STUDIES

FROM

THE ROCKEFELLER INSTITUTE

FOR MEDICAL RESEARCH

REPRINTS

VOLUME 71

NEW YORK
THE ROCKEFELLER INSTITUTE FOR MEDICAL RESEARCH
1930

25068

Results of the investigations conducted at The Rockefeller Institute, or elsewhere under its grants, are first reported in a variety of journals and publications. The reports are then assembled in volumes designated Studies from The Rockefeller Institute for Medical Research, of which this is Number 71. The Studies appear serially but at irregular intervals. The text of the original publications is in all respects followed in the Studies. The name, date, volume, number, and pages of the journal in which each article originally appeared are printed above the title. To insure uniformity and simplicity of reference, plates and illustrations repeat the numbers used in the place of first publication.

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WAVERLY PRESS
BALTIMORE, U. S. A.

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[Reprinted from THE JOURNAL OF IMMUNOLOGY, February, 1929, Vol. 16, No. 2, pp. 123–131]

ON THE RACIAL DISTRIBUTION OF SOME AGGLUTINABLE STRUCTURES OF HUMAN BLOOD

BY K. LANDSTEINER AND PHILIP LEVINE

(From the Laboratories of The Rockefeller Institute for Medical Research)

(Received for publication, July 1, 1928)

There are recorded in the literature some attempts to make a serological distinction of races with the methods that had proved successful in the differentiation of species. One may state that these efforts have not led to quite definite results. The claim of Bruck (1) that the sera of Caucasians, Chinese and Malays can be distinguished by means of complement fixation tests could not be confirmed by later workers.[1] Marshall and Teague (6) observed some differences in similar tests but in their opinion the data are not so clear cut as to permit of a practical application. Negative results were obtained by Fitzgerald (7) who feels justified in concluding that with the method employed "the existence of specific racial differences . . . . has not been proven." Taking into account the difficulty of distinguishing the sera of closely related species, e.g., man and chimpanzee (Nuttal (8)), one would scarcely expect a more favorable outcome without resorting to very sensitive methods.

A few communications deal with the distinction of races of animals. The paper of Glock (9) on chickens hardly calls for any discussion in view of the meagre evidence presented. The experiments of Lühning (10) appear to be better supported,[2] particularly those in which he employed Uhlenhuth's method of cross immunization. It is possible, however, that the races examined descended from diverse species as is considered by the author himself. Uhlenhuth (12), in experiments

[1] In view of the findings of Schiff (2), Dölter (3), Witebsky and Okabe (4), and others, on group specific substance in human sera related to the isoagglutinogens of the red cells, one has to consider the possibility that differences in precipitin reactions within a species may be dependent upon non-protein substances (cf. Friedberger and Lasnitzki (5)).

[2] These results were not properly acknowledged in a previous publication (11).

with the method just mentioned, was unable to differentiate the serum of tame and wild rabbits although in the same way he could easily distinguish between rabbit and hare. All things considered, it would seem that the method of cross immunization would deserve wider application for the question at issue. With the use of hemagglutinins, a technique serviceable for the distinction of closely related species (Landsteiner and van der Scheer (14)), no positive results on the differentiation of races have thus far been reported (cf. (15)).

A new line of research on the serology of races was initiated by L. and H. Hirschfeld (16). Their remarkable studies did not tend toward the detection of racial specificity but were concerned with the numerical distribution of serological characteristics among races and led to the discovery that the incidence of the human blood groups varies in different populations.

This work was continued by a great many investigators so that in the last few years there has accumulated a voluminous literature dealing with most human races. It is not intended to consider here these results in detail and to dwell upon the hypotheses put forth on the interrelationship and the origin of human races. For this we refer to the comprehensive reviews of Ottenberg (17), Bernstein (18), Snyder (19), Streng (20), and Hirszfeld (21). Suffice it to state that marked differences were found. The percentage distribution in some of the most striking instances was as follows:[3] English; O, 46.4; A, 43.4; B, 7.2; AB, 3.0: Hindus; O, 31.3; A, 19.0; B, 41.2; AB, 8.5: American Indians (pure blooded); O, 91.3; A, 7.7; B, 1.0; AB, 0.0: Australians; O, 46.0; A, 54.0.

An opportunity of pursuing, in a somewhat different manner, the investigations just outlined presented itself when a method became available to determine new serological characteristics of human blood (23, 24). The method consists in the use of immune agglutinins from rabbits. By exhaustion of various immune sera with particular human blood specimens, specifically reacting fluids were obtained which permitted the detection of three, more or less definite, agglutinable properties, termed as M, N, and P. These were found to occur ap-

[3] In the present paper the nomenclature by letters instead of numerals is used in accordance with the recommendation of the American Association of Immunologists (22).

parently with no preference in each of the four blood groups. The technique of performing the tests is described in the previous publication (24). As to the tests for N, it may be stated that the reactions of minor strength were not entirely uniform if several immune sera were used.[4] Accordingly two immune sera were chosen[5] which gave identical reactions and permitted of a sharp differentiation between positive and negative reactions.

TABLE 1

Incidence of M+ and M− Reactions

The figures in parentheses indicate the number of females.

	GROUP O		GROUP A		GROUP B		GROUP AB		TOTAL	
White										
Number of individuals...	578		648		226		76		1708	
Percentage.....	44.4		37.9		13.2		4.5			
	M+	M−	M+	M−	M+	M−	M+	M−	M+	M−
Number of individuals....	600	158	541	107	184	42	57	19	1382	326
	(184)	(57)	(137)	(36)	(44)	(16)	(15)	(3)	(380)	(112)
Percentage.....	79.2	20.8	83.5	16.5	81.4	18.6	75	25	80.9	19.1
Colored										
Number of individuals...	323		221		159		27		730	
Percentage.....	44.2		30.3		21.8		3.7			
	M+	M−	M+	M−	M+	M−	M+	M−	M+	M−
Number of individuals...	233	90	155	66	116	43	21	6	525	205
	(102)	(28)	(60)	(26)	(39)	(18)	(9)	(0)	(210)	(72)
Percentage.....	72.1	27.9	70.1	29.9	73.0	27.0	77.8	22.2	71.9	28.1

Our material was limited to the white and colored (Negro) populations in New York City and to a relatively small number of American Indians. Among these, differences were observed in the occurrence of the properties referred to.

For the factor M, 1708 white and 730 colored individuals were examined (table 1).

[4] See (24), page 769.

[5] The same two sera were used in a study on the heredity of M and N (25).

It appears from the table that 19.1 of the white and 28.1 per cent of the colored individuals were of the type M −. Using the formula[6] given by Yule (26) the standard error of the difference between M+ and M− is 1.8. The observed difference, 9.0, is 5 times the standard error of the difference and is therefore very probably significant.

The deviation is pronounced in the males, only slight in the females. Since in the latter the number of colored individuals is small this result may be a matter of chance.

In some experiments it seemed as if the positive reactions were often comparatively weaker with the blood of Negroes but the results were not conclusive.

The difference for M approaches that between the frequencies of group B in the colored and white races which is 8.6 in our material (table 1). The average frequency of group B was found to be 11.4 in about 10,000 native American white individuals examined (27, 28, 29) and 19.5 in 770 American Negroes (Lewis and Henderson (30) and Snyder (19)) a difference sufficiently close to our value.

Of Indians 205 individuals were examined; 124 (41 men, 83 women) from the Haskell Institute, Lawrence, Kansas, and 81 (57 men, 24 women) from the Choctaw-Chickasaw Sanatorium, Talihina, Kansas.[7] In the first series 82 (66.1 per cent) belonged to group O and 42 (33.9 per cent) to group A; none to group B or AB. In the second

[6] This formula is $e_{12} = \sqrt{p_0 q_0 \left(\frac{1}{n_1} + \frac{1}{n_2}\right)}$ in which e_{12} is the standard error of the difference of two sets of observations. In the case under consideration p_0 and q_0 are the percentages of M+ and M− reactions respectively, in the entire number of individuals of both populations; n_1 and n_2 indicate the number of individuals in each of the two populations. Thus

$$e_{12} = \sqrt{21.8 \times 78.2 \left(\frac{1}{1708} + \frac{1}{730}\right)} = 1.8.$$

[7] The blood specimens of the students of the Haskell Institute were collected by Miss C. Nigg; those from the Choctaw-Chickasaw Sanatorium were obtained by Dr. Van Cleave. Several drops of blood were taken from the finger and put into test tubes containing about 1 to 2 cc. of the mixture recommended by Rous and Turner (31). The blood was packed in ice and arrived in our laboratory in perfect condition. For their kind coöperation and the painstaking collection of the samples the authors are greatly indebted to Miss C. Nigg and Dr. W. E. Van Cleave.

series 74 (91.4 per cent) were in group O and 7 (8.6 per cent) in group A; none in B or AB.

The group distribution in the Lawrence series agrees with the findings of Coca and Deibert (32), Nigg (33), and Snyder (19) (see table 2) in that the incidence of O is rather high and B is lacking. The examination of the batch from Talihina showed a very high frequency of O, a low percentage of group A, and absence of B and AB, in agreement with the figures reported by Snyder for pure blooded Indians.

If one accepts the view, suggested by Coca and Deibert (32) and Snyder (19), that the incidence of A diminishes with the purity of the

TABLE 2

Incidence of the Blood Groups in Indians Examined by Various Authors

AUTHORS		O	A	B	AB	TOTAL NUMBER EXAMINED
Coca and Deibert		77.7	20.2	2.1	0	862
Nigg	Apparently pure	70.8	28.6	0.4	0.2	517
	Known to be impure	56.6	41.7	1.7	0	60
Snyder	Apparently pure	91.3	7.7	1.0	0	453
	Known to be impure	64.8	25.6	7.1	2.4	409
This paper	Specimens from Lawrence	66.1	33.9			124
	Specimens from Talihina	91.4	8.6			81

stock and the occurrence of A individuals is attributable to white admixture, one would conclude that the material from the Lawrence series, with its comparatively high incidence of A does not come from full blooded Indians although the records of the individuals gave no such indication (3 cases excepted). It is noteworthy that in the 205 Indians of our material there was no individual of group B.

The results of the tests for M are given in table 3.

As seen from table 3 the incidence of M− bloods among Indians was founded to be as low as 4.9 per cent (5.6 per cent in the Lawrence series, and 3.7 per cent in that from Talihina) as compared to 19.1

per cent in the white race and 28.1 per cent in the Negroes. Calculating as above, the difference between the Indian and the white man is 5 times the standard error of the difference and thus is probably due to real racial diversity, not to mere chance in sampling.

The examination of the blood of Indians for the agglutinogen N as compared with those of whites and American negroes, gave the figures

TABLE 3

Incidence of M+ and M− Reactions in the Blood of American Indians

	GROUP O		GROUP A		TOTALS	
Number of individuals	156		49		205	
Percentage	76.1		23.9			
	M+	M−	M+	M−	M+	M−
Number of individuals	152	4	43	6	195	10
Percentage	97.4	2.6	87.8	12.2	95.1	4.9

TABLE 4

Incidence of N+ and N− Reactions

The figures in parentheses indicate the number of females.

		GROUP O		GROUP A		GROUP B		GROUP AB		TOTAL	
		N+	N−	N+	N−	N+	N−	N+	N−	N+	N−
White	Number of individuals	187	59	164	59	34	18	8	3	393	139
		(73)	(15)	(63)	(17)	(17)	(2)	(1)	(1)	(189)	(35)
	Percentage	76.0	24.0	73.5	26.5	65.4	34.6	72.7	27.3	73.9	26.1
Colored	Number of individuals	58	24	31	14	35	12	7	0	131	50
		(22)	(8)	(9)	(6)	(14)	(3)	(3)	(0)	(48)	(17)
	Percentage	70.7	29.3	68.9	31.1	74.5	25.5	100	0	72.4	27.6
Indians	Number of individuals	56	100	26	23					82	123
	Percentage	35.9	64.1	53.1	46.9					40	60

summarized in table 4. Fewer tests for N in the latter races are recorded than for M because a more suitable technique—namely, absorption and testing at 37° to 40°C. was developed but late in the course of our studies, and only these tests appear in the table.

These results show that there is a considerable disparity in the fre-

quency of N in the Indians and the two other populations, the negative reactions for N being more than twice as frequent in the former. It is not accidental that in the Indians the percentage of N is high while at the same time the incidence of M is low for, as has been shown elsewhere (25), the properties M and N are not independent.

The examination for P was made with one immune serum. It was obtained from a rabbit injected with the blood of colored individuals of group O which were singled out in tests with absorbed normal rabbit sera.[8] While in the tests for N and especially for M there was, as a rule, a sharp break between positive and negative reactions, the intensity of the reactions in the P tests varied by degrees. Accord-

TABLE 5

Reactions for P

		INTENSITY OF REACTION*				TOTAL NUMBER
		++ and above	+± and +	± and tr.	F. tr. and 0	
White	Number of individuals	22	112	83	48	265
	Percentage	8.3	42.3	31.3	18.1	
Colored	Number of individuals	81	151	29	6	267
	Percentage	30.3	56.6	10.9	2.2	

* Tr. = trace; f.tr. = faint trace.

ingly the results were arranged into four classes depending upon the intensity of the agglutination (table 5). This arbitrary arrangement was compensated by examining in each experiment an approximately equal number of the specimens to be compared, namely, bloods of white and black races.

The group distribution (in percentage) of the 265 white individuals was as follows: group O, 41.5, group A, 46.8, group B, 9.8, group AB, 1.9; that of

[8] Other less active immune sera were obtained by injection with the same bloods. The specificity of these sera was similar to that of the serum mentioned above. However it was not established whether there was a complete agreement in the reactions. Absorbed normal sera of some animals seemed to give reactions that were somewhat parallel to those of our P immune sera (24).

the 267 colored individuals: group O, 46.4, group A, 34.1, group B, 17.2, group AB, 2.2.

The tests for P are recorded in table 5.

As seen in table 5 the strongest reactions (column 1) were almost four times as frequent in the colored as in the white individuals, whereas the weak reactions (columns 3 and 4) were much rarer in the former. Blood with negative reactions occurred only exceptionally among the colored.

It may be concluded, from the data reported, that the agglutinogens studied exhibit distinct racial variations and that it is possible to extend the use of the principle introduced by L. and H. Hirschfeld. The anthropological investigations made hitherto with normal isoagglutinins are limited to the examination of two independent characters only. This state of affairs is not decisively altered by the present contribution but it is possible that our observations do not exhaust the subject and that by finding methods of detecting a large number of serological properties, the scope of this sort of investigation will be widened.

Correlating all the known facts it is uncertain whether one will succeed in discovering immunological qualities entirely specific for races analogous to the serologic species characteristics; one is rather led to the idea that the serological make-up of races is determined by varying combinations of a number of characteristics (34).

For the material used in the present study we express our thanks also to the following: Drs. C. Floyd Haviland, I. J. Furman, and John R. Knapp, and Miss Frances W. Witte, of the Manhattan State Hospital; and Drs. J. G. M. Bullowa, A. K. Merkin, and H. M. Levin, of Harlem Hospital.

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[Reprinted from THE JOURNAL OF EXPERIMENTAL MEDICINE, May 1, 1929,
Vol. 49, No. 5, pp. 727–743]

STUDIES ON THE PRECIPITABLE SUBSTANCES OF BACILLI OF THE SALMONELLA GROUP

BY J. FURTH, M.D., AND K. LANDSTEINER, M.D.

(From the Laboratories of The Rockefeller Institute for Medical Research)

(Received for publication, January 9, 1929)

In earlier communications we (1) have described precipitable substances, probably of carbohydrate nature, isolated from *B. typhosus*, *paratyphosus* B, and *enteritidis*, and two antigenic protein preparations obtained from *B. typhosus*. These studies have also been extended to the main serological types of the Salmonella group of organisms.

For a general review of the subject we refer to the communications by P. Bruce White (2), Krumwiede, Cooper, and Provost (3), Tulloch (4), and to the references given in our earlier papers.

We would however like to mention several recent publications. Thus Happold (5) described a precipitable substance obtained from *B. aertrycke* which he considered to be of protein nature and "to be identical with the antigen which stimulates the production of agglutinins to a heat-stable antigenic form of the organism." Ecker and Rimington (6) report obtaining from *B. aertrycke* a carbohydrate containing material possessing toxic properties; while White (7) states that he has extracted a soluble specific substance from *B. aertrycke* similar to the specific soluble substances of Avery and Heidelberger. The precipitable solution obtained was acted upon by the sera of *B. aertrycke* and *paratyphosus* B but not by those of *B. Newport* and *suipestifer*. White's paper contains a full discussion of the serological properties of the rough *B. aertrycke* strains. Casper (8) prepared from *B. paratyphosus* B a carbohydrate containing substance which reacted with sera for *B. paratyphosus* B, *B. typhosus*, and *B. typhi murium*, to a lesser degree with sera for *B. enteritidis* Gärtner, but not with *suipestifer* serum. A report on the carbohydrate and protein fractions of *B. typhosus* was made by Heidelberger, Shwartzman, and Cohn (9).

Preparation of the Crude Precipitable Carbohydrate Substances.—Three methods were employed for the preparation of the crude substances:

1. Extraction by alkaline hypochlorite solution: Bacilli grown on agar for 48 hours were taken up in 0.9 per cent sodium chloride solution, and alkaline hypochlorite solution was added in a quantity sufficient to dissolve the bacteria at about 50°C. The solution was chilled and cold 95 per cent alcohol added until a heavy precipitate was formed carrying down most of the active substance. This

TABLE I

Precipitation Tests

Carbohydrates from *Bacillus*	Antigen dilutions	Immune sera obtained with *Bacillus*											
		Typhosus		*Enteritidis*		*Paratyphosus* B		*Derby*		*Newport*		*Hog cholera*	
Typhosus	5,000	+++	+++	++	++++	+	+±*	++	+++	0	0	0	0
	50,000	+±	++±	+	++	±	+	+	+	0	0	0	0
	500,000	±	±	f. tr.	±	0	0	0	tr.	0	0	0	0
Enteritidis	5,000	+±	+++	++	++++	tr.	±	+	+	0	0	0	0
	50,000	±	±	+	++	0	0	0	tr.	0	0	0	0
	500,000	0	tr.	f. tr.	±	0	0	0	0	0	0	0	0
Paratyphosus B	5,000	tr.	±	+±	++	+±	++	+++	++++	0	0	0	0
	50,000	0	0	±	+	+	+++	++	++±	0	0	0	0
	500,000	0	0	0	±	±	+	±	±	0	0	0	0
Derby	5,000	±	+	+	++	+±	+++	++++	++++	0	0	0	0
	50,000	f. tr.	±	±	+	+	+++	++	++±	0	0	0	0
	500,000	0	0	0	tr.	±	+	±	±	0	0	0	0
Newport	5,000	0	0	0	0	0	0†	0	0	++	++++	tr.	±†
	50,000	0	0	0	0	0	0	0	0	+	+++	0	0
	500,000	0	0	0	0	0	0	0	0	±	+	0	0
Hog cholera	5,000	0	0	0	0	0	0	0	0	+	+++	+++	++++
	50,000	0	0	0	0	0	0	0	0	±	++	++	++++
	500,000	0	0	0	0	0	0	0	0	tr.	++	+	+±

To 0.1 cc. of the antigen solution 0.05 cc. immune serum and 0.05 cc. physiological salt solution were added. The reactions were read after 2 hours at room temperature (Column 1) and after standing overnight in the ice box (Column 2).

The immune sera were obtained by injections of rabbits with heated bacilli, as stated above.

* With other sera of the same type the reactions were negative with the substance of *B. typhosus*.

† With some other sera of the same type marked reactions were obtained with the substance of *B. Newport*.

precipitate was dissolved in water, and, after removal of some insoluble material, was reprecipitated with 95 per cent alcohol. After washing with 95 per cent alcohol, absolute alcohol, and ether, it was dried *in vacuo*.

2. Extraction with saline solution: Bacilli grown on agar for 48 hours were taken up in 0.9 per cent sodium chloride solution, centrifuged,[1] the sediment washed with 95 per cent alcohol, and extracted with boiling 95 per cent, and absolute, alcohol. After filtration on a hot water funnel the bacillary mass was heated in the steam bath two or three times with 0.9 per cent sodium chloride solution for 1 to 2 hours and the extract separated each time by centrifuging. Much of the protein was removed by addition of hydrochloric acid in an amount sufficient to produce maximum precipitation and the fraction was reprecipitated to recover some of the active non-protein substance; the joint mother liquids were then precipitated with alcohol after addition of 1/20 volume of normal sodium hydroxide. The further redissolving, removal of insoluble material, reprecipitation, and drying of the preparation were done as described above.

3. Extraction by autoclaving and subsequent tryptic digestion (*cf.* Goebel and Avery (10)): Agar cultures were washed off and were autoclaved for 20 minutes and digested overnight with purified trypsin solution (Goebel and Avery) at 37°C. The subsequent alcohol precipitation, removal of insoluble material, reprecipitation, and drying of the substance were carried out in the manner given.

Cultures.—Besides our laboratory strains the following cultures obtained through the courtesy of the National Collection of Type Cultures, London,[2] were used: *B. paratyphosus* B, *Tidy No. 14;* type *Stanley, No. 92;* type *Reading, No. 72;* type *Derby, No. 1729;* type *Newport, No. 129;* type *Hog cholera, No. 356; B. enteritidis, Gärtner, No. 127; B. abortivo-equinus, No. 766;* and *B. aertrycke.*[3] They were plated on agar and colonies showing homogeneous growth in broth were selected.

Immune Sera.—Unless otherwise stated, immune sera were prepared by weekly injections of rabbits with about 1/10 to 1/20 of 24 hour agar slant cultures heated to 62–65°C. for 40 minutes. Immune sera prepared with various strains of the same type, *e.g. B. typhosus*, showed considerable variations in their capacity to precipitate the carbohydrate preparations.

Precipitin Reactions of the Carbohydrates and Their Parallelism to the "Small Flaking" Agglutination.—Precipitin reactions with the purified specific substances (see page 21) are presented in Table I. The crude substances had a lower titer but showed in cross-tests almost the

[1] In a control experiment the bacillary mass was washed several times with saline solution.

[2] We are indebted to Dr. R. St. John-Brooks, Curator of the National Collection of Type Cultures, London, for supplying us with the cultures mentioned.

[3] The numbers correspond to those given in the catalogue of the collection.

same range of specificity as purified preparations, no matter which of the methods described was employed.

It is seen that the strains can be divided roughly into three groups within which the strongest cross-reactions occur. The first group consists of *B. typhosus* and *B. enteritidis*, the second of *B. paratyphosus*

TABLE II*

Typhosus	*Enteritidis*	*Paratyphosus* B	*Derby, Reading, abortivo-equinus*	*Newport*	*Hog cholera*
III, (X), 8	III, 8	I, II, 7, 8	II, 7, 8	IV, VI, 7	V, VI

* Roman numerals are used by White to designate salient components, Arabic numerals for those of minor development.

TABLE III

Absorption Tests with Immune Serum for B. typhosus

Dilution of the carbohydrates	Serum, unabsorbed		Serum absorbed with alcohol-treated *B. enteritidis*	
	Precipitation tests with the carbohydrate of *B. typhosus*			
5,000	++	+++	0	0
50,000	++	++	0	0
500,000	f. tr.	tr.	0	0
Dilution of the immune serum	**Agglutination tests with *B. typhosus* (suspension preserved with chloroform)**			
300	+++	+++	+++	+++
900	+++	+++	++	++
2,700	++	++	+±	+±
8,100	+	+	±	tr.
24,300	0	tr.	0	0

The reactions were read after 1½ hours at room temperature (Column 1) and after standing overnight in the ice box (Column 2).

B, and *B. Derby*, and the third of *B. Newport* and *B. hog cholera*. Additional tests with crude preparations, not included in Table I, showed that the substances and sera of *B. Stanley*, *B. Reading*, and *B. abortivo-equinus* reacted like those of *B. paratyphosus* B.

A comparison of these results with the agglutinin reactions of the "stable agglutinogens" suggested itself. The distribution of the

agglutinable factors underlying the "small flaking" agglutination is summarized, according to White, in the scheme shown in Table II.

On comparing this scheme with the precipitin reactions an approximate correspondence between the stable agglutinogens and the precipitable carbohydrates appears (see White).

This relation is also illustrated by the following observations (Table III): An immune serum for *B. typhosus* was absorbed with alcohol-treated *B. enteritidis* and tested for precipitins and agglutinins to *B. typhosus*. Since the "stable" alcohol-resistant agglutinogens of *B.*

TABLE IV

Absorption Experiments

Dilution of the precipitable substance	Precipitable substance	Immune serum for *B. enteritidis*		Precipitable substance	Immune serum for *B. Newport*		Precipitable substance	Immune serum for *B. hog cholera*	
		Unabsorbed	Absorbed with *B. paratyphosus* B		Unabsorbed	Absorbed with *B. hog cholera*		Unabsorbed	Absorbed with *B. Newport*
2,000	*Enteritidis*	+++	+++	*Newport*	+++	+++	*Hog*	+++	+++
20,000		+++	+++		++±	++±	*cholera*	+++	+++
200,000		±	±		+	+		++±	++±
2,000	*Paraty-*	+++	0	*Hog*	++	0	*Newport*	+	0
20,000	*phosus* B	+++	0	*cholera*	+++	0		±	0
200,000		+±	0		++±	0		tr.	0

The immune sera were absorbed with bacillary suspensions kept in alcohol and washed once with saline solution.

typhosus and *B. enteritidis* are supposed to be very similar it was to be expected that by the exhaustion with alcohol-treated *B. enteritidis* the precipitins would be removed but not the "large flaking" agglutinins. The result of the experiment bore out this assumption.

In contrast with the absence of precipitating action of fluids with large flaking agglutinins of high titer (*cf.* Heidelberger, Shwartzman, and Cohn) are instances in which immune sera with a relatively low agglutinin content, mainly of the small flaking type, precipitated intensely the carbohydrate solutions. Such sera were prepared by the method of Douglas and Fleming (1).

Some discrepancies will be noted such as the absence of precipitin reactions of certain *B. paratyphosus* B sera on the substances of *B. Newport* (factor 7) and *B. typhosus* (factor 8), and the lack of precipitating capacity of most of the hog cholera sera for the substance derived from the strain *Newport* (factor VI). Since in these combinations some of the sera give positive reactions one may assume that the precipitable substances do not lack the properties in question. An explanation may perhaps be seen in the observation that the agglutinin reactions with alcohol-treated bacilli were comparatively weak in

TABLE V

Precipitable substance	Dilution	Immune serum for *paratyphosus* B				
		Unabsorbed, diluted			Absorbed with *B. Reading*, diluted	
		1:2	1:8	1:32	1:2	1:8
Paratyphosus B, not treated with alkali	5,000	+++	+++	+	++++	+++
	50,000	+±	++	++	++	++
	500,000	±	±	tr.	±	−
Paratyphosus B, treated with alkali	5,000	++	+	tr.	0	−
	50,000	+	+	0	0	−
	500,000	f. tr.	0	0	0	−
Reading (treated with alkali)	1,000	+++	++	tr.	0	−
	5,000	++	+±	f. tr.	0	−
	25,000	+	+	f. tr.	0	−

those instances in which precipitation was lacking. However, a thorough study of this question was not made.

In order to determine whether the multiplicity of precipitins corresponds to that of the agglutinins, absorption experiments were made in several cases (Table IV).

On the whole the tests were in agreement with the idea of a multiplicity of precipitins, corresponding to that of agglutinins. A striking exception was the following:

Immune sera for *B. paratyphosus* B or *B. Stanley*, when absorbed with *B. Reading* or *B. abortivo-equinus* did not precipitate the substances prepared from the homologous organisms, although they agglutinated the alcohol-treated bacillary suspensions. Search for

the missing precipitable property (attributable to factor I) revealed that it was present in the crude saline extracts, but disappeared after treatment with alkali. Substances exhibiting the property were prepared as follows:

Saline extracts obtained from alcohol-extracted bacilli, as described above, were precipitated with alcohol (without the addition of alkali), after previously removing the substances precipitable by dilute acid. The precipitate was dissolved in a small volume of water, some insoluble material discarded, and 50 per cent trichloracetic acid added to cause optimal precipitation. The unneutralized mother liquid containing most of the active substance was then precipitated with an excess of 95 per cent alcohol and dried after washing several times with 95 per cent alcohol, absolute alcohol, and ether.

Table V indicates that immune sera for *B. paratyphosus* B (or *B. Stanley*) contain a fraction of antibodies directed towards the alkali-labile property I. After absorption with *B. Reading* the immune serum for *B. paratyphosus* B still precipitates the substances derived from this bacillus but no longer acts on the substance from *B. paratyphosus* B after treatment with alkali. That this effect is not due to a diminution of one single antibody was shown by tests with diluted immune serum.

The reactivity of the substance was not altered by peptic and tryptic digestion at a pH not in itself injurious. It was not destroyed by treatment for 1 hour at room temperature, with normal hydrochloric acid, but it did not withstand under the same conditions the action of 0.01 normal sodium hydroxide (Table VI).

A preliminary investigation was made of the carbohydrates of "rough" strains of *B. paratyphosus* B and *B. aertrycke*. According to several authors such strains may contain special antigenic components. In view of these statements we prepared precipitable substances from "rough" strains by the method of dissolving the bacilli in alkaline hypochlorite solution (see page 11). The substance prepared from *B. aertrycke* "R" and purified as described below proved to contain large amounts of carbohydrates not further investigated. In precipitation tests (Table VII) the "R" substances reacted specifically with the immune serum to the "R" strains whereas the corresponding "S" preparations reacted also to "R" immune sera. It is uncertain whether the interaction can be explained by the presence of "R" forms in the "S" strains.

Attempts to Fractionate the Precipitable Substances by Means of Precipitins.—The resistance of the specific precipitable substances to the action of alkali seemed to offer a way of ascertaining whether the various serological properties of these substances are due to different

TABLE VI

Precipitin Tests Showing the Effect of Acid, Alkali, Peptic, and Tryptic Digestion on Factor I of B. paratyphosus B

Dilution of the precipitable substance	Control I	0.01 N NaOH	N HCl	Peptic digestion	Control II	Tryptic digestion I	Tryptic digestion II	Control III
5,000	++++	0	+++	+++	+++	+++	0	0
100,000	+	0	±	+	+±	+	0	0

To 0.1 cc. of the precipitable substance, 0.1 cc. of an antibody solution for factor I (immune serum to *B. paratyphosus* B absorbed with suspensions of *B. Reading*) was added.

Control I: untreated precipitable substance.

Control II: 0.5 cc. of a 1 per cent solution of the substance, 0.05 cc. N HCl, 0.45 cc. water; kept at 37° for 24 hours.

Control III: 0.5 cc. of a 1 per cent solution of the substance, 0.2 cc. of a 1 per cent solution of Na_2CO_3, 0.8 cc. water; kept at 37° for 24 hours.

Peptic digestion: 0.5 cc. of a 1 per cent solution of the substance, 0.05 cc. N HCl, 0.1 cc. of a 0.2 per cent pepsin solution, 0.35 cc. water; kept at 37° for 24 hours.

Tryptic digestion I: 0.5 cc. of a 1 per cent solution of the substance, 0.5 cc. of a 1 per cent trypsin solution, 0.05 cc. 1 per cent Na_2CO_3, 0.45 cc. water; kept at 37° for 24 hours.

Tryptic digestion II: 0.5 cc. of a 1 per cent solution of the substance, 0.5 cc. of a 1 per cent trypsin solution, 0.2 cc. 1 per cent Na_2CO_3, 0.3 cc. water; kept at 37° for 24 hours.

For the treatment with acid and alkali a 2 per cent solution of the substance was mixed with an equal volume of 0.02 N NaOH or 2 N HCl respectively and the tests kept for 1 hour at room temperature.

separable portions. Accordingly attempts were made to fractionate the precipitable substances in a manner analogous to the usual absorption experiments with immune sera.

By suitable exhaustion with heterologous bacilli fractions of immune sera were prepared acting only on part of the supposed precipitable factors. The precipitates produced were washed twice with saline

solution and boiled for a few seconds with a small quantity of 0.1 normal sodium hydroxide solution, the solutions obtained being neutralized and centrifuged.

When such solutions were tested with various antibodies they behaved like the original precipitable substance and not like qualitatively different fractions thereof.[4] With the procedure described no difficulty was encountered in separating precipitable substances of two different strains of bacilli after their solution had been mixed. It was

TABLE VII

Precipitation Test

Immune serum of *Bacillus*.....		*Aertrycke* "S"		*Aertrycke* "R"		*Paratyphosus* B "S"		*Paratyphosus* B "R"	
Precipitable substance	Dilutions								
Aertrycke "S"	2,000	+++	+++	+±	++±	+±	+±	+±	+++
	20,000	++	++	±	++	+	++	tr.	+
	100,000	±	+	f. tr.	+	±	+±	0	tr.
	500,000	0	0	0	0	0	0	0	0
Aertrycke "R"	2,000	0	0	+±	++±	0	0	++	++±
	20,000	0	0	0	0	0	0	tr.	+
Paratyphosus B "S"	2,000	+++	+++	++	++±	+	++	++	++
	20,000	++	++±	+	++	±	++	tr.	+±
Paratyphosus B "R"	2,000	0	0	++	+++	0	0	+++	+++
	20,000	0	0	++	+++	0	0	++	++±

Reactions read after 2 hours (Column 1) and after standing overnight (Column 2).

thought however that in these control experiments the mixture of the substances might not have been as intimate as that of the hypothetical fractions in the precipitable carbohydrates derived from one organism. We attempted therefore to separate by the same method precipitable substances after their solutions had been mixed, boiled, precipitated with alcohol, and dried in the usual manner.

[4] The results were the same in experiments aiming at the separation of Factors I and II of the precipitable substances of *B. paratyphosus* B. In this instance, instead of alkali, dilute acid was used for decomposing the precipitate.

TABLE VIII

Precipitable substance	Dilution	Immune sera for:							
		B. paratyphosus B absorbed with *B. enteritidis* (II-7)		*B. enteritidis* (8)		*B. hog cholera* absorbed with *B. Newport* (V)		*B. Newport* (VI)	
Mixture of the carbohydrates of *B. paratyphosus* B and *B. hog cholera*	1,000	+±	++++	+	++++	++	++++	++	++++
	10,000	++	++++	±	+±	+±	+++	+±	++++
	100,000	tr.	+	f. tr.	±	f. tr.	tr.	f. tr.	tr.
Factor II, 7	1,000	+±	+++	+	+±	0	0	0	0
	10,000	±	+±	tr.	±	0	0	0	0
	100,000	0	0	0	0	0	0	0	0
Factor 8	1,000	+±	+++	+	++	0	0	0	0
	10,000	tr.	tr.	0	f. tr.	0	0	0	0
	100,000	0	0	0	0	0	0	0	0
Factor V	1,000	0	tr.	0	0	+++	++++	++±	++++
	10,000	0	0	0	0	±	+	±	+±
	100,000	0	0	0	0	0	tr.	0	tr.
Factor VI	1,000	0	f. tr.	0	0	++	++±	+±	++±
	10,000	0	0	0	0	±	+	±	++
	100,000	0	0	0	0	0	0	0	0

To a 0.1 per cent solution of the mixture of *B. paratyphosus* B and *B. hog cholera* carbohydrates an equal volume of the immune sera (diluted 1:2) was added. After keeping the tubes for 2 hours at room temperature and overnight in the ice box, the precipitate formed was dissolved by sodium hydroxide, as described, and brought with saline solution to half of the original volume. (This dilution was arbitrarily designated 1:1000.)

Factor II, 7: precipitate formed by *paratyphosus* B immune serum absorbed with *B. enteritidis*.

Factor 8: precipitate formed by immune serum of *B. enteritidis*.

Factor V: precipitate formed by hog cholera serum absorbed with *B. Newport*.

Factor VI: precipitate formed by immune serum of *B. Newport*.

In these tests a considerable amount of carbohydrate was carried down non-specifically by the heterologous serum. The possibility that this effect was caused by imperfect solution of the mixture of precipitable substances led to the following modification of the experiment. The dried mixture obtained as before was dissolved in 0.1 normal sodium hydroxide, boiled for 1 hour, and neutralized. In this way again by specific precipitation an almost complete separation could be brought about.

In the following experiment this method was applied to the separation of fractions of single precipitable substances (Table VIII).

The experiment shows that while the two substances mixed together could be separated, apparently no fractionation of either of them was accomplished.

An analogous experiment was carried out with the substance of *B. Newport* and with the sera to *B. suipestifer* (factor VI), *B. paratyphosus* B (factor 7), and *B. Newport*, after exhaustion with *B. suipestifer* and *B. paratyphosus* B (factor IV). Comparing factors IV and VI the results resembled those just reported; the substance however precipitated by *paratyphosus* B serum gave a considerably stronger reaction with this serum than with the hog cholera serum. Conversely the solution obtained from the precipitate caused by the hog cholera serum reacted, like the original substance, more intensely with this serum than with *B. paratyphosus* B serum.

Chemical Data on the Precipitable Substances.—Several of the substances described were purified to a certain extent by a procedure very similar to that used for pneumococcus polysaccharides by Avery, Heidelberger, and Goebel (11).

The crude preparations were dissolved in water, some insoluble matter removed, and, after addition of normal sodium hydroxide to a concentration of about N/20, the active substance was precipitated with alcohol. This precipitation was repeated 4 to 6 times. Usually sodium acetate was added to aid flocculation. Then the solution was acidified with hydrochloric acid and precipitated with acidulated alcohol. When possible the precipitate was made in two steps, the first precipitate, containing most of the proteins and much carbohydrate, was removed by centrifugalizing and the supernatant fluid poured into an excess of acidulated alcohol. From the first pre-

cipitate, rich in proteins, part of the carbohydrate could be recovered by repeating the procedure.

The carbohydrate substance was reprecipitated in acid solution and dried after washing with alcohol and ether. The solutions were kept at low temperature while in acid solution.

These fractions were soluble in water, strongly reduced Fehling's solution after hydrolysis, and gave faint or negative protein reactions, particularly no precipitate with tannic acid or uranyl nitrate. The purified substance of *B. typhosus*, unlike the crude preparation, was not precipitated by barium hydroxide.

On analysis, the figures for the N content of the substances prepared from *B. typhosus*, *B. enteritidis*, *B. paratyphosus* B, *B. Derby*, *B. Newport*, and *B. hog cholera*, varied from 0.5 per cent to 1.4 per cent. The values for $[\alpha]^D$ were as follows: *B. typhosus*, +103; *B. enteritidis*, +95; *B. paratyphosus* B, +94; *B. Derby*, +76; *B. Newport*, +75; *B. hog cholera*, +48. For the sugar analysis the substances were hydrolyzed by heating 1 per cent solutions with an equal volume of normal hydrochloric acid in the steam bath for 5 hours, and the sugar content was determined by reduction of Fehling's solution. It was not established whether the time chosen was sufficient for complete hydrolysis. The values obtained were between 63 per cent and 74 per cent (calculated as glucose) for the six strains mentioned. During hydrolysis with acid some insoluble material, partly soluble in alcohol and of acid character, separated from the solutions as in the case of the specific substance from *V. choleræ* (12). No conclusion can be reached as yet whether these products form a part of the precipitable substances or are impurities.

Since it seemed possible that the precipitable substances contained carbohydrates derived from the agar used for cultivating the bacteria, two preparations were made from gelatin cultures of *B. typhosus* and *B. paratyphosus* grown in Blake bottles. They gave the following figures: *B. typhosus*, reducing sugar 69.5 per cent, $[\alpha]^D + 98°$; *B. paratyphosus*, reducing sugar 67.3 per cent, $[\alpha]^D + 99°$. It cannot be claimed that the substances were obtained in a state of purity and therefore all of the figures given are to be considered as preliminary.

On testing the action of alkali and acid it was found that the precipitable substances were remarkably resistant to alkali but readily destroyed by acids.

TABLE IX

Precipitin Tests with the Carbohydrates after Treatment with Acid and Alkali

Carbohydrates		Heating with N HCl for				Heating with N NaOH for				Control untreated	
From *Bacillus*	Dilution	3 min.		15 min.		½ hr.		2 hrs.			
Proteus O_{X19}	10,000	0	f. tr	0	f. tr.	0	0	0	0	+++	+++
	100,000	0	0	0	0	0	0	0	0	+	+±
V. choleræ	10,000	±	+++	0	0	0	+++	0	+	++	+++
	100,000	0	++	0	0	0	±	0	0	±	+
Pneumococcus Type III	10,000	++	++	+±	+±	++	++	++	++	++±	++±
	100,000	+	+±	±	+	±	+	±	+	+	+±
B. typhosus	10,000	0	0	0	0		−	++	+++	+++	+++
	100,000	0	0	0	0		−	±	++	+	++
B. enteritidis	10,000	0	0	0	0		−	++±	++±	++±	+++
	100,000	0	0	0	0		−	±	+±	+	+±
B. paratyphosus B	10,000	0	f. tr.	0	0		−	+±	++±	++	++±
	100,000	0	0	0	0		−	++	++±	++	++
B. Derby	10,000	0	0	0	0		−	+++	+++	+++	+++
	100,000	0	0	0	0		−	+	+±	+	+
B. Newport	10,000	0	0	0	0		−	++	+++	++	+++
	100,000	0	0	0	0		−	+	++	+	++
B. hog cholera	10,000	+±	++±	0	f. tr.		−	++±	+++	+++	+++
	100,000	0	+±	0	0		−	+±	++	+±	++

The substances of *Proteus* O_{X19} and cholera were prepared by extraction with boiling 75 per cent alcohol (1,10). The specific polysaccharide of Pneumococcus III was obtained through the courtesy of Dr. Avery.

The first reading of the precipitin tests was made after 2 hours at room temperature, the second reading after the tests were kept overnight in the ice box.

To a 2 per cent solution of the various preparations an equal volume of 2 normal sodium hydroxide or 2 normal hydrochloric acid was added and the solutions were kept in boiling water.

It is seen from the experiment (Table IX) that the preparations of the *typhosus-paratyphosus* group were similar as regards their resistance to acid and alkali, with the exception of the hog cholera substance which was somewhat more resistant to acid than the other preparations. The substances from the other organisms tested, behaved differently. Thus the preparation obtained from *Proteus* O_{X19} was destroyed by alkali as well as by acid, under the conditions of the experiment; the Pneumococcus III preparation was resistant to both, whereas the substance derived from *V. choleræ* was less resistant to alkali than the Salmonella carbohydrates. The differences found would seem to be significant even if one takes in account the variation in the method of preparation.

Observations on the Precipitable Proteins of B. typhosus.—A precipitable protein was prepared in a similar manner as the preparation P_2 *typhosus* described previously (1). The precipitation with alcohol of the extracted substance was omitted but after removal of the suspended bacilli by centrifugalizing, the saline extract was precipitated with dilute hydrochloric acid, redissolved by addition of a small quantity of alkali, filtered through a Berkefeld filter, and reprecipitated with acid. The precipitate was dried after washing with alcohol and ether.

This substance when injected into rabbits induced the formation of "large" and "small" flaking agglutinins aside from precipitins. Although this would seem to point to a relation between the substance P and "large flaking" agglutinogens there are observations which do not agree with this assumption. In the first place the titer of the large flaking agglutinins was relatively low in comparison with sera obtained with bacillary suspensions, and on prolonged immunization the increase of precipitins was not accompanied by a corresponding rise in the agglutinin titer. Furthermore in an absorption experiment the large flaking agglutinins of the sera for P_2 were apparently absorbed to a greater extent by typhoid bacilli treated with alcohol than the large flaking agglutinins of common typhoid sera.

It is as yet difficult to interpret these observations. They may possibly be ascribed to the presence in the P_2 preparations of a special substance responsible for the production of flagellar agglutinins, or to some flagellar material perhaps in an altered state which passed the filter candles. In this respect attention may be called to the observation that bacilli treated with alcohol are no longer agglutinable by large

flaking sera although they give rise to the formation of large flaking agglutinins (13).

The toxic action of the preparation P_2, mentioned previously, showed considerable variation. One preparation was toxic for rabbits in a dose of 0.5 mg. given intravenously. On repeated injections the animals tolerated doses up to about 20 mg. These animals as well as those immunized with digested bacilli (14) exhibited a typical Arthus phenomenon on intradermal injections of about 1 mg. of P_2 or the carbohydrate respectively. In cross-tests the reactions with the homologous substances were more pronounced. (*Cf.* the experiments on anaphylaxis by Tomcsik (15) and Avery and Tillett (16).) These tests were made on a small number of animals and therefore should be considered as preliminary.

DISCUSSION

The carbohydrate-containing preparations isolated from the main serological types of the Salmonella group gave on analysis figures for nitrogen of 0.5 to 1.4 per cent, but they showed only weak or negative reactions for proteins. One may assume that their serological activity is due to specific carbohydrates, for during purification there was a diminution of the nitrogen content and of the protein reactions along with an increase in the amount of sugar liberated by hydrolysis and an increase of the serological activity. The specific reactivity of the preparations to immune sera remained almost unimpaired after heating to about 100° with normal alkali for 2 hours but was quickly destroyed by boiling with normal hydrochloric acid; parallel with the disappearance of the serological activity reducing sugar and some insoluble material were set free. Aside from these observations the assumption that the substances are carbohydrates rests on the analogy of the results with those of Avery, Heidelberger, and Goebel (*cf.* 11).

The present studies support the view that the specific carbohydrates form an essential part of the "stable" agglutinogens of the bacilli (White (7)). We failed to establish a relationship to the phenomenon of large flaking agglutination.

The similarity of agglutination and precipitation is also shown by absorption experiments, which demonstrate that from one immune serum precipitins can be separated which correspond to agglutinin fractions (*cf.* Krumwiede (17)).

The explanation of these phenomena so far as the antigens are concerned is still an open one. The fact that certain antibodies are removed from an immune serum through successive absorptions and others are left behind gives evidence for a multiplicity of antibodies; but when the conclusion is drawn from the reaction of certain antibody fractions upon several antigens, that all of the positively reacting antigens contain a definite common substance or one clearly defined chemical group, it remains hypothetical so long as the assumed different elements have not been separated or established as individual structures by chemical methods (see 18). It could also be assumed that the phenomena are at least in part brought about by the action of one antibody on several antigens whose specific groups are similar but not identical (see 19, 4).

In order to examine the question raised, an attempt was made by specific precipitation to separate the carbohydrates into their hypothetical units. On the whole these experiments did not lead to obtaining fractions with different properties; and therefore the results did not support the idea of the existence of separable units in the single antigens. Indeed it would be desirable also to apply chemical methods for the purpose of fractionation of the specific carbohydrates.

A noteworthy difference between the observations reported and the results obtained with the carbohydrates of pneumococci by Heidelberger, Avery, and Goebel, is the following: The carbohydrates of the three fixed types of pneumococci exhibit very marked chemical differences in correspondence with their serological diversity. Such conspicuous chemical differences have not been found among the carbohydrate preparations from the Salmonella group, which, although serologically different, showed no very striking variation in sugar content and optical rotation, with perhaps one exception (*B. hog cholera*).

SUMMARY

Specific precipitable substances rich in carbohydrates, containing very little protein and small amounts of a material apparently of fatty nature, have been prepared from the main serological types of the typhoid-paratyphoid groups. The preparations in their present

state of purity do not exhibit very pronounced chemical differences in spite of serological dissimilarity. In this respect the results differ from those observed with the polysaccharides of pneumococci.

The specificity of the precipitin reactions of these substances parallels in a general way the so called small flaking agglutination.

Attempts to separate different fractions from the active substance serologically by means of precipitation with antibody solutions were on the whole unsuccessful.

The differences in resistance to the action of acid and alkali were found to be characteristic for various specific carbohydrates.

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[Reprinted from THE JOURNAL OF IMMUNOLOGY, July, 1929. Vol. 17, No. 1, pp. 1–28]

ON ISOAGGLUTININ REACTIONS OF HUMAN BLOOD OTHER THAN THOSE DEFINING THE BLOOD GROUPS

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(Received for publication, December 8, 1928)

The existence of reactions of human sera on human bloods other than those due to the group isoagglutinins α and β has been recognized for a long time (1, 2). A number of instances have been reported[1] among which the following may be mentioned as examples:[2] Meyer and Ziskoven (4), serum B acting on cells B; Sucker (5), serum agglutinating cells of group A, cells agglutinated by sera B; Beck (6), serum B agglutinating some cells O; Phillips (7), serum O reacting with two out of six blood samples of group O. The fact that also sera AB may contain agglutinins has been described by Landsteiner and Witt (8) and confirmed by Wiemer (9) and Lauer (10) (cf. Lattes and Cavazutti (11), and Dyke (12)).

Regarding the issue of the subgroups of group A (and AB) we refer to our recent discussion of this topic (8, 13, 3).

Parenthetically it may be mentioned that the two subgroups can often be distinguished by the difference not only in the strength of agglutination but also by isohemolysis.

A more general discussion of the irregular isoagglutinin reactions of human blood has been presented by several authors.

Unger (14) stated that he observed slight agglutinin reactions of bloods and sera within the same group due to what he designates as "minor" agglutinins. According to his experience, in transfusion of such cases untoward symptoms are apt to occur.

Guthrie and his coworkers (15) reported the following observations,

[1] For references see Levine (3), p. 136.

[2] Certainly among the cases in the literature cited as exceptions to the group rule, a good number are ascribable to mistakes or faulty technic.

in addition to those dealing with the subgroups described for the first time by v. Dungern and Hirschfeld (16). Out of 20 sera of group O one was found which acted on one only of 18 bloods of the same group. The agglutination was marked at 15 to 20°C. or below, and absent entirely above 32°C. The strength of the reaction as compared to common isoagglutination is not indicated. Further they observed that some sera of group A when absorbed with bloods AB (2 specimens) acted on the cells of group B in 7 cases tested. Similar experiments were made by Bunker and Meyers (17). The authors ascribe the phenomenon to qualitative differences but do not present sufficient evidence for their view. On the basis of their findings Guthrie and his coworkers reject the scheme of the four blood groups neglecting entirely, however, the undeniable practical value of this scheme and the differences existing between typical and abnormal isoagglutination. Their assumption of new agglutinogens and corresponding agglutinins analogous to the pairs A, α, and B, β, is not in conformity with the known facts (see Thomsen (18)).

A careful systematic investigation was carried out by Jones and Glynn (19). These authors tested the sera of 40 healthy individuals by cross agglutination and absorption tests. They found 48 positive reactions of low titer out of 1003 tests expected to be negative. On repetition of these tests with fresh samples of sera and cells all but 6 were negative.

In a paper on irregular reactions by Hübener (20), from Schiff's laboratory, mention is made of a blood weak in agglutinogen A. Also he describes a blood A, preserved for some time, which was agglutinated at low temperature, but not at 37°, by sera A and AB. The agglutinin was specifically absorbable. Apparently Hübener's case and similar ones of Schiff and Halberstaedter (21) are related to the findings of Thomsen (22) who described the following phenomenon. Occasionally bloods which when fresh showed nothing peculiar, become agglutinable on standing for some time at room temperature, by sera of all groups and also by their own sera. The agglutinin is absorbable by the altered cells. The abnormal property can be tranferred to fresh blood suspensions by addition of some of the altered blood. Thomsen and Friedenreich (23) discovered that the change is brought about by the action of a bacillus and that it can be produced

also by filtrates thereof. The authors urge the necessity of using fresh material in studies on the subject of isoagglutination.

Isoagglutinin reactions occurring only at low temperature have been studied by Bialosuknia and Hirszfeld (23a), and Landsteiner and Levine (24). At a temperature near 0°C. almost all sera agglutinate the cells of other individuals and also their own cells. Accordingly a specificity of these reactions is not easily demonstrable although certain specific differences have been observed.

Not infrequently abnormal reactions were found by Oppenheim and Voigt (see Lützeler) (25) in tests on the bloods of corpses. The highest incidence of these reactions was found in sera of group AB.

Instances of absence of isoagglutinins in sera O, A and B are probably not frequent and one may suppose that in some of these cases there were weak agglutinins which escaped detection. Five thoroughly investigated cases were found by Thomsen (18) among 3500 specimens. By means of family examinations the author was able to demonstrate that he dealt with a lack not of agglutinogens but of agglutinins, at least in 3 of his 5 cases. Examinations of this kind or perhaps the use of sera specific for blood O would be necessary to prove whether there actually exist cases in which an expected isoagglutinogen is absent.

Guthrie and Huck (15) observed sera of group B containing agglutinins active only for one of the two subgroups of group A.

In spite of the rather numerous observations on abnormal reactions it seemed desirable to undertake a new systematic investigation.[3] Indeed from the available reports it was not easy to judge how frequently distinct, irregular reactions occur at room temperature under well defined experimental conditions. Other points requiring additional information are the specificity of the abnormal reactions when the tests are made on a large scale, and the effect of variations in temperature.

While our work was in progress there appeared a paper by Thomsen (18) dealing with similar aspects of the subject. In investigations on about 3500 individuals he found 32 abnormal sera. His conclusions agree with our findings in essential points.

[3] See (26), p. 674.

For the experiments to be described it was indispensable to have a supply of material from a source enabling us to examine the blood of the same individual several times at different intervals. The specimens studied came from patients in the Manhattan State Hospital for mental diseases. The cases showing most marked irregular reactions were apparently free from syphilis and did not exhibit the autohemolysin described by Donath and Landsteiner (27).

To obtain the cells blood was drawn from the vein and was collected in the mixture of Rous and Turner (28).[4] Another part was allowed to clot and the serum was separated at room temperature. Soon after collection the material was stored in the ice box.

The tests were made by mixing in small test tubes (inside diameter 7 mm.) one drop each of saline, serum, and a 2.5 per cent suspension of twice washed corpuscles. The tubes were kept for two hours with occasional shaking in a water bath of 20°C., unless otherwise mentioned. The tests were read both with a hand lens (magnification 6 ×) and microscopically (magnification 100 ×). After shaking each tube gently, a drop of the mixture was withdrawn by means of a thin glass rod, placed on a glass slide and examined microscopically.

The experiments were carried out according to two different plans. One sort of tests was made by taking a number of bloods of the same group and examining the sera and cells in all their combinations, and also on cells of group O. This scheme, similar to that of Jones and Glynn, was adopted in order to obtain some statistical evidence as to the frequency of the abnormal reactions. In another set of experiments numerous (320 including 60 of group AB) sera were tested against a few bloods which had been found to be especially sensitive to some irregular agglutinins of human sera.

Frequency of Sera with Abnormal Agglutinins

Sera of groups O, A, and B, 60 of each, were tested in batches of 12 on as many bloods of their own group; in addition the same sera of groups A and B were tested on bloods of group O, again in batches of 12 sera and 12 bloods. All the tests were made on the day of the bleeding or on the following day.

[4] Five volumes of a 5.4 per cent aqueous solution of glucose and 2 volumes of a 3.8 per cent aqueous solution of citrate for 3 volumes of blood.

In order to summarize the results the sera are divided into three classes depending upon the strength of the most intense agglutination shown by each of the sera: (*a*) those with the most marked reactions, designated by the sign + (or stronger); (*b*) sera giving reactions recorded as ±; (*c*) sera with weak reactions, designated as trace (tr.) or faint trace (f. tr.).

The reaction + is barely visible to the naked eye; the reactions +± and ++ signify larger clumps, visible without magnification. The following schematic figure is given to illustrate the meaning of the signs:

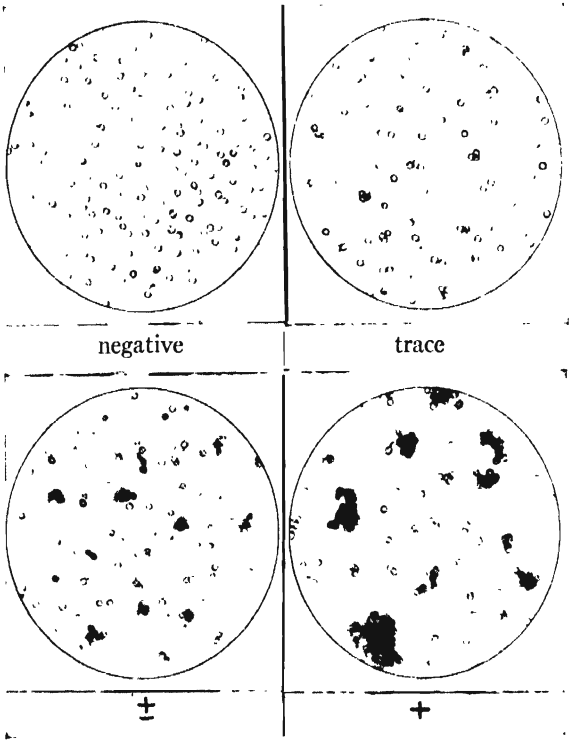


FIG. 1

Tables 1, 2, and 3 represent the results for the individual sets of 12 sera tested on the corresponding bloods, thus making a total of 720 tests for the 60 sera in each of the groups, O, A, and B, respectively. The first figure indicates the number of the sera which give abnormal reactions, the figure in parentheses the number of bloods showing agglutination with the particular serum.

TABLE 1

Sera O Tested on Bloods O

BATCH NUMBER	REACTIONS		
	+	±	tr. and f.tr.
1	1* (10)	1 (7)	4 (1) 1 (2)
2	1† (5)		1 (2) 1 (1)
3			1 (1)
4			1 (2)
5	1‡ (6)		1 (3) 1 (1)
Total 60.....	3	1	11
Per cent.....	5	1.6	18.3

* Serum 299.
† Serum 972.
‡ Serum 76.

TABLE 2

Sera A Tested on Bloods A

BATCH NUMBER	REACTIONS		
	+	+	tr. and f.tr.
1			1 (1)
2	1* (8)	1 (6) 1† (2)	1 (2)
3	1‡ (8)	1 (4) 1 (6)	1 (2) 1 (1)
4		1 (2)	2 (2) 1 (6)
5		1 (2)	1 (1)
Total.....	2	6	8
Per cent.....	3.3	10	13.3

* Serum 1135.
† Serum 1155.
‡ Serum 1219.

The results (not tabulated) of the tests of sera A and B on bloods of group O may be stated as follows. About twice as many sera A (see p. 39) and B gave agglutinations recorded as tr. or ± with bloods O as with bloods of their own group. Moreover the number of O bloods reacted upon was about three to four times as great as in the experiments in which an equal number of A or B bloods was tested with A or B sera respectively. Hence it may be said that in sera A and B

TABLE 3

Sera B Tested on Cells B

BATCH NUMBER	REACTIONS		
	+	±	tr. and f.tr.
1			1 (2) 1 (4)
2		1 (1)	2 (1) 1 (3)
3		1* (1)	2 (1) 1 (4)
4		1 (3)	4 (1)
5	1† (4) 1‡ (4)	1 (3) 1§ (11)	
Total.....	2	5	12
Per cent.....	3.3	8.3	20

* Serum 1450.
† Serum 1940.
‡ Serum 2038.
§ Serum 1979.

there occur frequently weak agglutinins acting by preference on bloods of group O ((24) p. 447 and 456). The more active sera of this type are mentioned below.

Sixty sera of group AB found in a material of more than one thousand individuals were tested only on two bloods each of group O, and subgroups[5] AA^1 and AA^2. In these tests two sera, nos. 850 and 995, were

[5] AA^1 designates the more frequent type, AA^2 the rarer type; α^1 and α^2 represent the corresponding agglutinins, (see (24)). In referring to the subgroups the letter A is omitted.

detected which gave marked reactions. The properties of these sera will be discussed presently, along with another serum, no. 535, group AB, found previously.

In addition there were numerous sera AB that showed minor reactions (tr. to ±). Most of these agglutinated both cells O and AA^2, but not AA^1. A small number of sera (3) agglutinated cells of subgroup A^1 (not O nor A^2). Only a few weak reactions were observed with B bloods.

Properties of the Abnormal Agglutinins

In the following a description is given of those abnormal agglutinins which showed the strongest reactions or those of a peculiar type. These sera were detected mostly in the two sorts of experiments mentioned above; some of them were known from previous work.

Group AB. Serum 850 showed a characteristic manner of action. At 20°C. it agglutinated distinctly every blood of group O tested, some more intensely than others; it had a somewhat weaker action on cells of group A belonging to subgroup A^2. Occasionally it agglutinated, mostly to a slight or moderate degree, blood cells of group A which from their other reactions belong to subgroup A^1. Accordingly and also on account of the varying intensity of the reactions with absorbed O and B sera, (cf. (16) p. 527) there exist bloods which form a transition, as it were, between A^1 and A^2. In group AB some specimens having the property A^2 were not agglutinated. In a small number of specimens of group B, reactions occurred much weaker than on bloods O and somewhat weaker than on AA^2.

The reactions of serum 995 in general ran parallel to those of 850 but they were somewhat weaker. Serum 995 did not act on any bloods of group AB. Reference has already been made to weaker sera showing the same type of specificity.

These statements concerning serum 850 are based upon an examination of the following numbers of specimens: 59 in group O, 61 in group A (37 of subgroup A^1 and 24 of subgroup A^2), 22 in group B, and 15 in group AB. Serum 995 was tested on 19 specimens of group O, 40 of group A (21 in subgroup A^1 and 19 in subgroup A^2), 20 in group B, and 6 in group AB.

