deficiency or vitamin A deficiency. Undoubtedly these children suffer from a combination of many nutritional deficiencies and it is not likely that therapy with any one nutritional factor will effect a complete cure. It was reported that biotin, 0.25 mg. two or three times daily, appeared to accelerate the cure of the nutritional alopecia and achromotrichia over that observed on the hospital diet alone, or with added thiamin and nicotinamide. Because the curative experiments were not controlled definite conclusions could not be drawn as to the effect of individual vitamins. It was clear, however, that the depigmentation was easily reversible by adequate diet in those children who survived this severe vitamin deficiency state.

Williams (77) in 1933 in South Africa was among the first to observe the changes in hair color associated with severe nutritional deficiencies in children on maize diets. Trowell later (78) suggested that the disease was infantile pellagra. Gillman and Gillman (79) in 1945 stated that the disease, which they accept provisionally as pellagra, is prevalent among the native children of South Africa. The latter workers,

in a careful review of the symptoms and treatment of the disease, state that nicotinic acid and other crystalline vitamins are ineffective and even appear to be contra-indicated in cases of severe infantile pellagra. The severity of the deficiency is indicated by a record of 40 to 60 per cent mortality, despite the administration of the then known vitamins and a full diet. It was noted that if the liver had not been severely damaged and if the children took their feedings they would recover slowly on any form of rational therapy. The greatest difficulty was the complete loss of appetite and inanition. Results with injectable liver extract and powdered stomach were far superior to those with vitamins. Powdered stomach, especially, was considered a life-saving drug in the treatment of the disease.

The fact that the above-mentioned nutritional deficiency disease is entirely resistant to nicotinic acid and is cured by natural materials known to contain folic acid and other yet unknown factors suggests further experimental approaches to the disease. For instance, it would be of significance to determine whether folic acid alone, and in combination with other vitamin B-complex factors, will cure the disease. For purposes of this review it would be especially interesting to know whether folic acid together with other known pigmentation factors would reverse the graying which apparently occurs as a usual symptom of the disease. The relationship between folic acid and the anti-pernicious anemia fraction of liver extract, and the relationship between the latter factor and dried stomach, are at present diffuse but many indications suggest that there is a rather close biochemical relationship between them.

Gillman and Gillman (80) very appropriately quote the suggestion made to them by personal communication by Trowell that the name for the disease, infantile pellagra, originally proposed by him be replaced by the term 'malignant malnutrition'.

ENDOCRINE FACTORS

Certain other investigations relating to the achromotrichia problem may be reviewed here. The fact that acute pantothenic acid deficiency results in adrenal damage and the fact that adrenalectomy stimulates hair growth (81) suggested the use of cortical hormones for prevention of graying. Mushett and Unna in 1941 (82) reported the ineffectiveness of cortin, desoxycorticosterone, thyroid extract, U.S.P., and anterior pituitary extract to replace pantothenic acid for prevention of graying or adrenal hemorrhage in rats on pantothenic acid deficient diets. The preparations assayed both curatively and prophylactically were without effect.

That the adrenals are in some way involved in pigmentation processes is clearly indicated from the work of Ralli and her associates. In 1941 Ralli, Clarke and Kennedy (51) reported that the amount of salt added to filtrate-factor deficient diets influenced the incidence, extent and degree of graying in rats. Rats on low salt intake became gray 55 days sooner than those on high salt intake. Changes were seen in the adrenals of all animals. In 1943 Ralli and Graef (83) discovered that adrenalectomy causes a marked transitory hyperplasia of the hair bulbs and follicles and increased hair growth and deposition of melanin both in normal and in pantothenic acid deficient rats. The effect was less marked in the deficient animals than in the normal controls. It was noted that pantothenic acid deficient animals lose skin pigment as well as hair pigment and that adrenalectomy stimulates the return

of pigment to both skin and hair. Histologic studies indicated that the graying of hair was associated with some change in the hair follicles and a cessation of melanin deposition. Ralli and Graef (84) later showed that injection of desoxycorticosterone acetate following adrenalectomy in large measure inhibited the increase in melanin deposition and hair growth both in normal and in pantothenic acid deficient animals. These experiments suggest involvement of the corticosterone hormones in the overall mechanism of pigmentation. Ralli and Graef (85) reported graying in 284 rats on a filtrate factor deficient diet, but only a minor incidence of adrenal damage. The graying was most pronounced in rats on low salt intake and in rats receiving extra nicotinic acid.

Willier (4) cites experiments which indicate that melanophores from chick embryos respond in a specific fashion to various hormones, such as the sex hormones, thyroxin and desoxycorticosterone. Such experiments furnish a basis for studying the wide differences in pigmentation between different species of birds, and between the two sexes in the same species.

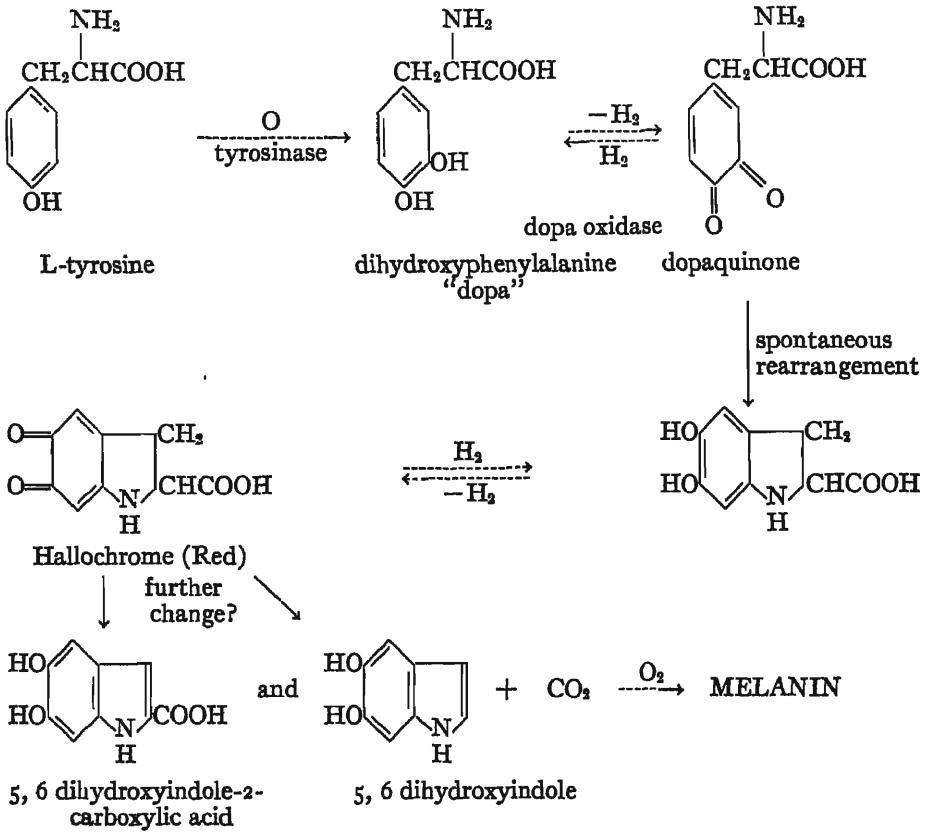
The effect of sex in mice on purified diets deficient in certain of the factors of the vitamin B-complex has been studied by Gerstl and Goldfarb (86). The incidence of graying was reported to be much higher in males than in females and the conclusion was drawn that in the strain of mice used hormonal factors play a part. Forbes (87) reported that estrogen implants caused local pigmentation of fur, but that testosterone propionate failed to do so.

CHEMISTRY OF PIGMENTATION

Melanin, the insoluble pigment of skin and hair, is amorphous and has not been well characterized chemically; however, the beginning steps leading to its final formation in plants and animals seem fairly well understood. Production of melanin may be observed in the blackening of cut potatoes on exposure to air or in the rapid blackening of catechol or tyrosine in the presence of certain plant extracts. Tyrosine is thought by Rothman (88) to be the unique beginning material for melanin formation both in plants and animals. *In vitro* experiments have shown that tyramine and adrenaline for which tyrosine is a precursor will yield pigments indistinguishable from melanin in presence of the enzyme tyrosinase.

The scheme on page 378 for melanin formation was postulated by Bloch (1) and studied in detail by Raper (89). The final agglomeration of the water-insoluble melanin granules which are deposited in the skin and hair by the melanoblast cells is not well understood.

The presence of a specific enzyme, dopa oxidase, in melanoblast pigmentation was postulated in 1917 by Bloch (90), but its actual existence was not proved. According to the work of Figge (91) tyrosinase may catalyze both the oxidation of tyrosine to dopa and the oxidation of dopa to dopa quinone. The second reaction is thought to be inhibited by the presence in excess of certain compounds having a low oxidation-reduction potential. Ascorbic acid and glutathione in excessive amounts hinder the forward progress of this reaction and may even reverse it. Only the first two steps in the above scheme are thought to require the intermediation of an enzyme since the remaining reactions proceed spontaneously. Arnow (92) has



shown that tyrosine can be converted to dopa by irradiation with ultraviolet light, and this may occur to some extent *in vivo*. Rothman (93, 94) has further elaborated the effect of ultraviolet light in production of melanin and has demonstrated the catalytic effects of both ferrous salts and ascorbic acid. *In vitro* experiments were reported which indicated a close similarity in melanin formation from tyrosine by tyrosinase and by actinic stimulation. Ascorbic acid was shown to stimulate melanin formation but to reduce preformed melanin to lighter colored products, both *in vitro* and *in vivo*.

Demonstration of the presence and functional significance of phenol oxidases in mammalian tissue has generally proved difficult and neither tyrosinase nor dopa oxidase has been isolated from normal skin. Bloch (1) in 1917 reported experiments wherein dihydroxyphenylalanine was incubated with skin and over a period of time melanin was formed. The microscopic dopa reaction described by Bloch as a specific enzymatic process was used in early work by him as an indicator of the potential capacity of pigment formation in single cells. Hogeboom and Adams (95) reported the presence of a phenolase in a mouse melanoma which oxidized both tyrosine and dihydroxyphenylalanine to melanin and suggested that this is probably a property of normal tissue, though in much smaller concentration. Although the experiments

were not always successful, Onslow (96) and Pugh (97) have confirmed the 'dopa' reaction in skin. According to Rothman (93), Bloch's theory of the dopa reaction as a specific enzymatic process is in accord with all facts in the physiology and pathology of the pigmentation process.

Richter and Clisby (98) made the interesting observation that phenylthiocarbamide, a compound which inhibits the copper containing oxidative enzymes of tissue, causes graying in one to two months when fed to black rats in amounts of 1 to 14 mg. per rat day. When the phenylthiocarbamide feeding was stopped the hair again became black in about three months. In light of the above work Bernheim and Bernheim (99) studied the effect of phenylthiocarbamide on the tyrosinase of potatoes and mushrooms, and on the oxygen uptake of rat tissue slices and cell suspensions. The compound was found to be a very effective inhibitor of tyrosinase, but relatively ineffective against various tissue enzymes. Although the toxic effects of phenylthiocarbamide in the rat cannot be explained by its action on tyrosinase, it appears highly probable that the compound causes graying by inhibition of a specific copper-protein enzyme system in the melanoblasts of the skin, the hypothetical dopa oxidase.

An explanation of a few of the forementioned nutritional effects can be attempted in light of the above chemical scheme. Since copper is known to be the prosthetic group of all of the various phenol and polyphenol oxidases (100–102, 95), a deficiency of this element might conceivably lead to an interruption of the production of melanin at its very beginning. The depigmentation of hydroquinone poisoning which has been studied in rats and cats by Oettel (103) may result from accumulation of hydroquinone in the melanoblasts, where its reducing effect might be expected to interfere with either of the reversible reactions shown in the scheme. The hyperpigmentation of Addison's disease has been alleviated with vitamin C (104) which is known to act toward reversal of the above scheme. Goodman (105) has reported initial stimulation of pigment formation in two patients with alopecia areata by topical application of anthralin (dihydroxyanthranol), a reducing compound. Persistent use resulted in a complete sudden loss of pigment. Thus conditions, which are thought to cause a reduction in the oxidative enzyme system associated with pigmentation and conditions which theoretically favor an inhibition or reversal of melanin formation, appear to be available for further exploration.

Martin, Wisansky and Ansbacher (106, 107) have reported that p-aminobenzoic acid has some effect in changing the normal course of melanin formation *in vitro*, but these results are not interpretable on the basis of the nutritional effects reported for this compound. In this regard we should bear in mind that the amounts of p-aminobenzoic acid which have been reported to cure achromotrichia in rats and humans are out of all proportion to the amount likely to be obtainable in food. The reported curative effect on achromotrichia may well prove to be an artifact which will have no counterpart in normal nutrition. How or where the remaining achromotrichia compounds might fit into the above scheme is beyond the limits of supposition at present. From the accumulated evidence it would seem that pigmentation in general is in part subject to proper hormone function and this in turn may depend on the adequacy and balance of certain nutritive factors.

SUMMARY

The fact that graying has been induced in all species of animals studied and has been observed in human infants under a variety of dietary deficiency conditions would seem to indicate that graying, particularly during growth, is a characteristic manifestation of certain types of dietary deficiency. The intent in the great majority of experiments has been to develop a specific deficiency of only one pigmentation factor. There is little chance that such extreme specific dietary imbalances often occur among humans, but multiple deficiencies undoubtedly do occur. That graying is ever actually induced under ordinary conditions by mild chronic deficiencies of various B-complex and mineral elements has not been proved. Since genetic, hormonal, and dietary influences have all been shown to play a part in pigmentation processes, the etiology of graying in individual people is unavoidably obscure. The practice of ascribing human curative or prophylactic properties to single pigmentation factors, which have been found effective in animals, under highly specialized and controlled conditions, is not well founded. It is not certain that graying in adult humans is ever caused by a dietary deficiency and, if such were indeed the case, the deficiency would almost certainly involve the lack of more than one of the factors thus far shown to be needed for normal pigmentation in animals.

Because of genetic influences, graying in adult human beings is not considered a manifestation of unnatural physiologic change. The fact that graying occurs in children on extremely poor diets is of considerable interest, but it is not very helpful in assessing the situation with regard to adults. The above-cited work with human beings was carried out before the need for pteroylglutamic acid for normal pigmentation was established in animals. Most of the human studies involved pantothenic acid. It is therefore of interest that there now appears to be physiologic relationship between these factors. The apparent functional relationship between pteroylglutamic acid and the anti-pernicious anemia principle of liver is also of interest, particularly in view of the fact that liver extract has been reputed to have an effect on graying in isolated instances. When more is known about about the nutritional rôle of the various pigmentogentic factors and about their interactions in essential enzyme systems, further study of thier effects in graying in human beings may be indicated.

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ORIGIN OF MELANOPHORES AND THEIR RÔLE IN DEVELOPMENT OF COLOR PATTERNS IN VERTEBRATES

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IT IS PROBABLE that no phenomenon of nature has attracted more attention over a longer period of time or has been investigated from more diverse points of view than that of pigment formation in the integument of animals. Among the vertebrates, color patterns may be produced in a number of different ways, but in the majority of cases melanin pigment is the principal color constituent. Although melanin pigment-forming cells have been known and described for more than a century, it is only within recent years that the fundamental question of the embryonic origin of these cells has been definitely settled within three groups—amphibians, birds and mammals. It is only through the knowledge that the pigmentary unit, the melanophore, has an extrinsic origin in the neural crest that it has become possible to apply experimental methods of causal analysis towards discovering the mechanisms of control in the development of the definitive system of pigmentation. Through such analysis, definite progress is being made towards an understanding of the complex interrelation of factors, genetic and environmental, involved in the development of the final color pattern.

The main purpose of the present article is to bring together in one summary the principal findings obtained so far from the work on amphibians, birds and mammals, which quite definitely are leading to a more unified concept of the rôle of the melanophore in the development of pigmentation in the vertebrates in general.

MELANIN PIGMENTS

The melanin pigments are produced in certain highly specialized, branched, migratory cells which bear a striking morphological similarity in all of the vertebrates. These pigment-forming cells have been designated by a variety of names—chromatophores, melanophores, melanoblasts, pigmentoblasts, dendritic cells—which has led to some confusion and misunderstanding. Some authors have, therefore, resorted to referring to them simply as pigment-cells. In the following pages the

terms melanophore and pigment-cell are used interchangeably to designate cells containing melanin granules which they themselves have synthesized; the term melanoblast is used to designate an undifferentiated, prospective melanophore or pigment-forming cell.

The melanin pigments produced by the melanophores are granular in nature, the granules being of definite shape and measurable size, ranging in color from pale yellow or buff through various shades of red-brown to black. Numerous histological studies of the definitive granules deposited in the feather cells of a variety of birds (106, 71, 63, 44, 87, 126, 17) and in the hair cells of various mammals (123, 103, 49, 26, 100) have revealed that within any one genotype, the size, shape and color of the granules exhibit a remarkable specificity. In general, the dark melanins occur in the form of rods, the light melanins in the form of spheres. Also, the dark colored granules are much less soluble in alkalis than those of lighter color, probably due to differences in degree of oxidation of the melanin precursors (44). Undoubtedly the so-called diffuse melanins mentioned in hair and feather studies, time and again by the older workers in particular, can be accounted for on the basis of partial solubility of the granules which occurred during the preparation of the material for microscopic study. According to the present status of our knowledge, melanin exists only in the form of discrete particles.

Melanin granules have usually been considered as inert deposits of the end product of cellular metabolism. That they can no longer be considered as such has been demonstrated recently by the work of Hermann and Boss (54) with pigment granules isolated from bovine ciliary bodies by differential centrifugation. Examination of these isolated melanin granules for certain enzymatic components has revealed the presence of three oxidative enzymes—dopa oxidase, cytochrome oxidase and succinic-dehydrogenase.

One of the most important problems in melanin pigmentation centers around the mechanism involved in the formation of the characteristic and uniformly shaped granules formed within a single melanophore. Ever since Reinke in 1894 (97) pointed out clearly that the melanin granules are composite structures made up of a colorless substrate, Pigmentgrundsubstanz or Pigmentträger, to which the coloring matter melanin is attached, numerous statements have been made in the literature regarding their origin. It was natural that the early investigators, in view of the importance assigned to the cell nucleus at the time, should seek to identify the necessary morphological ground substance (Pigmentträger or Pigmentbildner) with the nuclear chromatin elements. The chief exponent of this view was Aurel von Szily (117), who in 1911 published an extensive monograph on the subject. He described colorless bodies (Pigmentträger) extruded from the nucleus and derived directly from its chromatic elements, which, after wandering to a more or less peripheral position in the cell, became colored by the action of cell ferments. Others tried to derive the Pigmentträger from filaments of the protoplasm (38, 62) and Kromayer (62), thought he could observe a direct transition of protoplasmic fibers to pigment granules in epithelial cells. Later on, as various cytoplasmic constituents became investigated, these elements, particularly the mitochondria (95, 59) and the lipochondria (42, 74) were considered to be the morphological constituents from which the de-

finitive pigment granules arose. The diversity of opinion was no doubt due to the fact that all of the conclusions were drawn merely from fixed and stained material which varied considerably depending upon the techniques applied. Interestingly enough, with the discovery in recent years of submicroscopic particles of cytoplasmic origin and of highly complex chemical composition (phospholipids and ribonucleoproteins) by Bensley (7, 8), Claude (20, 21) and others, attention is now being focused upon them as possible sources of origin of melanin pigment granules (61).

The chief difficulty in formulating an hypothesis to account for the formation of melanin granules centers in the fact that the chemical composition of melanin is not known. In fact it is quite probable that all of the substances to which the term 'melanin' has been applied are not identical (77). It has been generally accepted since the discovery of tyrosinase by Bertrand, in 1896 (9), that a substrate or melanogen and an oxidative enzyme are involved in melanin synthesis; oxidation products resembling natural melanins have been demonstrated in many animals and plants. Histological studies on human and other mammalian skin led Bloch (15, 16) to the assumption that melanin is formed by a highly specific enzymatic oxidation of 1-dihydroxyphenylalanine (dopa). The enzyme became known as dopa oxidase. While Bloch's results have been substantiated by many workers the specificity attributed by him to dopa oxidase has met with severe criticism (see Franke (45) for a short summary). The criticism is based mainly on the contention that the ease with which the reaction occurs may depend on the ease with which dopa is known to oxidize rather than on the specificity of the enzyme present. The experimental results of Figge (41) suggest strongly that in certain amphibians melanin pigment formation is the result of the oxidation of tyrosine by tyrosinase and is regulated by a delicate balance of oxidative and reductive mechanisms within the melanophores.

In recent years Hogeboom and Adams (55) working with cell-free extracts of melanotic mouse tumors, which presumably arose from skin pigment cells, obtained two highly specific fractions: one containing an enzyme which catalyzes tyrosine to melanin (tyrosinase), another which catalyzes dihydroxyphenylalanine to melanin (dopa oxidase). Also, as previously mentioned, Hermann and Boss (54) have demonstrated the activity of dopa oxidase in purified melanin granules of bovine ciliary bodies. Their purified preparation failed to oxidize tyrosine, hydroquinone and catechol unless cytochrome *c* was added. The important point in this respect, however, is the fact that dopa could be oxidized to form melanin pigment without the added cytochrome *c*. These results indicate that dopa may be oxidized *in vitro* by at least two different enzyme systems, one of which is concerned primarily in the formation of melanin. The distribution of cytochrome-*c*, cytochrome oxidase and dopa oxidase as well as the relative reducing intensity of the cells appears to be of importance in the formation of melanin pigment. From numerous investigations but particularly those of Hogeboom and Adams, and Hermann and Boss, it appears quite probable that the oxidation of dopa through a system other than that of the generally distributed cytochrome oxidase system is the important reaction in melanin formation. If these interesting findings can be verified in other melanin pigment-forming tissues, we shall have gone a long way towards an under-

standing of the complicated problem of melanogenesis. It is of interest to note that in both of the aforementioned investigations the experimental material was relatively homogeneous and extremely rich in pigment. In the light of these findings, the failure heretofore to demonstrate either tyrosinase or dopa oxidase in mammalian skin extracts could very well be attributed to the relatively small quantity of the enzyme present with respect to the total volume of tissue concerned.

In view of the fact that during the past few years it has become increasingly evident that the submicroscopic granules (and the mitochondria) are seats of enzymatic activity and the fact that melanin granules of the ciliary body have already been shown to contain several enzyme systems, including dopa oxidase, it may not be going too far afield to suggest that the submicroscopic granules are the sites of melanin synthesis. Assuming that these specialized particulates in the cytoplasm of a prospective melanophore (melanoblast) contain the specific enzyme for the oxidation of the melanin precursor, the ultimate melanin granules could be built up by an accumulation and subsequent polymerization of the oxidation products; the specific size, shape etc. depending upon the nature of the polymer. (See Kopac (61) for further discussion in this new and interesting direction.) With the progress that is now being made in protein and cytochemistry we have every reason to believe that the time is not too far distant when we shall have a satisfactory solution to the as yet little understood process of melanin synthesis.

EMBRYONIC ORIGIN OF THE MELANOPHORES

In order to arrive at some understanding of the interplay of factors, genetic and environmental, which are necessarily involved in the development of a system of pigmentation, it is of the utmost importance to know the source of the pigmentary unit, the melanophore. For nearly one hundred years a wide variety of histological observations on embryonic and adult tissues of every class of vertebrates has been brought to bear upon the general problem of melanophore origin. The theories which have been advanced have been reviewed many times. See Post (84), von Szily (117), Fuchs (46), Biedermann (10–12), Strong (109) and more recently DuShane (32, 33). At the present time, it is so clearly established that the neural crest is the source of all pigment cells (exclusive of the retinal tapetum) in amphibians, birds and mammals that other hypotheses need no longer be considered.

The composite nature of the neural crest is well known. Cells destined to give rise to a variety of tissues, in addition to the pigment cells, such as visceral cartilages, ganglia and sheath cells have been shown by Harrison (51), Stone (108), Horstadius and Sellman (58), Adelmann (1), Holmdahl (56) and others to take their origin from the crest. In their early developmental stages the prospective pigment cells (melanoblasts) cannot be distinguished either morphologically or histologically from the other embryonic cells with which they are associated. They are at first without pigment and often reach remote positions in the body of the embryo before showing their characteristic differentiation. For this reason their true origin cannot be ascertained by the study of serial sections alone—the method used almost exclusively until recent years. With the introduction and perfection of precise techniques, by Spemann and his school, for isolating and transplanting regions of the embryo, it

became possible to analyze the inherent developmental capacities of embryonic tissues. It was primarily through the use of such methods that the neural crest origin of pigment cells was, finally, clearly demonstrated.

The amphibians were the first group in which information was obtained for linking pigment cell origin with the crest, and the evidence was gradually accumulated. Harrison (50), in his study of the growth of nerve fibers in tissue cultures, noted that pigmented cells frequently formed from pieces of medullary cord. He thought their origin from the ganglion crest likely. Later Mangold (75) and others and Holtfreter (57) obtained evidence from various transplantation experiments with Triton embryos, which pointed strongly to a neural crest origin of the pigment cells. Since the primary interest of these investigators was directed along lines other than those of pigmentation, it remained for other experimentalists to furnish the conclusive proof.