Sera 850 and 995 seem to be similar to the case of Wiemer on account of the action on bloods of group O, but differ from his case since

TABLE 4

SERA GROUP AB	SUBGROUP	CORPUSCLES																							
		Group O						Group A									Group B				Group AB					
								Subgroup A^1					Subgroup A^2								Subgroup A^1B				Subgroup A^2B	
		201	509	523	540	730	901	203	240	740	1172	1176	263	271	500	625	616	795	823	829	850	914	995	1428	535	1985
850	A^1	+ ±	+ ±	+ ±	+ +	+ ±	+ +	0	0	tr.	0	±	+ ±	+	+	+	tr.	0	±	f. tr.	0	0	0	0	±	0
995	A^1	+	+	+	+	+	+	0	0	0	0	0	+	±	±	+	0	0	0	tr.	0	0	0	0	0	0
535	A^2	0	0	0	0	0	0	+	+	+	+ ±	+	0	0	0	tr.	0	0	0	0	+	+	+	+	0	0

Tests with sera 535 and 850 were made at 23°C., with serum 995 at 20°C.

TABLE 5

SERUM NUMBER	GROUP	SUBGROUP	CORPUSCLES																														
			Group O										Group A																				
													Subgroup A_1										Subgroup A_2										
			34	78	97	99	200	214	255	299	538	972	203	245	740	1135	1155	1172	1176	1531	1611	1635	80	263	271	577	625	1173	1219	1509	1614	1628
1155	A	A_1	±	+	+	+	+	+	±	±	+	+	0	0	0	0	0	0	0	0	0	0	±	±	±	±	±	±	±	±	±	±
625	A	A_2	0	0	0	0	0	0	0	0	0	0	+±	+	+±	+	+	+±	+±	+±	+	++	0	0	0	0	0	0	0	0	0	0
1219*	A	A_2	f.tr.	+	±	tr.	0	0	0	0	0	tr.	++	+±	++	+±	+±	++	+±	+±	+±	++	±	0	0	f.tr.	tr.	±	0	tr.	f.tr.	0
1219†	A	A_2	0	0	0	0	0	0	0	0	0	0	+	+	+	+	+	+	+	+	±	+	0	0	0	0	0	0	0	0	0	0
535	AB	A_2	0	0	0	0	0	0	0	0	0	0	+±	+	+	+	+	+	+	+	+	+	0	0	0	0	0	0	0	0	0	0

Tests with serum 1219 were made both at 20°C. (*) and at room temperature (†), 25°C. on the day of the experiment.

TABLE 6

SERUM NUMBER GROUP A	CORPUSCLES																																		
	Group O												Group A																						
													Subgroup A_1										Subgroup A_2												
	97	99	201	255	299	1021	1501	1512	1514	1546	1554	1555	203	740	1135	1155	1172	1176	1531	1567	1590	1595	1613	1638	271	575	577	625	1173	1219	1553	1559	1599	1602	1620	1629
740	+	0	+	+	0	+	+±	0	+	0	0	0	+	0	0	±	0	0	+	+	0	+	+	+	+	±	+	±	0	±	+	0	0	+±	+	0
1135	+	0	±	+	0	±	+	0	+	0	0	tr.	±	0	0	±	0	0	+	+	0	+	+	±	+	tr.	+	±	0	tr.	+	0	tr.	+	±	tr.
1172	+	tr.	+	+	f.tr.	+	+	tr.	+	0	0	±	+	0	0	+	0	0	+	+	0	+	+	+	+	±	+	±	0	±	+	0	±	+±	±	0
1176	±	0	tr.	0	0	+	tr.	±	f.tr.	0	±	+	0	±	0	0	0	0	+	+	±	0	tr.	0	±	f.tr.	0	+	0	0	±	f.tr.	+	+	0	tr.

Wiemer did not notice any selective action on cells of group A. Actually he found reactions on bloods A, B, and AB which in his opinion may be attributed to pseudo-agglutination.

A serum with marked activity (+ to +± reactions), no. 535, and several weaker sera belong to a second type described by Landsteiner and Witt (8). This type is characterized by an almost specific action on cells of subgroups A^1 and A^1B. Apparently the 2 cases of Lauer belong to this category.

Serum 535 was tested with 37 bloods of group O, 28 of group A^2, 21 of group B, and 3 of A^2B with negative results aside from a few faint reactions. It acted on all 41 cells AA^1 and 10 cells AA^1B examined.

Three weak sera AB of similar specificity were not further studied.

Representative tests with the three most active sera are given in table 4.

Group A. Sera 625 AA^2 and 1219 AA^2 agglutinated almost specifically cells AA^1 (marked reactions of the strength + to ++), thus resembling serum 535 and others of group AB.

Serum 1155, AA^1, acted (± to +) on all bloods of group O examined and less intensely on cells AA^2. As with serum 850 of group AB there were weak reactions on exceptional bloods of subgroup AA^1.

Serum 625 gave weak reactions with a few bloods other than AA^1 which corresponded in their specificity with the reactions of sera 214 of group O, 577 of group A and others (see p. 43). At 20°C. serum 1219 sometimes acted to a lesser degree also on cells O and AA^2, but at 25° the reactions occurred only with cells AA^1.

Serum 625 was tested against 41 bloods O, 73 group A (46 AA^1 and 27 AA^2); serum 1219 was tested with 30 bloods of group O and 74 of group A (53 AA^1 and 21 AA^2).

Serum 1155 was tested on 29 cells of group O, and 41 cells of A (24 AA^1 and 17 AA^2).

Weak reactions especially on cells AA^1 were given by two other sera of subgroup A^2. Several sera AA^1, as mentioned above, behaved similarly but gave rather weak reactions, at most ±, with most cells of group O and some cells A, apparently more frequently on A^2. Tests made at lower temperature, 12° to 15°C., indicated that the specificity of these sera is similar to that of sera 850 and 995 in group AB and serum 1155 in group A.

In table 5 are recorded tests with the 3 most active sera, and also with serum 535, group AB.

Distinct reactions ± to +±, not correlated to the subgroups, were shown by 4 sera of group A (nos. 740, 1135, 1172, and 1176). An illustrative experiment (with selected blood specimens) with the 4 sera is given in table 6.

The results (table 6) show that there is a significant, although not perfect, parallelism in the reactions of 3 of the sera, 740, 1135, and 1172. In several instances not recorded in the table the discrepancies were

TABLE 7A

SERUM NUMBER	GROUP	CORPUSCLES OF GROUP O											
		34	78	97	99	200	201	214	232	255	299	538	972
34	O	±	+	±	tr.	tr.	±	±	±	tr.	0	tr.	tr.
299	O	+	+	+	0	+	+	±	0	+	0	0	0
972	O	±	f.tr.	+	0	f.tr.	f.tr.	0	0	tr.	0	0	0
740	A	+	±	+	0	+	+	f.tr.	0	+	0	0	0

TABLE 7B

SERUM NUMBER	GROUP	CORPUSCLES OF GROUP O											
		46	63	76	97	99	100	200	201	509	835	901	907
76	O	0	+	0	+	0	+	±	±	f.tr.	f.tr.	0	±
740	A	0	+	0	+	0	+±	+	±	f.tr,	tr.	0	+

more pronounced. The fourth serum, 1176, differs distinctly from the others in its reaction on a number of bloods, for instance, nos. 255 and 1554, in group O, and 203 and 740, in A.

Sera 740 and 1172 were tested on 503 and 217 bloods, respectively (groups O and A). They gave reactions of the intensity designated as ± or more on about 30 per cent of the specimens.

Serum 1176 was examined with 53 bloods of group O, 74 bloods of group A (44 A^1 and 30 A^2). Altogether it gave reactions of the intensity + on 17 per cent, weak reactions on 24 per cent.

Group O. Four sera (nos. 34, 76, 299, and 972) gave marked reactions on bloods of their own group.

TABLE 8A

SERA NUMBER, GROUP B	CORPUSCLES																						
	Group O												Group B										
	33	34	46	200	250	916	938	974	975	990	1139	1145	224	616	795	1037	1048	1438	1450	1505	1940	1979	2039	Menz.
1048	tr.	+	f. tr.	±	+	0	f. tr.	+	+	±	0	±	+	+	+	+	0	0	±	+	0	+	0	0
1438	0	tr.	tr.	f. tr.	f. tr.	0	tr.	±	0	tr.	f. tr.	f. tr.	+	f. tr.	f. tr.	f. tr.	0	0	tr.	+	0	±	0	0
1450	+	±	±	+	±	0	+	±	+	±	±	+	0	0	0	0	0	±	0	0	±	0	±	±
1940	tr.	tr.	±	tr.	±	0	f. tr.	+	tr.	±	f. tr.	f. tr.	+	±	tr.	tr.	0	tr.	f. tr.	+	0	+	0	0
1979	±	±	±	+	±	+	±	tr.	+	+	±	±	0	0	0	0	±	tr.	f. tr.	0	0	0	tr.	tr.
2038	tr.	+	tr.	tr.	±	0	f. tr.	+	±	tr.	0	f. tr.	+	+	±	0	0	0	f. tr.	+	0	+	0	0
Menz.*	±	+	+	0	+±	+	0	±	0	+	+	±	+±	+	tr.	f. tr.	±	+	±	+±	+	+	0	0

* The serum of this individual has been described by Ottenberg and Johnson (29).

Serum 76 (Japanese) could be tested on 26 specimens only; 74 bloods of group O were tested with serum 299; positive reactions were somewhat more frequent than with sera 740 and 1172 (group A). Serum 972 showed reactions of the intensity + on a small number of bloods (4 per cent), and more frequently (24 per cent) weaker reactions; it was tested on 54 bloods of group O. The fourth serum, no. 34, agglutinated numerous bloods, occasionally with an intensity +. This serum acted also on its own blood cells (tr. to ±).

Apparently, as is seen from table 7a and 7b, the specificity of sera 76 and 299 resembles that of 740, group A (and sera 1135 and 1172).

TABLE 8B

SERUM NUMBER	GROUP	CORPUSCLES OF GROUP O											
		33	34	46	250	299	538	901	915	916	938	974	1021
299	O	0	+	f.tr.	+	0	0	0	f.tr.	0	0	+	+
1172	A	0	+	f.tr.	+	tr.	tr.	tr.	f.tr.	0	0	+	±
1048	B	f.tr.	+	tr.	+	0	0	0	0	0	0	+	±

TABLE 9

SERUM NUMBER	GROUP	CORPUSCLES OF GROUP O											
		201	209	214	231	232	235	248	252	253	266	270	279
214	O	+	0	0	±	0	+	0	0	f.tr.	0	±	+
289	O	+	0	0	±	0	+	0	0	0	0	±	+
226	A	+	0	0	f.tr.	f.tr.	±	0	0	0	0	tr.	+
577	A	+	0	0	+	0	+	0	0	tr.	0	±	+±
616	B	±	0	0	tr.	0	±	0	0	0	0	tr.	+

The action of these sera on cells of other groups, after removal of the group isoagglutinins, has not been examined thoroughly.

Group B. Sera 1048, 1940, and 2038 reacted with bloods of groups O and B. The reactions of serum 1048 on bloods of group O ran somewhat parallel to those of sera 299 (group O), 740, 1172 (group A) and others of similar action (tables 8a and 8b). Also there was some agreement with these sera in the reactions of sera 1940 and 2038.

Serum 1438 showed agglutination of the intensity + on a small number of bloods and many reactions of weaker intensity. Sera 1450 and 1979 agglutinated more frequently and more intensely

bloods of group O than bloods B, in accordance with the phenomenon described above (see p. 35); the reactions of the two sera on bloods B did not agree.

With tests made at 12°C. one serum B, no. 1532, was found which agglutinated almost specifically all of the 85 bloods of group O tested; it gave only traces of reactions with some bloods of group B. Accordingly an effect with human serum can be obtained similar to that described by Schiff (30) with absorbed beef sera.

Serum 1048 was tested on 39 bloods of group O and 24 bloods B, and gave reactions of the intensity ± or more on 36 per cent of the specimens; with serum 1940, 37 bloods O and 25 bloods B were tested and it agglutinated about 40 per cent of the bloods; 70 bloods O and 65 bloods B were examined with serum 2038 and positive reactions occurred in 30 per cent of the specimens.

Serum 1438 was tested with 54 bloods O and the same number of bloods B; it reacted on about 20 per cent of the specimens. Seventy-six bloods O and 87 bloods B were examined with serum 1450; it reacted with 80 per cent of bloods O and 20 per cent of bloods B. With serum 1979 there were tested 31 bloods O and 33 bloods B and it agglutinated all bloods O, and about 30 per cent of bloods B.

The stronger positive reactions of serum 1438 and some other sera, e.g., serum 740 group A, and 1048 group B, occurred more frequently in the blood of negroes (cf. (7)).

Several sera (nos. 214 and 289 of group O, 226 and 577 of group A, 616 of group B) were found to give agglutinin reactions of a special sort.[6] On microscopic examination of positive reactions the field frequently showed a few rather large clumps but mostly free cells. Sometimes the clumps seemed to contain, beside the agglutinated red cells, particles the nature of which was not determined.

When tests made at 20°C. were warmed to 37° the clumps were, as a rule, not broken up and also, when tested directly at 37°, reactions occurred of similar strength as at 20° or weaker.

On account of the peculiar character of the reaction it was thought that the phenomenon may be distinct from real agglutination. Opposed to such a view, however, are the facts that the sera were better absorbable by sensitive than by non-sensitive cells and that sometimes the appearance of the reaction was not different from common agglutination.

Distinctly positive reactions occurred in about 16 per cent of the bloods. When

[6] Three of the sera were detected in some experiments which preceded the present investigation. The sera of this sort (with stronger activity) are not included in the percentage figures given above (see tables 1, 2 and 3).

several sera were tested with a number of cells the reactions were, on the whole, in agreement. In no case did the sera act on their own blood.[7] (See table 9.)

TABLE 10A

Variation of the Reactions at Different Temperatures

SERUM NUMBER	GROUP	TESTED ON BLOOD NUMBER	GROUP	20°	25°	30°	37°
625	A	240	A	+	+	tr.	0
		1172	A	+±	+	±	0
740	A	271	A	+	+	tr.	0
		1102	A	+	+	±	0
795	B	509	O	+	±	0	0
		901	O	±	0	0	0
1438	B	224	B	+	±	0	0
		1505	B	+	±	0	0
1450	B	33	O	±	±	±	±
		901	O	±	f.tr.	0	0
535	AB	1172	A	+	+	±	0
		1176	A	+	+	tr.	0
850	AB	540	O	++	+±	+	0
		901	O	++±	+±	+±	0
Menz.	B	248	O	+	+	+	±
		370	O	+	+	±	0

TABLE 10B

SERUM NUMBER	GROUP	TESTED ON BLOOD NUMBER	GROUP	10°	15°	20°	25°	30°	37°
87	A	370	O	+	±	f.tr.	0		
		938	O	+	±	tr.	0		
1125	AB	370	O	+	+	tr.	0		
		938	O	+	+	±	0		
740	A	370	O	++	+±	+	+	±	0
1438	B	370	O	++	+	±	±	0	

Influence of temperature variations; absorption experiments. The action of the sera described was considerably influenced by a change

[7] Blood 226 was acted upon by serum 577.

in temperature. Tests were made at 20°, 25°, 30° and 37°C. At 37° with some exceptions (sera 1450, Menz., (table 10a) serum 1176

TABLE 11

The sera were absorbed with one-half their volume of packed, washed, red blood cells. The first two absorptions with the negative reacting blood and the absorption with the positive reacting blood were carried out at 20°C.; the tubes were centrifuged for a short time at high speed in water of about 15°C., so that at the end of the centrifuging the temperature was not higher than 22°C. or 23°C. The third absorption was made at 5°C. In each case the tests were set up at 20°C.

SERUM NUMBER	ABSORBED WITH BLOOD NUMBER		TESTED WITH BLOOD OF GROUP O					
			99	605	834	250	257	334
740 Group A		Unabsorbed	0	0	0	+	+	+±
	834	First absorption, 20°C.	0	0	0	+	+	+
	834	Second absorption, 20°C.	0	0	0	+	+	+
	834	Third absorption, 5°C.	0	0	0	+	+	+
	257	First absorption, 20°C.	0	0	0	0	0	0
			TESTED WITH BLOOD OF GROUP A					
			Subgroup A_2			Subgroup A_1		
			259	577	1602	10	226	282
1219 Group A		Unabsorbed	0	0	0	+	+±	+
	259	First absorption, 20°C.	0	0	0	+	+±	+
	259	Second absorption, 20°C.	0	0	0	+	+±	+
	259	Third absorption, 5°C.	0	0	0	tr.	+	tr.
	282	First absorption, 20°C.	0	0	0	f.tr.	±	f.tr.
			TESTED WITH BLOOD OF GROUP A					
			Subgroup A_2			Subgroup A_1		
			263	271	1219	203	245	1155
625 Group A		Unabsorbed	0	0	0	+±	+	+±
	271	First absorption, 20°C.	0	0	0	+	±	+
	271	Second absorption, 20°C.	0	0	0	±	tr.	tr.
	271	Third absorption, 5°C.	0	0	0	0	0	0
	1155	First absorption, 20°C.	0	0	0	0	0	0

and the sera mentioned on p. 43) the agglutination did not occur or it disappeared when cells agglutinated at 20°C. were warmed to 37°C. For several sera the upper limit of activity was at 30°, for others at 25° or even 20°C. (table 10a).

In table 10b are given reactions of two sera which show very weak reactions at 20° but distinct and selective action at lower temperatures.

Absorption experiments (table 11) were performed with almost all the strongest reacting sera. In general the results gave evidence for specific absorption when the sera were treated with positively and negatively reacting cells. As in former similar experiments (8, 32) the specificity of the absorptions was not absolute. More particularly there were sera whose agglutinins were not absorbed by non-sensitive cells even when treated repeatedly at 20°, or once at 5°C. (e.g., sera 740, 299, 1048). Other sera, e.g., 1219, group A, were absorbed very little by non-sensitive cells at room temperature but distinctly at 5°C., while sensitive cells absorbed at room temperature. The absorption effect brought about by negatively reacting erythrocytes was still more pronounced in certain sera (e.g., 625) in that such cells removed agglutinins also at room temperature although not so well as sensitive cells.

With serum 850 a specific absorption could not be carried out. The agglutinins of this serum were in part or entirely removed both by sensitive and non-sensitive cells at 20°C. However this serum resembled the other sera, both in regard to specificity of agglutination and its sensitivity to temperature. Hence one would believe that the failure of specific absorption does not suffice to prove that the agglutinins in this serum are altogether different from the other irregular agglutinins. It would seem, rather, that the absorption effect merely represents to a higher degree the phenomenon which at low temperature takes place also with other sera.

The cases described were grouped in both ways, first by examining the cells with sera of all groups and secondly by testing the sera on several bloods of all groups. In all cases the blood cells behaved typically and so did the sera, excluding of course the abnormal reactions already discussed.

The above description is based upon repeated tests, including samples from several bleedings. A number of cases were under observation for a year. The results, on the whole, were consistent, i.e., bloods with distinctly positive reactions or those reacting negatively at one examination showed the same properties when tested again after an interval even of several months. In some instances

there were variations, considerable at times, in the intensity of the reactions, so that occasionally weak agglutinations were not reproducible on reexamination (cf. Jones and Glynn).

The stronger sera were found to be active when kept in the ice box for months. With sera of weak activity deterioration is noticeable after a few days. Several sera tested were still active after heating for thirty minutes at 55°C.

Thomsen (18) believes that the activity of the cold agglutinins is more pronounced in sera which exhibit also pseudo-agglutination, and may vary depending upon this circumstance.

DISCUSSION

Among the abnormal sera there are some which display a certain regularity in their reactions. In this class belong sera of group AB no. 535, and 625 and 1219 of group A. These are similar to the sera Barnett and Sn. formerly described (8) and are characterized by their action on cells of subgroup A^1. The cells of bloods 535 were of the type AA^2B; those of blood 625 and 1219 were of the type AA^2. It would seem that the cases of Lauer also belong to this type, though he does not accept the notion of the subgroups. For he states that his AB sera acted on those cells of group A which were more sensitive to isoagglutinin α.

Another type is represented by sera 850 and 995 of group AB, 1155 of group A, and some others of weaker activity. These sera agglutinated most intensely the cells of group O, to a lesser degree most cells AA^2 and, as a rule, not AA^1. A case, perhaps of this type, has already been reported by Wiemer although the author did not observe the distinction in group A.

These results are in keeping with our earlier findings on cold agglutinins (24) and furnish definite evidence for the view that the subgroups of group A (and AB) as described by v. Dungern and Hirschfeld and others (33, 15, 34, etc.) are qualitatively different (8), since the so-called less sensitive cells are acted upon more intensely by certain human sera than the "more sensitive cells." It must be admitted that the separation of subgroup A^1 and A^2 is certainly not as sharp as that between the isoagglutinogens A and B, since some cells are encountered which are reacted upon both by sera containing agglutinin

α^1 and α^2. Still the regularity in action of the above mentioned sera is unmistakable in contrast to some other varieties found by means of abnormal human sera that, at present, cannot be classified.

It has already been pointed out (8) that the subgroups discussed may be explained by the assumption either of special agglutinogens A^1 and A^2 in addition to A or, which is perhaps more likely, the existence of qualitatively different agglutinogens in each of the two subgroups.

It is noteworthy that the two AB sera and the one A serum acting on A^2 also agglutinated (and to a higher degree) the cells of group O. A similar phenomenon had been found in our former experiments (24) made at low temperature (5°C.) and in the present studies one serum of group B is described which acted almost specifically on bloods of group O. The facts demonstrate that, aside from the absence of the agglutinogens A and B, the cells of group O possess also positive qualities (cf. Schiff (30)).

A certain regularity was noticed with at least 6 sera (2 of group O and 3 of group A, 1 of group B). The property indicated by these sera, like the other reactions described (aside from the agglutinins acting specifically on O, AA^1 and AA^2) is apparently not limited to any group. The intensity of the agglutination of different bloods varied by degrees as in the case of the other irregular sera. Also the reactions of the 6 sera in question, when tested on numerous bloods, did not agree entirely. Consequently the agglutinable factor indicated by these sera is not a constant but a somewhat changeable quality. Instances of this sort are not uncommon. Taking into consideration only the individual differences in human blood, we may cite the agglutinogens N and P demonstrable by immune sera both of which are fluctuating, the latter more than the former (32).

The most active sera with one exception (see p. 40) did not agglutinate their own cells (at 20°C.).

In a total of about 500 sera examined there were at least 16 that gave reactions designated as +, i.e., about 3 per cent. Of 180 sera (tables 1, 2, 3) 12 (6.6 per cent) reacted weakly and 31 sera (about 17 per cent) showed traces of agglutination within the same group. This high frequency may seem unexpected but it must be recalled

that the tests were not made under conditions which obtain in the usual grouping of blood. In order to make the tests very sensitive dilute blood (2.5 per cent) was used. The readings were made microscopically at the end of two hours and the temperature was kept constant at 20°C.; also each serum was tested on 6 to 12 bloods.

There are also other workers who reported a considerable frequency of irregular sera. In tests on a relatively small number (40) of normal individuals Jones and Glynn found 6, and probably more sera which gave atypical, mostly weak agglutination. Thomsen (18) found 32 sera giving irregular reactions at room temperature, in a material of 3500 individuals. He states that more such sera would have been detected had they been tested on a greater number of blood specimens.

A survey of our results yields some information as to the rôle played in the abnormal reactions by the sera on the one hand and the erythrocytes on the other. Given a serum with sufficiently active irregular agglutinins, it will in most cases react, not on exceptional but on numerous bloods though with varying intensity. A converse statement does not hold; that is, if a certain specimen of blood cells displaying irregular agglutination be tested with numerous sera only a few will be found to give distinct abnormal agglutination.[8] Accordingly one may speak of abnormally reacting sera but hardly of abnormal blood cells;[9] otherwise practically every blood cell ought to be considered as irregular. It is obvious from the results reported that there is by no means a regular relation between the absence of agglutinable properties and the presence of corresponding agglutinins.

On examining two sera chosen at random (one of group A and one of group B) on more than 125 bloods each of the same group or group O, no abnormal agglutination was found. The same negative result was obtained on testing numerous sera with 24 bloods each (see Tables 2 and 3).

From this and the results of the two kinds of experiments reported in this study, we believe that for detecting abnormal sera it is more convenient not to make complete cross tests but to examine many sera with few bloods including, if possible, such as give distinct, irregular reactions.

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[8] Faint reactions due to subgroups or the frequent faint reactions of many sera on bloods of group O are here left out of consideration.

[9] This is not contradicted by the fact that some cells are more sensitive than others to certain agglutinins.

It may also be advisable to include tests at a temperature of 12° to 15°C. in order to detect irregular agglutinins particularly those differentiating the subgroups.

It must be stated that the present observations cannot be explained by pseudoagglutination. The dilution of the serum in the tests was such that pseudoagglutination would rarely occur. Besides, the phenomenon is ruled out by the influence of temperature on the reactions, the selectivity and the absorption experiments.

Since our material was taken from patients in a hospital for mental diseases, most of them cases of dementia praecox, one must consider the possibility that the incidence of the reactions described may be attributable to pathological conditions. However, the distribution of the various diseases among the cases with irregular sera was about the same as that in the patients of the hospital in general (cf. Jones and Glynn). Pathological conditions other than psychiatric were not included in our study and it is quite possible that in such cases different results would be obtained (cf. Kramar and Reiner (31)).

To decide whether or not the agglutinable properties demonstrable by the various atypical sera are constitutional, will require studies on heredity. The first alternative would seem more likely from previous studies (35, 36) and from some results pointing to a racial difference in the distribution of one of the agglutinable properties here described.

The evidence already presented for the existence of many serological varieties of human blood (24, 32, cf. 16) is corroborated by the present studies.

As an illustration an experiment may be cited (table 12), made on the bloods, previously tested with immune agglutinins and atypical human agglutinins, of the nine individuals working in our laboratory. Five of these belonged to group O and four to group A (two A^1, two A^2). The specimens were labelled by numbers only. Using the same reagents as before it was not difficult to establish to which individual each of the specimens belonged.

Comparing the properties of the irregular reactions and typical isoagglutination and considering only the intensity of the reactions and the influence of variations in temperature, an absolute distinction between the two sorts of reactions cannot be made, although, in general, the differences are striking.

The intensity and the titer of the strongest irregular reactions (at

20°) is considerably weaker than that of ordinary isoagglutination so that in the great majority of cases the appearance of the reactions alone almost always permits of recognition. In exceptional instances the titer of an irregular reaction may be as high as 1:40 (serum 1219, group A) and on the other hand we found an isoagglutinin β (serum 52, group A) which had a titer of not more than 1:4.

As to the effect of changes in temperature again the separation is not perfectly sharp. (For instance, the reactions on bloods B of the isoagglutinin β just mentioned were very weak at 37° and disappeared entirely with several bloods.) But it is possible and seems advisable

TABLE 12

	BLOOD								
	v.d. Sch.	Ldst.	Henry	Mock	Ph.L.	Hdy.	Bl.	Kr.	A.W.
Group	O	O	A	O	A	A	A	O	O
Reaction for M†	+±	0	++±	++±	0	++±	++±	0	+±
Reaction for N†	+	++	0	+	++±	+	0	++±	+±
Serum 1219 AA^2 (Agglutinin α')	0	0	+	0	0	+	0	0	0
Serum A, no. 740	+	tr.	0	±	+	+	tr.	+±	0
Serum B, no. 2038	±	0	*	0	*	*	*	+	0
Serum B, Menz	0	+	*	0	*	*	*	0	±

* Isoagglutination.

† See (32).

to classify the reactions in a general way. The two extremes are represented by isoagglutination which is little influenced by an increase from 20° to 37°C. (cf. (37)) and typical cold agglutination, i.e., reactions taking place only at low temperatures (Bialosuknia and Hirszfeld (23a), Landsteiner and Levine (24)). The latter are not encountered in the usual blood grouping performed at room temperature or are so faint as not to attract attention.

Of significance for the practical work are the reactions of an intermediate type which are distinct under ordinary conditions, i.e., at room temperature of about 20°C. Only in a minority of the cases do they persist at 37°C.

While the properties discussed do not suffice in every case to separate the irregular reactions from the typical isoagglutination yet the general characteristics of the latter, namely, the almost perfect regularity of the blood group scheme, the sharp difference between the strongly positive and entirely negative reactions and particularly the reciprocal relation of agglutinogens and agglutinins put the two phenomena on different levels. Indeed, the existence of reactions other than typical isoagglutination does not at all interfere with the scheme of the four groups, so that with the abnormal sera here reported, there was no difficulty whatever in determining the group of the blood cells and even of the sera, provided they were tested not with single but with several blood specimens of each group. For instance, an abnormal serum of group A would react moderately or weakly on some but not on all blood cells O and A but intensely on all cells B and AB.

Our views are, on the whole, in agreement with the conclusions of Thomsen (18) (cf. Schiff (38), p. 32, Jones and Glynn (19)) who, on the basis of extensive investigations, stresses the essential differences between the group properties and the reactions within the groups or on bloods O. He especially excludes the existence of another agglutinogen allelomorphic and analogous to A and AB. In Thomsen's opinion a classificationof the irregular agglutination phenomena would not seem possible.

With regard to the practical aspect of the experiments reported, tests were made to determine whether the reactions are manifest with the technique commonly employed for blood grouping. The method selected for this purpose consisted in mixing on glass slides one drop each of serum with both 5 and 33 per cent washed blood suspensions. The tests showed that, with the most active of the irregular sera, the reactions were noticeable also under these conditions.

The significance of the abnormal reactions for transfusion has been discussed repeatedly and several authors, e.g., Unger, Guthrie and his co-workers are inclined to ascribe the symptoms which occasionally occur after transfusion with blood of the same group to such lesser degrees of incompatibility. In our opinion this assumption is doubtful at least if the agglutination is not very strong since hemolysis does not seem to occur in such cases (cf. Oehlecker (39)) and because in most instances agglutination does not take place or is very weak at

body temperature (18, 40, cf. 41). Actually we observed (together with Dr. Janes) uneventful transfusions in five patients whose sera agglutinated distinctly the cells of the respective donors, some of the sera acting even at 37°C. (see (26)). It is more probable that harmful effects would result from repeated transfusions which may perhaps stimulate the formation of stronger agglutinins. A final decision on these questions must be postponed until more complete information has been gathered.[10]

SUMMARY

A study was undertaken on the occurrence of atypical isoagglutinin reactions of human blood. Such a technic was purposely chosen as would detect sera of even slight activity. In this way rather numerous irregular reactions were found in a material obtained from patients with mental diseases.

Most of the reactions observed may be put under the heading of cold agglutination, that is, the sera are active only at low temperature and give very weak agglutination at room temperature (20°). A smaller number of sera (about 3 per cent) contained agglutinins of a type intermediate between cold agglutinins and typical isoagglutinins since their reactions were distinctly noticeable also under the usual conditions of blood grouping. In all of the cases with atypically reacting sera the blood cells showed no abnormal behavior *and could readily be grouped.* Also the grouping of the sera was not interfered with by the presence of the irregular agglutinins. Hence the irregularities are superimposed upon the much more prominent group properties, and are not in conflict with the scheme of the four blood groups, the usefulness of which cannot be questioned (cf. Thomsen (18)).

With regard to the practice of transfusions, it may be stated that in several instances in which the serum of the recipient contained irregular agglutinins for the blood of the donor no untoward symptoms at all were observed.

[10] The case of Ottenberg and Johnson is peculiar in that death followed transfusion with a blood of the donor containing agglutinins for the cells of a recipient of the same group. Hence this instance is comparable to a transfusion with a universal donor.

Several sera of groups O, A, and B, described, had agglutinins which were similar with regard to their specificity.

In groups A and AB two types of agglutinins were found specific in general for cells of one or the other subgroup of group A; those agglutinating blood AA^2 showed in addition a specific action on cells of group O (cf. Schiff (30)) The properties of these sera demonstrate again quite definitely the existence of subgroups in group A, a distinction hardly significant at present for practical purposes.

The results with the abnormal agglutinins of human sera give further support to the conclusion that there exists a great number of individual differences of human blood.

As on previous occasions, the authors are again indebted to the following persons for their coöperation: Dr. C. Floyd Haviland, Superintendent, Drs. I. J. Furman and John R. Knapp, First Assistant Physicians, and Miss Frances W. Witte, Superintendent of Nurses, of the Manhattan State Hospital, New York City.

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[Reprinted from THE JOURNAL OF EXPERIMENTAL MEDICINE, May 1, 1929,
Vol. 49, No. 5, pp. 745–758]

THE PRODUCTION OF PARTIAL LIVER INSUFFICIENCY IN RABBITS

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(Received for publication, January 29, 1929)

Reasons enough for the existence of the liver are known. A host of functions have been ascribed to the organ already. Nevertheless the clinical condition of liver insufficiency is still a riddle. For the better understanding of it one must distinguish between functions which are essentially hepatic and those which can be carried out somewhere else in the body when the liver fails.

The recent work on dogs by Mann (1–9) and his colleagues has greatly increased our knowledge of liver functions. But much remains to be learned, and the method of these authors is laborious. For this reason we venture to submit a simple procedure for inducing extreme liver insufficiency in rabbits.

To approach the problem of liver insufficiency in animals three general types of procedure or their combination are available; total extirpation, partial ablation, and derangement of the organ by tying the bile duct or by chemical injury. The method of total extirpation, though it has served most excellently (1–9), still leaves much to be desired. Death follows the operation so rapidly that scant time remains for study of its onset. The use of drugs to bring about liver derangement is questionable for one must assume that the substance employed acts only on the liver parenchyma and not elsewhere as well. The evidence for and against these methods has been reviewed by Marshall and Rowntree (10) and by Mann (9).

The employment of partial ablation has also been open to objection. Investigators are agreed (9, 11, 12) that the liver possesses a "factor of safety" so great that it is well-nigh impossible to remove enough parenchyma to produce insufficiency without fatally obstructing the portal flow. Workers partially ablating the liver in dogs and rats

have found this difficulty insurmountable (9, 11, 13). In the rabbit our efforts have led to success. One can remove slightly more than 90 per cent of the animal's liver without untoward obstruction to the portal blood flow, with result in an extreme degree of hepatic insufficiency. The animals unless treated succumb in 12 to 18 hours, with convulsions and low blood sugar. If given glucose they live for varying periods up to 5 days, eventually dying with symptoms like those exhibited by dogs deprived of the entire liver (2, 9). For a study of the problems of liver insufficiency the method presents many advantages. Not the least of these is the ease of the ablation. It can be accomplished in less than 10 minutes, by a single operator, unassisted.

Anatomical Peculiarities of the Rabbit's Liver

The arrangement of the rabbit liver in well-nigh separate lobes permits the removal of much of the hepatic substance by simple ligations and excision. Three large cephalad lobes, comprising what has been termed the "main liver" (14–16), constitute about 70 per cent of the organ. The remainder, almost separate from these, the "posterior lobe mass" (14, 15), is situated further from the head and to the right of the portal vein. Unlike the lobes of the "main liver" the "lobe mass" is intimately attached to the vena cava and cannot be wholly ablated without injury to this vessel. But a deep cleft exists on the cephalad surface of the mass and here a ligature may be thrown about the lobe and tied in such a way that it lies close to the vena cava but yet not close enough to impede the flow of blood. The half of the lobe beyond this ligature can now be removed, leaving a neat small stump. The small caudate lobe on the other side of the cava possesses a flattened, tail-like portion springing from a narrow base which can be readily severed after ligation. There remains about half the substance of the lobe, that portion which extends along the sheath of the portal vein between it and the spinal column.

In our attempts to induce liver insufficiency in the rabbit we employed three types of sub-total hepatectomy. Ablation of the "main liver" was the first. To this operation there was next added excision in the removable part of the caudate lobe, leaving the stump and the whole "posterior lobe mass" intact. Finally removal of the "main

liver" and half of the "posterior lobe mass" was practiced, leaving only the stump of this latter and the small caudate lobe intact. This last operation alone resulted in true liver insufficiency.

Estimation of the Relative Amounts of Liver Tissue Removed by the Three Types of Sub-Total Hepatectomy in the Rabbit

In twenty-five normal rabbits we have separated the portions of the liver, discussed above, weighing the "main liver," the excisable portion of the "posterior lobe mass," its remaining stump, the removable bit of the caudate lobe, and its stump. Table I shows, in terms of percentage of total liver, the relative amounts of the tissue remaining and removed by each of the three procedures outlined above. Further, the table gives some idea of the relative variability in individual instances. Simple ablation of the "main liver" (see Column I of the table) withdraws on the average about 73.6 per cent of the liver tissue, a figure well in accord with the findings previously published by Rous and Larimore, 72.3 per cent (15), and by Ponfick, 74.7 per cent (16). As will be seen below, this procedure does not induce apparent liver insufficiency. "Main liver" removal plus partial ablation of the caudate lobe offers no great additional advantage over the former method, for the caudate lobe adds but little to the percentage of tissue removed (Column II). Ablation of the "main liver" and half the "posterior lobe mass" is the method of election (see Column III of the table). By this procedure an average of slightly over 90 per cent of the liver tissue is removed, the remaining stump of the "posterior lobe mass" and the intact caudate lobe constituting but 9.6 per cent of the original total.

Method

Rabbits weighing 1500 to 4500 gm., previously kept on a mixed diet, were fasted but allowed water for periods of 24 to 48 hours. They were then operated upon under ether anesthesia. The three large cephalad lobes of the liver, the "main liver," were removed together with that portion of the "posterior lobe mass" which lies to the right of the vena cava.

To accomplish this, the "posterior lobe mass" of the liver was exposed and the ligament attaching its lower medial pole to the sheath of the vena cava severed. A heavy silk ligature was thrown around the lobe, placed in the deep incisura on its cephalad surface, and tied, care being taken to avoid all puckering of the vena cava or inclusion of its sheath in the knot. The liver portion to the right of the

TABLE I

The Relative Amounts of Liver Removed by the Three Methods of Sub-Total Hepatectomy Described in the Text

	No.	Body wt.	Liver percentage of body wt.	I: "Main liver" out, Per cent of total liver		II: Posterior lobe in, Per cent of total liver		III: Caudate lobe in, Per cent of total liver	
				Out	In	Out	In	Out	In
		gm.							
	1	2570	2.32	76.8	23.2	78.6	21.4	93.4	6.6
	2	1960	2.70	72.7	27.3	76.1	23.9	90.8	9.2
	3	2150	2.21	68.1	31.9	72.2	27.8	90.7	9.3
	4	2957	2.41	76.5	23.5	81.2	18.8	90.2	9.8
Freshly fed	5	2250	2.88	81.2	18.8	83.9	16.1	89.4	10.6
	6	2010	2.22	76.2	23.8	81.0	19.0	89.1	10.9
	1a	4275	2.48	73.7	26.3	77.2	22.8	91.9	8.1
	1b	2375	2.36	80.1	19.9	84.2	15.8	91.2	8.8
Average.....		2568	2.45	75.7	24.3	79.3	20.7	90.8	9.2
	1	1980	4.38	73.1	26.9	76.8	23.2	91.4	8.6
	2	2310	4.61	75.3	24.7	80.2	19.8	90.6	9.4
Fasted 24	3	2621	4.74	69.8	30.2	74.9	25.1	90.6	9.4
hrs.	4	2210	3.67	72.0	28.0	77.2	22.8	90.3	9.7
	x	1800	3.99	70.4	29.6	75.2	24.8	88.7	11.3
	y	2100	3.80					90.6	9.4
	z	2050	3.76					90.2	9.8
Average.....		2153	4.14	72.1	27.9	76.9	23.1	90.3	9.7
	1	2250	3.17	75.2	24.8	78.4	21.6	92.4	7.6
	2	1770	2.53	69.8	30.2	73.6	26.4	90.1	9.9
Fasted 48	3	1450	3.12	66.8	33.2	71.8	28.2	89.5	10.5
hrs.	4	1960	3.20	68.1	31.9	73.7	26.3	88.1	11.9
	*	2150	2.25	76.8	23.2	82.2	17.8	88.3	11.7
Average.....		1916	2.85	71.3	28.7	75.9	24.1	89.7	10.3
	1	1509	2.83	75.9	24.1	78.1	21.9	92.1	7.9
	2	1500	2.87	74.2	25.8	77.9	22.1	91.3	8.7
Fasted 5	3	1550	3.02	74.3	25.7	80.3	19.7	90.3	9.7
days	4	1600	2.63	72.6	27.4	75.5	24.5	89.5	10.5
	5	1750	2.75	72.8	27.2	76.6	23.4	89.1	10.9
Average.....		1582	2.82	74.0	26.0	77.7	22.3	90.5	9.5
Average of all.....				73.6	26.4	77.7	22.3	90.4	9.6

* Fasted 3 days.

ligature was then cut away. After severing the ligaments attaching the "main liver" to the diaphragm, a ligature was placed about it. This ligature, tied just ventral to the diaphragm without inclusion of the vena cava, permitted excision of the "main liver."

In some instances interference with the circulation of the remaining liver tissue resulted in necrosis of these parts. Rarely the vena cava was obstructed by the ligatures about the "posterior lobe mass" and very rarely a bile leak or hemorrhage occurred. Occasionally, too, animals developed a postoperative pneumonitis. Such instances have been ruled out in considering the evidences of liver insufficiency induced by this method. They have amounted in all to about 15 per cent.

After excision of nine-tenths of the liver tissue, in the rabbit the remaining portions appeared slightly congested but never turgid and tense as they do after the removal of but three-fourths of the organ in dogs and rats (11, 13). The venous channels became moderately dilated and a fatty infiltration of the parenchyma developed. Furthermore changes indicative of a fatty degeneration were found histologically, this serving to increase further the degree of liver insufficiency obtained by the operative procedure. Autopsy yielded no evidences of severe portal obstruction. There was no pronounced dilatation of the portal tributaries, and no marked congestion of the gastric or intestinal mucous membranes or engorgement of the spleen. Even the remaining portions of liver were not greatly distended with blood. Nevertheless we have asked ourselves, Can death have been due to obstruction to the flow of portal blood occasioned by the operation? To answer this question a series of control experiments were carried out in which an equivalent or greater obstruction of the portal blood flow was induced with but a negligible removal of liver parenchyma.

Control Experiments

In seven rabbits, the portion of the "posterior lobe mass" of the liver lying to the right of the vena cava was removed under ether anesthesia as in the operation for nine-tenths liver ablation. The portal vessels to the "main liver" were then ligated, leaving intact all branches of the bile ducts and the hepatic artery. In this way the portal blood to 90 per cent of the liver was shunted through the caudate lobe and the remaining stump of the "posterior lobe mass." 4 to 8 days later, India ink was injected into the portal vein of the surviving rabbits to determine how great an amount of portal obstruction had actually been obtained. In

five instances the ink flowed only to the 10 per cent of liver which, according to the conditions of the experiment, should have received portal blood. In the two remaining rabbits some ink reached the main liver, showing that the portal blood had some access to it.

These animals in which a diversion of 90 per cent of the portal blood to the liver had been successfully effected had all survived in good condition.

In another group of four rabbits the branch of the portal vein which leads to the "posterior lobe mass" was ligated following ablation of the "main liver." This procedure caused an even greater blocking of the portal blood flow than that above described; yet the animals did not die until about 5 days after the operation. The sole road for the portal blood had been through the small caudate lobe. Marked hypertrophy of this lobe was found with dilatation of its channels and one may infer that obstruction to the portal flow had progressively diminished from the time of the operation. Death was preceded by the typical signs of liver insufficiency, to be outlined below.

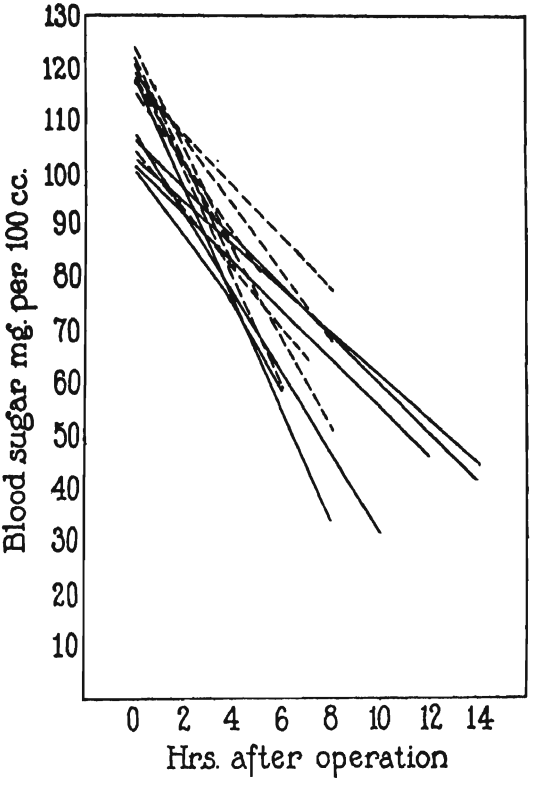
It is evident that death of rabbits when 90 per cent of the liver has been removed by our method cannot be ascribed to the portal obstruction incident to the operation. As will be reported in a succeeding paper, we have found it possible to occlude in the rabbit from 9/10 to 19/20 of the lumen of the portal vein together with 4/5 to 9/10 of the lumen of the vena cava without jeopardizing the animal.

The Evidences of Liver Insufficiency after Ablation of 90 Per Cent of the Rabbit Liver

The Fall in Blood Sugar.—Following removal of nine-tenths of the liver the animals made a rapid recovery from the anesthetic, drank water, and appeared to be in excellent condition. In a few hours, however, they were to be found with drooping head, sprawling legs, and obvious muscular weakness. The heart rate was rapid, rising to 285 beats per minute from a preoperative average of about 140. In 6 to 12 hours the animals became prostrate and convulsions supervened, in one of which death occurred about 8 to 18 hours after operation. Mann and Magath have described (2, 3) a rapid fall in the blood sugar concentration of dogs deprived of the entire liver, with result in convulsions and death a few hours after the operation. So too in our rabbits deprived of 90 per cent of the organ the blood sugar fell rapidly to a level below the minimum compatible with life.

In numerous instances we have followed the blood sugar concentration in rabbits before and after removal of nine-tenths of the liver.

employing for the purpose the Hagedorn-Jensen method (18, 19). Text-fig. 1 shows the rapid decrease of blood sugar resulting from the liver loss, in fourteen of these animals. In the instances depicted by the solid lines, the blood sugar concentration fell to a level incompat-



TEXT-FIG. 1. *Fall in the Blood Sugar Concentration in Rabbits Following Ablation of 90 Per Cent of the Liver.*

The blood sugar findings in fourteen rabbits deprived of 90 per cent of the liver are plotted in terms of milligrams of glucose per 100 cc. of blood. In all the animals a rapid fall in the amount of blood sugar occurred. In the instances depicted by continuous lines, typical hypoglycemic symptoms developed and the animals died. The dotted lines show that a similar fall in blood sugar concentration took place in eight animals, until they were given glucose at the points where the lines are discontinued.

ible with life; convulsions and death followed. The dotted lines show an equally rapid initial fall in the blood sugar of other rabbits, which was checked by injections of glucose given at the points indicated by the discontinuance of the lines.

The Insufficiency in Rabbits Given Glucose after Ablation of 90 Per Cent of the Liver

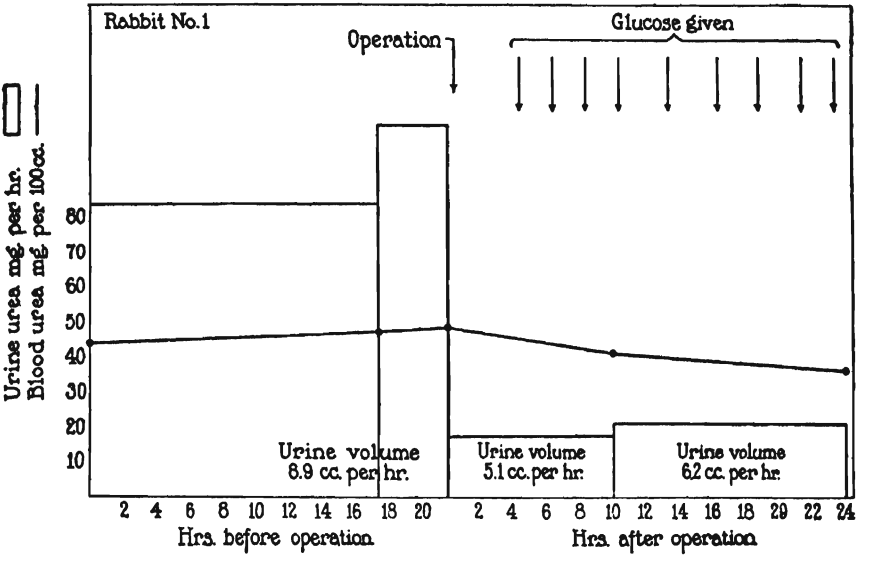
Mann and Magath showed (3) that administrations of glucose to dogs deprived of the total liver preserved life for many hours. Eventually the animals died, in spite of the maintenance of a high blood sugar level, in a condition characterized by weakness and flaccid paralysis. The same phenomenon more prolonged was found in our rabbits given glucose after ablations of about 90 per cent of the liver. The substance was usually administered by stomach tube, but often by intravenous injection, and at times subcutaneously, in varying doses and at various intervals of time.

The effect of glucose, especially by intravenous injection, in rabbits deprived of 90 per cent of the liver is as startling as in completely hepatectomized dogs (3, 9). Prostrated, moribund animals will be found sitting up or moving about freely a few minutes after a single injection of 10 to 20 cc. of 5 or 10 per cent dextrose solution. The pulse and respiratory rates are slowed and the animal appears normal again. The effect, however, is not long lived. 30 minutes to several hours later the rabbit again becomes weak, the pulse and the respiration rate are increased, the blood sugar is low, reflexes are exaggerated, and convulsions may occur. Again and again the animal may be restored by administrations of glucose but finally, in spite of these, death comes on, heralded by extreme asthenia, coma, and respiratory failure.

Derangements in Urea and Uric Acid Metabolism.—It is now well known that urea formation ceases in the liverless dog (20). This phenomenon is accompanied by an accumulation of uric acid in the blood, with a consequent increased excretion of the substance if kidney function is maintained. The change from a normal urea metabolism does not appear in dogs deprived of as much as 70 per cent of the liver after an Eck fistula operation (9, 21), although uric acid destruction may possibly fail to occur. Indications of a lack of urea formation or of cessation of uric acid destruction in dogs may be taken as definite evidence of liver insufficiency (9). Such evidence has been obtained in our partially hepatectomized rabbits.

Procedure.—Freshly fed rabbits were fasted for 24 hours, given 50 to 75 cc. of 5 per cent glucose solution by gavage, and later allowed to drink the same solution

at will. A copious secretion of urine resulted. 12 to 20 hours later the animals were catheterized, the bladder washed thoroughly with water, and a blood sample taken from an ear vein. They were then placed in clean metabolism cages for 17 to 24 hours. At the end of this period another blood specimen was taken, and the catheterization and bladder washing repeated. The urine thus obtained and that collected during the previous 17 to 24 hour period was analyzed for urea and uric acid. At once after taking the second catheter specimen 90 per cent of the liver was removed under ether anesthesia, and for varying periods thereafter

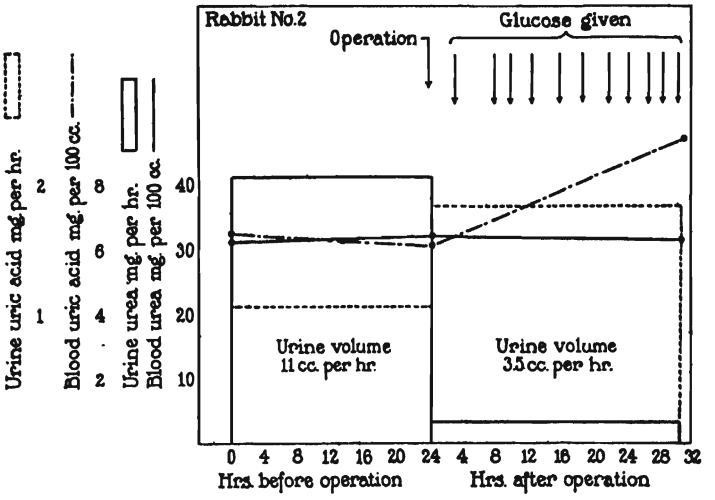


TEXT-FIG. 2. *Changes in Blood Urea Concentration and Urea Output in the Urine after Removal of 90 Per Cent of the Liver.*

Uric acid studies were not made. The changes shown are similar to those described in Text-figs. 3 to 6.

the animals were kept in metabolism cages. From time to time they were given water or 5 per cent glucose solution to maintain diuresis. At intervals they were catheterized and the urine and bladder washings added to the cage urine specimens to be analyzed for uric acid and urea. Blood specimens taken during or immediately before the catheterizations were used for blood urea and blood uric acid analyses. For the determinations of urea concentration in blood and urine, the method described by Addis (22) was employed. In some instances (Text-figs. 2, 4, and 5) the technique of Van Slyke (23) was used as well. Folin's method (24) served for the estimation of uric acid in the blood, and the procedure of Folin and Wu (25) for its determination in urine.

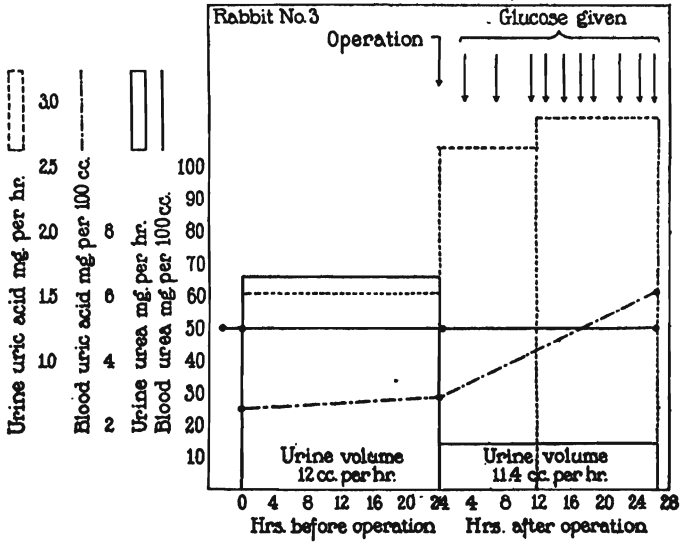
In all five instances depicted in Text-figs. 2 to 6 adequate diuresis was maintained. In all a striking decrease of urea output in the urine



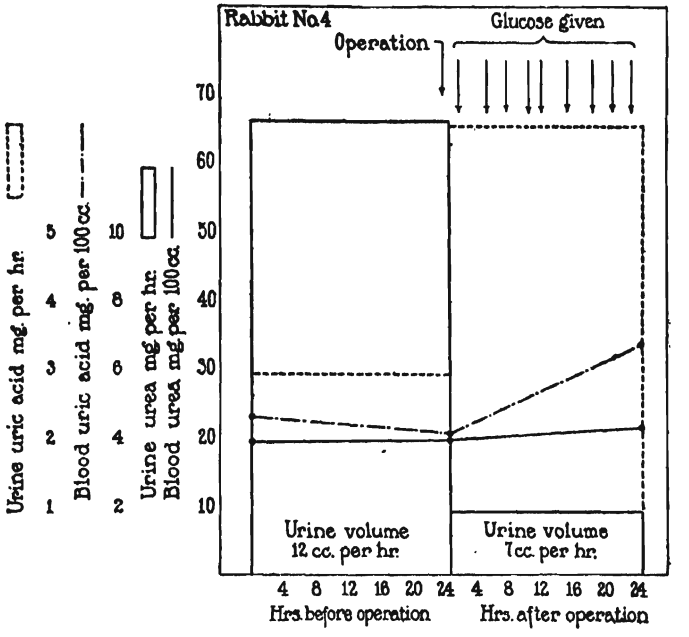
TEXT-FIG. 3

TEXT-FIGS. 3 to 6. *Changes in the Urinary Output and Blood Concentration of Urea and Uric Acid in Rabbits Deprived of 90 Per Cent of the Liver.*

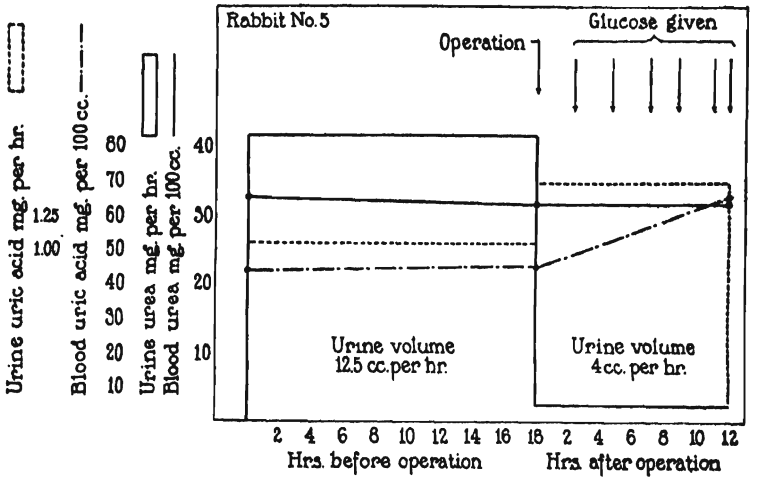
Text-figs 3 to 6 depict the uric acid and urea findings in the blood and urine of rabbits before and after ablation of 90 per cent of the liver. In all, the urea output of the urine decreased profoundly after the ablation while the blood urea concentration remained almost unchanged. In all, an increased urinary output of uric acid appeared, together with a greater concentration of the substance in the blood.



TEXT-FIG. 4



TEXT-FIG. 5



TEXT-FIG. 6

occurred. Despite this lack of urea output the blood urea concentration remained unchanged in three of the animals, fell in one (Text-fig. 2), and rose but slightly in two, affording thereby evidence of greatly decreased urea formation. The uric acid studies made on four of these animals, Text-figs. 3 to 6, showed an increase of this substance in both blood and urine after ablation of 90 per cent of the liver.

In considering the evidences of liver insufficiency in these animals it is to be noted too that a mild tissue icterus appeared, accompanied by an output of bile pigment in the urine, as shown by the Van den Bergh test.

Liver Insufficiency in Rabbits Deprived of 80 Per Cent of the Liver

Similar studies were made on three animals deprived of but 80 per cent of the liver. The findings were not clear-cut. The almost complete absence of urea excretion observed after ablation of 90 per cent of the liver was not present in these instances. In two of the animals the urinary output of urea per hour amounted to about half that of the preoperative period, while in the third animal no decrease was observed. Despite this the blood urea concentration of all rose notably, yielding evidence of a normal formation of urea in the case of the animal last mentioned, and of only a slight decrease in the others. In but one of the three experiments was an increase in urinary uric acid noted and this appeared in the instance showing normal urea formation. From these findings one might suppose that by removal of 80 per cent of the organ a borderline condition had been obtained in which there might or might not be liver insufficiency, in respect to these functions.

The Degree of Liver Insufficiency in Rabbits Following Ablation of 70 Per Cent of the Organ

Prior to the adoption of the method described above, the "main livers" of several rabbits were ablated in the hope that the animals would develop a true liver insufficiency, for the operation entails a loss of 70 per cent of the organ. In 1889 Ponfick (16) reported his results with this procedure, stating that practically all the animals died. Of twenty-one rabbits operated upon in our series, seven survived in

good health for several weeks, when they were autopsied. Two others died of postoperative pneumonia and five others lived about 6 days, succumbing from undetermined causes. In the remaining seven instances, death occurred in about 20 hours accompanied by very low blood sugar concentration and convulsions. Ordinarily one finds after removal of the rabbit's "main liver" a transient fall in the blood sugar which may be sufficient to bring about death. The other evidences of liver insufficiency, the clinical picture of asthenia and prostration with faulty urea and uric acid metabolism, are in our experience lacking.

DISCUSSION

The evidence presented herein clearly shows that a fatal liver insufficiency develops in rabbits deprived of 90 per cent of the organ. Following this great reduction in parenchyma the blood sugar falls to the lethal point. If this be avoided by the administration of dextrose the insufficiency manifests itself in a partial failure of the organ to form urea and to transform uric acid. Eventually the animal dies, of causes unknown.

After ablation of 90 per cent of the liver a mild jaundice appears, showing that there is a deficiency in the function of bile elimination. Earlier work from this laboratory, on the biliary obstruction required to produce jaundice in dogs, is in agreement with this finding. McMaster and Rous showed that jaundice of biliary obstruction does not appear after ligating several of the branches of the hepatic duct unless the drainage from 95 per cent or more of the liver is obstructed (12).

The fact is important that the rabbit deprived of 90 per cent of its liver behaves like the liverless dog in all ways that have thus far been tested. The blood sugar concentration falls, convulsions occur, urea formation ceases, uric acid metabolism is disturbed, and even when the blood sugar is artificially maintained at a high level death occurs, with symptoms similar to those arising in the liverless dog (9).

SUMMARY

A rapid and simple method for the production of marked liver insufficiency in rabbits has been described. The necessary operation can be carried out by an unassisted operator in a few minutes. The method should further the study of liver physiology.

The changes as concerns blood sugar, urea formation, and uric acid metabolism would appear to be the same in the rabbit suffering from hepatic insufficiency as in the dog.

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[Reprinted from THE JOURNAL OF EXPERIMENTAL MEDICINE, May 1, 1929,
Vol. 49, No. 5, pp. 759–764]

TOTAL SURGICAL REMOVAL OF THE LIVER IN RABBITS

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(Received for publication, February 28, 1929)

Total surgical removal of the liver in the rabbit without impairment of the portal and caval circulation has not hitherto been reported. A method has been devised for the purpose of work in this laboratory. It would seem to be desirable to report its technique not only because of the wide use of the rabbit as an experimental animal but because of the frequency with which it has been employed in studies upon the relation of the liver to carbohydrate metabolism (1).