DuShane (31), working with Amblystoma, found that removal of the neural folds (the primordia of the neural crest) in the neurula stage resulted later in a total lack of pigment cells in the operated (trunk) region; and the isolated folds produced numerous pigment cells when grown in culture medium or transplanted to the ventral region of another embryo. After closure of the neural folds, in tail-bud stages, the extirpated crest region transplanted heteroplastically between two pigmented embryos invariably produced pigment cells of the donor type. Similar observations were made by Twitty (111) and Twitty and Bodenstein (114) on several species of Triturus and by Raven (86) on Triton, and confirmed many times by subsequent workers not only for the crest of the trunk region but also for that of other body regions (82). Bytinsky-Salz (18) and Baltzer (3) extended the investigations to include the anurans. By means of xenoplastic transplantations, they were able to show that the neural crest of the head and trunk regions gives rise to all types of pigment cells, guanophores as well as melanophores. Also, Barden (5) working with the amphibian eye demonstrated conclusively that the xanthophores, guanophores and melanophores found in the chorioidea and iris arise from the neural crest. For a time there was some question among amphibian workers as to whether the melanophores found in the epidermis, which become very conspicuous at metamorphosis and play the major rôle in the pigmentation of the adult in certain species, were also of neural crest origin. The experiments of Rosin (99) as well as the recent experiments of Stearner (107), in which special consideration was given to this question, would seem to leave no further doubt that in the urodeles the epidermal melanophores do originate in the neural crest. Stearner's observations have shown further that these melanophores are the sole source of the melanin pigment granules which are deposited in the epidermal cells. At the present time, then, the large body of evidence agrees in showing that the neural crest is the source of all types of pigment cells—melanophores, xanthophores, guanophores—that develop in various parts of the body of an amphibian. While the crest as a whole is potentially capable of producing pigment cells it does, however, exhibit conspicuous differences along its cranio-caudal extent with respect to the relative number of pigment cells produced; the crest of the trunk region is by far the richest source (82).

In birds, the evidence pertaining to the origin of melanophores follows similar

lines. Dorris (29) found that explants including the neural crest of the pre-otic region of early chick embryos of several breeds produced typical melanophores in culture medium. Later (30) she showed that similar isolates transplanted to the leg bud of three-day host embryos produced donor coloration in the down feathers of the host leg (cf. 34, 36, 126). Ris (98), working with intracoelomic and other grafts from various axial levels of embryos of several varieties of fowl and other birds, was able to correlate the presence of pigment cells in the grafts with the morphological development of neural crest at the time the isolation was made. Thus, it was conclusively demonstrated that only those isolates known to contain neural crest, the region from which it arises, or its migratory cells, are capable of producing melanophores in grafts. So reliable has this correlation proved that pigment-free (white) feathers can be produced, at will, from any potentially pigmented variety of bird by the simple precaution of excluding neural crest cells from the desired region (90). In an appropriately designed experiment, Ris (98) was also able to determine the origin of the retinal and chorioidal pigments of the eye in fowl. The retinal pigment does not occur in branched cells. It arises *in situ*. The pigment of the chorioidea (and iris) is, however, produced in characteristically branched cells, melanophores, identical with those in the skin and mesoderm and, like them, derived from the neural crest. It is worth mentioning that pigment cells comparable to xanthophores, guanophores, liphores etc. in amphibians do not exist in birds. The important and widely occurring lipochrome pigments found within the feather cells of birds are not produced by specialized migratory pigment-forming cells. These pigments are diffuse rather than granular and appear to be of exogenous (plant) origin. They are dissolved in the fat droplets deposited inside of the cells of the barb ridges prior to the onset of keratinization (28, 116).

The only mammalian embryo in which the origin of melanin pigment-forming cells from the neural crest has actually been demonstrated is the mouse (89, 92). The experimental procedure was similar to that used for demonstrating the origin of melanophores in birds, namely, the isolation of tissues from various axial levels at different developmental stages and their subsequent transplantation to the embryonic coelom of White Leghorn chick hosts. Data obtained from an extensive series of experiments with embryos of a homozygous black strain of mouse have demonstrated, clearly, that only those tissues containing presumptive neural crest, histologically recognizable neural crest or cells migrating from the neural crest, can produce melanophores. The fact that well-developed, structurally normal hairs entirely without pigment (white) can be obtained from a potentially pigmented (black) strain of mouse, if certain body regions are grafted at a particular developmental stage, shows very definitely that the ectoderm and its derivatives, the hairs, have no inherent capacity for autonomous melanin-pigment formation.

In the remaining vertebrate groups, the fishes and the reptiles, the origin of melanophores has received little attention in spite of the fact that in both of these classes of animals melanophore activity has been investigated intensively with respect to color changes. (See Parker (83) for a survey of the investigations on animal color changes from the years 1910 to 1943.) From the recent transplantation experiments of Lopashov (73) on teleost embryos, it is evident that the melanophores

are derived from the anlage of the central nervous system. It is highly probable that melanophore origin in fishes and in reptiles, also, is linked with neural crest differentiation.

While there is no doubt that in the vertebrates investigated the pigment-forming cells arise from the neural crest, it should be pointed out that the organization of the crest as an embryonic structure is, as yet, little understood (53). It is not known, for example, whether the cells of the crest are pluripotent from the beginning and differentiate according to their later positions or whether their potentialities are already fixed before migration begins. The question of cell differentiation is one of the most fundamental and at the same time one of the most obscure problems of all biology. The neural crest would seem to offer especially favorable material for a study of the differentiation of embryonic cells into specific cell types.

MIGRATION OF MELANOPHORES

Having demonstrated that melanophores originate in the neural crest, the question next arises as to how and when they reach those parts of the body, the skin and its derivatives, visceral membranes etc., in which they are later found. Conclusive evidence that prospective pigment cells (melanoblasts) reach their destinations in the feather germs of birds by migration has been obtained by implanting small pieces of embryonic tissue containing neural crest cells in the early wing buds of host embryos of a different breed (126). At hatching, the plumage of the entire host wing, and often adjacent regions of the breast, invariably exhibit the color pattern characteristic of the donor individual. Furthermore, the melanin granules deposited by the grafted pigment cells in the host feathers are always of the size, shape and color characteristic of the donor breed, even when the donor represents another order of birds (87).

In the absence of suitable cytological criteria for recognizing pigment cells in their early unpigmented stages, their exact migratory pathways are not known. It has been possible, however, to ascertain with a fair degree of accuracy the direction of migration and the time when these cells reach their definitive locations in both chick and mouse embryos. The general procedure has been to isolate systematically various portions of the embryo from different axial levels at successive developmental stages (during somite formation) and to test the capacity of such isolates for pigment cell production in grafts. For example, if the forelimb bud is removed from a 9- to 10-day mouse embryo of a homozygous black strain and allowed to continue its development in a site, such as the coelom of a White Leghorn chick embryo, known to be favorable for further growth and differentiation, it will produce hairs entirely without pigment (white). If taken a little later, the identical isolation will produce pigmented (black) hairs. In the interval of time between the two isolations, prospective pigment cells have migrated into the limb bud, as evidenced by the presence of pigment in the hairs (89, 92). Similarly, the wing bud of a potentially pigmented chick embryo will produce white feathers if isolated before approximately 80-hours' incubation; pigmented feathers after that time (34, 98). The hind limb bud will not produce pigmented feathers until still later, 96 to 103 hours etc. (120, 126).

Thus by testing not only limb buds, but somites, skin ectoderm, mesoderm, neural tube etc., alone and in combination, it has been possible to demonstrate 1) that

the direction of migration of melanoblasts is mediolateral and 2) that melanoblasts have reached their definitive locations in all parts of a chick embryo by the 4th day of incubation and in all parts of a mouse embryo by the 12th day of gestation.

In amphibians, the experimentation has been carried out from a somewhat different point of view. Nevertheless, it is evident from the transplantations of Du-Shane (31) that melanoblasts have reached their definitive locations in the limb buds by the late tail bud stage and, from the experiments of Twitty (111) and Twitty and Bodenstein (115), it is clear that in normal development melanoblasts show a general tendency to migrate in a medio-lateral direction, just as they do in birds and mammals.

That pigment cells migrate extensively in their early, unpigmented stages is quite evident from the foregoing account. In birds there is no evidence that a fully differentiated (pigmented) melanophore can shift its position to any great extent, even when grown *in vitro* (47). In amphibians the relationship is not necessarily so rigid, at least when the cells are cultivated under certain conditions *in vitro* (112, 113). However, the observed movements initiated after the onset of melanin granule formation in cultures are by no means comparable to the extensive migrations occurring normally when these cells are in their unpigmented phases.

It would be important to know the nature of the factors which influence the pathways of migration. Are they intrinsic to the pigment cells themselves or are they extrinsic, i.e. imposed by the tissue environment, or both?

Intrinsic factors have been demonstrated in amphibians (114, 115, 112) and in birds (126). Melanoblasts of certain species of amphibian embryos exhibit a marked inherent tendency to spread more extensively under identical experimental conditions *in vivo* and *in vitro*, than those of other species, and among the white varieties of fowl, White Leghorn melanoblasts show far less ability to migrate when implanted into the wing bud of a pigmented host than melanoblasts from any of the so-called recessive white varieties (Wyandotte, Plymouth Rock, Silkie).

Among extrinsic factors affecting the migratory activities of grafted melanoblasts is the age of the host embryo. Melanoblasts from a three-day chick donor embryo implanted to the wing bud of an older (4–5 days) host, after the host's neural crest cells have already migrated into the wing bud, as a rule do not leave the implant to produce the extensive area of donor-coloration in the plumage of the wing of the host, which is observed when younger hosts are used (126). Likewise, the migration of melanoblasts from transplanted neural crest in amphibians is much less extensive in older than in younger hosts (115).

Other conditions in the host, apart from age, have been shown to influence migration. A conspicuous example is found in Triturus (111). Whenever *T. torosus* is host to either *T. rivularis* or *T. simulans*, the grafted melanophores do not descend below the dorsal margin of the yolk mass of the host while in their normal environments or in other host environments they do not stop at this point. Even more pronounced examples of host influence on grafted melanophores may be found when donor and host represent different orders. Under the influence of Hyla hosts, Triton melanoblasts surpass their normal migratory boundaries and become distributed in the ventral body region (2, 4, 64). In birds there is, also, good evidence that consti-

tutional differences in hosts may either favor or restrict the extent of migration of donor melanoblasts introduced into the wing bud (126).

The extent of the area of donor-colored plumage, hence the degree of pigment cell migration, in wing bud grafts is controlled to a great extent by the quantity or concentration of melanoblasts originally introduced. Similar observations have been made in amphibians, particularly by growing neural crest cells in cultures under especially devised experimental conditions (112). In fact, Twitty considers this observed quantitative relationship between size of cell colony (population density) and extent of migration important supporting evidence for his hypothesis that migration is a response to mutual influences exerted by the melanoblasts themselves through the release of metabolic substances. According to this conception, melanoblasts would continue to move away from their neighbors until spaced beyond the effective range of influence. Hence, the larger the original population the greater the distance travelled by the individual cells in achieving such spacing.

The possibility of purely mechanical hindrances to the movements of melanoblasts should not be entirely overlooked. In birds there is good evidence that a sudden change in the arrangement of cells in adjacent tissues can effectively block melanoblast entry (98, 121, 90); and, with the failure of development of the dorsal fin in amphibians, melanoblasts, which normally stop at this point, now migrate freely across the mid line (113). It is quite probable that the accumulation of melanoblasts at the margin of the yolk mass in certain species of Triturus and in Triton belongs in this category.

While it is evident that pigment cells are capable of independent movements, much of their migratory activity is unquestionably dependent upon their contact with certain other cell strains and tissues. The fact that melanophores are found primarily along surface membranes, e.g. the basal layer of the epidermis, parietal membranes, meninges, walls of blood vessels, walls of sex and urinary ducts etc., is significant. (For a discussion bearing upon the problems of selective cell association, tissue affinities etc., the reader is referred to a recent paper of Weiss (122) dealing with the general problem of cell specificity in growth and development.) The possibility that the final distribution of melanophores may be affected by morphogenetic movements and by growth movement of their tissue substrates must be considered. Watterson (121), in his studies on the development of the feather germ, describes a succession of changes in melanophore distribution which are definitely correlated with morphogenetic movements of the epidermal cells in the organization of barb ridges. Among amphibian workers it appears to be rather generally accepted that melanophores of the larva can be carried along passively, to some extent, by the rapid growth of their tissue substrates (99, 113). Other aspects of pigment-cell migration in relation to pattern formation will be considered in the following section.

INTERACTION OF MELANOPHORES AND TISSUE ENVIRONMENT IN THE FORMATION OF DEFINITIVE PIGMENTARY PATTERNS

Most of our knowledge concerning the development of patterns of pigmentation in the vertebrates has come in recent years through experimental studies in two widely different groups, amphibians and birds. As the investigations have gradually

broadened it has become more and more evident that the underlying principles involved are remarkably similar in both groups. In the analyses of pattern formation, both in the embryo and in the adult animal, the investigations have centered around two fundamental problems: *a*) the part played by the individual pigment cells and *b*) the part played by the tissues with which the pigment cells become associated. Both, as we shall see, figure importantly in the formation of the definitive pigmentary pattern.

Larval Amphibian

Among larval amphibians there is a general tendency for the melanophores to be either uniformly distributed over the dorsal and lateral surfaces of the somites or confined more or less sharply into bands or stripes which have a definite relation to certain regions. These observed differences in melanophore distribution do not necessarily reflect corresponding differences in the original dispersal of prospective melanophores from the neural crest. The most recent evidence indicates that in Triton and Triturus the characteristic longitudinal stripes are formed by a secondary rearrangement of melanophores, originally more widely scattered over the lateral surfaces of the somites (99, 113). Aside from characteristic differences in melanophore arrangement or pattern we find also among the various species differences in melanophore number. There is evidence that many of the prospective melanophores which migrate from the crest are not self-differentiating but require some stimulus or material contribution from other cells, notably the ectoderm, in order to produce pigment granules (52, 31, 27, 111). Finally, characteristic differences exist in the morphology of the pigment cells themselves—qualitative differences such as size, type of branching, color or tone, number of granules etc.

By exchanging segments of neural folds containing the crest, in early developmental stages, it is possible to introduce melanoblasts of species exhibiting one characteristic type of pigmentation into individuals exhibiting a different type without changing the topographical arrangement of the tissue environment. Thus it can be determined to what extent the pattern produced by the foreign pigment cells develops according to origin and to what extent it is influenced by the foreign host tissues. When such transplantations are made between embryos of three closely related species of Triturus and their hybrids, the resulting pattern produced by the grafted melanophores in the region of the host into which they are introduced (usually the trunk) is invariably a duplicate of the type found in the donor individual (111, 114). Prospective pigment cells of *T. torosus*, for example, form their normal banded pattern in either *T. similans* or *T. rivularis* hosts; similarly, pigment cells of *T. similans* or *T. rivularis* form their characteristic non-banded patterns in *T. torosus* hosts. The only evidence of any environmental influence on the donor melanophores in these transplantations is seen in their distribution at the border of the yolk mass. In *torosus* hosts, grafted melanophores from either *rivularis* or *similans* fail to descend below the dorsal margin of yolk mass as they would in their normal environments. From these experiments it would appear that the pattern of melanophore distribution is determined primarily by properties intrinsic to the pigment cells.

When such studies were extended to include intergeneric combinations between

either Amblystoma and Triton (99) or Amblystoma and Triturus (114, 115) interesting modification of the basic pattern not observed in the above interspecific transplants became apparent. Under such conditions the pattern produced by the grafted cells does not follow so closely that of the donor type. The paired bands produced by Triton pigment cells in the black axolotl, Amblystoma, for example, are less sharply defined than those of the normal Triton donor; and in the reciprocal combination, the axolotl pigment cells, which in their normal environment become uniformly distributed, show a slight tendency to aggregate in Triton hosts in the regions where Triton bands would normally form. In transplantations from Amblystoma to Triturus the grafted pigment cells show a decided tendency to migrate even beyond the graft region into adjacent host areas where they tend to suppress the development of the host's melanophores, thus bringing about subsequent modifications in melanophore arrangement.

When the exchange of crest is made between donor and host still further apart phylogenetically, such as between anurans and urodeles (3, 4, 64), the pattern formed by the grafted cells bears even less resemblance to that of the donor type. In such cases the final distribution of the grafted melanophores is tremendously influenced by the environment of the foreign host. In all of the aforementioned experiments the morphology of the individual, grafted melanophores is not altered. They retain their specific characteristics as regards size, color intensity, branching etc. and can be readily distinguished from those of the host. The primary change which occurs, therefore, is one affecting their orientation into distinctive patterns.

From the results of transplantations, the xenoplastic type especially, it is evident that factors extrinsic to the melanophores do play an important part in the formation of the definitive pigmentary pattern. What are these factors? Undoubtedly they are many and varied; a few have been somewhat clarified.

By appropriate transplantations between *T. torosus* and *T. torosus-T.similans* hybrids, it has been shown that the factors which condition the peculiar distribution of melanophores at the dorsal margin of the yolk mass are located in the mesoderm of the flank (27). The nature of the 'barrier' is not known. The possibility of its being a purely mechanical block to pigment cell movements has already been mentioned.

It is to be expected that normal organic relationships would be of great importance in pattern formation and this has been shown to be true. In the absence of somites, for example, the melanophores of *Triturus torosus* cannot assume their normal orientation into bands, but remain scattered (111, 113). This clearly indicates that the specific orientation of melanophores, e.g. rearrangement into bands in torosus, is a result of interaction between them and their normal tissue substrate. On this basis it may be possible to explain some of the unusual arrangements occurring when melanophores are grafted to widely unrelated individuals where great differences in substrate must exist. The results of such transplantations indicate that melanophores respond to foreign tissues in a particular way which differs from the response normally given under their own environmental conditions. The response, however, that can be given is in accordance with the specific set of reaction potencies inherent to the melanophore—i.e. provided by its genic composition.

Additional evidence that the rearrangement of melanophores is conditioned by the character of the substrate has been obtained from *in vitro* experiments (113). Outgrowths of *T. torosus* melanophores adhering to the glass cover slip do not undergo rearrangement into dense clusters nearly so readily as those adhering to the inner surface of a hanging drop of medium. Such experiments further show that rearrangements do not take place at all unless the melanophores attain their normal degree of differentiation, i.e. have become fully pigmented. In physiological salt solution *T. torosus* melanophores differentiate slowly and incompletely and remain permanently scattered. In peritoneal fluid, on the other hand, where conditions for complete and normal pigment differentiation are provided, a subsequent rearrangement into dense clusters, never observed in the saline cultures, occurs. This rearrangement appears to be brought about by tensions exerted by the melanophores upon one another by the retraction of the filamentous processes connecting the neighboring cells (112). How do such findings apply to the living organism?

It has been demonstrated that in the normal embryo prospective pigment cells differentiate in a definite sequence (115). Pigmentation begins anteriorly and proceeds gradually posteriorly. This sequence appears to be referable to a corresponding antero-posterior differential in the organic environmental factors influencing melanin pigment formation, since melanophores originating from posterior pieces of crest grafted to anterior levels differentiate at the time appropriate for their new position. Similarly it has been demonstrated that conditions affecting the time of pigment formation are graded along the dorso-ventral axis. If the rearrangement of melanophores is dependent upon a requisite degree of differentiation, as the *in vitro* experiments would seem to indicate, then melanophores situated along the dorsal margins of the myotomes would be the first to fulfill the conditions necessary for rearrangement. Outlying melanophores as they in turn become differentiated would gradually be drawn dorsally towards the region where the tensions, brought about by the retraction of the interconnecting processes, first develop, i.e. along the dorsal margins of the myotomes (113).

In *T. rivularis* the melanophores retain their initial diffuse arrangement throughout larval life. They are inherently incapable of a bandforming response under all of the *in vivo* and *in vitro* conditions so far tested. In view of the results with *T. torosus*, it has been suggested (113) that in *T. rivularis* the development of the pigment cells remains arrested below the level at which the mechanism of reaggregation can be set into play. According to such an interpretation the contrasting behavior of the two species of cells is based on differences which are purely quantitative or relative in nature. Whatever the differences may be, they are clearly referable to factors within the pigment cells themselves, since the melanophores of each species reproduce their characteristic pattern when transplanted to a host of the other species.

The distribution of melanophores is markedly affected by differences in growth rates between donor and host species. Pertinent evidence may be found in examples already cited, where crest is exchanged between Amblystoma and Triturus embryos of the same age. The identical effect, i.e. the spread of donor melanophores beyond the graft region and the subsequent prevention of host melanophores from entering the areas occupied by the grafted melanophores, can be reproduced in embryos having

the same inherent growth rates if crest from older embryos is grafted orthotopically to younger hosts (115). One is led to suspect that at least some of the unusual distribution of pigment cells arising in the anuran-urodele transplantations is related to species differences in growth rate.

The importance of local, environmental factors in the development of the definitive pigmentary pattern in another body region, the eye, has been demonstrated by Barden (5). By combining pigment cells and optic vesicles between species exhibiting marked differences in iris pattern, he was able to show that the resulting pattern followed that of the individual which furnished the structural elements of the eye i.e. the pigment pattern in the iris is controlled by factors located in the iris.

ADULT AMPHIBIAN

The adult amphibian pigmentary pattern is conspicuously different from the larval. In general the adults are more intensely pigmented. Some like *Triturus torosus* darken more or less uniformly and no longer show any indication of the larval stripes. Others like *Amblystoma tigrinum* and *punctatum* develop characteristic light spots on the darkened background. Recently Stearner (107) has studied histologically, in several species of Urodeles, the sequence of changes in the development of the cutaneous pigmentary system, prior to and during metamorphosis, which lead to the formation of the definitive adult pattern. She has been able to identify certain stellate, unpigmented cells in the subdermis and dermis, which occur in close association with the melanophores. The evidence, although somewhat indirect, indicates that some if not all of these cells are potential melanophores. Some differentiate before they migrate into the dermis but the majority enter in the undifferentiated state and later form melanin. The ratio of pigmented to unpigmented cells (obtained from cell counts of a unit area at various developmental stages) is found to be smaller in the advanced larva than it is after metamorphosis is completed. During metamorphosis, the number of melanophores increases rapidly while the number of unpigmented cells shows a general decrease. The developmental changes in pigmentation described by Stearner, both in a normal series and in a series with transplanted neural folds, indicate that a simple relation exists between subdermal, dermal and epidermal melanophores which makes it highly probable that all of these cells arise from one cell type. Accordingly, the only distinction between melanophore types is one based on their position at a given time. Whether this holds for certain anurans where a peculiar type of adepidermal melanophore, functioning as a supporting net work in larval stages, has been described (18, 39) is not clear. Studies on anurans such as those made by Stearner on urodeles are obviously needed to settle this point.

The increase in melanophores at metamorphosis and their appearance in regions where they had hitherto not appeared has been observed in animals with exchanged segments of neural folds (114). For example, transplanted melanoblasts of *Amblystoma punctatum* origin remain undifferentiated, hence produce no pigment in the ventral region of the trunk of *Triturus torosus* hosts until metamorphosis. At this time they differentiate in large numbers and form the typical donor spotted pattern in *T. torosus* hosts which are normally uniformly pigmented. Such results show, without any question, that the melanophores which appear at metamorphosis are

derived from the neural crest and indicate, further, that many are held over in an undifferentiated (unpigmented) state until activated by the metabolic changes accompanying metamorphosis. Although mitoses have been observed in melanophores, it is doubtful whether the phenomenon is common enough to account for the rapid increase observed at this time.

Histological study of the development of the pigmentary system reveals that many important changes take place before the onset of metamorphosis. The development of the dermis, for example, is completed before metamorphosis is begun (107). In some forms (Amblystoma), the striking color change is brought about not only by an increase in the number of dermal melanophores but by their rearrangement within the dermis, just below the epidermis, to form a more complete layer instead of a loose network. Although some melanophores are present in the epidermis they are relatively few in number. The typical yellowish spots result from an aggregation of lipophores in the epidermis and a slight decrease in number of underlying melanophores of the dermis. In other forms (*T. torosus*) the uniform brown pattern, characterizing the metamorphosed animal, is brought about by a large increase in the number of epidermal melanophores which in this species play the major rôle in adult pigmentation. Dermal melanophores are present but they are usually contracted and are masked by the heavy pigmentation of the epidermis.

In *T. torosus*, Stearner observed migration of melanophores from the dermis into the epidermis throughout the larval period, but found it to be particularly prominent at the time when the increase of epidermal melanophores is most rapid. It appears that the morphology of these melanophores is modified by the epidermal environment. Their highly branched processes extend for long distances between the epidermal cells. Most interesting is the observation that the ends of the processes penetrate the epidermal cells, pass over the outer side of the nucleus and form a caplike arrangement of pigment granules. This intimate relation, together with the close correlation between the presence of epidermal melanophores and melanin in epidermal cells, suggests strongly that the melanophores are the source of the melanin pigment granules occurring in the epidermal cells and, thus, strengthens the relationships in this respect between the amphibians and birds and mammals, including man. In view of the observations of Rahn (85) on the deposition of melanin pigment in the epidermal cells of the skin of the rattlesnake by melanophores, reptiles may, also, be added to the list. On the basis of our present knowledge it is quite improbable, in fact, that the epidermal cells of the integument of any vertebrate are inherently capable of autonomous melanin pigment formation.

Birds (Fowl)

The plumage of birds is characterized by a succession of feather types pertaining to definite stages of the life history. In all birds there is a sharp distinction between down feathers and feathers of the definitive plumage (contour feathers). Down feathers are nearly always uniformly colored while definitive feathers may display a wide variety of coloration.

The present status of our knowledge leads to the view that melanin pigmentation

patterns are the result of the interaction of two separate and distinct components: the melanoblasts which produce the pigment granules and the feather germ or papilla which produces the structural elements of the feather. When a feather begins to form, these two components are for the first time brought together. Their relationships in the pigmentation process may be more clearly understood by considering briefly the mechanism of feather formation.