Excision of the liver necessitates occlusion of the portal vein. Moreover the intimate attachment of the organ to the vena cava requires the removal of a segment of this vessel as well, if a complete and satisfactory liver ablation is to be obtained. In the normal rabbit the obstruction of either, or both, of these veins is followed by death so rapidly that in a method for hepatectomy channels must be provided for the immediate return of caval and portal blood to the heart. Markowitz and Soskin (2) have reported a technique for inducing a collateral circulation in dogs without an Eck fistula. At a preliminary operation the portal vein and vena cava are partially occluded with ligatures which constrict the lumina of these vessels to about one-fifth the original size. After some weeks collaterals to the veins are so well developed that the liver may be removed without impairment of the portal circulation. The present method is a modification of this technique.

Our first attempts with the rabbit were unsuccessful. The vena cava and portal vein had been approached directly, and exposed on the right side of the abdomen. The consequent injury to the ventral surface of the liver and the peritoneum of this region resulted in massive adhesions among the injured parts, preventing removal of the liver without great blood loss. It therefore became imperative to devise a method whereby these veins could be approached, and

partially ligated, from the left side of the abdomen, without exposing the liver, leaving the right side free of adhesions that would hinder the later hepatectomy.

Anatomical Peculiarities of the Rabbit's Liver

The liver of the rabbit consists of two relatively separate masses connected by a thin isthmus of parenchyma. The larger mass, comprising the three cephalad lobes, has been termed the "main liver" and the smaller portion, the "posterior lobe mass" (3). Each possesses its own arteries, ducts and branches of the portal vein. The main portal trunk on approaching the liver divides to send its first branch to the "lobe mass." At about this level it also receives a tributary—the superior pancreatico-duodenal vein—and in this region too the hepatic artery comes to lie close to the vessel and accompany it to the liver. Still another tributary, the left gastro-epiploic, enters the portal vein about 1 cm. cephalad to the juncture of the portal and superior pancreatico-duodenal veins. The relationship of these structures is by no means constant. Thus, for example, the superior pancreatico-duodenal vein may enter the portal stream either below or above the great branch of the portal to the "posterior lobe mass" of the liver. As the aim of the preliminary operation is the establishment of a portal obstruction so great that it will induce the development of a collateral circulation, it is necessary to produce this obstruction caudad to the first branch of the portal vein, that to the 'lobe mass." Unfortunately the tributaries mentioned above, the superior pancreatico-duodenal and the left gastro-epiploic, usually enter the portal vein above this site of election and, as our experience has shown, will serve as by-passes, whereby portal blood reaches the "main liver." To cut off this source of supply it is necessary to ligate the two small vessels individually.

The Preliminary Operation

The shaved skin of the rabbit is swabbed with 60 per cent alcohol and under ether anesthesia the abdominal cavity is exposed from the level of the ensiform to the umbilicus by an incision 1 cm. to the left of the mid-line. The relatively bloodless mid-region is thus left intact for the incision of the secondary operation. The stomach is pressed upward, covering the liver, and the portal vein is approached from the left side and sufficiently freed of the surrounding tissue for a

silk ligature, soaked in petrolatum, to be passed about it. The ligature is placed just caudad to the branch to the "posterior lobe mass" and to the junction of the superior pancreatico-duodenal and portal veins. Great care should be exercised to place the ligature around the vein only, leaving intact between it and the liver the peritoneum adherent to its right side, thus preventing all contact between silk and the liver. Only if this is successfully accomplished will adhesions fail to develop.

The ligature is tied down so as to include not only the vein but the end of a glass rod. This end is bent at a right angle to the main shaft and is laid parallel to the vessel for ease in tying. It should taper slightly so that it can be readily withdrawn after the tie has been accomplished leaving the vein partially ligated, with a lumen equal to the size of the rod. The diameter of the latter should be 2 mm. for a 2 kilo animal.

The superior pancreatico-duodenal vein is tied near its junction with the portal, and likewise the small vein which arises on the caudad surface of the pylorus and leads into the portal about 1 cm. above the entrance of the superior pancreatico-duodenal vein. At times this vessel may enter the portal vein just dorsal to the pylorus. Both these procedures, carried out without injury to the peritoneum of the right side of the abdomen, are essential; for as already mentioned these veins, unless occluded, enlarge with extreme rapidity and soon provide an adequate supply of blood to the liver. The ligation of the small left gastro-epiploic vein can be carried out only by a dissection along the posterior wall of the pylorus. The vein receives, as a rule, one or more tributaries. It should be inspected along its entire course and the ligature placed below the last tributary, a delicate procedure which exposes the bile duct and portal vein in this region as well.

Finally partial occlusion of the vena cava is effected from the left side of the abdomen. As a first step the superior mesenteric artery is identified and the peritoneum perforated on the left side of the mesentery just cephalad to this artery and just ventral to the aorta. A small glass spatula covered with vaseline is introduced into the aperture and worked between the vessel and the peritoneum anterior to it. A threaded ligature passer introduced along the track thus made is turned posteriorly about the vena cava, thus carrying a ligature around the vessel, just caudad to the right adrenal body. The lumen of the vena cava is then reduced to 2 mm. by tying the ligature about the removable glass rod as in the case of the portal vein.

Recovery from the operation is usually rapid and a collateral circulation soon develops about the liver. In a few days, if the procedure has been successful, evidences of this fact appear, in a notable enlargement of the veins of the abdominal wall. The development of this collateral circulation is so rapid that one can totally occlude the portal vein and the vena cava 5 days after the primary operation without the appearance of any untoward symptoms. This has been done in 4 instances as a control experiment to demonstrate the existence of an adequate by-pass for the blood about the liver. In our later work, however, we have gener-

ally allowed an interval of 3 weeks to elapse between the primary and secondary operations. This not only affords ample time for the development of the new vascular bed about the liver but suffices for the animal's complete return to general health. Liver extirpations have been done successfully as late as 6 months after the preliminary operation.

Hepatectomy in the Rabbit

The incision for hepatectomy in the rabbit should run in the mid-line from ensiform to umbilicus. A ligature is placed around the entire gastrohepatic omentum, including in the one tie the portal vein, bile duct and hepatic artery. The vena cava is ligated just cephalad to the right adrenal gland, and again just cephalad to its union with the hepatic veins from the "main liver," and severed between. The structures in the gastrohepatic omentum are cut cephalad to the tie placed about them. The liver can now be removed *in toto*, after cutting its ligaments to the diaphragm and dividing the peritoneum on either side of the vena cava in the regions where this is intimately attached to the liver. No stump of hepatic tissue need be left.

A diaphragmatic vein enters the right side of the vena cava slightly caudad to the entrance of the hepatic veins from the "main liver." This small vessel must be tied and cut between ligatures.

In certain instances it may be of advantage to remove the "main liver" early in the operation, immediately after tying the gastrohepatic omentum, thereby gaining working space for the more difficult steps. This is done by snipping the ligamentous attachments between the "main liver" and diaphragm and throwing a stout ligature around the former, tying it down closely upon the vena cava. The "main liver" is then cut away above this ligature. In the final stage of the operation, its stump is removed.

DISCUSSION

The clinical picture in the rabbit, deprived of the entire liver, needs only brief mention for in all important particulars it is like that in the liverless dog, described by Mann (4). As is now well known, hepatectomy in the dog is followed by pronounced hypoglycemia with an accompanying train of typical symptoms. Further, this condition fails to appear when the diminution of blood sugar is prevented by administrations of glucose. The postoperative state in the liverless dog may be divided into two clearly defined stages, the first characterized by the hypoglycemic symptoms, the second, even if glucose be given, by coma terminating in death.

All these phenomena have their counterpart in the rabbit, as our observations upon these animals deprived of 90 per cent of the liver

(5) have already shown. Completely hepatectomized animals treated by the administration of dextrose in 25 per cent solution *per os* or in 5.4 per cent solution intravenously in sufficient amounts to keep the blood sugar concentration at or about 125 mg. per cent behave normally for from 12 to 32 hours. In this first stage no untoward symptoms appear. As a rule only slight variations of the pulse and respiratory rate are noticed, together with an increase in body temperature of 1 to 1.5°. Finally, in spite of the glucose administrations, certain phenomena usher in what may be called the second stage. Now the animal when placed on the floor no longer investigates its surroundings in the ordinary way but hops blindly in a straight line, striking any object in its path, even a brilliantly lighted wall. Later on evidences of muscular weakness and ataxia appear; the animal sits with sprawling legs and drooping head. Left in the cage it will be found with nose pressed in a corner. In the next few hours, although care is taken that the blood sugar level does not fall below normal, the weakness progresses and the movements become more ataxic. The animal falls to one side in attempting to move and cannot rise again, or it lies with sprawling legs unable to raise its head or draw the limbs under the body. The temperature becomes subnormal, the respiratory movements are labored and less frequent, and the pulse is slower too. The corneal reflex and knee jerk previously present are lost and complete flaccid paralysis, save of the respiratory muscles, sets in. For a period Cheyne-Stokes respiration may occur, after which the respiratory rate falls to 8 per minute or even lower, and while the pulse rate remains at about 30 per minute the heart sounds progressively weaken. After varying periods up to 40 hours death comes on, with respiratory failure, the heart continuing to beat for 10 minutes or more after respiration has ceased.

At the present time we can venture no explanation of the later symptoms. It is of importance now merely to call attention to the close similarity of the postoperative events in the liverless rabbit and dog, a similarity which permits us to infer perhaps that the major functions of the organ are much the same though the one creature is herbivorous and the other omnivorous.

SUMMARY

A technique is described for total removal of the liver of the rabbit without circulatory difficulties as a result. The method requires a preliminary operation to induce a development of portal and caval collaterals.

Rabbits deprived of the liver in this manner if given glucose live for varying periods up to 40 hours. Before death they show the same disturbances as do hepatectomized dogs. They die early, of hypoglycemia, unless provided with sugar.

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[Reprinted from THE JOURNAL OF EXPERIMENTAL MEDICINE, May 1, 1929,
Vol. 49, No. 5, pp. 765–778]

THE RELATION OF THE LIVER TO FAT METABOLISM*

I. EFFECT OF LIVER LACK ON FAT COMBUSTION AND THE RESPIRATORY QUOTIENT

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(Received for publication, February 28, 1929)

It is generally agreed that the liver is active in fat metabolism in many ways. As early as 1886 this became evident to Nasse (1). Subsequent systematic studies by Noel Paton (2), Leathes (3, 4), Leathes and Raper (5), Raper (6), Mottram (7), Rosenfeld (8), Bloor (9, 10, 11) and others, have resulted in a mass of evidence, demonstrating various liver activities in the metabolism of fat. But are these functions performed solely by the liver and are they vital to the organism? Can fat combustion be carried on at all in the absence of the organ? These are the chief questions concerning the rôle of the liver in fat metabolism. Their answer waits upon a demonstration of the absence or continued presence of fat combustion in animals deprived of the liver or otherwise brought into a state of extreme hepatic insufficiency. In the present communication we will report experiments upon this theme. Rabbits were chosen for the work and total liver ablations done (12) by a method already described. For the induction of a partial but pronounced liver insufficiency, removal of approximately 90 per cent of the liver was practiced (13).

The Respiratory Quotient of Rabbits with Partial Liver Insufficiency

Earlier workers have shown that the respiratory quotient of fasting animals, previously well fed, is low (14), indicating a body metabolism largely that of fat. What now will the quotient be after removal of the liver, or in conditions of hepatic insufficiency?

* A preliminary report upon some of the findings given herein has already appeared in the *Proceedings of the Society for Experimental Biology and Medicine*, 1927, XXV, 151.

Well nourished rabbits on a mixed diet, and weighing 2 to 3 kilos, were fasted 48 to 72 hours and subjected to a preliminary determination of the respiratory quotient by the method outlined below. The respiratory quotient was low in such fasting animals indicating a pronounced combustion of fat. Then liver insufficiency was induced.

Method

The Respiratory Quotient. The Apparatus.—All respiratory quotient determinations were made by the closed circuit method with the rabbits sealed in an air tight respiration chamber, a very large desiccator, submerged in a constant temperature bath. The chamber was just large enough to allow the animals the normal crouching posture. The closed circuit consisted of the animal respiration chamber, two parallel absorbing systems with shut-off clamps, a mercury pump which afforded a continuous circulation of air, and a set of valves permitting the air to pass through the circuit in one direction only. The total volume of the apparatus was 11 liters. The absorbing systems, for the removal of water vapor and CO_2, were each made up of an ascarite[1] tube connected at both ends with sulfuric acid absorption bottles. The weight of the ascarite tube in one of these systems was carefully determined, and so too was that of the second sulfuric acid bottle which the air entered after passing through the ascarite. The circulating air could be shunted at will through either of these absorption circuits. From a reservoir of known volume, oxygen entered the closed circuit between the absorbers and the pump. At this point too, connections were made with an auxiliary oxygen reservoir. At frequent intervals the apparatus was tested for leaks, by raising the pressure by an amount equal to that of 100 cm. of water in addition to the atmospheric pressure and ascertaining that no volume change occurred in the circuit within 5 minutes.

The Respiratory Quotient Determination.—The respiration chamber containing the experimental animal was submerged in the constant temperature bath and the air within the system circulated at the rate of 3 liters a minute. But one of the absorption systems was employed in the circuit and oxygen was used from the auxiliary oxygen reservoir. After 20 minutes the pump was stopped and the pressure within the system adjusted to that of the room atmosphere. The barometric pressure was noted at the same time. Upon starting the circulation of air again the current was deflected through the second train of absorbers containing the weighed ascarite tube and weighed sulfuric acid adsorption bottles. Oxygen was now allowed to enter the closed system from the measured reservoir until about 1.5 liters had been consumed. This required about 1 hour and 20 minutes with the average 2 to 2.5 kilo rabbit. At the end of this period the pump was

[1] "Ascarite," a mixture of asbestos and sodium hydroxide, distributed by A. H. Thomas Company, Philadelphia.

stopped, the pressure in the closed system adjusted again to that of the room atmosphere, the barometric pressure observed, and the volume of oxygen added to the system during the period was noted. By reweighing the ascarite tube and second sulfuric acid bottle the amount of CO_2 produced was determined. The volume of oxygen added during the period of the experiment, plus or minus the correction for changes in barometric pressure, temperature and vapor pressure, represented the oxygen consumed.

The rabbits remained quiet within the respiration chamber. It was not necessary to prevent all motion on the animal's part for calorimetry studies were not contemplated and only the gas exchange was measured. However the animals sat so tranquilly that we were able, in later work, to give them continuous intravenous injections of glucose while in the chamber, despite the fact that the injecting needle once placed in an ear vein could not be readjusted during the period of the respiratory quotient determination. In over 20 such experiments no animal ever moved sufficiently to dislodge the needle from the vein. The method will be described further on.

Technique.—When it had been established that the respiratory quotient of a fasted rabbit was definitely low, indicating that fat combustion was taking place, blood specimens were taken from an ear vein for sugar, hemoglobin and hematocrit estimations, and immediately thereafter 90 per cent of the liver was ablated under ether. 5 to 7 hours later, when the immediate effects of operation and anesthesia had worn off, a second determination of the respiratory quotient was made. Immediately preceding the animal's entrance into the respiration chamber and at once following its removal therefrom, samples of venous blood were taken by cardiopuncture from the right ventricle for CO_2 and sugar analyses.

As we have shown in a preceding paper the blood sugar concentration falls rapidly after removal of 90 per cent of the liver, reaching the lethal minimum 6 to 10 hours after the operation. As the second respiratory quotient determinations were made in the latter part of this period the blood sugar level was invariably found low, between 60 and 70 mg. per cent. Often the animals collapsed and became moribund while in the respiration chamber the second time. In such instances the experiments were discontinued.

In four instances a third respiratory quotient was determined on the following day, 24 hours or more after the removal of 90 per cent of the liver. These animals, of course, received glucose during the night in amounts just sufficient to keep the blood sugar level slightly above the minimum required to keep them free from convulsions. Blood sugar analyses were made before the final respiratory quotient was taken to rule out instances in which the giving of too much glucose might have brought the blood sugar concentration to normal or above. It seemed conceivable in this event, indeed it has been shown by our later work, that the utilization of glucose by the animal might mask or entirely supplant the combustion of fat. In one instance, Table I, No. 4, the blood sugar concentration was found high (0.099 per cent) on the day following operation and the respiratory quotient determination was duly postponed several hours, until it had fallen to 0.079 per cent.

TABLE I

Respiratory Quotient, Metabolic Rate and Blood Sugar Findings in Rabbits Deprived of 90.6 Per Cent of the Liver*

No.	Preoperative findings			6 to 8 hours after operation			24 hours after operation		
	Respiratory quotient	O_2 consumption	Blood sugar	Respiratory quotient	O_2 consumption	Blood sugar	Respiratory quotient	O_2 consumption	Blood sugar
		mg. per min.	*mg. per cent*		*mg. per min.*	*mg. per cent*		*mg. per min.*	*mg. per cent*
1	0.740	27.5	0.114	0.720	27.8	0.064	—	—	—
2	0.766	20.7	0.119	0.757	18.5	0.069	—	—	—
3	0.783	19.3	0.118	0.720	18.9	0.077	—	—	—
4	0.729	28.0	0.122	—	—	—	0.731	32.6	0.079
5	0.782	33.6	0.128	—	—	—	0.745	31.9	0.056
6	0.768	30.0	0.125	—	—	—	0.735	29.5	0.046
7	0.760	25.5	0.122	0.739	25.0	0.059	0.743	26.1	0.066
8	0.826	29.9	0.128	0.751	27.7	0.085	0.714†	29.6	0.051

* Expressed in terms of O_2 consumption per minute.

† 10 hours after operation.

TABLE II

Blood CO_2 of Rabbits Immediately before and after the Period of Respiratory Quotient Determination

No.	Before entering respiration chamber	After entering respiration chamber	Hours after operation
	vol. per cent	*vol. per cent*	
1	48.1	48.3	6
2	46.8	47.6	6
3	39.6	39.9	6
4	47.5	47.2	24
5	40.1	40.5	24
6	42.7	43.1	26
7	44.4	44.4	5
7	41.6	40.6	24
8	46.0	45.6	6
8	45.8	45.5	10

As an additional routine measure, blood specimens from the ear vein were taken before operation and at variable intervals thereafter for sugar estimation by the Hagedorn-Jensen (15) method. After operation, blood specimens for CO_2 analysis were taken immediately preceding, and at once following the sojourn of

the animals in the respiration chamber. They were obtained by cardiopuncture of the right ventricle, under oil, and in paraffined tubes. The CO_2 estimations were carried out by the method of Van Slyke and Sendroy (16, 17). These latter findings for the instances shown in Table I are presented separately in Table II. As the respiration chamber was small and the animals quiet, differences in the metabolic rate could be roughly determined in terms of oxygen consumption per minute.

Only those instances have been considered in which the metabolic rate remained constant before and after operation (see Table I). All experiments have been ruled out, too, in which any significant change in the concentration of blood CO_2 was found. The results given below must be attributed to the true gas exchange of the animal and not to mere retention or blowing off of CO_2 during the periods of experimentation.

In these experiments, which were of relatively brief duration, no significant hemoglobin or hematocrit changes were observed.

Findings

The preliminary respiratory quotients of 25 fasted rabbits averaged 0.755, with variations between 0.722 and 0.826, indicating a great combustion of fat. In Table I the respiratory quotient, blood sugar and oxygen consumption data are given in 8 of these instances which constitute experiments free from objection, in which neither blood CO_2 changes nor significant variations in the metabolic rate appeared. The magnitude and character of the individual variations are self-evident. When about 90 per cent of the liver of the animals was ablated, the respiratory quotient 6 to 8 hours later averaged 0.737, with individual variations between 0.720 and 0.757. The respiratory quotients of 4 of these rabbits taken again the following day averaged 0.738, with only slight variations,—0.731 to 0.745.

In 4 other successful experiments, not shown in the table, the respiratory quotient and blood sugar findings were similar but the metabolic rate showed some retardation, the animals requiring $1\frac{3}{4}$ to 2 hours, even $2\frac{1}{2}$ hours in one instance, to consume the quantity of oxygen used prior to operation in about $1\frac{1}{4}$ to $1\frac{1}{2}$ hours. These latter instances are merely corroborative and hence need be mentioned no further.

From the findings reported so far we conclude that fat combustion

can be carried on as readily and as rapidly in the rabbit after ablation of 90 per cent of the liver as before, in spite of the fact that the animals suffer from extreme liver insufficiency, eventually dying therefrom (13).

The Respiratory Quotient of Rabbits Deprived of the Entire Liver

With these facts established, it became essential to determine whether fat combustion could continue in the absence of the entire liver. In the experiments just described, there was the possibility that the findings depended upon some activity of the small remnant of the liver. For this reason we repeated the experiments, as described above, but employed rabbits deprived of the entire organ according to the method described in an earlier paper (12).

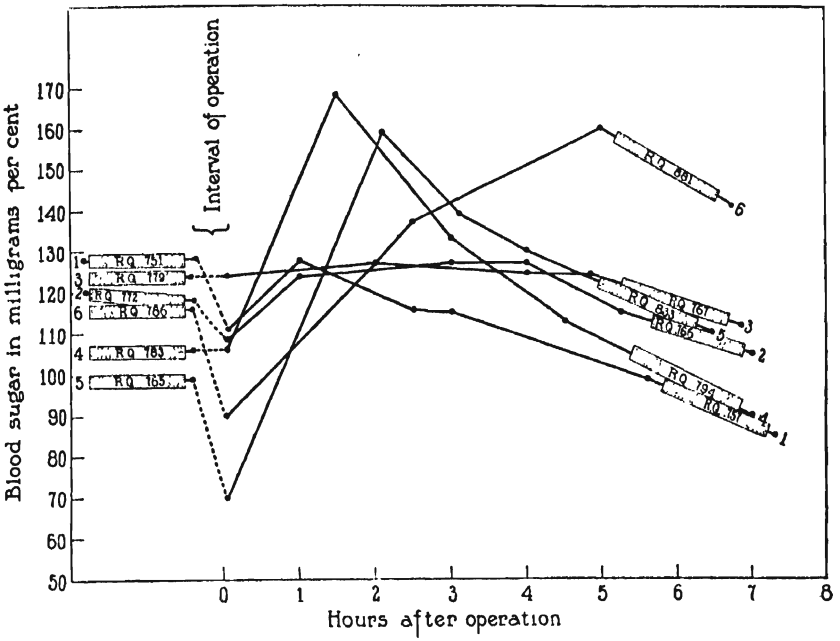
One important change in technique was forced upon us. The rabbits deprived of the entire liver required glucose prior to the postoperative estimation of the respiratory quotient. For the fall in blood sugar concentration took place more rapidly in them than in those animals retaining 10 per cent of the organ. In the liverless rabbit given no glucose death from low blood sugar occurs in some instances within 2 to 3 hours after operation; and it seemed wise to wait at least 4 hours before determinations of the respiratory quotient since both metabolism and respiratory quotient might have been disturbed by the ether anesthesia and laparotomy. Preliminary experiments were made to determine the minimum amount of glucose which would maintain hepatectomized rabbits. These experiments will be detailed in a following communication. Suffice it to say here that 100 to 130 mg. of glucose per kilo of body weight per hour answers the purpose, when given by continuous injection during the first 8 hours after hepatectomy. This small amount of glucose only was given in the later work, since a larger dosage might conceivably have acted to mask an existing fat consumption.

Technique

For the respiratory quotient experiments a technique was used similar to that just described, with certain minor modifications.

Rabbits of about 2 kilos were subjected to subtotal ligations of the portal vein and vena cava to establish a collateral circulation about the liver (12). 3 to 5 weeks later the animals were fasted for 72 hours and given water which they drank freely. The long fasting period was found necessary, for the respiratory quotient of a previously well nourished rabbit, which is fasted, does not fall in less than 3 days. At the end of this time if a preliminary respiratory quotient determination was found to be low, as it usually was, blood specimens were taken for sugar estimations and hepatectomy done under ether anesthesia.

At once after operation blood sugar determinations were again made and a continuous intravenous injection, of isotonic (5.4 per cent) glucose solution, begun. The change in the blood sugar percentage found at this second estimation deter-



TEXT-FIG. 1. *The Blood Sugar Concentration of Rabbits Deprived of the Entire Liver and Given Small Amounts of Glucose during the Interval between the Preoperative and Postoperative Respiratory Quotient Determinations.*

The blocked in areas indicate the period during which respiratory quotient determinations were made and their height above the base line the blood sugar levels. Blood sugar estimations were done after each of the preoperative respiratory quotient determinations and their level plotted accordingly. In each instance a blood sugar estimation was made again immediately after the operation for hepatectomy. The changes in concentration are shown by the dotted lines. At various intervals thereafter the blood sugar concentration was determined in each animal and the variations depicted by the continuous lines. As the blood sugar levels were known both before and after the postoperative respiratory quotient determinations were made, the chart shows the approximate level of blood sugar concentration during those periods. It is obvious that the blood sugar concentration fell during the postoperative respiratory quotient determinations.

mined how much glucose was later given, the amount being that deemed necessary just to maintain the blood sugar concentration above the level of 100 mg. per cent. Experience soon taught us that it was safer to give too much glucose than

too little, for in two experiments the animals entered the state of hypoglycemic collapse while in the respiration chamber before the respiratory quotient determinations could be made. These experiments, of course, were ruled out. Three of the instances plotted in Text-fig. 1 received slightly too much glucose and a distinct rise in the blood sugar level followed. At various intervals blood sugar determinations were made as guides to the rate at which glucose was injected later.

After the operation, during the continuous glucose injection, the animals sat quietly on a warmed pad, with only occasional shifting movements.

About 5 to 6 hours after removal of the liver a second estimation of the respiratory quotient was begun; and in one instance a third was made 24 hours after hepatectomy. Ordinarily the animals remained in the respiratory quotient chamber about 1½ hours. The glucose injection was continued during this period, the fluid entering the chamber through a glass tube connected in turn with fine rubber tubing about 8 inches long, to which the needle was attached. The needle inserted in the ear vein was kept in place by two weak bulldog clips and the rubber tube supported along the animal's back by an adhesive strip to prevent its occlusion by a kink should the rabbit shift its position. In all of the experiments the glucose injection was successfully carried out.

To determine changes in the blood CO_2 and sugar, specimens of venous blood were taken in every instance, immediately before and again after the animal's sojourn in the respiration chamber. To obtain blood samples in these experiments cardiopuncture was not necessary since the animals presented greatly enlarged collateral abdominal veins from which blood was taken, under oil, in paraffined syringes.

As in the previous experiments, we shall consider only the cases in which the metabolic rate remained high after operation and no significant blood CO_2 changes could be demonstrated.

Findings

It was deemed sufficient to carry on experiments with hepatectomized rabbits until 6 instances had been obtained which fulfilled the conditions mentioned above. Table III gives a survey of the findings with the 6 different animals, on one of which (No. 4) a third respiratory quotient was obtained 24 hours after operation. In Text-fig. 1, we have charted the blood sugar curves of these animals in such a way as to show the *probable* blood sugar level during the period of respiratory quotient determinations.

Of the 6 instances, 4 showed practically no change in the respiratory quotient after liver removal, although in 1 of these (No. 4) a late determination was made as long as 24 hours after the operation. In

TABLE III

The Respiratory Quotient and Metabolic Rate in Rabbits Deprived of the Entire Liver*

The respiratory quotients of 6 fasted rabbits before and after removal of the liver are shown, together with the rate of oxygen consumption during each respiratory quotient determination. After hepatectomy all these animals received glucose. In Columns 4 and 9 the "noncarbohydrate" respiratory quotient has been calculated as explained in the text.

No	Preoperative		Postoperative							
	Preliminary respiratory quotient	O_2 consumption	Second respiratory quotient	"Noncarbohydrate" respiratory quotient	Hours after operation	O_2 consumption	Third respiratory quotient	"Noncarbohydrate" respiratory quotient	Hours after operation	O_2 consumption
	A	B	C	D	E	F	G	H	I	J
		mg. per min.				*mg. per min.*				*mg. per min.*
1	0.751	17.22	0.757	0.715	6	16.40	0.862†	—	8†	15.91
2	0.772	20.53	0.766	0.749‡	5½	19.35	—	—	—	—
3	0.779	18.03	0.767	0.709	5½	17.08	—	—	—	—
4	0.783	19.84	0.794	0.729	5½	20.18	0.786	0.715	24	18.84
5	0.765	17.82	0.833§	0.765	5	15.48	—	—	—	—
6	0.786	21.12	0.881§	0.748	5½	16.92	—	—	—	—

* Expressed in terms of O_2 consumption per minute.

† Excess of glucose given as described in text.

‡ Did not receive enough glucose during respiratory quotient determination.

§ Received too much glucose before and during respiratory quotient determination. Blood sugar high.

TABLE IV

Blood CO_2 of Rabbits Deprived of Total Liver Immediately before and after the Period of Respiratory Quotient Determination

No.	Before entering respiration chamber	After entering respiration chamber	Hours after operation
	vol. per cent	*vol. per cent*	
1	43.35	43.14	6
2	39.35	38.98	5½
3	40.18	39.95	5½
4	41.63	41.34	5½
4	44.80	45.40	24
5	37.16	37.58	5
6	45.60	45.42	5½

2 instances an appreciable rise in the respiratory quotient occurred. It may here be remarked again that of necessity all the animals received continuous intravenous glucose injections during the interval between the operation and the completion of the respiratory quotient estimation.

In both of the animals in which a rise in the respiratory quotient occurred, Nos. 5 and 6, a marked fall in blood sugar was noticed immediately after the liver ablation (see Text-fig. 1). To prevent the development of hypoglycemic symptoms, much more glucose was given to these animals than to the others. In Rabbit 6, far too much glucose was given, 200 mg. per kilo per hour, and the blood sugar level stood at 159 mg. per cent at the beginning of the respiratory quotient determination. The other instance showing a rise in the respiratory quotient (No. 5) also received much more glucose per kilo per hour than those in which no rise was found. As Text-fig. 1 shows, the blood sugar concentration in this instance was abnormally low after operation. The animal was consequently given 185 mg. of glucose per kilo per hour instead of the usual 100 to 130 mg. Although the blood sugar concentration in the animal was not high immediately before the period of respiratory quotient estimation, it is to be noted that the animal had received this excess sugar.

The findings show beyond doubt that fat combustion continues actively in the animal without a liver.

The Effect of Increased Sugar Administration on the Respiratory Quotient of Hepatectomized Rabbits

During the respiratory quotient determinations, the period that is to say when the blood sugar level could not be ascertained, the amount of glucose given was arbitrarily cut down to avoid the production of a sugar combustion sufficient to mask any existing one of fat. On the assumption that this glucose, given during the periods of respiratory quotient estimation, was burned we have calculated the hypothetical "non-carbohydrate-burning respiratory quotients," shown in Columns D and H of Table III. These approach closely in some cases the figure 0.71, that of a wholly fat burning. respiratory quotient. As Text-fig. 1 shows, the blood sugar concentration of each animal was lower at the end of the respiratory quotient determination than before,

a finding which would support the assumption that the sugar introduced had been used up.

In calculating the hypothetical "non-carbohydrate-burning respiratory quotient" of Columns D and H in Table III we are for the moment assuming that the administration of glucose raised the respiratory quotient. To test the point one experiment was done.

In the experiment on Rabbit 1 a respiratory quotient of 0.757 was obtained between the 6th and 7th hours after operation. For 6 hours prior to this the animal had been given 100 mg. of glucose per kilo per hour by continuous intravenous injection. The blood sugar level fell from 126 mg. per cent 1 hour after liver removal to 0.098 just before the respiratory quotient determination. During the period of the respiratory quotient estimation the glucose dosage was further decreased to 80 mg. per kilo per hour, causing a further fall in the blood sugar level to 0.086, as Text-fig. 1 shows.

The animal was then given an intravenous injection of 5 cc. 5.4 per cent glucose and the rate of the continuous injection increased to the level of 300 mg. per kilo per hour. 20 minutes later another respiratory quotient determination was begun. This required 1½ hours, during which glucose was given at the rate of 300 mg. per kilo per hour. The quotient rose to 0.862. To avoid confusion with the findings in the other instance the latter part of the experiment has been omitted from Text-fig. 1.

DISCUSSION

It is not to be inferred from our findings that the respiratory quotient must be low under all conditions after removal of the entire liver or a 90 per cent fraction of it. In this paper we wish to emphasize merely that the organism deprived of the liver is still capable of burning fat adequately for its needs. An existing fat combustion may be easily masked, as shown by our experiments in which too much glucose was administered, purposely or inadvertently to the liverless animals. And the respiratory quotient may be high in a variety of circumstances after hepatectomy. Thus, for example, if it is high before operation, it will tend to remain so thereafter. One of our "unsuccessful" experiments serves to illustrate this point.

Ablations of 90 per cent of the liver were carried out on two rabbits which had been fasted but 36 hours. This was done following the preliminary respiratory quotient estimations but before the results had been calculated. At operation the stomachs of the animals were found moderately distended with food. The preliminary respiratory quotients were 0.865 and 0.851, respectively. In both

instances, the respiratory quotient was found high 6 to 8 hours after operation, 0.811 and 0.826, respectively. Autopsy disclosed quantities of undigested food in the stomachs, sufficient reason for the high quotients. In another experiment, a freshly fed rabbit on a carbohydrate diet was subjected to the same procedure. The respiratory quotient which was close to unity (0.951) remained at this figure 6 hours later.

In another rabbit with a preliminary respiratory quotient of 0.731 the second determination was purposely taken 2 hours after operation and found to be 0.840. Guided by this finding we considered it advisable as routine to wait several hours after hepatectomy before attempting the second respiratory quotient estimations.

From the evidence given here one can only say with certainty that fat combustion continues unaffected after removal of the liver of animals already burning fat before operation. The state in which they are burning fat can be brought about by fasting, for about 3 days, animals previously well fed. The freshly fed rabbit on a carbohydrate diet shows as a rule a high respiratorv quotient, and so does one fasted for less than 48 hours. On the other hand the fasting period should not be unduly prolonged. For one would of course expect a respiratory quotient indicative of protein combustion in an animal suffering from severe inanition.

Some experimenters have in the past employed the respiratory quotient as an indicator of the type of metabolism existing in animals after attempted exclusion of the liver from the general circulation by the ligation of vessels. In 1910 Porges (18) and Porges and Solomon (19) found a rise in the respiratory quotient of rabbits and depancreatized dogs following ligation of the abdominal aorta, the inferior vena cava, the portal vein and the hepatic veins. They concluded that the oxidation of fat and protein proceeds within the body only in the presence of the liver. In these experiments the animals lived but a few hours, rapidly becoming moribund. Rolly (20), too, reported changes in the respiratory quotient after interferences with the circulation of the liver but found the n inconstant. Verzar (21, 22) obtained inconstant results after partial exclusion of the liver by anastomosis of the portal vein with the inferior vena cava. Grafe and Fischler (23) reported no change in the respiratory quotient of dogs with Eck fistulas. Fischler and Grafe (24) ligated the hepatic artery in Eck fistula dogs and observed usually a rise in the respiratory quotient within 6 hours of the operation. But it is to be noted that the respiratory quotient returned to the preoperative figure in the animals surviving more than 6 hours. Bohm (25) excluded the abdominal organs in depancreatized dogs and found but little rise in the respiratory quotient. Still later Grafe and Denecke (26) extirpated the liver in dogs several weeks after an Eck fistula had been formed and found the respiratory quotient low—in one instance 0.774 after operation.

Several explanations for these varying results have since come to hand. As is now well known, from the work of Mann (27) and others, an evident liver lack can only be brought about by almost complete removal or destruction of the organ. Rich (28) has recently demonstrated the futility of attempted exclusion of the liver from the circulation through the methods practiced by most of the earlier workers. Murlin, Edelmann and Kramer (29) have pointed out that radical ligations, excluding as they do much blood and tissue, should be carefully controlled. These authors have demonstrated that changes in the relationships of the blood gases follow sudden clamping of the abdominal aorta which are of themselves sufficient to account for the results of the earlier workers without the need to invoke, as explanation, a change in the metabolism of the animals.

Although no radical circulatory interference, such as complicated these various findings, has been brought about in our experiments we have thought best to rule out the possibility of errors due to changes in the blood CO_2 and have done so by considering only instances in which no variations of the sort occurred.

Mann (27, 30) has reported a series of carefully conducted studies upon the respiratory quotient of hepatectomized dogs. In his experiments the respiratory quotient tended to approach unity after removal of the liver. That the quotient in dogs need not necessarily be close to unity after hepatectomy is shown by the work of Markowitz (31).[2] From our findings on rabbits and the figures given in his paper we believe that the respiratory quotient of hepatectomized dogs would be found low 6 hours after the operation if fat combustion were duly established before liver removal and the giving of glucose afterwards reduced to a minimum.

SUMMARY

Fat combustion is carried on adequately in rabbits deprived of the liver or brought into a condition of extreme liver insufficiency. Even 24 hours after hepatectomy fat combustion goes on as well as in the normal animal. Evidently the liver plays no essential part in the breaking down of fat.

[2] Following the completion of this work, F. C. Mann and W. M. Boothby reported experiments in the *American Journal of Physiology*, 1928, lxxxvii, 486, which show an increase in the respiratory quotient of dogs immediately after removal of the liver. A subsequent return of the respiratory quotient 17 hours later to a figure but slightly above the preoperative one was also observed.

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[Reprinted from THE JOURNAL OF EXPERIMENTAL MEDICINE, May 1, 1929,
Vol. 49, No. 5, pp. 859–882]

BIOMETRY OF CALCIUM, INORGANIC PHOSPHORUS, CHOLESTEROL, AND LIPOID PHOSPHORUS IN THE BLOOD OF RABBITS

III. INFLUENCE OF VARIOUS TYPES OF LIGHT ENVIRONMENT

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(Received for publication, January 24, 1929)

In previous papers (1, 2) the calcium, inorganic phosphorus, cholesterol, and lipoid phosphorus found in the blood of rabbits as they were received from the dealer were compared with results obtained for animals living under laboratory conditions. The difference in mean values, in the variations that occurred, in the trends of the curves, and in the chemical relationship seemed to warrant the conclusion that all of these substances were affected by light. Since it had been found that calcium and inorganic phosphorus are affected by various types of light environment (3), it seemed desirable to extend the observations which had been made for the purpose of determining whether cholesterol and lipoid phosphorus were also affected by light and to study the action of a wider range of light conditions. The comparisons were made with complete exclusion of light, continuous exposure to light supplied by Cooper Hewitt lamps, exposure to diffuse filtered sunlight of variable intensity, and exclusion of light interrupted by brief periods of exposure to ultra-violet light. The results of these experiments will be reported in the present paper.

Materials and Method

In the experiments presented, 3 animal rooms with similar environmental conditions other than those of lighting were employed. One group of 10 animals which may be designated as the open laboratory group, were kept in a well lighted (sunlight), well ventilated room with a southern exposure.

Two groups of 10 animals each were kept in a room from which all light was excluded. Other living conditions were the same as those of animals living in the open laboratory. During the course of the experiment, beginning on Feb. 1, 1928,

one group of animals was exposed for an hour each day to the unfiltered radiations of a quartz-mercury arc lamp (80 volts, 4.2 amperes) at a distance of 4.5 meters. These animals shall be designated as the ultra-violet group. The other animals which remained in the dark room throughout the experiment shall be designated as the dark group. Both of these groups of animals received some light from two other sources. For a brief period each day a 30 watt Mazda lamp was used in the dark room for cleaning cages, feeding, and making certain necessary observations. The animals were also exposed to diffuse filtered sunlight for a brief period once a week when they were brought into the laboratory for bleeding purposes.

The fourth group of animals which shall be designated as the light group was kept in a room similar to the others with sunlight excluded. In this room a *constant* source of light was furnished by 13 Cooper Hewitt low pressure mercury arcs, Type P, in crown glass arranged in 3 superimposed rows on an iron frame placed in the center of the room. The animals in individual cages were placed in racks on either side of and parallel with the mercury arcs at a distance of 1 meter. The average intensity of the light was calculated to be 2000 foot candles.

The spectrogram of this type of mercury arc in crown glass shows that the range of light rays is from 3022 to 5790 Ångström units with the majority falling in the 3650 and 5790 portion of the spectrum. The exposure to the Cooper Hewitt mercury arcs was begun on Jan. 20, 1928.

The doors of the cages were opened to permit free access of light but no depilatory measures were used for any group of animals, and at no time throughout the experiment could there be demonstrated evidence of dermatitis, conjunctivitis, or any other inflammatory process which might be attributed to the action of either ultra-violet or Cooper Hewitt radiation.

The temperature of the 3 rooms was satisfactorily maintained at 70° to 75°F. and the humidity of each varied with that of the outside atmosphere.

The animals selected for this experiment were all male rabbits of mixed grey and brown breeds from 6 to 8 months old. They were sexually mature but had not yet attained their full growth. All animals were caged separately and fed a uniform diet of hay, oats, and cabbage.

The data presented in this paper are derived from experiments, the procedures of which were described in detail in a preceding paper (1).

The determinations of calcium and inorganic phosphorus of blood serum, cholesterol and lipoid phosphorus of the whole blood were made on the same sample of blood. Determinations were made, beginning on Jan. 5, 1928, at weekly or biweekly intervals until May 31, 1928, resulting in 15 recorded observations for the light, dark, and open laboratory groups; 14 observations were made on the ultra-violet group.

In all text-figures and tables the lipoid phosphorus is calculated and presented as lecithin.

RESULTS

The results of the observations made in this experiment are presented in the form of tabulated summaries, Tables I to VI, which are supplemented by a series of graphs, Text-figs. 1 to 14. The mean values contained in Tables I to VI inclusive have been smoothed by the formula $\frac{A + 2B + C}{4}$ and are presented in the graphs in terms of per cent variations from standard mean values, using for this purpose values obtained for animals living in the open laboratory throughout the year. These results were reported in a previous paper (2) and shall serve also as a basis of comparison for certain ratio values obtained in the present experiment. The average values for this group of animals were calculated to be for calcium 15.7, inorganic phosphorus 4.65 mg. per 100 cc. of blood serum, and for cholesterol 58.2, lecithin 118.4 mg. per 100 cc. of whole blood. Text-figs. 1 to 4 inclusive represent the per cent variation of the 4 blood constituents; Text-figs. 5 to 10 inclusive, the per cent variation of the 6 possible ratios of the same 4 blood constituents; and Text-figs. 11 to 14 inclusive, the distribution of individual values for calcium, inorganic phosphorus, cholesterol, and lecithin.

TABLE I

Calcium Values for Consecutive Determinations

Date	Open laboratory			Dark			Cooper Hewitt			Date	Ultra-violet		
	Mean	Standard deviation	Coefficient of variation	Mean	Standard deviation	Coefficient of variation	Mean	Standard deviation	Coefficient of variation		Mean	Standard deviation	Coefficient of variation
1928	*mg. per 100 cc.*	*mg.*	*per cent*	*mg. per 100 cc.*	*mg.*	*per cent*	*mg. per 100 cc.*	*mg.*	*per cent*	*1928*	*mg. per 100 cc.*	*mg.*	*per cent*
Jan. 5	16.4 ±0.23	1.10	7.06	15.5 ±0.17	0.80	5.16	15.6 ±0.17	0.82	5.26				
" 19	15.2 ±0.14	0.67	4.45	15.3 ±0.20	0.95	6.22	15.2 ±0.11	0.55	3.62	Jan. 12	14.5 ±0.09	0.46	3.22
" 26	15.6 ±0.17	0.80	5.16	16.4 ±0.17	0.80	4.87	16.6 ±0.19	0.92	5.19	" 31	15.2 ±0.34	1.60	10.58
Feb. 2	15.3 ±0.10	0.48	3.15	15.3 ±0.10	0.48	3.16	15.7 ±0.15	0.72	4.58	Feb. 7	15.8 ±0.16	0.75	4.75
" 9	15.4 ±0.16	0.75	4.88	14.9 ±0.14	0.65	4.42	15.2 ±0.10	0.51	3.36	" 14	14.6 ±0.08	0.38	2.60
" 16	14.8 ±0.11	0.52	3.52	14.6 ±0.11	0.53	3.64	15.1 ±0.12	0.58	3.90	" 21	15.4 ±0.20	0.95	6.17
" 23	16.4 ±0.32	1.50	9.14	16.9 ±0.33	1.59	9.51	17.0 ±0.35	1.65	9.70	" 28	15.7 ±0.22	1.05	6.72
Mar. 1	15.8 ±0.04	0.19	12.34	16.3 ±0.14	0.67	4.11	16.1 ±0.11	0.54	3.35	Mar. 6	16.0 ±0.14	0.66	4.15
" 8	15.6 ±0.09	0.45	8.68	15.7 ±0.12	0.58	3.70	15.5 ±0.15	0.71	4.58	" 13	15.5 ±0.14	0.69	4.47
" 22	14.7 ±0.07	0.34	23.74	15.2 ±0.12	0.57	3.75	14.7 ±0.11	0.55	3.74	" 26	15.6 ±0.14	0.70	4.58
" 29	14.8 ±0.10	0.48	3.25	15.8 ±0.16	0.74	4.75	15.2 ±0.08	0.40	2.66	" 29	15.4 ±0.19	0.91	5.92
Apr. 12	14.8 ±0.13	0.62	4.15	15.0 ±0.12	0.57	3.82	14.9 ±0.16	0.61	5.35	Apr. 12	15.1 ±0.13	0.61	4.08
" 26	14.9 ±0.13	0.62	4.20	15.1 ±0.20	0.94	6.27	15.0 ±0.14	0.69	4.64	" 26	14.6 ±0.16	0.75	5.16
May 10	14.8 ±0.13	0.63	4.29	15.9 ±0.20	0.96	6.09	15.2 ±0.20	0.96	6.36	May 10	14.8 ±0.32	1.53	10.53
" 31	14.9 ±0.14	0.64	5.10	14.7 ±0.14	0.67	4.44	14.7 ±0.13	0.63	4.29	" 31	14.2 ±0.30	0.96	6.78

TABLE II

Inorganic Phosphorus Values for Consecutive Determinations

Date	Open laboratory			Dark			Cooper Hewitt			Date	Ultra-violet		
	Mean	Standard deviation	Coefficient of variation	Mean	Standard deviation	Coefficient of variation	Mean	Standard deviation	Coefficient of variation		Mean	Standard deviation	Coefficient of variation
1928	*mg. per 100 cc.*	*mg.*	*per cent*	*mg. per 100 cc.*	*mg.*	*per cent*	*mg. per 100 cc.*	*mg.*	*per cent*	*1928*	*mg. per 100 cc.*	*mg.*	*per cent*
Jan. 5	5.43 ±0.10	0.48	8.87	5.64 ±0.11	0.53	9.55	5.50 ±0.11	0.55	10.10				
" 19	5.26 ±0.11	0.55	10.60	4.75 ±0.08	0.39	8.24	4.87 ±0.06	0.30	6.34	Jan. 12	6.10 ±0.16	0.78	12.85
" 26	5.40 ±0.06	0.32	6.01	4.53 ±0.10	0.48	10.63	5.27 ±0.08	0.37	7.13	" 31	5.17 ±0.10	0.49	9.61
Feb. 2	5.52 ±0.07	0.33	6.03	5.26 ±0.06	0.27	5.30	5.05 ±0.08	0.37	7.48	Feb. 7	4.99 ±0.11	0.52	10.46
" 9	5.58 ±0.07	0.34	6.24	4.73 ±0.05	0.26	5.68	5.01 ±0.07	0.34	6.96	" 14	4.84 ±0.13	0.62	12.83
" 16	5.97 ±0.09	0.46	7.75	5.13 ±0.09	0.45	7.91	5.44 ±0.07	0.33	6.22	" 21	4.43 ±0.03	0.17	3.90
" 23	5.73 ±0.11	0.51	9.95	5.18 ±0.16	0.79	15.36	5.26 ±0.07	0.36	6.93	" 28	5.40 ±0.15	0.71	13.14
Mar. 1	5.48 ±0.10	0.48	8.89	4.91 ±0.07	0.37	7.53	5.01 ±0.11	0.53	10.75	Mar. 6	5.96 ±0.13	0.64	11.31
" 8	5.15 ±0.05	0.24	4.83	4.73 ±0.08	0.41	8.74	5.01 ±0.06	0.27	5.55	" 13	4.94 ±0.09	0.43	8.76
" 22	4.78 ±0.07	0.34	7.18	4.71 ±0.08	0.40	8.58	4.65 ±0.06	0.32	6.99	" 26	5.14 ±0.08	0.39	7.65
" 29	5.05 ±0.10	0.48	9.52	4.67 ±0.06	0.30	6.61	5.18 ±0.06	0.30	5.92	" 29	6.58 ±0.20	0.94	14.29
Apr. 12	4.46 ±0.08	0.40	9.90	4.57 ±0.07	0.33	7.35	4.62 ±0.07	0.36	7.83	Apr. 12	4.99 ±0.08	0.40	8.18
" 26	4.47 ±0.07	0.34	7.77	4.57 ±0.07	0.34	7.48	4.77 ±0.10	0.47	9.84	" 26	5.02 ±0.09	0.42	8.54
May 10	4.99 ±0.10	0.51	10.26	4.92 ±0.09	0.43	8.81	4.92 ±0.09	0.43	8.92	May 10	5.53 ±0.08	0.41	7.55
" 31	4.80 ±0.08	0.36	8.10	4.27 ±0.08	0.40	8.61	5.07 ±0.08	0.36	6.96	" 31	4.87 ±0.09	0.43	8.72

TABLE III

Cholesterol Values for Consecutive Determinations

Date	Open laboratory			Dark			Cooper Hewitt			Date	Ultra-violet		
	Mean	Standard deviation	Coefficient of variation	Mean	Standard deviation	Coefficient of variation	Mean	Standard deviation	Coefficient of variation		Mean	Standard deviation	Coefficient of variation
1928	*mg. per 100 cc.*	*mg.*	*per cent*	*mg. per 100 cc.*	*mg.*	*per cent*	*mg. per 100 cc.*	*mg.*	*per cent*	*1928*	*mg. per 100 cc.*	*mg.*	*per cent*
Jan. 5	65.6 ±1.23	5.76	8.78	63.4 ±1.18	5.54	8.74	61.8 ±0.75	3.54	5.74				
" 19	65.2 ±1.20	5.66	8.68	61.8 ±0.92	4.32	7.00	59.3 ±0.77	3.63	6.12	Jan. 12	73.3 ±1.35	6.36	8.68
" 26	61.9 ±1.39	6.56	10.59	57.0 ±1.18	5.56	9.75	56.1 ±0.82	3.88	6.82	" 31	57.5 ±1.23	5.76	10.03
Feb. 2	61.5 ±1.07	5.04	8.20	60.8 ±1.58	7.43	12.22	57.3 ±1.37	6.45	11.26	Feb. 7	60.3 ±0.84	3.71	6.33
" 9	61.2 ±1.26	5.92	9.67	58.3 ±1.25	5.89	10.11	55.2 ±0.73	3.45	6.25	" 14	65.8 ±0.94	4.41	6.70
" 16	61.6 ±1.28	6.04	9.81	58.3 ±1.10	5.18	8.88	62.1 ±0.91	4.29	5.90	" 21	71.2 ±2.18	10.24	14.38
" 23	60.0 ±1.77	8.30	13.83	58.5 ±0.83	3.93	6.72	55.6 ±1.31	6.14	11.04	" 28	66.0 ±1.63	7.64	11.58
Mar. 1	55.2 ±1.36	6.39	11.57	56.5 ±0.69	3.22	5.69	54.2 ±0.82	3.83	7.06	Mar. 6	57.0 ±0.66	3.10	5.96
" 8	58.7 ±1.47	6.89	11.73	65.8 ±1.54	7.20	10.94	58.0 ±0.80	3.74	6.45	" 13	60.9 ±1.20	5.63	9.24
" 22	59.5 ±1.46	6.84	11.49	65.7 ±1.32	6.20	9.43	64.4 ±1.11	5.19	8.05	" 26	59.1 ±1.15	5.40	9.14
" 29	63.9 ±1.91	8.96	14.02	57.7 ±1.00	4.70	8.14	59.7 ±1.05	4.90	8.21	" 29	73.4 ±1.10	5.15	7.02
Apr. 12	65.8 ±2.32	10.88	16.55	61.2 ±0.73	3.43	5.61	60.2 ±0.99	4.64	7.71	Apr. 12	69.6 ±0.96	4.51	6.47
" 26	71.2 ±2.60	12.20	17.13	64.0 ±1.81	8.48	13.25	66.9 ±1.95	9.15	13.67	" 26	68.3 ±1.43	6.72	9.84
May 10	64.7 ±1.59	7.46	11.53	67.8 ±1.66	7.76	11.45	61.7 ±1.56	7.30	11.80	May 10	80.3 ±1.72	8.03	10.01
" 31	66.2 ±1.22	5.69	8.72	59.9 ±0.92	3.84	6.38	83.8 ±1.36	6.38	10.49	" 31	67.7 ±1.30	5.82	8.76

TABLE IV

Lecithin Values for Consecutive Determinations

Date	Open laboratory			Dark			Cooper Hewitt			Date	Ultra-violet		
	Mean	Standard deviation	Coefficient of variation	Mean	Standard deviation	Coefficient of variation	Mean	Standard deviation	Coefficient of variation		Mean	Standard deviation	Coefficient of variation
1928	*mg. per 100 cc.*	*mg.*	*per cent*	*mg. per 100 cc.*	*mg.*	*per cent*	*mg. per 100 cc.*	*mg.*	*per cent*	*1928*	*mg. per 100 cc.*	*mg.*	*per cent*
Jan. 5	129.8 ±2.97	13.11	10.10	137.9 ±2.38	11.17	8.10	143.2 ±0.15	7.07	4.93				
" 19	145.4 ±1.69	7.94	5.46	158.7 ±1.46	6.86	4.32	154.8 ±1.95	9.14	5.90	Jan. 12	125.1 ±1.63	7.65	6.12
" 26	112.1 ±2.16	10.17	9.07	110.7 ±2.60	12.19	11.01	119.2 ±2.09	9.80	8.22	" 31	109.6 ±1.10	5.19	4.74
Feb. 2	87.7 ±1.57	7.37	8.41	86.1 ±1.88	8.84	10.27	96.1 ±1.64	7.69	8.01	Feb. 7	124.6 ±2.91	13.65	10.95
" 9	145.3 ±2.21	10.39	7.15	155.4 ±2.37	11.15	7.18	151.5 ±3.90	18.29	12.07	" 14	141.9 ±1.90	8.92	6.29
" 16	116.8 ±2.75	12.90	11.04	108.9 ±4.21	19.75	18.13	121.2 ±3.46	16.23	13.39	" 21	144.9 ±3.51	16.48	13.73
" 23	138.4 ±3.18	14.89	10.75	129.1 ±6.34	29.75	23.04	115.5 ±3.87	18.15	15.71	" 28	95.7 ±2.24	10.53	11.00
Mar. 1	137.7 ±3.55	16.66	12.10	109.2 ±4.76	22.30	20.42	109.4 ±4.31	20.21	18.47	Mar. 6	96.1 ±2.43	11.39	11.85
" 8	77.8 ±4.02	18.85	24.22	75.1 ±3.69	17.31	23.05	73.6 ±2.37	11.10	15.08	" 13	134.5 ±3.16	14.81	11.01
" 22	147.2 ±2.34	10.95	7.43	162.5 ±4.30	20.15	12.40	159.0 ±4.61	21.60	13.52	" 26	96.7 ±2.13	9.97	10.32
" 29	139.1 ±2.71	12.70	9.13	122.3 ±3.09	14.50	11.85	130.4 ±3.84	18.01	13.81	" 29	111.0 ±1.86	8.72	7.85
Apr. 12	124.0 ±1.87	8.75	7.05	159.0 ±2.09	9.82	6.17	145.3 ±2.57	12.05	8.29	Apr. 12	126.9 ±4.39	20.62	16.34
" 26	129.2 ±3.48	16.33	12.63	134.4 ±2.65	12.45	9.26	126.6 ±4.73	22.19	17.53	" 26	128.4 ±3.27	15.35	11.95
May 10	129.5 ±4.51	21.15	16.33	135.4 ±3.73	17.48	12.91	135.0 ±2.55	11.95	8.85	May 10	123.6 ±3.30	15.45	12.50
" 31	119.9 ±1.66	8.31	7.68	126.1 ±1.44	6.21	5.37	117.1 ±1.67	8.93	7.52	" 31	135.4 ±1.81	10.11	9.46

TABLE V

(*a*) *Coefficients of Correlation for Consecutive Group Means (Trend)*

Group	rP. Ca.	rP. Chol.	rP. Lec.	rCa. Chol.	rCa. Lec.	rChol. Lec.	
Open laboratory	+0.498 ±0.13	−0.566 ±0.12	−0.097 ±0.18	−0.328 ±0.15	−0.057 ±0.18	+0.117 ±0.17	N = 15
Dark room	+0.021 ±0.21	+0.065 ±0.18	−0.178 ±0.17	−0.180 ±0.17	−0.256 ±0.16	+0.147 ±0.17	N = 15
Light	+0.491 ±0.13	−0.146 ±0.17	−0.238 ±0.16	−0.627 ±0.10	−0.619 ±0.10	+0.776 ±0.07	N = 15
Ultra-violet	+0.027 ±0.21	+0.016 ±0.21	−0.722 ±0.08	−0.575 ±0.12	−0.625 ±0.10	+0.404 ±0.14	N = 14

(*b*) *Coefficients of Correlation for Individual Mean Values*

Group	rP. Ca.	rP. Chol.	rP. Lec.	rCa. Chol.	rCa. Lec.	rChol. Lec.	
Open laboratory	−0.398 ±0.18	−0.110 ±0.21	−0.309 ±0.19	+0.034 ±0.22	+0.150 ±0.21	+0.064 ±0.22	N = 10
Dark room	−0.559 ±0.15	−0.008 ±0.22	−0.226 ±0.20	−0.028 ±0.22	+0.451 ±0.17	+0.061 ±0.22	N = 10
Light	−0.225 ±0.20	+0.331 ±0.19	−0.320 ±0.19	−0.607 ±0.13	−0.169 ±0.20	+0.403 ±0.18	N = 10
Ultra-violet	−0.258 ±0.20	+0.137 ±0.21	−0.683 ±0.11	+0.626 ±0.13	+0.646 ±0.12	+0.415 ±0.17	N = 10

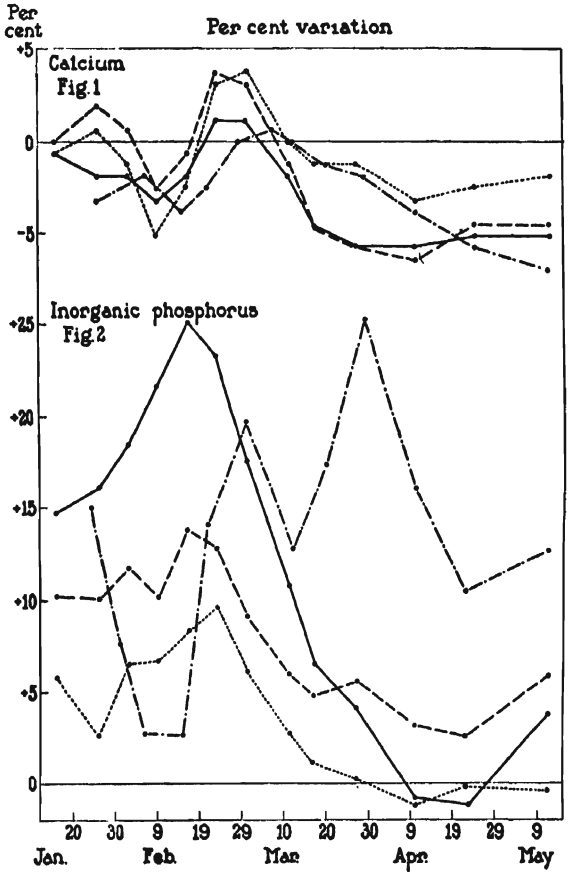
(*c*) *Coefficients of Correlation for Entire Series*

Group	rP. Ca.	rP. Chol.	rP. Lec.	rCa. Chol.	rCa. Lec.	rChol. Lec.	
Open laboratory	+0.655 ±0.03	−0.241 ±0.05	−0.084 ±0.06	−0.069 ±0.06	−0 001 ±0.06	+0.027 ±0.06	N = 150
Dark room	−0.051 ±0 06	+0.045 ±0.06	−0.102 ±0.06	−0.075 ±0.06	−0.015 ±0.06	+0.169 ±0.05	N = 150
Light	+0.154 ±0.05	−0.052 ±0.06	−0.112 ±0.06	−0.398 ±0.04	−0.220 ±0.05	+0.113 ±0.05	N = 150
Ultra-violet	+0.031 ±0.05	+0.152 ±0.05	−0.306 ±0.04	+0.023 ±0.06	−0.115 ±0.06	+0.224 ±0.05	N = 140

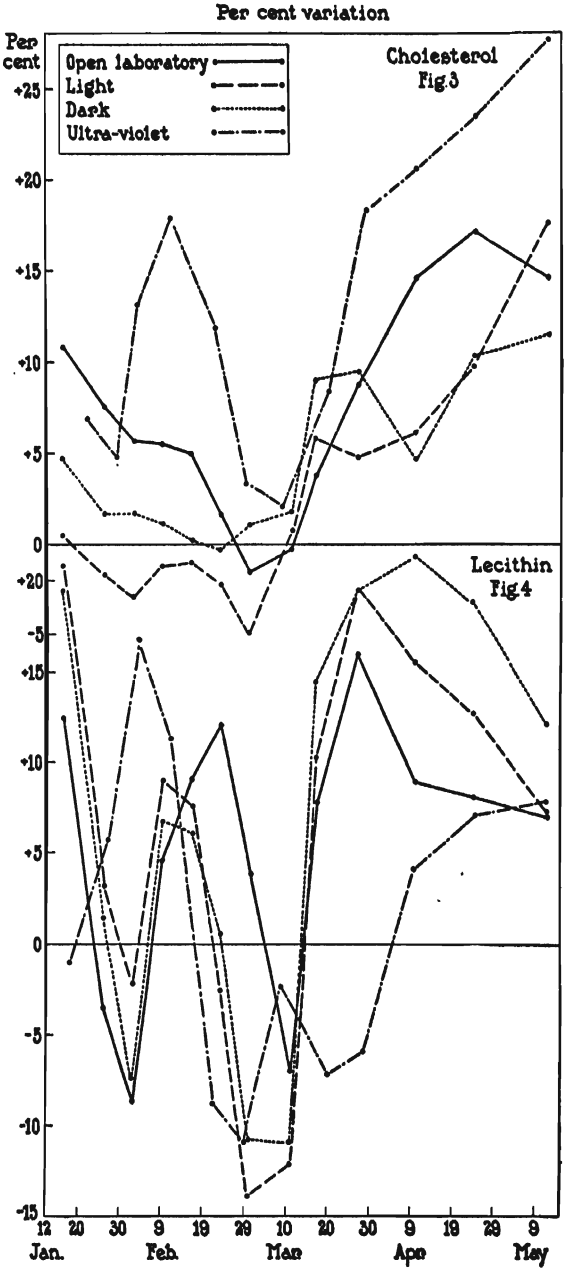
TABLE VI

Mean Values for Each Group

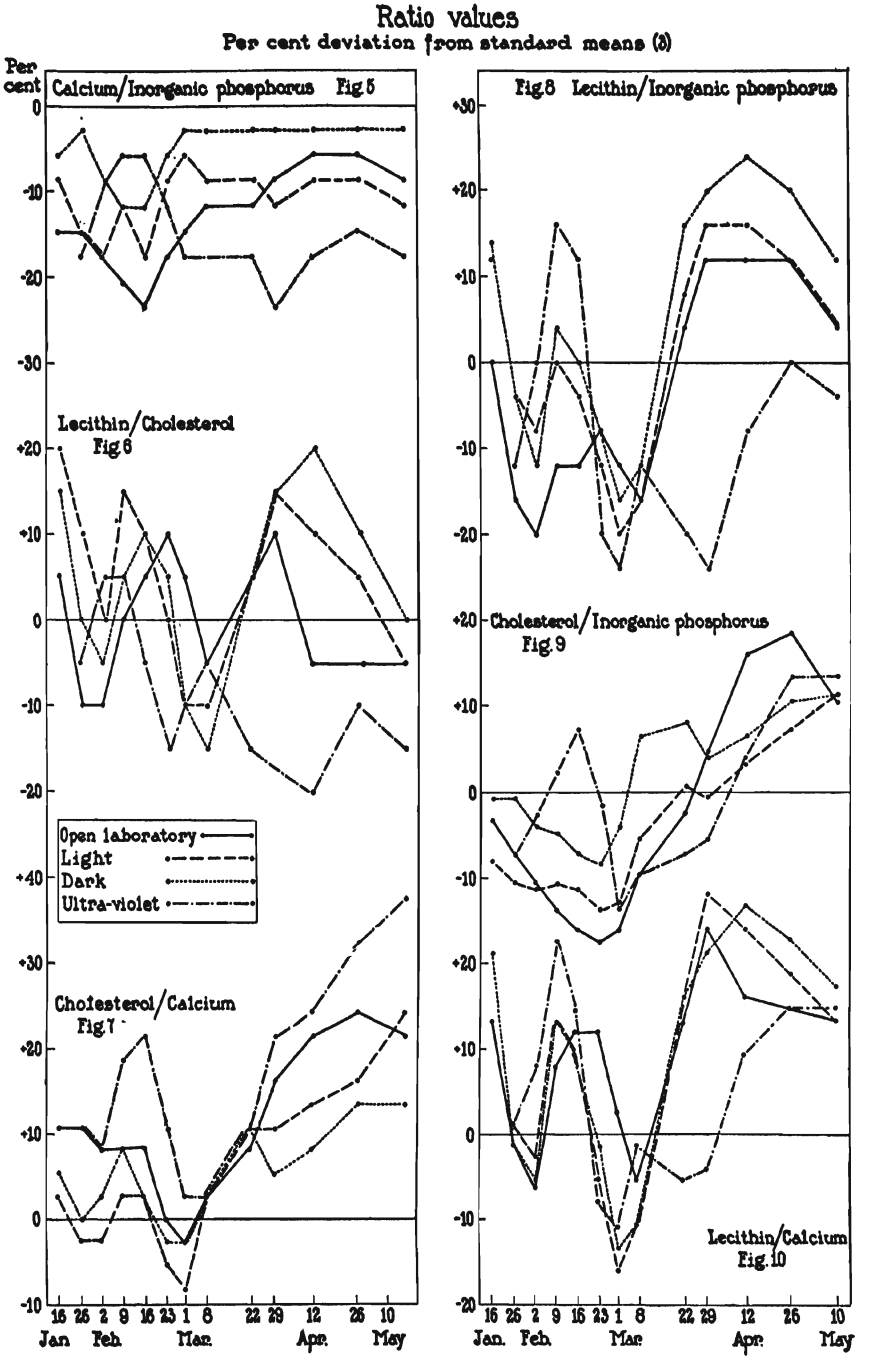
Group	Calcium	Phosphorus	Cholesterol	Lecithin	N
Open laboratory.....	15.3 ±0.05	5.21 ±0.01	62.7 ±0.45	124.0 ±1.33	150
Dark room.....	15.6 ±0.05	4.83 ±0.02	60.9 ±0.38	126.0 ±1.65	150
Light.....	15.5 ±0.05	5.02 ±0.02	59.8 ±0.42	125.0 ±1.35	150
Ultra-violet.....	15.3 ±0.05	5.26 ±0.04	65.9 ±0.51	120.0 ±1.22	140



FIGS. 1 AND 2


Per cent variation
Per cent
Open laboratory
Light
Dark
Ultra-violet
Cholesterol
Fig.3
Lecithin
Fig.4
+25
+20
+15
+10
+5
0
-5
-10
-15
12 20 30 9 19 29 10 20 30 9 19 29 9
Jan. Feb. Mar Apr May

FIGS. 3 AND 4


Ratio values
Per cent deviation from standard means (δ)
Per cent
Calcium/Inorganic phosphorus Fig.5
Lecithin/Cholesterol Fig.6
Cholesterol/Calcium Fig.7
Fig.8 Lecithin/Inorganic phosphorus
Cholesterol/Inorganic phosphorus Fig.9
Lecithin/Calcium Fig.10
Open laboratory
Light
Dark
Ultra-violet
16 25 2 9 16 23 1 8 22 29 12 26 10
Jan. Feb. Mar. Apr. May

FIGS. 5 TO 10

Per cent
Open laboratory
Dark
Cooper-Hewitt
Ultra-violet
Phosphorus distribution Fig 11
Calcium distribution Fig 12
Cholesterol distribution Fig 13
Lecithin distribution Fig 14
Mg

FIGS. 11 TO 14

DISCUSSION AND CONCLUSIONS

Variation

Calcium.—The element calcium (Text-fig. 1) proved to be the most stable and least variable of the 4 blood constituents. This was true for each of the 4 groups of animals.