The feather arises from a specialized unit of skin, the papilla, located at the base of the follicle. All feather parts, shaft, barbs and barbules, are derived from the epidermal component of the papilla which becomes specialized to form a ring or collar of embryonic cells encircling the rather massive dermal component. Differentiation of the feather parts progresses from the apical margin of the epidermal collar. The apex of the feather is formed first; successively more basal regions are added by the continuous proliferation of the cells of the collar until the entire feather is formed (see Lillie and Juhn (68, 69), Lillie and Wang (70), Lillie (66, 67) for details). When a feather is shed or plucked the dermal papilla covered by a thin layer of epidermal 'regeneration' cells is left behind. From these cells a new collar forms which gives rise to new feather portions. In contrast to the epidermal component of the papilla which forms anew with each feather regeneration, the dermal papilla remains as a permanent body. If it is removed, the epidermis of the follicle cannot regenerate a feather (70). The dermal papilla functions as a feather 'inductor' and determines the symmetry and orientation of the feather (70). The specific type of feather induced is dependent upon the tract origin of the overlying epidermis (118). A saddle feather papilla, for example, from which the epidermal cells have been removed, transplanted to an empty breast follicle produces a breast feather from the epidermal 'regeneration' cells of the breast follicle and vice versa. Intact breast or saddle papillae, on the other hand, retain their specificity in follicles of either tract (118).

If pigmentation is to take place, prospective melanophores must enter the epidermal primordia of the feather elements. Their entry may be blocked, experimentally, in which case the feather develops normally, but remains unpigmented (90). The most recent evidence indicates that neither the epidermis nor the dermis of the papilla harbors melanoblasts permanently, but each successive generation of feathers acquires a new complement via the dermal papilla from a 'reservoir' of stem cells (melanoblasts) located within the dermis external to the follicle (43). (There is some evidence from recent *in vitro* experiments (76) which would lead us to believe that the dermis exerts an inhibitory influence on the differentiation of melanoblasts into melanophores.) Since pigment cells enter the epidermal collar in an undifferentiated, colorless stage they cannot be detected until pigment granules begin to form. This occurs in a particular region, the zone of differentiation (125) just anterior to the distal portion of the collar where the proliferating epidermal cells are being organized into barb ridges. Above this zone melanophore arrangement becomes definite and orderly. As the cells of the barb ridges gradually attain a certain degree of differentiation, characterized by the onset of cell elongation and keratinization, pigment granules are released from the tips of the melanophore processes or branches into the

cytoplasm of the feather cells (109, 121). Each melanophore deposits pigment granules in only a single small region of the feather during a limited time in development, after which the melanophore ceases to function as a living cell. The completed feather, then, represents a mosaic of enormous numbers of pigment granules laid down by many separate and distinct melanophores.

The respective rôles of the melanophores and the feather germ in the formation of the definitive color pattern have been analyzed by combining melanophores of individuals of species exhibiting one type of color and pattern with feather germs of individuals exhibiting a different color and pattern. Such combinations have been accomplished by means of a variety of methods: 1) By grafting melanoblasts from numerous sources (neural crest, prospective neural crest, skin ectoderm, mesenchyme and from developing barb ridges of regenerating feathers) to host limb buds, prior to the entrance of migratory neural crest cells (30, 35, 126, 87, 88, 80, 110). 2) By grafting prospective limb buds (prior to the entrance of crest cells) to the coelom of pigmented hosts, thus introducing prospective melanophores from the parietal lining into wing skin and feather germs (34, 91). 3) By grafting areas of wing skin and down feathers, experimentally freed of melanoblasts, to the backs of newly hatched host chicks (90). 4) By exchanging areas of back skin between two newly hatched chicks (25, 22). 5) By transplanting feather germs (papillae) between adult individuals (119). Irrespective of the immediate source of the melanoblasts or the method of introducing them into foreign feather germs, the results have been consistent in showing that the melanophores produce their specific color and pattern in homologous feathers of varieties normally exhibiting an entirely different color and pattern. Combinations have been made between all of the common varieties of domestic fowl (including bantams) and other fowl such as pheasants, guinea fowl etc. and even several of the common song birds. In all cases melanophores from varieties with uniformly colored or white plumage (Black or Buff Minorca, Black or White Silkie, etc.) always produce their characteristic uniform color patterns. Melanophores from varicolored varieties (Brown Leghorn, Pheasant, Guinea, Robin, etc.) likewise produce their characteristic varicolored patterns; interestingly enough, melanophores from males and females of varieties showing sex-linked differences in plumage pattern (Barred Plymouth Rock; Rhode Island Red—Barred Plymouth Rock cross) also produce their respective male and female types of pattern in whatever host feather germs they are introduced. The sex genotype of the feather germs in which the melanophores function has no effect on the final pattern produced (127, 128).

Microscopic examination of the barbules of the resulting feathers of the graft-region revealed that the transplanted foreign melanophores deposit in the feather cells melanin granules characteristic of their own particular species (126, 87). It should be pointed out that all of the white varieties of fowl tested (Leghorn, Wyandotte, Plymouth Rock, Silkie) possess melanoblasts which, upon being transplanted to feather germs of pigmented hosts, enter and occupy definite positions in the feather germ where they differentiate into melanophores. However, such melanophores die at a low level in the feather germ before they can deposit pigment in the feather barb or barbule cells; hence the feather is white. The *in vitro* studies of Hamilton (47) have shown that the melanoblasts of the white feathered varieties are all potentially

capable of synthesizing black melanin granules, but are characterized by a lower viability and a higher sensitivity to adverse environmental conditions than those from pigmented varieties. For a discussion of the causal relationships between viability differences and whiteness in feathers see Hamilton (47), Willier and Rawles (126) and Willier (125).

While the large body of data agree in showing that the genotypic constitution of the melanophores is the controlling factor in phenotypic expression of color and pattern, it is also clear that the migration of precursor melanophores into the feather germ and their differentiation and arrangements into definitive patterns are not autonomous but influenced to a great extent by a variety of extrinsic factors. Skin grafting experiments have demonstrated that the invasion of melanoblasts is controlled by the skin and feather germs. Areas of melanoblast-free skin, transplanted soon after hatching to normal host chicks, are readily invaded by melanoblasts from the surrounding host skin (90). But, melanoblasts from the host do not invade a skin graft containing its normal complement of melanoblasts (except occasionally at the margins as evidenced by the presence of mosaic colored feathers, Danforth (22, 23). Such results indicate that some constant ratio between the cells of the skin (and feather germs) must be established and maintained during the process of pigmentation. Foulks (43) has shown that in a normal regenerating feather germ the rate of melanoblast-migration from the dermis differs at different stages during the process of regeneration and, in down feathers, Watterson (121) found that pigmentation is due solely to descendants of melanoblasts which penetrate the feather-forming epidermis during a very limited interval of time in development. It has been established further that conditions within the feather germ determine the orderly arrangement of melanophores, the direction of outgrowth of melanophore-branches and the time of acceptance of pigment granules (Watterson (121) and others).

Other physiological properties of the individual feather germs such as position on the body (tract location), growth rates etc. act as modifying factors and affect the details of the basic type of pattern produced by the melanophores of a particular genotype. Regional differences in feather pattern may be readily observed by examining the plumage of any bi- or multicolored bird. In a normal adult Barred Plymouth Rock, for instance, all the feathers are barred; yet the width and clarity of the bars, relative amounts of black and white etc. vary considerably in feathers from different regions such as wing, tail, breast or back. Each shows its own characteristic type of barring. When melanoblasts from a Barred Plymouth Rock (embryo or adult) are introduced into the developing feather germs of a non-barred individual (Black Minorca, N. H. Red, White Leghorn) these same characteristic differences in the quality of the barring appear in homologous feathers of the graft-region. Similarly, melanoblasts from a non-barred individual, e.g. New Hampshire Red, migrating into feather germs of Barred Rock or White Leghorn origin, produce in saddle feathers the solid red uniform pattern characteristic of the normal New Hampshire Red saddle feathers; in wing feathers the black and red splotched pattern characteristic of homologous New Hampshire Red wing feathers etc. (90).

The black and red response always given by New Hampshire Red melanoblasts in certain wing feathers of different genotypes (Barred Plymouth Rock, White Leg-

horn), in contrast to the all red response in either breast or saddle feathers, is of particular interest as regards the course of differentiation of red and black melanophore types. The presence of these two distinct types of melanophores in the plumage of many other varieties of domestic fowl such as Brown Leghorns, Rhode Island Reds, Silver Campines and various hybrids, and the marked changes in their relative numbers and distribution which may occur with age, hormonal and nutritional changes, have led to much speculation regarding their developmental relationships. Since this topic has been discussed in detail by Willier (124, 125) and Willier and Rawles (127), it need only be mentioned here that the available evidence supports the view that the two types, red and black, differentiate directly from a common precursor cell (melanoblast) rather than the view that red melanophores are merely blacks whose development has been arrested before they reach the black stage. It would appear, then, that melanoblasts of any black-red genotype are potentially capable of differentiating into either black melanophores synthesizing and depositing black, rod-like melanin granules in the feather parts or red melanophores synthesizing and depositing reddish spherical granules: the locus of differentiation, i.e. the epidermal substratum of the particular feather germ or region of the feather germ in which the melanoblast differentiates, determines which of these two potencies is realized. Once a melanoblast becomes fixed (segregated) as to type, black or red, it cannot change to the opposite type. Transitional types have never been observed (24, 48, 81, 110).

It should be emphasized that there is no correlation between the locus of origin of the melanoblasts and their differential response to feather germs of various body regions. Melanoblasts from any region or level—in fact, even melanoblasts from the parietal lining which normally would never enter feather germs—introduced into an area of wing skin, produce in the feathers the color and pattern characteristic of wing feathers (91). The same principle holds for feathers of other regions, breast, saddle etc. Such results lead to the conclusion that in the undifferentiated, melanoblast-stage, pigment cells of any one genotype are all alike.

Rhythmic Patterns—Barring. Not only is there a constant reaction between melanoblasts and feather germ, but also reactions between the individual melanoblasts within the feather germ. The importance of such reactions in the formation of rhythmic, barred patterns has been emphasized by Nickerson (80) on the basis of results from a special study of the Silver Campine and the Barred Plymouth Rock, varieties of fowl exhibiting two genetically and morphologically distinct types of black and white barring. In view of the attempts that have been made to explain the rhythmic pattern formation in barred fowl without considering the melanophores as a distinct part of the pattern-producing system, the recent work of Nickerson deserves more than passing mention.

Nickerson found that melanoblasts from the Silver Campine and Barred Plymouth Rock introduced into feather germs of White Leghorns always produced barred patterns with the barring-period characteristic of the donor. These findings indicated strongly that the periodicity is intrinsic in the melanophores. It was observed further, by appropriate isolation tests, that the prospective white bands or bars of the developing feather contained melanoblasts potentially capable of differentiating and depositing pigment granules in feathers, i.e., the whiteness of the white bands is not

due to an absence of pigment cells, but to their failure to differentiate and deposit pigment granules. In the light of these observations it was suggested that the barring rhythm is controlled through the medium of some diffusible, metabolic substance, produced by the active melanophores in the black band, which inhibits pigment synthesis by the melanoblasts in the subjacent differentiating barb ridges of the prospective white band. As the barb ridges form and increase in length, the source of the inhibitor (black-band) becomes progressively removed from the zone of differentiation with a consequent diminution in concentration of the inhibiting substance. When the concentration falls below the threshold of inhibition, the next series of melanoblasts begins to differentiate and to synthesize and deposit pigment in the feather parts. With the production of pigment, inhibitor substance will again be formed and the cycle repeated until feather differentiation is completed. The above mechanism possesses the characteristics necessary for the production of a time rhythm which must arise as the resultant of the action of two opposed forces: 1) the production of the inhibitor which fluctuates with pigment production and 2) its gradual decrease in concentration with time and with distance from the center of production. Also, the epidermal cylinder of the feather germ, which is devoid of vascular circulation, is an ideal medium for the action of such a mechanism. Strengthening evidence that the production of pigment by the melanophores is an important factor in the control of barring rhythms is found in the fact that the phase of bar formation in a feather germ at any given time is dependent upon the length of time from the beginning of pigment deposition in the feather rather than upon its total age.

In contrast to the Silver Campine, the bar gene of the Barred Plymouth Rock is sex-linked—the female is the hemizygous sex. Since the white bars or bands are wider in the male than in the female pattern of the Barred Plymouth Rock, it would appear that the melanoblasts are inhibited over a greater expanse of developing barbs when the genotype is male than when it is female. This indicates that either the threshold of inhibition is lower in male melanoblasts than in female or that the former produce the inhibitor in greater quantity than the latter. It is to be expected that some such differences in reactivity could show up with differences in genotype (see Willier and Rawles (128) for a discussion of the effects of sex-linked genes on the expression of melanophores in the formation of sexually different pigmentation patterns of feathers of the Barred Plymouth Rock).

As mentioned previously, the details of the barred pattern are modified by many factors involving both the melanophores and the epidermal substratum of the feather germ in which they function. Nickerson tested the validity of his diffusion hypothesis by an analysis of the effects of variations in growth rates, barb ridge size, pigment intensity etc. on the definitive barring pattern. In general, the hypothesis appears to be adequate for explaining the observed differences in barring exhibited by different individuals and by different feathers of the same individual. The nature of the inhibiting substance, however, remains for subsequent work to elucidate. As Nickerson suggests, it may prove to be non-specific, perhaps some substance or substances which act by altering the redox potential and consequently the melanin pigment production of the melanophores (cf. 41).

Mammals (Mouse, Rat, Guinea Pig)

While the development of pigmentation pattern in mammals has not been analyzed nearly as thoroughly as that of birds the experimental results as far as they go are in complete agreement. This is to be expected on the basis of similarities existing in the origin and properties of the melanophores and in the formation and function of the specialized integumentary derivatives, the feathers and the hairs, which in both of these groups are the structures primarily concerned in the development of the definitive pigmentation. It has already been pointed out that prospective melanophores of the mouse arise from the neural crest and are distributed to all parts of the body by extensive migration during early stages in embryonic development. Transplantation experiments have shown that by the twelfth day of gestation these cells are present in all portions of the body (92). Many potential melanophores undoubtedly do not undergo differentiation. Since they cannot be distinguished from accompanying embryonic cells prior to melanin synthesis little is known of their history. In order to participate in hair pigmentation, prospective pigment cells must make their way into the epidermal primordia of the hair elements. In their absence a structurally normal hair can be formed but it cannot become pigmented (89, 92). Hairs like feathers are being constantly shed and replaced throughout life. Each follicle gives rise to a succession of hair generations, each of which requires a new complement of melanophores. So far, little is known of the immediate source of this new complement or how and when during hair regeneration the melanoblasts enter. Presumably they enter via the dermal papilla since it is the only permanent component of the follicle. The pigment cells make their first appearance in the rapidly dividing matrix cells of the hair bulb surrounding the papilla. They deposit pigment granules into the hair cells in a manner similar to that described for the feather and for other epithelial cells (96, 60, 37, 78, 6, 130). After completing their pigmentary function the melanophores degenerate as keratinization of the hair cells sets in. In order to provide pigment for each additional part of the hair as it in turn differentiates, melanophores, obviously, must be continually supplied at the growing base. The completed hair, like the feather, contains numberless melanin granules deposited by many different melanophores functioning at different time intervals during hair development. Thus, any variations or fluctuations in pigment-forming activity of the melanophores will be recorded in the deposition of the pigment granules. It was pointed out by Lillie (67) several years ago that "There is no more convenient or accurate record than the feather once one has learned how to identify the locus of reaction in the germ with the result in the finished feather; in short, to read its autobiography." The same statement holds for the hair. The usefulness of the pigment-granule-record in the finished hair for analyzing the nature of the action of various genes on the pigmentation process in the mouse has been demonstrated recently by Russell (100). In view of the numerous methods that have been employed in the past to analyze pigment-gene action in mammalian skin and hair, this method of approach is of particular interest and promise.

In the mouse as in fowl, melanoblasts, by virtue of their migratory capacity, invade areas of grafted normal skin under proper experimental conditions. Proof has come from homoplastic transplantations of undifferentiated mouse skin. By

removing areas of dorsal and ventral skin from colored and albino donors at birth (or several days earlier) and transplanting them to ventral and dorsal regions, respectively, of new-born hosts of a different color genotype, Reed (93) and Reed and Henderson (94) succeeded in combining pigment-cells of one genotype with hair germs of a different genotype. In all cases, hairs at the periphery of the grafted area of skin, in a zone a millimeter or more in width, developed the color characteristic of the host genotype. (Other hairs of the graft developed the original, donor color showing conclusively that donor melanoblasts were already present in the skin at the time of its removal.) Such results can be explained only on the basis of melanoblast migration. That the host-colored hairs of the graft are not the result of replacement of the grafted epithelium by that of the host is evident from distinct differences in the morphology of hair types developing from dorsal and ventral skin. The hairs of the grafted skin develop always according to origin. These and more recent experiments with other epithelia (13) show that the replacement hypothesis of earlier workers is no longer tenable.

Owing to the fact that many of the genotypes investigated by Reed, and Reed and Henderson displayed color differences in the hair coat of dorsal and ventral body regions, the data (if the writer may interpret them) illustrate further an important point that might otherwise have been overlooked, namely, that the color pattern produced in the grafted hairs by the invading host melanophores is a replica of that found in the hairs of homologous regions of the host. For instance, melanoblasts from the ventral, tan, region of a black-tan host invading a graft of dorsal skin from a colored or an albino donor, produce in the dorsal hairs black, not tan, coloration; similarly melanoblasts from the dorsal, black, region of a black-tan host invading a graft of ventral skin of a different genotype, produce in the ventral hairs tan, not black, coloration. Thus the genotype of the melanoblast provides it with the capacity to develop into a melanophore which synthesizes either black or tan pigment; the epidermal cells of the hair primordia determine which one of the two capacities is realized. The same principle holds for the various other genotypes tested.

As we have already seen in the foregoing section, the identical situation exists in fowl. It will be recalled that host melanoblasts from the saddle region of a New Hampshire Red invading the wing feathers of a wing skin graft of Barred Plymouth Rock origin, placed in the saddle region, produce in the wing feathers the characteristic red-black mottled color pattern of the wing, not the solid red color pattern of the saddle feathers (see page 399).

In hooded rats the migration of melanoblasts into the prospective white hairs developing from a piece of skin from the ventral region of the body grafted autoplastically at birth to a dorsal, prospective black region has been demonstrated by experiments of the writer now in progress. Owing to the fact that hairs developing from dorsal and ventral areas of skin exhibit certain morphological differences (size, texture etc.) and retain these structural characteristics after transplantation to another location, the grafted area is easily distinguishable. Results show that the pigment cells which in dorsal hairs produce a jet-black color pattern, produce in the adjacent grafted hairs of ventral origin a dark-grey color pattern. This latter color is characteristic of the hairs of small ventral spots or flecks occurring now and then in normal

individuals of this strain. So, here again, as in the mouse, we have a demonstration of a variation in pigment-cell response elicited by hair germs originating in a different body region.

In contrast to the results with undifferentiated skin of new-born mice and rats, the hairs regenerating from unpigmented areas of adult (or nearly adult) guinea pig skin transplanted autoplastically to pigmented regions do not become invaded by melanoblasts from the surrounding skin (105, 40, 65). In many cases, in fact, the hair does not regenerate at all (40, 65). The difference in results can no doubt be attributed to the tremendous difference in the developmental age and the physiological condition of the skin at the time of transplantation. Even at birth a guinea pig is much 'older' than a new-born rat or mouse. The work with new-born skin grafts in both the mouse and the rat suggests that melanoblasts from the surrounding skin become incorporated in the grafted follicles at a particular time in development, i.e. during the process of follicular organization. Evidence of a similar nature is available in birds. Watterson (121), for instance, found that melanoblasts enter developing feather germs only during a very limited period of time during the formation of the feather germ. Danforth's beautiful skin transplantation experiments have shown, further, that feathers regenerating from areas of skin exchanged at hatching between two chicks of different color genotypes, retain their original color and pattern indefinitely. (In the chick the organization of the feather papillae is completed approximately 10 days prior to hatching.) At the margins of the graft, however, where certain follicles occasionally undergo reorganization during the process of 'healing in', melanoblasts which have invaded the grafted skin do get a chance to enter and become incorporated in the feather germ, as evidenced by the formation of mosaic feathers of both donor and host color. The evidence, then, points to the view that during the early differentiation of feather or hair papillae some balance or equilibrium between the melanoblasts and the hair- and feather-forming epidermal cells is established and maintained. Following this period of stabilization melanoblast entry is, somehow, blocked.

While the experimental evidence indicates that melanoblasts can enter hair (and feather) papillae only under certain physiological conditions correlated with developmental age, this does not necessarily hold for the more generalized epithelium of the skin which is not concerned with hair formation. In fact, numerous investigators (19, 72, 101, 104, 102, 40, 65, 13, 14) have reported that white skin epithelium from a spotted black and white guinea pig becomes black after it is grafted to a black region; black skin grafted to a white area slowly blackens that of the white area surrounding it. Such experimental evidence indicates that the epidermal epithelium of the skin proper is potentially capable of receiving melanoblasts from an adjacent area after the skin is fully differentiated.

In regard to the development of melanin pigment in the epidermal epithelium of the skin, attention must be called to certain differences that are found normally between mice and rats and guinea pigs which show up, also, in skin grafts. In the mouse and rat the epidermal epithelium does not become pigmented. Except for certain relatively hairless regions, such as ears, nose, tail, soles etc., melanophore differentiation is restricted to the specialized epidermal cells concerned with hair

formation. In the guinea pig, by contrast, the epidermis of the entire body as well as that destined to form hair may become pigmented or, at any rate, is potentially capable of permitting melanophore differentiation. Since most of the workers with guinea pig skin transplants, with the exception of Seevers and Spencer (105), appear to agree that the unpigmented skin epithelium of a white area of the body grafted to a pigmented area elsewhere on the same individual does gradually become pigmented, the question arises as to whether this result is due to the migration of undifferentiated (colorless) melanophores from the surrounding skin or to an 'activation' of those already present in the white region. The principal argument which has been brought against melanoblast migration (79) appears to be based on the assumption that melanoblasts already present in an area of skin prevent the entrance of others. This does not necessarily follow. In the mouse, for instance, we know that melanoblasts are present in the prospective skin of all regions of the body by the twelfth day of gestation. Yet, skin transplanted several days before birth, before any follicular differentiation has begun, and which certainly contains melanoblasts, does receive other melanoblasts from the surrounding skin of the host. Proof has come from transplanting skin between donors and hosts of different color genotypes (homoplastic transplantations) and has already been discussed (pp. 402–403). That host melanoblasts invade the graft is, of course, evidenced by the fact that donor hairs develop the color-pattern of the host genotype. The fact that colored hairs developing in an albino skin graft, for example, are invariably the color of the host and not the masked color of the albino genotype (94) is extremely 'damaging' to any 'activation' conception. The question obviously cannot be settled for the guinea pig by the use of autoplastic grafts in the adult, near-adult or juvenile animal. In view of present-day trends the 'infective cellular transformation' hypothesis of Billingham and Medawar (14, 14a) and Medawar (79) has a certain appeal particularly in explaining their specific results; yet this hypothesis has most certainly not disproved or brought into discredit the known facts regarding melanophore migration clearly established by much careful and painstaking research with amphibians, birds and other mammals.

CONCLUSION

From the data reviewed in this paper we are led to conclude that in the vertebrates, in general, melanin pigmentation in its great variety of forms is produced by one type of highly specialized cell, the melanophore, which originates in the embryonic neural crest. That the melanophores found in all of the vertebrates are homologous cells is evidenced by their origin, by their capacity for extensive migration in their early undifferentiated (melanoblast) stages when indistinguishable from mesenchyme cells, by their general morphology and physiology and, above all, by their capacity to synthesize melanin pigment granules and deposit them in epidermal cells. The data indicate strongly that all melanoblasts of any one individual are initially alike and potentially capable of synthesizing melanin granules. Whether or not this potentiality is realized depends to a great extent upon the particular tissue substrate with which they become associated, in other words, their locus. In the lower, cold-blooded vertebrates, differentiation of melanoblasts into melanophores is, in general, widespread and occurs in a variety of deeper visceral tissues, in the dermis and in the

epidermis. In the higher, warm-blooded vertebrates melanophore differentiation is much more limited and occurs primarily in the epidermis and its specialized derivative, feathers and hair. Associated with the development of these keratinized epidermal structures is the loss of the ability of the melanophore to shift its contents in effecting color changes.

Like any other embryonic cell of the individual, the melanoblast is endowed with a complement of genes and its development and ultimate differentiation are dependent upon the activity of these genes, subject at all times to correlative influences from adjacent or contiguous tissues and to influences from the external environment. While the action of genes in the control of morphological and color patterns in general has not been completely clarified, there is much evidence that genes act by determining the presence or absence of specific enzymes which guide metabolic processes along particular paths or channels (129).

The interpretation, towards which recent investigations on the development of melanin pigmentation in the vertebrates appear to be leading, is that color patterns arise through a constant interaction between melanoblasts and their tissue substrates and to reactions between the melanoblasts themselves. The complement of genes with which a melanoblast of a particular genotype is endowed, provide it with certain reaction potencies which determine the particular type of response that can be elicited by a particular tissue substrate (125). The physiological condition of the substrate may be influenced or 'conditioned' by numerous factors, hormones, vitamins and various stimuli from the external environment, such as temperature, light etc. Changes in the physiological condition of the substrate can elicit changes or modifications in melanophore response, but the specific response is always in accordance with the genic constitution of the melanophore.

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ANTICONVULSANTS[1]

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THE NUMBER AND VARIETY of antiepileptic therapies suggested in the past (200) reflect the frustration of generations of healers confronted by the 'Sacred Disease'. Today, when considerable hope can be extended to the epileptic patient, the armamentarium of clinically acceptable anticonvulsants does not exceed 10 useful preparations. As yet the laboratory methods for screening potentially useful agents are highly empirical, and the probability that any one such agent will receive clinical validation is small.