The trend of calcium in the blood serum of animals living in the open laboratory showed a gradual decrease from the beginning of the experiment on Jan. 5 until about Feb. 9. Throughout the next 3 weeks the calcium in this group of animals increased in amount until on Feb. 29 it reached its highest level. From this high value the calcium decreased until about Mar. 20 at which time it had reached a more or less stable level which was maintained during the remaining 2 months of the experiment.

The animals living in the dark and those exposed to Cooper Hewitt light showed similar trends for the first 3 months of the experiment but exhibited a degree of variation as to the general levels of calcium metabolism. These groups of animals showed first an increase in calcium and then a rather rapid decline, reaching their minimum level at the same time as those animals living in the open laboratory, the dark group slightly lower, and the light slightly higher than those living in the open laboratory. From the low values of Feb. 9, the calcium of both groups of animals increased in amount, reaching their maximum on Feb. 29. Throughout the initial period of the experiment, the animals in the dark room maintained a lower level of calcium than those exposed to the Cooper Hewitt light. This difference, however, was reversed, and from Feb. 29 throughout the remaining time of the experiment, the animals living in the dark room maintained a higher level of calcium than any of the others, while the light group followed closely the trend of the animals living in the open laboratory.

The animals exposed to ultra-violet rays showed first, a slight increase of calcium and then a decrease, this decrease occurring at a time when the trend of calcium for the 3 other groups was on the increase. The ultra-violet group reached its highest level of calcium on Mar. 6, this occurring at a time when the trend of all other groups was downward. From this time until the end of the experiment, the calcium for the ultra-violet group continued to decrease.

During the last month of the experiment the 4 groups of animals exhibited 4 distinct levels for the calcium content of blood serum. The animals living in the dark room had the highest amount of calcium, those exposed to Cooper Hewitt light ranking next with a smaller amount, the third group, those animals living in the open laboratory with a slightly lower amount of calcium than the light group, while the least amount of calcium during this last 4 weeks' period was found to be in the animals exposed to the ultra-violet light.

Inorganic Phosphorus.—The inorganic phosphorus of the blood serum (Text-fig. 2) ranked next to calcium with a higher degree of variation. This fact held true for each of the 4 groups of animals. The greatest variation occurred in the group of animals living in the open laboratory while the animals in the dark room showed the least variation. The most abrupt changes in inorganic phosphorus were noted to occur in the blood serum of those animals that were exposed to ultra-violet light.

Analyzing the curves for the individual groups, it will be noted that with the animals living in the open laboratory there occurred a gradual increase in inorganic phosphorus which reached its maximum amount on Feb. 16. This gradual increase was not unlike that which occurred in the group of animals previously reported (2) and it will also be noted that this maximum occurred at a time when the previously reported group gave its minimum values, *i.e.*, at about 6 weeks from the beginning of the experiment. From this high value, the inorganic phosphorus in the serum of animals living in the open laboratory exhibited a gradual and uninterrupted decrease, reaching its lowest level on Apr. 12. The last 3 determinations gave only slight increases in inorganic phosphorus over this minimum value.

The animals exposed to Cooper Hewitt light showed the same initial rise in inorganic phosphorus as the animals living in the open laboratory. It will be noted, however, that at the beginning of the experiment the light group maintained a decidedly lower level than those in the open laboratory, but the maximum values for the 2 groups of animals occurred at about the same time. From this high point on Feb. 16, the inorganic phosphorus showed a gradual decrease which continued until Apr. 12, the last 3 determinations giving increasing amounts over this minimum value. At the beginning of the experi-

ment the level of inorganic phosphorus for the light group was far below that of the open laboratory group, but this order was reversed on about Mar. 20, and from this time until the end of the experiment the light animals maintained a higher level of inorganic phosphorus than those living in the open laboratory.

Except for a slight decrease at the beginning of the experiment, the trend of inorganic phosphorus in the serum of animals living in the dark room was the same as that found in the light and open laboratory groups. With the dark group the maximum value, which occurred Feb. 23, came 1 week later than the maximum of either the light or open laboratory groups. From this high amount, the inorganic phosphorus in the blood serum of the dark animals gradually decreased in amounts until about Apr. 9, when the minimum for the entire experiment was obtained. It will be noted that the general level of inorganic phosphorus maintained by the dark group was below that of the light, and except for one determination, that of May 19, was far below the level maintained by the animals living in the open laboratory. The animals living in the dark room showed the least variation.

The inorganic phosphorus of the ultra-violet group exhibited trends and variations in a direction opposite to that of the 3 other groups of animals. At the beginning of the experiment this group of animals gave the highest value but during the succeeding 4 weeks the inorganic phosphorus decreased, reaching its minimum on Feb. 21. It may be noted that this continued decrease occurred over a period of time when the 3 other groups of animals were exhibiting trends of increasing amounts. From this minimum, the ultra-violet group showed a gradual increase in inorganic phosphorus and except for the 2 determinations on Mar. 10 and 20, reached their maximum on Mar. 30. This high value for the ultra-violet group was reached at a time when the 3 other groups had more or less stabilized at their respective minimum levels. From this maximum, the inorganic phosphorus showed decreasing amounts for the 2 next determinations and was followed by increasing amounts for the 2 last examinations.

During the last 10 weeks of the experiment, the 4 groups of animals maintained distinct levels of inorganic phosphorus, these levels being independent and except for one value found in the dark group, showed no overlapping. The highest level of inorganic phosphorus was main-

tained during the last 10 week period by the ultra-violet group followed in order by the light group and animals living in the open laboratory, with the dark group at the lowest level.

Cholesterol.—The variability exhibited by the cholesterol (Text-fig. 3) in the blood of the 4 groups of animals was practically the same as that of inorganic phosphorus.

The group of animals living in the open laboratory showed a gradual decrease in cholesterol from the beginning of the experiment, reaching their minimum on Feb. 29. This low value occurred at a time when animals living out of doors, *i.e.*, just received from the dealer, were maintaining their maximum level of cholesterol for the entire year. From this minimum the cholesterol in the blood of the open laboratory group rapidly increased, the maximum value occurring on Apr. 19. The 2 last determinations showed only a slight decrease from this maximum value.

At the beginning of the experiment the group of animals exposed to the Cooper Hewitt light showed the least amount of cholesterol, but during the last 10 weeks gave values which were only slightly less than those for the group living in the open laboratory. The light group at the beginning of the experiment showed a gradual decrease in cholesterol which was followed by a slight increase during February, and a second decrease which reached its minimum on Feb. 29. Throughout this entire period the light group showed the least cholesterol of any of the 4 groups. During the remainder of the experiment the trend of cholesterol for this group of animals was upward, except for the one determination on Mar. 29, and a level below the open laboratory group was maintained until the last examination was made.

The animals living in the dark room maintained a level of cholesterol which in general was between the values shown by the light and open laboratory groups, but during the last 4 weeks was just below the animals exposed to Cooper Hewitt light. From the beginning of the experiment the cholesterol decreased until a minimum was reached on Feb. 23. This was followed by a gradual increase until Mar. 9 at which time a marked decrease in cholesterol was noted. The following determinations, however, gave values which represented the maximum for this group for the entire time of the experiment.

The group of animals exposed to the ultra-violet light gave 2 values

for cholesterol at the beginning of the experiment which were slightly below that obtained for the group in the open laboratory. The ultra-violet group then showed a rapid increase in cholesterol, reaching a maximum for this period on about Feb. 14. This increase occurred at a time when the cholesterol of all other groups was decreasing. The trend of cholesterol following this high point was downward until Mar. 10 when it reached its minimum. From this minimum the cholesterol exhibited a very rapid increase which continued throughout the remainder of the experiment. It will be noted that except for the 2 values at the beginning of the experiment and the one obtained on Mar. 20, the general level of cholesterol for the ultra-violet animals was markedly higher than that for any of the 3 other groups of animals. For the last 6 weeks of the experiment, in the order of decreasing levels, the ultra-violet group ranked first; the group living in the open laboratory came next with practically 10 per cent less cholesterol; the light and dark groups followed with the light group slightly higher in cholesterol than the dark.

Lecithin.—The abruptness and degree of variation proved to be greater for lecithin (Text-fig. 4) than for any of the 3 other blood constituents. This fact held true for each of the 4 groups of animals. The animals living in the open laboratory, the dark and light groups, listed in the order of increasing values for lecithin at the beginning of the experiment, showed a decreasing trend which reached a minimum on Feb. 2. From this point, the 3 groups exhibited a rather abrupt increase in lecithin, the group living in the open laboratory reaching its maximum about Mar. 1 which was 10 days later than the maximum for the light or dark groups. During the following 4 weeks, there occurred another decrease in lecithin in each of these 3 groups of animals which terminated with minimum values for the 3 groups on Mar. 10.

During the last 8 weeks of the experiment, the 3 groups of animals showed a rapid increase in lecithin, the dark group showing the greatest amount throughout this period; the light group next with slightly less; and the open laboratory group with the lowest level for the 3 groups.

The animals exposed to the ultra-violet light maintained the highest level of lecithin during the first part of the experiment, but during the

last 8 weeks they were the lowest of the 4 groups. Beginning with lecithin far below the 3 other groups, the ultra-violet animals showed a rapid increase which reached its maximum about Feb. 21. This occurred at a time when the 3 other groups were exhibiting decreasing trends. From this high point, the lecithin decreased and reached a minimum about the same time as the light and dark groups, this being the lowest point attained by the ultra-violet group throughout the experiment. A slight increase in lecithin was found at the next examination, but this was followed by 2 determinations of decreasing values. It will be noted that this decrease occurred at a time when the 3 other groups exhibited their greatest per cent increase. From this low level the lecithin in the ultra-violet group gradually increased and at the last determination was slightly higher than for the group living in the open laboratory or the group exposed to Cooper Hewitt light. The general level of lecithin maintained by the ultra-violet animals throughout the last 8 weeks of the experiment, however, was markedly lower than that of the 3 other groups.

Considering the calcium, inorganic phosphorus, cholesterol, and lecithin throughout the course of the experiment in all groups of animals, it will be noted that the curves representing the trend of these 4 blood constituents may in general be divided into two periods. The first 8 weeks of the experiment showed the greatest degree of irregularity in trend and frequency of variation. This period, as suggested by Brown (3) may be called the period of adjustment or accommodation to changed environment. In this experiment the period of accommodation was about 2 weeks longer than that required by the group of animals previously reported (2) but this difference in time may be attributed to the difference of environmental conditions. The second period, comprising the last 8 weeks of the experiment, was characterized by relative stability. During this last 8 weeks, each group of animals exhibited and maintained more or less distinct levels for each of the 4 blood constituents which can only be explained on the basis of differences in light environment, since all other factors for the 4 groups of animals were, as far as possible, the same.

The mean values for calcium, inorganic phosphorus, cholesterol, and lecithin maintained by these 4 groups of animals over the entire period of the experiment are presented in Table VI. These values

show differences of the same general order as those indicated in the text-figures.

Ratios

The 6 possible combinations of calcium, inorganic phosphorus, cholesterol, and lecithin in the form of ratios are presented in Text-figs. 5 to 10 inclusive. In order that each group might serve as a control for the others all values were reduced to terms of a percentage deviation from a standard mean as explained above.

Each of the 6 ratio text-figures is divisible into two sections as in the case of the curves showing the variations in the amounts of the 4 substances. The first period of some 8 weeks represents the period of accommodation to the changed environment. It will be noted here, as in the previous curves, that all ratios during the first 8 weeks exhibited wide degrees of variation and rather abrupt changes, and that it was not until the last 8 weeks of the experiment that any degree of stability was maintained by all groups of animals. Since the true effect of the conditions studied is shown best by the results obtained during the last 8 weeks of the experiment, the discussion will be limited to this period.

The most consistent and the highest calcium-inorganic phosphorus ratio (Text-fig. 5) was maintained by the animals living in the dark room. While both calcium and inorganic phosphorus showed a certain degree of variation during this period of the experiment, the ratio between the 2 substances was practically constant. A change in relative position took place between the light and open laboratory animals during the last 5 weeks' period. At the beginning, the light group exhibited a higher ratio of calcium-inorganic phosphorus, but fell slightly below the open laboratory groups after Mar. 22. This change in ratio value was due primarily to the drop in inorganic phosphorus of the animals in the open laboratory below that maintained by the light group.

The lowest calcium-inorganic phosphorus ratio during this period of the experiment was maintained by the animals exposed to ultraviolet light. This low ratio was due to both a decrease in calcium and an increase in inorganic phosphorus. The order into which these 4 groups of animals aligned themselves with respect to the calcium-

inorganic phosphorus ratio was first, the animals living in the dark with the highest ratio; second, the open laboratory group; third, the light group; and fourth, the ultra-violet group with the smallest ratio value. It will be noted that all values were lower than those for the standard group (3). This was due mainly to a difference in calcium values.

Stability in the lecithin-cholesterol ratios (Text-fig. 6) for all groups of animals did not occur until some 10 days after the calcium-inorganic phosphorus ratios had been established at more or less definite levels. The delay in lecithin-cholesterol stabilization was due chiefly to the variation which occurred in the cholesterol content of the blood during this period.

The highest lecithin-cholesterol ratio was maintained by the animals living in the dark room. At the beginning of the last 8 weeks' period, the ratio maintained by the light group coincided with that of the dark, but during the final 5 weeks, there was a separation of the 2 groups, the ratio of the light falling below that of the dark group.

The animals living in the open laboratory showed the least variation in the lecithin-cholesterol ratio. With the beginning of the last 8 weeks' period, this group gave ratio values below both the dark and light groups. The final ratios were somewhat lower than that maintained by either the light or dark groups.

The animals exposed to the ultra-violet light maintained the lowest lecithin-cholesterol ratio. This low value was maintained throughout the last 8 weeks. Listing the 4 groups of animals in the order of the relative magnitude of the levels maintained, the animals in the dark room came just with the highest lecithin-cholesterol ratio; the light group second; the open laboratory group came third; and the ultra-violet animals fourth with the smallest ratio values. The low position of the ultra-violet group was the result of the high cholesterol (Text-fig. 3) and the comparatively low lecithin values (Text-fig. 4) exhibited throughout this last 8 weeks' period.

The cholesterol-calcium ratios (Text-fig. 7) for the 4 groups of animals showed levels which were just the reverse of the lecithin-cholesterol ratios. The ultra-violet groups maintained the highest cholesterol-calcium ratio throughout the last 8 weeks; the open laboratory group, except for the last value, came next with a slightly lower

ratio; then the light group; and the animals in the dark with the lowest value for this period of the experiment.

The ratio of lecithin to inorganic phosphorus (Text-fig. 8) was highest in animals living in the dark room. The light and open laboratory groups came next with a slight decrease in ratio values, and it will be noted that at the time of the 2 last determinations these groups gave values which coincided.

The ratio of lecithin to inorganic phosphorus maintained by the ultra-violet group was markedly below that found in any other group of animals. This low ratio was due mainly to a high inorganic phosphorus, but the lecithin was also lower than in the other animals.

The cholesterol-inorganic phosphorus ratio (Text-fig. 9) showed marked delay in stabilization with more overlapping of the trends than any of the other ratios. This condition may be ascribed to the wide variation in cholesterol content of the whole blood than to the inorganic phosphorus which exhibited a much greater degree of stability and maintained a definite separation of levels for each of the 4 groups of animals.

The animals living in the open laboratory gave the highest cholesterol-inorganic phosphorus ratio except for the last determination at which time these animals were the lowest of the 4 groups. The ultra-violet group, which at the beginning of this period gave values below those for all other animals, came next with a slightly lower ratio; the final value, however, was slightly above that obtained for the 3 other groups.

The animals in the dark room exhibited a greater stability with respect to their cholesterol-inorganic phosphorus ratio than the other groups of animals. While at the beginning of this period the ratio values of the dark group were well above those of other animals, the subsequent increase was less than that of the open laboratory and ultra-violet groups, both of which rose to a higher level than the dark group. At the last determination, the ratio for the animals in the dark coincided with that found for the light group. The animals exposed to Cooper Hewitt light maintained a cholesterol-inorganic phosphorus ratio below that of the dark group throughout the experiment, except as pointed out, at the time of the last examination when these 2 ratios were the same.

The results of the 6 ratio curves (Text-figs. 5 to 10) with respect to relative position of each group of animals during the last 8 weeks of the experiment are presented on a comparative basis in the following table, the figure 1 representing the highest and 4 the lowest ratio value.

Group	Ca./P.	Lec./Chol.	Chol./Ca.	Lec./P.	Chol./P.	Lec./Ca.
Dark room	1	1	4	1	2	1
Cooper Hewitt	3	2	2	2	3	2
Open laboratory	2	3	3	3	1	3
Ultra-violet	4	4	1	4	4	4

These results show definitely that the group of animals in the dark room and those exposed to ultra-violet light occupied diametrical positions with respect to their ratio levels. Except for 2 ratios, the animals in the open laboratory maintained values next to that of the ultra-violet group, while the light group gave results nearer those of animals in the dark.

Distribution

The distributions of all values obtained for each of the 4 substances studied are presented according to groups in Text-figs. 11 to 14 inclusive. These curves are similar in form to those previously reported (1) except for the one curve representing the distribution of cholesterol for the ultra-violet group of animals. The irregularity in this curve is probably due to the extreme high values obtained for these animals during the last 6 weeks of the experiment. The differences in position of the other curves correspond to the results presented in Text-figs. 1 to 4.

Correlation

The coefficients of correlation for the 6 possible combinations of calcium, inorganic phosphorus, cholesterol, and lecithin are presented in Table VI. Section (a) gives the results obtained by calculating the correlation coefficients using mean values for each group and, therefore, presents the relationship with respect to trend. Except for

rP.Ca. which gives a positive correlation for the 4 groups of animals, the remaining 5 correlations were the same with respect to sign as those presented in a previous report (1). The highest degree of correlated trend was found in the rChol. Lec. of animals exposed to the Cooper Hewitt light. In the rP. Lec. the ultra-violet group gave the highest degree of correlated trend.

In section (b) of the same table the correlation coefficients are calculated on the basis of the mean values maintained by each individual animal throughout the experiment. The signs of the rP.Ca. in this section are negative, while in the previous section they are positive. This is due to the fact that while individual animals maintained negative relationships in their corresponding sigma values, the general trend of the mean values for calcium and inorganic phosphorus tend to parallel each other in degree and direction of variation.

The correlation coefficients in section (c), calculated on the basis of a comparison of single determinations with the means for the entire series, give values which in the majority of cases are the same as section (a).

Throughout the 3 sections it will be noted that the rChol.Lec. were all positive in sign, the magnitude of the coefficients varying somewhat with respect to the different groups and method of calculation. These results correspond with those reported in a previous paper (1) and seem to indicate that the 2 lipoid substances, cholesterol and lecithin, parallel each other in variation and trend.

The relationship existing between the inorganic phosphorus of the blood serum and the lecithin of the whole blood is of especial interest. Reviewing the results so far obtained with respect to these two substances, it will be noted that lecithin in all cases has proved to be the most variable constituent and has shown the most abrupt and most frequent change of trend. The coefficient of variation and standard deviation of inorganic phosphorus emphasize the stability of this substance when compared to the lipoid fraction. It seems probable, therefore, that there is a reciprocal relationship existing between inorganic phosphorus and lecithin and also the lipoid fraction may serve as a reservoir or source of supply for the inorganic form.

The results show that animals when brought into the laboratory pass through a period of adjustment or accommodation which in this

experiment covered a period of about 8 weeks. The mechanism by which these effects are induced is unknown.

The results obtained from this experiment are interpreted as furnishing evidence that exposure to the ultra-violet light for a comparatively brief period daily results in a definite change in levels of calcium, inorganic phosphorus of the blood serum and cholesterol and lecithin of the whole blood. Animals excluded from the effects of all light, as far as is practicable, maintained levels of the 4 blood constituents diametrical to the group of animals exposed to ultra-violet rays. The animals living in the open laboratory and those exposed to the Cooper Hewitt light exhibit levels of the 4 blood constituents which are somewhat similar in position, but both groups maintained values just between those of the dark and ultra-violet groups of animals.

SUMMARY

Experiments are reported in which it was shown that the calcium, inorganic phosphorus, cholesterol, and lecithin in the blood of normal rabbits were influenced by 4 types of light environment. The results of the experiments seem to warrant the following conclusions:

1. Animals exposed to the ultra-violet light for a brief period each day give results which are diametrical to those obtained for animals living in total darkness.

2. The results obtained for animals exposed to the Cooper Hewitt light and for those living in the open laboratory are somewhat similar but occupy a position between those of the dark and ultra-violet groups.

3. Animals do not develop immediately the characteristic effects of a particular environmental condition, but pass through a period of accommodation which varies somewhat with different environmental conditions.

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[Reprinted from THE JOURNAL OF EXPERIMENTAL MEDICINE, July 1, 1929,
Vol. 50, No. 1, pp. 109–120]

BIOMETRY OF CALCIUM, INORGANIC PHOSPHORUS, CHOLESTEROL, AND LECITHIN IN THE BLOOD OF RABBITS

IV. EFFECTS OF A MALIGNANT TUMOR

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(Received for publication, May 11, 1929)

The present paper is part of a study of the relations of certain blood constituents in animals as affected by various types of environment and disease (1, 2, 3). The experiments to be reported deal with the variations of calcium, inorganic phosphorus, lecithin and cholesterol in the blood of animals inoculated with a malignant neoplasm (4).

Material and Methods

The animals used were male rabbits from 6 to 8 months old at the beginning of the experiment. While under observation, the animals were caged separately, and kept in a well lighted (sunlight), well ventilated room with a southern exposure. The rooms were heated during the colder weather. The diet of the animals consisted of hay, oats, and cabbage.

Three groups of 10 animals each were inoculated in one testicle with 0.3 cc. of a tumor emulsion. Examinations of the blood were made at weekly intervals for a period of 2 months. During this time, some animals died; those that survived were then killed and autopsied.

The results for the control animals will be omitted as the general trend and variation of results are comparable to the group previously reported (2). Moreover, a comparison of the values obtained for animals that recovered with those for animals that died is more significant than a comparison made with normal values.

At the conclusion of the experiment, the 30 animals comprising this series were divided into 2 groups: (1) those animals in which death was due to the neoplasm, and (2) those recovering from the tumor inoculation. The results obtained from these 2 groups of animals are presented in the present paper. The results for the

12 animals in which death was due to the tumor (Group I) are presented in Table I, while the results of the 18 animals recovering from the tumor inoculation (Group II) are presented in Table II. Each table is divided into 7 dates of record, the first recorded examination, −14 days, are the results obtained 14 days preceding inoculation; second, the results obtained on the day of inoculation; third, the results +14 days after inoculation. The subsequent 4 determinations were made at 7 day intervals. The recorded values at "time of death" does not mean all animals died 42 days after inoculation, but summarizes the results obtained from these animals a day or two preceding expiration. The data presented in this paper are derived from experiments, the procedures of which were described in detail in a preceding paper (1). In all text-figures and tables the lipoid phosphorus is calculated and presented as lecithin.

The pertinent features of the experiment may be summarized as follows:

Group	No. of animals	No. of examinations	Date of inoculation	Observation period
A	10	99	Nov. 17, 1927	Oct. 27, 1927 to Jan. 12, 1928
B	10	99	Jan. 19, 1928	Jan. 5, 1928 to Mar. 22, 1928
C	10	76	Mar. 27, 1928	Mar. 15, 1928 to May 22, 1928

Results

The results of the observations made in this experiment are presented in the form of tabulated summaries, Tables I to III, which are supplemented by a series of graphs, Text-figs. 1 to 10. The mean valuescontained in Tables I and II have been smoothed by the formula $\frac{A + 2B + C}{4}$ and are presented in the graphs in terms of per cent variations from standard mean values, using for this purpose values obtained for animals living in the open laboratory throughout the year. These results were reported in a previous paper (2) and shall serve also as a basis of comparison for certain ratio values obtained in the present experiment.

TABLE I

Group I. Tumor Deaths

Days	Inorganic phosphorus			Calcium			Cholesterol			Lecithin		
	Mean	Standard deviation	Coefficient of variation	Mean	Standard deviation	Coefficient of variation	Mean	Standard deviation	Coefficient of variation	Mean	Standard deviation	Coefficient of variation
	mg. per 100 cc.	*mg.*	*per cent*	*mg. per 100 cc.*	*mg.*	*per cent*	*mg. per 100 cc.*	*mg.*	*per cent*	*mg. per 100 cc.*	*mg.*	*per cent*
−14	5.59 ± 0.22	1.11	19.87	16.5 ±0.29	1.47	9.03	60.6 ±1.67	8.59	14.17	129 ±5.08	26.1	20.33
Inoculated	5 52 ± 0.25	1.29	23.37	15.0 ±0.17	0.87	5.80	60.0 ±1.61	8.26	13.77	113 ±4.30	22.1	19.56
+14	5.37 ± 0.12	0.63	11.75	14.8 ±0.18	0.95	6.42	63.7 ±1.27	6.51	10.22	118 ±5.47	28.1	23.81
+21	5.96 ± 0.22	1.11	18.62	14.7 ±0.13	0.67	4.56	64.4 ±1.75	8.97	13.93	108 ±5.82	29.9	27.68
+28	7.19 ± 0.91	4.65	64.67	14.4 ±0.23	1.17	8.13	69.0 ±2.09	10.72	15.54	123 ±3.29	16.9	13.74
+35	6.34 ± 0.38	1.95	30.76	15.4 ±0.37	1.88	12.21	80.5 ±6.09	31.28	38.86	110 ±1.42	7.3	6.64
Time of death	8.67 ± 1.10	5.67	65.39	13.9 ±0.26	1.33	9.57	91.5 ±6.42	32.98	36.04	120 ±3.82	19.6	16.33

TABLE II

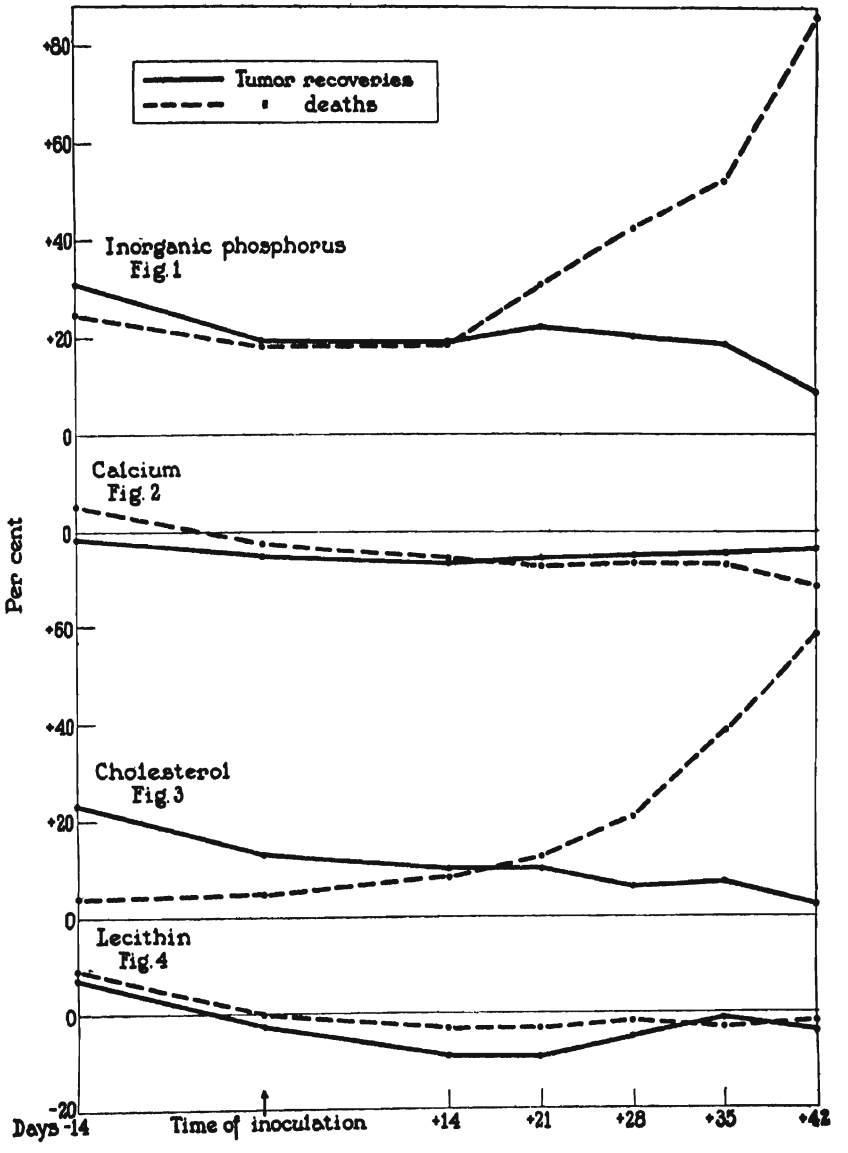
Group II. Tumor Recoveries

Days	Inorganic phosphorus			Calcium			Cholesterol			Lecithin		
	Mean	Standard deviation	Coefficient of variation	Mean	Standard deviation	Coefficient of variation	Mean	Standard deviation	Coefficient of variation	Mean	Standard deviation	Coefficient of variation
	mg. per 100 cc.	*mg.*	*per cent*	*mg. per 100 cc.*	*mg.*	*per cent*	*mg. per 100 cc.*	*mg.*	*per cent*	*mg. per 100 cc.*	*mg.*	*per cent*
−14	6.13 ±0.11	0.67	10.92	15.4 ±0.29	1.85	12.01	71.6 ±1.90	11.93	16.62	127 ±4.27	26.87	21.16
Inoculated	5.29 ±0.10	0.65	12.29	15.0 ±0.15	0.96	6.40	64.1 ±1.45	9.13	14.24	114 ±4.79	30.16	26.46
+14	5.67 ±0.15	0.93	16.40	14.5 ±0.14	0.91	6.28	63.1 ±0.76	4.80	7.61	107 ±4.38	27.57	25.77
+21	5.75 ±0.09	0.54	9.39	15.0 ±0.18	1.07	7.13	65.3 ±1.29	7.86	12.04	105 ±3.11	19.02	18.11
+28	5.81 ±0.08	0.50	8.61	14.7 ±0.11	0.68	4.63	62.0 ±1.39	8.48	13.68	115 ±3.49	21.36	18.57
+35	5.68 ±0.16	0.95	16.73	15.2 ±0.26	1.57	10.33	63.8 ±1.04	6.33	9.92	120 ±2.89	17.59	14.66
+42	5.05 ±0.14	0.83	16.44	15.1 ±0.17	1.03	6.82	59.7 ±1.34	8.21	14.18	114 ±2.52	15.40	13.51

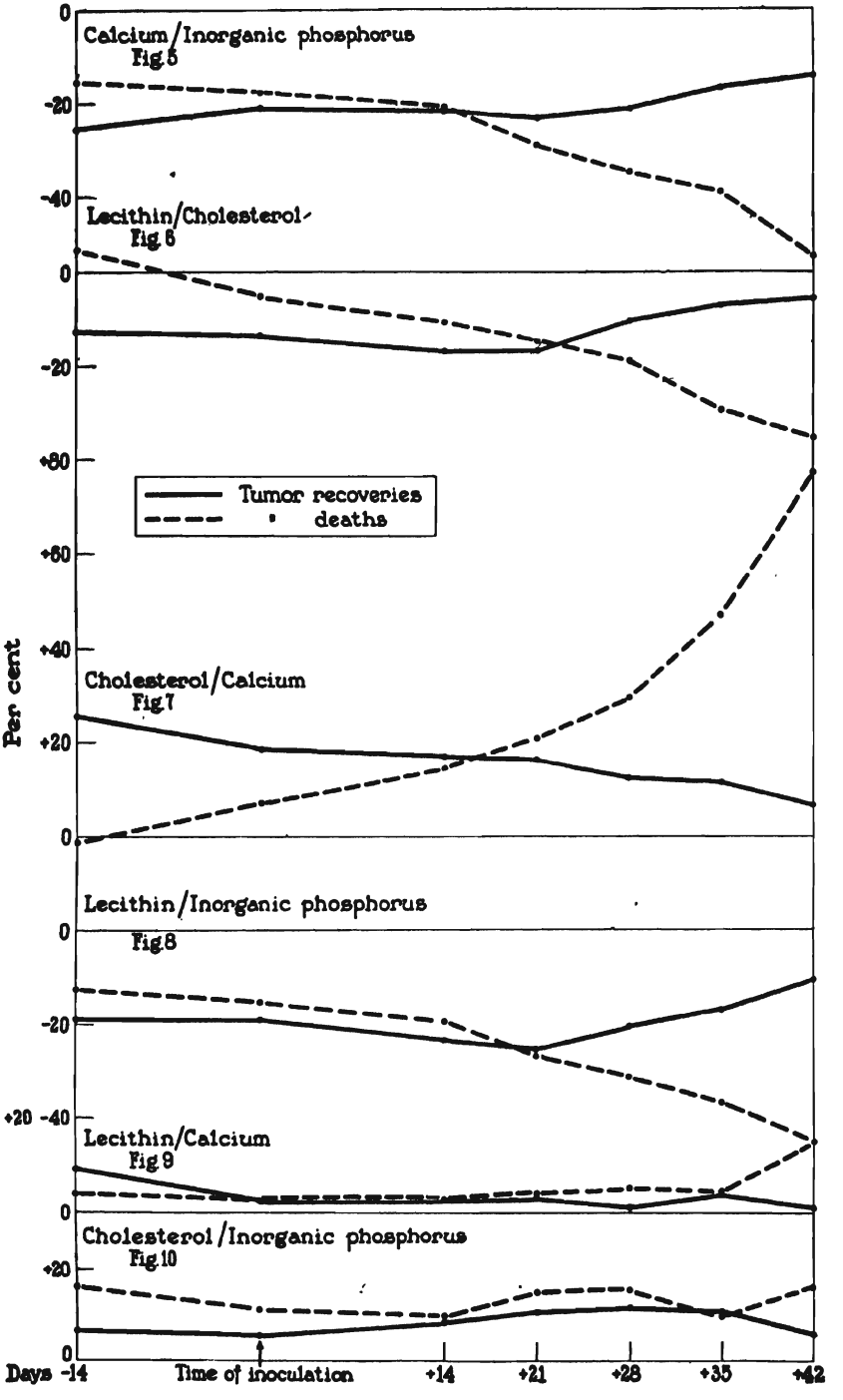
TABLE III

Coefficients of Correlation

	rP. Ca.	rP. Chol.	rP. Lec.	rCa. Chol.	rCa. Lec.	rChol. Lec.	
Deaths from Tumor. Group I							
Trend	−0.654 ±0.12	+0.867 ±0.05	+0.165 ±0.21	−0.511 ±0.16	+0.290 ±0.20	−0.083 ±0.22	N = 7
Individual mean	−0.197 ±0.18	+0.653 ±0.12	−0.571 ±0.13	−0.200 ±0.18	+0.768 ±0.07	−0.066 ±0.20	N = 12
Series	−0.477 ±0.06	+0.537 ±0.05	−0.138 ±0.08	−0.319 ±0.07	+0.277 ±0.08	−0.072 ±0.09	N = 83
Recoveries from Tumor. Group II							
Trend	+0.113 ±0.21	+0.329 ±0.19	+0.337 ±0.19	+0.542 ±0.16	+0.729 ±0.11	+0.546 ±0.16	N = 7
Individual mean	−0.400 ±0.13	−0.428 ±0.13	−0.350 ±0.14	+0.171 ±0.15	+0.618 ±0.10	+0.066 ±0.22	N = 18
Series	−0.188 ±0.06	+0.206 ±0.05	−0.192 ±0.06	+0.086 ±0.07	+0.305 ±0.05	+0.029 ±0.07	N = 122


Tumor recoveries
deaths
Inorganic phosphorus
Fig. 1
Calcium
Fig. 2
Cholesterol
Fig. 3
Lecithin
Fig. 4
Per cent
+80
+60
+40
+20
0
0
+60
+40
+20
0
0
-20
Days -14
Time of inoculation
+14
+21
+28
+35
+42

FIGS. 1–4


Calcium/Inorganic phosphorus
Fig.5
Lecithin/Cholesterol
Fig.6
Tumor recoveries
deaths
Cholesterol/Calcium
Fig.7
Lecithin/Inorganic phosphorus
Fig.8
Lecithin/Calcium
Fig.9
Cholesterol/Inorganic phosphorus
Fig.10
Per cent
Days -14
Time of inoculation
+14
+21
+28
+35
+42

FIGS. 5–10

DISCUSSION AND CONCLUSIONS

An examination of the results given in Tables I and II and the curves in Text-figs. 1 to 4 show certain striking differences between animals which died from the tumor and those that recovered. The differences were exhibited chiefly in the variation and trend of inorganic phosphorus, calcium, and cholesterol, the lecithin remaining more or less constant in both groups of animals.

Inorganic Phosphorus.—At the beginning of the experiment, the inorganic phosphorus (Text-fig. 1) in Group I was slightly lower than that obtained for Group II. At the time of inoculation both groups showed a decrease in inorganic phosphorus but the relative position was the same. The next examination, 14 days after inoculation, gave values of inorganic phosphorus which coincided. From this period, Group I gave increasing values for inorganic phosphorus and at the end of the experiment the results were 60 per cent higher than those recorded at the time of the first examination. Group II showed a steady decrease in inorganic phosphorus, the final level for this substance being 20 per cent lower than that obtained at the first examination. The inorganic phosphorus exhibited the highest per cent variation.

Calcium.—The calcium (Text-fig. 2) in Group I, at the −14 day period, was slightly higher than that found in Group II. With a gradual decrease in trend, this position was maintained until after the +14 day period at which time the position of the 2 groups was reversed. From this time, the results for Group I showed a more or less constant level until at the time of the last examination the values obtained for calcium were decidedly lower than those obtained for Group II. The calcium in the blood serum of Group II exhibited an uninterrupted increase beginning 14 days after inoculation and continuing throughout the remaining period of the experiment.

Cholesterol.—The cholesterol (Text-fig. 3) content of the whole blood in Group I was practically 20 per cent higher than that found in Group I at the −14 day examination. From this high level, the cholesterol in Group II showed a marked decrease which continued throughout the remainder of the experiment. At the

time of inoculation, the cholesterol content of the whole blood in Group I was practically the same as that of the preceding examination. From this time, however, the cholesterol increased rapidly, the last value being 55 per cent higher than that obtained for Group II.

Lecithin.—The lecithin (Text-fig. 4) level for Group I was slightly higher than that found in Group II throughout the entire experiment, with the exception of the results obtained at the +35 day period. At this time, the values for Group I were slightly lower than those for Group II. From the beginning of the experiment, both groups exhibited a gradual decrease in lecithin which continued until 14 days after inoculation. During the next 7 days both groups remained practically constant. From this period, Group II showed a gradual increase in lecithin, reaching a maximum on the +35 day examination. At the time of the last examination, however, the values were again lower than Group I. The lecithin for Group I throughout this same period maintained an almost constant level.

Ratios

The ratio curves (Text-figs. 5 to 10 inclusive) are presented in terms of per cent variation of the smoothed values from standard ratios (2).

The calcium-inorganic phosphorus ratio (Text-fig. 5) of Group I at the −14 day period was almost 10 per cent higher than that found in Group II. At the time of inoculation, the ratio for Group I showed a slight decrease while in Group II the ratio was markedly increased. From the time of inoculation throughout the remainder of the experiment, the ratio of calcium to inorganic phosphorus for Group I continued to decrease, the last determination being 36 per cent lower than that found in Group II. This low value was due to both an increase in inorganic phosphorus and a decrease in calcium. The trend of the ratio for Group II following inoculation showed a decrease of about 2 per cent at the +21 day period. From this time on, however, the ratio in this group continued to rise, reaching its highest value at the time of the last examination.

The lecithin-cholesterol ratio (Text-fig. 6) for Group I began with

a value about 4 per cent above the standard. From this time throughout the entire experiment, the ratio for Group I showed an uninterrupted decrease and at the last examination was 29 per cent below Group II, the decrease being affected chiefly by the marked increase in cholesterol occurring in this group of animals. At the —14 day examination, the lecithin to cholesterol ratio in Group II was some 16 per cent lower than that found in Group I. Throughout the next 28 day period, the ratio trend showed a slight decrease. During the next 7 days there was a tendency toward stabilization. Following this period of stabilization, however, the ratio trend in Group II showed a marked increase which continued throughout the remaining period of the experiment.

The cholesterol-calcium ratio (Text-fig. 7) for animals in Group I began with a value slightly under the standard. There occurred a gradual increase in the ratio up to the +14 day period. Following this, the ratio increased very rapidly and at the time of expiration the value for the cholesterol-calcium was 80 per cent higher than that found at the —14 day examination. This high ratio value was due to both an increase in cholesterol and a decrease in calcium. The cholesterol-calcium ratio for animals in Group II at the —14 day examination was 25 per cent higher than that found in the animals of Group I. From this initial value, the ratio trend continued to decrease gradually until the +21 day period. Following this date, the decrease which continued throughout the remainder of the experiment, was more rapid and at the final examination was practically 18 per cent lower than the first determination. A more detailed discussion of calcium-cholesterol relationship will be presented in a subsequent paper.

The trend of the lecithin-inorganic phosphorus ratio (Text-fig. 8) for both groups was somewhat similar to that of calcium-inorganic phosphorus. At the —14 day examination, Group I showed a ratio level about 5 per cent higher than Group II. With a slight decrease in trend, the 2 groups maintained practically the same relationship until the +21 day period when their ratio positions were reversed. From this date, the ratio for animals of Group I showed a rapid decrease while that for Group II gave increasing

values. This change in position for both groups of animals can be attributed chiefly to the variation of inorganic phosphorus.

The lecithin-calcium ratio (Text-fig. 9) for animals in Group I was slightly lower than that found in Group II. At the time of inoculation and throughout the following +14 day period, the ratios for both groups coincided. At the time of the +21 day examination, the ratio for Group I was slightly higher than that found in Group II. This position continued throughout the remainder of the experiment and at the time of the last examination the animals of Group I gave a ratio for lecithin-calcium 14 per cent higher than that of Group II.

The cholesterol-inorganic phosphorus ratio (Text-fig. 10) showed the least per cent variation. Group I which at the —14 day examination gave a ratio value 10 per cent higher than Group II, showed a gradual decrease in trend until the +14 day period. During the next 14 days a slight increase occurred to be followed by a rapid decrease. This decrease occurred at the +35 day examination and was the only time during the experiment when the cholesterol to inorganic phosphorus ratio for Group I was below that of Group II. This final examination resulted in a value for Group I which was 10 per cent higher than that found in Group II. During the 14 days preceding inoculation, the trend of the cholesterol-inorganic phosphorus ratio for Group II paralleled that of Group I. Following inoculation the ratio for Group II gradually increased, reaching its highest level on the +28 day examination. From this time, the ratio decreased, the decrease being more rapid throughout the last 7 days of the experiment.

It will be noted that in all ratio curves the change in position or trend occurred about 21 days after inoculation. This corresponds in a measure with the clinical course of the disease, for it is about this time that the ultimate fate of the animal is determined; from this point onward the tumor either pursues a malignant course or is gradually brought under control and recovery begins.

In the following table a comparison is made between the two groups of animals with respect to their ratio positions at the end of the experiment, 1 representing the highest and 4 the lowest value.

Ratio Positions of Tumor Animals

Group	Ca./P.	Lec./Chol.	Chol./Ca.	Lec./P.	Chol./P.	Lec./Ca.
II	1	1	4	1	4	1
I	4	4	1	4	1	1

When compared with a previous report on the influence of light environment (3), it will be noted that with the exception of the cholesterol to inorganic phosphorus ratio, the animals in Group I occupy a position similar to that of animals exposed to the ultra-violet light, while Group II occupies a position similar to animals living in total darkness.

Correlation

The coefficients of correlation for both groups of animals calculated on the basis of trend, individual mean values, and for each group series, are presented in Table III. It will be noted that the highest negative r inorganic phosphorus-calcium occurred in the animals of Group I. In Group II the r calcium-cholesterol were all positive while in Group I these same coefficients were all negative and of a somewhat greater magnitude. The r cholesterol-lecithin were all positive in Group II. These values while rather small were all negative in Group I.

Summarizing the results of this experiment, it will be noted that animals in which the tumor proved most malignant (Group I) there occurred a marked increase in inorganic phosphorus of the blood serum and cholesterol of the whole blood and a decrease in serum calcium. The ratios of cholesterol to calcium and lecithin to calcium increased in trend following inoculation, while the ratios of calcium to inorganic phosphorus, lecithin to cholesterol, and lecithin to inorganic phosphorus showed a marked decrease in trend. The animals in Group II exhibited trends and ratios of these 4 blood constituents similar to the control animals (2).

SUMMARY

Experiments are reported in which it was shown that the calcium, inorganic phosphorus, cholesterol, and lecithin in the blood of

normal rabbits were influenced by inoculation with a malignant tumor (4).

Animals in which death was due to tumor gave results on which the following conclusions are based:

1. A marked increase in inorganic phosphorus of blood serum and cholesterol of whole blood followed inoculation.

2. The ratios of cholesterol to calcium and lecithin to calcium increased in trend, while the calcium to inorganic phosphorus, lecithin to cholesterol, and lecithin to inorganic phosphorus ratios showed a marked decrease in trend.

3. The r cholesterol-lecithin were all negative.

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[Reprinted from THE JOURNAL OF THE AMERICAN MEDICAL ASSOCIATION, May 25, 1929, Vol. 92, No. 21, pp. 1725–1727]

RELATION OF STREPTOCOCCI TO THE SPINAL FLUID IN EXPERIMENTAL POLIOMYELITIS

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(From the Laboratories of The Rockefeller Institute for Medical Research)

Rosenow and his collaborators and others[1] have insisted on the identity of streptococci with the filtrable virus of poliomyelitis, a view that has not been generally accepted. For example, Bull[2] expressed the opinion that the bacteria are secondary invaders in the disease, and Smillie[3] and Amoss[4] viewed the cocci as being agonal invaders.

Recently Long, Olitsky and Stewart[1] showed that there may be still another source of the streptococci; namely, the air of the place in which the cultures are made. Furthermore, their experiments revealed that cultures of other organisms, such as staphylococci, diphtheroids, spore-bearing rods, and other miscellaneous, familiar microorganisms, can be obtained frequently from the ground up brains of monkeys with poliomyelitis, the source of these being also the air. Certain biologic tests were reported which demonstrate the wide variation of the effects of streptococci from those of the true filtrable virus of poliomyelitis.[5]

1. References are given by Long, P. H.; Olitsky, P. K., and Stewart, F. W.: The Rôle of Streptococci in Experimental Poliomyelitis of the Monkey, J. Exper. Med. **48**: 431 (Sept.) 1928.

2. Bull, C.G.: The Pathologic Effects of Streptococci from Cases of Poliomyelitis and Other Sources, J. Exper. Med. **25**: 557 (April) 1917.

3. Smillie, W. G.: Cultivation Experiments on the Globoid Bodies of Poliomyelitis, J. Exper. Med. **27**: 319 (March) 1918.

4. Amoss, H. L., in Rivers, T. M.: Filtrable Viruses, Baltimore, Williams & Wilkins Company, 1928, p. 173.

5. Additional recent tests on the distinctive properties of streptococci and the virus of poliomyelitis are given by Long, P. H., and Olitsky, P. K.: Comparison of the Resistance of Streptococci and of Poliomyelitic Virus in Glycerol, Proc. Soc. Exper. Biol. & Med. **26**: 337 (Jan.) 1929. Similar experiments on the source of streptococci in another virus, that of herpes virus encephalitis, are described by Olitsky, P. K., and Long, P. H.: The Relation of Streptococci to Herpes Virus Encephalitis, J. Exper. Med. **48**: 199 (Aug.) 1928.

Shortly after the publication of the latter article, a paper by Rosenow[6] appeared in THE JOURNAL in which he stated that streptococci were obtained from the spinal fluid of five patients during life, of two patients after death, and of each of three monkeys in which typical poliomyelitis followed intracerebral inoculation of virus from three fatal human cases. Rosenow states futher: "The freshly isolated strains of streptococci, like those obtained in previous epidemics, produce in rabbits flaccid paralysis and other symptoms which I have come to regard as more or less characteristic of poliomyelitis in these animals."

We had not made cultures of spinal fluids because such material is, as a rule, free from virus, as we shall show immediately. In view of Rosenow's recent observations, however, an obvious discrepancy was apparent between his results and the prior conclusions obtained in this laboratory. We therefore undertook anew a study of the relationship of streptococci to the virus of poliomyelitis. This study concerned especially the cultivation of the spinal fluids of poliomyelitic monkeys according to the methods used by Rosenow.

Methods

The following method of preparation of the selective yeast medium employed by Dr. Rosenow[6] was kindly supplied at our request:

> Routinely, we prepare an emulsion of this (Fleischmann's) yeast by adding 500 cc. of distilled water to one-half pound of yeast. The suspensions made from different samples which we have worked with have all been acid to litmus. This acid suspension or emulsion is then autoclaved at 17 pounds pressure for twenty minutes, in 8 ounce bottles about 5 cm. in diameter, which fit the cups in our centrifuge. After sterilization the mixture is thoroughly centrifugated and the supernatant opalescent and cloudy material is decanted in a sterile manner and then rendered slightly alkaline to litmus, with a 40 per cent solution of sodium hydroxide. This is then used as the culture medium in varying amounts and in varying dilutions. It is difficult to say precisely what dilution is best to use, for the results are so irregular; but we have obtained growth most constantly when the undiluted extract is distributed in small amounts of 2 cc. in ordinary sized test tubes, to which varying amounts of the spinal fluid are added. In some instances growth has occurred when one part of this extract is added to 9 parts of the spinal fluid itself.

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6. Rosenow, E. C.: Streptococci in the Spinal Fluid in Acute Epidemic Poliomyelitis, J. A. M. A. 91: 1594 (Nov. 24) 1928.

Dr. Rosenow also stated that:

The centrifugated extract often contains a few gram-positive streptococci which must be differentiated from the growth obtained from the spinal fluid.

and further that:

In the instances where we succeed in growing the streptococcus in subculture from the primary growth in the yeast medium inoculated with the spinal fluid of cases of poliomyelitis, there is, of course, no question as to the source of the streptococcus. In those instances in which we fail to obtain growth in subculture, differentiation is made between the small number of streptococci found in the autoclaved yeast extract and those which we believe represent growth from the spinal fluid, by the increased cloudiness of the inoculated tube as compared with the control tube of the uninoculated extract of the same batch and in like amount, and incubated side by side; by the presence of a large number of organisms in smears of the inoculated medium, as compared with incubated control tube, and by differences in morphology and staining reaction. The streptococci that occur in the yeast extract are smaller, more slender, more uniform in size, less gram-positive, and the margin is less distinct than the streptococci which we feel represent a primary growth.

Stained-Film Preparations and Cultures

With these instructions kindly supplied by Dr. Rosenow and with the directions in his paper[6] as bases for procedure, we made twenty-four examinations of "smear" or stained-film preparations of the spinal fluids of nineteen monkeys suffering from experimental poliomyelitis. In five instances the spinal fluids of the same monkeys were studied during early and late periods of infection. In addition, twenty-two cultivation tests were made with these fluids from seventeen of the monkeys, the test being repeated in the early and late stages of the characteristic disease in five.

Stained-Film Preparation.—From gram-stained preparations of the sediment of the twenty-four centrifugated spinal fluids, no definite evidence of streptococci was detected. In two instances, single diplococcoid bodies were seen, but in view of the fact that a similar picture was obtained from the materials of the gram stain alone and also that the fluids were sterile by culture, the bodies could be considered only as artefacts.

Cultivation Tests.—Twelve cultures were made in a series of long

tubes of ascitic broth, in ascites-tissue fluid, and on blood agar—material also employed by Rosenow[6] for cultivation of streptococci from poliomyelitis. Ten tests were made in the yeast medium already described. Each series of the ten comprised from three to four tubes. As a rule, 2 cc. of the yeast medium was inoculated with different amounts of spinal fluid, and in two instances nine parts of spinal fluid were added to one of the yeast medium, a procedure sometimes followed by Rosenow. Because the yeast medium was opaque to start with, it was thought that both inoculated and uninoculated tubes might fail to reveal by visual inspection a sparse growth of bacteria. Hence the material from all the tubes was examined microscopically by means of stained films after twenty-four hours' and fourteen days' incubation.[1] Transfers were then made to broth and to rabbit blood dextrose-agar plates.

The results of the cultivation tests were as follows:

In mediums other than the yeast, there was no evidence of visible growth of bacteria. In the series of cultures with yeast medium, however, another picture was seen. The yeast itself contained, as foretold by Rosenow, a number of streptococci, revealed by an examination of gram-stained film preparations. The process of autoclaving was evidently sufficient to kill these bacteria, for repeated transfers of the yeast medium to other mediums favorable to the growth of streptococci failed to yield any living micro-organisms. The dead streptococci were in the form of diplococci and of chains, the individual cocci being small and lanceolate. In fresh yeast "extract" they were gram-positive, but after the medium was kept for fourteen days in the cold, the micro-organisms became fewer in number and showed several gram-negative forms. After sixteen days the streptococci had disappeared from the medium, probably as a result of autolysis. Precisely similar conditions were noted in the cultures of spinal fluids in this medium. Streptococci could be seen matching exactly those in the yeast extract itself, in respect both to numbers and to morphology. These showed the same degree of degeneration, corresponding to that of the bacteria in the yeast medium used in the particular culture. When an older medium was employed, that is, one in which the bacteria had completely disappeared, then the spinal fluid cultures were also free from organisms in gram-stained film preparation. Moreover, no growth was obtained after the inoculated or the control, uninoculated yeast medium was subplanted to broth or to blood agar.

Characteristics of Rosenow's Strain of Streptococcus

The next step in this study concerned the characteristics of the streptococcus obtained from spinal fluids in cases of poliomyelitis.

In view of our failure to isolate this micro-organism from the spinal fluids of the poliomyelitic monkeys which we have studied, we requested a culture of Dr. Rosenow. He kindly sent us a culture derived from the spinal fluid of a patient with poliomyelitis, with the reservation that the streptococcus may have lost its specific infecting power and immunologic properties. The following studies were carried out with this culture:

Cultural Characteristics.—The streptococcus grew in solid medium in the form of diplococci or short chains, but in fluid medium the lanceolate cocci became somewhat more evenly rounded and appeared in much longer chains. In plain or dextrose broth there was a diffuse clouding of the medium with a heavy, whitish, precipitated sediment. On blood agar the colonies were round, small and greenish and were surrounded by a very narrow zone of cleared medium. The cocci grew profusely in ordinary peptone water containing different carbohydrates. In such mediums, acid was formed with dextrose, maltose, lactose, saccharose and salicin, but no acid was noted with raffinose or mannitol. With inulin there was shown either no acid or at times a slight degree of acidity.

In all these respects the streptococcus corresponded closely with a streptococcus obtained from the brain of a normal monkey and with another derived from the air of the laboratory, which have already been described.[1]

Rabbit Inoculation.—In earlier papers, a series of intracerebral injections of rabbits with streptococci derived from poliomyelitis and other sources was reported.[7] The present Rosenow spinal fluid strain acted in a similar manner to the other streptococci, as the following protocol shows:

Two rabbits were inoculated[8] intracerebrally with 0.4 cc. of a broth culture of Rosenow's spinal fluid streptococcus strain. One was found dead after twenty hours; the other, at this time, was moribund. The latter animal showed generalized spasmodic, muscular movements, with spasmodic head movements. The eyes rolled upward and the animal was on its side in a state of opisthotonos. Frequent spastic muscular contractions of the limbs were noted. This animal was killed by etherization. Stained-film preparations of the brains of both rabbits

7. Long, Olitsky and Stewart (footnote 1). Long and Olitsky (footnote 5). Olitsky and Long (footnote 5).

8. All operative procedures were done under full ether anesthesia.

revealed numerous polymorphonuclear cells and a few streptococci. Cultures of the brain and of the heart's blood of both yielded pure cultures of streptococci. Microscopic examination of the two rabbits showed the purulent type of meningoencephalitis, often associated with streptococcic septicemia. It is needless to repeat in detail the microscopic observations, for they have already been given.[7] No difference was detected in the effects of the Rosenow spinal fluid streptococcus in the rabbit and the effects of strains obtained from the air, from poliomyelitic and nonpoliomyelitic tissues, from medium, or from ground normal or herpes virus-infected brain.[7]

Contrary to Rosenow's finding, the rabbit is considered by other investigators as resistant to poliomyelitic infection.[9] Thus, in our experience, the following massive doses of fresh and filtered virus failed to infect rabbits: 20 cc. of a Berkefeld V filtrate of a 5 per cent suspension of active monkey brain injected intravenously, plus 0.5 cc. of a 50 per cent emulsion of the same brain injected intracerebrally in both hemispheres, plus 20 cc. of a 40 per cent emulsion injected intraperitoneally, plus 6 cc. of a 40 per cent emulsion injected intraspinally (cervical region), plus cotton plugs soaked in 50 per cent emulsion and packed into both nostrils. The monkey responds to this virus when inoculated with far smaller doses.

Inoculation of Monkeys

A study was then undertaken to determine the effects of Rosenow's spinal fluid strain of streptococci in normal monkeys and monkeys recovered from infection with the poliomyelitic virus. The following protocol is illustrative of the results obtained:

Two monkeys were used. Animal A had been injected intracerebrally with active poliomyelitis virus six months previously and developed the characteristic disease, which progressed to prostration. The monkey recovered but showed marked residual paralysis, with contracture of the leg muscles and moderate atrophy of certain muscle groups of the arms. This animal resisted subsequent intracerebral inoculation of active virus and his blood serum was shown to be capable of neutralizing poliomyelitis virus in vitro. Monkey B was a normal animal taken from stock. Each was inoculated intracerebrally with 1 cc. of the centrifugated sediment of a 50 cc. twenty-four hour broth culture of the streptococcus. Twenty-four hours after inoculation, both animals were somewhat less

9. Flexner, Simon; and Lewis, P. A.: Epidemic Poliomyelitis in Monkeys: The Activity of the Virus, J. A. M. A. **54**: 45 (Jan. 1) 1910.

active than usual and tended to tire easily. They appeared quite normal in every other respect. Forty-eight hours after inoculation, the symptoms were about the same. Lumbar puncture revealed a very cloudy spinal fluid. The fluid from monkey A contained 12,600 cells per cubic millimeter and that from monkey B, 12,000 cells. In both cases the majority of the cells were polymorphonuclear leukocytes. The spinal fluids were spread on slides and stained by Gram's method. No organisms were observed and no growth was obtained on culture. Seventy-two hours after inoculation the animals appeared essentially normal and no further symptoms developed during the period of observation. At no time were any symptoms observed suggesting those appearing in monkeys infected with the virus of poliomyelitis. This experiment was repeated with similar results.

A normal monkey was injected intracerebrally with 1 cc. of the sediment of a twenty-four hour broth culture of Rosenow streptococci. Twenty-four hours after inoculation the animal was somewhat ataxic and moved rather slowly. Spinal fluid withdrawn by lumbar puncture was very cloudy and contained 8,200 cells, mostly polymorphonuclear leukocytes. There was no essential change forty-eight hours after inoculation, and after seventy-two hours the animal appeared normal. It was killed and the central nervous system examined histologically. The microscopic examination revealed an acute, purulent meningitis, without changes in the nerve cells, in their processes, or in the supporting structure. Scattered throughout the meninges of the brain and cord were large clumps of polymorphonuclear cells and a few mononuclears, often associated with masses of fibrin and a moderate number of erythrocytes. The characteristic nerve cell and other lesions of poliomyelitis were absent.

Spinal Fluid As a Source of Virus

The opinion of Rosenow that spinal fluid is a tissue in which the virus of poliomyelitis (identified as the streptococcus) exists is not in accord with the results of other workers.[10]

Recent tests with the spinal fluid used in the cultivation experiments already mentioned have again shown that the fluid is free from virus. For example, 2 cc. of spinal fluid which had been removed by lumbar puncture from a monkey acutely ill with poliomyelitis was without effect when injected intracerebrally into a normal monkey.

10. Clark, P. F., and Amoss, H. L.: Intraspinous Infection in Experimental Poliomyelitis, J. Exper. Med. **19**: 217 (Feb.) 1914. Flexner, Simon; and Amoss, H. L.: Penetration of the Virus of Poliomyelitis from the Blood into the Cerebrospinal Fluid, J. Exper. Med. **19**: 411 (April) 1914.

SUMMARY AND CONCLUSIONS

In twenty-four examinations of stained-film preparations of centrifugated spinal fluids from nineteen monkeys showing characteristic experimental poliomyelitis, streptococci were not found. Twenty-two cultivation tests, including the use of Rosenow's yeast medium, were made with the fluids from seventeen of the monkeys. These also failed to yield streptococci. The yeast medium itself contains streptococci; it therefore cannot be regarded as a medium of choice for the isolation of this micro-organism.

The properties of a streptococcus isolated by Rosenow from the spinal fluid of a patient with poliomyelitis correspond in essential characteristics with those of a streptococcus obtained from the brain of a normal monkey and from the air of the laboratory.

The inoculation of Rosenow's streptococcus into rabbits, which animals are generally considered as resistant to the virus of poliomyelitis, induces a purulent meningo-encephalitis, such as is often associated with streptococcic septicemia. The microscopic appearance of the brain of such rabbits differs essentially from that of poliomyelitis in man and the monkey.

Inoculation effects in the rabbits similar to those produced by the Rosenow strain can be obtained with streptococci derived from poliomyelitic tissues and from other sources.

The inoculation of Rosenow's streptococcus 'intracerebrally in monkeys does not bring out any difference in reaction in normal animals and those recovered from poliomyelitis. This is in direct conflict with the susceptibility of normal and the insusceptibility of recovered monkeys to the virus of poliomyelitis. The microscopic change in the monkeys injected with the streptococcus is that of an acute purulent meningitis, which differs wholly from the nervous lesions present in poliomyelitis in man and the monkey.

If the streptococcus of poliomyelitis loses on cultivation its specific infecting power, this fact is in disagreement with the well known observation that the filtrable virus of the disease retains its specific infecting power as long as it is viable.

Attempts to infect monkeys by means of intracerebral inoculation of spinal fluid derived from monkeys and human beings suffering

from poliomyelitis have been uniformly unsuccessful. It is therefore apparent that spinal fluid cannot be regarded as a source of the infectious agent.

Finally it may be stated that this report offers additional support to the view that the streptococcus cannot be regarded as being identical with the filtrable virus of poliomyelitis.

[Reprinted from THE JOURNAL OF EXPERIMENTAL MEDICINE, June 1, 1929,
Vol. 49, No. 6, pp. 959–973]

INTRADERMAL VERSUS SUBCUTANEOUS IMMUNIZATION OF MONKEYS AGAINST POLIOMYELITIS

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(Received for publication, March 2, 1929)

Early experiments of Flexner and Lewis (1), Levaditi and Landsteiner (2), and Römer and Joseph (3) showed that monkeys once recovered from poliomyelitis are immune to subsequent intracerebral inoculations of poliomyelitis virus. This immunity was apparent no matter how slight had been the symptoms of the initial infection. The refractory state was of long duration and was absolute within the limit of infecting doses employed. These same workers noted that sera of convalescent monkeys, when mixed with poliomyelitis virus *in vitro*, rendered the material, otherwise infective, inactive when introduced intracerebrally in test animals.

These observations became the basis for numerous efforts to immunize animals against experimental poliomyelitis. Flexner and Lewis (4) injected monkeys subcutaneously with living active virus, beginning with a dose of 0.05 cc. This amount was given daily for four days and the series was repeated twice with a four day rest period between each individual set of injections. After the last interval the animals received on successive days 0.1, 0.5, and 1.0 cc. of virus, and after one month 5.0 cc. A week's rest period then followed, after which time the animals were tested intracerebrally with 2.0 cc. of fresh Berkefeld filtrate of poliomyelitis virus. Control monkeys which received 0.1 to 0.01 cc. of a similar filtrate intracerebrally developed typical poliomyelitis, whereas the vaccinated animals remained free from symptoms.

Levaditi and Landsteiner (5) attempted to immunize monkeys by a single subcutaneous inoculation of 0.5 cc. of virus suspension previously heated to 50°C. for 30 minutes. They failed to produce any immunity by this treatment. In another experiment glycerinated virus was heated to 50°C. for 2 hours; this heated virus was still active in producing disease when inoculated intracerebrally, but did not infect when given daily in subcutaneous doses of 2 cc. each over a period of one month. Nine days after the last subcutaneous injection two treated monkeys were tested intracerebrally. One of these showed slight prodromal symptoms of

poliomyelitis and the second gave no evidence of disease, whereas the control developed typical poliomyelitis.

Kraus (6) attempted the attenuation of poliomyelitis virus by phenolization and found that virus treated by 1 per cent phenol was rendered ineffective in four days, even when inoculated subdurally. He then endeavored to immunize monkeys by subcutaneous injection of 5 to 10 cc. of virus treated with varying concentrations of phenol for different periods of time. Of fifteen animals subjected to intracerebral test inoculations, twelve were immune. It is interesting to note that three animals immunized with virus treated with 1.5 per cent phenol for five days—a procedure calculated by Kraus to render virus inactive—were completely protected. In a second communication Kraus (7) reports results on two animals, one of which received 5 cc. of fresh virus cord emulsion subcutaneously, followed fifteen days later by 6 cc. of 0.5 per cent phenolized cord; a second monkey received 6 cc. of 0.5 per cent phenolized cord. Ten days later both monkeys resisted a test intracerebral inoculation with paper filtrate, whereas a control developed the typical disease.

Olaf Thomsen (8) gave monkeys daily sub-infective inoculations subcutaneously for twelve days and subsequently at weekly intervals, 0.06, 0.2, 0.4, 1.0, and 2.0 cc. of virus suspension. All animals were then resistant to intracerebral test but the author states that every animal showed symptoms such as excitement, tremor, and ataxia, during immunization. A second group was treated, using considerably smaller immunizing doses; of this series no animal showed symptoms during the immunizing procedure, yet all resisted test inoculation. The initial immunizing dose in this second series was only one hundredth of the estimated intracerebral infecting dose.

Zappert, Wiesner, and Leiner (9) attempted to immunize four monkeys by means of subcutaneous injections of gradually increasing doses of active virus emulsions. During the immunization, two of the animals died of intercurrent infection, one of typical poliomyelitis, and one of a supposedly marantic type of the disease. They attempted to induce an artificial immunity in one animal by the use of phenolized virus. The monkey developed the disease during the treatment designed to immunize against it.

Flexner and Amoss (10) described a so-called immunizing strain of poliomyelitis virus. To free a contaminated glycerinated brain from organisms, the tissue was immersed in 0.5 per cent phenol for a few hours and replaced in glycerine. This procedure was repeated once. The animals were subsequently infected with 1.0 cc. of 10 per cent suspension, their disease running an average eleven day course. In a series of passages the virulence of the strain decreased until a point was reached where the monkeys showed very few symptoms,—such as ataxia, tremor, and slight convulsive seizures—recovered, and were subsequently immune to strong virus.

Abramson and Gerber (11) treated emulsions of brain and cord of poliomyelitic monkeys for four hours with 0.5 per cent formaldehyde; this material was infective

when introduced subcutaneously in monkeys. They then endeavored to immunize by heated virus. On five successive days monkeys were injected with cord emulsion; the emulsion was heated to 55°C. for thirty minutes the first and second day, to 45°C. for thirty minutes the third day, to 37°C. for thirty minutes the fourth, and was used without preliminary heating on the fifth day. The dose was 5 cc. on each day. Three weeks after treatment the animals were bled and their sera tested for its power to neutralize virus. Of eight sera, three neutralized, four led to prolonged incubation period, and one failed. Intracerebral tests indicated that five of the treated monkeys were resistant to three to six minimum lethal doses of virus, whereas three proved susceptible.

In another series of three monkeys, Abramson and Gerber gave daily injections subcutaneously of 5 cc. of 10 per cent cord emulsion previously heated to 55°C. for one hour. On intracerebral test three weeks later, all developed poliomyelitis; of the three sera tested, one monkey showed no symptoms and two a delayed incubation period but eventually developed the disease.

McKinley and Larson (12) inoculated monkeys intracerebrally with 0.15 cc. of filtrate of a mixture of 5 per cent emulsion of castor oil soap and virus emulsion. The animals remained well and later resisted intracerebral inoculation of 0.7 cc. virus filtrate. Four more monkeys received 4 cc. of the virus-soap mixture intraperitoneally; none developed poliomyelitis, whereas a control with virus alone became paralyzed in a typical manner. Eleven days after the intraperitoneal virus-soap treatment, all four monkeys were tested intracerebrally; three remained well and one developed poliomyelitis.

The largest and most varied series of tests of poliomyelitis immunization is that of Aycock and Kagan (13). These investigators attempted to immunize with virus attenuated by various methods. The old experiments of Kraus with phenolized virus were repeated using material treated with 1.0, 0.75, 0.50, and 0.25 per cent phenol. The mixtures were kept for seven days in the icebox. Monkeys were then given four injections every other day of from 8 to 10 cc., beginning with the 1.0 per cent phenolized virus, and ending with the 0.25 per cent. Of four animals so treated, two became paralyzed during the process of vaccination, one failed to resist intracerebral test inoculation, and one resisted. In a second experiment monkeys were injected subcutaneously with virus cords dried over caustic potash from one to twenty-six days. Two of six monkeys became paralyzed during treatment, two failed to show protection on intracerebral inoculation, and two proved resistant. Next, virus cord was exposed to different glycerol-water dilutions (5 to 50 per cent glycerol) for seven months at ice box temperature. Monkeys were injected daily subcutaneously, beginning with virus from 5 per cent glycerol and ending with 50 per cent glycerol. Three animals developed paralysis during immunization; three failed to resist an intracerebral test; one resisted. In another group, virus in agar was introduced subcutaneously in eight animals; the total virus emulsion given ranged from 20 to 96 cc. of 5 per cent suspension in

from three to seventeen injections; two animals became paralyzed during treatment, two failed to show subsequent immunity, and two resisted.

In a fifth experiment virus was introduced intracutaneously in from 1 to 2 cc. amounts but was distributed in 0.05 cc. blebs, thus making from twenty to forty piqures each day of inoculation. The total amount of virus injected ranged from 5 to 76 cc. in six to forty-three inoculations, given during a period ranging from fifteen days to five months. Twelve monkeys were used; one became paralyzed during treatment; one failed to resist intracerebral inoculation; ten resisted one intracerebral test, but of these, two failed to withstand a second such test. Serum from eight resistant monkeys neutralized virus twenty-one times; one monkey's serum protected in one test, although the animal itself was not immune to intracerebral test inoculation.

From the review of the literature, it is apparent that the results of experiments designed to immunize monkeys against poliomyelitis have been inconclusive. Two facts stand out clearly; first, that it is impossible to protect monkeys by the use of killed virus, and second, that a definite though inconstant resistance to poliomyelitis can be brought about by the intradermal and subcutaneous introduction of the living virus. It was therefore deemed advisable to compare the results of the two routes of inoculation in order to gain information as to their relative efficacy. The following experiments were carried out with this point in view.

Experimental

Eight monkeys (Table I) were immunized by the intracutaneous route, following in general the procedure of Aycock and Kagan. The injections were made biweekly and the total amount of a single day's dosage (1.5 to 2.0 cc. of 5 per cent glycerolated virus) was distributed in some twenty small blebs. The duration of the immunizing period was variable, lasting from three to five months. The total amounts of virus administered ranged from 42 to 66 cc. Before intracerebral test inoculation, all animals were bled in order to test their sera for virus-neutralizing power. The test inoculations were made with fresh virus injected intracerebrally in doses of 0.5 cc. of 5 per cent suspension. During the immunization period all animals were observed daily in order to detect possible abortive symptoms of disease and were exercised to bring out masked weaknesses.

Eight more monkeys (Table II) were treated in an analogous fashion but received their immunizing virus subcutaneously instead of intracutaneously. The amounts of virus used and the time intervals were comparable with those of the intracutaneous series, and bleedings and test inoculations were done in the same manner. Both tests for active immunity and for passive serum protection were rigorously controlled. The results in the two series are best seen in the tables.

DISCUSSION

The primary purpose of this series of experiments was to determine whether the intradermal or the subcutaneous introduction of poliomyelitis virus was most effective in protecting monkeys against virus inoculation. Reference to Tables I and II shows that the degree of immunity produced is strikingly in favor of the intradermal method. Of the eight animals subjected to that procedure, all but one showed slight symptoms of the disease when tested by intracerebral inoculation of an amount of virus sufficient to cause characteristic poliomyelitis in the controls. No animal, however, developed more than the mildest abortive symptoms, such as tremor or excitement. No definite paralysis developed in any instance, and no subsequent muscle atrophy was observed. These results are sharply at variance with those of intracerebral inoculation of the group of monkeys treated by subcutaneous inoculation of virus. Four of the eight animals of this series developed typical poliomyelitis which progressed to prostration in two instances, and to well-marked paralysis in the other two. The remaining four animals proved to be completely refractory to the intracerebral tests.

During the process of immunization, the animals were closely observed to determine whether or not they developed an abortive form of poliomyelitis which might explain the subsequent immunity to the disease. Wickman (14), during the Swedish epidemic of 1905, noticed a considerable number of cases in man, which showed slight, transient symptoms, without developing the outspoken disease. Caverly (15), in the Vermont epidemic of 1904, saw six children with fever, nausea, and convulsions, whose illness never progressed further. Medin (16) also observed such abortive cases. Aycock (17) mentions the possibility that mild attacks of poliomyelitis are responsible for the development of immunity. In view of these observations we were on the alert to detect slight symptoms referable to the treatment. However, no deviation from the normal was discovered. Subcutaneous inoculation of virus has in our experiments on eight animals failed to produce the disease, although it has given rise to poliomyelitis in the hands of others (Flexner and Lewis, Aycock, and Olaf Thomsen). As evidence of the relative safety of intradermal inoculation of virus, in experiments to be reported, as much as 16 cc. of virus suspension

TABLE I

Intradermal Immunization

Monkey	Immunization		Total virus inoculated, 5 per cent suspension	Strain	Intracerebral test			Result of test		Serum neutralization	
	Begun	Ended			Date	Amount	Strain	Tested animal	Control	Test	Control
			cc.								
1	9/21/27	12/27/27	42	M.A.	Died of tuberculosis during immunization						
2	9/21/27	1/19/28	66	M.A.	1/19/28	0.5 cc. 5 per cent suspension	M.A.	No symptoms	Typical poliomyelitis Prostrate in 6 days	Pooled neutralized	Typical poliomyelitis Prostrate in 11 days
3	9/21/27	1/ 3/28	48	M.A.	1/19/28	0.5 cc. 5 per cent suspension	M.A.	Slight excitement			
4	3/ 5/28	5/21/28	42	M.A.	5/31/28	0.5 cc. 5 per cent suspension	Aycock	Slightly slow	Typical poliomyelitis Prostrate in 6 days	Neutralized	Typical polio
5	3/ 5/28	5/21/28	42	M.A.	5/31/28	0.5 cc. 5 per cent suspension	Aycock	Tremor and ataxia; weak deltoid		Neutralized	Typical polio
6	3/ 5/28	5/21/28	42	M.A.	5/31/28	0.5 cc. 5 per cent suspension	Aycock	Slightly slow		Neutralized	Typical polio

7	6/ 8/28	11/ 1/28	56	Aycock	11/20/28	0.5 cc. 5 per cent suspension	M.A.	Slow and excited	Typical poliomyelitis Paralyzed in 8 days	Neutralized	Typical polio
8	6/ 8/28	11/ 1/28	56	Aycock	11/20/28	0.5 cc. 5 per cent suspension	M.A.	Excitement and tremor		Neutralized	Typical polio

TABLE II

Subcutaneous Immunization

Monkey	Immunization		Total virus inoculated, 5 per cent suspension	Strain	Intercerebral test			Result of test		Serum neutralization	
	Begun	Ended			Date	Amount	Strain	Test animal	Control	Test	Control
			cc.								
9	10/27/27	1/27/28	45.9	M.A.	2/11/28	0.5 cc. 5 per cent suspension	M.A.	No symptoms	Prostrate 9th day	Pooled neutralized	Paralyzed 17th day
10	10/27/27	1/27/28	42.8	M.A.	2/11/28	0.5 cc. 5 per cent suspension	M.A.	No symptoms			
11	10/27/27	1/27/28	38	M.A.	2/11/28	0.5 cc. 5 per cent suspension	M.A.	No symptoms			
12	3/ 5/28	5/21/28	42	M.A.	5/31/28	0.5 cc. 5 per cent suspension	Aycock	Prostrate 10th day	Prostrate 11 days	Neutralized	Typical polio
13	3/ 5/28	5/21/28	42	M.A.	5/31/28	0.5 cc. 5 per cent suspension	Aycock	Paralyzed 18th day. Recovered		Serum contaminated	
14	3/ 5/28	5/21/28	42	M.A.	5/31/28	0.5 cc. 5 per cent suspension	Aycock	Prostrate 9th day		Typical polio	

15	6/ 8/28	10/16/28	56	Aycock	11/20/28	0.5 cc. 5 per cent suspension	M.A.	No symptoms	Prostrate in 7 days	Not done	
16	6/ 8/28	10/16/28	56	Aycock	11/20/28	0.5 cc. 5 per cent suspension	M.A.	Paralyzed on 9th day. Recovered		Neutralized	Typical polio

has been given intracutaneously at one time without producing symptoms, while 0.005 cc. of Berkefeld filtrate of virus of the same strain inoculated intracerebrally consistently produced characteristic poliomyelitis in six days.

The question of the degree of protection conferred by the treatment proved to be an extremely interesting one. It has often been observed that different strains of poliomyelitis virus vary markedly in their power to produce the disease in susceptible animals. We therefore attempted to detect degrees of immunity by testing animals by intracerebral inoculation, not only with virus of the strain with which they had been immunized, but also with other strains. Thus monkeys treated with the M.A. strain of virus were tested with a fairly recent virus isolated in Vermont by Aycock, and animals immunized with Aycock strain were tested with the M.A. virus. The difference in the results is well-marked; monkeys treated in exactly the same way proved totally resistant to the relatively weak M.A. virus and not totally immune to the stronger Aycock virus. A group of three animals immunized with M.A. strain is described in Table III. All withstood subsequent intracerebral inoculation with both M.A. and Aycock virus but one of the three developed typical poliomyelitis on inoculation with a very active pooled, mixed virus derived from material of the original M.A. and K. strains which had been preserved in glycerol since 1920 (18, 19, 20).

That the immunity induced in the monkeys in these experiments is relative only, is more strikingly shown by tests employed to determine the power of the sera to neutralize the virus. The results of these determinations are shown in Table IV. The sera of Monkeys 1, 2, and 3 were pooled in one and those of 9, 10, and 11 in a second group. These two mixed sera neutralized, as was to be expected, since on intracerebral test the monkeys had proved resistant. Moreover, Sera 6, 7, and 8, derived from monkeys which had presented definite symptoms of poliomyelitis on intracerebral inoculation, were found also to neutralize completely a small, though ample dose, approximately 50 M.L.D. of a highly active virus filtrate of the pooled mixed virus strain. The results of the neutralization tests of sera 12 and 16 are especially significant. Although the monkeys from which they had come had proved ordinarily susceptible to intracerebral

TABLE III

Results on Reinoculation

Monkey	Method of immunization	Immunization ended	Intracerebral test			Result	
			Date	Amount	Strain	Test animal	Control
First intracerebral inoculation							
2	Intradermal M.A.	1/19/28	6/11/28	0.3 cc. 5 per cent suspension	Aycock	No symptoms	Prostrate on 30th day
9	Subcutaneous M.A.	1/27/28	6/11/28	0.3 cc. 5 per cent suspension	Aycock	No symptoms	
10	Subcutaneous M.A.	1/27/28	6/11/28	0.3 cc. 5 per cent suspension	Aycock	No symptoms	
Second intracerebral inoculation							
2	Intradermal M.A.	1/19/28	12/ 5/28	0.2 cc. 5 per cent suspension	Pooled mixed virus	No symptoms	Prostrate on 7th day
9	Subcutaneous M.A.	1/27/28	12/ 5/28	0.2 cc. 5 per cent suspension	Pooled mixed virus	Prostrate on 12th day	
10	Subcutaneous M.A.	1/27/28	12/ 5/28	0.2 cc. 5 per cent suspension	Pooled mixed virus	No symptoms	

TABLE IV

Serum Neutralizations

No.	Treatment		Test		Result	Neutralization				Result	
	Dose	Virus	Date	Virus		Virus	Virus treatment	Route	Amount serum	Test	Control
						Intradermal					
1	42	M.A.	Died intercurrent infection			cc. 0.3 M.A. Pooled	2 hrs. incubator. Overnight icebox	Icer.	cc. 0.9	No symptoms	Typical polio
2	66	M.A.	1/19/28	M.A.	No symptoms						
3	48	M.A.	1/19/28	M.A.	Slight excitement						
4	42	M.A.	5/31/28	Aycock	Slow	0.1 M.V.		Icer.	0.9	No symptoms	Typical polio
5	42	M.A.	5/31/28	Aycock	Tremor, ataxia, weak deltoid	0.1 M.V.		Icer.	0.9	No symptoms	Typical polio
6	42	M.A.	5/31/28	Aycock	Slow	0.1 M.A.		Icer.	0.9	No symptoms	Typical polio
7	56	Aycock	11/20/28	M.A.	Slow and excited	0.3 M.A.		Icer.	0.9	No symptoms	Typical polio
8	56	Aycock	11/20/28	M.A.	Excitement and tremor	0.2 M.V.		Cist.	0.8	No symptoms	Typical polio

Subcutaneous

9	45	M.A.	2/11/28	M.A. 0.5	No symptoms	0.3 Pooled	2 hrs. incubator. Overnight icebox	Icer.	0.9	No symptoms	Typical polio
10	42	M.A.	2/11/28	M.A. 0.5	No symptoms						
11	38	M.A.	2/11/28	M.A. 0.5	No symptoms						
12	42	M.A.	5/31/28	Aycock 0.5	Prostrate	0.1 M.V.		Icer.	0.9	No symptoms	
13	42	M.A.	5/31/28	Aycock 0.5	Paralyzed	0.1 M.V.		Icer.	0.9	Died intercurrent disease. Serum contaminated	
14	42	M.A.	5/31/28	Aycock 0.5	Prostrate	0.2 M.V.		Cist.	0.8	Late polio	Typical polio
15	56	Aycock	11/20	M.A. 0.5	No symptoms	Not done					
16	56	Aycock	11/20	M.A. 0.5	Paralyzed	0.2 M.V.		Cist.	0.8	No symptoms	Typical polio

inoculation of the active pooled virus, their sera neutralized the same potent material. It is interesting to note that in one instance of the 16 animals tested did the serum fail to exhibit neutralizing power.

The results of the serum neutralization tests show that degrees of immunity to poliomyelitis virus exist not only in monkeys but suggest that the same condition exists in man. The employment of relatively small doses of filtrate of a virus strain, whose potency is quite constant, brings out degrees of specific protection in monkeys. Such variations would have been totally obscured by the ordinary means of determining immunity by the intracerebral inoculation of considerable amounts of suspension of virus possessing varying degrees of infective power. It is conceivable that the past failures of certain efforts to induce immunity in monkeys may be explained, not by the inadequacy of the methods employed, but rather by the overwhelming inoculation which the animal was required to withstand, doubtless far greater than that to which any human would be exposed. The intracerebral test inoculation particularly, with its associated damage to nervous tissue, makes demands upon the immune reaction of an animal many times greater than that arising in any natural method of infection.

CONCLUSIONS

1. The introduction of considerable amounts of living, active poliomyelitis virus into the skin and subcutaneous tissue of monkeys protects the animals against intracerebral inoculations of similar virus material.

2. The degree of protection conferred by intradermal is greater than by subcutaneous injection.

3. During intradermal and subcutaneous inoculations, no local or general pathological signs were observed.

4. The degree of protection produced by the immunization methods used is not absolute, since a percentage of the inoculated monkeys respond to intracerebral injections of highly potent virus.

5. The sera of the animals inoculated intradermally or subcutaneously neutralized poliomyelitis virus *in vitro*, irrespective of the result of intracerebral inoculation, in all except one instance.

6. The power of the serum of treated monkeys to neutralize virus

in vitro is a more delicate test of immunity than is the intracerebral inoculation.

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[Reprinted from THE JOURNAL OF EXPERIMENTAL MEDICINE, June 1, 1929,
Vol. 49, No. 6, pp. 993–1008]

ETIOLOGY OF OROYA FEVER

XIV. THE INSECT VECTORS OF CARRION'S DISEASE

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PLATES 45 TO 47

(Received for publication, December 5, 1928)

It is desirable to present the two parts of this investigation in a single paper, since they bear on each other so closely that to publish them separately will call for considerable repetition of statement. The origin of the studies which have led to the results here presented is to be found in the earlier papers of this series (1), and in the work of Townsend (2), who concerned himself especially with the insect vector of the disease embraced under the names of Oroya fever and verruga peruana. Since the observations given in this paper were completed after the death of Dr. Noguchi, who planned the experiments, we wish to state briefly the circumstances surrounding the investigation.

The earlier papers of the series established *Bartonella bacilliformis* as the bacterial incitant of Oroya fever (Carrion's disease) and verruga peruana. The etiology and much of the pathology of these manifestations of a single infectious disease were made clear by the experimental studies (Noguchi) carried out between 1925 and 1928. The essential fact which remained to be determined was the precise mode of infection in the two maladies for which the history and clinical observation had indicated an insect vector. Townsend's studies of the distribution of the disease and the nocturnal nature of its origin had led him to the decision that the vector belonged to the class of phlebotomi. Indeed, he had gone so far as to designate the vector as *Phlebotomus verrucarum*.

The cooperation of the International Health Division of the Rockefeller Foundation was secured in the field investigation of insects in the verruga zones in Peru. Various insect species were collected by one of us (Shannon), identified as far as possible on the grounds, the identifications being completed afterwards in the United States, and sent by ship to New York, where they were tested for infectivity on monkeys according to Dr. Noguchi's plans (Tilden and Tyler). The procedure employed in this testing was as follows:

The insects were collected without the use of chemicals and sealed alive in sterile tubes, which were either dry, or contained a piece of absorbent cotton moistened with sterile citrate solution (about half of each shipment was sent dry. the other half in moist condition). Collections were made near the time of sailing of the fast boats to New York, and shipments were placed in the steamer's refrigerator during transit.

The method of determining the presence of *Bartonella bacilliformis* in the insects was to inject a saline suspension of the crushed bodies intradermally, sometimes also intravenously, into monkeys (*Macacus rhesus*) and to make cultures of the blood at intervals of from one to four weeks later, irrespective of the occurrence of local lesions or fever. A given lot of insects was crushed in 0.9 per cent sodium chloride and injected intradermally into one or two sites on the shaved abdominal skin of two monkeys at least. Because of the differences in susceptibility of individual monkeys, duplicate tests were necessary.

The culture technique was the same as that used in earlier work on Carrion's disease (3). The blood was withdrawn from the monkey into an equal part of 2 per cent citrate in 0.9 per cent sodium chloride, and ascending dilutions in saline (1:10, 1:100, 1:1,000, 1:10,000, and 1:100,000) were inoculated into the semisolid leptospira medium (4) in amounts of 0.2 cc. One tube of the medium was inoculated with a drop of the undiluted citrated blood. The cultures were kept at 30°C. By the end of a two to three week period the positive cultures can usually be picked out by their macroscopic appearance, but even microscopic examination may fail to reveal a positive culture, and subculture is often desirable (5). The growth may be so slight in the initial culture as to be easily mistaken for the haze which develops in a tube of sterile medium after standing at 30°C.

Although *Bartonella bacilliformis* was detected only in the phlebotomi, it is desirable to state that other insects collected (Shannon) in the verruga zone were inoculated into *rhesus* monkeys in the manner of the phlebotomi and the blood cultures carried out in the same way as for the latter. In no instance was *Bartonella bacilliformis* isolated in these cultures. A list of the insects with which no results were obtained follows:

Ticks.—Ornithodorus megnini (on burros), *Argas* sp.? (on burros, birds, bats), Tick larvae (genus and species?) on lizard.

Mites.—Tarsotomus sp. (on ground), *Trombidium* n.sp. (on ground), *Geckobia* sp. (on lizard), *Geckobiella* sp. (on lizard and geckos).

Lice.—Trichodectes ovis (on sheep).

Fleas.—Pulex irritans (on man), *Ctenocephalus canis* (on cat, dog, and man), *Rhopalopsyllus* (on dog and guinea pig).

Bedbugs.—Cimex lectularius.

Mosquitoes.—Anopheles pseudopunctipennis, Culex quinquefasciatus (*fatigans*).

Buffalo gnats.—Simulium escomeli, Simulium sp. (on burros).

Midges.—Forcipomyia utae, Forcipomyia townsendia.

Muscidæ.—Stomoxys calcitrans.

Hippoboscidae.—Melophagus ovinus (on sheep).

Streblidae.—3 genera, one species each (on vampire bats).

Note should be made of the fact that Townsend (2) regarded the lizard as the natural reservoir of the incitant of verruga peruana, for the reason that he detected intracorpuscular bodies in the blood cells, which he identified with Barton's rods. Hence the red mites (*Trombidium, Tarsotomus, Geckobia, Geckobiella*), some of which were obtained from geckos (*Phyllodactyus reisii*), as well as the blood of two geckos, were injected into monkeys. Cultures prepared from the blood of these monkeys remained sterile.

Phlebotomi of Verrugas Cañon

One of us (Shannon) spent from March to July, 1928, in the Rimac verruga zone, Peru. As many varieties of insects as possible were collected (6) from this zone and sent to The Rockefeller Institute in New York to be tested upon monkeys. It is desirable to state that Townsend was the first to implicate phlebotomus with the transmission of verruga peruana. His studies, conducted between 1912 and 1914, led him to decide, on ecological and experimental grounds, that a species of gnat, later called *Phlebotomus verrucarum* Townsend, was the vector of the disease.

Our studies revealed three species of *Phlebotomus* in the verruga zone. Two of the species had a wide and the third a limited distribution only in the zone. It is significant that of the three, only the two which occurred in considerable numbers were found on inoculation to yield *Bartonella bacilliformis.*

In this paper brief descriptions only will be given of the three species, based upon the characters of the males. All three species belong to the subgenus *Brumptomyia* (França and Parrot), which may be described as follows:

Basal segment of the upper appendage of the male terminalia with a well defined sub-basal tuft of hairs on the inner surface; the distal appendage either with four well developed spines, the fifth one weak, or with five strong spines; median appendage simple, without spines, lower appendage unarmed. The abdominal hairs are suberect to erect; length of the upper branch of upper forked vein longer than the petiole preceding the fork.

Ph. noguchii and *Ph. peruensis* are described here for the first time (Shannon).

Key to the Males

1. Distal segment of upper appendage with four well developed spines, the fifth (an apical one) being very slender and hair-like; petiole of upper forked

cell slightly longer than that section of the first longitudinal vein which overlaps the upper branch of the second vein..... *Ph. verrucarum* Townsend.

2. Distal segment with five well developed spines, both apical ones being equally strong.

(*a*) Distal segment with two submedian spines, a third located slightly distal of the middle of the segment, the fourth and fifth forming an apical pair; petiole of the upper forked cell much longer than that section of the first vein which overlaps the upper branch of the second vein. (Type locality, Verrugas Cañon, Department of Lima, Peru)

Ph. noguchii Shannon.

(*b*) Distal segment with two submedian spines, a subapical one and an apical pair; that section of the first vein overlapping the upper branch of the second is distinctly longer than the length of the petiole of the upper forked cell. (Type locality, Matucana, Department of Lima, Peru)

Ph. peruensis Shannon.

The females of *Phlebotomus peruensis* can be separated from those of *verrucarum* and *noguchii* by differences in the arrangement of the wing veins, but definite characters have not yet been found whereby the females of *verrucarum* and *noguchii* may be positively identified. Approximate identification of the females which were sent to New York for bacteriological study was made on the basis of (1) average differences in size, *verrucarum* being in general smaller than *noguchii*, and (2) habitat. All males found in houses proved to be *verrucarum*, hence all females found in-doors were assumed to belong to this species. All the *noguchii* males were found out-of-doors, in excavations and natural cavities, where they were three times as numerous as *verrucarum* males.

Before leaving this description, it may be well to bring together certain accepted facts with reference to the epidemiology of Carrion's disease and certain known habits of the phlebotomi. It is admitted that the disease is contracted only in certain limited areas in Peru, and that infection, with possibly rare exceptions, takes place only at night. This infection may be acquired indoors or in localities remote from human habitations and at any time of the year.

With these facts in mind, it would seem to follow that the insect vector must be (a) common blood sucker of man; (b) restricted to the verruga zone; (c) nocturnal in habit; (d) capable of breeding in varied localities, so that adults, which have restricted flight, may be everywhere present, and (e) active throughout the year.

All these conditions are fulfilled by phlebotomi and not, as far as determined, by other insects of the verruga zones.

Finally, it may be recorded that we (Shannon and assistant) safely

spent from 2 to 5 nights a week for 4 months in the verruga zone after taking adequate precaution to protect ourselves from bites of phlebotomi.

Experiments with Phlebotomi

Eighteen special lots of phlebotomi, prepared and shipped as described, were inoculated into monkeys (Tilden and Tyler). The material in each instance was introduced intradermally at several sites on the shaved skin of the abdomen, and was also applied to a scarified area of the abdominal skin. Occasionally an intravenous injection was also made.

Lots 1, 2, 9, and 14 were pooled, ground in a mortar with 0.9 per cent saline solution, and injected, Apr. 25, 1928, into two monkeys (*Macacus rhesus* I-3 and I-4).

Lot 1, collected Mar. 9, 1928, out-of-doors in Matucana. 3 females and 4 males of *Ph. noguchii*, *Ph. verrucarum*, and *Ph. peruensis*.

Lot 2, collected Mar. 9, 1928, out-of-doors in Matucana. 2 females, 18 males, Phlebotomus, chiefly *noguchii*.

Lot 9, *Ph. verrucarum* collected Mar. 20, 1928, in house in Verrugas Cañon. 40 females.

Lot 14, *Ph. verrucarum*, collected Mar. 26, 1928, in Verrugas Cañon. 24 females.

Lot 20. *Ph. noguchii*, with possibly a few *verrucarum* intermixed, collected Apr. 9, 1928, consisted of about 20 females. A saline suspension of the crushed insects was injected May 31, 1928, into two monkeys (*M. rhesus* I-7 and I-8). I-8 also received 1 cc. of the suspension intravenously.

Lots 27 and 28. *Ph. noguchii*, with possibly a few *verrucarum* intermixed, collected May 1 and May 8, 1928. 10 to 12 females. A saline suspension of the crushed insects was injected June 13, 1928, into two monkeys, *M. rhesus* I-17 and I-5.

Lots 29, 30, and 38. *Ph. verrucarum*, collected May 1 and May 8, 1928, 15 to 20 females. Saline suspension injected June 13, 1928, into *M. rhesus* I-16 and *M. rhesus* I-6.

Lots 40, 41, and 44. *Ph. verrucarum*, collected June 9, 11, and 18, 1928, both in houses and out-of-doors, in Verrugas Cañon. These lots comprised about 100 females. Saline suspension injected July 14, 1928, into *M. rhesus* I-26 and I-27. All the specimens which came in moist condition were covered with green mold.

Lots 39 and 45. *Ph. noguchii*, collected June 6 and June 19, 1928, out-of-doors in Verrugas Cañon. The number of insects was small (about 25 females), and the specimens which came in moist condition were covered with green mold. Saline suspension inoculated July 14, 1928, into *M. rhesus* I-28 and I-29.

Lots 42 and 46. *Ph. peruensis*, collected June 12 and 19, 1928, out-of-doors in Matucana. The specimens which came in moist condition were covered with green mold. 8 females. Saline suspension inoculated July 14, 1928, into *M. rhesus* I-30 and I-31.

Lot 43. *Ph. noguchii*, with possibly a few *verrucarum* intermixed, collected June 12, 1928, out-of-doors in Matucana. Saline suspension inoculated Aug. 13, 1928, into two monkeys, I-33 and I-34.

Lot 51. *Ph. verrucarum*, collected during the last week in July, 1928. 6 females. Saline suspension inoculated Aug. 13, 1928, into *M. rhesus* I-37.

Lot 54. *Ph. noguchii*, with possibly a few *verrucarum* intermixed, 8 to 10 females, collected during the last week in July out-of-doors in Verrugas Cañon. Saline suspension inoculated Aug. 14, 1928, into *M. rhesus* I-38 and I-39.

The first material inoculated, which contained all three species of Phlebotomus (Lots 1, 2, 9, and 14) yielded positive results.

Strain 1, from Lots 1, 2, 9, and 14

M. rhesus I-3 and I-4, inoculated intradermally Apr. 25, 1928. No local lesions developed at the sites of inoculation. *M. rhesus* I-3 had a temperature of 104°F. on May 14, 19 days after inoculation, and blood was withdrawn on that day. *Bartonella bacilliformis* was obtained in culture from 1:10, 1:100, and 1:1,000 dilutions of the blood. The temperature reached 104°F. again several times, but blood culture was negative on May 29 and June 30. Blood was taken from *M. rhesus* I-4 at the same time as from I-3, but cultures remained negative.

Inoculation of Cultures from M. rhesus I-3.—*M. rhesus* I-14 and *M. rhesus* I-15 (Fig. 1) were inoculated June 5, 1928, with the 20 day culture of *Bartonella bacilliformis* obtained from the blood of *M. rhesus* I-3 and a 5-day subculture. The culture, which was, as usual, diluted with an equal part of saline for inoculation, was also applied to a scarified area on the abdominal skin. Tiny nodules were observed in *M. rhesus* 15 at the sites of intradermal inoculation on June 11 (16 days after inoculation), and 5 days later they were well developed, and one was excised[1] for examination and transfer. *Bartonella bacilliformis* was obtained from a 1:1,000 dilution of blood withdrawn June 16, and from a 1:100,000 dilution of the nodule suspension. By June 22 the scarified area showed small nodules. The photograph was taken 3 days later (Fig. 1). By June 28 the lesions had disappeared. *M. rhesus* I-14 had almost continuous high fever (104° to 106°F.) from June 8 to 28 and again from July 16 to 23, but no local lesions developed, and blood culture was negative 13, 31, and 55 days after inoculation.

The strain of *Bartonella bacilliformis* obtained from these first lots of phlebotomi was carried through two animal passages by direct transfer and has since been maintained by alternate generations in culture and

[1] All operations were carried out under ether anesthesia.

monkey. The usual course of verruga of moderate severity (7) has been observed in the animals (Fig. 8), with the exception of one monkey of the first passage (*M. rhesus* I-1), which had an unusually severe cutaneous reaction (Figs. 6, 7), not unlike that which had been induced in one of the monkeys (*M. rhesus* 18) of an early experiment (Noguchi (8)). This animal was acutely ill over a period of two weeks but recovered.

Histological study of the nodular tissue from I-15 and I-1, made by Dr. Henry R. Muller, shows the characteristic proliferation of endothelial cells and the formation of new capillaries. *Bartonella bacilliformis* was detected in some instances within the endothelial cells.

The second lot of phlebotomi tested, which probably consisted chiefly of *Ph. noguchii* but may have contained a few *verrucarum*, also yielded a strain of *Bartonella bacilliformis*.

Strain 2 from Lot 20

M. rhesus I-7 and I-8 were inoculated May 31, 1928, intradermally, and I-8 received 1 cc. of the suspension in the left saphenous vein. The animals were bled on June 11 and again on June 30. The blood of I-8 yielded cultures of *Bartonella bacilliformis* in 1:100,000 dilution on June 11 and in 1:100 dilution on June 30; no cultures were obtained from the blood of I-7. Neither animal developed lesions at the sites of inoculation, but I-8 had almost continuous fever (104° to 105.2°F.) from June 4 to June 28.

Inoculation of Culture from M. rhesus I-8.—*M. rhesus* I-22 and I-23 were inoculated on June 22, 1928, intradermally, with a 10-day-old culture from the blood of *M. rhesus* I-8. The culture was also applied to a scarified area on the abdomen. The nodules showed at the sites of intradermal inoculation after about 10 days, and blood withdrawn after 2 weeks yielded cultures of *Bartonella bacilliformis* in 1:10,000 dilution. The nodule excised[1] on July 6 for examination and transfer yielded cultures in a 1:100 dilution. The lesions were considerably larger and more extensive in I-23 (Fig. 3), and the edema of the abdominal wall developed early and became marked. Regression of the lesions began about four weeks after inoculation and within four weeks recovery was practically complete. The course of disease was almost afebrile in both animals.

Further inoculations, first with nodular tissue from I-22 and I-23, and later with cultures from the blood of passage animals, showed that this strain of *Bartonella bacilliformis*, like Strain 1, was moderately virulent, inducing pronounced local lesions and moderate anemia.

One animal of the series died in 33 days, after an afebrile course of disease, during which moderately severe anemia had been observed.

Lots 27 and 28 (chiefly *Ph. noguchii*) and Lots 29, 30, and 38 (*Ph. verrucarum*) yielded negative results, as did also Lots 40, 41, 44, and 51 (*Ph. verrucarum*), Lots 42 and 46 (*Ph. peruensis*), and Lot 43 (*Ph. noguchii*).

From Lots 39 and 45, which probably consisted of *Ph. noguchii* alone, a third strain of *Bartonella bacilliformis* was obtained.

Strain 3, from Lots 39 and 45

M. rhesus I-28 and I-29 were inoculated intradermally on July 14, 1928. Monkey I-28 showed no fever at any time, and blood cultures were negative 1, 2, and 3 weeks after inoculation. Monkey I-29 had fever (104.2° to 105.2°F.) 3 days after inoculation, which continued for a week with one day of remission. The blood yielded cultures of *Bartonella bacilliformis* in a dilution of 1:100 on four occasions, 7, 13, 23, and 31 days after inoculation, but there was no reaction at the sites of injection.

Inoculation of Cultures from I-29.—M. rhesus I-44 was inoculated intradermally and by scarification on Aug. 16, 1928, with a culture 14 days old from the blood of *M. rhesus* I-29. 1 cc. of the culture was also injected into the saphenous vein. Nodules developed in 2 weeks (Figs. 5 and 9), and the scarified area presented the characteristic miliary eruption. The blood was positive in a dilution of 1:10,000 at this time. From Sept. 7 to 19 there was marked fever (104° to 105.2°F.).

M. rhesus I-45 was inoculated at the same time and in the same manner as I-44. The intradermal nodules attained a diameter of only 0.5 cm., and the blood was positive in a dilution of 1:100. Fever existed (104.2° to 105.4°F.) from Sept. 10 to 13, was followed by two days of subnormal temperature, and death of animal on Sept. 16. Autopsy (Dr. Muller) revealed nothing abnormal except in the spleen, which contained numerous pale areas 2 to 3 mm. in diameter. Film preparations were negative for tubercle bacilli, and microscopic examination disclosed infarcts such as are found in the spleen in human (9) cases of Oroya fever, and in cases of the experimental disease (10).

Later passage of Strain 3 produced local lesions of very large size (2 to 3 cm. in diameter), but no unusual systemic effects.

Lot 54, which consisted chiefly, perhaps wholly, of *Ph. noguchii*, also yielded *Bartonella bacilliformis*.

Strain 4, from Lot 54

M. rhesus I-38 was injected intradermally on Aug. 14, 1928, and intravenously (1 cc. of the saline suspension into the left saphenous vein). From Aug. 22 to

Aug. 29 the temperature was 104°F., but blood culture made Aug. 28 was negative. It was also negative on Sept. 10, but blood taken on Sept. 25, when the temperature was 104.2°F. yielded *Bartonella bacilliformis* in 1:10 and 1:100 dilutions after 13 days incubation. The intradermal mixtures produced no lesions. *M. rhesus* I-39, inoculated at the same time as I-38, and with the same material, showed a rise of temperature (104.2° to 104.8°F.) on three occasions, but blood cultures made on Aug. 28, Sept. 10, and Sept. 25 were negative.

Inoculation of Cultures from I-38.—M. rhesus I-58 was inoculated on Oct. 10, 1928, with 15-day culture from the blood of *M. rhesus* I-38. Small nodules appeared at the sites of intradermal injection after 7 days and were well advanced after 16 days (Fig. 5). The abdominal wall became oedematous and the area of scarification showed miliary nodules in addition to which three or four small eruptions arose outside the inoculated areas. Blood culture was positive in dilutions up to 1:10,000, 12 days after inoculation. The animal died on the 18th day, when the local lesions were still actively progressing. Histological examination of tissues by Dr. Muller revealed the characteristic zonal necrosis around the central vein in the liver, with extensive invasion by polymorphonuclear leucocytes. The spleen showed no lesions. The various skin nodules were histologically characteristic of verruga in the monkey.

Further inoculations with cultures of Strain 4 yielded similar results. In one animal (*M. rhesus* S-7) the local lesions reached large size.

The results of the inoculations are summarized in Tables I to V.

Exposure of Monkeys to Bites of Phlebotomi

Six *rhesus* monkeys were exposed (Shannon) for several weeks to natural infection, three in an excavation in Verrugas Cañon, where *Ph. noguchii* was fairly common, and three in a house where *Ph. verrucarum* was abundant. These animals were brought to The Rockefeller Institute on Aug. 13. Blood withdrawn on three occasions failed to yield cultures of *Bartonella bacilliformis*, and only one of the animals failed to respond to subsequent inoculation of virulent cultures or passage virus. The result therefore was regarded as negative.

Immunity

Seven of the monkeys which had developed verrucous lesions and blood infection with *Bartonella bacilliformis* following inoculation with the Phlebotomus strains and in which the lesions had regressed, were subsequently tested for immunity by reinoculation. Similar immunity tests were made on three monkeys which had received crushed

TABLE I

Inoculations of Crushed Phlebotomi

M. rhesus No.	Date 1928	Lot No.	Method of inoculation	Local lesions	Blood culture
I-3	Apr. 25	1, 2, 9, 14 *verrucarum* *noguchii* *peruensis*	Multiple intradermal Scarification	−	+
I-4	Same	Same	Same	−	−
I-7	May 31	20 *noguchii* (few *verrucarum?*)	Multiple intradermal Scarification	−	−
I-8	Same	Same	Same, also intravenous	−	+
I-16	June 13	29, 30, 38 *verrucarum*	Multiple intradermal Scarification	−	−
I-6	Same	Same	Same	−	−
I-17	Same	27, 28 *noguchii* (few *verrucarum?*)	Same	−	−
I-5	Same	Same	Same	−	−
I-26	July 14	40, 41, 44 *verrucarum*	Same	−	−
I-27	Same	Same	Same	−	−
I-28	Same	39, 45 *noguchii*	Same	−	−
I-29	Same	Same	Same	−	+
I-30	Same	42, 46 *peruensis*	Same	−	−
I-31	Same	Same	Same	−	−
I-33	Aug. 13	43 *noguchii* (few *verrucarum?*)	Same	−	−
I-34	Same	Same	Same	−	−
I-37	Same	51 *verrucarum*	Same	−	−
I-38	Same	54 *noguchii* (few *verrucarum?*)	Same, also intravenous	−	+
I-39	Same	Same	Same	−	−

TABLE II

Strain 1, from Lots 1, 2, 9, 14 (Ph. verrucarum, Ph. noguchii, Ph. peruensis)

M. rhesus No.	Date 1928	Material inoculated	Mode of inoculation	Local lesions	Blood culture
I-14	June 5	Culture from I-3	Intradermal Scarification	−	−
I-15	Same	Same	Same	+++	+
		First passage			
I-18	June 16	Nodule susp. I-15	Same	++++	+
I-19	Same	Same	Same	++++	+
I-1	Same	Same	Same	++++	+
		Second passage			
I-12	July 6	Nodule susp. I-1	Same	++++	+
I-13	Same	Same	Same	+++	+
		Third passage (via culture)			
I-40	Aug. 16	14-day culture I-12	Same, also intravenous	++++	+
I-41	Same	Same	Same	++++	+

TABLE III

Strain 2 from Lot 20 (Ph. noguchii—Few verrucarum?)

M. rhesus No.	Date 1928	Material inoculated	Mode of inoculation	Local lesions	Blood culture
I-22	June 22	10-day culture from I-8	Intradermal Scarification	++++	+
I-23	Same	Same	Same	++++	+
		First passage			
I-24	July 6	Nodule susp. I-23	Same	++++	+
I-25	Same	Same	Same	+++	+
		Second passage (via culture)			
I-42	Aug. 16	14-day culture from I-24	Same, also intravenous	++++	+
I-43	Same	Same	Same	++++	+

TABLE IV

Strain 3 from Lots 39 and 45 (Ph. noguchii)

M. rhesus No.	Date 1928	Material inoculated	Mode of inoculation	Local lesions	Blood culture
I-44	Aug. 16	14-day culture from I-29	Intradermal Scarification Intravenous	++++	+
I-45	Same	Same	Same	++++	+
		First passage			
I-55	Sept. 13	Nodule susp. I-44	Intradermal Scarification	+++	+
I-56	Same	Same	Same	++	−
I-34	Oct. 22	20-day culture from I-45	Same	+++	+
		Second passage (via culture)			
S-6	Dec. 15	25-day culture from I-34	Same	++++	+

TABLE V

Strain 4 from Lot 54 (Ph. noguchii—Few verrucarum?)

M. rhesus No.	Date 1928	Material inoculated	Mode of inoculation	Local lesions	Blood culture
I-58	Oct. 10	15-day culture from I-38	Intradermal Scarification	+++	+
		First passage (via culture)			
I-17	Nov. 5	14-day culture from I-58	Same	++++	+
		Second passage (via culture)			
S-7	Dec. 15	18-day culture from I-17	Same	++++	+

TABLE VI

Immunity Tests

M. rhesus No.	First inoculation				Immunity test			
	Date, 1928	Material inoculated	Local lesions	Blood culture	Date, 1928	Material inoculated	Local lesions	Blood culture
I-18	June 16	Nodule susp. I-15 (Str. 1)	++++	+	Sept. 13	Nodule susp. I-41 (Str. 1)	−	−
I-13	July 6	Same	++++	+	Same	Same	−	−
I-3	Apr. 25	Phlebotomi Lots 1, 2, 9, 14	−	+	Same	Same	++++	−
I-53 Control					Same	Same	++++	+
I-8	May 31	Phlebotomi Lot 20	−	+	Sept. 13	Nodule susp. I-43 (Str. 2)	++++	+
I-25	July 6	Nodule susp. I-23 (Str. 2)	++++	+	Same	Same	−	−
I-23	June 22	Blood culture I-8 (Str. 2)	++++	+	Same	Same	−	−
I-11	July 6	Nodule susp. P. 5*	++++	+	Same	Same	−	−
286	June 1	Culture P. 5*	++++	+	Same	Same	−	−
I-54 Control					Same	Same	+++	+
I-1	June 16	Nodule susp. I-15 (Str. 1)	++++	+	Sept. 26	Culture P. 5*	−	−
I-19	June 16	Same	++++	+	Same	Same	−	−
I-29	July 14	Phlebotomi Lots 39, 45	−	+	Same	Same	+++	+
I-57 Control					Same	Same	+++	+

* Noguchi, H., *J. Exp. Med.*, 1927, xlv, 175.

phlebotomi without developing skin lesions which reacted as do previously untreated animals. Table VI summarizes the results.

Morphology

No morphological or cultural differences could be detected between the Phlebotomus strains and the human strains of *Bartonella bacilliformis*. Cultures seven days old of the four strains, grown on horse blood agar slants, were stained to bring out the unipolar flagella (one to four) which are characteristic of *Bartonella bacilliformis* (Figs. 11, 13, 15, 17), the films being made on the same slide, in order that the stained preparations might be comparable. Cultures of the same age but grown on leptospira medium were used for similar comparative preparations which were stained by Gram's method, with fuchsin as the counterstain (Figs. 10, 12, 14, 16).

SUMMARY AND CONCLUSIONS

With a view to determining the mode of infection in Carrion's disease, a study of the blood-sucking insects found in the districts of Peru where the disease prevails has been carried out, through the cooperation of The Rockefeller Institute and the Rockefeller Foundation. The material studied included ticks, mites, midges, lice, fleas, bedbugs, mosquitoes, buffalo gnats, horse-flies, "sheep ticks," 3 species of Streblidae, and 3 species of Phlebotomus, including *Phlebotomus verrucarum* Townsend and two new species which have been named *Phlebotomus noguchii* and *Phlebotomus peruensis*. The insects were collected without the use of chemicals, were prepared for transportation in such a manner as to prevent drying, and were shipped under conditions of refrigeration to New York, where they were inoculated into monkeys. The plan followed was to inject saline suspensions of the crushed insects intradermally into *rhesus* monkeys and to make cultures of the blood of the animals at intervals of 1 to 6 weeks after inoculation.

The only class of insects in which the presence of *Bartonella bacilliformis* could be detected were phlebotomi. No cutaneous lesions were induced in monkeys injected with the crushed insects, but in the case of four different lots of phlebotomi the blood of the animals so injected yielded cultures of *Bartonella bacilliformis* which produced typical verrucous lesions on inoculation into other monkeys.

The morphology and cultural characteristics of the Bartonella strains obtained from phlebotomi proved identical with those of strains

isolated from human blood and skin lesions. Monkeys which had recovered from infection with the phlebotomus strains resisted inoculation with a human strain of *Bartonella bacilliformis*, and, conversely, monkeys which had passed through an infection induced by the human strain resisted inoculation with the strains obtained from phlebotomi.

The experimental observations described in this paper lead us to conclude that certain phlebotomi act as insect vectors of Oroya fever and verruga peruana. The phlebotomi which have been shown quite certainly to carry the *Bartonella bacilliformis* are those of the species *Phlebotomus noguchii*. *Phlebotomus verrucarum* is also probably a vector, while *Phlebotomus peruensis* remains doubtful in this respect.

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EXPLANATION OF PLATES

PLATE 45

FIG. 1. Cutaneous lesions induced in *M. rhesus* I-15 by Phlebotomus Strain 1. Photograph taken 20 days after inoculation. One intradermal nodule had been excised 2 days previously.

FIG. 2. The appearance of the lesions in *M. rhesus* I-1, a Strain 1 first passage animal, 18 days after inoculation. The sacrified area already shows characteristic minute nodules. All the lesions reached considerable size (Figs. 6 and 7).

FIG. 3. Strain 2. Early culture lesions (two weeks after inoculation) in *M. rhesus* I-23.

FIG. 4. *M. rhesus* I-44, 21 days after inoculation with cultures of Strain 3 from Lots 39 and 45 of *Ph. noguchii*.

FIG. 5. *M. rhesus* I-58, 16 days after inoculation with Strain 4 phlebotomus cultures. The eruption was more general and the edema extensive in this animal. Death occurred 3 days after the photograph was made.

PLATE 46

FIGS. 6 AND 7. Late lesions in *M. rhesus* I-1 (Strain 1) as they appeared 31 days after inoculation. The most pronounced lesion occurred at the scarification site (center).

FIG. 8. *M. rhesus* I-19, 29 days after inoculation in the same way and at the same time as *M. rhesus* 1.

FIG. 9. *M. rhesus* I-44, 21 days after inoculation with cultures of *Phlebotomus noguchii* Strain 3.

PLATE 47

Magnification × 1,000

FIG. 10. Phlebotomus Strain 1, from Lots 1, 2, 9, 14. Gram's stain, counterstained with saturated alcoholic solution of fuchsin.