It is not the purpose of this review to recount the vast literature on clinical effects of the host of drugs which have been tried, in order to rank them in a sort of popularity poll, nor even to cover all the known physiological effects of the most useful agents, seeking a thread of consistency in the tangle of unrelated and desultory observations. At the moment it seems more profitable first to make a frontal attack on the problem of the seizure itself—its mechanism rather than its treatment; then to examine the crucial points in this mechanism which might be advantageously blocked by drugs; and finally to discuss some of the typical actions and structural chemical relations of the clinically useful antiepileptic agents.

MECHANISM OF SEIZURES

That excessive electrical stimulation can produce seizures was demonstrated in the 1870's by Fritsch and Hitzig (62, 103) and by Ferrier (56) in their classical explorations of the excitable cortex of experimental animals. Such studies gave strong confirmation to the speculations of John Hughlings Jackson (106) on the initiation and spread of convulsive activity in the human brain. After more than 70 years, one can hardly improve today upon Jackson's deductions, based on his exhaustive inquiries into aura, onset and pattern of seizures, post-ictal recovery of function and associated interseizure signs of neurological lesions. His primary emphasis was upon the rôle of focal lesions in the various convulsive disorders (pp. 185, 255). The functional aberration of the discharging lesion was well defined by Jackson half a century before the advent of electroencephalography. "Epilepsy is the name for occasional, sudden excessive, rapid and local discharges of grey matter" (p. 100). He inferred that normal brain tissue could participate in the convulsive activity initiated by the abnormal focus, leading to generalization of a seizure (p. 239). He removed the problem of loss of consciousness during petit mal or major seizures from the realm of metaphysics and concentrated instead on the effect of excessive discharge upon the anatomical sub-

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stratum required for more complex nervous function (p. 205). The occurrence of post-seizure depression of function became, in Jackson's hands, a useful anatomical tool and also gave theoretical support to the concept of excessive discharge during a seizure (pp. 145, 183).

Other contemporary advances in the analysis of mechanism of seizures came largely from animal experimentation. Both electrical (7, 60, 141) and chemical methods (64, 125) were used focally for the production of experimental seizures in the latter half of the last century. These experiments raised a new problem. Why, in view of the ease with which seizures could be initiated, did not the activity of neurones in ordinary behavior result in wholesale cerebral explosions? It was necessary to invoke the concept of inhibition. The possible rôle of inhibition in preventing the appearance of seizures was apparently first recognized in 1881 by Bubnoff and Heidenhain (25). They reasoned that stimulation of the gray matter ordinarily produced excitation and inhibition in equal amounts and that the latter restricted the spread and cut short the duration of excitation. According to this concept, seizures occurred either because of excessive stimulation or because, for other reasons, the ratio of excitation to inhibition became greater than unity.

The idea that inhibitory processes must be exceeded by excitatory events to permit the transition from normal behavior to seizures has an inner logic independent of the mechanism of the inhibitory process. A revolutionary conception of the nature of this transition was proposed in 1906 by Sherrington (181). He postulated a transformation of inhibition into excitation under the influence of convulsant drugs or excessive stimulation (p. 112). His examples were drawn not only from spinal cord experiments but also from studies on cerebral cortex (p. 292).

In spite of the most refined modern methods of electrical recording, the mechanism of inhibition has continued to be highly controversial (24, 135, 172). The Brooks-Eccles (24) hypothesis of inhibition contains many implications for the problem of seizures, since it contemplates the possibility that the same short cells which ordinarily inhibit passively can excite actively if they are made to transmit impulses. Thus an anatomical basis is laid for the Gargantuan leap of Sherrington from inhibition to excitation.

The rôle of inhibition in the self-regulation of cortical excitability through reentrant circuits has been vigorously championed by McCulloch (145). The existence of gross inhibitory areas in the cerebral cortex, operating upon cortex through extracortical mechanisms, has been convincingly demonstrated by Dusser de Barenne and McCulloch (42). It would be more difficult to demonstrate a much finer mechanism of inhibition within cortex itself and yet the variety of human experience demands an intracortical mechanism such as that postulated by Bubnoff and Heidenhain and anatomically specified by Brooks and Eccles. Certainly one feature which distinguishes a major seizure from normal behavior is the cataclysmic loss of anything resembling normal reciprocal inhibition even at the lowest levels of nervous integration (96).

The development of methods for the recording of the electrical activity of the human brain has provided convincing evidence for Jackson's surmise that abrupt and excessive discharges are characteristic of seizures and of epileptogenic lesions (18, 71, 108). It is interesting to note that an earlier enthusiasm for classification and inter-

pretation of convulsive disorders on the basis of wave-form in the electroencephalogram (71) has progressively yielded to the Jacksonian emphasis on the value of localization of the discharging lesion (72, 108). Thus psychomotor or psychical seizures have come to be recognized as partial seizures with a focus in either the temporal or frontal lobes (67, 158, 161). Likewise seizures of the petit mal triad have been referred to subcortical midline foci (104, 109, 161) which may be either the seat of a lesion or activated secondarily from other sites.

Not only has electroencephalography confirmed Jackson's concept of occasional sudden, excessive and local discharges, but the more refined electrical studies by Adrian and Moruzzi (2, 155) on single neurones of the pyramidal tract have shown that the seizure discharges are also of high frequency, thus completing Jackson's definition. The importance of these high frequency discharges, elicitable by chemical or excessive electrical stimulation, has been emphasized by Moruzzi, particularly with reference to the manner of invasion of normal brain by excessive activity from a focal lesion.

The anatomical pathways for spread of seizure discharges have been most extensively studied by Rosenblueth *et al.* (175, 176), who used focal stimulation and electrical recording in animals; their work provides an excellent basis for understanding of the march of an aura and other seizure prodromata, as well as for the generally recognized tendency of lesions in some cortical areas to produce convulsive disorders more readily than in others.

Since lesions of the brain do not invariably result in convulsive disorders and because the lesion may be constantly present whereas the seizures are intermittent, the special character of the epileptogenic lesion has received sporadic attention. Certain investigators have stressed instability of the brain as a whole, characterized by sensitivity to changes in such factors as blood gas tensions (68, 71). The Montreal group has placed emphasis on the vascular abnormalities which are demonstrable at the frontiers between normal brain tissue and areas of scarring, etc.; they view seizures as the end-result of progressive neural injury produced by vascular spasm (160). Another point of view is that of Cannon and Haimovici (27), who extended the principle of sensitization by denervation to include the effects of endogenous acetylcholine upon neurones sensitized by loss of afferent connections within the brain. Evidence for this view is not lacking (156, 192).

Most discussions of the nature of seizure foci seem to imply that there is present a collection of pathologically altered neurones which fire excessively when exposed to normal stresses. Another possibility involves the concept of normal neurones exposed to the abnormal stresses of restricted blood supply, etc. and thus forced to discharge excessively. But a third possibility might well be kept in mind. If previous focal injury should have permanently destroyed the smallest (and usually most vulnerable) neurones of a portion of a nerve net, then one might predict from the Brooks-Eccles hypothesis (24) a stable hyperactive region which is deprived of its internal inhibitory mechanisms and therefore capable of responding excessively. This view obviates the necessity for invoking abnormal stress or pathological alteration of function in the individual neurones. When one considers the monotonous regularity of seizures in many patients, continuing for years with no evidence of pro-

gressive neurological damage, one is tempted to consider the possibility of a stable focus of this architectural type.

Penfield and Erickson (159) accept Foerster's characterization of epilepsy as not a disease *sui generis*, but rather as a characteristic response of the central nervous system to a noxious stimulus, which for its development ordinarily requires a predisposing lesion and a precipitating factor. Among the physiological precipitating factors should be considered changes in the *p*H, pO_2, pCO_2, total osmotic pressure and electrolyte composition of the fluid environment of brain cells, as well as changes in body temperature. Endocrine disturbances, nutritional deficiencies and exogenous chemical substances may activate a dormant lesion Thus a multiplicity of factors may play upon the brain, predisposed by acquired injury or inherited defect, to produce the manifestations of a seizure. Therefore it should not perplex the physician that patients with identical seizure patterns may often respond quite differently to treatment.

In summary and in extrapolation, both clinical and experimental evidence suggests that even small groups of neurones, variously damaged, may give rise to excessively rapid and uncontrolled bursts of discharges. Under certain favorable circumstances these may cause neighboring cells or related centers to discharge excessively. The critical step in the process of secondary involvement may be the appearance of high frequency discharges, the transformation of passive inhibitor cells into excitors, or both. By such a process of self-propagation the entire brain may be set afire in a tonic-clonic seizure, or the conflagration may be retained within bounds to produce manifestations dependent upon its anatomical base. Other centers may then be driven indirectly without themselves participating in the production of seizure discharges. In such a case the centers being driven will not be expected to show post-seizure depression. Perhaps this is the meaning of the characteristic cortical electrical signs and unconsciousness of petit mal, which disappear as abruptly as they began, leaving behind no sign of cortical involvement.

METHODS FOR STUDY OF ANTICONVULSANT DRUG ACTION

The ultimate test of anticonvulsant action must necessarily use patients as test material. The difference in response of different convulsive disorders to a particular medication, well illustrated by the specificity of trimethadione for seizures of petit mal type (128, 131), makes accurate diagnosis an absolute prerequisite for adequate evaluation of the clinical worth of new drugs, particularly in patients with multiple seizure types. Certain complications of clinical trial may tend to discriminate for or against a particular anticonvulsant. For example, the effects of addition of another agent to an established schedule of medication may give an exaggerated impression of the separate worth of the added drug, since synergism may occur, as with trimethadione and diphenylhydantoin (17) or phenobarbital and diphenylhydantoin (32). If the previous medication is too abruptly withdrawn, particularly in the case of phenobarbital, the increased frequency of seizures (113) may be erroneously credited against the new drug (88). An initial exacerabation of seizures, such as may occur with trimethadione (88) or with fasting (132) in cases of petit mal, may prompt the patient or his physician to discontinue medication after an inadequate trial of no

more than a day or two, when perseverance might have resulted in complete control of seizures. The tendency to reserve clinical trial of new agents for refractory patients whose seizures have resisted all ordinary therapy (147) places an undue burden of proof on the test agent.

Since it is highly impracticable and potentially dangerous to test the anticonvulsant action of new drugs directly upon patients without preliminary screening and assay, various laboratory methods have been evolved for such evaluation. For the most part, they have been based on the unquestioned assumption that a clinically effective antiepileptic agent must raise threshold for experimental seizures however induced. Only to a limited extent have methods been varied in order to examine more critically the possible mechanisms of anticonvulsant action.

In general, the methods for initiation of experimental seizures fall into three classes: 1) electrical, 2) chemical and 3) the production of chronic epileptogenic lesions.

1) *Electrical.* For studies of drug action, Albertoni (7) and Bikeles and Zbyszewski (19) used faradic stimulation of the cerebral cortex through the trephined skull; the latter investigators employed relatively prolonged stimulation (30 sec.). Schilf (178) adopted Jellinek's method (110) of stimulation through corneal electrodes and measured seizure threshold for alternating current applied for 0.5 seconds. Spiegel (185) also used corneal electrodes, but measured threshold with both time and intensity as variables, expressing threshold in ampere-seconds. Merritt and Putnam (148) applied rectangular pulses of direct current for 10 seconds through skull and mouth electrodes, as did Knoefel and Lehmann (120). Kozelka and Hine (122) and later Alles *et al.* (8, 9) employed alternating current of fixed strength; shock duration rather than current was varied in their threshold determinations. Bárány and Stein-Jensen (14, 15) passed alternating current through electrodes placed in the external auditory meatus and, in contrast to previous workers, determined the effects of drugs on seizure threshold for both long and short periods of stimulation. In addition, they observed the action of drugs on seizure pattern as well as upon seizure threshold. The reviewers (205) also directed attention to the effect of anticonvulsant agents upon seizure pattern elicited by supramaximal shocks, independently of their effect on threshold, and introduced an assay method based on their observations; they later extended this method to studies on the action of drugs on seizure pattern in non-epileptic human patients undergoing electroshock therapy for psychiatric disorders (204). Hemphill and Walter (99), Kalinowsky and Kennedy (114) and Garciadiego (65) had previously studied the effect of antiepileptic drugs on electroshock seizures in man, but directed their attention to changes in threshold rather than seizure pattern. Swinyard *et al.* (198) introduced the technique of artificially lowering seizure threshold by hydration of experimental animals in order to study the protective action of anticonvulsant drugs.

2) *Chemical.* Albertoni (7) should probably be credited with the first attempt to analyze the efficacy of an anticonvulsant drug (bromide) by means of convulsant agents as well as by electrical methods According to Moruzzi (155), Landois (125) applied creatine to the cerebral cortex of experimental animals, in the earliest attempt to produce focal seizures by chemical means. Baglioni and Magnini (11) introduced

the topical application of strychnine, which had been shown by Magendie in 1819 to produce seizures by its central nervous action. The literature on strychnine action has been reviewed by Dusser de Barenne (41). Recent usage of convulsant drugs in laboratory assay of anticonvulsant potency is examplified in the work of Everett and Richards (50), Pollock and Finkelman (166) and the reviewers (82, 88, 203). The latter have used various subconvulsive EEG phenomena as well as seizures for laboratory anticonvulsant tests. The use of metrazol for activation of EEG dysrhythmia in epileptic patients (116, 210) has also permitted observations of anticonvulsant drug action. Metrazol seizure threshold has been used by Goldstein and Weinberg (77) and Frost (63) to test anticonvulsant drug action in patients.

3) *Chronic Focal Seizures.* Speransky *et al.* (184) introduced the method of focal freezing the cerbral cortex to produce chronic epileptogenic lesions in dogs, a method later adopted by Keith *et al.* (118) Kopeloff *et al.* (121, 157) have produced focal lesions by implantation of alumina cream and other substances. The method has been used only sporadically for observation of anticonvulsant drug action by the latter group of investigators, although it obviously offers a useful method, particularly when combined with EEG studies.

MECHANISMS OF ANTICONVULSANT ACTION

The conceivable mechanisms by which anticonvulsant drugs might prevent, abort, mollify or reduce frequency of clinical seizures should include at least the following broad categories: *a*) action upon the non-neural lesion; *b*) action upon the abnormally altered neurones to prevent their excessive discharge; *c*) action upon normal neurones to prevent their detonation by excessive discharge.

In the first category should be included the possible actions of drugs in reducing the sensitivity of the abnormal vascular supply of an epileptogenic focus. If Penfield's (159, 160) concept of the rôle these vessels occupy in precipitating seizures has even a limited application, then the possibility of anticonvulsant drug effects through vascular action cannot be neglected. This in turn admits the possibility of anticonvulsant effects of a variety of autonomic stimulants and blocking agents, of which few have been studied (199). Aird (3, 4) has interpreted the anticonvulsant action of certain dyes and of desoxycorticosterone acetate as involving stabilization in permeability of cerebral vessels, to add still another possible vascular mechanism.

The second category assumes that, as a result of disease or injury, neurones may exist for a period in a state of hyperactivity, perhaps analogous to that produced by focal application of convulsant substances. It assumes also that drugs which have little effect upon normal neurones may have quantitatively important effects upon those which are pathologically hyperactive. No sharp examples of such a differentiation have yet been presented, although one might point to the ability of diphenylhydantoin to prevent abnormal susceptibility to electroshock seizures in hydrated animals (198), or the general ability of many other anticonvulsant drugs to prevent seizures induced by metrazol, picrotoxin, etc. A pertinent clinical example may be the considerable specificity of trimethadione in eradicating both the spike and wave response and the clinical seizures of petit mal in response to hyperventilation (88, 128, 162). But in general it can be said that agents which modify abnormal excita-

bility or activity of neurones may also be shown to have actions upon normal neurones (202).

Therefore one is led to consider more extensively the third and last category of anticonvulsant action. Clinical experience presents many illustrations of drug action in severing the chain of convulsive activity somewhere between seizure focus and widespread involvement of the normal brain (202). It becomes pertinent therefore to inquire concerning the action of the common anticonvulsant drugs upon properties of normal brain in general and of neurones in particular.

The reported actions of anticonvulsant drugs upon normal brain can be broadly divided into two major groups: *a*) increased threshold for an exciting agent; *b*) decreased responsiveness of the excited system.

The first group has received by far the most attention. The laboratory studies of Merritt and Putnam (148, 168), resulting in the introduction of diphenylhydantoin to clinical practice, have done much to propagate the idea that increased threshold is the *sine qua non* of anticonvulsant action. The concept is implicit in the procedures of all investigators who have attempted to assay new anticonvulsant drugs and it must be said that almost without exception they have succeeded in demonstrating by various methods that the most commonly accepted anticonvulsant drugs may raise threshold either for electrical or chemical stimulation or both (7–9, 14, 15, 19, 26, 29, 33, 38, 44, 48–51, 63, 65, 70, 75–77, 80–88, 99, 114, 119, 120, 138, 147, 148, 150–152, 164, 166, 168, 169, 173, 174, 185–187, 189, 190, 195–199, 202, 203, 216).

The reviewers (203) have contested this simple concept, particularly with regard to the action of diphenylhydantoin upon electrical threshold for production of minimal or focal seizures, but this does not negate the fact that something akin to an increase in threshold may be demonstrated if only the threshold for more severe seizures is observed.

Less attention has been given to the problem of drug action upon the ability of the brain to respond. Bárány and Stein-Jensen (13, 14) have found that shortening of the tonic phase of experimental seizures is a characteristic action of all the common anticonvulsants. The reviewers (205) have shown that abolition of the extensor component of the tonic phase of seizures produced by supramaximal shocks is a common property of the clinically accepted antiepileptic agents. The possible significance of this phenomenon will be discussed in more detail in relation to the action of diphenylhydantoin (see below).

Bikeles and Zbyszewski (19) postulated that the action of depressant drugs in preventing convulsive responses to faradization of the cerebral cortex was attributable to their ability to reduce temporal summation of repetitive stimuli. Bárány (12), after studying the effects of various anticonvulsants on seizure threshold for shocks of different durations, has found no evidence for such a mechanism.

Bárány (13) discusses another interesting possibility bearing on the problem of anticonvulsant action. He considers that the effect of a small degree of synaptic depression may have little importance in a short reflex chain but that the same effect at each synapse in a reverberating long chain system will be amplified in accordance with the total number of synaptic links in the circuit. Thus small increases in threshold might have little effect upon many normal functions, yet at the same time

make impossible the long-chain reverberation supposedly involved in the spread and maintenance of convulsive activity. An extension of this concept also raises the possibility that small increases in threshold may cause previously excitatory links to become inhibitory, in accordance with the Brooks-Eccles theory of inhibition (24).

One further type of anticonvulsant drug action seems to be sufficiently general to merit description. It has been recently found (202) that peripheral nerve, when subjected to excessive stimulation, may produce double or multiple action potential spikes, somewhat akin to the high frequency discharges described for pyramidal neurones by Adrian and Moruzzi (2, 155). The phenomenon is apparently dependent upon a prolonged depolarization and reduction in threshold, resulting in an extreme degree of supernormal excitability after passage of a single volley of impulses. These effects are prevented by the common anticonvulsants in relatively physiological concentrations. In addition, the repetitive and hypersynchronized discharge of peripheral nerve which is produced by immersion in high concentrations of phosphate is also abolished. Thus direct neuronal actions of anticonvulsant drugs can be demonstrated under conditions of excessive excitability.

An utterly different mechanism of anticonvulsant action must be considered for those drugs, such as amphetamine, which excite rather than depress the central nervous system and which, until the advent of trimethadione, were among the most effective agents in the treatment of petit mal (78, 128, 129, 214). Perhaps glutamic acid (167, 207) should also be considered in this group. When it is recalled that sensory stimulation may occasionally abort cortical seizures (106, 159) and that seizure discharges are much more common in sleep than in the waking state (67), it seems possible that increased activity of normal brain tissue may inhibit discharges from seizure foci. This may be in itself an adequate explanation of the anticonvulsant action of excitant drugs.

CHARACTERISTICS OF SOME COMMON ANTIEPILEPTICS

Bromide. Bromide was the first of the modern antiepileptic drugs. Its introduction by Locock in 1857 (136) for the treatment of catamenial seizures marked a sharp historical break from earlier inadequate methods of treatment (182). Samuel Wilks in 1859 (212) apparently was the first to use bromide (but with iodide) in ordinary epilepsy, thus dissociating the use of bromide therapy from the problem of supposed sexually exciting factors in convulsive disorders. The clinical use of bromide preparations became popular within a short time (89) and introduced an era in which the greatest emphasis was placed on the use of sedative drugs (191). Although progressively superseded by phenobarbital since 1912 and eclipsed by diphenylhydantoin since 1938, bromide continues to find some useful application in the treatment of grand mal seizures in the absence of other seizure types (165, 193). It is less useful in psychic seizures and may exacerbate petit mal attacks (127). The blood levels required to control patients (110–125 mg. %) tend to produce mild sedation and at somewhat higher levels bromide psychosis, neurological disturbances, dermatitis and other signs of bromide intoxication are apparent (79).

Experimental studies of bromide action have been relatively few. Albertoni (7) demonstrated in dogs that bromide antagonized the effects of convulsant agents and

of direct electrical stimulation of the motor cortex. Merritt and Putnam (148), Tainter *et al.* (199) and the reviewers (205) showed that bromide could raise threshold or modify seizure pattern in animals only in doses causing neurological signs. Kalinowsky and Kennedy (114) and the reviewers (204) obtained similar results in human patients undergoing electroshock therapy. Thus the anticonvulsant effect of bromide is relatively poor compared to that of diphenylhydantoin and other agents studied by the above authors. The mechanism of bromide action is unknown. It cannot be solely a matter of replacement of extracellular chloride ion (159) which can be materially reduced in peripheral nerve (139) or in the intact animal (101) without impairment of function.

Phenobarbital. Phenobarbital (5,5-phenyl ethyl barbituric acid) was introduced for clinical trial in 1912 as a sedative and hypnotic on the basis of the studies of Loewe (137), Juliusburger (111), and Impens (105) and in the same year was shown by Hauptmann (97) to be superior to bromide in the treatment of grand mal. Dercum (40) and Grinker (92) were among the first to popularize the use of phenobarbital as an anticonvulsant in the United States. It remained the chief pharmacological weapon against epilepsy until superseded by diphenylhydantoin within the last decade.

The principal limitation upon the use of phenobarbital as an antiepileptic has been its sedative property. Unfortunately the sedative action of bromide and phenobarbital was long considered a requisite for anticonvulsant action, a concept which until recently tended to impede progress in the search for more potent and specific drugs.

Another disadvantage of phenobarbital, but probably not peculiar to this drug alone, is the exacerbation of seizures or even the appearance *de novo* of convulsions after abrupt withdrawal of the barbiturate following a long period of treatment. The phenomenon was commented upon by Hauptmann (97) and has been studied more recently by Kalinowsky (113). Whenever a drug is capable of causing a withdrawal syndrome, one ordinarily expects to find evidence of development of drug tolerance during treatment. Experimentally the occurrence of barbiturate tolerance and cross tolerance has been shown by Green and Koppanyi (90) and Gruber and Keyser (94). Schulz (179) has shown that during phenobarbital treatment there is a progressive decline of plasma cholinesterase, which regenerates more slowly than the rate of disappearance of anticonvulsant action following abrupt withdrawal of phenobarbital. However, this interesting parallelism is not in itself an adequate explanation of phenobarbital tolerance and withdrawal signs and it would be presumptuous to conclude from the work of Schulz that the mechanism of action of phenobarbital is primarily anticholinergic.

The specificity of phenobarbital when compared with other barbiturates has received only occasional attention. Keller and Fulton (119) found that phenobarbital was the only one of several barbiturates examined which could completely abolish the electrical excitability of the motor cortex of monkeys when anesthetic doses were employed. Merritt and Putnam found that phenobarbital in sedative doses was much more effective than other barbiturates or bromide in preventing electrically induced seizures in cats. The reviewers (88, 195, 204, 205) have found

that the outstanding anticonvulsant effect of phenobarbital, as with diphenylhydantoin, is its ability to modifye lectroshock seizure pattern, in which respect it far surpasses various barbiturates having sedative but not clinical antiepileptic action. Taking species differences into account, in general it may be said that phenobarbital is somewhat less effective than diphenylhydantoin in ability to modify seizure pattern. Unlike diphenylhydantoin, phenobarbital possesses definite ability to increase minimal electroshock seizure threshold and to protect animals against metrazol convulsions. In these respects it is in turn inferior to trimethadione. Like diphenylhydantoin but unlike trimethadione, phenobarbital is highly effective in preventing repetitive firing in a supramaximally stimulated peripheral nerve (202).

Mebaral. Mebaral (3-methyl-5,5-phenyl ethyl barbituric acid) is the N-methyl derivative of phenobarbital; it is the barbiturate homolog of mesantoin. Mebaral was first reported by Heyde (100) and Blum (20) in 1932 as an effective antiepileptic in comparison with phenobarbital and in the same year pharmacological studies by Weese (209) suggested a superior margin of safety for mebaral (cf. 177, 204).

Other Barbiturates. Among other barbiturates which have been tried in convulsive disorders, 5-ethyl-5-(1-methyl-1-butenyl) barbiturate (vinbarbital; 'delvinal') has been reported by Davidoff and Doolittle (36) to give favorable results.

Trimethyl barbituric acid and 1-methyl-5-methyl-5-ethyl barbituric acid, particularly the former, have been found effective against grand mal but not petit mal in a preliminary report by Everett (48) who has also demonstrated that these compounds have a high degree of protective action against metrazol seizures in laboratory animals (48, 49, 51). The reviewers (81, 205) found these substances relatively ineffective in modifying seizure pattern.