FIG. 11. Same, stained for flagella, by a combination of Zettnow's mordant and Fontana's ammoniac silver solution.

FIG. 12. Phlebotomus Strain 2, from Lot 20. Gram's stain, counterstained with fuchsin.

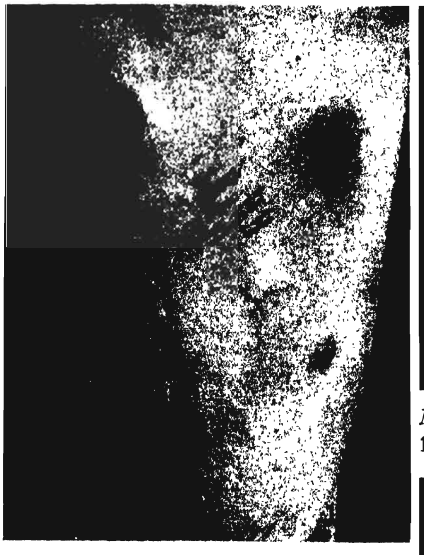
FIG. 13. Same, Zettnow-Fontana flagella stain.

FIG. 14. Phlebotomus Strain 3, from Lots 39 and 45. Gram's stain, counterstained with fuchsin.

FIG. 15. Same, Zettnow-Fontana flagella stain.

FIG. 16. Phlebotomus Strain 4, from Lot 54. Gram's stain, counterstained with fuchsin.

FIG. 17. Same, Zettnow-Fontana flagella stain.



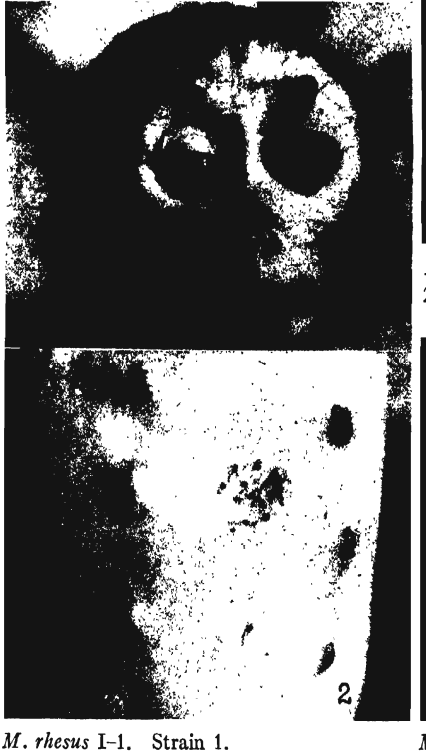
M. rhesus I–15. Strain 1.
20 days after inoculation.



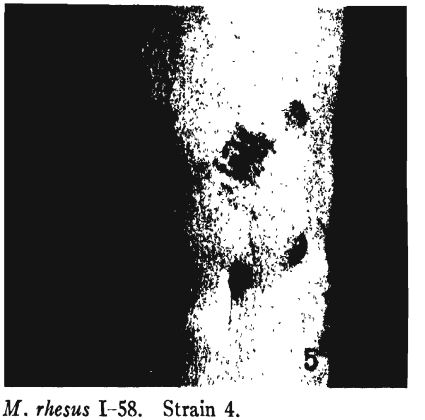
M. rhesus I–23. Strain 2.
14 days after inoculation.



M. rhesus I–44. Strain 3.
21 days after inoculation.



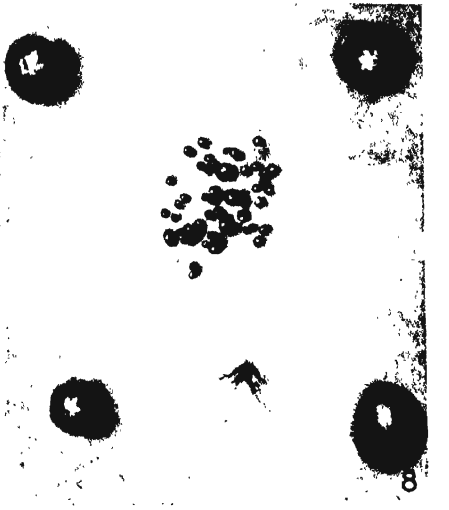
M. rhesus I–1. Strain 1.
18 days after inoculation.



M. rhesus I–58. Strain 4.
16 days after inoculation.



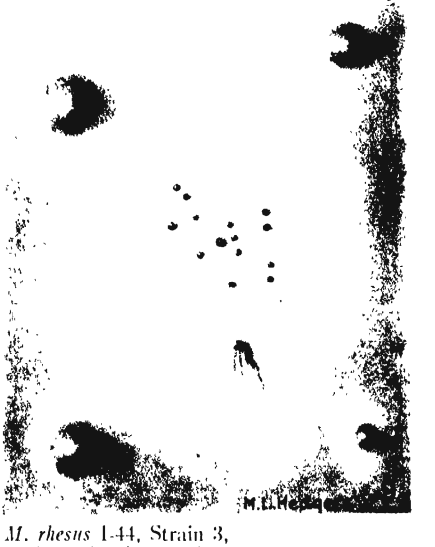
M. rhesus I-1, Strain 1, 31 days after inoculation



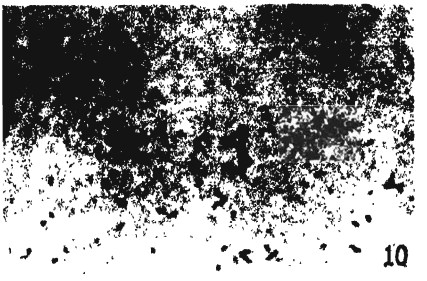
M. rhesus I-19, Strain 1, 29 days after inoculation. Scarified area in center.



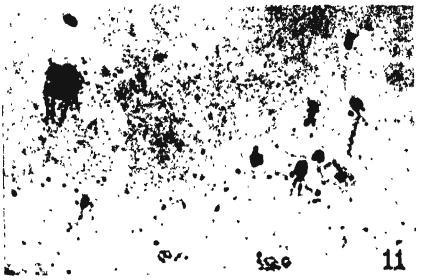
M. rhesus I-1, Strain 1, 31 days after inoculation. The largest lesion arose on the area inoculated by scarification (center).



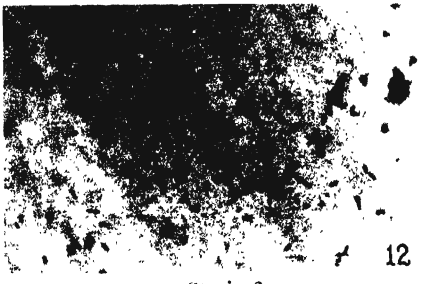
M. rhesus I-44, Strain 3, 21 days after inoculation. Scarified area in center.



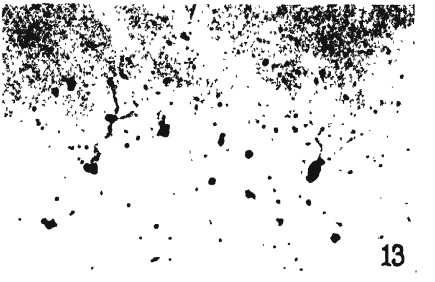
Strain 1.



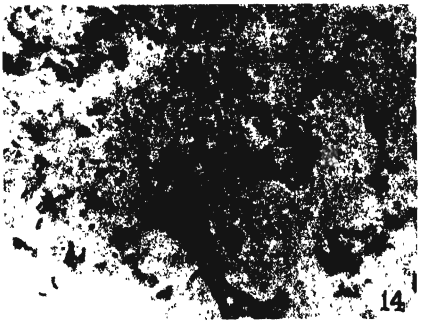
Strain 1.



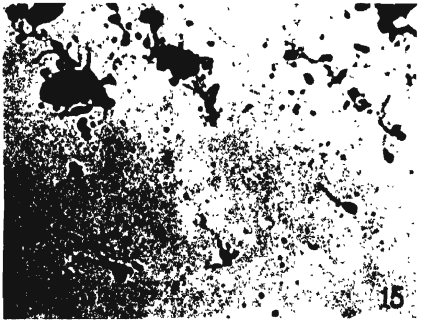
Strain 2.



Strain 2.



Strain 3.



Strain 3.



Strain 4.

Gram, counterstained with fuchsin.



Strain 4.

Zettnow-Fontana combination stain.

× 1,000.

(Noguchi *et al.*: Etiology of Oroya fever. XIV.)

[Reprinted from THE JOURNAL OF EXPERIMENTAL MEDICINE, July 1, 1929,
Vol. 50, No. 1, pp. 121–141]

BLOOD AND BONE MARROW CELLS OF THE DOMESTIC FOWL

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PLATE 6

(Received for publication, April 25, 1929)

The development of the supravital technique for the study of living cells has provided a new and interesting method for the cytological study of various animal tissues. This paper concerns the application of such methods to the blood and bone marrow cells of the domestic fowl. White Leghorn, Plymouth Rock and Black Jersey Giant roosters have been used in these experiments.

Direct Method of Counting

The problem of securing an accurate count of the white blood cells of the fowl has presented three difficulties. The avian red blood cells are nucleated, hence any method using the usual diluting fluids containing acetic acid will not permit discrimination between the red blood cell nuclei and the white blood cells. The second obstacle to an accurate count is the character of the thrombocytes. These structural elements in the fowl are about the size of lymphocytes and some of them may be confused either with red blood cells in which there is little hemoglobin, or with lymphocytes. The third factor is the rapidity with which coagulation occurs in the blood of the fowl.

Blain (1) has recently introduced a direct method for making total white blood cell counts on avian blood. He used two diluting fluids; Solution 1 contained neutral red 1:5000 made up in Locke's solution and adjusted to a pH of 7.4. The blood was first diluted with this solution at a temperature of 39°C., until the red cell pipette was one-half full. The mixture was shaken for 15 seconds, at the end of which time the pipette was filled with Solution 2, consisting of 12 per cent formalin in Locke's solution at pH 7.4. Shaking was then resumed for from 2 to 3 minutes. In this way he stated that every white cell had taken up sufficient neutral red to make possible its identification from the red blood cells. The latter took up no neutral red.

Prior to Blain's work the only method of estimating the number of white blood cells of avian blood was by indirect calculation, in which the total number of red and white corpuscles was determined in the counting chamber. Then, in films, the ratio between the number of red and white blood corpuscles was determined. From the total number of blood cells and from this ratio the number of white blood corpuscles per cubic millimeter may be calculated. This indirect method is subject to great error chiefly for two reasons. It presupposes an absolutely even distribution of red and white blood cells, which actually rarely obtains. The second source of error is in the thrombocytes. The size and character of these elements make their separation from lymphocytes in the counting chamber exceedingly difficult or impossible.

Blain (2) stated in a further communication that he identified no structures in avian blood corresponding to the platelets of mammals. It would appear, then, that the structures which other investigators, among them Ellermann (3), Albertoni and Mazzoni (4), Sugiyama (5), and which we ourselves consider to be thrombocytes have been included either in the red blood cell counts or in the white blood cell counts of Blain.

The method employed for the counting of the white blood cells in the studies reported in this paper has also made use of the vital staining properties of neutral red. A single diluting fluid has been devised, consisting of 25 mgm. of neutral red in 100 cc. of 0.9 per cent sodium chloride solution. This solution is filtered once and is kept at room temperature. It is preferable, but not essential to have the fluid warmed to body temperature at the time of use. The ordinary red blood cell pipette is used. The blood from a puncture of the wing vein or of the comb is drawn to the 0.5 mark and immediately diluted to the 101 mark, giving a dilution of 1:200. The pipette is shaken for 4 or 5 minutes as in ordinary blood cell counts. The counting chamber is filled in the usual manner. We have found it quite unnecessary to have the fluid at a definite pH or to introduce other complicated procedures.

It has been found that with this diluting fluid the polymorphonuclear elements and the monocytes stain in a characteristic manner, so that they can be recognized in the counting chamber with an 8 mm. lens and No. 10 objectives. The lymphocytes and thrombocytes are

not appreciably stained with the dye. The polymorphonuclear cells are characteristic in that the cytoplasmic border stains heavily and gives the appearance of a very dark, heavy rim to these cells. The monocytes take the dye more diffusely and do not have the deeply stained border. The lymphocytes and thrombocytes can be seen in the chamber but remain almost entirely unstained. Those red blood cells which have a small amount of, or no hemoglobin likewise fail to stain. The last three elements, lymphocytes, thrombocytes, and red blood cells without appreciable hemoglobin cannot be distinguished with any degree of accuracy in the counting chamber. The white blood cell count is made in the following manner: The total number of polymorphonuclear cells and monocytes is counted in the whole area of the ruled portion of the chamber (0.9 cu. mm.). This value taken together with the percentage of these combined elements obtained from the differential count permits a calculation of the total leucocyte count, exclusive of thrombocytes. An example will clarify this explanation.

Total number of polymorphonuclear cells and monocytes in 0.9 cu. mm.	7200 cells
Total number of polymorphonuclear cells and monocytes in 1 cu. mm.	8000 cells
Differential leucocyte count	
Polymorphonuclear eosinophiles	50 per cent
" neutrophiles	4
" basophiles	2
Monocytes	10
Total polymorphonuclear cells and monocytes	66 per cent
Small lymphocytes	30
Large "	4
	100 per cent

Therefore, 66% of total count = 8000 cells

$$100\% \text{ " " " } = \frac{8000}{66} \times 100 = 12{,}121+ \text{ white blood cells per cu. mm.}$$

By this method the difficulties of confusing the thrombocytes with the white blood cell count or with the erythrocyte count are overcome. There is also the advantage that the red blood cells may be counted in the same chamber as the leucocytes. This is practicable in the chicken because of the relatively low erythrocyte and relatively high leucocyte counts.

It should be stated here that the above method has been employed in this laboratory on many animals over a period of two years with con-

sistent results. The accuracy has also been tested by using the method on human blood, where entirely consistent results were obtained. The method was tried on a case of human lymphatic leukemia with a count of 60,000 cells. None of the lymphocytes contained any significant amount of dye, whereas the polymorphonuclear leucocytes and monocytes stained as they do in chickens' blood. It is pointed out that the method is readily adaptable for the counting of human blood, in which there are many normoblasts. I recall a case of sickle cell anemia in which there were 40,000 to 70,000 normoblasts per cubic millimeter of blood. The usual acetic acid diluting fluid does not distinguish these cells from leucocytes and thus a false leucocyte count was obtained. Likewise the normoblastic showers occurring in pernicious anemia are apt to lead to an erroneous white blood cell count unless a method of the type here outlined is resorted to.

Supravital Studies

Because of the diversity of opinion concerning the character of the cells in the blood of the domestic fowl, it seems necessary to give in some detail descriptions and illustrations of the types of cells encountered. It should be pointed out at this time that the following descriptions of cells apply only to the domestic fowl. Other kinds of birds, as pigeons, doves, etc., show a somewhat different picture.

Detailed observations on the blood and bone marrow cells of the chicken have been made by the use of the supravital technique and are reported in this communication. This is now a generally accepted method of great value to the cytologist for the study of living cells. It yields information concerning the motility, phagocytic power, mitochondrial content, fragility, and life history of the cells, which cannot be obtained by studying fixed smears or sections. The method has been used for many years by isolated investigators and has been more recently advocated and described by Simpson (6), Sabin (7), and others. For detailed descriptions of the technique, their papers should be consulted. In these studies neutral red and Janus green have been used as in the method given by Sabin (7).

Descriptions of Blood-Cells

Red Blood Corpuscles (Fig. 11).—These cells in the chicken are all nucleated. They are oval in shape and considerably larger than the

red blood cells of man. The nucleus is usually slightly irregular and roughly oval in shape, and is smaller than that of a small lymphocyte. It is about the same size, or somewhat smaller than that of a thrombocyte. The nuclei of the normal red corpuscles are distinct from those of any other cells in that most of them are clear and show very little nuclear structure. Occasionally one finds a nucleus of a red blood corpuscle which is somewhat shrunken, more round than usual, and which has a more distinct, reticular structure. Around the nuclei of red blood corpuscles one almost invariably finds a few bluish-green, rod-shaped mitochondria which stain specifically with Janus green. The cytoplasm also contains from none to several small, reddish-brown bodies which are usually near the nucleus, but they often move about and may be far out near the cell border. In the supravital film, cells are sometimes found which obviously belong to the erythrocyte series, but in which there is no hemoglobin or a barely perceptible amount (Fig. 8). These cells have been confused with thrombocytes (Figs. 7, 9). However, careful scrutiny will always reveal an intact cellular membrane. The cytoplasm is much more abundant than that of the thrombocyte and the nucleus is distinct. These we have called degenerating forms of red blood cells. Clinging to their nuclei one can often see refractive vacuoles which are rarely stained and which are different from the stained vacuoles one sees in thrombocytes.

Thrombocytes (Figs. 7, 9).—These cells in the fowl are nucleated and are about the size of small or intermediate lymphocytes. They are distinctly smaller than erythrocytes. Their configuration is usually irregular in contrast to the round lymphoid cells and the oval red blood cells. The nuclei are distinguished by a very pronounced splotching which is so marked that it often appears vaculated. This is in contrast to the clear, almost structureless nuclei of the erythrocytes, and to the softer, more cloudy appearance of the nuclei of the lymphocytes. The cytoplasm of thrombocytes is often quite clear at first (Fig. 9), but after a few minutes' exposure to neutral red and Janus green, vacuoles (one or a few) develop at one or both ends of the nuclei (Fig. 7). These vacuoles stain a muddy brown color, are non-refractive, and increase in size with longer exposure to the dye. Sometimes a few very delicate, barely perceptible mitochondria appear in the perinuclear area. These bodies are much less constantly found and are not as large or as conspicuous as in lymphocytes.

Thrombocytes occur characteristically in clumps of two, three, or many cells, but are also found singly. In this respect they behave as do the blood platelets of mammals.

Polymorphonuclear Eosinophiles (Fig. 1).—These cells are numerous in the blood stream and, as has been shown by Moore (8) and others, are the elements which are specifically increased in fowl typhoid. They have a lobulated nucleus with a reticular chromatin network. The cytoplasm is filled with rod-shaped, specific granules which stain red with eosin in fixed preparations and stain a golden-yellow with neutral red in supravital films. These rods are not very uniform in size and shape. Some of them are short and almost round; others are sometimes club-shaped. The majority of them, however, are uniform in their size and staining reactions. After a few minutes' exposure to the dye, one or more vacuoles may appear in the cytoplasm. These vacuoles stain a reddish-orange color and increase in size with longer exposure to the dye. Mitochondria are rarely seen. When present, they are delicate filaments or dots staining specifically with Janus green. The polymorphonuclear eosinophiles are actively motile cells. They are probably comparable, in their function, to the polymorphonuclear neutrophiles of human blood.

Polymorphonuclear Cells with Pseudo-eosinophilic Granules (Fig. 2).—These elements have been commonly referred to in the literature as true eosinophiles, as eosinophiles with round granules, and as neutrophiles. They differ from the eosinophilic cells with rods not only in the shape of the granules, but also in their chemical reaction to the dye. The granules are really pseudo-eosinophilic. They take very little of the color in neutral red preparations. The granules are uniform in size and color and are quite small. They rarely fill the cytoplasmic area. The granules are very similar to the granules of amphophiles or neutrophiles of rabbits. The nucleus is usually bilobed. Mitochondria are commonly present among the specific granules. The cells are rarely as actively motile as the cells with eosinophilic rods.

Basophiles (Fig. 4).—The cells with specific basophilic granules are about the same size as those with eosinophilic rods. The nucleus is usually a single, irregular or round mass, but may be lobulated. The nucleus shows a diffuse chromatin distribution. The cytoplasm is filled with red specific granules. The cells are only slightly motile.

Monocytes (Fig. 5).—These are conspicuous cells in the chicken's blood. They vary somewhat in size from that of the polymorphonuclear cells to slightly larger. The nucleus is commonly indented or horseshoe-shaped but may be round or lobulated. Mitochondria are abundant, small, and may be in the shape of filaments, rods, or dots. The cytoplasm possesses a hazy appearance in contrast to the clear, glassy cytoplasm of lymphocytes. Neutral red bodies are prominent features and are often grouped together, forming a rosette in the bay of the nucleus. These neutral red bodies (segregation apparatus) increase in size with longer exposure to the dye. They stain a brick-red color and are almost all non-refractive. These cells are motile and have the same type of motility as has been described by Sabin (7) for the monocytes of human blood.

Lymphocytes (Figs. 6, 10).—Small, intermediate, and large lymphocytes are easily distinguished in fowl blood. The small lymphocytes are much more numerous than the larger forms. They are about the size of human small lymphocytes, but appear much smaller because of the larger red blood cells with which to compare them. They are similar in all respects to the lymphocytes of human blood. The nucleus is round or slightly indented and is relatively large in proportion to the amount of cytoplasm. The nuclear structure is made up of masses of chromatin which fade off gradually into the surrounding nuclear structure, giving a soft, cloud-like effect in contrast to the nuclear structure of thrombocytes and erythrocytes. In the cytoplasm of lymphocytes one finds large, coarse mitochondria as a constant feature. These Altmann bodies, which stain specifically with the Janus green, are apt to be perinuclear in their distribution and are sometimes grouped on one side of the nucleus. Many of the lymphocytes possess one or several small, red bodies in the cytoplasm. These structures stain a dark red in contrast to the brick-red of the monocyte granules and the brown of the thrombocyte segregation bodies. These neutral red bodies of the lymphocyte are round and are refractive, whereas the neutral red bodies of the monocytes and thrombocytes are non-refractive. The lymphocytes are only slightly motile in very fresh preparations. They move slowly with the nucleus near the front of the cell.

The accompanying table (Table I) gives the average total and per-

TABLE I

Blood Cell Counts of Normal Chickens

Fowl No.	Date	Total erythrocytes per cu. mm.	Total thrombocytes per cu. mm.	Total leucocytes per cu. mm.	Hemoglobin (Newcomer)	Eosinophiles with rods	Pseudo-eosinophiles	Basophiles	Monocytes	Lymphocytes	Myelocytes	Unclassified	Total eosinophiles with rods per cu. mm.	Total pseudo-eosinophiles per cu. mm.	Total basophiles per cu. mm.	Total monocytes per cu. mm.	Total lymphocytes per cu. mm.
						per cent	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*					
55 B	11/ 2/27	3,270,000		17,670	65	31		10	14	42			6,008		1,767	2,474	7,421
	11/ 9/27	3,310,000	101,090	25,920	56	25	1	2	9	62		1	6,480	259	518	2,333	16,070
	11/12/27	3,220,000	46,137	11,830	60	26		3	10	60	1		3,076		355	1,183	7,098
	11/14/27	3,520,000	40,800	12,000	60	18	3	4	13	62			2,160	360	480	1,560	7,440
	11/19/27	3,020,000	17,961	8,553	65	32		6	8	54			2,737		513	684	4,619
	11/21/27	3,600,000	13,793	13,793	65	23	3	3	18	52		1	3,172	414	414	2,483	7,172
	11/26/27	2,960,000	23,716	26,351	70	31		6	23	40			8,169		1,581	6,061	10,540
	11/28/27	2,300,000	29,716	11,607	70	25	1	2	15	57			2,902	116	232	1,740	6,616
	12/ 1/27	3,540,000	39,130	13,043	70	19	1	3	8	69			2,478	130	390	1,043	9,000
	12/ 6/27	3,660,000	52,800	28,225	68	23	1	7	19	50			6,492	282	1,976	5,363	14,113
64 B	4/ 7/28	4,620,000	9,420	15,710	60	49	1	5	19	26			7,700	157	786	2,985	4,085
	4/11/28	3,700,000	17,976	29,960	60	43	1	10	9	37			12,882	300	3,000	2,700	11,085
51 B	10/20/27	2,810,000	35,156	9,375	60	37	3		7	53			3,468	281		656	4,970
54 B	11/ 2/27	3,070,000	47,823	17,390	57	33	8	5	5	48			5,739	1,391	870	870	8,347
65 B	4/ 7/28	3,760,000	23,280	23,280	75	66		1	13	20			15,365		233	3,026	4,656
	4/11/28	3,620,000	20,882	20,882	70	48	11	5	21	15			10,023	2,297	1,044	4,385	3,132
66 B	4/11/28	3,600,000	5,408	6,760	77	44	2	1	14	39			2,974	135	68	946	2,636
	4/16/28	3,390,000	25,025	22,750	72	24	2	5	8	61			5,460	455	1,137	1,820	13,878
1 C	10/19/28	3,750,000	10,672	26,686	46	48	1	1	17	32			12,809	267	267	4,536	8,540

2 C	10/22/28:																
	11:45	2,810,000	52,382	33,795	52	33	2	3	34	28			11,152	676	1,014	11,490	9,463
	2:15	2,835,000	30,640	30,640	48	32		2	32	34			9,805		613	9,805	10,417
	2:45	2,730,000	28,192	26,597		40	4	2	20	34			10,639	1,064	532	5,319	9,043
	3:15	3,030,000	34,513	33,508		31	2	7	23	37			10,387	670	2,345	7,708	12,398
	3:45	3,080,000	21,912	27,390		44		4	25	27			12,052		1,096	6,847	7,395
	4:30	2,830,000	42,543	39,030		30	3	7	30	29		1	11,709	1,171	2,732	11,709	11,319
	10/23/28	2,970,000	11,556	29,630	52	38		2	38	19		3	11,259		593	11,259	5,630
4 C	10/29/28	2,780,000	27,960	41,730	58	46		4	24	26			19,196		1,669	10,015	10,850
5 C	1/24/29	3,610,000	142,048	73,600	57	29	1	2	3	65			21,344	736	1,472	2,208	47,840
6 C	1/25/29	3,150,000	27,306	33,300	69	39		10	17	34			12,987		3,330	5,661	11,322
Average		3,267,000	34,990	24,586	62.9	34.72	1.76	4.21	17.1	41.79			8,642	385	1,069	4,444	9,900

centage values of each of the structural elements found in the blood of eleven domestic fowls. The total number of counts reported is 29. The average red blood cell count was 3,267,000 cells per cubic millimeter. The highest and lowest counts were 3,760,000 and 2,300,000 erythrocytes per cubic millimeter respectively. The average thrombocyte value was 34,990 cells per cubic millimeter, with a variation from 5,408 to 142,048 thrombocytes per cubic millimeter. Total leucocytes averaged 24,586 cells per cubic millimeter and varied between 6,760 and 73,600 cells per cubic millimeter. The hemoglobin (Newcomer method) varied from 46 per cent to 77 per cent. The average was 62.9 per cent.

Eosinophiles with rods varied between 18 and 66 per cent in different animals and averaged 34.72 per cent. Pseudo-eosinophiles with small, round granules were not encountered in ten of the counts. The highest percentage obtained was 11, and the average 1.76 per cent. Basophiles were found in all except one animal. The highest percentage value obtained was in Rooster 64, where they were 10 per cent. The average value was 4.21 per cent. Monocytes were found to be high in the chicken, varying from 3 to 38 per cent and averaging 17.1 per cent. Lymphocytes were usually more numerous than any of the white blood cells except thrombocytes. They also showed a wide variation between 15 and 69 per cent. The average value was 41.79 per cent. Myelocytes were rarely encountered and unclassified cells were few in number.

The total numbers per cubic millimeter of each of the types of leucocytes are given in Table I. Their average values obtained were:

	per cubic millimeter
Eosinophiles with rods	8,642
Pseudo-eosinophiles	385
Basophiles	1,069
Monocytes	4,444
Lymphocytes	9,900

Discussion of Blood Cells

The literature records the observation of numerous investigators on the blood of the fowl. Some of the more important of these studies have been summarized in Table II.

TABLE II

Blood Counts of Domestic Fowls as Recorded in Literature

Authors	Erythrocytes per cu. mm.	Leucocytes per cu. mm.	Thrombocytes per cu. mm.	Eosinophiles with rods	Pseudoeosinophiles	Basophiles	Monocytes	Lymphocytes		Hemoglobin	Degenerating types	Unclassified	Clasmatocytes
								Large	Small				
				per cent	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
Stöltzing (9)	3,860,000												
Malassez (10)	3,100,000												
Hayem (11)	2,400,000	26,300											
Moore (12)	3,637,000	20,081											
Ward (13)	3,283,000	36,185											
Mack (14)	3,017,000	55,272								87.3			
Albertoni and Mazzoni (4)	2,460,000	32,300	45,566										
Klieneberger and Carl (15)	3,117,000	35,000 to 60,000	22,900 to 130,000	29.5	4.5	2.2	None	63.8		62.0			
Blain (2)		18,630	None	49.4	8.7	3.6	5.7	32 8					
Warthin (16)	2,000,000 to 3,000,000	12,000 to 29,000		21.5	10.0	2.0	None	35.5	14.5		16.5		
Burnett (17)	3,324,000	17,921		28.8	3.3	4.3	5.5	58.0		76.0			
Schmeisser (18)	2,500,000 to 4,500,000	20,000 to 80,000		29.6	4 3	2.2	19.4	42.3		45.0 to 75.0 (Sahli)		2.2	
Breusch (19)	3,468,000	33,300		17.7	4.1	2 5	9.2	66.5					
This study (average values)	**3,267,000**	**24,586**	**34,990**	**34.7**	**1.8**	**4.2**	**17.0**	**41.8**		**62.9 (Newcomer)**	**Rare**	**Rare**	**Rare**

It will be seen that Mack (14) records 55,272 leucocytes per cubic millimeter as the normal count, whereas Blain's (2) and Burnett's (17) data show 18,000 cells per cubic millimeter as the average normal value. Likewise the thrombocytes have been variously recorded. Most of the investigators have failed to enumerate them. Klieneberger and Carl (15) state that they vary from 22,000 to 130,000 per cubic millimeter. On the other hand, Blain (2) has failed to find any of these structures. The percentages of the types of leucocytes have yielded markedly different figures in the hands of different investigators. Eosinophiles with rods are reported by Breusch (19) as constituting 17.7 per cent of the total leucocytes, whereas Blain (2) found 49.4 per cent of these cells. Klieneberger and Carl (15) identified no monocytes or transitional cells in normal domestic fowls. On the other hand, Schmeisser (18) states that there are 19.4 per cent of these elements in the blood.

An analysis of the literature reveals four causes for most of the discrepancies. These causes are: study of insufficient numbers of animals, failure to make a sufficient number of observations on single animals, lack of an adequate method of counting white blood cells, and confusion of thrombocytes with other elements of the blood. It must also be borne in mind that there are large individual fluctuations of the leucocyte counts of fowls which are considerably greater than those encountered in mammals.

Still another factor which has led to confusion in the interpretation of blood cells of the fowl is the method of classification of the granular leucocytes.

Niegolewsky (20), for example, described two types of oxyphil granular cells, one with large refractive granules and another with fine oxyphil granules. The former were numerous and the latter few in number. In addition he found two types of basophiles and also neutrophilic leucocytes. The latter had round or polymorphous nuclei and fine granules. Grünberg (21) described two types of eosinophilic leucocytes: (a) cells with crystalloid granules, and (b) cells with globular-like granules. These latter he stated were similar to the polymorphonuclear cells of man. Hirschfeld-Kaszmann (22) stated that three types of eosinophilic granular cells occurred: (a) cells with segmented nuclei and crystalloid granules, (b) cells with single nuclei and pale rod-shaped granules, and (c) cells similar to (b) but having round granules. They found no neutrophilic granular cells. Burnett (17) did not call the polymorphonuclear cells with rod or spindle-shaped granules eosinophiles. He believed that these cells resembled in their staining reaction and biological properties the polymorphonuclear neutrophiles of mammals. He found eosinophilic leucocytes with round granules which were similar to the eosinophiles of mammals. Kasarinoff (23) described the same types

of eosinophiles as did Grünberg (21). Klieneberger and Carl (15) found two types of eosinophiles. Those with rod-shaped granules they called pseudo-eosinophiles and those with small granules they labelled as eosinophiles with small granules.

Descriptions of Bone Marrow Cells

The method which has been applied in these studies to the bone marrow has been a modification of that employed for blood and is the same as that which has been used by Sabin and Doan (24) and others for the study of bone marrow, lymph nodes, and other tissues of animals. Neutral red and Janus green films are prepared as for making supravital studies on blood, except that the concentrations of the dyes are increased about three fold. Small pieces of representative bone marrow are then removed from an anesthetized or freshly killed animal. A suspension is made on the prepared slide by mixing the small piece of marrow in a drop of normal saline, or better yet, with the animal's own serum. This material is spread out on the slide over an area the size of the coverslip. The coverglass is then allowed to fall gently on the prepared marrow. The preparation is quickly rimmed with vaseline or melted paraffin and can be studied immediately in a constant temperature box at about 38 or 39°C. Material prepared in this way gives a fairly uniform distribution of the cells and a thin enough film so that the individual cells can be studied and counted. The cells survive for hours if kept at body temperature.

A complete review of the histology of the bone marrow has recently been written by Sabin (25). For this reason it is unnecessary to review again the literature on this organ.

The types of cells found in the bone marrow of the chicken are, in addition to the fully matured formed elements of the blood, immature forms of all the leucocytes and red blood corpuscles. The relative numbers of the various types of cells are recorded in Table III. In this table the numerical values are given of the cells found in the bone marrow of four normal, Plymouth Rock roosters, about 8 months of age.

The cells present in largest numbers in films of the bone marrow are mature red blood corpuscles. They constitute an average of 53.8 per cent of all the cells present. Mature polymorphonuclear eosinophiles, on the other hand, constitute only 3.9 per cent of all the cells and 8.18

TABLE III

Survey of Cells of Bone Marrow of the Domestic Fowl

Rooster No.	Marrows examined	Total number of cells counted			Mature R. B. C. Per cent of all cells	Eosinophiles								Neutrophiles			
		Mature		All other cells		Mature P. M. E.		Myelocytes "A"		Myelocytes "B"		Myelocytes "C"		Mature P. M. N.		Myelocytes	
		R. B. C.	P. M. E.			Per cent of all cells	Per cent of cells exclusive of mature R. B. C.	Per cent of all cells	Per cent of cells exclusive of mature R. B. C.	Per cent of all cells	Per cent of cells exclusive of mature R. B. C.	Per cent of all cells	Per cent of cells exclusive of mature R. B. C.	Per cent of all cells	Per cent of cells exclusive of mature R. B. C.	Per cent of all cells	Per cent of cells exclusive of mature R. B. C.
2 C	Rt. femur Lt. " Rt. tibia	769	80	798	46.86	4.85	9.11	0.48	0.91	2.31	4.33	8.74	16.40			0.48	0.91
1 C	Rt. femur Lt. " Rt. tibia Lt. "	839	49	375	66.43	2.69	8.02			0.95	2.83	8.16	24.29			0.24	0.71
4 C	Rt. femur Rt. tibia Lt. " Rt. radius	828	12	660	55.20	0.80	1.79	0.47	1.04	2.20	4.91	9.93	22.17			0.13	0.29
5 C	Rt. femur Lt. " Rt. tibia Lt. "	876	133	862	46.70	7.41	13.80	0.75	1.40	1.17	2.20	11.30	21.2	0.16	0.30	0.21	0.40
Average.....		828	70	674	53.8	3.94	8.18	0.42	0.84	1.66	3.57	9.50	21.02	0.04	0.08	0.26	0.58

TABLE III—*Concluded*

Rooster No.	Marrows examined	Basophiles		Monocytes		Small, round cells, probably lymphocytes		Undifferentiated cells—megalo-, mono-, lympho-, myeloblasts		Erythroblasts		Clasmatocytes		Osteoclasts		Unclassified	
		Per cent of all cells	Per cent of cells exclusive of mature R. B. C.	Per cent of all cells	Per cent of cells exclusive of mature R. B. C.	Per cent of all cells	Per cent of cells exclusive of mature R. B. C.	Per cent of all cells	Per cent of cells exclusive of mature R. B. C.	Per cent of all cells	Per cent of cells exclusive of mature R. B. C.	Per cent of all cells	Per cent of cells exclusive of mature R. B. C.	Per cent of all cells	Per cent of cells exclusive of mature R. B. C.	Per cent of all cells	Per cent of cells exclusive of mature R. B. C.
2 C	Rt. femur Lt. " Rt. tibia	0.31	0.58	0.67	1.25	13.78	25.85	4.37	8.09	16.15	30.29	1.09	2.05			0.12	0.23
1 C	Rt. femur Lt. " Rt. tibia Lt. "					6.65	19.81	7.67	22.87	7.05	21.00					0.16	0.47
4 C	Rt. femur Rt. tibia Lt. " Rt. radius			5.93	13.24	10.93	24.40	2.74	6.11	10.13	22.62	0.48	1.64	0.13	0.30	0.67	1.49
5 C	Rt. femur Lt. " Rt. tibia Lt. "	0.05	0.10	3.09	5.80	14.71	27.60	3.04	5.70	11.46	21.5						
Average.....		0.09	0.17	2.42	5.07	11.52	24.41	4.46	10.69	11.20	23.85	0.39	0.92	0.03	0.07	0.24	0.55

per cent of all the cells, exclusive of mature red blood corpuscles. The accompanying table has been arranged to give the percentage value of each constituent element both in relation to the total of all the cells present and in relation to the number of cells, exclusive of mature red blood corpuscles. The reason for this is that most of the mature erythrocytes probably represent extravasations from traumatic hemorrhage and are not true parenchymal elements of the marrow.

Myelocytes (Figs. 14, 15, 16).—In this paper the myelocytes have been grouped arbitrarily into A, B, and C classes, according to the plan employed by Sabin, Austrian, Cunningham, and Doan (26). Myelocytes A are of the earliest type and represent the next stage of development after the myeloblast. Myelocytes A include cells with 1 to 10 specific granules in the cytoplasm. Myelocytes B (Figs. 14, 15) have more specific granules than Myelocytes A, but do not possess their full quota. Myelocytes C (Fig. 16) represent the next stage of development in which there is the full quota of specific granules in the cytoplasm, but the cells still retain their mononuclear characteristics.

The myelocytes of the chicken's blood are not only distinguished by the number of their specific granules, but also by their character. The granules are almost always round or globular, whereas the fully mature cell possesses elongated, rod-shaped granules. Moreover, the younger the specific granules are the more intensely they stain with neutral red. The mature granules of the adult polymorphonuclear cell and of the very late myelocytes stain a yellowish copper color with neutral red. The earlier myelocyte granules (Fig. 15) are darker in color, staining a brownish red. Mitochondria are readily seen in the early myelocytes. They are scattered through the cytoplasm and are delicate rods or dots which stain specifically with Janus green. With greater accumulation of specific granules, the mitochondria become less conspicuous and sometimes cannot be seen. Myelocytes A are are few in number in normal bone marrow. The average value was slightly less than 1 per cent of the cells, exclusive of mature erythrocytes. Myelocytes B composed 3.57 per cent. These cells were present in about the same relative proportions as Sabin and Doan (24) found them in normal rabbit bone marrow.. Myelocytes C were represented by 21.02 per cent of the cells, distinctly less than their proportion in rabbit bone marrow.

Pseudo-eosinophilic myelocytes and polymorphonuclear pseudo-eosinophiles were only occasionally found in the bone marrow, where they constituted one-half of 1 per cent of the cells. They were identical with the pseudo-eosinophilic cells of the blood.

Basophiles.—These cells were likewise rare in the bone marrow. They, too, were identical with the basophilic cells which were found in the blood and have been described in an earlier part of this paper. In two of the animals no basophiles were seen in the marrow films. The average value for basophiles in the bone marrow was less than 0.2 per cent. Sabin and Doan (24) found in rabbits that they constituted from 0.29 to 2.02 per cent of the bone marrow cells.

Monocytes.—About 5 per cent of the parenchymal elements of the marrow are monocytes. They are identified by the criteria previously given in the section on blood cells, and need no further description here. It is not difficult to distinguish them from the early myelocytes because of the larger and more refractive granules of the latter.

Lymphocytes.—Contrary to the findings in bone marrow of mammals, there is a considerable number of small round cells (Fig. 3) in every film of bone marrow equivalent to about 25 per cent of the cells. They are probably small lymphocytes. It is often difficult in the marrow to differentiate these elements from primitive cells and thrombocytes. The characteristics which render the distinction easy in a thin film of blood are often obscured by the crowding of the cells and lack of perfectly uniform staining.

Undifferentiated Cells (Fig. 13).—The early undifferentiated cells of the bone marrow I have chosen to group together under one heading for the reason that I have not been able to say in numerous instances whether given cells were megaloblasts, monoblasts, lymphoblasts, myeloblasts, or primitive cells. The problem of sub-classifying these "blast" cells in the marrow of the chicken is much more difficult than to recognize and classify them when they occur in the blood. The reasons are obvious. The staining is not as uniform, the cells are more crowded, and one cannot have as an aid the character of the other accompanying cells, which aid is often of great value in a study of blood films. In the bone marrow, spleen, and lymph nodes of mammals it is usually not difficult to segregate the cells into the various types of blasts, but I do not believe that there is sufficient evidence in the

chicken for such a rigid sub-division of the undifferentiated marrow cells. This group of blast cells represents about 10.5 per cent of the parenchymal elements. They are about the size of myelocytes. The nucleus may be central or peripheral and frequently contains a large nucleolus. The cytoplasm usually contains no stainable material in supravital films.

Erythroblasts (Fig. 12).—Immature red blood cells are separated from the mature red corpuscles by their larger and more conspicuous nuclei, by the presence of more neutral red bodies in the cytoplasm, and often by a decreased amount of hemoglobin. The immature red corpuscles are frequently less oval and more round than the adult forms. The total number of erythroblasts represents about 24 per cent of the cells, exclusive of mature red blood cells, and about 11 per cent of the total cells present.

Clasmatocytes (*Macrophages*).—The characteristic by which these cells are recognized is dependent largely on their phagocytic properties. They engulf leucocytes, red corpuscles, and débris. The vacuoles of the living cells stain brilliantly with various shades of red, yellow, and brown. These vacuoles increase in size with longer exposure to the dye, and are not arranged in any definite pattern. The nucleus is usually round and placed near the center of the cell. The shape of macrophages is often irregular. They vary in size, but are commonly about the size of monocytes or other large leucocytes. These cells constitute about 1 per cent of the marrow elements.

Osteoclasts (Fig. 17).—The surveys of the bone marrows of four chickens reported in this communication show osteoclasts to be present in only one. In this animal they represented 0.3 per cent of the marrow cells. The ends of the long bones often contain many spicules of bone, and in these regions osteoclasts are more numerous than in the shafts. They are very large, multinucleated cells. The nuclei do not appear to have any definite pattern, but are of about equal size and scattered through the cytoplasm. The cytoplasm often contains considerable numbers of vacuoles or granules which are quite homogeneously distributed throughout the cytoplasm and may tend to obscure the nuclei. These vacuoles stain brown with neutral red.

Thrombocytes have not been observed in the marrow of the long

bones of these chickens. Their origin is obscure and work is now in progress to determine more about the life history of these elements.

SUMMARY AND CONCLUSIONS

1. A simple, direct method of counting leucocytes of the fowl is described.

2. Twenty-nine complete, morphological studies of the blood of eleven domestic fowls are recorded.

3. The characteristics of the cells found in the blood and bone marrow are described in detail and their relative numbers reported.

4. The supravital technique, using neutral red and Janus green, enables one to separate and classify accurately the confusing cells of the blood and bone marrow.

5. These studies provide a basis for future experimental studies on the blood and bone marrow cells of the fowl.

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EXPLANATION OF PLATE 6

FIG. 1. Typical polymorphonuclear eosinophile with rods from peripheral blood. Stained with neutral red.

FIG. 2. Pseudo-eosinophile from peripheral blood of domestic fowl. This cell is slightly larger than the typical pseudo-eosinophile. Stained with neutral red and Janus green.

FIG. 3. Small, round cell of bone marrow of the domestic fowl, probably a lymphocyte.

FIG. 4. Basophilic leucocyte of the peripheral blood. Note that the granules vary somewhat in their affinity for neutral red.

FIG. 5. Monocyte of the peripheral blood. Mitochondria stained with Janus green and segregation bodies stained with neutral red. Note the tendency for grouping of neutral red bodies about the centrosphere, a common, but not invariable characteristic of these cells.

FIG. 6. Large lymphocyte from peripheral blood. Stained with neutral red and Janus green. There were no neutral red bodies in this cell, a fairly common finding. Mitochondria are large.

FIG. 7. Two thrombocytes grouped together in the peripheral blood. No mitochondria were seen in these cells, but there were numerous neutral red vacuoles. Stained with neutral red and Janus green.

FIG. 8. A degenerating red blood corpuscle of peripheral blood. Note the absence of hemoglobin, but the preservation of the cellular membrane. A few refractive, unstained bodies are near or on the nucleus. These cells have been confused with thrombocytes. Compare with Figs. 7 and 9.

FIG. 9. Two thrombocytes of peripheral blood. Mitochondria are more conspicuous than in the usual thrombocyte. A neutral red vacuole has begun to develop. Stained with neutral red and Janus green.

FIG. 10. Intermediate lymphocyte of peripheral blood. Slightly larger than a small lymphocyte. Stained with neutral red and Janus green. Two small neutral red bodies are present.

FIG. 11. Typical erythrocyte of peripheral blood.

FIG. 12. Erythroblast from the bone marrow. Note the smaller amount of hemoglobin than in the mature erythrocyte.

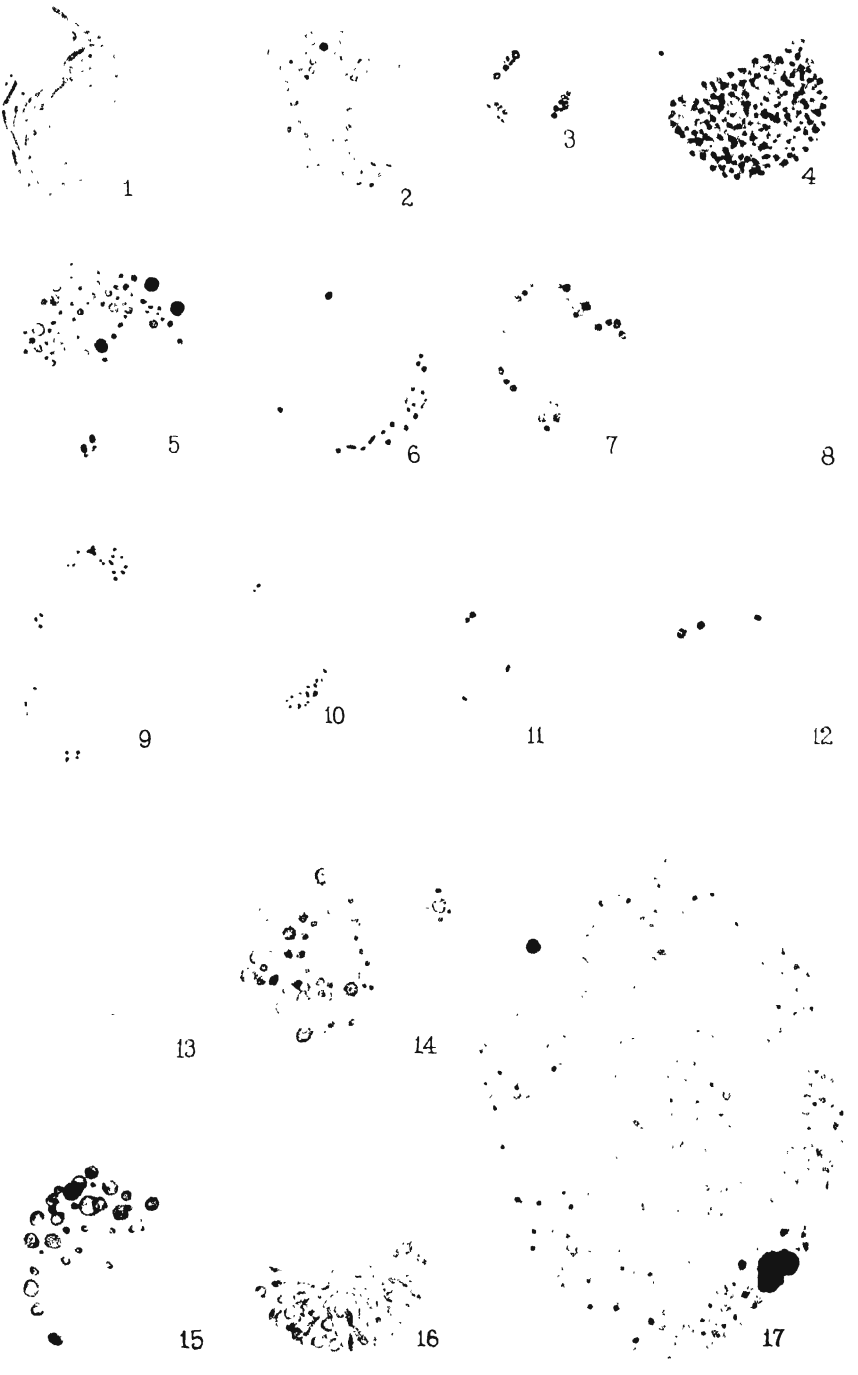
FIG. 13. An undifferentiated cell of the bone marrow. This cell has an eccentric nucleus. Many of them have nuclei in the center of the cell. Neutral red and Janus green stain.

FIG. 14. A myelocyte "B" of the bone marrow. Specific granules are refractive and round. Stained with neutral red. Mitochondria are often abundant, but are not shown in this drawing.

FIG. 15. Myelocyte "B" of a somewhat earlier stage than that shown in Fig. 14. Specific granules vary considerably in size and take a somewhat deeper color than in the later myelocytes. Stained with neutral red.

FIG. 16. Myelocyte "C" of bone marrow. Some of the specific granules are beginning to assume the color and shape of those of the fully mature polymorphonuclear eosinophile shown in Fig. 1. Neutral red stain.

FIG. 17. Osteoclast of the bone marrow. Nuclei are round and almost obscured by the neutral red bodies.



(Forkner: Blood and bone marrow cells of domestic fowl)

[Reprinted from the ARCHIVES OF NEUROLOGY AND PSYCHIATRY, July, 1929, Vol. 22, No. 1, pp. 135–138]

STAINING OF TISSUES OF THE CENTRAL NERVOUS SYSTEM WITH SILVER

THE INFLUENCE OF THE STRENGTH OF THE REDUCING AGENT

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In studies of the so-called "silver staining methods," emphasis has usually been laid on the rôle of the fixative and on the nature of the silver solution, while relatively little attention has been paid to the action of the reducing agent. Perhaps the first investigator to make use of wide variations in this step was del Rio-Hortega. In the gradual development of his method of staining microglia, he parted from the traditional reduction with 10 or 20 per cent commercial formaldehyde solution and found that selective staining of the cells could be secured by immersing the silver-impregnated sections, without washing, directly into a formaldehyde solution of only 1 per cent (1 cc. of concentrated liquor formaldehydi—approximately 37 per cent—in 100 cc. of distilled water). He also made certain interesting observations on reduction after a variety of intervening washes with, for example, alcohol, pyridine and other liquids. These observations, which I was privileged to share with him in his laboratory during the spring of 1926, have not yet been published.

OBSERVATIONS

My purpose in this article is to discuss the effects of varying the strength of the formaldehyde alone. These effects are most clearly seen when the sections are taken directly from the ammoniacal silver bath into the formaldehyde solution, without any intervening wash—as in the method of staining microglia. This procedure will therefore be discussed first.

The concentration of the formaldehyde solution has a primary effect on the speed with which the reduction occurs. On theoretical grounds this must be true; and, when tested either on solutions alone or on

frozen sections, it is readily demonstrated that a weak solution reduces the silver slowly, while a highly concentrated formaldehyde solution of the same p_H reduces the silver rapidly. From this simple fact, many secondary results ensue.

1. Sections that are stained in any of the usual ammoniacal silver solutions will emerge from the silver bath with a color that varies from pale yellow to deep tobacco brown, depending on the duration, concentration and temperature of the stain. If such sections are then placed directly, without any washing, into an undiluted, fresh, approximately neutral solution of liquor formaldehydi (about 37 per cent formaldehyde), an almost instantaneous reduction occurs. This rapid reduction is characterized by three results: 1. The color of the sections after reduction is the same as before the reduction—at most becoming a trifle darker. 2. Under the microscope no precipitate is visible either on the sections or in the solution; this is true whether or not the sections are stirred during the reduction. 3. The tissues of the sections are homogeneously stained, so that little structural detail can be made out. The silver appears to have been taken up in an almost undifferentiated way, forming with the tissues a colloidal combination, the metallic units of which are smaller than the limits of microscopic visibility. In the flat field of yellow-brown, the cellular structures can sometimes be seen dimly as pale outlines or occasionally as slightly denser, browner bodies; but neither nerve cells nor fibers nor neuroglia are differentially stained.

2. If, on the other hand, some of the same sections are carried directly from the silver bath into dilute formaldehyde solutions (as, for example, a 1 per cent solution of liquor formaldehydi), a different picture results. In the first place, unless the section is vigorously stirred from the very instant that it enters the formaldehyde, an abundant gray-black precipitate will gradually form in the reducer, welling in clouds from the section. (With vigorous stirring, less precipitate will form in the formaldehyde.) During this process, the section itself slowly changes color—taking on a gray-black tone—the final intensity of which depends on the depth of the initial staining in the silver bath and on the dilution of the formaldehyde. There is a certain concentration of the formaldehyde at which this gray-black color reaches its maximum, while at dilutions stronger than this the sections will be yellow-brown, and at weaker dilutions they will be paler gray.

Under the microscope, these sections are seen to carry a gray, dustlike coating of finely divided silver against an almost colorless background. To some extent, this silver dust may be diffusely scattered over the surface of the sections. (This is particularly likely to occur if too weak a reducing bath has been used, or if the stirring has been inadequate during the reduction.) To a great extent, however, the fine granules of reduced metallic silver are seen to outline the cellular elements—nuclei, cytoplasm and processes.

COMMENT AND CONCLUSIONS

From these observations, it is legitimate to make several deductions:

1. The yellow-brown "argyrol-like" color of sections, after reduction in concentrated formaldehyde, is due to a true colloidal combination of the tissue and the silver, with the silver dispersed into units smaller than the limits of microscopic vision.

2. The gray-black color of sections after reduction in very dilute formaldehyde solutions is due (a) to the fact that during the slow reduction in weak formaldehyde the silver has had time to diffuse partially out of its loose union with the tissue elements, leaving a more or less colorless background; and (b) to the fact that with slow reduction the silver has had time to aggregate into particles that are of sufficient size to be visible under the microscope. Neither of these processes can occur under the more rapid action of the concentrated formaldehyde.

3. During the diffusion of silver out of the tissues which occurs in a weak formaldehyde reducing bath, the relatively loose and permeable structures of the background lose silver more readily than do the denser cellular elements. Reduction tends, therefore, to take place within the cells and on their processes and at their surfaces. (This interpretation is in harmony with the theories and experiments of von Möllendorf on the influence of diffusibility of dyes and density of tissue elements on the processes of differential staining by ordinary histologic dyes. It would be rash, however, to deny any influence to chemically selective forces in the differential staining of neurons and neuroglia with silver.)

4. Whether the reduced silver is visible as discrete granules, or is too finely divided to be seen as such, will depend on the density of the particular cells observed, the thickness of the section and the concentration of the reducer. This point makes it necessary to be extremely cautious in interpreting intracellular silver granules as "gliosomes," and granules or spicules of silver along the course of processes as true protoplasmic structures.

5. It is far harder to avoid the formation of misleading artefacts and of superficial encrustations and precipitates with the use of dilute reducing agents than with the use of stronger ones.[1] On the other hand,

1. Under appropriate conditions, cell-like and fiber-like formations can be approximated in coagulated egg-white by these methods.

it is easier, with the dilute reduction agent, to pick out the parenchymatous elements for intensive staining. For any group of sections, therefore, it is possible to find an optimal reducing strength by trying out a series of formaldehyde solutions of different concentrations.

This optimal reducing strength is usually that at which the color-change of the sections from yellow-brown to gray-black is well discernible but not complete. In sections thus treated there is only a partial washing out of the background; and although there is some tendency for the silver to concentrate at the cell surfaces, there should be little aggregation of diffusing silver into microscopically visible particles of metallic silver dust. Occasionally, however, for special purposes (as for staining of microglia) a much weaker reduction than this will give the best results. In fact, the staining of microglia depends largely on this superficial deposit of metallic silver. With different sets of sections and different solutions, the actual optimal strength has been found to vary from a liquor formaldehydi concentration of 100 per cent (37 per cent formaldehyde) to one of 0.25 per cent (0.25 cc. of concentrated liquor formaldehydi in 100 cc. of water).

The optimal reduction must be determined by a few tests for each set of sections. One reason for this is that a number of variables other than the concentration of the formaldehyde enter into the reaction, variables which are difficult to control; for example, the density and exact thickness of the sections, the p_H of the formaldehyde, the possible buffer content of the formaldehyde, the concentration and p_H of the silver solution, the excess ammonia of the silver bath, the heat and duration of the silver bath and so forth.

The same principles apply when the sections are washed between the silver bath and the reduction. In this case, however, much diffusion of silver out of the sections will occur in the washing, and the main effect that differences in concentration of formaldehyde exert is on the state of aggregation of the reduced silver. Here, too, however, optimal concentrations can be found which produce final stains that are superior to those produced by stronger or weaker reductions.

[Reprinted from THE PHYSICAL REVIEW, July 15, 1929, Vol. 34, series 2, No. 2, pp. 296–299]

ON THE ABSENCE OF CHANGE IN MAGNETIC SUSCEPTIBILITY WITH CRYSTALLIZATION IN STRONG MAGNETIC FIELDS

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(Received for publication, April 25, 1929)

ABSTRACT

Lead, tin, bismuth, nickel chloride and potassium ferricyanide were crystallized in strong magnetic fields and examined for changes in the magnetic susceptibilities. The changes, if any, were not more than 0.16 percent in Pb, 0.29 percent in Sn, 0.32 percent in Bi, 0.42 percent in $NiCl_2$ and 0.04 percent in $K_3Fe(CN)_6$.

This paper describes attempts to alter the magnetic susceptibilities of some diamagnetic and paramagnetic substances by crystallization in fairly strong magnetic fields. The substances were lead, tin, bismuth, nickel chloride and potassium ferricyanide. The choice of the metals was determined partly by their conveniently low melting points and partly by the fact that they represent, according to Honda,[1] three types of susceptibility changes at the melting point. The susceptibility of lead changes very little (-0.12×10^{-6} to -0.07×10^{-6}); that of tin changes sign although the absolute value of the change is small ($+0.025 \times 10^{-6}$ to -0.038×10^{-6}) while that of bismuth exhibits a large discontinuity (-1.00×10^{-6} to -0.07×10^{-6}). $NiCl_2$ is a typical paramagnetic salt and in working with it one is less likely to meet with chemical complications than with, say, a salt like $FeCl_3$. $K_3Fe(CN)_6$ represents salts showing the magnetic anomalies often observed when a transition element appears in a complex ion.

Preparation of the Material

The metals were cast as thin disks 7 mm in diameter and 1 mm thick in a special form of graphite mould. The central part of the mould

[1] K. Honda, Ann. d. Physik **32**, 1027 (1910).

where the disks were formed had walls only 1 mm thick, so that it was possible to work with gaps of 3 mm. The pole face diameter was 8 mm. When lead and tin were cast the fields were 25100 gauss. After the completion of the work with lead and tin, the pole tips were found to be partly hollow, the bolts not having been completely turned up. New pole tips were made before the final work on bismuth was done and fields of 28900 gauss were obtained.

The disks for comparison (cast without field) were formed in the same mould and with the mould mounted between the pole pieces.[2] This insured that the rates of cooling were the same and that the only difference was the presence or absence of the magnetic field. A pile of 10 disks was used when comparing the susceptibilities. Both sets of 10 disks were made from the same melt. To eliminate any possible non-homogeneity of the melt the disks were cast alternately, with field, without field, with field, etc. until the required number of 20 was secured. The magnetic field was turned on before pouring and left on until the mould had cooled to about 100°C. The castings were trimmed with broken pieces of glass instead of a file.

$NiCl_2$ was crystallized out of an alcoholic solution by the circulation of dry, dust-free air above the solution at the bottom of a small test tube. $K_3Fe(CN)_6$ was crystallized out of an aqueous solution in the same way. The gap and the pole faces were larger than when casting the metals. The fields were 18500 gauss. $NiCl_2$ came down as a fine powder. The $K_3Fe(CN)_6$ crystals were powdered before measuring.

No attempts were made to grow single crystals.

The Method of Comparison

For the measurements a torsion balance was constructed, following, in the main, the design of Terry.[3] With this balance the suscepti-

[2] This procedure has raised the question as to the magnitude of the residual fields and the possibility that they might produce as much effect as the maximum fields applied. The residual fields were 298 gauss in the work with bismuth and 91 gauss in the work with the salts. The pole pieces that were used in the study of lead and tin were destroyed so that the residual fields could not be determined, but they were certainly less than 298 gauss because of the cavities. The present negative results do not, therefore, exclude possible effects within the range 0–300 gauss in the metals and within the range 0–90 gauss in the salts.

[3] E. M. Terry, Phys. Rev. 9, 394 (1917).

bility is proportional to the product of the two currents necessary for compensation; I, the current in the fixed coils and i the current in the suspended coil. In the general case the observed value

$$(Ii) = (Ii)_s + (Ii)_c + (Ii)_f + (Ii)_a$$

where the subscripts s, c, f and a refer, respectively, to the substance being studied, the container, the stray field and the air correction.

The last three terms are not altered by replacing the ten disks cast in a magnetic field by the ten cast without the field. Also, the two sets of disks can be adjusted to equal masses. Then the condition that crystallization in magnetic fields has no effect on the susceptibility is

$$(Ii)_m = (Ii)_{nm}, \tag{1}$$

the subscript m referring to the set cast with magnetic field and nm to the set cast with no field.

With the powdered salts it was more practicable to determine the masses as M_m and M_{nm} instead of adjusting to equality. The correction for the container, for the stray field and for the air having already been made we have as condition for absence of effect

$$\frac{(Ii)_{sm}\, M_{nm}}{(Ii)_{snm}\, M_m} = 1 \tag{2}$$

RESULTS

Lead. The first observations gave the result $(Ii)_m < (Ii)_{nm}$. A second double set (10 disks cast with magnetic field and 10 without) showed an inequality in the same direction but of smaller amount. The absolute values of (Ii) were larger in the second double set which indicated that paramagnetic or ferromagnetic contaminations were present in the first. Their absence from the second double set was not certain so that still a third double set was cast, with more rigid precautions against the entrance of dust while melting and pouring, and while handling the finished castings. With this third double set $(Ii)_m = (Ii)_{nm}$ within the limits of the errors of measurement.

Measurements were made with the disk axes both parallel and perpendicular to the field. In all, 16 determinations of $(Ii)_m$ were obtained, the disks being piled in the container and mounted in the balance 4 times and measurements made for each mounting at 4 different

positions in the field. In the same way 16 determinations of $(Ii)_{nm}$ were made. These measurements yielded 16 values of $(Ii)_m - (Ii)_{nm}$ for which the mean was +0.00011. The mean value of $(Ii)_s$ (not (Ii)) was 0.06934 so that the diamagnetic susceptibility of lead cast with the magnetic field was not increased by more than

$$\frac{0.00011}{0.06934} \times 100 = 0.16 \text{ per cent.}$$

Tin. Three double sets of tin castings were made of which two gave $(Ii)_m = (Ii)_{nm}$ within the limits of error. For one of these, comparisons were made with the disk axes parallel to the field with readings at 5 different positions. A similar comparison was made with the disk axes perpendicular. The mean of 10 values of $(Ii)_n - (Ii)_{nm}$ was −0.000024. The mean value of $(Ii)_s$ was −0.00838 so that the increase in susceptibility of the paramagnetic tin cast with a magnetic field of 25100 gauss was not more than

$$\frac{0.000024}{0.008380} \times 100 = 0.29 \text{ per cent.}$$

In the work with tin difficulties were expected because of magnetic anisotropy since it is not cubic but tetragonal in structure. They did not appear so that the crystals were either small and numerous enough to be oriented at random or else, tin is magnetically isotropic. I am not aware of any studies on magnetic susceptibilities of single tin crystals as a function of the orientation.

Bismuth. In bismuth the difficulty of magnetic anisotropy[4] did appear. With the disk axes parallel to the field I observed $(Ii)_{sm} = 0.1836 = N_1$ and $(Ii)_{snm} = 0.1902 = N_2$ as the means of 5 measurements on each set, repiling and remounting in the balance each time. The means of 9 measurements with the disk axes perpendicular were, repiling and remounting each time: $(Ii)_{sm} = 0.1890 = N_3$ and $(Ii)_{snm} = 0.1848 = N_4$. (The adjustment to equal values of $\int H(dH/dx)\,dv$ was made).

It can be shown, if there is no effect while crystallizing in a magnetic

[4] C. Nusbaum, Phys. Rev. **29**, 905 (1927).

field and if enough measurements are made, repiling each time so that the disks are oriented at random, that the identity

$$N_1 + 2N_3 = N_2 + 2N_4$$

should exist. This identity is satisfied by the foregoing values of N_1, N_2, N_3 and N_4 to within 0.32 percent, the change in diamagnetic susceptibility, if any, being an increase.

Nickel chloride. Only one sample of $NiCl_2$ was prepared with a magnetic field and one without it. Also, only one measurement was made on each of these samples, the value of $(Ii)_s$ taken being the maximum as the position of the sample was changed with respect to the field. Placing the observed values of $(Ii)_s$ and M in Eq. (2) there was obtained

$$\frac{0.2024 \times 0.2515}{0.1730 \times 0.2930} = 1.0042$$

so that crystallizing $NiCl_2$ in a magnetic field of 18500 gauss did not increase the susceptibility by more than 0.42 percent. Care was taken that the comparison was made at the same temperature.

Potassium ferricyanide. As with $NiCl_2$, only one set of samples was prepared and only one measurement made with each. It was found that

$$\frac{0.08964 \times 0.3408}{0.09064 \times 0.3369} = 1.0004$$

so that the effect, if any, was very minute.

Professor E. P. Adams suggested these experiments and I am indebted to him for much helpful advice.

[Reprinted from the PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES, July, 1929, Vol. 15, No. 7, pp. 609–611]

A METHOD FOR THE DETERMINATION OF THE ACTIVITY OF ANTIPOLIOMYELITIC SERUM

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(Read before the Academy, April 22, 1929)

Continued outbreaks of infantile paralysis (poliomyelitis) during the summer and autumn seasons in America and Europe keep this serious disease in the foreground of medical and public interest. The nature of the inciting microörganism and the usual mode of infection of the disease are known, and this knowledge has led to the employment of certain methods of communal control.

The clinical course of the disease has been closely studied in recent years, so that its early diagnosis, often before pronounced injury has been inflicted on the central nervous organs, is now possible. Side by side with this more precise study of the manifestations of the disease in man has gone the progress of the more fundamental experimental disease produced by inoculation in monkeys. The latter disease, which so exactly reproduces the human malady, has indeed opened the latter to experiments in treatment and prevention which have now reached the stage of practical application in man.

It is now established that when human beings and monkeys recover from attacks of poliomyelitis, they carry in their blood neutralizing substances against the incitant, so-called virus, of poliomyelitis, which are effective not only when the serum derived from the blood is mixed with the virus in a test tube (*in vitro*), but also when the virus is first inoculated into the monkey and the serum injected subsequently. The observations here summarized have led first to the serum treatment of early cases of poliomyelitis in man (Flexner and Lewis, Amoss and Chesney) and also to the preventive inoculation of persons exposed to the disease (Flexner and Stewart).

Since there are still many problems relating to immunity in poliomyelitis awaiting solution, improvement in the manner of inducing

the experimental disease in monkeys is an important matter. In the past the method of choice for inducing infection in monkeys was to introduce the virus directly into the forepart of the brain. This slight operation carried out under ether anesthesia produces no immediate effect, and unless the virus employed is active, no ultimate effect. The method possesses, however, an inherent defect, namely, that the operation cannot be carried out without gross injury to the brain structures themselves, which places the inoculated animal at an abnormal disadvantage in dealing with or combating the virus, compared with the general way in which in the human disease the reparative forces of the body are enabled to act.

This defect works with particular disadvantage when the purpose is to study prevention by or treatment in monkeys with convalescent serum. Hence efforts have been made to secure a method which, while leading to consistent experimental infections, avoids the difficulty of tissue injury. The employment of the device of lumbar puncture for the injection of the virus suffers from the same general defect as that of intracerebral inoculation. When the puncture needle is introduced low down in the spinal cord region, injury is inflicted either on the nerves of the cauda equina or the tip of the cord itself.

These essential drawbacks have been recently overcome by the perfection of a technical method of injecting the virus into the membranes about the brain and spinal cord by way of the cisterna magna, at the junction of the skull and the upper vertebral column. When this mode of injection is used, no injury whatever is inflicted on the nervous structures, and when a virus is employed for injection which is active or virulent, infection of the monkeys regularly ensues. The experimental disease which arises under these circumstances reproduces, indeed, even more accurately than after intracerebral inoculation, the human malady, since the paralysis tends to appear first rather in the lower than in the upper extremities. This result is in conformity with the observations made by Flexner and Amoss that when effective quantities of the virus are injected into the blood, its presence in the cerebrospinal fluid on the way to the nervous tissues can be determined by the withdrawal of fluid and its inoculation into other monkeys.

The significance of the intracisternal method of inoculating the virus arises from the fact that it provides a superior means of testing the efficiency of immune poliomyelitic serum in the prevention and treatment of experimentally produced poliomyelitis in monkeys. The results being obtained and to be obtained will undoubtedly prove more accurately applicable to the human disease than any thus far secured. Moreover, it is believed that other theoretical problems relating to the immunity of poliomyelitis are brought more completely under investigative control than has up to now been the case.

[Reprinted from THE JOURNAL OF EXPERIMENTAL MEDICINE, August 1, 1929,
Vol. 50, No. 2, pp. 189–211]

STUDIES OF TISSUE MAINTENANCE

I. THE CHANGES WITH DIMINISHED BLOOD BULK

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PLATE 8

(Received for publication, June 10, 1929)

The present paper is the first of a series dealing with the service rendered to the tissues by the blood under various conditions. As indices to such service we have utilized the extravascular spread of easily recognizable, innocuous materials thrown into the blood stream. For the purpose of the work here reported certain highly diffusible vital dyes have been employed.

It goes without saying that the multifarious activities of the blood in relation to the tissues cannot be adequately comprehended through observations on the passage from and into the vessels of any single substance or set of substances. But one can at least obtain in this way a knowledge of the general problem in some of its quantitative aspects. Most of the information thus far accumulated concerning it is inferential in nature, being the outcome of observations on rates of blood and lymph flow, on the relative abundance of capillaries in different organs, on capillary pressures, and the state of the local circulation as determined by direct observation. Hooker, Krogh, Richards and others have made studies of the small blood-vessels which illumine the general field; and some investigators have followed directly the diffusion from or into individual capillaries. Our aim has been to determine what the blood does under pathological conditions for the various organs of the body as a whole.

The Choice of Materials

The peculiarities of living cells as manifested in what they reject or accept, and accepting, utilize, secrete, or store, renders the problem of tissue maintenance highly diverse. But it should be possible to

find out in a general way whether the circulation is adequately serving the cells in bringing materials to them and away from them, irrespective of what the cells do with these materials. Susbstances that are let pass by the capillaries must, of course, be employed. True, these will be let pass in varying degrees. But it would seem to be a general law that diffusible, non-toxic "acid" stains penetrate the capillary wall at approximately the same relative rates as when they spread through gelatin (1). If a number that differ widely in this latter respect are selected for animal test it should be possible to gain an insight into how the tissues are served with materials normal to them. At first thought one would say that normal substances should be used. But not only are many of these subjected to change or removal through cell activities, with result that the gradient of permeability is altered locally, but their situation and quantity cannot be gauged with the eye as it can in the case of vital stains of intense color. The best of these stains have special affinities and are stored or excreted in ways that alter interpretations. But with the recognition of such peculiarities, errors due to them can be ruled out. The monographs of Schulemann (1) and of von Möllendorff (2) give one access to a large series of vital dyes. From amongst them and from other sources it should be possible to select test-substances covering the diffusion range of most materials normally purveyed to the cells, with exception of the gases.

The recent history of vital staining is an instructive one, illustrating as it does how rapidly the uses of a scientific tool of wide applicability can become stereotyped as result of success with it in a special field. Ehrlich brought vital staining to modern attention, making significant observations on the nervous system with the aid of methylene blue about thirty years ago (3). But with the subsequent discovery that certain of the poorly diffusible dyes are taken up and stored within living cells, interest turned almost wholly in the direction of the disclosures thus made possible. Not very often since, despite the immense gamut of available dyes, has diffuse vital staining been employed, and then, with a few noteworthy exceptions, for highly specialized purposes. Furthermore the range of dyes utilized in the study of cell-storage has of late narrowed instead of broadening, the mass of investigations nowadays being conducted with one or another of but a few slightly diffusible stains. An observation of the earlier investigators should be mentioned because of its bearing on our own problem, namely that dye storage is especially abundant in the eye muscles, diaphragm, and heart,—whence these workers inferred the existence of an especially great fluid interchange in the organs mentioned (4). Recently Okuneff (5) and Kusnetzowski (6), using the same

criterion, have concluded that much more dye reaches the cells of regions that are inflamed or heated than is the normal case.

For the present work highly diffusible stains devoid of confusing tissue affinities have been selected—Patent Blue V, brom phenol blue, phenol red and sodium indigotate. In enlargement of some of the observations india ink has been pressed into service. Ordinarily we have followed only the distribution of the dyes from blood to tissues, not their subsequent removal from the latter.

Patent Blue V is an intense stain of great diffusibility (7).* Within a few seconds after it has entered the blood stream white animals become brilliantly blue; and by pressing out the blood from the tissues the extravascular situation of the dye can readily be demonstrated. To obtain data on its rate of diffusion into the various organs is difficult, so quickly does it enter most of them. One can always tell, though, where it is and where it is not; and it has the great advantage of rendering visible the walls of the arterioles. Unanesthetized rabbits injected with 1½ cc. per kilo of a warmed, unbuffered 8 per cent solution of Patent Blue V (Hoechst), of pH 6. approximately, manifest no symptoms whatever; and etherized ones show no disturbances of heart beat or blood pressure that are observable with the kymograph, other than those fleeting ones produced by a similar quantity of salt solution. The dye retains its color within the organism. More than three-fourths of it can be recovered as such from the urine of the first twenty-four hours, and most of the rest later on.

Brom phenol blue, somewhat less diffusible and leaving the blood not quite so rapidly, is almost as intense a vital stain. Its range as indicator lies too far to the acid side for the blood and tissue reactions to affect its hue. For our purposes it has proved well-nigh ideal, being devoid, when properly purified†, of action on blood vessels or heart, and diffusing at such rate that the stages in its distribution

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* A correlation of the diffusibilities of the dyes *in vivo* and *in vitro* is being carried out in our laboratory by Dr. Frederick Smith who will report upon them later. It will suffice here to state that a watery solution of Patent Blue V diffuses through a porous glass disc of the sort employed for diffusion measurements by Northrop and Anson (*J. Gen. Physiol.*, 1929, **12**, 541) about as quickly as does dextrose. When the pores of the disc have been filled with gelatin it passes only about one fourth as rapidly as dextrose.

† As ordinarily sold for pH determinations brom phenol blue often contains contaminants affecting the blood pressure. Hynson, Westcott and Dunning have most kindly made for us a purification of their product, which has proved innocuous and devoid of cardiac or vascular effects in cats and rabbits.

can be followed in animals killed at brief intervals after injection.* Furthermore its color so dominates over that intrinsic to the tissues that direct comparisons of degrees of staining become possible. It is best employed in 4 per cent solution, and for intense staining a somewhat greater fluid bulk than of Patent Blue V must be injected intravenously, 3 cc. per kilo of cat or rabbit.† Our usual technic, as in the case of other stains, has been to inject the warmed dye solution during the course of one minute. By the end of this time the body surface is already an even, intense blue; and the failure of the tissues to change color when the blood is driven from them by pressure shows that the dye has already largely passed from the vessels. Neither then nor later are symptoms evident. Rabbits suddenly stained deep blue and placed on the floor sniff about inquiringly and when food is placed before them at once fall to. Three minutes after the dye injection the staining is nearly at a maximum throughout the animal, though accurate color comparisons show that it deepens slightly within another five minutes, remaining constant then for approximately 4 hours and gradually fading later. By next day the animal is practically decolorized, the dye lingering only where the circulation is poor, as in the cartilage of the ear which is still light blue, the tendons (paler blue), the sclerotics (faintly blue); or about a locus of special retention as in the case of the blue gall-bladder wall through which resorption is still taking place secondarily from bile blue-black with the dye excreted with it. At this time the blood plasma no longer is colored. The phthalein leaves the body chiefly by way of the kidney though a little escapes in the feces. As much as 94 per cent of the amount injected has been recovered in the urine of the first 48 hours. The liver takes much out of the blood at an early period, secreting it into the bile, but, as happens with so many other stains, it passes into the circulation again from the gall bladder and intestinal tract, and ultimately escapes in the urine after all. No evidence has been obtained of the least decolorization of the dye within the organism. If any occurs it is negligible.

Phenol red diffuses twice as fast as does brom phenol blue‡ and somewhat more rapidly than Patent Blue V. Much of what has just been said applies to it. Because of the current utilization of it in renal tests it is readily available in pure

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* Dr. Smith has found it to pass through the porous disc of Northrop and Anson at a speed slightly more than half that of Patent Blue V irrespective of whether the disc has been filled with gelatin or not.

† We are indebeted to Dr. MacInnes for freezing point determinations which show that the solution we have employed is isotonic with 0.92 per cent NaCl, and to Dr. Mirsky for observations with the glass electrode which show the pH to be approximately 7.24 at 37°C. The method of preparing the solution has been given in a previous paper.

‡ Unpublished observations of Dr. Smith.

form; and the injection of a freshly made, warmed, isotonic 4 per cent solution of it at pH 7.4, to the amount of 3¾ cc. per kilo, the optimal quantity for staining, causes no symptoms or cardiac or vascular manifestations that a corresponding amount of Ringer's solution would not elicit. It can be used to study blood service to the connective tissue and the other relatively alkaline matrix tissues (cartilage, fascia and tendon(8)) where it appears ruddy, but not for observations on the muscles and viscera, since in them it assumes various shades of orange and yellow that are not readily perceived and evaluated when only a small amount of the dye is present.

Sodium indigotate is poorly soluble at best, only to 2 per cent in water at body temperature; and as much as 7½ cc. per kilo must be injected if the color native to the organs is to be drowned in blue. It is reduced to indigo white in many of the tissues, though it turns blue again on exposure to air; and it is rapidly excreted by liver and kidneys. For all these reasons it has proved unsatisfactory in the study of blood service, although it can be used in confirmation of certain phenomena.

General Procedure

Rabbits and cats have been used for most of the work, and light ether as the anesthetic when one has been necessary. Many of the rabbits have been unanesthetized. To begin with, the normal distribution to the tissues of brom phenol blue was ascertained, and a routine method of examination was worked out. In order that the spread of the dye to the superficies might be studied the fur was in many instances removed from a large part of the trunk and thighs some days prior to the observations, the animal being guarded against chilling thereafter. Barium sulphide proved better for the purpose than shaving. Areas accidentally inflamed were rapidly discriminated by the special intensity of the staining. Ordinarily the animals were fasted 24 hours in order to avoid a digestive hyperemia of the gastro-intestinal tract (Bier); but they were allowed water. Etherized ones were kept on an electrically warmed pad, and were not stretched out but laid on the back or side, without ties. Tracheal cannulation ensured a more even anesthesia. The dye was injected into an ear vein of rabbits and into the basilic vein or internal saphenous of cats, these vessels frequently being cannulated for the purpose. Oiling the body surface brought out brilliantly the surface hues. Since the abrupt introduction of even as little as 1 cc. of fluid into the circulation of a large rabbit brings about compensatory readjustments (Tigerstedt) the dye solution was given gradually in the course of one minute, as already stated. Ordinarily three further minutes were let elapse and then the animal was killed, by cutting both carotids, or,—when no anesthetic had been used,—by decapitation at a blow. The complication of stained blood within the tissues was minimized by the rapid exsanguination. The autopsy was carried out at once and very rapidly, by two workers, with the animal on a slanting board, head down. The organs of special interest were first looked at, the order of

inspection being purposely varied to rule out the possibility of errors due to post-mortem diffusion of the dye. The intensity of the staining as viewed in the gross, was frequently recorded in terms of Ridgway's color standards (9) according to the method used in previous investigations (10). Save in special instances we have not concerned ourselves with the precise location of the dye within the tissues, the main point being that it should have left the circulation, have been served up to the cells, so to speak, irrespective of acceptance or rejection by them.

Not infrequently an amount of blood equivalent to that of dye was removed just prior to injection of the latter. But needless to say this proceeding merely complicated the issue. For not only must some vasoconstriction have been invoked by it, but the salt solution containing the dye must practically at once have been removed from circulation. Whatever the importance of these various factors they did not suffice to bring about differences in the picture. The animals as a group yielded consistent findings, irrespective of the stain employed.

The Staining in Normal Animals

The phenomena observed after the injection of brom phenol blue were essentially the same in rabbits and cats, and were unaffected by etherization. By the time the injection was completed the *hairless tip of the nose*, the *gums*, *conjunctivae* and *fauces* had stained deeply and evenly. The pads of the feet became blue only a little more slowly and less markedly. The *general body surface* took on color progressively and evenly except for intensifications where vessels were abundant, as over the heels and about the mammae. After three minutes the surface hue was brilliant (between "cadet blue" and "diva blue"—Ridgway), the stain lying in the connective tissue. The thin sheet of *voluntary muscle* coming away with the pelt was but palely blue, much paler than the external and internal oblique and the pectoral and leg muscles. These in turn were not nearly so well stained as the *diaphragm*, *intercostals* and *lingual muscles*, the differences being especially plain when muscle layers of the same thickness were compared. To the unaided eye the staining of *subcutaneous tissue* and muscles appeared diffuse, and this still held true when the animals were killed only a few seconds after the injection. For example the tissues of a rabbit killed within 15 seconds after an injection lasting 30 seconds appeared diffusely stained, as did those of a cat killed 15 seconds after an injection which had itself required 30 seconds. But in certain noteworthy instances of animals kept for the routine period of 3 minutes there were indications in the muscles of a latticing or transverse banding with blue, a phenomenon later found to be pronouncedly present when staining was done after the blood bulk had been reduced by bleeding or through the production of anhydremia (11).

The *connective tissue* and *fascia* were everywhere of a medium blue, the *cartilage*, —except for the rib cartilages which fail to stain,—a lighter blue, and so too with the *tendons* and the newer portions of *bone*. Old bone remained uncolored.

Though the supporting framework of the *adipose tissue* was well colored the fat itself did not stain, nor did the white matter of the *central nervous system*, the gray matter being dubiously tinged. The *nerves* to the muscles were beautifully visible in grayish blue to their finest ramifications. The *red bone marrow* appeared deeply colored, but the dye was localized to the blood content. There was intense staining of the media of the larger *arteries* suggesting a special affinity, though the subsequent decolorization took place nearly if not quite as rapidly as elsewhere save in the aorta where it noticeably lagged. The latticing and banding in the muscles mentioned above was not due to this vascular staining. The walls of the *veins* stained poorly. The fibrin of post-mortem blood clots stood out in deep blue. Embolism and thrombosis were sought for but never found.

The tissues thus far described were only moderately blue as compared with most of the abdominal viscera. These were so suffused with the dye as to afford a startling contrast. The *intestines*, large and small, were a deep purplish blue, and so too with the *oesophagus* and *gall-bladder*. The bladder bile of rabbits was already definitely blue after three minutes, but that of cats only later. The peritoneal surface of the *stomach* appeared rather light blue, though finely stippled with darker blue points in the case of the cat; but when the organ had been slit open the mucosa and submucosa proved to be deeply stained, like the gut further down. It was the gastric muscularis into which relatively little of the dye had gone. Occasionally there were to be noted in it areas of local contraction which had not stained at all though the inner and outer layers of these areas had stained as well as ordinary. In this connection the fact deserves mention that the segments of large intestine distended with fecal masses were as excellently stained as the empty, contracted regions between. A notable example was furnished by the rabbit colon wherein fecal pellets are usually distributed at nearly regular intervals with a thinned wall over them and contracted gut between, like coarse beads on a thick, gristly string. Such a colon when slit longitudinally, emptied and inspected between glass plates had the same color intensity everywhere.

So rapidly did stain pass into the *mesenteric lymphatics* that they were distended with deep purply-blue fluid within 15 seconds after the dye injection. The glands at the root of the mesentery contained a similar fluid. In view of the extremely rapid diffusion the viscera were ordinarily inspected first of all, often before the heart had stopped.

In the gross the *liver* appeared deeply and evenly colored, but Valentine knife sections disclosed minor variations in hue, the periphery of the lobules being more intensely colored than the center, and both appearing greenish as compared with the clear blue of the interlobular connective tissue. In rabbits killed after three minutes some stain had already reached the bile. The *spleen* was a more or less deep blue,—less when the organ was somewhat contracted. The *kidney* cortex was dark blue, the medulla lighter, and brom phenol blue was present in the cortical tubules. The *urinary bladder* was medium blue, irrespective of whether it was full or empty, the hue approximating that of the superficial connective tissue. The *omentum* of the cat was lighter. That of the rabbit proved too filmy for useful

observations. Both in omentum and *retroperitoneal* fat the stain was localized to the connective tissue framework. The *pancreas* appeared evenly and rather lightly stained. Here too the color was principally in the connective tissue.

The *ovaries* stained rather intensely, and so too with the medulla of the adrenals, the cortex staining scarcely at all. The *pregnant uterus* was deeply blue, but into amniotic fluid and well-developed fetuses the dye had not penetrated during the few minutes following the injection.

The *lungs* were evenly and lightly blue, and the *thymus* too. The *heart wall* appeared deep purple blue. We have not attempted to determine how much of the color was due to contained blood.

In significant contrast to the mesenteric *lymphatics and glands*, those of the limbs contained fluid that was at most but palely blue.

Substantially identical findings were obtained with Patent Blue V, though no banding or lattice work was disclosed in the muscles, so rapidly did the dye diffuse. With sodium indigotate, the far greater intensity of the staining in the viscera was readily demonstrated, but minor differences were not easily to be apprehended owing to the reduction of the dye to a colorless form.

A number of the organs considered above will not be referred to again in the course of the present paper, notably the heart, kidneys, thymus, ovaries and adrenals. We have described them merely to round out the picture.

In summary one can say that certain organs or tissues (brain, lamellated bone) are entered practically not at all by the dyes of our experiments, that others receive but little of them (cartilage, tendon, new bone, nerves), others show them in considerable quantity (connective tissue, urinary bladder, muscle, pancreas), while others yet become so suffused as to constitute a group apart (liver, gall-bladder, intestines, stomach). The deep color of the spleen and red bone marrow is deceptive, the dyes lying for the most part still in the blood contained in these organs. In not a few others they are localized almost entirely to connective tissue scaffolding and interstitial fluid. With the binocular microscope one can readily make out that the epithelium of the gut is stained only faintly if at all, and that in voluntary muscle the color lies in general between the fibres. But, as has already been stated, our object has been merely to determine whether the dyes are purveyed to the cells, not whether they are taken up. The observations just recounted yielded a norm for the distribution from the circulation.

Certain affinities of brom phenol blue require recognition at this time. It has some affinity for the media of arteries, as also for medul-

lated nerves. In common with many other dyes it is rapidly removed from the blood by liver and kidneys. Möllendorff has correlated the rate of excretion of such dyes into the bile with their physical properties (12); yet the activities of the liver cells in secretion of them are still not wholly understood. The failure of the phthalein to penetrate old lamellated bone is evidently due to the constitution of the latter. The failure of the brain to stain with vital dyes in general has never been satisfactorily explained. The intense color of the diaphragm, intercostals and tongue as compared with the other skeletal muscles is understandable on the basis of their more abundant circulation (13).

The Technique of Depletion

When the blood bulk has been suddenly and considerably reduced, by bleedings or procedures causing anhydremia, profound alterations take place in the service rendered to the various organs by the circulation. Some of the changes have been briefly described in a report on the local, extravascular acidoses arising out of the state of affairs (14). We shall here consider them more fully.

To deplete the cats and rabbits used in the present observations successive bleedings have been employed. Since the circulatory alterations disclosed by the staining method are seen in pronounced form only when the blood bulk is not restored through readjustments within the body, the animals were fasted from 18 to 24 hours prior to experiment in order to lessen the utilization of fluid from the gut, as further, to avoid digestive hyperemia. Usually they had access to water; but under the circumstances they drank little. Those that were etherized and connected with the kymograph by way of the carotid were bled, either from this carotid by puncture of the rubber tubing just above the cannula, or preferably from an axillary artery cannulated for the purpose. Rabbits were bled with the aid of local anesthesia (novocain) by a method which involves a preliminary operation under general anesthesia to bring the carotid to the surface, cannulate it, and place about it an elastic clamp. The clamp was made from a short piece of rubber tubing about 3 mm. in outside diameter which was bent upon itself and tied so tightly at the bend that its limbs did not lie in parallel but sprung somewhat apart. The contrivance was slipped about the artery, and the free ends of the tubing were pulled through a constricting ring of rubber formed from the segment of a larger, thick-walled tube. To shut off the vessel the ring was rolled toward it along the pieces of tubing, the pressure of these latter upon each other sufficing for the purpose. For bleeding the ring was rolled away. There was an optimal position of the latter at which the vessel was held gently shut, to be opened merely by pressing the ends of the contrivance toward each other, thus

springing its sides apart. Successive bleedings were readily carried out with the animal on its feet.

It was early found that a large depletion, especially in the absence of general anesthesia, was required to bring about deviations in blood service so pronounced that certain regions which ordinarily color well failed to stain. No effort was made to determine the least loss of blood that would suffice for this purpose. When it was accomplished by a progressive anhydremia the blood pressure often varied little from the normal (15); but when bleeding was employed it usually fell to about 100 mm. Hg in cats, and 60 mm. Hg in rabbits, in the absence of any efforts to conserve it. After a first hemorrhage, as is well known, fluid from the tissues enters the vessels; but this readjustment is ordinarily completed within a few minutes, and takes place to but a slight extent after later bleedings (16). To rule out its influence upon the findings, as also to permit conditions to become relatively stable, three or four bleedings were done in all, at ten to twenty minute intervals, and the dye injection was ordinarily deferred until twenty minutes after the last one. Great care was taken that the depilated, depleted animals should not grow cold during the experiments. The unanesthetized ones were kept in warm rooms, and those under ether were in addition placed on electrically warmed pads. With the successive bleedings the skin and mucous membranes became pallid, and the superficial veins more or less collapsed. Very occasionally an ill-defined surface mottling could be made out.

Ordinarily almost half, if not quite half, of the calculated blood volume (which is approximately 7.5 per cent in the cat, 5.5 per cent in the rabbit) was removed. The rabbits depleted with local anesthesia still kept their feet but the respirations were exaggerated. That the alterations in the staining were not traceable to low blood pressure, as such, had been disclosed by the observations on anhydremic animals (17), and was now further shown by the fact that the longer the interval elapsing between the final bleeding and the injection the more pronounced were the deviations from the ordinary staining, although in the interval the pressure often tended to recover. The same amount of dye per kilo was given as under normal conditions, and in the same way. It sometimes caused a partial recovery of the blood pressure, but no symptoms. As in the case of the controls, the animals were killed three minutes after the injection.

The Alterations after Reduction of the Blood Bulk

The phenomena occurring in the *superficial tissues*, in the pelt that is to say, of animals receiving brom phenol blue have been briefly described in a preceding paper. The cat or rabbit turned blue more slowly than usual, the rate depending on the degree of depletion and how long after it the dye was injected. In extreme instances only a faint blue staining developed and this was limited to the regions where large vessels entered the skin. In the majority of instances the staining was patchy, unstained areas being everywhere interspersed amidst others that became brightly and diffusely blue. This patching proved in the

cat to be essentially similar to that already described for the rabbit and rat (18) but the white areas tended to be larger. They were irregular in outline, with serpiginous margins, frequently confluent, and varied in size according to the depletion conditions, the arrangement being sometimes of blue on a white ground and again of white on blue. In poorly marked instances there was merely a scattered sprinkling of small white spots on the blue expanse. The patching was especially well seen in well-nourished white cats with an underlying panniculus. In extreme instances, in which phenol red had been used instead of a blue dye, there took place a very gradual orange staining,—the color indicative of acidosis,—about the largest arteries entering the skin, while elsewhere the surface was unstained. The orange-red hue of the conjunctiva in such cases pointed to a blood acidosis. In less severely depleted animals the injection of the phthalein was followed by a brilliant mottling of red on white or white on red. Where the dye crept in later, at the edges of the unstained regions, it was seen to be orange.

Slight differences in local pressure sometimes exerted a great influence to determine the situation of patches in the depleted animal (19). Crouching rabbits often showed a broad, unstained strip along the ridge of the backbone and other large unstained areas over the bulge of the knees. It was necessary to allow for such localizations, as further for the influence of isolated masses of fat to make an overlying, thin, translucent skin appear poorly stained. Where errors of interpretation from these causes could be ruled out one saw that the patches were not only highly irregular in contour but without trace of symmetry. On reflecting the pelt the relation of them to the blood vessels could be made out; and the arrangement of the patching was found to be wholly independent of the vascular pattern visible in the gross, save in the extreme instances above mentioned in which only the tissue immediately about the largest vessels was stained. This independence was especially well to be seen where a number of arteries supplied the subcutaneous tissue in parallel, the lumbar vessels of the cat, for example. Here some of the arterial twigs ran to patches that were white, other corresponding ones to areas that were blue, and yet others to areas that were irregularly blue and white (Fig. 2).

The patching was not essentially dependent upon cooling of the skin, though frequently developing when it was cool. It appeared pronouncedly in one of our animals which was accidentally overheated on the pad; and it can be regularly elicited in rats submerged in oil at body temperature (20). If nothing occurred to relieve the depleted organism the white areas persisted for long periods, though tending gradually to diminish in size by a peripheral encroachment of the dye. When they were very large dye sometimes appeared secondarily at spots here and there within them. Obliteration of them occurred in both ways when the blood volume was restored by reinjection of the portion removed.

The *gums, nose tip, conjunctivae* and *mucous membranes of the mouth* colored promptly and deeply even when they had appeared absolutely bloodless prior to injection of the dye. They showed no patching, nor did the pads of the feet, which also stained rapidly but somewhat less well.

The *skeletal muscles* exhibited a remarkable reticulation or transverse banding with blue as has already been briefly recorded. And the pattern, unlike that of the skin, was regularly ordered, and did not vary in its dimensions with the degree of depletion. It could be studied directly under the binocular dissecting microscope in certain of the flat muscles (tibialis anticus, gracilis, pectorals, extensor longus digitorum) wherein it took the form of a transverse banding, and in others (as e.g. those of the abdominal wall) where a blue reticulum separated oval, unstained areas. To obtain specimens glass plates were slipped over and under the muscle which had been loosened from the tissues sufficiently for this purpose, and the attachments were then cut. The banding was now seen with the unaided eye to be discontinuous, consisting of many short blue segments displaced regularly a little to this side or that of the main axis of the band. When the muscle was thick (quadratus lumborum) the segments were frequently superimposed at different levels. The blue reticulum was likewise discontinuous. For the present it will suffice to say that the arrangement of the staining was determined by that of the vascular tree, and that it occurred about vessels of a special magnitude, several times larger than capillaries. The average length of the latter in rabbit muscle is 0.69 mm. (21), while the unstained regions between the blue bands (or blue reticulum) were more than 2.0 mm. across in this species, and even broader in cats.

Greater differences were evident in the staining of the various muscles than under normal circumstances. The cutaneous muscle layer did not color at all after severe depletion, and the sheet muscles of the abdomen but slightly as compared with the brilliantly banded quadratus lumborum and gracilis. Even these were pale in comparison with the diaphragm, intercostals and tongue muscles which were diffusely stained and almost if not quite as deep blue as ordinary. Sometimes the intercostals showed a slight relative pallor midway between the ribs; but the blue was uniformly distributed in diaphragm and tongue even when the animal had been sacrificed only a few seconds after injection.

The findings thus far described bear witness to pronounced and peculiar empoverishments of blood service. Not so with those in the *gastro-intestinal tract.* Here the staining appeared to have the same intensity as ordinary, and only on recording the hues in terms of Ridgway's book did one perceive it to be slightly less. As result of the fasting the duodenum and jejunum were wholly collapsed as a rule. The *intestines*, large and small, were always a diffuse, even, purply blue, and so too with the *gall-bladder* wall, *esophagus* and the mucosa of the *stomach.* In extreme instances the muscularis of this last organ showed an ill-defined, slight, pallid blotching but as a rule the coloration was diffuse. The staining was as good where the intestines were stretched over fecal masses as in the contracted lengths between. All this was true even when the animal was killed practically at once after the dye injection. Already the *lymphatics* of the mesentery and of the gall-bladder wall were distended with deep blue lymph.

The *liver* was, as in the normal animal, diffusely and deeply blue. The *pancreas*

was stained about as well as ordinary and showed no patching. The *urinary bladder*, on the other hand, was decidedly paler than in controls, often indeed almost unstained, but without any patching. And the condition of the spleen showed frequently that it had been shut off *in toto* from the circulation, even in etherized animals. Contracted and small the organ stood forth in vivid red against deep blue surroundings. When depletion had not been severe small spreading areas of blue were found scattered amidst the red of the surface exposed on section.

The *omentum* of cats presented a singular picture, being splotched with blue and white wherever it was thick enough for the presence or absence of staining to be made out. The distribution of the stain was irregular, as in the case of the skin, and independent of the vascular patterning visible in the gross. It was confined to the connective tissue. The unstained patches were often several centimeters in greatest diameter. In the retroperitoneal fat a similar splotching or marbling with blue was brilliantly evident. The omentum of the rabbit proved too filmy for satisfactory study.

A contrast to these evidences of ischemia was furnished by the *uterus* of cats far pregnant. The organ was found markedly and evenly stained despite the severest depletion, and the veins coming away from it contained much deeply colored blood although there was little elsewhere owing to the exsanguination at death. In this connection mention may be made of the fact that inflamed areas in the skin of depleted animals stained excellently.

The *kidneys* were diffusely blue, to the naked eye at least. *Tendon* and *cartilage* (ear, knee joint) colored somewhat less well than in controls, but there was no patching. The deep hue of the *red bone marrow* (legs, ribs) on the other hand showed that the dye had reached this tissue abundantly. The *lungs* stained lightly, as in controls, save at their wedge edges which were often wholly uncolored. The tissue at the apices was no paler than that elsewhere.

The lymphatics and glands of axilla and groin, in contrast to those draining the intestines, contained a fluid only faintly tinged with the dye.

These various findings were readily confirmed with Patent Blue V, and some of them with phenol red. The patching of the skin could be demonstrated with sodium indigotate, as also the profound staining of the gastro-intestinal tract, gall-bladder included.

In summary of the observations it can be said that under the circumstances of a markedly reduced blood volume certain of the organs continue to be well and evenly served by the circulation, whereas in others a pronounced patchy ischemia occurs. The conjunctiva, lips, gums, pharynx, oesophagus, stomach, intestines, gall-bladder and liver all become rapidly and deeply stained; and the dye appears in quantity in the lymph from the gut and gall-bladder, even

when the carotid pressure has been greatly lowered by the bleedings. Liver lymph was not studied. The red bone marrow continues to be excellently served by the blood in animals at the extreme of depletion, and so too does the pregnant uterus. The skin on the other hand and the voluntary muscles, with certain notable exceptions, are largely deprived of effective circulation. The deprivation takes a singular form, regions showing it being interspersed amidst others in comparison excellently served by the blood as evidenced by the staining. The size and number of the patches in the skin varies directly with the degree of depletion and the length of time it endures. Their distribution is entirely unsymmetrical and they seem unrelated to the vascular patterning visible to the eye. In the muscles on the other hand there is a regular disposition of unstained and stained regions, the latter situate about vessels of a special, and not inconsiderable magnitude. The unstained regions have a diameter several times greater than the length of the individual capillary. Not all of the muscles suffer this neglect. The diaphragm, intercostals and tongue muscles continue to be excellently supplied by the blood, as the depth and evenness of the staining attest.

Though blood service is remarkably well sustained in most of the abdominal organs there are certain significant exceptions. The spleen is largely, sometimes entirely, deprived of circulation, as shown by the failure of dye to enter it, and the urinary bladder, lightly colored in the controls, is often practically unstained in the bled animals. In the omentum pallid, ischemic regions are scattered irregularly amidst others well supplied from the blood as shown by their brilliant blue color.

Observations with India Ink

It has seemed important to determine the precise relation of the blood vessels to the ischemic patching just described. For this purpose we have resorted to india ink injections. A principal result of the work has been the recognition of how far the method falls short of demonstrating circulatory conditions within the body.

The distribution of india ink by the blood stream has been followed by a host of workers. Krogh has made large use of it in his studies of capillary regulation. Our own observations with ink of the sort he employed (Pelikan Perl Tusch,

Günther Wagner), dialyzed against Ringer's solution and filtered in the way he describes (22), would seem to indicate that his findings were obtained in the face of serious technical draw-backs. When the amount of ink that Krogh employed is injected intravenously into a rabbit or a guinea-pig, and the vessels of the ear are watched under a microscope, one can perceive that the foreign particles tend to agglomerate into lumps as they are carried along. These lumps soon lodge here and there within or at the entrance to capillaries, effectually blocking them. Using such material one can be certain only that where it passes the vessels are open. There is, fortunately, no difficulty in demonstrating with it that many more such vessels are open in the diaphragm of the normal animal than in most of the other skeletal muscles, a phenomenon emphasized by Krogh. But to assure oneself that where the ink does not penetrate no circulation had existed is quite another matter. And this holds true even when an ink far better for the purpose is used, Higgins' American Drawing Ink (non-waterproof), dialyzed against Ringer's solution, filtered and centrifuged—the last two processes being unnecessary in our experience. On injection of this the particles can be seen to circulate separately during the brief period before they are removed from the blood by the sessile phagocytes.

Normal white animals receiving either Higgins' ink, or Pelikan Perl Tusch, become transiently gray. We had supposed that in depleted ones patchily but intensely colored with brom phenol blue, injected ink particles would pass into the stained regions of skin and muscle in sufficient quantity for histological recognition if the animals were killed while they still circulated. But this did not prove to be the case. To all intents and purposes the particulate matter was shut off from the organs mentioned, and this proved to be the case as well in animals that had been merely depleted, not stained. The skin did not turn gray anywhere. The abdominal viscera on the other hand were dark with ink, except for the spleen which contained practically none. The liver was black with that which had been taken up by the Kupffer cells. In contrast to the pallid state of the skin generally, the tip of the nose, and the pads were gray with ink, lying doubtless within the arterio-venous anastomoses there known to exist (23, 24). The quantity injected was only $2\frac{1}{2}$ cc. per kilo, because of the need to avoid large increases of the blood bulk. Further findings will be detailed in a succeeding paper.

DISCUSSION

The method of the present work would seem to be validated by the disclosures it has yielded. The alterations we have observed in the

service rendered by the blood to the skin, muscles, and certain other organs of the depleted animal cannot be apprehended by ordinary laboratory procedures; nor are they demonstrable with india ink. In animals depleted by large hemorrhages a rapid and deep staining occurs in some regions (lips, gums, tip of the nose) which under such conditions in unstained animals appear wholly bloodless; and furthermore staining occurs in skin regions to which india ink particles are not carried by the blood. It seems probable that in some of these situations only a stained plasma may have circulated, red cells being removed by the "skimming" that Krogh first described. The great diffusibility of the dyes we employed proved in some ways a disadvantage; for they were so readily distributed that only drastic reductions in service to the tissues were recognizable with their aid. To appreciate less considerable changes it will be necessary to employ vital stains that do not leave the blood so rapidly.

Can it be said that where brom phenol blue failed to go all interchange between the blood and tissues had ceased? Scarcely. For Patent Blue V gradually penetrated where brom phenol blue did not. And carbon dioxide, which passes through the tissues with unexampled ease (25), reaches situations inaccessible to other substances. To all intents and purposes nevertheless the circulation had ceased to be effective in the skin areas which brom phenol blue or phenol red failed to penetrate. For an acidosis developed in such areas, one referable to the local accumulation of acid metabolites (26).

In appraising our results a first question is, how far they were conditioned by special affinities of the dyes employed? By employing several of highly different constitution we have tried to minimize this factor; but in another and better way it has been proven unimportant, namely by following the process of decolorization. Those organs which stain most rapidly and deeply with brom phenol blue are, with exception of the liver and kidneys,—which actively excrete the dye,—precisely those which lose color soonest, as could scarcely happen were it fixed upon them as result of a special affinity. The gastro-intestinal tract and the diaphragm become colorless long before the skin does. Brom phenol blue stains the media of arteries with a special intensity; but even here decolorization does not lag notably.

A principal alteration in blood service after hemorrhage is a peripheral vascular shut-down, the blood supply to the viscera being maintained at the expense of that to the superficial tissues and the muscles. This readjustment has long been recognized as one of the means whereby the vital forces are conserved in individuals "bled white." Other changes disclosed by the staining method impress one with their purposefulness. Not all of the abdominal organs continue to be well served by the blood. Those which are essential, and which can be safely neglected,—the omentum, urinary bladder and spleen,—are neglected. By contrast the whole gastrointestinal tract —from which alone help can come to the organism under natural conditions,—continues to be well served. Special mention may be made of the maintenance of the circulation to the gall-bladder, since the realization is recent that a very active resorption takes place through the walls of the organ into the blood and lymph. Even the esophagus and fauces are well served, and the gums and lips are much better maintained than is the skin. The red bone marrow continues to be excellently supplied with blood, though situated within limbs that are for the rest largely deprived of it.

The amount of blood which would be conserved to the organism were there complete ischemia of the skin is but slight (2 to 3 per cent) (27), but the saving of heat is far more considerable. And the cooling of the neglected tissues lessens the formation of waste products within them. In the voluntary muscles, large, enduringly bloodless patches do not develop as in the skin, but there is a regular arrangement of smaller ones into which a certain amount of diffusion gradually occurs, as shown by the findings with Patent Blue V. But not all of the muscles suffer in this way. Those which are essential to respiration (diaphragm, intercostals) and to swallowing (tongue muscles) continue to be well served by the blood. The question whether this is true because these muscles go on working need not be taken up at the moment, though the fact may be mentioned that the muscles of the tongue continue to be well served from the blood, as shown by a diffuse, deep staining, even when the organ lies flaccid in animals anesthetized through a tracheal cannula.

Under normal circumstances much more stain has been found to pass from the blood into the tissues of the gastro-intestinal tract than into

the skin and muscles. Numerous reasons for this can be thought of. The amount of blood passing in a unit of time through the skin and resting muscles is many times less than through the portal circulation. Capillaries are far more numerous in the viscera while furthermore a great proportion of those existing in the skin and resting muscles are ordinarily shut. There is an active flow of lymph from the blood into the mesenteric lymphatics, but practically none into the lymphatics of resting muscles (28, 29), whence it follows that a dye circulating in the blood penetrates into the muscles only by diffusion, whereas the process of distribution to the tissues of the gut is actively aided by a streaming of fluid out of the vessels. Furthermore the distribution to the gut is aided by a high capillary blood pressure, and the barrier offered by the capillary wall itself is imperfect, as shown by the presence of blood proteins in lymph collected from the mesenteric channels (30). Considering all this one cannot wonder that the stomach and intestines showed a specially intense and rapid staining with vital dyes.

From what is known of the physiological readjustments which take place in the bled animal one might expect the staining in the gastro-intestinal tract to disclose wide deviations from the normal. The capillaries of the region are known to be actively contractile; and a narrowing of the portal channels through vaso-constriction is deemed one of the most important compensatory changes occurring when the blood volume is diminished (31, 32). Even losses of blood which do not suffice to lower the arterial pressure cause some blanching of the intestines (Starling); and when such a lowering has taken place the rate of formation of lymph in the gut and liver is markedly reduced (33). Not a few functional conditions have been described in which blanching of the intestines was so great that the tissue seemed to all intents and purposes bloodless. For every *a priori* reason, then, except the teleologic, one might suppose that blood service to the gut after hemorrhage would be greatly lessened, perhaps to the extent of ceasing in some regions. But the dye experiments showed quite another state of affairs to prevail. Staining of the gut, gall-bladder and liver was always deep, though the animal was at the extreme of depletion and was killed but a few seconds after the dye injection; and the staining was diffuse save occasionally in the muscularis of the

stomach where an ill-defined blotching could be made out. The mesenteric lymphatics were always distended with deep blue fluid. The pancreas likewise was colored as usual. One must conclude that the compensatory constriction was never so great as to interfere seriously with blood service to the digestive organs.

Mention has been made of the fact that the staining of the intestine where it was stretched over fecal masses was of precisely the same intensity as in the empty, contracted segments lying between. The pressure condition where feces distend the gut is probably much like that in the full bladder, of which Sherrington remarks (34) that it "enfolds its contents in the same light grip whether these contents be ample or little." Owings, McIntosh, Stone and Weinberg (35) have ascertained that in normal dogs the greatest intraintestinal pressure is equivalent to only 2–4 cm. of water.

The amount of dye injected into the depleted animals was the same as in normal ones. Since the blood volume had been reduced by nearly half in most instances, it follows that the dye circulated in unusually great concentration. The intensity with which the abdominal viscera stained despite the untoward conditions, must be attributed in considerable part to this cause. But it will not explain the patching of the omentum, the more or less complete failure of the urinary bladder to stain, or the neglected state of the skin and muscles, which, so far as they received blood at all, received the same sort as did the viscera.

Starling has pointed out that normally absorption goes on from the digestive tract irrespective of whether there is a body need for the materials absorbed, the sole recourse of the organism being a regulation through the excretory organs which remove at an appropriate pace that which has been taken into the body willy-nilly (36). It is plain from our findings that even when the blood bulk has been diminished to the limit of tolerance, adequate circulatory conditions are maintained for absorption from the gut, a process which frequently acts to sustain life. Robertson and Bock have proved that salt solution introduced into the intestine is far more effective in permanently restoring the blood pressure of human beings after hemorrhage than when it is thrown directly into the circulation or injected into the tissues (37). The reason for this is not yet clear.

The dyes we have used are rapidly excreted into the bile; and this

of course makes for a deep staining of the liver. Even within so brief a period as three minutes after the injection of brom phenol blue or Patent Blue V, much dye had reached the finer bile ducts. The dyes yielded no evidence that after hemorrhage some hepatic regions were better off than others. Yet the vaso-motor regulation within the liver is far from negligible (38); and a "stroking reaction" can be elicited on the surface of the organ, like the cutaneous one so much studied (39). In view of all this one of us has made a special study of the character of the hepatic blood service after hemorrhage. The results are detailed in the paper which follows.

The mackerel-sky or lattice work staining in the muscles of bled animals was obviously related to the arrangement of the vascular tree. That it was largely dependent upon contraction of the vessels, was shown by experiments in which vaso-constriction was prevented from occurring in the muscles of a leg by cutting the nerves to it just prior to injection of the dye. In the muscles of a limb so treated staining took place diffusely whereas in those of the control leg the usual mackerel-sky patterning was found. Our many experiments of the sort will be described in detail on another occasion. Not infrequently normal, stained cats and rabbits exhibited traces of the patterning here or there, more especially in the gracilis and quadratus lumborum. It may with good reason be attributed to that partial vaso-constriction on which maintenance of the normal blood pressure depends, but there are other important conditioning factors as will be shown subsequently. In not a few cases, just before the introduction of the dye an equivalent amount of blood was removed from the circulation. There was no more pronounced patterning in such instances.

The factors responsible for the patching of the skin are less readily to be explained. They too are dealt with in a subsequent paper. The possibility has already been ruled out (40) that the patching depends on a differing intrinsic permeability of vessels of like magnitude supplying tissue of the same general sort, a difference becoming effective only when the blood flow has been cut down by vaso-constriction.

Some foreshadowings of the changes we have observed in the service to the tissues after depletion can be found in previous work. Meek

and Eyster, watching directly the circulation in the dog's ear, noted that after a considerable loss of blood there suddenly occurs an active contraction of the capillaries and small venules (41). They suggest that possibly "when the circulation is at the breaking point as it is when the bleeding equals 2 per cent of the body weight the venules and capillaries are constricted in widespread areas." The pronounced restriction of blood service occurring in the skin and muscles under such conditions is not uniform, as our experiments show, some regions being still fairly served while neighboring ones are wholly passed by. Langley (42) noted that the circulation continues through a few arterioles in the muscles of the frog after hemorrhages severe enough to stop it in the generality. He believed that a similar state of affairs would be found to exist in mammals. Gesell and Moyle (43), who ascertained the volume flow through the muscles of dogs repeatedly bled, found that at late stages of the gradual depletion it was reduced to an extent out of all proportion to the drop in blood pressure.

The vascular shut down in the spleen after hemorrhage is no new phenomenon (44). One may contrast therewith the state of affairs in the red bone marrow as disclosed by our experiments. Not only does dye still reach this tissue in quantity but india ink does as well.

The conditions as concerns blood service to the kidney under pathological conditions are complicated and we have made no attempts to study the organ. Richards states that relatively few kidney glomeruli are open to the circulation in frogs that have lost blood but that the number can be greatly increased by restoring the blood bulk (45).

SUMMARY

The spread through the living animal of various highly diffusible dyes has been utilized as an indicator of the ability of the circulation to serve the tissues under various conditions. The method is direct and searching. Blood service to the viscera, as demonstrated by it, is normally far more profuse than to the skin and muscles, for evident physiological reasons. After hemorrhages which greatly reduce the blood bulk service to the viscera is in general still well maintained even though the animal be *in extremis*. However great the compensatory contraction of the splanchnic vessels may be,—and physiologists have long supposed it to be very great,—it certainly does not suffice to

hinder blood service anywhere in the digestive tract. On the other hand the service to certain unessential abdominal organs (spleen, omentum, urinary bladder) is cut off in large part or wholly; and in comparison with the essential viscera, the skin and most of the skeletal muscles of the bled animal are largely deprived of circulation. This neglect takes a curious form, some regions being still fairly served by the blood while others next them are no longer ministered to. In the skin the areas served, or not served, are highly irregular but are to some extent determined in situation by local pressure factors. Within the muscles the neglect is orderly in arrangement and is largely referable to compensatory vaso-constriction. Certain of the muscles, those used in respiration and in swallowing, furnish significant exceptions to the general rule, being excellently served despite the serious general state. The red bone marrow of the depleted organism continues to be well served by the blood even though situated in limbs that are, for the rest, almost devoid of a circulation. The pregnant uterus also is excellently maintained despite the serious general state.

The changes are such as would tend to conserve the forces of the depleted organism and to contribute to its recovery.

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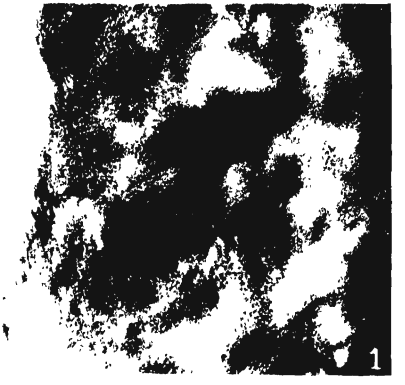
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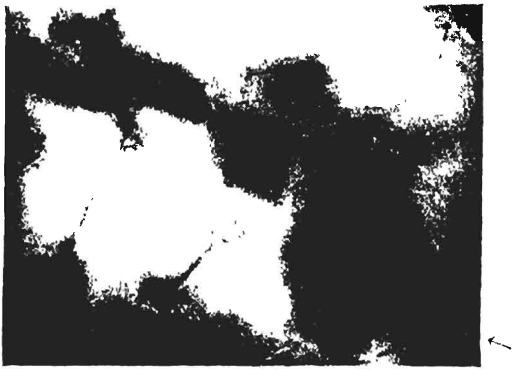
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EXPLANATION OF PLATE 8

Fig. 1. Skin of the side of a white cat injected with brom phenol blue after reduction of the blood volume by repeated bleedings under ether. The brilliant blue and white mottling is only moderately well shown in the photograph; yet the contrast is sufficiently great to suggest that the white patches were raised above the blue, as was not the real case. The hair had been removed by shaving.

Fig. 2. Reflected skin of the same cat showing three parallel distributions to the subcutaneous tissue from the series of lumbar vessels. Some of the blue patches in this tissue occupy the regions supplied by one or another of the secondary arterial branchings; but of others this is not true. Three branches that correspond in situation are indicated by arrows. Two of them run to colorless patches whereas the third enters tissue that is heavily stained.





(Rous and Gilding: Studies of tissue maintenance. I)

[Reprinted from THE JOURNAL OF EXPERIMENTAL MEDICINE, August 1, 1929,
Vol. 50, No. 2, pp. 213–226]

STUDIES OF TISSUE MAINTENANCE

II. THE SERVICE TO THE LIVER AND DIGESTIVE TRACT AFTER HEMORRHAGE

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PLATE 9

(Received for publication, June 10, 1929)

The service rendered to the various organs by the blood can be gauged by the distribution to them of diffusible vital stains. In a previous study by the method, there have been observed certain well-marked alterations occurring after the blood bulk has been reduced by hemorrhage. The service to the organs immediately essential to life and to recovery (heart, lungs, respiratory muscles, alimentary canal, liver, red bone marrow) was maintained at the expense of that to certain others (skin, voluntary muscles in general, spleen, urinary bladder). But these latter organs,—with the exception of the urinary bladder and sometimes of the spleen,—did not suffer to an equal degree throughout, regions in which the circulation was ineffective being interspersed amidst others still served by the blood, as shown by the stain that entered the tissues. It has seemed possible that a similar, if less pronounced, patchy neglect might after all have been present in the splanchnic viscera that appeared well and uniformly served, a neglect masked by rapid diffusion of the dyes, or by a rapidly intermitting circulation, now to this region and again to that. The present work was undertaken to cover the point thus brought up.

One does not have to look far in the literature to find that the vessels of the gut and liver are sufficiently contractile to render the assumption reasonable that a functional ischemia may exist in these tissues on occasion.

Mall showed in 1892 that during stimulation of the splanchnics the blood flow from the systemic arteries to the portal vein is greatly cut down. There occurs

also an active contraction of the portal twigs within the liver (1). These observations on vasomotor activities within the hepatic tissue have been repeatedly confirmed (2). In anaphylaxis of the dog an interference with the circulation through a spasm of the small hepatic vessels leads to the characteristic drop in blood pressure (3). Mautner and Pick (4) have adduced experiments to show that the liver of cats possesses a mechanism whereby contractile spasm of the liver capillaries is produced. According to these authors the action of epinephrin or barium chloride upon the vessels causes obstruction to the blood flow through the cat's liver as not through that of rabbits. Ebbecke brought about a capillary dilatation with edema on stroking the hepatic surface, which he deemed comparable with the cutaneous stroking phenomenon that has of late been so much studied (5).

The fact is well attested that contraction of the portal bed through vaso-constriction is largely responsible for the maintenance of blood pressure after hemorrhage. Krogh has advanced evidence on this point (6). Starling states that a loss of blood too slight to reduce the general blood pressure will cause blanching of the intestines (7). In experiments upon some lower forms an extreme blanching has been elicited in various ways. Bayliss states, in summary of general knowledge, that the innervation of the abdominal viscera is predominantly vaso-constrictor whereas that of the peripheral tissues is vaso-dilator (8). And yet, if one may judge by observations from our laboratory, the vascular readjustment which takes place after large hemorrhages in cats and rabbits, cuts down and may almost abolish the effective circulation to the periphery while interfering but little with that to the viscera.

Method

For the present work india ink was mainly employed. It was mixed with a 4 per cent solution of brom phenol blue or preceded by an injection of the stain, or of phenol red, when information was desired of the state of blood service to the skin and muscles. Though this is brilliantly disclosed with the dyes it cannot be with ink, which, in animals that have been bled, frequently fails entirely to reach the tissues mentioned. Higgins' American Drawing Ink (non-waterproof) was employed. For injections during life this is far superior to the Pelikan Tinte (Günther Wagner) so much used by Krogh, since the particles circulate separately, not in agglomerates of various size. The ink was dialyzed against Ringer's solution for some days and was then both filtered and centrifugalized, processes which separate out few if any particles. 2½ cc. per kilo was introduced at body temperature into a vein, during the course of one minute. There followed only the rise in blood pressure which an equivalent amount of salt solution would have produced. The animals employed were rats, rabbits and cats. In some of the early work general anesthesia was induced with urethane, but this proved unsatisfactory for reasons to be given later. Light ether was employed with all of the cats and they were bled from a cannulated axillary vein. Rabbits can be

repeatedly bled from the carotid under a local anesthetic (9), but most of those employed were etherized. Kymograph records of the blood pressure were taken in many cases. The rats were bled from the carotid while under the influence of urethane or ether.

To learn the distribution of the ink in the stomach and intestines direct inspection during life was necessary; for needless to say much of it might be forced here and there through post-mortem contraction of the vessels. So rapidly are the ink particles removed from circulation by phagocytes sessile within various organs that the inspection has to be carried out within the first minutes after the injection. For these reasons the animals used for the study of the gut were under general anesthesia; and in order to rule out the effects of exposure, the abdomen was opened only when the time had come to look at the organs. In the case of the liver the state of affairs could be studied at leisure after the animal had been killed,—by exsanguination from the carotids, or by decapitation in the case of unanesthetized rabbits; for the ink is so rapidly taken out of the blood by the Kupffer cells that the amount of phagocytosis will serve as an index to blood service. But in order to rule out a possible post-mortem distribution of the ink, with such phagocytosis as result, it was essential to remove the liver from the body immediately upon death. Livers thus treated empty themselves of blood as they do not when they have been left in situ for even a few minutes with the large vessels uncut (10). In the present work the capillaries regularly proved empty. Microscopic preparations were obtained with the Valentine knife or the freezing microtome, and cleared in glycerine. If there was brom phenol blue in the organ, the complication of its intense blue hue was done away with by placing the sections in a solution possessing that slight degree of acidity necessary to turn the phthalein yellow.

The Maintenance of the Liver

In a series of preliminary experiments urethanized rats were bled repeatedly from a carotid; injected with ink; killed after various brief periods of time; and compared with controls merely receiving ink. It was a surprise to find that under these circumstances the spleens of the depleted rats contained as much ink as those of the controls; for Barcroft has proved that the spleen contracts after hemorrhage. Our finding was traced to the urethane, which acts to prevent contraction of the spleen, as Henning showed (11). When the rats had been bled under a local anesthetic, almost no ink reached the spleen. It was everywhere equally distributed to the liver lobuli, irrespective of the character of the anesthesia; but in the bled animals the peripheral Kupffer cells contained vastly more than did the central ones,—which was not the case in controls. In rabbits that were depleted under light ether a pronounced splenic contraction occurred, as proved both

by the appearance of the organ and the almost complete failure of ink to enter it. The rabbit liver on the other hand showed an abundant and even distribution of ink to all of the lobuli, but within these units the same pronounced divergence from the normal was observed as in rats. The peripheral Kupffer cells were swollen with phagocyted ink particles, while those further in had less and less the nearer they were to the center. Identical findings were obtained when the bleedings had been carried out with the help of local anesthesia.