Pharmacological actions of other barbiturates on the cerebrospinal axis have been extensively studied, but unfortunately for the most part pentobarbital and other sedative barbiturates without strong anticonvulsant potency have been used. The studies are therefore probably more relevant to an understanding of the nature of sedation, sleep and anesthesia than to antiepileptic action. The peripheral nerve studies of Heinbecker and Bartley (98) indicate that while pentobarbital increases threshold it is particularly effective in prolonging recovery time of neurones after impulse propagation. This property has also been demonstrated in thalamic synaptic transmission by Marshall (144), while Jarcho (107) has recently presented evidence that the prolongation of recovery cycle is even more pronounced at the cortical level.

The most direct studies of synaptic depression by barbiturates have been those of Eccles (43) who showed that pentobarbital blocked two-neurone transmission in the spinal cord primarily by increasing the extent of local depolarization required for initiation of propagated impulses by the motoneurone soma; he also presented evidence that the local increase in threshold was related to an increase in membrane resistance. The two-fold action of barbiturates in increasing threshold and prolonging recovery time is probably adequate to account for most of the various types of central depressant actions which have been studied by many investigators (1, 16, 21–23, 39, 59, 73, 126, 210). The central depression caused by most barbiturates may be related in turn to their ability to depress oxidative metabolism of brain in animals and man (47, 55, 91, 171).

Diphenylhydantoin. Diphenylhydantoin (5,5-diphenylhydantoin; dilantin; phenytoin; epanutin) was recommended for clinical trial as an antiepileptic by Putnam and Merritt in 1937 as a result of their careful laboratory studies of the anticonvulsant action of a large number of substances (168). In the following year they reported its superiority over bromide and phenobarbital, thus validating the importance of a rational experimental laboratory search for new antiepileptic drugs (148, 149). Other investigators promptly verified the clinical value of diphenylhydantoin (cf. 147) and it is now generally accepted to be at least the equal of phenobarbital in the therapy of grand mal, as well as an effective agent in the treatment of some cases of psychomotor epilepsy (69, 129, 208), related behavior disorders (61, 170) and symptomatic epilepsies (154).

The successful use of diphenylhydantoin established the important fact that an antiepileptic drug need not be a hypnotic (169). The effects of overdose of diphenylhydantoin both in animals (93, 120) and in man (10, 57, 149) were found to be in general excitatory rather than depressant.

In retrospect, it seems unfortunate that the laboratory methods of Putnam and Merritt (168) were based on the supposed ability of drugs to increase threshold for electroshock convulsions. In the particular case of diphenylhydantoin, the reviewers have been unable to demonstrate any threshold-raising ability in animals when minimal seizures or EEG signs of seizures are taken as the end point (83, 203, 205). Most other investigators (9, 26, 29, 50, 120, 199) have reported increases in threshold of varying degrees, but it is interesting to note that the criteria of convulsive activity taken by these investigators are varied and are much more rigorous than those which one would apply to a patient. Thus the results are complicated by drug-induced changes in seizure pattern. Furthermore the use of long periods of stimulation operates to conceal the occurrence of drug-modified seizures (203, 204). However, when the end point taken is that of minimal seizures, it has been demonstrated that diphenylhydantoin can increase threshold in animals whose excitability has been raised by hydration, although other anticonvulsant agents are much more potent in this respect (198).

There is more general agreement that diphenylhydantoin does not protect animals against convulsant drugs (50, 88, 120). Here again some authors have reported slight protection against metrazol (80, 216) which is more evident with chronic medication (80). But a large number of drugs, including some which could not be classed as useful anticonvulsants, are clearly superior to diphenylhydantoin in ability to protect against metrazol seizures (see fig. 1 for examples).

The same controversy has arisen in connection with the effects of diphenylhydantoin on electroshock seizure threshold in man. Hemphill and Walter (99) and Kalinowsky and Kennedy (114) have reported that diphenylhydantoin increases threshold in patients undergoing electroshock therapy, while the reviewers (204) have interpreted similar observations as representing a change in seizure pattern rather than threshold. Some investigators have reported that pretreatment with diphenylhydantoin necessitates an increase in the quantity of metrazol required to produce seizures (77) or subconvulsive EEG dysrhythmia (216) in epileptic patients, but others have published negative findings (63).

Another problem which has received attention is the effect of diphenylhydantoin

on pattern of seizures. Hemphill and Walter (99) and Kalinowsky and Kennedy (114) observed that atypical seizures were frequent among patients treated with diphenylhydantoin prior to receiving electroshock therapy. Delay and Soulairac (38), Bárány and Stein-Jensen (15) and the reviewers (205) observed that anticonvulsant drugs in general and diphenylhydantoin in particular modified electroshock seizures by reducing or abolishing the tonic phase in animals and in man (204). It was noted that with diphenylhydantoin, in contrast to many other drugs, the modified clonic seizures in animals were quite prolonged (15, 205).

Bárány and Stein-Jensen interpret the change in pattern as representing a greater action of diphenylhydantoin upon subcortical mechanisms responsible for the tonic phase of seizures than upon cerebral cortex (14, 15). Since the medulla in particular has been shown to have a high seizure threshold (95, 96), a selective vulnerability to anticonvulsant drug action might be expected. However, Gley *et al.* (75, 76) have observed that larger doses of diphenylhydantoin are required to modify or prevent seizures in the decerebrate than in the intact animal, while Knoefel and Lehmann (120) failed to find any effect of diphenylhydantoin on seizures in decerebrate preparations. The more rapid post-seizure recovery of animals pretreated with diphenylhydantoin (15, 205) has recently been shown to occur to an equal degree for responses at all levels of integration from medulla to cerebral cortex (202). This finding suggests the possibility that diphenylhydantoin may reduce seizure activity at all levels of the brain, in which case the modification of seizure pattern need not be interpreted on a specific anatomical basis.

Turning now to the effects of diphenylhydantoin upon non-convulsive responses of the brain, the experimental evidence is even less enlightening than in the case of seizures. Gley (cf. 115) found that diphenylhydantoin increased threshold for unsustained motor responses more than for seizures elicited by cortical stimulation. Although the reviewers failed to find an increase in threshold for non-convulsive cortical EEG responses elicited by cortical electrical stimulation, it has recently been demonstrated in their laboratory (202) that thresholds for unsustained movement may be increased particularly when the rate of repetition is slow. No effect of diphenylhydantoin on the recovery process in cerebral cortex was found in these studies.

In an effort to find some neural counterpart of the central actions of diphenylhydantoin, the reviewers (202) have studied various properties of frog nerve. They found that diphenylhydantoin had little effect on various properties including threshold and the recovery process. However, relatively low concentrations (0.04 mmol/l.) prevented the reduction in threshold and the spontaneous firing produced by immersion in isotonic neutral sodium phosphate solutions and also prevented the appearance of repetitive firing in normal nerves subjected to supramaximal shocks. Thus there would seem to be some tangency between the observed effects of diphenylhydantoin upon peripheral nerve and upon brain.

Diphenylene Hydantoin. In addition to diphenylhydantoin, several other hydantoinates tested by Merritt and Putnam (150) were found to possess sufficient experimental anticonvulsant activity to suggest clinical trial (147). For example, 5,5-diphenylene hydantoin showed antiepileptic activity approaching that of di-

phenylhydantoin and similar toxicity except for the appearance of frequent skin rashes and the lack of gingival hyperplasia. This agent had previously been shown to be effective against experimental seizures by Knoefel and Lehmann (120) and also found on preliminary trial to be an effective clinical antiepileptic by Fabing *et. al.* (53).

Mesantoin. Mesantoin (3-methyl-5,5-phenyl ethyl hydantoin) may be considered the hydantoin homolog of the barbiturate mebaral. Initial clinical trial by Loscalzo (140), Clein (30), Kozol (124), Lennox (130) and Marburg and Helfand (143) showed that mesantoin could control many grand mal and psychomotor cases which were refractory to diphenylhydantoin or other medication. Only the last-named authors reported favorable results in petit mal. The toxicity of mesantoin differs from that of diphenylhydantoin in that sedation and skin rashes occur more frequently, while gingival hyperplasia has not been reported. Thus combinations of mesantoin and diphenylhydantoin can be employed for summation of their therapeutic properties with lessened likelihood of toxicity (124).

The clinical use of mesantoin was suggested by the studies of Tainter *et al.* (199), who found that it was highly effective in raising electroshock threshold in experimental animals. It was found more effective than diphenylhydantoin in altering seizure pattern in experimental animals (197), and resembled diphenylhydantoin in similar tests with human electroshock seizures (204). The reviewers have also shown that it differs from diphenylhydantoin in exhibiting some protective action against metrazol seizures, a property apparently related to N-methylation in several heterocyclic anticonvulsants (fig. 1).

Phenyl Thienyl Hydantoin. The thienyl derivative of diphenylhydantoin (5-phenyl-5-thienyl hydantoin) differs from diphenylhydantoin only in the substitution of a more unstable thienyl ring for one of the 5-phenyl groups. Peterman (163) has considered this compound to be superior to diphenylhydantoin in grand mal and petit mal, but clinical studies are not yet adequate for comparison. The reviewers have noted that it may be successfully substituted for diphenylhydantoin when gingival hyperplasia limits the tolerated dosage of the latter. Their laboratory studies indicate that there are no important differences between the two drugs when compared by various anticonvulsant tests (85).

Trimethadione ('Tridione'). The specificity of trimethadione ('tridione'; 3,5,5-trimethyl-oxazolidine-2,4-dione) in the treatment of petit mal seizures was briefly reported by Perlstein in 1945 (162, 174) and confirmed in the extensive studies of Lennox (128, 131, 133) and within a short time by many other clinical investigators. It was also reported to be useful in at least some cases of grand mal (201), psychomotor seizures (37) and cerebral palsies (35, 162) and was shown to have clinical analgesic properties (173). Its usefulness in major seizures has been seriously questioned by Lennox (131), but the reviewers have convinced themselves of its general antiepileptic value when it is combined with diphenylhydantoin in cases which yield to neither drug alone (17); dramatic results may frequently be obtained in cases of grand mal particularly when associated with petit mal EEG (88, 202). Even petit mal seizures are occasionally exacerbated during the first few days of therapy in cases which ultimately respond favorably to trimethadione (88).

Toxic signs have been reviewed by Lennox (131). This side-effect most frequently reported by all investigators is hemeralopia (photophobia), an unusual form of toxicity which seems to depend upon the retinal rather than central action of trimethadione, according to Sloan and Gilger (183). As with many other drugs, frequent skin rashes and occasional blood dyscrasias are observed; reversible renal damage has also been reported. In large doses the drug has a sedative action (162).

Trimethadione was synthesized and first reported to have analgesic properties by Spielman (188). Other oxazolidine-2, 4-diones had previously been shown by Erlenmeyer (46) to have central nervous depressant actions and in particular propazone (5,5-di-*n*-propyloxazolidine-2, 4-dione) had been tried in animals and man as a hypnotic and anticonvulsant by Luton *et al.* (142) and Stoughton and Baxter (194). Everett and Richards in 1944 (50) reported that trimethadione raised thresholds both for chemically and electrically induced seizures. The reviewers (81, 86, 88) extended these laboratory observations using a number of assay methods and concluded that the outstanding property of trimethadione was its protective effect against metrazol-induced seizures and subconvulsive phenomena. Although as effective as phenobarbital in raising electrical seizure thresholds, it was found far inferior to phenobarbital and diphenylhydantoin in ability to modify maximal seizure pattern in man (204) as well as in animals (205). This latter feature would seem to correlate with its relative ineffectiveness in grand mal. In acute spinal animals it was found to depress multineuronal more than two-neuronal reflexes (88), an action possibly correlated with its known clinical analgesic properties (173) In peripheral nerve studies (202) it was found much inferior to the usual anticonvulsants in ability to prevent repetitive firing.

It will be seen that trimethadione differs from the anticonvulsants previously discussed in clinical specificity and toxicity and in laboratory tests. But as yet there seems to be no adequate laboratory test for distinguishing drugs which would be effective against petit mal, although attempts to reproduce the laboratory counterpart of petit mal have not been lacking (28, 88, 109).

Paradione. Paradione (3,5-dimethyl-5-ethyl oxazolidine-2,4-dione) was reported by Everett (48) to have high antimetrazol potency and to possess clinical effectiveness against petit mal. The studies of Lennox and Davis (133) indicate that there is little clinical difference between paradione and tridione except perhaps for a somewhat less frequent occurrence of hemeralopia with the former. From the investigations of Spielman and Everett (190) on structure-activity relations of trimethadione congeners, it seems improbable that longer alkyl substituents will bestow greater clinical effectiveness against petit mal.

Epidon. Epidon (5,5-diphenyl oxazolidine-2,4-dione) has been reported by Ellermann (44, 45) to be clinically effective in grand mal but not in petit mal and to have anticonvulsant properties comparable to those of diphenylhydantoin in laboratory studies. As may be seen in figure 1, epidon is superior to trimethadione and paradione in ability to modify seizure pattern but is relatively ineffective against metrazol. Pfeiffer (164) has recently observed that 5-phenyl oxazolidine-2,4-dione is superior to the diphenyl congener (epidon) and a number of others in raising metrazol seizure threshold and in modifying electroshock seizure pattern. Epidon

has been found comparable with diphenylhydantoin rather than with trimethadione in peripheral nerve studies (202).

Phenacetylurea (Phenurone). Phenacetylurea ('phenurone') has recently been been reported effective in selected refractory cases of grand mal, petit mal and psychomotor seizures by Gibbs, Everett and Richards (70). Observed toxic signs were few, with anorexia, cachexia, headache, insomnia, palpitation and questionable drug rash occurring in diminishing order of frequency. A most interesting result of phenurone therapy was an exacerbation of preexisting personality disorders in patients with psychomotor seizures. The drug is one of a large series of conceivable degradation products of anticonvulsant substances. It was synthesized by Spielman (189) and is essentially 5-phenyl hydantoin with an opening of the hydantoin ring between the 1 and 5 positions. It was found by Everett (49) to be one of the most effective agents yet tested against chemically and electrically induced seizures in laboratory animals. The heuristic implications of the as yet limited experimental and clinical results obtained with phenurone are many.

Glutamic Acid. Price *et al.* (167) reported that glutamic acid was effective in some cases of grand mal and psychomotor seizures and subsequently the *l*+ form was shown by Waelsch and Price (207) to be the active isomer. Other central nervous actions were also reported, including enhanced maze learning ability in rats (215) and improved behavior and performance in subjects with mental deficiency (5). That any favorable action should be found with small oral increments of the most common dietary amino acid seems surprising. Laboratory studies by the reviewers (82) have failed to show any anticonvulsant action of glutamic acid in various species of experimental animals.

Steroids. McQuarrie, Anderson and Ziegler (146) have reported a reduction in frequency of grand mal seizures in patients treated with desoxycorticosterone acetate, but their results were not confirmed by Aird (4). Aird was able to detect a protective action of the steroid against procaine convulsions, as had Selye (180) against metrazol seizures; but, in contrast to Woodbury and Davenport (213), he was unable to confirm Spiegel's (186, 187) demonstration of the ability of desoxycorticosterone to raise seizure threshold. Since Spiegel has found that several steroids without common physiological effects have laboratory anticonvulsant potency, and in view of the paucity of clinical data, the significance of the desoxycorticosterone studies cannot yet be assessed.

Carbon Dioxide. The ease with which seizures of the petit mal group may be precipitated by hyperventilation and suppressed by CO_2 inhalation (71) places CO_2 in the category of a physiological anticonvulsant. Cerebral blood flow may be increased by CO_2 excess (74), but a direct neural effect should also be considered in the light of observations that carbon dioxide may increase threshold in peripheral nerve and prevent repetitive firing induced by a wide variety of stimulating substances (139).

Miscellaneous Antiepileptic Therapies: Fasting; Ketogenic Diet; Dehydration. The use of dietary measures is among the most ancient of all therapeutic attempts to control the 'Sacred Disease' (200). Simple fasting was one of the more prominent of these measures and has even had a desultory trial in the present century. It is

interesting to note in some seizure charts published by Lennox and Cobb (132) that with fasting there may be an initial exacerbation of seizures particularly of the petit mal group, even in patients who subsequently show complete remission of attacks with continued rigorous fasting. However, starvation is at best a barbaric practice in the eyes of most patients. Geyelin (66), who reintroduced the method in the modern period, suggested that the favorable effects were due to acidosis. Wilder (211) proposed and successfully used a high fat diet in an effort to produce acidosis. Keith (117) has recently reevaluated the efficacy of the ketogenic diet after a trial of several decades in a large series of patients and concluded that it compares favorably with the use of anticonvulsant drugs.

The possibility that the success of the ketogenic diet was attributable to products of intermediate fat metabolism was considered by Wilder, who suggested acetoacetic acid as a possible physiological anticonvulsant. Recently the glycerol moiety has been tested by Kajdi and Livingston (112) with results equal to those of the ketogenic diet itself.

In general, the literature suggests that dietary measures are more useful in convulsive disorders of the petit mal group. Therefore it is not surprising that laboratory studies fail to reveal obvious anticonvulsant effects of dietary changes. In fact, Davenport and Davenport (34) have found an increased susceptibility to electrical and chemical seizures in rats which were starved as well as in those given a high fat diet.

Rigorous fluid restriction was introduced by Fay (54) in 1929 for the therapy of convulsive disorders, concomitant with the introduction by McQuarrie (see 146) of a pitressin-hydration test for precipitating seizures as a diagnostic procedure. The reviewers (203), in evaluating previous studies upon the relation between water balance and electroshock seizure threshold (198), have concluded that total extracellular electrolyte concentration is an important determinant of seizure threshold. An excess of sodium ion, the predominant ion of extracellular fluid, has recently been shown by Woodbury and Davenport (213) to increase seizure threshold in laboratory animals.

STRUCTURE-ACTIVITY RELATIONS

The search for more effective anticonvulsants passed from the era of trial and error with the publication by Merritt and Putnam of their extensive studies on hydantoinates and other related chemical nuclei, culminating in the clinical trial and validation of diphenylhydantoin (148, 168, 169). Although other investigators have studied the effects of modification of molecular structure on anticonvulsant activity (8, 9, 26, 29, 33, 48, 51, 87, 120, 164, 187, 189, 190, 195, 197, 199, 205), the most extensive contribution has continued to come from Merritt and Putnam and their associates (138, 147, 150–152).

Before attempting to analyze the structural relationships necessary for anticonvulsant activity, it might be well to set down certain general principles for orientation:

(a) *The ultimate value of an anticonvulsant drug must be determined in clinical practice.* Only to the extent that laboratory methods of assay correlate well with the

clinically established potency of a series of drugs can the laboratory methods be validated. Even then it is not always possible to forsee prohibitive toxic side-actions which are peculiar to the human species.

(b) *The convulsive disorders differ radically in their responses to therapy.* To cite but one example the specificity of trimethadione for seizures of the petit mal triad and the lack of effect of diphenylhydantoin in most such cases (128) illustrate the futility of a search to find a single agent for the treatment of 'epilepsy' in the singular, or of reliance upon a single laboratory test to determine the potential anticonvulsant activities of a given substance.

(c) *There are many possible mechanisms of anticonvulsant action.* Some of these mechanisms have already been discussed and it is obvious that each of them requires a different type of laboratory analysis for its detection. Therefore, as emphasized also in the preceding paragraph, there are cogent reasons for using a battery of laboratory tests.

(d) *The measure of usefulness of an agent is not its absolute potency but its therapeutic index.* The comparison of drugs on a weight or molar basis for their laboratory anticonvulsant potency may have a limited theoretical value, but a more important consideration, especially to the patient, is the ratio between the dosage of a particular drug which causes untoward effects and that which adequately controls seizures. The higher the ratio, the more useful the drug, regardless of the absolute dose levels.

The reviewers have attempted to carry out these principles by developing a battery of tests and expressing the specificity of each drug as a 'protective index' (84, 87, 203). Table 1 illustrates the contrast between two such anticonvulsant tests when used for analysis of a series of drugs including both clinically effective and ineffective agents. The reviewers limit themselves to data from their laboratory (87, 195, 196, 197, 205), since the literature does not provide adequate information for cross comparison of indices. The table gives only a few representative examples in the three most widely-studied heterocylic groups. The tests are roughly comparable to those of Pfeiffer (164) and Spielmann (189). The supramaximal test may also be cautiously compared with electroshock threshold tests of the many other investigators who have used severe seizures as end points. Accepted clinical uses of the drugs are indicated where possible. The structural modifications are also given, starting from the basic configuration shown in figure 1.

It should be emphasized that the nuclei represented in table 1 are not the only structures having anticonvulsant activity (cf. 150). However, members of these three groups together with their degradation products (189) comprise most of the clinically useful antiepileptic drugs. It should also be emphasized that experimental data derived from the various types of electroshock seizure tests are extensive, while metrazol studies are relatively meager. With these reservations in mind, it is possible to draw several tentative conclusions from the literature and to illustrate a few of them with the findings given in table 1.

(a) A 5-phenyl group is important but not critical for antielectroshock activity. It may be replaced by a naphthyl ring (138, 150) but not by cyclohexyl, benzyl or various other rings or by substituted or unsubstituted alkyl chains without considerable loss in activity (8, 9, 150, 164, 169, 189). For example, a comparison of the

TABLE 1. COMPARISON OF CHEMICAL STRUCTURE, LABORATORY ASSAY AND CLINICAL USEFULNESS

NUCLEUS AND DRUG	SUBSTITUENTS[1]			CLINICALLY USEFUL IN:[2]	EXPERIMENTAL PROTECTIVE INDEX	
	R_1	R_2	R_3		Supramaximal electroshock[3]	Metrazol[4]
Hydantoins:						
Mesantoin	C_6H_5	C_2H_5	CH_2	GM, PsM	11.1	0.9
5,5-phenyl thienyl hydantoin	C_6H_5	C_4H_3S	H	GM, PsM	4.5	0
Nirvanol	C_6H_5	C_2H_5	H		3.4	0.6
Diphenylhydantoin	C_6H_5	C_6H_5	H	GM, PsM	2.4	0
3-methyl diphenylhydantoin	C_6H_5	C_6H_5	CH_3		0.9	0.6
Barbiturates:						
Mebaral	C_6H_5	C_2H_5	CH_3	GM	2.5	2.5
Phenobarbital	C_6H_5	C_2H_5	H	GM	3.6	0.9
Diphenyl barbituric acid	C_6H_5	C_6H_5	H		1.8	0.4
Trimethyl barbituric acid	CH_3	CH_3	CH_3	GM	1.0	1.5
Barbital	C_2H_5	C_2H_5	H		0.7	1.1
Oxazolidine-2,4-diones:						
Epidon	C_6H_5	C_6H_5	H	GM	1.8	0
Trimethadione	CH_3	CH_3	CH_3	PM	1.2	1.5
Propazone	C_3H_7	C_3H_7	H		1.0	0
Paradione	C_2H_5	CH_3	CH_3	PM	0.7	1.2

[1] See fig. 1.

[2] *GM:* Grand mal. *PsM:* Psychomotor seizures. *PM:* Petit mal.

[3] Supramaximal index determined by dividing dosage of drug causing minimal neurological signs in 50% of rats by dosage abolishing extensor tonic component of seizure in rats subjected to a shock of five times threshold strength (150 mA alternating current delivered for 0.2 sec. through corneal electrodes).

[4] Metrazol index determined by dividing minimal neurological dose as above by dose preventing seizures in 50% of rats after subcutaneous injection of metrazol (70 mg./kg.).

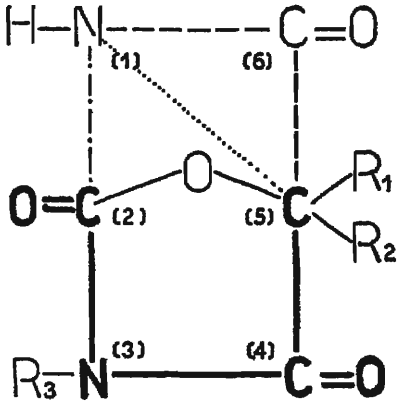


Fig. 1. STRUCTURAL RELATIONSHIPS of clinically available antiepileptics. *Bold face:* Common denominator. *Dash line:* Barbiturate nucleus. *Dotted line:* Hydantoin nucleus. *Thin solid line:* Oxazolidine-2,4-dione nucleus. Opening of the hydantoin nucleus between positions (1) and (5) yields the corresponding acetylurea.

supramaximal electroshock indices of phenobarbital and barbital in table 1 shows the clear superiority of the former.

(b) The addition of a second 5-phenyl group reduces antielectroshock activity in comparison with the effect of a short 5-alkyl chain, as may be seen by contrasting nirvanol with diphenylhydantoin or phenobarbital with diphenylbarbituric acid in table 1. Similar results for these and other pairs are to be found in the literature (9, 150, 164, 169, 189). Optimum activity among the 5-phenyl-5-alkyl compounds seem to occur with short alkyl groups (9, 138). Alkyl substitutions are more effective than their alkoxy- or alkylthio-homologs (26, 152).

(c) A reactive group at position 4, and particularly a carbonyl group, increases antielectroshock activity (150, 169).

(d) N-methylation at position 3 has been shown to reduce antielectroshock potency and specificity at least among the barbiturates (196). Although this effect may be seen by comparing mebaral with phenobarbital in table 1, the opposite effect is illustrated by two hydantoinates, nirvanol and mesantoin. N-methylation seems to improve the antimetrazol potency of all three groups of compounds in table 1. Indeed this is the only tentative correlation that can be made between chemical structure and metrazol index in this series.

(e) A reactive group at position 2 may not be essential (168), but a 2-keto group contributes to high activity (150) and is superior to the homologous 2-thio derivative (26) in electroshock tests.

(f) Beyond position 2, the optimal structure is yet to be determined. In addition to various heterocyclic rings, several straight-chain compounds and particularly the phenacetylureas possess high indices in metrazol as well as in electroshock tests (189). Although it is conceivable that the heterocyclic rings are opened *in vivo* to yield a common anticonvulsant substance (169), available information on the metabolism of anticonvulsants (cf. 102, 103) is too sparse to justify this unitarian concept.

From the above considerations, it can be said that 5-phenyl-5-alkyl compounds having the common denominator shown in figure 1 possess superior ability to modify electroshock seizure pattern and to raise threshold for generalized seizures. Since the indices obtained by various investigators using these tests have correlated to a fair degree with clinical usefulness in the treatment of grand mal, continued study of this group of substances is warranted by the clinical need for new effective antiepileptics of low toxicity.

No such general conclusions can yet be drawn concerning the relation between chemical structure, laboratory assay methods, and effectiveness against convulsive disorders of the psychomotor or petit mal types. The apparent specificity of hydantoinates for psychomotor seizures and of oxazolidine-2,4-diones for petit mal, suggested by table 1, is belied by the reported effectiveness of phenacetylurea in both of these disorders (70). If further progress is to be achieved other than by trial and error in the treatment of these seizure types, it becomes most urgent to study their basic pathology more intensively, and if possible to reproduce them experimentally in laboratory animals.

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GASTRIC ABSORPTION

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ALTHOUGH EXPERIMENTAL EVIDENCE regarding gastric absorption was presented as early as 1808 (71, cf. 70), the general impression prevails that relatively little work has been done on the problem of stomach permeability and that only few substances are absorbed from the gastric cavity in amounts which might lead to the conception of the stomach as an absorptive organ (8, 9, 67, 101). Yet, since the gastric mucosa and the stomach walls are permeable to ethyl alcohol, for example (53), it becomes apparent that gastric absorption may be of some physiological significance in nutrition and therapeutics and of special significance in toxicology and allergy, despite the fact that the capacity of the stomach for absorption is not nearly so great as that of the intestine.

No review of what has been accomplished in the studies of absorption from the stomach would be complete without mention of the procedures used, particularly since divergent conclusions regarding absorption are often the consequence of differences in methods.

Among the experimental techniques in determining gastric permeability, closure of the pyloric region in order to obviate contact between the material investigated and the highly absorptive small intestine has been one of the most frequently used (7, 10, 16, 17, 29, 38, 68, 69, 78, 79, 97, 99, 104, 109), the substances being introduced into the stomach by injection, by ingestion, by stomach tube or by fistula (2, 57, 60–66, 74, 86, 93, 105, 108, 111, 112). Even more frequently employed in investigations has been ligation of both the cardiac and pyloric regions of the stomach, the test material being introduced by means of a small-bore hypodermic needle (4, 33–35, 39, 40, 42, 50, 53, 54, 56, 71, 75, 76, 77, 98, 107, 114).

Of these two methods, the latter is preferable, since in its use one does not have to be concerned with the possibility of absorption by the esophagus, as the test material is passing along it to the stomach, or with the problem of regurgitation. On the other hand, ligation of cardia and pylorus introduces the possibility of creating abnormally high 'filtration' or hydrostatic pressures in those instances where appreciable volumes of fluid may be injected (cf. 26, 27, 53). However, where ligation techniques are to be employed, cannulation at either the cardiac or pyloric end of the stomach is an obvious way of solving the potential problems of either esophageal absorption or increased hydrostatic pressure.

Aside from the objections mentioned, there are two others: one pertains to the condition of the stomach following ligation; the other, to the element of time. While it is true that the ligated or the ligated and cannulated stomach or the stomach with fistula is unphysiological, it will be conceded that abnormal conditions must of necessity be the case in many instances of biological experimentation and that, where comparative data are obtained under identical conditions, the validity of the comparisons is not impaired. More serious, however, is the criticism that in attempts to demonstrate gastric permeability, many investigators have allowed the substance tested to remain in contact with the gastric mucosa for inordinately long periods, even considering the length of time necessary for complete emptying of such materials as fats, proteins and carbohydrates.

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Still another technique employs the principle of blocking the opening between stomach and duodenum by use of an inflatable balloon (12, 93, 111, 113). While this method has the advantage of being more physiological than the preceding ones, reproducibility of closure is more exact in the former, in which, also, leakage is less likely to occur.

Some investigators have closed the pylorus by introducing acid into the duodenum just below the pylorus, the pylorospasm thus produced being said to last for several hours (22, 94). Here, too, the questions of leakage and of exact reproducibility of extent of closure from animal to animal have yet to be satisfactorily answered.

The Pavlov and Heidenhain pouch methods have also been frequently employed (18, 19, 30–32, 55, 59, 72, 89). Although the problems inherent in the previously mentioned methods are not encountered here, absorption studies by these procedures have been usually confined to only a part of the stomach; whereas, frequently, conclusions have been referred to the entire stomach.

The method of introducing two substances simultaneously, the gastric absorption characteristics of one being known, has been used occasionally, although this procedure is not popular. In the latter method, the pyloric opening is left unligated. After a given time interval, the stomach contents are withdrawn and the amount of 'unknown' absorbed is determined and expressed as a percentage based on the amount of the 'known' present and the amount previously determined as being absorbed under the experimental conditions (49, 51, 83). This method fails to account for the possibility of differential expulsion into the intestine.

Finally, in studies on the absorption of inorganic iron, fats and dyes, a few investigators have employed the histological technique, examination being made of the gastric mucosa (1, 41, 44, 48, 78, 92).

WATER

The gastric absorption of water has been studied in human beings, dogs, cats, sheep and calves. Of the various investigators involved in these studies, Bönninger (10), Edkins (26), Jaworski (51) and von Mering (112) presented data which purported to show that absorption of water does not occur or occurs only to a negligible extent, while the results of Brandl (12), Feitelberg (30), Klimov and Kudriavtsev (55), Pfeiffer (85) and Trautmann (108) suggested the contrary.

Little credence, however, should be given to the results of the former group, since either the methods employed were faulty or the conclusions did not logically follow the experimental data. In the latter group, too, criticism may justifiably be leveled at the findings of Brandl (12), because of his use of the balloon technique, and especially at those of Pfeiffer (85). The evidence presented by the others in the second group, particularly that appearing in the report of Feitelberg (30), is, however, convincing.

Finally, in 1943, Cope, Blatt and Ball (18) investigated the exchange of water across the gastric membrane by observing the absorption of heavy water (deuterium oxide—D_2O) from stomach pouches in the dog. One animal had a pouch of the fundus, or acid-secreting portion of the stomach and another, a pouch of the gastric antrum. Under the conditions of the experiment, half of the D_2O was absorbed in approximately 20 minutes. There was no significant difference between the body and the antral pouches in the rate of absorption. Whether the body pouch was in a secreting or resting state also made no difference in absorption. If it is assumed that the gastric membrane handles D_2O as it does H_2O, it can be concluded that there is a rapid exchange of H_2O between the gastric contents and body fluids.

ACIDS

That the absorption of HCN from the stomach is rapid was reported by Meltzer (76) who found that in unstarved rabbits weighing about 2.2 kg., the introduction

of 60 mg. and 100 mg. of HCN (as the salt) into stomachs ligated at cardia and pylorus resulted in death in 12 and 15 minutes, respectively. Meltzer felt that the absorption of HCN may have been due to diffusion of the highly volatile HCN (in acid gastric juice) and/or the production of a hemorrhagic surface on the mucous membrane, which would facilitate absorption.

Regarding other inorganic acids, Teorell (105a) in 1933 showed that in decerebrate or anesthetized cats the stomachs of which were tied off at both pylorus and cardia, with a cannula being inserted into the pyloric end, a very active disappearance of HCl occurred and that this absorption of the acid was a consequence of rapid diffusion of the hydrogen ion into the gastric mucosa. Although Klimov and Kudriavtsev (55) found that natural gastric juice and 0.2 per cent HCl can be absorbed to the extent of 35 per cent to 45 per cent in calves and Feitelberg (31), likewise, found natural gastric juice to be absorbed by the stomach of the dog, Hollander (48a) and Wilhelmj (113a) in their studies on gastric secretion came to the conclusion that absorption of HCl from the stomach could not have occurred as stated by earlier workers. Support to the latter view was rendered by Shay *et al.* (94a) who were unable to find evidence of absorption of 0.5 per cent to 1.0 per cent HCl in 30 minutes from human stomachs. Nevertheless, Teorell (105b) again reported that the gastric mucosa of the cat was permeable to both weak and strong acids.

Of the experimental data presented by the proponents and the opponents of the theory that the gastric mucosa is permeable to inorganic acids, the most cogent arguments are to be found among the proponents. Added support to the validity of the data and conclusions regarding positive gastric permeability to acids is afforded by the experimental results with both volatile and non-volatile organic acids reported in the following paragraphs.

Inouye and Kashiwado (50) concluded that the gastric mucosa of the dog absorbs salol (phenyl salicylate) and salicylic acid. They stated that it is probable that not salol but its hydrolytic product, the acid, is actually absorbed from the stomach. Prior to the work of these authors, Stein (102) had also reported the permeability of the stomach to salol. Confirmation of the gastric penetration of salicylic acid came from Carnot, Papaconstantinou and Simonnet (15) who reached the conclusion that absorption of sodium salicylate was positive when the stomach contents were acid and negative when the gastric contents were neutral or alkaline. The absorption in the acid stomach was so rapid that salicylate was detected in serum from the third minute after the salt was introduced into the dog stomach ligated at both ends. The total amount absorbed was about 30 per cent of 0.50 gm. given as a 10 per cent aqueous solution. The serum concentration of salicylate was increased when the stomach was in the fasting state and when there were gastric lesions (gastritis) in the mucous lining.

Studying the absorption of volatile acids from the rumen of sheep, on the basis of the acid content of blood drawn from veins of the rumen, McAnally and Phillipson (73) introduced sodium acetate, propionate and butyrate into fistulas in the rumen. They found that introduction of sodium acetate into the fistula caused an immediate increase in the concentration of volatile acid in the blood from the rumen; whereas an equimolecular solution of the butyrate caused no significant increase. This observation suggested that the rate of absorption depends on the size of the molecule.

Continuing the study of absorption of volatile acids, Barcroft, McAnally and Phillipson (3) reported that when 25 grams of acetate in 2 liters of H_2O and equimolecular concentrations of propionate or butyrate were introduced into the rumen of sheep, the acetate and propionate appeared in the blood within five minutes; while the butyrate was hardly at all absorbed within this period. Positive results similar to those obtained in the sheep were obtained in the pig also.

In the rabbit and pony, however, Barcroft *et al.* (3) were unable to demonstrate absorption of acetate, propionate or butyrate; and in the dog, Klemperer and Scheurlen (54) were unsuccessful in their attempts to find evidence of absorption of oleic acid.

ALCOHOLS

Much of the work relating to absorption from the stomach has been conducted with ethyl alcohol. Regardless of the techniques and animals employed, there has been essential agreement concerning the significant disappearance of ethyl alcohol by virtue of its ability to penetrate into the stomach wall (7, 11, 12, 16, 22, 28, 31, 32, 40, 55, 81, 93, 104, 105, 112). Only the data of Rasmussen (87) and of Dybing and Rasmussen (25) obtained on rats indicate that absorption of alcohol is insignificant and these workers attribute the poor absorption to the fact that the stomach of the rat differs from that of other species in that two thirds of the rat stomach consists of a membrane which has a multi-layered, glandless, relatively impermeable, 'horny' epithelium. However, Haggard, Greenberg and Lolli (38) and Karel and Fleisher (53) have offered evidence of the consistent and significant absorption of ethyl alcohol in this species.

Investigations of the gastric absorption of alcohols other than ethyl have been limited to methyl alcohol, shown by Haggard *et al.* (38) to be rapidly absorbed, and to propylene glycol. Lehman and Newman (58) introduced 6 cc. of propylene glycol per kg. of body weight into the isolated stomach of the dog and found, at the end of 120 minutes, that the blood propylene glycol was only about 1.0 mg. per cent. However, according to van Winkle's data (109) when 10 cc. of 10 per cent propylene glycol per kilo of body weight was injected into the ligated stomachs of cats and rabbits, about 8 per cent was absorbed in 10 minutes, about 18 per cent in 30 minutes, 21 per cent in 60 minutes and 30 per cent in 120 minutes. Rats showed a somewhat more rapid absorption of the glycol than cats and rabbits, but the quantitative relations were similar.

ALKALOIDS

Confirmation of the original observations by Magendie (71), that strychnine can be absorbed from the ligated stomach of the dog, has come from several investigators and has been extended to include other species as well. Thus, Colin and Bouley (17) were able to demonstrate in dogs that death could occur within five minutes after the injection of 5 to 7 grams of an alcohol extract of nux vomica into the ligated stomach, although alcohol itself apparently did not significantly increase permeability (13, 76, 77). Similar results were obtained by Colin and Bouley (17) in the rabbit and pig; in the cow, spasms did not occur until 4½ hours after the injection of 32 grams into the fourth stomach (abomasum), while in horses even 32

grams produced no symptoms in 35 hours. Negative results on the horse were also obtained by Perosino *et al.* (84) and by Berard (6) with 5 grams of strychnine sulfate.

In cats, according to Dixon (23), injection of strychnine into the ligated stomach was followed by symptoms of poisoning in 30 minutes.

In rabbits, although active absorption from the gastric cavity was demonstrated by Brooks (14), Meltzer (76) found that absorption of strychnine, with both cardiac and pyloric ends ligated and with blood vessels carefully excluded, did not occur even after 19 hours with a dose of 60 mg., whereas, when the drug was injected into the stomach wall, the tetanic effects were quickly evident. Even 60 mg. in 10 cc. of a 40 per cent alcohol solution given to a 0.98-kg. rabbit fasted for three days produced no effects in 15 hours. Meltzer (77), in a subsequent paper, reported that in dogs with pylorus and cardia ligated, $2\frac{2}{3}$ mg/kg. of strychnine had no effect in $2\frac{1}{4}$ hours and 3 mg. had no effect in 2 hours, but 20 mg/kg. caused tetanus in 42 minutes. The absorbent power of the fundus seemed to be even lower than that of the entire stomach. Likewise, Inouye and Kashiwado (50) were unable to demonstrate absorption even after 50 hours following the introduction of 5 cc. of atropine in 1 per cent and 2 per cent solutions in fasted dogs or cats with both cardia and pylorus ligated, when the end point used was dilatation of the pupil, although the unabsorbed drug was found active when placed in the eye of a test animal.

Using goats, Trautmann (108) prepared fistulae in the paunch, reticulum and abomasum, and inserted cannulae into the fistulae. Atropine and pilocarpine were found to be rapidly absorbed from all three compartments.

In a study of the influence of the hydrogen ion concentration on the absorption of alkaloids from the stomach, Travell (107) showed that in the cat and dog, with ligated cardia and pylorus, alkaloids, including atropine, were not absorbed to any extent from the stomach when the reaction of the gastric juice was strongly acid. But, when the gastric juice was rendered alkaline with $NaHCO_3$, alkaloids were rapidly absorbed from the ligated stomach. Similar results were obtained with cocaine, nicotine, strychnine and physostigmine. In the case of strychnine, the rate of absorption varied in general with the p_H and the dose, decreasing with increase in acidity. Observations were conducted on the ligated organ for as long as 24 hours or more.

However, previously, when Ryan (89) introduced about one mg/kg. of strychnine into a miniature Pavlov pouch of such nature that the alkaloid was limited to the gastric mucosa, and the acidity of the stomach was 0.35 per cent in terms of HCl, a sufficient amount of a 0.5 per cent aqueous solution of the nitrate was absorbed in nine minutes to cause changes in the reflexes of the animal.

Although the data from the several investigations tend to confuse the picture regarding the inherent rôle of p_H in gastric permeability, it is, nevertheless, clear that sufficient absorption of alkaloids may occur to produce toxic symptoms in the animal.

CARBOHYDRATES

Perhaps the most controversial issue in the field of gastric absorption is the question of the permeability of the stomach to carbohydrates. Aligned on the side of positive absorption of glucose are Brandl (12), Delhougne (22), Edkins (27),

Freund and Steinhardt (36), Holtz and Schreiber (49), Klemperer and Scheurlen (54), Segall (93), Smith (97), Strauss (103), Tchekounow (105), Teorell (105b), von Anrep (111) and von Mering (112); and, regarding inulin, Heupke and Blanckenburg (47). However, only the data of Edkins (27), Klemperer and Scheurlen (54), Smith (97) and Teorell (105b) appear to be clearly unequivocal.

London and his co-workers (60, 62, 64), on the contrary, are in complete disagreement, stating that carbohydrates, including glucose, sucrose, starch, amylodextrin and erythrodextrin definitely are not absorbed by the stomach of the dog. Confirming the latter data on the impermeability of the gastric mucosa of the dog to carbohydrates is the more recent report of Maddock and his colleagues (69). Fundamentally in agreement with the previously enumerated authors who believe that sugars cannot penetrate the mucosa of the normal stomach are Fenton and Pierce (32a) and MacLeod *et al.* (68) whose investigations were conducted with rats, and Martini *et al.* (72), who worked with dogs. All three groups present data indicating that while absorption may occur under certain conditions (see below), even when it does occur, it is not appreciable.

MacLeod, Magee and Purves (68) determined the extent of absorption of glucose in the stomach of rats, using the technique of ligating the pylorus, but leaving the esophageal opening to the stomach untouched. To prevent regurgitation, the esophagus was ligated before excising the stomach. The animals, anesthetized with urethane, were given 1 to 2 cc. of glucose solutions varying in strength from 14 to 51.5 per cent. The time of contact between glucose solution and gastric mucosa was varied between 0.1 hour and 2 hours. No appreciable absorption was found. The glucose recovered ranged from 95.5 per cent for 1 cc. of a 20 per cent solution left in the stomach for 1 hour to 99 per cent for 1 cc. of a 51.5 per cent solution left in the stomach for 0.1 hour. There was no apparent correlation between concentration of glucose solution, time of sojourn in the stomach and percentage absorbed.

Maddock, Trimble and Carey (69) measured absorption by determining the sugar content of blood from the gastric veins. Amytal was used as the anesthetic, since it has no hyperglycemic effect. As it is known that losses of gastric secretions may occur under anesthesia, these authors confirmed their experiments conducted with the animal under anesthesia by a preliminary surgical procedure which enabled them to close the pylorus after the animal had fully recovered from the operation. At the beginning of the absorption experiment, the pylorus was blocked and a solution of glucose was introduced into the stomach. After a known interval, the animal was killed by amytal, the stomach was removed, its contents were collected and the sugar present was determined. The esophagus was ligated at its upper end before recovery of glucose from the stomach was attempted. The blood sugar was measured by the microferricyanide method of Folin and Malmros. The determinations of blood sugar were made in samples—drawn 20, 40 and 60 minutes after the sugar solution was given to the animal. The authors stated that the sugar content of blood from the small veins of the stomachs of 4 dogs was not definitely in excess of the probable errors of sampling and of measurement when amytal anesthesia was used. In six experiments without anesthesia, where absorption was determined by the difference between the amount given and the amount remaining, the recovery

of sugar one to two hours after its introduction averaged 99.3 ± 1.2 per cent of the glucose administered. In two control trials, 99.1 ± 0.2 per cent was recovered immediately after its introduction had been completed. The quantities given ranged from 5 to 47 grams in concentrations ranging from 2.8 to 38 per cent. These results were interpreted by Maddock and his colleagures as demonstrating that the absorption of glucose in significant quantities from the stomach of the dog does not take place.

In 1936 Feitelberg (31) conducted a series of studies on 2 dogs with isolated Pavlov stomachs. The stomachs contained a metal fistula tube and the pouches were formed from the fundic portions of the gastric cavity. The tests were made on an empty stomach following 16 to 18 hours of fasting. The carbohydrates tested were introduced through the fistula tube and were left in the stomach for 1½ hours. The strength of the solutions varied between 5 and 30 per cent and the amount introduced ranged between 20 to 30 cc. The solutions were warmed to 38° C. prior to their use. Recoveries were estimated by means of a Zeiss-Volny refractometer. Blood sugar both before and after introduction of the carbohydrates into the gastric cavity was estimated by the Hagedorn-Jensen method. The results obtained by Feitelberg were as follows:

The absorption of glucose begins only with the introduction of solutions of 10 per cent or greater, the higher concentrations producing somewhat greater absorption, although the amount of absorption after one hour is not significant. As would be expected, the longer the sojourn in the stomach, the greater the absorption. In one experiment, 25.95 per cent of 27 cc. of an 11.8 per cent glucose solution was absorbed in 120 minutes. Controls to determine the extent of decomposition of sugar in the stomach were negative after 12 hours contact of gastric mucus secreted for 2 hours from the isolated stomach and glucose in Hiss' Medium. The resorption of galactose was found to be considerably higher than that of glucose, 23.98 per cent of 22 cc. of a 21.5 per cent solution being absorbed in one hour and 40.64 per cent in two hours. Blood sugar increase could not, however, be detected. In solutions weaker than 22 per cent, levulose was not absorbed as well as glucose. With 22 to 25 per cent solutions, the absorption almost equalled that for glucose. Again, hyperglycemia—as in the case of glucose—was not demonstrable. The resorption of lactose was poorer than that of galactose and was almost equal to that of glucose. No increase in blood sugar could be detected for the solutions used, which ranged from 6 to 20 per cent in strength. There was no absorption of low concentration sucrose solutions in one hour, but increasing the concentrations to 38 per cent markedly increased absorption. However, the absorption of sucrose was poorer than that of other sugars; as in the previous instances, blood sugar increase could not be detected.

Resorting to the technique of closing the pylorus by duodenal stimulation, Shay *et al.* (94) studied the absorption of glucose in the stomach, as affected by duodenal stimulation by HCl, olive oil, $NaHCO_3$, saline and glucose. Five minutes after the duodenal instillation of the stimulant, 225 cc. of about 40 per cent glucose mixed with 2 ounces of $BaSO_4$ were ingested. The instillation was continued for 30 minutes. Fluoroscopic observations and roentgenographic records were made at

frequent intervals in order to make certain that none of the gastric contents had left the stomach. In addition, duodenal contents were checked for sugar every 10 minutes. On the basis of their data, these authors concluded that glucose, at least when in high concentration, may be absorbed by the human stomach. In a subsequent paper on the absorption of glucose from the human stomach, Shay *et al.* (95) again concluded that considerable amounts of glucose may be absorbed by the stomach alone from solutions of high concentration. From concentrations below 15 per cent, no appreciable absorption could be demonstrated.

Determining the absorption of glucose one hour after its introduction into the ligated stomachs of dogs, Morrison *et al.* (79) found that glucose is absorbed from the stomach when present in high concentrations and that the rate of absorption seems to bear a relationship to the concentration of the solution in the stomach.

In 1940, Rankin (86) working with sheep reported that when 2 molar dextrose was placed in the isolated rumen in a ratio of 4 gm/kg. body weight, the absorption of dextrose as determined by increase in blood sugar levels was marked.

By using a modification of the balloon technique to close the pylorus, Warren *et al.* (113) were able to study the absorption of glucose in the human stomach. They stated that when concentrated glucose solutions were ingested, a certain amount left through the stomach wall during the first short period of contact. The indirect evidence was supported by experiments in which the pylorus was mechanically closed. The strength of the glucose solutions varied between 2.46 and 60.3 per cent and the interval between injection of the glucose through the stomach tube and withdrawal of samples by washing varied from 4 to 37 minutes. In no instance was more than approximately 13 per cent of the sugar absorbed.

In general, the following conclusions may be drawn from the data of the authors mentioned: *a*) Little, if any absorption of glucose or sucrose from solutions of low concentrations (<15%) occurs during the first hour or two; *b*) where the concentration of glucose, sucrose and some other carbohydrates is greater than about 20 per cent, absorption for some, but not all, can be decisively demonstrated, particularly when contact between carbohydrate solution and gastric mucosa is greater than one to two hours (when concentrated solutions are used, however, much of the permeability may be the consequence of mucosal congestion produced by the hypertonic solution (cf. Brandl)); *c*) often, although the data are acceptable, an insufficient number of experiments has been reported to justify general conclusions; and *d*) species variation may play an important rôle in the divergence of results.

FATS

In 24-hour fasted dogs in which the pylorus and cardia were ligated, Klemperer and Scheurlen (54) found that olein was not absorbed in three or in six hours. A similar conclusion was reached by Volhard (110), who reported that unsplit egg and milk fat does not permeate the stomach even after contact of two and one-half hours, and by Delhougne (22), who found no absorption of triolein in three hours. While the work of both Volhard and Delhougne is subject to the criticism that some of the experimentally employed fat may have escaped into the intestine, the negative results obtained even with the possibly faulty technique strengthen the conclusions of

Klemperer and Scheurlen. On the other hand, Schilling (92) reported that in calves fed fats the gastric mucosa was thickly imbedded with fat droplets, thereby indicating absorption. The droplets were found not only in the epithelium, but also in the tunica propria and in the parenchyma. A similar observation regarding the plaice (a member of the flounder family) was made by Dawes (21), who likewise resorted to histological examination as evidence of absorption.

Following earlier experiments by Baumann (5), Mendel and Baumann (78) asserted that, although from the histological findings it appeared that an increase in the amount of fat in the gastric mucosa and submucosa resulted when fat was ingested and that the appearance strongly resembled the microscopic picture obtained during intestinal fat absorption, their experiments on cats and dogs, in which the stomach was ligated at the pyloric end and in which they followed fat content of the blood and traced oil soluble dyes, indicated that no active absorption of fat occurred from the ligated stomach. The materials used were cream, peanut oil emulsion (peanut oil—45%, lecithin—5%, water—50%), olive oil and Sudan III stained fat. Experiments continued for 6 to 12 hours during which the thoracic and common bile ducts were cannulated, but no fat was ever found in the lymph channels or in the portal circulation.

Although the weight of the currently available evidence (22, 54, 110) is such as strongly to suggest the validity of the statements made by Mendel and Baumann (78), this evidence does not refute the histological. Rather, it merely implies that not enough fat is absorbed to yield positive proof of its absorption by blood examination. Were it possible to sensitize animals to the various fats as it is to proteins, it might be found by sensitization phenomena that some fats are able to penetrate the normal mucosa.

PROTEINS AND PROTEIN CLEAVAGE PRODUCTS

From experiments with the ligated stomachs of cats and dogs into which, at different times, peptone, taurine, albumoses, acid-albumin (*syntonin*), fibrin and glycine were introduced and allowed to remain in contact with the stomach tissues for periods which varied from 30 minutes to 4 hours, depending on the investigator, it was concluded by Brandl (12), Delhougne (22), Lang (57), Salaskin (90), Tappeiner (104), Teorell (105b), Tobler (106), von Anrep (111), von Mering (112) and Zunz (114) that the gastric mucosa is permeable to proteins and their cleavage products—although Delhougne also reported that no absorption of glycine occurred prior to 2½ hours and that even at 4 hours, peptone remained unabsorbed. With the exception of the work by Tappeiner (104), Teorell (105b) and Zunz (114), however, the likelihood of leakage into the highly absorptive duodenum was present in the studies reported.

In regard to other species, Smith (97) published data which was indicative of marked absorption from the ligated frog stomach, while Scheunert (91), whose findings are questionable, stated that the absorption of protein in horses was to a degree dependent on the digestibility of each individual substance and was comparable to that in the dog. Klimov and Kudriavtsev (55), using stomachs isolated by the method of Heidenhain, found definite gastric permeability of glycine in bull

calves; Davey (20), on the basis of osmotic data obtained by the freezing point determination procedure of Beckmann, suggested that absorption occurs from all four stomach compartments of the sheep (reticulum, rumen, omasum and abomasum), although only the abomasum has a lining epithelium of secretory cells and is comparable with the stomach of the non-ruminant.

Using stomach and pyloric fistula animals, London *et al.* (60), London and Sulima (61) and London and Polowzowa (62, 63, 65, 66) emphatically disclaimed the likelihood that there could be any absorption of proteins or protein cleavage products by the non-pathologic canine stomach; although in an earlier investigation, Abderhalden, Prym and London (2) made the statement that absorption, if it does occur, is slight; and in part of London's subsequent data there is evidence, which the authors choose to doubt, that absorption, though small in extent may, nevertheless, occur. The various substances investigated included egg albumen, glycine, l (+) alanine, dl-leucine, casein, serum, gelatin, glutamine and edestin. Comparable results with glycine were achieved by Martini *et al.* (72), who employed the Pavlov pouch technique in dogs.

Following heated controversies during which many laboratories were overtly involved in the question of protein permeability, Folin and Lyman (34, 35) reported on their work with cats in which ligatures were placed at both the cardiac and pyloric ends of the stomach. After the substance to be tested was injected into the gastric cavity, the abdomen was closed. At suitable intervals, 5-cc. samples of blood were taken from the carotid and femoral arteries and analyses for digestion products were made. Also, at the end of the experiment, splenic and enteric tributaries of the portal vein were ligated and a sample was taken from the portal vein which was by this means robbed of all but its gastric branches. They found that the non-protein nitrogen of the blood steadily increased (up to about 180 minutes after injection) for the following, while the urea nitrogen remained constant: glycine (61 gm. in 40 cc. of warm water were injected into the ligated stomach), alanine (6.4 gm. in 50 cc. of warm water were injected), Witte's peptone (8.0 gm. in 40 cc. of warm water were injected). With creatinine, the N.P.N. did not increase up to 5 hours and 10 minutes after injection of 2.8 grams in 50 cc. of warm water. The creatinine was quantitatively recovered. The experiment was repeated with the same result, showing no absorption of creatinine. When 1.8 grams of urea in 25 cc. of warm water were tested, it was found that urea was absorbed. The earliest time of increase in N.P.N. for the various substances was alanine—30 minutes, Witte's peptone—30 minutes, glycine—38 minutes and urea—90 minutes.

Recently, Harten *et al.* (42) used a qualitative test of absorption dependent on the prior sensitization of a cutaneous site to the antigen to be studied. The urticarial reaction at the sensitized cutaneous site within a few minutes marked the entrance of unaltered protein into the circulation. In this manner, the absorption of unaltered cottonseed protein was experimentally demonstrated to occur from the esophagus and from the fasting, ligated stomach in the rhesus monkey, the absorption time from the stomach having been 11 minutes in one monkey and 2 minutes in another.

A critical interpretation of the physiological and the chemical analytical methods

employed in the experimental evaluation of the absorption of proteins and their cleavage products from the stomach inevitably leads to the conclusion that not only may absorption occur, but that it can occur quickly following ingestion.

GASES

With the exception of the conclusions in the paper of Bassal and Uteau (4) cited below, which may be discounted, it is agreed that gastric absorption of gases may occur. Von Mering (112) noted that when soda water was introduced through a fistula into the stomachs of dogs, H_2CO_3, presumably as CO_2, was absorbed in large amounts. Much later, Edkins and Murray (28) stated that in decerebrate cats, with esophagus ligated and pylorus cannulated, the rate of absorption of CO_2 was increased in the presence of alcohol and vice versa. In 1926, McIver, Redfield and Benedict (75) reported that a study of the behavior of CO_2 in the stomach, as a typical case of gaseous exchange, resulted in strong evidence that the movement of the gas into and out of the stomach was not due to secretory factors, but was governed by the physical laws of diffusion (cf. von Mering, 112). The gas to be studied was introduced into the stomach, ligated at the cardia and pylorus, of cats fasted 24 to 48 hours but allowed free access to water. The stomach was washed out with warm water before the experiment and the body temperature was measured and maintained by heating pads. They found that of 50 cc. of O_2 introduced, 6.7 cc. were absorbed in one hour; while 19.4 cc. were absorbed in three hours. The absorption of CO_2 was greater, 39.0 cc. of the 50 cc. being absorbed in 15 minutes. On the other hand, N_2 was absorbed only to the extent of about one cc. in one hour. One hour after the introduction of 60 cc. of air, the analysis of the gas withdrawn was CO_2—5.2 per cent, O_2—15.3 per cent and N_2—79.5 per cent.

In 1912 Bassal and Uteau (4) ligated the cardia and pylorus of guinea pigs and rabbits. Results with $CHCl_3$, $(C_2H_5)_2O$ and CO were negative for periods of 20 to 25 minutes, but the analytical data are questionable.

Singh (96) estimated from his experiments in which the pylorus was not ligated that the possible maximum rates of absorption of O_2 from the stomach were 0.2 to 0.4 cc/min. as measured from change in oxygen consumption through the lungs.

According to Fine, Sears and Banks (33), when the ligated stomach of a cat breathing O_2 was distended with N_2, the total gas volume was as a rule about as great after periods varying from 6 to 24 hours as at the beginning of the experiment. When H_2 was used instead of N_2, the total gas volume was reduced about 10 per cent after 12 to 24 hours. Since other gases diffused into the stomach until an equilibrium was established, it appeared that on the basis of gas analyses, both N_2 and H_2 were absorbed from the stomach.

DYES

Kobayashi (56) ligated the cardia and pylorus of dogs by the standard technique in which the blood vessels leading to and away from the stomach are left intact. To determine positively that any dyestuff not recovered from the stomach contents had been absorbed rather than adsorbed, he collected control samples of bile, urine and serum. Then, 50 cc. of 0.5 per cent dye solution was introduced by a thin needle

into the stomach and, to compensate for loss of body fluids withdrawn as samples, an equivalent volume of saline was injected subcutaneously. Every hour, bile, urine and serum were collected for comparison with the controls. Each animal was observed for more than eight hours. At the end of an experiment, the animal was sacrificed and the stomach contents were analyzed. Kobayashi concluded that, in general, a dyestuff which has great diffusibility can be absorbed by the stomach, whereas a dyestuff which has little diffusibility cannot be absorbed by it. This fact is not influenced by many conditions, for example, acid or base reaction, chemical structure, solubility in lipoids or vital staining. Diffusibility was determined by placing 0.5 per cent dye in agar-agar gel and measuring the diffusion of the dye into the gel for an eight-day period. Those dyes, according to Kobayashi, having a diffusibility greater than 5.5 cm. can be absorbed by the stomach. Those with less than 5.0 cm. cannot be absorbed. In all, 31 dyes were investigated, including mono- and diazo-triphenylmethane, xantheine, azine, oxazine and thiazine derivatives. It is regrettable that data for periods much less than the selected observation period of eight hours are not given.

Several years later, Henning (45) conducted a series of experiments in which he allowed one per cent aqueous dye solutions to remain in contact with the gastric mucosa of the frog for, usually, 5 minutes. At the end of this period, the dye was washed off with Ringer's solution. If a dyeing of the cells was not microscopically evident, the dye was permitted to remain in contact with the stomach for 20 minutes. *Uranin*, a fluorescent dye, was strongly absorbed, especially in the uppermost portion of the glands. The rapidity of absorption was such that one minute after wetting the mucosa, the dye could be detected, by its fluorescence, in the plasma. *Eosin, erythrosin, magdalarot* and *trypan* blue, like *uranin*, could be ultimately detected in the blood. On the other hand, *Chicago blue* and *diamine black* did not dye the cells of the stomach even after contact of 30 minutes, indicating no absorption. Several basic dyes investigated, like *malachite green* and *alizarin blue*, were readily absorbed, selecting the edges and lacunae, but could not be found in glandular or interstitial cells. There was some absorption of *methylene blue* and *congo red*, but other dyes like *neutral red* and *acid violet* were not absorbed. This author also stated that the chemical relationship of the individual dyes cannot be the basis of their similar biological behavior, inasmuch as compounds of entirely different chemical constitution dyed similar cell-complexes.

IRON

Although Hári (41), using reduced iron, at first found that even after 3½ hours there was no absorption of iron as determined histologically, he subsequently obtained positive results at the level of the fundus and the pars pylorica in one experiment in which about one gram was administered; in four experiments in which 0.2 gram was given, absorption from the fundus and from the pars pylorica was positive three times.

Later, in 1900, Abderhalden (1) investigated the absorption of inorganic iron in rats, rabbits, guinea pigs, cats and dogs. Using microchemical-histological methods, he concluded that no iron was absorbed by the stomach during experiments

lasting 24 to 120 hours. Hofmann (48) was also unable to show by histological means that iron is absorbed from the stomachs of human beings or of guinea pigs.

However, in 1928, Starkenstein (99), after working with frogs, rabbits and rats, stated that all parts of the gastro-intestinal tract are capable of the absorption of iron. Finally, in 1943, Hahn *et al.* (38a), employing radioactive iron, were able to show that very active absorption of Fe could occur from a gastric pouch of a completely isolated stomach of a dog. While only one animal was used in the experiment of Hahn and his colleagues, the evidence is such that the successful repetition of their findings is quite likely. Meanwhile, in simultaneous studies not reported until a year or so later, Vahlquist *et al.* (108a) established that in individuals with artificial stenosis of the pylorus iron is absorbed from the stomach in rabbits and in man. Significant increases in serum Fe^{++} following the administration of the lactate occurred in all subjects in 45 minutes or less.

MISCELLANEOUS

While much of the data on the absorption of salts may be criticized because of inadequate control observations, there is, as with alcohol, no fundamental disagreement regarding the permeability of the gastric mucosa to salts. Moreover, enough sound positive evidence (cf. 17, 19, 29, 39, 43, 55, 74, 86, 105b) is available to obviate doubts concerning the absorption of salts in general and of inorganic salts in particular.

In 1880, Tappeiner (104) asserted that although Na_2SO_4 could be absorbed, of 1.57 grams of the salt introduced into the ligated stomachs of cats and of dogs, only about 16 per cent had disappeared from the stomach after $3\frac{1}{2}$ hours, whereas chloral hydrate was absorbed only from alcoholic solution and not at all from its aqueous solution. Subsequently, absorption of salts, among them NH_4Cl, NaCl, $CaCl_2$, $NaHCO_3$, Na_2CO_3, CH_3COONa, KI (or NaI) and KBr were reported by Bönninger (10), Brandl (12), Jaworski (51), Klimov and Kudriavtsev (55), Roth and Strauss (88), Teorell (105b) and von Mering (112). More recently, McDonald (74a) has shown that absorption of ammonia occurs from the rumen of sheep.

Brandl (12) noticed that in all instances where absorption had occurred, the gastric mucosa was measurably erythematous. After investigating the effect of several stomachics on the gastric permeability of aqueous solutions of different substances, he concluded that the increased intragastric penetration was probably the consequence of irritation of the mucosa.

On the basis of positive urine tests following the introduction of 0.5 gram quantities into the stomach, Delhougne (22) stated that the absorption of KI and of sodium salicylate had occurred within two hours, while that of KBr, pyramidon and quinine had occurred within one hour.

Following the study of Starkenstein and Hahnel (100) on factors affecting the absorbability of magnesium salts, Hay (43) studied the absorption of saline cathartics in the cat and concluded that Na_2SO_4 was absorbed to a slight but significant extent. Jaworski (51), using his previously described technique, listed the decreasing order of absorbability as *a*) $H_2Mg_2(CO_3) > NaHCO_3 > Na_2SO_4 > MgSO_4 > NaHPO_4$

> KCl > $FeCl_2$ > NaCl and *b*) Na_2SO_4 > Na_2CO_3 > $MgCl_2$ > KCl > $FeCl_2$ > NaCl.

Otto (82) reported only negligible absorption of $MgSO_4$; according to Myant (80) loss of SO_4 in the quiescent stomach of the cat cannot be observed when the concentration of the solution is 4 per cent or less. Henning (44) stated that the normal mucous membranes of the human stomach are impermeable to iodide ion in aqueous solution but are permeable to iodide in alcoholic solution. Hanzlik (39), however, found that marked absorption of NaI in water solution occurred from the ligated stomachs of dogs and of cats after one-half hour.

In the experiments of Eisenman *et al.* (29), isotonic solutions of radioactive isotopes of Cl^-, Na^+ and K^+ were introduced into the stomach of rabbits with ligation at the pylorus. Sometimes non-radioactive solutions containing Br^- and I^- were introduced as well. In a parallel series of observations, solutions were placed in an isolated gastric pouch of the stomach of a dog. By comparison of the specific radioactivity of serum or tissue at the end of an hour with that of gastric juice, an estimate of the degree of absorption was obtained. Na^+, K^+, Cl^-, Br^- and I^- placed within the stomach were all transferred to some extent across the stomach wall into the general circulation. The transfer after one hour, however, was too slight to permit an approach to equilibrium. The transfer of Na^+ and K^+ was much slower than that of Cl^- which, in turn, was slower than that of Br^- and I^-.

In 1943, Cope, Cohn and Brenizer (19) reported on the absorption of radioactive Na^+ from pouches of the body and antrum of the stomach of the dog. They found that the absorption of radioactive Na^+ as NaCl within 15 minutes from the body or acid-secreting area was small but significant. Two to three times as much was absorbed when the mucosa was in the resting rather than in the secreting state. The gastric antrum absorbed 100 times as much per unit of surface area as the acid secreting body. Whether the stomach was fasting or secreting made less difference to the absorption from the antrum than to absorption from the body. Variations in the osmotic pressure of the Na^+ solution and in electrolytic concentration in the blood serum, within the limits observed, had no significant effect on the rate of absorption of the Na^+.

According to the data of Rankin (86), when 4 grams of KI were dissolved in 200 cc. of warm water and placed in the rumen of sheep, the saliva gave a positive test for I^- within 5 minutes. Methylene blue which had been mixed with the iodide indicated complete localization of the latter. Using the same technique with NaCN, Rankin gave 0.4 gram dissolved in 200 cc. of saturated methylene blue. In 20 minutes, the animal showed labored respiration with its head extended and tongue blue and, in 43 minutes, the sheep was dead. It may be recalled that in these experiments, the rumen was exposed by fistula and isolated from the other compartments.

Colin and Bouley (17) reported that the stomachs of the dog, the cat and the pig absorb $K_4Fe(CN)_6$ while those of the deer and the horse do not.

By means of radiographs of the bladder before and after dosing, McAnally and Phillipson (74) determined that the radio-opaque salt sodium ortho-iodo hippurate, when administered in the quantity of 2 gm/kg. body weight into the isolated rumen, was absorbed, although absorption was slow. A distinct bladder shadow was ob-

tained one hour after administration of the hippurate, but was not dense until the fourth to fifth hour.

Sollman, Hanzlik and Pilcher (98) introduced one gm/kg. body weight of phenol into the doubly ligated stomachs of cats and dogs. Even after several hours, a large proportion could be removed by gastric lavage. Dunn and Perley (24), working with rabbits in which the pylorus was open, concluded that the effect of alcohol on the gastric absorption of phenol was slight. The appearance time of convulsions and the time of death were essentially the same with or without alcohol.

Levyn and Beck (59) introduced 3 gm. of tetraiodophenolphthalein into the Pavlov pouch of a dog weighing 48 pounds. Seven, 17, 48 and 53 hours later roentgenograms were made of the gall bladder, but no shadows were obtained in any of the trials. Introduction of 3 grams of tetraiodophenolphthalein in 30 cc. of water and of 8 grams of dye in 60 cc. also gave negative results; the latter two doses placed into the duodenum, however, yielded positive results.

Shortly after the appearance of the report by Penner and Hollander (83) on the non-absorption of phenol red, Shay *et al.* (95) confirmed the fact that phenol red is neither adsorbed nor absorbed by the human stomach.

SUMMARY

It is unfortunate that many investigators who employed the technique of ligating both cardia and pylorus in their studies of gastric absorption overlooked the possibility that increases in hydrostatic pressure resulting from complete ligation and introduction of relatively large amounts of fluid would tend to produce greater 'absorption' than might occur were the stomach contents allowed to remain at atmospheric pressure (cf. Karel and Fleisher, 53). Nevertheless, data so obtained are not invalid even though the essential comparative data are lacking, for similar increased hydrostatic pressure may occur from a variety of not only pathological circumstances but, also, normal conditions such as the ingestion of large quantities of food and carbonated beverages.

In all but a relatively few studies, absorption has been studied over periods of time which are abnormally long and during which, particularly in ligated stomachs, the possibility of the occurrence of some degenerative changes, perhaps, as a result of neurological disturbances leading to increased permeability, has not been excluded. However, where, under the circumstances of unduly prolonged experiments, no absorption of a particular substance was shown to occur, one may be reasonably certain that under normal, physiological conditions absorption of that same substance is very unlikely.

It is regrettable that almost no work has been done on the complete quantitative recovery of test materials placed intragastrically. Experiments have largely been confined to determining the amount of substance disappearing from the stomach and further proof of its absorption by detection of the original compound in blood or other body fluids. That work on complete recovery which has been done has been limited almost wholly to ethyl alcohol studies.

While the need for quantitative definitive studies on the extent of gastric absorption occurring during short periods of contact, for example less than 30 minutes, is

obvious, sufficient positive evidence has been presented to indicate that the importance of absorption from the stomach must not be lightly discounted. While the stomach is definitely not an absorptive organ in the same sense as the intestine and cannot be considered of especial importance in supplying the nutritional needs of the normal organism, its absorptive ability, particularly as regards substances physiologically active in minute quantities, has been grossly underestimated. Investigations into the relationship of chemical and physical structure to gastric absorption should yield rich rewards in the increased knowledge concerning allergenic phenomena, toxicology and rapidly effective, oral therapeutics.

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THE PHYSIOLOGY OF ADIPOSE TISSUE

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THE PHYSIOLOGY OF ADIPOSE TISSUE has received comparatively little attention by physiologists. Howell (1), in his textbook of physiology, comes to the conclusion that "it is to be borne in mind that we know little or nothing concerning the physiology of the tissue most directly concerned in the deposition of fat, namely the fat cells themselves." A recent review by Wells (2) on the anatomical and pathological aspects of adipose tissue is rightly named "The Neglected Subject." The neglect is mainly due to the fact that adipose tissue has generally been regarded as an inert site of fat storage, without any specific metabolic activity. This point of view is, however, no longer tenable in face of data assembled during the last 20 years. The present review will endeavor to compile data concerning the specific physiological activity of adipose tissue and to show that the activity of this tissue constitutes an important link in the physiology of animal metabolism. Special consideration will be given to the more recent literature, in which references to older papers can be found.

CELL ORIGIN OF ADIPOSE TISSUE

The view that adipose tissue is merely ordinary connective tissue, in which fat has been deposited, was first expressed by Flemming (3) and is still generally taught, in spite of the fact that most recent histologists do not agree with this. Data obtained during the last 20 years show that adipose tissue develops from special primitive fat cells and that the cells of this tissue have a specific structure, entirely distinct from the fibroblasts of connective tissue.

The specific structure of adult adipose tissue has been clearly demonstrated by Maximow (4) in the case of the fat cells of the omentum. This specific, gland-like structure is not always evident in the fat-laden tissue and is restored only after depletion of the fat.

Direct proof of the occurrence of primitive 'fat organs' has been presented in a short note by Hausberger (5). Embryonic tissue, from a site that develops into adipose tissue, yields a typical fat tissue when transplanted into normal adult rats. Upon similar transplantation of embryonic connective tissue, no adipose tissue is formed. The embryonic fat tissue is fat free and is morphologically undistinguishable from embryonic connective tissue. The tissue is recognizable only by its specific location and development. Wassermann (6) demonstrated the affinity of fat tissue to reticular perivascular mesenchyme cells. The conception that adipose tissue is part of the reticulo-endothelial system is in harmony with the glandlike histological structure of the unfatted fat organ, as well as with the functions adipose tissue can fulfill along with its usual fat-storing function. Portis (7) showed that the cells of the omentum are able to form antibodies and that this ability is correlated with the

occurrence, in this adipose organ, of aggregates of reticulo-endothelial cells. Histologically, these cells closely resemble primitive adipose cells, before fat storage sets in. According to Wassermann (6), the blood-forming function may be reestablished in depleted adipose tissue under appropriate conditions. This is in analogy with the behavior of bone marrow, which sometimes adopts the function of fat storage in addition to its blood-forming function. Vital dyes are stored by adipose tissue just as in the reticulo-endothelium. This was shown by Dogliotti (8) in the depleted fat cells and by Bremer (9) even in the thin protoplasmatic ring about fat distended cells. McCullough (10) showed that Trypan blue is included in fat cells, especially after their depletion of fat. This is regarded as evidence for the macrophagial origin of adipose tissue. A critical review of the histological and embryological aspects of adipose tissue has been given by Wells (2).

It may be concluded that adipose tissue can no longer be regarded as connective tissue, fulfilling the function of fat storage. It seems to be part of the histiocyte system and is able to fulfill other tissue functions in addition to its specialization in fat storage.

INNERVATION OF ADIPOSE TISSUE

It had long been taken for granted that adipose tissue does not possess a nervous supply. Hofmeister (11), in a lecture in 1912, stated that fat mobilization is not governed by innervation, since no nerves are found in adipose tissue. The same attitude was adopted in a later research on brown fat tissue by Rasmussen (12). Goering (13), on the other hand, arrived at the opposite conclusion on the basis of clinical experience, which showed that localized fat deposition and depletion is governed mainly by nervous factors. Wertheimer (14) produced experimental evidence for the dependence of fat mobilization on the innervation of adipose tissue. The innervation of the blood vessels supplying the adipose tissue was demonstrated by Nordmann (15). This author believed that adipose tissue is influenced by the nervous system only through the vascular system. However, Hausberger (16) was able to show that the functional activity of the fat organs is regulated by an abundant nerve supply to both the vessels and the parenchyma. The best histological preparations demonstrating the nervous supply of fat cells are those of Boecke (17). In these preparations, fat cells of the interstitial tissue of the parotis are shown to be supplied by nerve fibers of the sympathetic nervous plexus of the surrounding tissue. It could not be proved with complete certainty, in a microscopic preparation, whether the nerve fibers penetrate the thin plasma membrane, but the curving of the fiber ends around the cell nucleus make this conclusion highly probable. Earlier histological papers, especially those of Stoehr and Reiser, are discussed by Boecke.

It may be concluded that adipose tissue is controlled in its activity by the nervous system.

BLOOD SUPPLY OF ADIPOSE TISSUE

The blood vessels of adipose tissue were the subject of several studies in the older literature (3, 6). Nordmann (15) tried to base a theory on the capacity of fat deposition and mobilization of adipose tissue on the sparsity of its blood supply. This

paper was later criticized by Hausberger (16), who concluded that Nordmann observed only the greater vessels and not the capillaries. The extent of the blood supply of adipose tissue was examined quantitatively in a recent paper by Gersh and Still (18). It was stressed that in the well developed fat sizes, generally examined by earlier authors, the capillary net appears to become coarser and inconspicuous, especially if the sections of the tissue are fixed in conventional ways. However, by a new method of fixing the fat and its contained blood by freezing and drying the tissue in unaltered condition or by arterial injection of the animal with India ink, the number and dimension of the capillaries present in the tissue could be estimated. It was found that the capillaries of the fat depots of the rat, unlike those in muscle, show no particular orientation, but form loose meshes which run in all directions in the tissue that encloses the fat cells. No marked quantity of fat cells escapes close contact with at least one capillary. The capillary density increases as the fat cells become smaller and there is considerable variation in the number of capillaries in different regions. The ratio of the surface of the capillary bed to the volume of the tissue supplied is about 52 in fat-rich tissue and about 220 in fat-poor tissue. The first value is about one-third as great as in muscle, the second is of the same order as in the most poorly supplied muscle. For metabolic purposes, however, the data are better calculated on the basis of surface area of capillaries to volume of protoplasma of fat cells. On this basis, a ratio of about 1000 is found in fat rich tissue for surface of open capillaries and of about 2160 for total capillary surface. This means that for metabolic purposes, the capillary bed of fat tissue is comparatively richer than that of muscle.

DEPOSITION OF FAT IN ADIPOSE TISSUE

The nature of the fat deposited in adipose tissue is determined by the balance between the fat in the diet, the fat synthesized in the body and that broken down in metabolism. Animals deposit a fat specific to their species, owing to their relatively specific feeding habits and because most of the body fat is that synthesized within the body, the diet of most animals consisting of predominantly carbohydrate foods. Only upon ingestion of large amounts of fat of specific composition is the fat store altered so that it approaches in composition that of the fat fed. An additional factor contributing to the constancy of fat composition is the ability of the body to carry out at least minor alterations in the fatty acids. It has been shown by Schoenheimer and Rittenberg (19) that upon ingestion deuteriostearic acid, deuteriopalmitic and deuterioleic acid are found in the fat deposited, proving the ability of the body to carry out desaturation, as well as shortening of the hydrocarbon chain. The site of these transformations in the body is not determined by these experiments. Previously it was generally accepted that adipose tissue does not play an active part in these processes. This can no longer be maintained, since the ability of adipose tissue to carry out desaturation of fatty acids has been repeatedly demonstrated (20–23). Moreover, the assumption that at least part of the desaturation is carried out in adipose tissue itself serves as a convenient explanation for the well known fact that the fat deposited in various parts of the body is not identical in its iodine value, the deeper layers being more saturated that the outer parts (24). The correlation between the temperature at the site of storage and the degree of saturation may be due to a de-

pendence upon the temperature of the equilibrium between saturated and unsaturated fatty acids in the fat body. An alternative explanation, proposed by Hilditch (25), that all the fat is synthesized and transformed in other tissues, especially in the liver, and that the adipose tissue at various sites selects a specific mixture of glycerides from the blood, also requires active work on the part of the adipose tissue However, no experimental basis is as yet available to suppose such an elective ability.

The source of fat deposited can be divided into *a*) preformed fat from the diet and *b*) fat synthesized in the body from carbohydrates and proteins.

a) *Deposition of Fat from Preformed Fat.* Good evidence is on hand for the assumption that food fat is first deposited in the adipose tissue, after being absorbed from the intestine. When fat labelled in various forms, i.e. as elaidic acid (26) or as deuterium marked fat (27), is fed, a large part of the labelled fatty acids can be recovered in the adipose tissue. According to Frazer's 'partition theory' (28) only that part of fat, resorbed as unhydrolyzed glycerides, goes directly to the depots, while fatty acids are absorbed into the portal vein and are transported to the liver. The classical theory, that fat is deposited in the adipose tissue only when given in excess of the caloric requirement, has been finally disproved by the experiments of Schoenheimer and Rittenberg (27) with deuterio-fatty acids. Fifty per cent of the deuterium ingested could be found in the fat stores four days after feeding. This is true even when no change in the total quantity of fat occurs and, as was shown by Bernhard and Steinhauser (29), even when fat disappears from the stores in the fasting mouse. Mobilization and deposition of fat go on continuously, without regard to the nutritional state of the animal. The lowering of the fat content of the tissue during hunger is the result of mobilization exceeding deposition. This can hardly be due to changes in the concentration of fat in the blood, which is on the normal level or even somewhat elevated. In fasting rats treated with phloridzin, the mobilization of fat from the depots is even generally accompanied by a marked elevation in the concentration of blood lipids. The fat level of the blood cannot, therefore, be regarded as the regulator of fat deposition and mobilization.

The balance between deposition and mobilization must thus be controlled by a factor acting directly on the fat cell. In a recent study, we (30) were able to show that the penetration of fat into adipose tissue is an active process, depending upon the metabolism of the cells. Fat-depleted adipose tissue, incubated with serum at 38° C., takes up fat from the medium. This uptake is prevented by heating the tissue to 80°C., by the addition of sodium fluoride and sodium cyanide and by lowering the incubation temperature to 20°C.

b. Fat Synthesized from Carbohydrates and Proteins. The ability of the animal body to build its fat from carbohydrates has been demonstrated in the older literature by balance experiments, and more recently by the recovery of deuterium, given to the animal in the form of deuterium oxide, from the fatty acids in its depots (31). The newly synthesized fat contains mainly C_{16} and C_{18} fatty acids (32). It is generally accepted that this newly synthesized fat is formed in the liver and that no synthesis occurs in adipose tissue itself. Quantitative data, obtained by Schoenheimer and Rittenberg (31) and by Stetten and Grail (33), on the velocity of exchange of fat in the liver and in the depots are very difficult to reconcile with this assumption.

From the results of Stetten and Grail it has been calculated that the actual rate of synthesis of fatty acids by a group of 5 mice was in the neighborhood of one gm/day. This is about four times as much as was present in the livers of the normal mice. If the liver be assumed to be the major site of synthesis, the half life time of fatty acids in the liver becomes a matter of hours and not of days. Actually the half life time of fatty acids in the liver was found to be between 2.6 to 2.8 days and that in the carcass between 5 to 6 days.

The studies of Tepperman, Brobeck and Long (34) suggest that the conversion of carbohydrates into fat may take place both in the liver and in the extrahepatic tissues. These workers compared rats which were trained to eat their entire daily ration in one hour with control animals. Since the trained animals exhibited a mean R.Q. of 1.22 after the administration of glucose, whereas the untreated ones showed, under these conditions, a R.Q. of about 1.05, it was concluded that one effect of the dietary training was an augmentation of the rate of fatty acid synthesis from carbohydrates. The difference in R.Q. between the two groups persisted even in the functional absence of the liver, though it was not so striking as in the intact animal.

The correlation between the appearance of glycogen in adipose tissue and the deposition of fat led Tuerkischer and Wertheimer (35) to suggest that adipose tissue itself is capable of converting carbohydrates into fat. This suggestion is corroborated by the finding that adipose tissue, containing glycogen, exhibits a respiratory quotient exceeding 1.0 (36, 37). Recently, we (38) were able to show that adipose tissue, incubated *in vitro* with serum enriched with deuterium oxide, contains fatty acids with stably bound deuterium. It thus seems established that adipose tissue participates in the synthesis of fatty acids in the body.

DEPOSITION OF GLYCOGEN IN ADIPOSE TISSUE

The presence of glycogen in adipose tissue was first observed in histological studies by v. Gierke (39). Hoffmann and Wertheimer (40), Wertheimer (41) and Tuerkischer and Wertheimer (35) showed that glycogen cannot be demonstrated by chemical methods in normally fed healthy dogs and rats. When, however, the animals were fasted or undernourished and afterwards placed for a short time on a carbohydrate rich diet, glycogen could be found regularly in adipose tissue. This glycogen deposition is transient. The glycogen disappears after a certain time if feeding is continued. The conditions favoring glycogen deposition are generally the same as those enhancing fat deposition. Glycogen deposition precedes fat deposition and comes to an end with the completion of fat deposition (35). Glycogen deposition has been established (35) in the following cases, in addition to refeeding after undernutrition: *a*) in rats trained to consume their entire daily ration in one hour (34); *b*) in rats fed and starved on alternate days (42, 43); *c*) in rats after a certain time of excessive carbohydrate consumption (35); *d*) in rats after injection of insulin in appropriate doses (45); *e*) in new-born rats (immediately after birth the glycogen content of presumptive adipose tissue decreases concurrently with the accumulation of fat, 44); and *f*) in rats after suture of the nerves supplying adipose tissue (45).

The deposition of glycogen is most marked in the interscapular brown fat tissue of the rat. The amount of glycogen deposited in this tissue under suitable conditions

is of the same order of magnitude as that in the liver. Comparatively high quantities are found in mesenteric fat as well. Groin fat and perinephric fat generally contain the smallest quantities of glycogen.

According to the view that adipose tissue is a quasi dead reserve material, the glycogen deposited must be assumed to reach the tissue via the blood stream. The glycogen would then have to diffuse through the cell membrane, as well preserved adipose tissue preparations can be shown to contain glycogen within the cells but not in the intercellular spaces. This is highly improbable; a more plausible explanation is that glycogen is synthesized in the adipose cells themselves.

It has been shown that glycogen accumulation occurs only under suitable conditions and that it is always correlated with an enhanced fat deposition. It thus seems probable that glycogen is directly concerned with fat synthesis in adipose tissue, possibly as an intermediary product in the transformation of carbohydrates into fat. This would explain why glycogen appears only when this synthetic process is especially active.

Adipose tissue cannot be regarded as an additional carbohydrate store for replacing blood sugar, as the findings of Mirski show that no glucose is formed upon breakdown of adipose glycogen (37). The conclusion that adipose glycogen is transformed into fat is corroborated by the high respiratory quotient of glycogen-containing adipose tissue.

REGULATION OF FAT AND GLYCOGEN DEPOSITION AND MOBILIZATION

In the regulation of fat metabolism, liponeogenesis must play as important a rôle as gluconeogenesis in carbohydrate metabolism. This is especially so, since the diet of most animals consists mainly of carbohydrates and fat constitutes the main reserve material of the body. The conversion of food carbohydrates into stored fat goes on continuously. The older view, that fat formation from carbohydrates commences only after the glycogen stores are fully loaded, can no longer be held (31). According to Stetten (46), in the normal rat 30 per cent of the carbohydrates fed are stored as fat and only about 3 per cent as glycogen. In view of the fact that adipose tissue is the main site of fat storage and has been shown to be an actively metabolizing tissue, with a comparatively dense vascular and nervous supply, it is presumable that nervous and endocrine factors influence the deposition and mobilization of fat.

Nervous Regulation

The first observations, indicating nervous regulation of fat metabolism, came from clinical sources. Goering (13), summarizing clinical experience, came to the conclusion that excessive nervous irritation brings about fat loss, while paralysis causes deposition of fat. Mansfeld-Mueller (47) found that upon scission of the femural nerve the denervated side becomes fatter than the intact one. Wertheimer (14) showed that scission of the spinal cord, above the sixth thoracic segment, prevents the formation of fatty liver in phloridzin-treated fasting dogs. Scission below this region allows the fatty infiltration of the liver to proceed. Scission of the liver nerves does not prevent fat infiltration. It thus seems presumable that, in the absence of nervous irritation of the fat tissue, fat mobilization is inhibited. This

conclusion was corroborated by Erben and Hasselbach (48) in experiments with phosphorus poisoned rats and by Mill (49) in experiments of fat mobilization following hemorrhagia. Hausberger (50) was able to cut the nerve connections supplying the symmetrical interscapular fat body of the mouse on one side only, thus setting up conditions in which the normally innervated side of this bilateral symmetrical body served as an ideal control for the otherwise identically situated but denervated other side. Influx of glycogen and, in its wake, an accumulation of fat were noticeable in the denervated side of the interscapular fat body some 10 hours after denervation. The fat content of the denervated side exceeded that of the control side in the normally fed as well as in the starved mouse. The denervated side was depleted of its fat only after long periods of hunger. Beznak and Harris (51) showed, by scission of the splanchnicus, that sympathetic nerve fibers are involved in the mobilization and deposition of fat in the perinephric adipose tissue. Sympathetic control is also shown by the occurrence of facial hemiatrophy on irritation of sympathetic trophic fibers (13). The trophic influence of the autonomous nervous system on fat tissue was also shown by Kuré *et al.* (52) by isolated scission of the sympathicus and of the parasympathicus in dogs. The sympathicus was found to act as an inhibitor of fat deposition and the parasympathicus as an accelerator. After complete denervation, both fat deposition and fat mobilization were reduced. The denervated fat tissue was found to possess a reduced metabolism.

It seems well established that obesity can constantly be induced in rats by lesions of the hypothalamus (53, 54). It is presumed that the neurones involved in the disturbance lie in the region of the ventromedial nuclei and that their axons run into the region above and lateral to the mammillary bodies and from there into the mesencephalic tegmentum (54). From this center, fibers may enter the spinal cord and from there innervate the fat tissue by sympathetic nerve fibers, which leave the spinal cord mainly above the sixth thoracic segment.

Normal innervation seems to be requisite for the attainment of a dynamic equilibrium in fat tissue. Secession of the innervation is followed by elevation in the fat content of the tissue. The nervous influence on adipose tissue may be of high importance in the etiology of obesity and leanness, as well as in localized occurrence of fattening. Adipose tissue seems to possess a certain 'tonus' due to constant innervation. This 'tonus' may be either lowered or raised by pathological conditions.

Endocrine Regulation

1. *Anterior pituitary.* The regulation of fat mobilization from adipose tissue by the pituitary has been proved by Barett, Best and Ridout (55) by the use of deuterio-fatty acids and by Stetten and Salcedo (56) by labelling the newly synthesized fatty acids with deuterium from the body water enriched with heavy water. Both groups found that the fatty livers, produced by the injection of anterior pituitary extracts into fasting mice, are the result of excessive mobilization of depot fat and its migration to the liver. These findings suggest a direct influence of anterior pituitary hormones on adipose tissue activity. The hormone concerned has not been clearly defined. Weil and Stetten (57) showed that the urine of fasting rabbits contains a factor, which acts similarly to the pituitary factor, causing fatty liver upon

injection into a fasting mouse. Depletion of the fat depots and replacement of the fat by a translucent gelatineous material was observed by Dobyns (58) upon injection of a preparation of thyreotropic hormone of the pituitary. The changes were found to take place even in the absence of the thyroid, showing that it was not the thyreotropic hormone proper that caused fat depletion, but another factor present in the preparation. Recently it has been shown by Reiss (59) that lactogenic hormone also reduces the fat content of fat tissue in rats and in man. This mobilization of fat may be connected with lactation. Clement showed that scission of the nerves supplying the interscapular fat body and the perinephric fat abolished the fat mobilizing effect of pituitary extracts (60). The same method was used to prove the dependence of the adrenaline effect on fat mobilization upon the sympathetic system (61).

2. *Adrenal cortex.* The observation of Verzar and Laszt (62), that the deposition of fat in the liver of rats is inhibited in adrenalectomized animals, has been repeatedly confirmed (63–66). All experimental methods used to obtain fatty livers were less successful in adrenalectomized rats. Recently, Hartman, Brownell and Thatcher (67) succeeded in isolating a new factor from cortical extracts, distinct from the sodium and carbohydrate factors, which causes deposition of fat in the liver of starved adrenalectomized animals. The defect in adrenalectomized animals is generally attributed to inadequate mobilization of the fat depots. However, if the lack of fat mobilization were the main factor in the disturbances of fat metabolism in adrenalectomized animals, the fat depots would be rich in fat. Tuerkischer and Wertheimer (35) found that fat deposition in adrenalectomized rats is also markedly below normal. Together with the lack of fat deposition, only negligible amounts of glycogen are deposited, under conditions normally causing a marked deposition of glycogen. Glycogen deposition could be induced, under these conditions, by the administration of fresh cattle adrenal preparations and by large doses of desoxycorticosterone acetate (2 mg.). That the loss of body fat in adrenalectomized rats is not due to reduced food intake was shown by Schiffer and Wertheimer (78) in paired-fed rats, in which the normal always markedly exceeded the adrenalectomized rat in its fat content. Administration of 2 mg. of DCA or of adrenal cortical extracts reestablished fat deposition. These experiments suggest a direct influence of cortical hormones on the fat tissue and on the conversion of carbohydrate into fat. The lack of fatty livers in adrenalectomized rats may partly be due to this disturbance in fat synthesis. Another explanation points to the inability of the liver of adrenalectomized animals to store fat or to a higher fat metabolism in the liver. MacKay (65) showed that adrenalectomized rats store less fat in the liver than normal rats, upon ingestion of cream or pure fat. When adrenalectomy is performed after the establishment of fatty liver, the fat leaves the liver more quickly than in normal rats.

It may be concluded that in adrenalectomized animals, fat deposition in both the liver and the depots is inhibited.

3. *Insulin.* Insulin is known to prevent fatty livers occurring in cases of pancreas or phloridzin diabetes (69). It has recently been shown that insulin also partially inhibits fatty liver production, which occurs in response to the administration of anterior pituitary extracts in fasting rats, mice and guinea pigs (70). It is generally taken for granted that this insulin effect is indirect, acting through the influence of

insulin on carbohydrate metabolism. It has been found by Wertheimer (43) that, under suitable conditions, insulin injection brings about an appearance of glycogen in adipose tissue of the normally fed rat. Glycogen disappears from fat tissue simultaneously with the disappearance of the insulin effect on blood sugar. This insulin effect is not due to the increased appetite induced by insulin, since glycogen deposition in intrascapulary fat precedes the augmentation of appetite. Insulin induces glycogen deposition in intrascapulary adipose tissue even in hunger. However, no glycogen deposition is induced by insulin in rats on a protein rich diet. In adrenalectomized rats, insulin has no effect on appetite, but renews the capacity of adipose tissue to store glycogen. It may be concluded therefore that insulin exerts a direct influence on glycogen synthesis in adipose tissue. Alloxan diabetic rats are unable to store glycogen in their adipose tissue, under conditions which in normal rats regularly bring about such a deposition. Administration of insulin fully restores this ability in alloxan diabetic rats (71).

Experiments of Pauls and Drury (72) as well as those of Stetten and Kleiner (73) proved the deficiency in fat synthesizing capacity of diabetic animals. According to the latter authors, only 5 per cent of the normal amount of carbohydrate is converted into fat in the diabetic animal. Since adipose tissue participates in fat synthesis in the body (38) and insulin is obligatory for normal glycogen and fat deposition in adipose tissue, the obstruction of metabolism in this tissue may be of importance in the pathology of diabetes.

4. *Thyroidea.* Adipose tissue glycogen deposition is enhanced in thyreotoxic rats during the first days of recovery feeding (35). After this period, the glycogen values fall rapidly to zero, whereas in normal rats the maximum glycogen levels are attained only on the second day of recovery feeding. The consumption of glycogen in adipose tissue is enhanced in thyreotoxic rats, *in vitro* as well as *in vivo*. The influence of thyroxin on fat tissue seems to be due to a generally accelerated metabolism.

MacKay and Sherril (74) reported that thyroidectomy in the adult rat, fed with a fat rich diet, causes a marked reduction in body fat content. The animals do not appear less plump nor do they weigh appreciably less than the controls. This is contrary to the general assumption that thyroidectomy should cause fattening. However, in no case could obesity be induced experimentally by thyroidectomy.

METABOLISM OF ADIPOSE TISSUE IN VITRO

Respiration and Respiratory Quotient

The respiratory activity of adipose tissue was measured by Fleischmann (75) and by Scoz (76). The latter author found respiratory quotients of approximately 1.0 for normal adipose tissue, as well as for adipose tissue from fasting animals. High R.Q. values were also frequently found by Ruska and Quast (77). Henle and Szpingier (78), on the other hand, found R.Q. values of 0.75 for normal fat tissue. Mirski (37) showed that the respiratory rate and the respiratory quotient depend upon the condition of the tissue and upon the medium. With fat tissue from fasting rats, suspended in phosphate Ringer's solution, an average R.Q. of 0.64 and an oxygen consumption Q_{O_2} (mm^3 O_2/hr./mg. of fresh tissue) of 0.12 were found. Fat tissue

containing glycogen. obtained after recovery feeding, showed an average R.Q. of 1.05 and a Q_O of 0.18. The heightened oxygen consumption and the high R.Q. were transient under these conditions and tended to fall after one to two hours. Addition of glucose to hunger fat raised the R.Q. to 0.73. When the milieu was serum, instead of phosphate Ringer's solution, the Q_O, and R.Q. values of hunger fat were 0.18 and 0.78 respectively. With added glucose, the corresponding values were 0.21 and 1.15. In fat tissues containing glycogen, the R.Q. values exceeded 1.0 (av. 1.27) in all cases and the Q_O value was 0.38. The high R.Q. was maintained for many hours and in some cases rose to 1.6. These experiments lend additional proof for the conversion in adipose tissue of glycogen into fatty acids (38).

Hook and Barron (79) measured the respiration of brown adipose tissue of the hibernating and non-hibernating ground squirrel. They calculated their results on the basis of fat free tissue and found a Q_{O_2} of 17.1, i.e. of the same order as that of kidney tissue. During hibernation, adipose tissue retained a large proportion of its chemical activity at low temperature (3° C.). Respiration was 36 per cent of the value for normal temperature, as against a residue of 15 per cent in kidney and liver. The R.Q. found was 0.80 and fell after starvation for eight weeks to 0.67.

Enzymatic Activity

The presence of diastase, phosphatase, lipase and dehydrogenases in adipose tissue has been repeatedly reported (80). However, the demonstration of diastatic or phosphatatic activity in extracts of adipose tissue does not serve as conclusive proof for the presence of the enzymes in the tissue itself, since they may be derived from the blood included in the tissue. Mirski (37) was able to show that 'adipose diastase' is similar in its properties to blood diastase, converting glycogen into low polysaccharides which are not fermentable. Only 5 per cent of the glycogen broken down was accounted for as glucose. Hausberger (80), on the other hand, tried to prove that diastase and phosphatase originate in the tissue itself, by showing that the first enzyme is concentrated in denervated adipose tissue and that the activity of the second is enhanced on recovery feeding. An alternative path for glycogen decomposition in adipose tissue was demonstrated by Mirski (37), who showed that adipose tissue phosphorylates glycogen The product of phosphorolysis is Cori-ester No phosphoglucomutase was found in white adipose tissue. Brown adipose tissue, on the other hand, was found to contain this enzyme. The lack of glucomutase in white adipose tissue leaves the mechanism of glycogen synthesis in this tissue unexplained.

Among the various dehydrogenases present in adipose tissue, special interest pertains to the dehydrogenases of higher fatty acids. Their presence in adipose tissue was repeatedly demonstrated (20–23). According to Yosii (21) adipose tissue is the richest source of dehydrogenase of the higher fatty acids, exceeding the liver 2 to 3 times and the muscle and kidney up to 30 times. Shapiro and Wertheimer (22) showed that dialysed water extracts of adipose tissue dehydrogenate fatty acids only in the presence of phosphate and a boiled extract of adipose tissue or yeast. In agreement with Lang and Mayer (81), it was found that the co-factor present in the boiled extracts can be replaced by adenosine-5-phosphoric acid. The enzyme, studied by Lang and Mayer in liver extracts and by Shapiro and Wertheimer in adipose tissue,

seems to be different from other fatty acid dehydrogenase found (82), serving only for interconversions of fatty acids and not for their total breakdown.

Other substrates found to be attacked by adipose tissue extracts are phospholipids, succinic acid, glycerophosphoric acid and lactic acid (22).

BROWN ADIPOSE TISSUE

The brown adipose tissue is in many of its morphological and physiological properties quite distinct from ordinary, white, adipose tissue (83). It shows a much higher respiratory and metabolic activity than white adipose tissue. Under appropriate conditions, brown adipose tissue is capable of storing glycogen in concentrations comparable with those in the liver (35). On the other hand, fat mobilization from brown adipose tissue during hunger is much slower than that from white fat tissue (84). Due to these structural and metabolic peculiarities, various special functions have been ascribed to brown adipose tissue. Since the interscapular brown fat body is especially developed in hibernating animals, this tissue has been connected with hibernation and has been named 'hibernating gland'. The older literature on this aspect can be found in a review by Ferdmann and Feinschmidt (85) and in an article by Rasmussen (86). In the more recent literature two reports can be found that bring evidence pointing to a function of brown adipose tissue in hibernation. Wendt (87) showed that the injection of extracts of brown fat of the hibernating hedgehog into rats lowered the metabolism of the rats by 20 to 30 per cent. After an initial unrest, the animals became quiet and apathetic. Similar results were obtained by Hook (88) who injected white rats with an extract of brown fat of woodchucks and ground squirrels. Extracts of fat from the omentum or of perinephric fat tissue had no such influence. No experiments were made with extracts of brown fat of non-hibernating animals. The experiments prove only the presence in brown fat of a fat soluble substance that depresses the metabolism of the white rat.

In man and in the rabbit, the brown adipose tissue exists only in the embryo. In the rat and in the mouse, the brown fat in its embryonic form remains at various sites during adult life. White fat tissue, after depletion of most of its fat, becomes morphologically and functionally very similar to brown fat tissue. Brown fat tissue may therefore be looked upon as an adipose tissue that did not develop beyond its embryonic state.

RÔLE OF ADIPOSE TISSUE IN THE DEVELOPMENT OF OBESITY

As in other fields of physiology, much may be learned about the normal function of a tissue from the elucidation of disturbances during pathological conditions. The state of knowledge about experimental obesity has been recently reviewed (89, 53, 54). However, the place of adipose tissue in these disturbances has received very little attention. In one case, of congenitally obese mice, it could be clearly demonstrated by Salcedo and Stetten (90) that the retardation of fat mobilization from the depots is an important factor in the pathology of obesity. The mice were fed for five days on bread crumbs, to which ethyl esters of deuterio-fatty acids were added. Analysis of the deuterium content of body water and fatty acids showed that the storage of fatty acids, in these mice, was normal. The turnover of depot fatty acids, however,

was found to be considerably lower than in normal animals. A similar study, in the case of the best studied form of experimental obesity, in hypothalamic obesity (53, 54) would be of great interest.

CONCLUSIONS

Adipose tissue is a tissue with a special structure and a special type of cell. It is supplied by a comparatively dense capillary net and innervated by sympathetic nerve fibers. Deposition and mobilization of fat in adipose tissue is an active process, involving the metabolism of the tissue. Under conditions favoring fat deposition, adipose tissue accumulates glycogen, which is presumably built in the tissue cells themselves. Synthesis of new fatty acids from carbohydrates as well as transformation of one fatty acid into another proceed continuously in this tissue. All of these metabolic activities are regulated by nervous and endocrine factors.

The present knowledge points to the possible rôle played by adipose tissue in the development of various pathological conditions in the metabolism of the body.

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