In all of the foregoing instances depletion was purposely carried far, about half of the blood being taken as a rule. Sometimes 20 minutes had elapsed between the last bleeding and the ink injection, sometimes a less period, the plan being not to exhaust the activity of the vaso-motor center. The unanesthetized rabbits maintained the ordinary crouching posture. In every instance in which brom phenol blue had been mixed with the ink a pronounced patchy ischemia of the peripheral tissues was disclosed.

Identical findings were obtained in cats bled and injected under light ether, save for the fact that the ink was almost evenly distributed to the phagocytes within the hepatic lobuli, just as in the controls. Only when the depleted cats were sacrificed within a few seconds after the ink injection, with result that but little had been phagocyted, was more visible in the peripheral Kupffer cells.

The Service Rendered by the Hepatic Artery

The existence of two sources of hepatic blood renders uncertain the interpretation of the foregoing observations. That the two vascular trees supplying the hepatic tissue differ in their vaso-motor responses is known (12); and it might well be that the effects of a contraction of portal radicles here and there would be masked by a freer flow from the end arteries, or vice versa. For this reason observations were made on the distribution of ink after diversion of the portal stream from a part or all of the liver.

In some etherized cats and rabbits the branch of the portal vein running to the "main liver"—the mass lying between stomach and diaphragm—was tied, the result being that all of the portal blood was diverted through the "lobe mass"—the right posterior and caudate lobes. This abrupt change in the path of the blood is well tolerated by the organism, as an abundant experience has shown (13); and

in the course of the present work it has been observed to have no effect on the carotid pressure. Needless to say the necessary operation was performed with the least possible trauma and exposure of the viscera, only a short segment of the vein being dissected free for ligation.* The hepatic artery, in special, was not touched; and it was seen by direct inspection to continue to beat strongly. The abdomen was then closed with a running suture in two layers. After 2 to 18 minutes had been permitted to elapse, for purposes of readjustment within the organism, the bleedings were carried out as usual, and ink injected. When the animal was killed a little later the lobe mass was found coal black with phagocyted ink particles whereas the main liver was of a light or dark brown. In both portions of the organ the ink had been evenly distributed to the lobuli. To control the effectiveness of the ligation milk was injected under pressure into the portal vein below the tie. It flowed only into the lobe mass.

One of the animals, a rabbit, had been vitally stained with phenol red just prior to the portal diversion and its body surface had colored a uniform red. After the bleedings, at the time of the ink injection, the surface had become pronouncedly patched with buff upon red, plain evidence that there existed in the superficial tissues local areas of outlying acidosis, such as result from an ineffective circulation.

In some additional experiments (on rabbits) a preliminary operation was performed to induce a development of portal collaterals by partial obstruction of the flow to the liver, after Drury's modification of Moskowitz' method (14). Some weeks later when the collaterals were well developed the portal vein was completely tied off under ether. There resulted no alteration in the carotid blood pressure. After a brief interval to allow for readjustments the animal was depleted by successive bleedings according to the usual method, and ink, or ink and brom phenol blue, was injected. On sacrifice it was found that the effective blood pressure within the liver had been so greatly lowered by depleting the blood bulk that the slight pressure exerted on the organ by the stomach or ribs of the prone animal had sufficed to prevent the ink from entering certain regions. Elsewhere, though, it had been distributed evenly to the lobuli by the hepatic artery. The milk test showed that the portal stream had been wholly diverted from the liver.

In a final experiment the portal vein in a normal male cat weighing 3025 gm. was tied off, with result, of course, that the animal gradually bled into its own splanchnic vessels, the blood accumulating in the portal regions back of the ligature. The abdomen was closed as usual. The carotid blood pressure sank progressively and within 8 minutes the mucous membranes of the mouth, previously pink, had become pallid. After 18 minutes the animal was bled 20 cc. from the carotid cannula to hasten the depletion. After 35 minutes the carotid blood pressure was very low, having fallen from 150 mm. Hg prior to ligature to 40 mm. Hg. Now

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* The separation from its sheath of the portal vein of the cat or rabbit is a nice procedure, best accomplished after longitudinal incision of the sheath, by pressure with a pledget of cotton or gauze held in the grip of a mosquito forceps.

8 cc. of ink was injected intravenously during 35 seconds. The skin failed to color with it at all, but the mucous membranes of the mouth became gray in patches. 19 minutes later both carotids were cut. But little blood came away, nearly all having collected in the portal viscera, behind the ligature. The liver was slaty black with ink which had been evenly distributed to the lobuli by way of the hepatic artery. There was slightly more in the peripheral Kupffer cells than near the center. The milk test showed that the ligature had been occluding.

These experiments demonstrate that arterial blood is uniformly distributed within the liver even when the organism is at the extreme of depletion.

The Service Rendered by the Portal Vein

The literature on contraction of the portal radicles within the liver has already been reviewed. There is much to indicate that it exerts an important regulatory influence on the blood supply to the heart, notably after hemorrhage. The following tests were designed to show whether under such circumstances some parts of the hepatic tissue are served by the blood and others neglected.

In etherized cats and rabbits the hepatic artery was dissected free and ligated, at a single point in the earlier experiments, in the later at two points, above and below the origin of the A. gastro-duodenalis, in order to prevent all flow through collaterals. Two ligatures were placed at each situation, the abdomen was closed as usual, and bleedings and injection were carried out in the ordinary way. It was found, after the bleedings, that the intrahepatic blood pressure had been so greatly lowered that the slight pressure of the ribs sufficed to prevent the blood-laden ink from entering the tissue lying next them. Elsewhere, though, it was regularly distributed to the lobuli. The milk test showed that the ligatures had wholly cut off the arterial flow.

Again no evidence was encountered of a patchy maintenance of the hepatic tissue in animals with a reduced blood bulk. Some little arterial blood may have reached the liver by way of the diaphragm. The supply of arterial blood from this source is so small in the rabbit that necrosis of the liver and death regularly follow ligation of the hepatic artery. In the dog the supply is sufficient to avert this catastrophe.

The Service Rendered to the Intestines

When white animals (rats, rabbits and cats) are injected with ink after marked reduction of the blood bulk the body surface does not

become ashy gray as in normal controls. So few ink particles reach the skin and muscles that the regions of total ischemia in these tissues cannot be discriminated with their aid. As the foregoing experiments on the liver have sufficiently shown, they circulate in abundance through the portal bed. Special care had been taken in these experiments not to expose the intestines, in order to avoid vaso-dilatation due to trauma. The short incision necessary to tie one or the other of the hepatic vessels had been made high up to the left of the mid-line where only the upper surface of the stomach and the under surface of the liver came into view. Nevertheless a dilation of the capillaries of the gastro-intestinal tract sufficient to let ink through into the portal vein might have been an indirect result of the operative procedure. To learn whether in the absence of any such stimulus these vessels let ink through, as further to find whether they are patent everywhere, some animals were not laparotomized until after ink had been placed in circulation. The examination was made during the brief interval before the particles had been taken out of the blood; and heed was given only to the condition when the gut was first exposed. The animals had been fasted to rule out all possibility of the digestive hyperemia described by Bier. The stomach and small intestines of the cats were empty. In rabbits the stomach and large bowel were still distended with roughage after several days fasting.

Always it was found that despite the preliminary depletion the intestines and stomach were uniformly gray with circulating ink; and the blood of the portal trunk black. Such vascular contraction as may have taken place in readjustment for the blood loss had nowhere closed off the capillaries to such extent that ink particles could not course through them; and there was no sign of a patchy distribution of the material. The findings confirmed those with diffusible dyes (15). It can be concluded that such compensatory contraction of the small vessels of the alimentary canal as may occur after hemorrhage does not anywhere even nearly shut these vessels, and that the service rendered by the blood to the tissues is far less affected as a whole than is that to the skin and muscles.

Contractility of the Vessels of the Alimentary Tract

That the failure of the small vessels of the alimentary tract to undergo an occlusive contraction after hemorrhage is not due to any

inherent inability to contract has been shown by tests with epinephrin and pituitrin. After one of these has been given intravenously the blood supply to certain portions of the bowel may be so completely cut off that highly diffusible dyes fail to stain them. Service to the liver, though, as judged by this criterion, is still well and evenly maintained.

A sufficient amount of 1–1000 solution of suprarenin (Metz) (0.05 cc. per kilo, diluted with an equal part of 0.9 per cent saline) was injected into a vein of rabbits or cats to cause a great rise in the carotid blood pressure, and while this rise still endured brom phenol blue in the quantity usual for vital staining (16) was run into the circulation. As a rule a little of the suprarenin solution was mixed with it, the object being to sustain the high blood pressure. To avoid the possibility of cardiac default under the conditions the dye was injected more slowly than usual, in the course sometimes of as much as 2½ minutes. In rabbits the blood pressure rise was succeeded by an irregular fall toward the previous level throughout the subsequent period of 3 to 4 minutes before the animal was killed. In cats on the other hand vagal inhibition of the heart beat caused the pressure to drop before it had risen very far, and frequently brought it below the previous normal. This, of course, did not mean that vaso-constriction had been relaxed. At the time when the carotids of the anesthetized rabbits were cut they were hugely distended, their diameter being often twice the normal,—clear evidence of a peripheral obstruction to blood flow.

In both the species used the service rendered by the blood to the skin and muscles was greatly interfered with by the action of epinephrin, as shown by the slow, slight, and irregular staining. The small intestine, colon, and rectum on the other hand stained as well as normally, becoming deep blue within so short a time as a minute after the dye injection. The liver and gall bladder were also rendered diffusely blue and the phthalein promptly appeared in the bile. On the other hand the mucosa of the stomach of rabbits showed large, pallid, serpiginous areas on a blue ground. These contained neither dye nor blood. They had no obvious relation to the vascular arrangement, and the overlying muscularis was as well stained as ordinary. The caecum showed irregular unstained patches involving the whole thickness of the wall. These likewise were unrelated to the patterning of the vessels. In cats such evidence of visceral ischemia was wholly lacking, although in skin, muscles, and omentum it was pronounced. The cat spleens were not contracted whereas those of the rabbits were.

Very singular was the condition of the mesenteric lymphatics of the rabbits. Their contents became colored with blue as rapidly and deeply as ordinary; but after this had occurred the epinephrin must have induced an irregular spasm of the lymph channels themselves, the consequence being that they had the appearance of broken segments of blue thread, or,—in cases in which they were larger, with bulgings between the valves,—of irregularly contracted blue tapeworms. So great had the spasm been in two animals that the channels had ruptured in numerous places near the gut and there were spreading extravasations of blue lymph. Falta and Priestley (17) have made injections comparable to mine into dogs and have studied the vascular state by direct inspection of the organs. They found the intestines as a whole to be greatly blanched, and inferred that the circulation is diverted elsewhere. In my animals only the stomach and large intestine manifested any ischemia and in them its distribution was patchy. The interference with service to the gastric mucosa may have been attributable, in part at least, to contraction of the muscularis mucosae. Adrenalin stimulates this layer as it does not the muscularis proper (18). The ischemia involving the entire wall of the caecum can only be explained on the basis of vascular contraction.

Pituitrin yielded far more pronounced findings.

Two preparations were employed, Pituitrin (Obstetrical), Park, Davis and Co. and Infundin (Burroughs Welcome and Co.). As in the case of epinephrin, a preliminary intravenous injection was made to bring about vaso-constriction, and when a pronounced rise of the carotid blood pressure showed that this had taken place in the etherized animals brom phenol blue or Patent Blue V was run into the blood stream,—very slowly, else the heart failed and death occurred. For the pituitrin had caused an unexampled rise in the general blood pressure. At the time when the carotids were cut in order to kill the animal they were found distended to double the ordinary diameter. In two unanesthetized rabbits in which the blood pressure was not ascertained, the same injection procedures were successfully followed and with the same effects, as disclosed at autopsy. The alterations in the service rendered to the tissues were profound. The skin and voluntary muscles in general stained almost not at all during the routine 3 minute period after the dye injection (Figs. 1 and 2). The liver and gall bladder on the other hand were intensely and evenly stained and so too with the esophagus and small intestine. The stomach on the other hand was sometimes almost unstained throughout (Fig. 2), and again exhibited serpiginous pallid patches which were or were not limited to the mucosa. The large intestine sometimes showed

patching throughout and again was completely unstained. The omentum was marbled with stain or completely unstained. The spleen was sometimes blue, and again was contracted and not stained. The lymphatics deriving from the stained gut held deep blue fluid, whereas the contents of those from the pallid large bowel was colorless. No great contraction of any of these channels was discernible.

Both the preparations employed caused an interruption of service to the stomach and large bowel as shown by the more or less complete failure of these viscera to stain. The small intestine was by contrast deeply colored. The "Pituitrin" caused vigorous peristalsis with expulsion of feces, whereas the "Infundin" did not elicit this activity, the feces being retained, and the stomach and bowel, examined before life had wholly ceased, showing no abnormal contraction. It is conceivable that in some situations contraction of the muscular layer of the gut may have reinforced vascular contraction, with result in the ischemia encountered. But staining with the dyes employed is so extraordinarily swift that even a temporary relaxation of the muscle, as during peristalsis, would have sufficed to permit it, especially since the blood pressure was abnormally high. The inference is that the ischemia was due to the same cause as that in the skin and muscles, namely to vascular contraction.

It is possible that had the blood volume of the animals been reduced by bleeding just prior to the experiments, or had an amount of pituitrin been given which caused a less intense vascular contraction in the peripheral tissues some diminution in blood service to the small intestine might have come to light. The bloodless state of the skin and muscles has already been commented upon. With the narrowing of the circulatory channels everywhere the blood and the dye added thereto would inevitably be forced to the regions of least resistance, quite irrespective of whether the vessels within these regions,—which might well have been intestinal,—tended to contract. In our experiments the lungs can scarcely have served as reservoirs for blood forced out from elsewhere. They were less stained even than ordinary. Room for some of the excess blood was obtained in the large vessels, as the distended condition of the carotids proved; and some was held in the dilated heart. But in the circumstances of the case, with even the vessels of the stomach and large intestine closed to some extent,

much blood may have been foisted, so to speak, upon the small intestines and liver. Our interest did not lie in determining whether this was actually the case, but merely in the demonstration that the blood vessels in some parts of the gut, at least, are capable of such contraction as to prevent service to the tissues by way of them.

COMMENT

The experiments demonstrate that such vaso-constriction as may take place in the alimentary canal and liver to compensate for a lessened blood bulk never goes so far as to prevent the blood from rendering service to these organs. Not only do diffusible dyes continue to be rapidly distributed to them, but their capillaries fail to offer any obstacle to the passage of india ink particles. In contrast to the state of affairs in the skin and muscles, where well served regions are interspersed amidst others wholly neglected, the service to the hepatic parenchyma and the wall of the gut is evenly distributed. The maintenance of this uniformity was put to an extreme test in the case of the liver by cutting off the venous or arterial blood supply to the organ in animals with blood volume largely reduced. Under these circumstances the remaining stream to the parenchyma flowed with so little force that the slight pressure of ribs and stomach sufficed to turn it aside here and there, with result in patches of total ischemia. Had there been any local differences in the degree of constriction of the small vessels elsewhere in the organ surely these should have been evident in differences in the staining. None were observed, the lobuli being all served to precisely the same extent. Yet the existence of a vaso-motor mechanism within the liver is a fact that cannot be gainsaid. One must suppose it to be so admirably balanced in the healthy animal that all parts of the liver share equally in the blood; for otherwise local hypertrophy, and a concomitant atrophy, would occur (19). Mall observed that a colored mass injected into the freshly extirpated organ reaches the capillaries of the lobules everywhere at the same moment, and this no matter whether introduced by hepatic vein, portal vein or hepatic artery (20). The present experiments prove that the regulation continues undisturbed during the compensatory readjustments which follow reduction of the general or local blood bulk. In view of all these facts the active hyperemia followed by edema

(stroking phenomenon) which Ebbecke elicited on the liver surface must be thought of as a distinctly pathological manifestation.

It had seemed barely possible that under the circumstances of a general depletion the liver lobuli might be intermittently supplied with blood, the rate of intermittence being so rapid that staining with a vital dye or the scattering of india ink to the Kupffer cells would soon appear to have been broadcast. To settle the point thus raised some animals were killed only a few seconds after injection of the test material. Even in them the findings showed an even distribution of blood to the individual lobuli, though within these units one could see that the cells first reached by the stain or ink particles, namely those at the lobular periphery, shared in these substances more abundantly than those nearer the central vein. In the case of normal animals receiving ink,—which substance can be followed with special ease,—this difference disappeared after a few minutes, the Kupffer cells all appearing to have shared equally in the particulate matter, even when the amount was small. So too with cats which had been bled. In bled rabbits, on the contrary, there was a startling change in the distribution, almost all the ink particles being held in the peripheral Kupffer cells, which were swollen and black with the engorged material. A number of reasons for this suggest themselves, amongst them that of the known differences in the intrahepatic vascular responses of the rabbit and cat (21).

The maintenance of a well distributed and effective blood service to the gastro-intestinal tract after hemorrhage is necessary if the gut is to function in aid of recovery. Conditions would be doubly disastrous were service greatly cut down. Yet in view of all that has been written on intestinal blanching and compensatory vaso-constriction within the portal system after hemorrhage one might have expected some regions of local ischemia at the least. In this connection the demonstration of a previous paper may be recalled, that blood service is often still going on in tissues which, to the eye, appear wholly bloodless.

Bayliss has made the generalization that the vaso-motors to the viscera are predominantly vaso-constrictor in type, and those to the periphery vaso-dilator. While this generalization holds for the ordinary circumstances of life, certainly when the blood bulk is reduced the compensatory vaso-constriction is far more effective at the periphery than in the liver and alimentary canal.

SUMMARY

The vascular readjustments in compensation for a greatly reduced blood bulk affect the service rendered by the blood to the gastro-intestinal tract and liver far less than they do that to the skin and muscles. Into these latter tissues india ink is carried almost not at all, whereas it circulates in quantity through the capillaries of the bowel and liver. Evidently vaso-constriction is much less effective in these viscera. Nowhere in them does one find a patchy ischemia like that so wide-spread in the peripheral tissues. Blood service is maintained to the same extent everywhere throughout the liver even when one of its two sources (hepatic artery or portal vein) is obstructed, and the intrahepatic blood pressure brought very low.

A pronounced patchy ischemia of the stomach and large bowel can be induced by intravenous injection into normal animals of sufficient epinephrin to cause the systemic blood pressure to mount to an abnormally high level. Pituitrin used in the same way has a greater effect; blood service to the organs mentioned may be completely abolished by means of it. In both instances, though, service to the small gut and liver is still excellently and evenly maintained.

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EXPLANATION OF PLATE 9

Fig. 1. Organs of an unanesthetized white rabbit receiving brom phenol blue intravenously and decapitated two minutes later.

Fig. 2. Organs of a white rabbit treated in the same way but receiving pituitrin four minutes prior to the stain.

It will be seen that the skin of the right side of the pituitrin animal is unstained whereas that of the control was colored, and though the subcutaneous muscle (seen attached to the skin on the left side) was stained that of the control was much more deeply so. The stomach of the pituitrin rabbit though mottled with dye was largely unstained, whereas the stomach of the control was intensely colored. The small intestines of both animals were stained to the same great degree, but the caecum of the pituitrin animal showed some splotchy, partial pallor. The blue of the animals photographed so poorly that the brilliant character of the differences is far from evident.

Fig. 3. A more pronounced instance of pituitrin ischemia. The stomach of the rabbit injected with pituitrin followed by brom phenol blue has stained almost not at all, and so too with the descending colon,—which is recognizable by its content of fecal pellets. The small intestine, on the other hand, is intensely colored.



(Gilding: Studies of tissue maintenance. II)

[Reprinted from THE JOURNAL OF EXPERIMENTAL MEDICINE, August 1, 1929, Vol. 50, No. 2, pp. 233–244]

A STUDY OF PNEUMONIA IN A RURAL AREA IN SOUTHERN ALABAMA

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(Received for publication, April 4, 1929)

The present study was undertaken to determine certain basic facts in relation to pneumonia in a rural community in the South; it covers a period of 6 months, October to April, 1927–1928. Our field laboratory[1] was established in Andalusia, Alabama, a town of about 4,000 inhabitants, the center of a rather sparsely settled rural community where farming and lumbering are the chief industries. The area covered in the study comprises approximately a thousand square miles, and has a population of about 35,000, the great majority of whom are white.

Types of Pneumococci Isolated

Fifty-eight cases of pneumonia were seen in the Andalusia area during the course of the study. A summary showing the types of pneumococci isolated from these cases is given in Table I.

The striking feature of this series of cases is the small number of fixed types of pneumococci that were isolated. Pneumococci of the heterogeneous Type IV group were the prevailing organisms.

Severity of the Disease

In Table II we have classified the cases of pneumonias according to their severity as correlated with the type of pneumococcus isolated.

Lobar pneumonia takes a much heavier toll in the northern than in the southern United States. In 1925, for example, the death rates

[1] We are greatly indebted to Col. C. A. Reasoner of the U. S. Army Medical Service, for help and advice in selecting equipment for our field laboratory.

per 100,000 from all types of pneumonia in whites and negroes for certain typical states were as follows:

	White	*Colored*
New York	105.3	367.4
Massachusetts	116.4	230.5
Pennsylvania	116.7	366.4
Alabama	81.4	135.1

TABLE I

Types of Pneumococci Isolated Compared with Data from New York City (Monograph 7, Rock. Inst.)

Pneumococcus Type	No. of cases	Alabama	New York City
		per cent	*per cent*
I	7	12.3	33
II	0	0.0	31
IIx	2	3.5	—
III	1	1.7	12
IV	40	68.5	24
Pneumococcus not isolated	8*	14.0	—

* Two of this group had a pneumonia due to staphylococcus, one following a periostitis—the other a case of senile dementia, while one case had a hemolytic streptococcus in pneumonia following an automobile accident. The remaining five were children from 1 to 8 years of age, from whom it was difficult to get sputum. They were presumably pneumococcus pneumonias, Type IV.

TABLE II

Severity of the Disease

Type	Mild	Moderate	Severe	Fatal
I	0	3	4	0
IIx	0	0	0	2
III	0	0	1	0
IV	21	11	6	2
Pneumococcus not isolated	4	1	1	2*
	25	15	12	6

* Staphylococcus pneumonia.

Since no morbidity data are available, it is not certain whether pneumonia is less prevalent in the south than in northern states or equally prevalent but less fatal.

Tables I and II suggest that pneumococcus pneumonia may be as frequent in the isolated rural districts in the south as in the large cities in the north, but is much less fatal.

Age Distribution

In Table III we have summarized the distribution of cases of pneumonia by age groups. The striking feature of Table III is that pneumonia due to Type IV pneumococcus occurred chiefly in children under 15 years of age, whereas pneumonias due to the fixed types of pneumococci were seen chiefly in adults.

TABLE III

Age Distribution of Cases of Pneumonia in Southern Alabama

Age	Total cases	Fixed types of pneumococci	Type IV	Pneumococcus not isolated
Under 1 year	1	0	1	0
1– 4 years	9	0	6	3
5–14 "	26	4	19	3
15–44 "	15	4	10	1
45–64 "	4	2	2	0
65 and over	3	0	2	1
	58	10	40	8

Seasonal Distribution of Pneumonia

The community studied was free to a great extent from acute respiratory disease until the first week in January, though there had been mild outbreaks of "colds" in neighboring counties. Daily records were made of maximum and minimum temperature, relative humidity and rainfall. The exact day of onset of each case of pneumonia was recorded. Directly following a week of low temperature, Jan. 1, there occurred a widespread epidemic of colds. Aerobic cultures from the nasopharynx of many individuals during this outbreak showed (a) a great preponderance of pneumococci Type IV, of low virulence to white mice, (b) a large number of influenza bacilli of various types. The time relationship between the cold weather and the prevalence of pneumonia is shown in Chart I. The data seem to

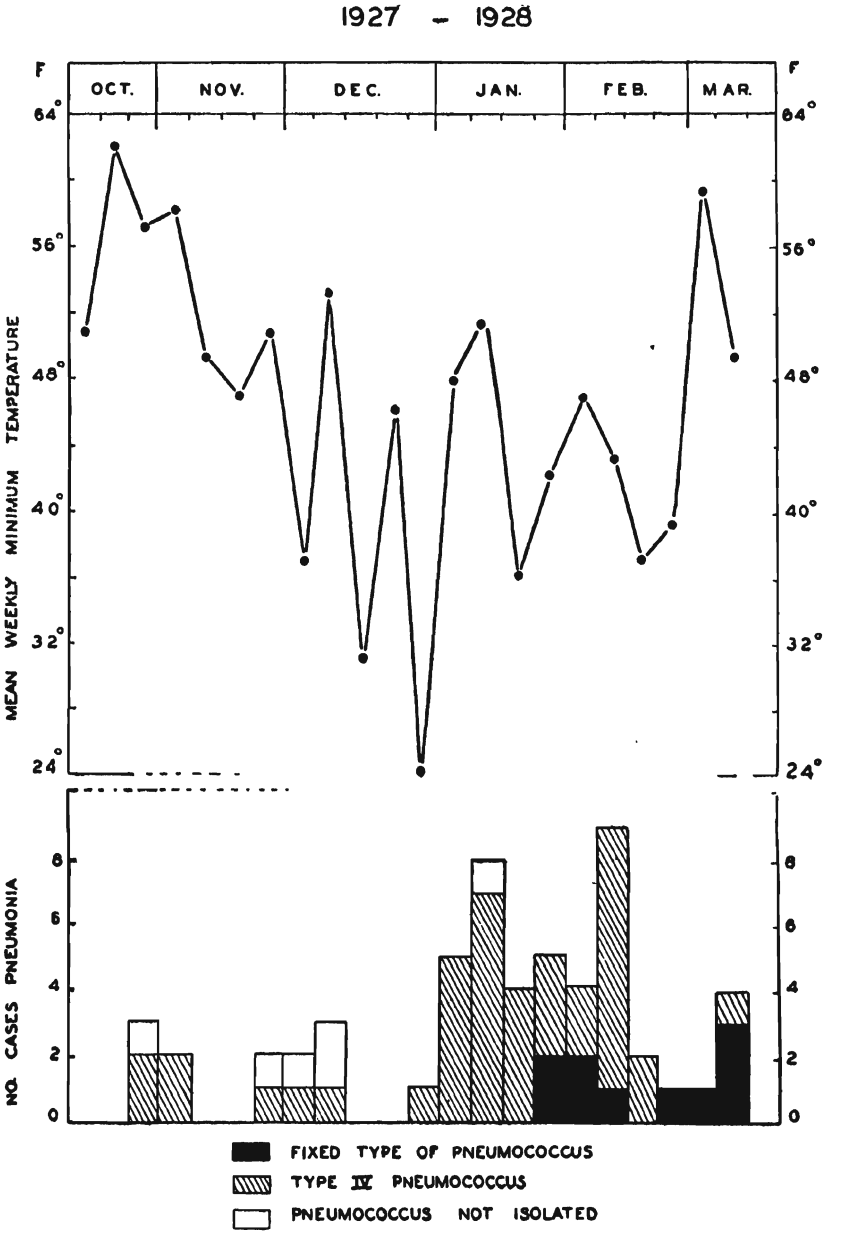


CHART I. Seasonal distribution of pneumonia, Andalusia, Alabama

indicate that a period of cold weather with attendant suffering and exposure bore a definite relationship to the onset of pneumonia. The sequence was first, cold weather; second, an epidemic of colds; third, pneumonia. The cases of pneumonia did not occur during the coldest weather but 2 to 4 weeks after the weather became warmer.

In individual cases the acute colds developed into pneumonia, usually on the sixth or seventh day of the cold, often following exposure while riding several hours in a wind, or getting rain-soaked and thoroughly chilled when returning from school. The great proportion of the cases occurred in children under 15 years of age and the invading pneumococcus was usually Type IV. Frequently two or more members of the family developed pneumonia at the same time.

TABLE IV

Relationship of Exposure and of Acute Colds to Pneumonia

	Type of pneumococcus			
	Fixed types	Type IV	Pneumococcus not isolated	
Sudden onset—no exposure or "acute cold".....	2	4	2	
Exposure but no "cold".....	1	1	0	Total
	3	5	2	10
"Acute cold" antedating pneumonia 5–10 days.....	6	25	3	
"Acute cold" plus exposure to chilling rain, etc.....	1	10	3	Total
	7	35	6	48

The relationship of the onset of pneumonia to chilling and exposure and also to acute colds is given in Table IV.

The clinical symptoms in these cases of Type IV pneumonia were typical of lobar pneumonia, but the physical signs were not characteristic of those found in cases of lobar pneumonia due to the fixed and more virulent types of pneumococcus.

The signs of consolidation in the lung were typical but the area involved did not conform to the anatomical outlines of a single lobe. Often areas of consolidation in two different lobes in the same lung were found—sometimes both lungs were affected. The cases could not be called broncho-pneumonia, for small multiple areas of con-

solidation were not found, but rather large areas with typical massive consolidation, sometimes in the axilla but most often in the right base.

The epidemiology of these cases of pneumonia in a sparsely settled rural area is in direct contrast to pneumonia in our larger northern cities where adults are more commonly affected than children and family epidemics are rare; where the attack usually runs a severe course, may or may not be preceded by a cold, and where the physical signs are usually confined sharply to the anatomical outline of a lobe of lung.

It seems a tenable supposition that the isolation, poor roads, lack of frequent contact with one another and with the outside world, have produced in a community of people a low resistance to relatively avirulent pneumococci, whereas under more crowded conditions, there develops a community resistance to avirulent pneumococci, where pneumonia occurs as a rule following an invasion of one of the virulent fixed types of pneumococci.

The mode of life, customs, and habitations of the community studied are closely comparable with those of our forefathers one hundred years ago or more. It is interesting to note that the medical literature of 1810 to 1840 is full of references to epidemic pneumonia.

Warner (1), 1814, describes the epidemic prevailing in New England and New York State. He states "the disease is characterized by an initial chill and prostration and seems related to exposure to wet or cold." Cartwright (2) describes an epidemic in Natchez in 1826 and notes that atmospheric vicissitudes, exposure to inclement weather and intemperance predispose to the disease. MacBride (3), 1813, in describing an epidemic in St. John's Parish, South Carolina, states that most of the deaths occurred in field negroes, but the disease was also seen in whites of the lower classes. When an individual in a family was attacked, nearly all other members developed the disease. McCall (4), 1823, describes a family epidemic in Kentucky; Williamson (5) described an epidemic in 1813 in North Carolina and felt that cold and rain predisposed to the disease. Smith (6), Stearns (7), Mott (8), Mann (9), Eights (10), LeComte (11) and many others describe epidemics of lobar pneumonia in various parts of the eastern United States in the early part of the last century.

It seems probable that the pneumonia in the isolated rural community studied is comparable to the type of pneumonia of pioneer days in the United States, and that the increase of population and the

industrialization of the northern states, producing crowded conditions and frequent contacts, have resulted in the development of a population largely immune to avirulent strains of pneumococci, but responding to certain of the fixed virulent strains.

Family Epidemics of Pneumonia

In seven different families two or more members of the family were ill with pneumonia at the same time. This is contrary to the usual finding in the northern United States, where family epidemics of pneumonia are rare.

The intimate relationship of one case of pneumonia with another suggested the possibility of transmission of the disease from one member of a family to another by immediate contact. If this were the case, then one would expect to find that the various contacts with a case of pneumonia would harbor in their nasopharynx the type of pneumococcus corresponding to that found in the sputum of the patient. To determine this point, a study was made of the nasopharyngeal flora of 26 families in which one or more cases of pneumonia had developed.

Technique

Practically every case of lobar pneumonia which occurred in the area during the winter was seen in consultation with the family physician[2] as soon as the diagnosis of pneumonia was made (usually the second day of the disease). Cultures of the nasopharynx of all contacts were made at once, using the West tube. They were placed directly in blood broth, kept warm during transportation, and plated on 3% horse blood hormone agar plates as soon as possible. The plates were searched after 24 to 36 hours, and typical colonies were isolated and the organism identified. A total of seventy-three contacts were cultured, each of whom had been in close and frequent communication with the pneumonia patient. A summary of the findings is given in Table V.

It might be assumed from Table V that the thirty-two contacts with cases of Type IV pneumococcus who harbored type IV strains in their nasopharynx were infected by actual contact with the patient who was ill with pneumonia in the household. If this were the case, contacts with Type I and Type II pneumonias should harbor corresponding strains. This occurred in only five instances, whereas ten of

[2] There are about twenty-five general practitioners in the area.

the seventeen contacts with Type I pneumonia harbored not Type I but Type IV pneumococcus. It seemed probable, therefore, that some other factor than contact with a case of pneumonia was responsible for the prevalence of pneumococci in the nasopharynx of these individuals.

TABLE V

Prevalence of the Pneumococcus in the Nasopharynx of Individuals in Direct Contact with Pneumonia Patients

	Type of pneumonia in patients to whom contacts were exposed		
	Type I	Type II	Type IV
Contacts harboring pneumococcus:			
Type I	2*	0	0
Type II	0	3	0
Type III	0	1	0
Type IV	10	1	32
No pneumococci found	5	2	17

* Both these contacts developed Type I pneumonia within 3 days after their throat cultures were taken.

TABLE VI

Correlation of the Prevalence of Pneumococci in the Nasopharynx with an Acute Respiratory Infection

Type of pneumococcus found	Acute cold when examined	Just recovered from cold	No history of recent cold
I	2*	0	0
II	1†	0	2†
III	0	0	1†
IV	25	15	3
No pneumococcus found	6	13	5
Total	34	28	11

* Contact with Type I pneumonia.

† Contact with Type II pneumonia.

We have noted that practically all cases of pneumonia were preceded by an acute upper respiratory infection and that these colds occurred as family epidemics affecting all members. It seemed possible that these epidemic colds might be associated with an in-

creased prevalence of pneumococci. An analysis was made, therefore, to determine this point. See Table VI.

Table VI shows clearly that a large proportion of the individuals in this study who were harboring pneumococci had colds at the time or had just recovered from them. This is particularly true of the group of individuals who made up the families in which there was a case of Type IV pneumonia. Furthermore, the relative number of pneumococcus colonies on the blood agar plate cultures of the nasophaynx seemed to bear some relationship to the course of the cold. See Table VII.

Tables VI and VII give a very definite indication as to what occurred in these epidemics of colds and the relationship of the cold to pneu-

TABLE VII

Group of Contacts with Type IV Pneumonia; Table Showing Relative Prevalence of Pneumococci Type IV in Acute "Colds"

Proportion of pneumococcus colonies on the blood agar plate in relation to all other colonies	Number of cases			
	1st 3 days of cold	4th to 8th day	1st week after recovery	2nd week after recovery
50 to 100 per cent	1	5	3	0
25 to 50 per cent	0	3	5	1
Few (less than 25 per cent)	5	3	1	2
No pneumococci	2	3	3	7

monia. Within the first few days of the cold, pneumococci Type IV are found in the nasopharynx of those affected. From the fourth to the eighth day, the pneumococci have so increased as to outnumber all other organisms. As the patient recovers from the cold, the pneumococci disappear and the normal flora of the throat reappear, though pneumococci are frequently found for 10 days to 2 weeks after symptoms of cold have disappeared.

It seems probable that the contacts with cases of pneumonia, particularly the Type IV group, were not infected by their direct contact with a pneumonia patient. The more probable history is as follows: An epidemic of acute colds occurred in a family, usually affecting all members of the family. Coincident with the cold, pneumococci occurred in the nasopharynx in large numbers. In the great majority

of the colds, nasopharyngeal symptoms disappeared after 7 to 10 days, and gradually the pneumococci disappeared also. A small proportion of the individuals who had an infection with pneumoccci of the upper respiratory tract developed pneumonia on the fourth to eighth day of their cold. The attack often followed an exposure or chilling of the body surface, with a resultant extension of the infection from the upper to the lower respiratory tract. The pneumonia, therefore, was an incident in the course of an epidemic of colds.

If this is true, family epidemics of colds associated with pneumococci must have occurred in which no case of pneumonia developed. Such indeed was found to be the case. A complete epidemic of acute colds was studied in a small rural school—cultures being taken before the colds began, during the epidemic, and after the epidemic had ceased. A report of this epidemic, together with other similar studies, will be made in a subsequent communication.

The assumption might be made that the incitant of these colds is the pneumococcus—especially since there is some contributory evidence in addition to that already presented. One bit of evidence is that these colds were associated with a high leucocytosis—a white blood count of twelve to fifteen or even eighteen thousand being commonly found. Furthermore, one of the present investigators developed a clear-cut nasopharyngeal infection due to pneumococci which was acquired in the laboratory while working with the pneumococcus cultures and which ran a course similar to the family epidemics of colds. The appearance and disappearance of symptoms in this infection were closely correlated with the appearance and disappearance of pneumococci in the nasopharynx.

In one instance, a pure culture of pneumococci Type I was found in the nasopharynx on the second day of a cold in a person who was nursing a patient with Type I pneumonia. 3 days later the nurse developed pneumonia Type I. In two other instances mothers who were caring for children with pneumonia Type IV developed typical colds, and almost pure cultures of pneumococci Type IV were isolated from the nasopharynx on the first or second day of the cold.

But there is evidence also that pneumococci were merely contributory causes and perhaps did not initiate the colds at all. It will be noted from Table VII that pneumococci were not abundant in the

nasopharynx during the first 2 or 3 days of the cold, but were most prevalent from the fourth to the eighth day. This observation suggests that the pneumococci may be secondary invaders and that the cold was initiated by some unknown factor.

Another interesting observation in connection with these family epidemics of colds associated with pneumococci is that Pfeiffer bacilli were frequently found in abundance in association with the pneumocci. In general these organisms appeared later in the cold than the pneumococcus, and were present for a longer period after symptoms had disappeared. No constant type of Pfeiffer bacilli was encountered. Both hemolytic and non-hemolytic forms were found—

TABLE VIII

Table of Correlation between Incidence of "Colds" and Prevalence of Pneumococci and Pfeiffer Bacilli in the Nasopharynx

	Number of cases			
	Acute cold at present	Just recovered from cold	No history of recent cold	Total
Pfeiffer bacilli and pneumococci	11	6	0	17
Pfeiffer bacilli only	4	8	1	13
Pneumococci only	15	11	5	31
Neither pneumococci nor Pfeiffer bacilli	5	3	6	14

some requiring V + X factors for growth, others requiring only V factor. Some produced indol—others did not. There was some consistence in the type found in members of the same family but not in the group as a whole. A brief summary of these findings is given in Table VIII.

Table VIII indicates that Pfeiffer bacilli were frequently associated with pneumococci in the acute colds which occurred in the group of contacts with cases of pneumonia.

CONCLUSIONS

1. Pneumococcus Type IV of low virulence was the prevailing organism in fifty-eight cases of pneumonia studied in southern Alabama. Fixed types of pneumococci were not common.

2. Pneumonia was more prevalent in children from 5 to 15 years of age than in adults. As a rule, the disease ran a mild course.

3. Most of the cases of pneumonia gave a definite history of an acute cold antedating the attack of pneumonia by a period of 5 to 8 days. Exposure alone did not seem to predispose to pneumonia, but those with an acute cold who were exposed to chilling of the body surface frequently developed pneumonia.

4. There were seven "family epidemics" of pneumonia. In each instance there was a family epidemic of colds antedating the pneumonia. The pneumococcus was found in large numbers in the nasopharynx of those suffering from colds as well as in the pneumonia patients.

5. The epidemiology of pneumonia in the pioneer days of American history has many points in common with the epidemiology of pneumonia in a rural isolated area in southern Alabama today. This suggests that the crowded conditions and frequent contacts of modern city life have built up a community resistance to avirulent strains of pneumococci.

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[Reprinted from BERICHTE DER DEUTSCHEN CHEMISCHEN GESELLSCHAFT, June 5, 1929, Vol. 62, No. 6, pp. 1475–1482]

MAGNETISCHE UND KRYSTALLOGRAPHISCHE UNTERSUCHUNGEN: ÜBER EISEN(III)-OXYDHYDRATE

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(Received for publication, April 3, 1929)

In zwei früheren Mitteilungen zeigten E. Wedekind und der Verfasser[1]), daß bei der langsamen Entwässerungderroten Eisen(III)-oxydhydrate die Magnetisierbarkeit sich in eigentümlicher Weise ändert. Mit abnehmendem Wasser-Gehalt wächst die Susceptibilität bis zu einem nicht scharf zu bestimmenden Maximum, um dann bei weiterer Entwässerung bis in die Nähe der Susceptibilität des Eisenoxyds zu sinken. Es erschien deshalb angebracht auch an auf andere Weise dargestellten Hydraten des Eisens und ihren Abbauprodukten magnetische Messungen vorzunehmen und sie gleichzeitig, ebenso wie die in den oben erwähnten Arbeiten dargestellten Hydrate, der Röntgen-Analyse zu unterwerfen.

Es gibt in der Natur zwei krystallisierte Hydrate des Eisens, die beide der Formel Fe_2O_3, H_2O entsprechen, obwohl sie verschiedene Krystallform haben. Die sehr eingehenden Untersuchungen von J. Böhm[2]) machen es ziemlich sicher, daß weitere krystallisierte Hydrate in der Natur nicht vorkommen. van Bemmelen[3]) hatte bereits die Existenz eines dieser Hydrate wahrscheinlich gemacht, und vor einiger Zeit hat dann J. Böhm[4]) durch Röntgen-Aufnahmen an einem gelben Hydrat, das nach einer modifizierten van Bemmelenschen Methode dargestellt worden war, die Existenz dieses Hydrates bewiesen. Für die Darstellung des anderen in der Natur vorkommenden Hydrates fehlte aber bisher eine geeignete Methode.

[1]) B. **59**, 1726 [1926], **60**, 2239 [1927].
[2]) Ztschr. Krystallogr. **68**, 567 [1928].
[3]) J. M. van Bemmelen, „Die Absorption," S. 145–174.
[4]) Ztschr. anorgan. allgem. Chem. **149**, 203 [1925].

Die beiden chemisch gleichen, aber krystallographisch verschiedenen Hydrate werden im folgenden mit α- und γ-Hydrat bezeichnet nach dem Vorschlag von F. Haber[5]), da die mineralogischen Bezeichnungen nicht einheitlich sind. Das α-Hydrat ist identisch mit dem Boehmschen Goethit und das γ-Hydrat mit dem Lepidocrocit, auch Rubinglimmer genannt.

Über das α-Hydrat

Bei der Oxydation einer wäßrigen Lösung von Ferrobicarbonat entsteht ein gelbes Hydrat, das sich nach der chemischen Zusammensetzung, der Röntgen-Analyse und der magnetischen Messung als identisch mit dem in der Natur vorkommenden α-Hydrat erweist.

Etwa 20 l fassende Flaschen wurden mit sauerstoff-freiem Wasser gefüllt und luftdicht verschlossen. In das Reaktionsgefäß führten drei Glasröhren, eine zur Zuleitung, eine zur Ableitung von CO_2 und eine zum Abheben der Lösung. In das mit CO_2 gesättigte Wasser wurde sehr feines Eisenpulver[6]) gebracht (etwa 1 g pro Liter) und während einer Reihe von Tagen CO_2 durchgeleitet. Bei sorgfältigem Ausschluß von Sauerstoff blieb die entstehende Lösung von Ferrobicarbonat klar. Die Lösung enthielt nach einigen Tagen immer ca. 0.65 g Eisen im Liter (durch Titration mit $\frac{1}{10}$-*n*. Permanganat festgestellt).

Die Oxydation wurde auf folgende Weise ausgeführt: 1) Durch Zufließenlassen von ca. 20 l Lösung zu 40 ccm konz. H_2O_2-Lösung (Perhydrol, Merck) bei Zimmer-Temperatur. 2) Durch Durchleiten eines raschen Stromes von Luft oder Sauerstoff durch die Lösung. 3) Durch langsames Einwirkenlassen des Sauerstoffes der Luft beim Stehen der Lösung bei 73° im Brutschrank.

1. Die Reaktion des H_2O_2 mit der Lösung setzt stürmisch ein unter Bildung eines rötlichgelben Niederschlages, der sich innerhalb von 24 Stdn. absetzt. Derselbe wurde dekantiert, zentrifugiert und im Trockenschrank (100°) ca. 3 Tage getrocknet. Der Niederschlag

[5]) Naturwiss. 1925, 1004.

[6]) Das Eisenpulver wurde durch die Vermittlung von Hrn. H. Hill, New York, von der I.-G. Farbenindustrie A.-G., Abteil. Ludwigshafen, an Hrn. Prof. O. Baudisch gesandt, der es mir zur Verfügung stellte. Nach Angaben der Firma war das Pulver durch Zersetzung von $Fe(CO)_5$ dargestellt und enthielt außer 0.02% C keine Verunreinigungen. Die Größe der Sekundärteilchen betrug 5.10^{-4} bis 2.10^{-2} cm. Den Genannten danke ich auch an dieser Stelle.

ließ sich jedoch auch leicht auf einem Büchner-Trichter absaugen. Beim Trocknen erhielt man leicht zu einem feinen Pulver zerreibbare, leuchtend gelbe Krusten. Mit frisch bereiteter 10-proz. Ferricyankalium-Lösung ließen sich keine Ferro-Ionen nachweisen. Das Pulver löste sich in 2 Min. beim Kochen in überschüssiger 10-proz. HCl und ziemlich leicht in überschüssiger kalter konz. HCl. CO_2 ließ sich nicht nachweisen. Der Glühverlust betrug bei 3 verschiedenen Präparaten: 10.8%, 11.2% und 11.6%. Theoretischer Wasser-Gehalt von Fe_2O_3, H_2O ist 10.4%. Die spezif. Susceptibilität war bei allen drei Präparaten dieselbe: $\chi = 35.6.10^{-6} \pm 0.5.10^{-6}$. Die Pulver-Aufnahmen zeigten alle Linien des α-Hydrates (siehe Tafel S. 266/267).

TABELLE 1

Oxydations-mittel	Trocknung	Fe''-Gehalt	Glüh-verlust	$\chi.10^{-6}$ T
Sauerstoff	3 Tage bei 100° im Dampfschrank	0	11.4%	36.12
"	Aceton und Äther	0	12.3%	36.47
"	Luft von 37°, 11 Tage	+	14.4%	40.15
Luft	3 Tage bei 100° im Dampfschrank	+	14.2%	43.85
"	Luft bei 37°, 8 Tage	0	14.4%	35.66
"	Aceton und Äther	++	17.1%	61.25
"	"	0	16.7%	36.90
"	"	0	16 4%	37.21

2. Beim Einleiten von Sauerstoff oder Luft in die Lösung entsteht sofort eine milchige Trübung; nachdem die Oxydation beendet ist, setzt sich rasch ein leuchtend gelber Niederschlag ab, der wie unter 1. beschrieben behandelt wurde. Doch wurde bei einigen Präparaten die Trocknung bei 37° im mit Wasserdampf gesättigten Brutschrank oder mit Aceton und Äther durchgeführt. Die Analysen-Ergebnisse sind in Tabelle 1 zusammengestellt. In keinem Präparat ließ sich Kohlensäure nachweisen.

Die Prüfung auf Ferro-Ionen geschah auf folgende Weise: 0.1 g Substanz wurde in 150 ccm H_2O + 25 ccm konz. HCl, die in einem mit Bunsen-Ventil versehenen Kölbchen $\frac{1}{2}$ Stde. ausgekocht war, gelöst. 10 ccm dieser Lösung wurden mit 5 ccm einer frisch bereiteten 10-proz. Lösung von Ferricyankalium versetzt. Eine nach einiger

Zeit einsetzende Bläuung der Lösung ist in der Tabelle mit + bezeichnet. ++ bezeichnet sofort einsetzende Blaufärbung.

Der Wasser-Gehalt liegt durchgängig über dem theoretischen Wert für FeO.OH, wie die angeführten Beispiele zeigen. Die Art der Trocknung und die Höhe des Wasser-Gehaltes lassen keinen Einfluß auf die Höhe der Susceptibilität erkennen, ebensowenig das Alter der Verbindung, wohl aber der, wenn auch geringe, Ferro-Gehalt. Alle Präparate zeigen die Röntgen-Interferenzen des α-Hydrates.

3. Die Lösungen wurden längere Zeit im Brutschrank bei 37° sich selber überlassen. Die Ergebnisse sind in Tabelle 2 zusammengestellt.

Die Tabelle zeigt, daß alle Präparate zweiwertiges Eisen enthalten. Bei Präparat 1 und 2 waren die Lösungen in offenen Schalen aufbe-

TABELLE 2

Nr.	Dauer der Oxydation	Trocknung	Fe''-Gehalt	Glühverlust	$\chi \cdot 10^{-6}$
1	3 Monate	3 Tage bei 100° im Dampfschrank	+	12.6%	55.8
2	80 Tage	8 Tage über konz. H_2SO_4	1.2%	14.34%	118
3	110 Tage	Aceton und Äther	++	12.3%	38.25
4	8 Monate	3 Tage bei 100°, 6 Monate an der Luft in einer Flasche	2.65%	24.0%	32.1
5	10 Monate	"	1.59%	26.9%	*)

*) Die Susceptibilität war so hoch, daß das Präparat bereits bei der kleinstmöglichen Feldstärke von einem Pol des Magneten angezogen wurde, also nicht gemessen werden konnte.

wahrt worden, Nr. 3 in einer 20-l-Flasche und Nr. 4 und 5 in großen Ballons mit ca. 60 l Lösung. Es wurde versucht, einen der ferrohaltigen Niederschläge, bevor er von der Lösung getrennt wurde, sowohl mit H_2O_2 als auch mittels Durchleitens von Luft und Sauerstoff vollständig zu oxydieren, doch ohne Erfolg. Meistens bedingt die Anwesenheit von zweiwertigem Eisen eine Erhöhung des Magnetismus. Die Schwankungen im Magnetismus können mit der Bildung von Ferriten in Zusammenhang gebracht werden. A. Krause[7]) hat gezeigt, daß bei der Oxydation von Ferrohydroxyd an der Luft unter gewissen Bedingungen Ferrite entstehen. Die Beständigkeit der Niederschläge gegen nachträgliche Oxydation spricht ebenfalls dafür.

[7]) Ztschr. anorgan. allgem. Chem. **174**, 145 [1928].

Weitere Versuche über diese Erscheinung sind im Gang. Alle Präparate geben bei der Röntgen-Aufnahme die Linien des α-Hydrates.

Es wurden nun einige Versuche über die Beständigkeit des α-Hydrates gemacht. Ein im März 1928 dargestelltes Präparat mit einem Wasser-Gehalt von 12.3% und einer spezif. Susceptibilität von $\chi = 36.47.10^{-6}$ wurde in einer mit einem Korken verschlossenen Flasche aufbewahrt und im Januar d. J., also nach etwa 10 Monaten, in einem scharf getrockneten Luftstrom bei stufenweiser Erhöhung der Temperatur entwässert. Während der 10 Monate hatte das Präparat Feuchtigkeit aus der Luft absorbiert, sein Wasser-Gehalt betrug 19.42%. Der Magnetismus hatte sich nicht verändert ($\chi = 36.2.10^{-6}$). Bis 100° verlor es innerhalb 48 Stdn. 8.92%. Weitere 24 Stdn. bei derselben Temperatur brachten eine Gewichts-Verminderung von 5 mg auf 2.7502 g Einwage, es war also praktisch

TABELLE 3

Behandlungsweise	Trocknung	Glüh-verlust	$\chi.10^{-6}$
150°, Bombenofen, 1 Stde.	3 Tage, 100°	11.9%	34.0
150°, " 20 Stdn.	3 Tage, 100°	11.2%	30.7
150°, " 65 "	3 Tage, 100°	11.2%	31.8
100°, Wasserbad, 24 "	Aceton und Äther	14.5%	32.4
100°, " 5 Tage	"	14.86%	33.3
100°, " 3 Monate	10 Tage, 37°, Brutschrank	16.02%	32.2

konstant. Mit 10.62% entsprach sein Wasser-Gehalt also fast genau dem theoretischen Wert für Fe_2O_3, H_2O. Nach 2 Stdn. bei 150° war der Wasser-Gehalt 8.92%. Durch langsames Steigern der Temperatur bis 260° innerhalb 8 Stdn. wurde der Wasser-Gehalt auf 2.21% reduziert. Der Rest konnte erst durch Steigerung der Temperatur auf 600° nach 4 Stdn. entfernt werden. Das Oxyd hat eine Susceptibilität von $\chi = 23.10^{-6}$. Es zeigt die Interferenzen des α-Oxyds in der Pulver-Aufnahme.

Ein anderer Teil wurde gleich nach der Darstellung auf 6 Einschlußröhren verteilt, mit Wasser versetzt und im verschlossenen Rohr erhitzt. Die Ergebnisse sind in Tabelle 3 zusammengestellt.

Alle Präparate gaben das Pulver-Diagramm des α-Hydrates. Aus der Tabelle ergibt sich die Beständigkeit des Hydrates gegenüber den angewandten Temperatur-Erhöhungen, die, wie in den früheren

Arbeiten gezeigt wurde, ausreichen, um das rote Eisenoxydhydrat zu entwässern. Die Pulver-Aufnahmen zeigen stark verbreiterte Linien (siehe Tafel), die mit der geringen Größe der Krystalle zu erklären sind. Die Präparate sind sehr empfindlich gegen Luft-Feuchtigkeit, worauf auch die immer zu hoch gefundenen Werte für den Wasser-Gehalt zurückzuführen sind. Bemerkenswert ist die durch die Temperatur-Erhöhung bedingte, über die Fehlergrenze hinausgehende Erniedrigung der Susceptibilität.

Über das γ-Hydrat

In einer kurzen Mitteilung über ein leicht filtrierbares Eisenoxydhydrat haben Fr. Hahn und F. Hertrich[8]) gezeigt, daß bei der Oxydation von stark verdünnten Ferrosalz-Lösungen mit der äquivalenten Menge von Natriumjodat bei Gegenwart von Natriumthiosulfat sich langsam rötlichgelbe Niederschläge absetzen, die leicht auswaschbar sind und das Eisen quantitativ enthalten. An einigen auf diese Weise dargestellten Präparaten wurden magnetische Messungen und Röntgen-Aufnahmen gemacht, ferner Alterungs- und Entwässerungs-Versuche angestellt. Die Pulver-Aufnahmen ergaben bei 4 Präparaten die Linien des γ-Hydrates, während ein weiteres Präparat sich als amorph erwies. Beim Entwässern im trocknen Luftstrom zeigte sich das Hydrat weniger stabil als das α-Hydrat. Das Ausgangsmaterial verlor beim Verglühen 23.84% Wasser. Das Präparat war lufttrocken. Nach 24 Stdn. bei 100° im trocknen Luftstrom hatte es genau die Zusammensetzung des Fe_2O_3, H_2O und gab auch die Interferenzen des γ-Hydrates; nach weiteren 24 Stdn. bei 100° hatte es noch 3.4% Wasser verloren, und nach weiteren 48 Stdn. enthielt es nur noch 6.73% Wasser. Das Präparat zeigte jetzt neben den Interferenzen des γ-Hydrates bereits einige sehr breite andere Linien.

Die Susceptibilität des Ausgangsmaterials war $\chi = 48.10^{-6}$. Nach der Trocknung bei 100° war die Susceptibilität so stark gestiegen, daß sie sich in der magnetischen Wage nicht mehr messen ließ. Nach 3-stdg. Erhitzen auf 250° enthielt es nur noch 1.75% Wasser und zeigte die sehr verbreiterten Interferenzen des γ-Oxydes; überdies war es stark ferromagnetisch. Der Rest des Wassers wurde durch 4-stdg. Erhitzen auf 600° vertrieben. Die Susceptibilität betrug jetzt

[8]) B. **56**, 1729 [1923].

$\chi = 25.6.10^{-6}$, und das Oxyd gab in Übereinstimmung mit dem Magnetismus die Interferenzen des α-Oxydes. Eine Reihe von Präparaten wurde, wie oben beschrieben, im Einschlußrohr im Wasserbade verschieden lange erhitzt. Sie veränderten sich dabei nicht. Die Susceptibilität war praktisch immer dieselbe, und alle Präparate gaben die Interferenzen des γ-Hydrates.

In der Tabelle 4 sind die Daten für eine Reihe von Präparaten zusammengestellt.

In einer kürzlich erschienenen Arbeit[9]) haben E. F. Herroun und E. Wilson gezeigt, daß bei der Ertwässerung von in der Natur vorkommendem γ-Hydrat (Lepidocrocit, Siegen und anderen) die Magnetisierbarkeit sich in folgender Weise ändert: Das Ausgangsmaterial ist para-

TABELLE 4

Nr.	Trocknung	Glühverlust	$\chi.10^{-6}$	Struktur
Hydrat				
1	Aceton und Äther	15.61%	48.6	γ-Hydrat
2	"	16.6%	63	"
3	"	16.7%	74	"
1	10 Stdn. unter H_2O im Einschlußrohr	14.5%	46	"
1	24 Stdn. bei 100° im trocknen Luftstrom	10.5%	45	"
1	3 Jahre im Präparatenglas aufbewahrt	37.6%	43.5	"
2	"	34.8%	37	"
Oxyd aus Nr. 1 durch Glühen bei 600° in 4 Stdn.		—	37	α-Oxyd
Oxyd aus Nr. 1 durch Glühen bei 800° in 3 Stdn.		—	25	"

magnetisch ($\chi = 42.10^{-6}$), bei $\frac{1}{2}$-stdg. Erhitzen auf 375° wird das Präparat ferromagnetisch ($\chi = 39500.10^{-6}$). Nach $\frac{1}{2}$ Stde. bei 750° war die Susceptibilität auf $\chi = 129.10^{-6}$ gesunken, um bei $\frac{1}{2}$-stdg. Erhitzen auf 1000° wieder auf $\chi = 392.10^{-6}$ zu steigen. Dieses Ergebnis steht mit den in dieser Arbeit beschriebenen Messungen insofern nicht in Einklang, als das synthetische-Hydrat nach dem Glühen ein Oxyd mit bedeutend niedrigerer Susceptibilität ergibt, als das natürliche Produkt und auch durch längeres Glühen bei erhöhter Temperatur die Susceptibilität nicht erhöht, sondern erniedrigt wird. Die Autoren nehmen an, daß die starken Änderungen in der Magnetisier-

[9]) Proceed. physical Soc. London **411**, 100 [1928].

barkeit beim Glühen des Hydrates bei verschiedenen Temperaturen auf einem in allen untersuchten Präparaten gefundenen Mangan-Gehalt von ca. 3–4% beruhten, da das Mangan bei den verschiedenen Temperaturen verschiedene magnetische Formen von Manganferriten bildet. Sie zeigten, daß ein synthetischer Manganferrit im ungeglühten Zustand para- und im geglühten Zustand (1000°) ferromagnetisch ist. Auf meine Bitte sandte mir Hr. Prof. Herroun eine Reihe der von ihnen untersuchten Präparate zur Röntgen-Analyse[10]). Einige der gemachten Aufnahmen sind in der Tafel wiedergegeben. Es zeigte sich, daß der Manganferrit vor dem Glühen α-Oxyd-Struktur (Hämatit-Struktur) und nach dem Glühen γ-Oxyd-Struktur hat. Die Pulver-Aufnahmen des nicht geglühten, des bei mittlerer Temperatur geglühten und des bei höher Temperatur geglühten γ-Hydroxydes zeigen dieselben Ergebnisse wie bei dem synthetischen γ-Hydrat, doch sind bei dem stark geglühten Produkt einige Linien des kubischen, ferromagnetischen Manganferrites zu sehen, womit die oben beschriebenen Unstimmigkeiten erklärt erscheinen. Auch das bei 375° entwässerte Mineral zeigt ein Gemisch der beiden Oxyde.

BESPRECHUNG DER RESULTATE

F. Haber hat vor einigen Jahren[11]) die Theorie entwickelt, daß die Wechselwirkung zweier Komponenten, der „Häufungs-Geschwindigkeit" und der „Ordnungs-Geschwindigkeit", die Bildung von krystallinen oder amorphen Niederschlägen entscheidend beeinflußt. Ist die Häufungs-Geschwindigkeit groß gegenüber der Ordnungs-Geschwindigkeit, so erhalten wir amorphe Niederschläge, im umgekehrten Falle krystalline Körper. Durch die langsam verlaufende Oxydation und die große Verdünnung, in der die Reaktion stattfindet, ist in den oben beschriebenen Fällen die Häufungs-Geschwindigkeit herabgesetzt. Wir können also krystalline Niederschläge erwarten. Die Röntgen-Aufnahmen haben diese Erwartung bestätigt. Bei der Darstellung des γ-Hydrates war in einem Falle das Reaktionsgemisch höher als vorgeschrieben erwärmt worden (auf ca. 80°). Bei dieser

[10]) Hrn. Prof. Herroun danke ich auch an dieser Stelle bestens für die freundliche Übersendung der Präparate.

[11]) B. **55**, 1717 [1922].

Temperatur trat die Fällung rasch ein, und wie die Pulver-Aufnahmen zeigen, ist das Präparat, selbst 3 Jahre nach der Darstellung, noch amorph.

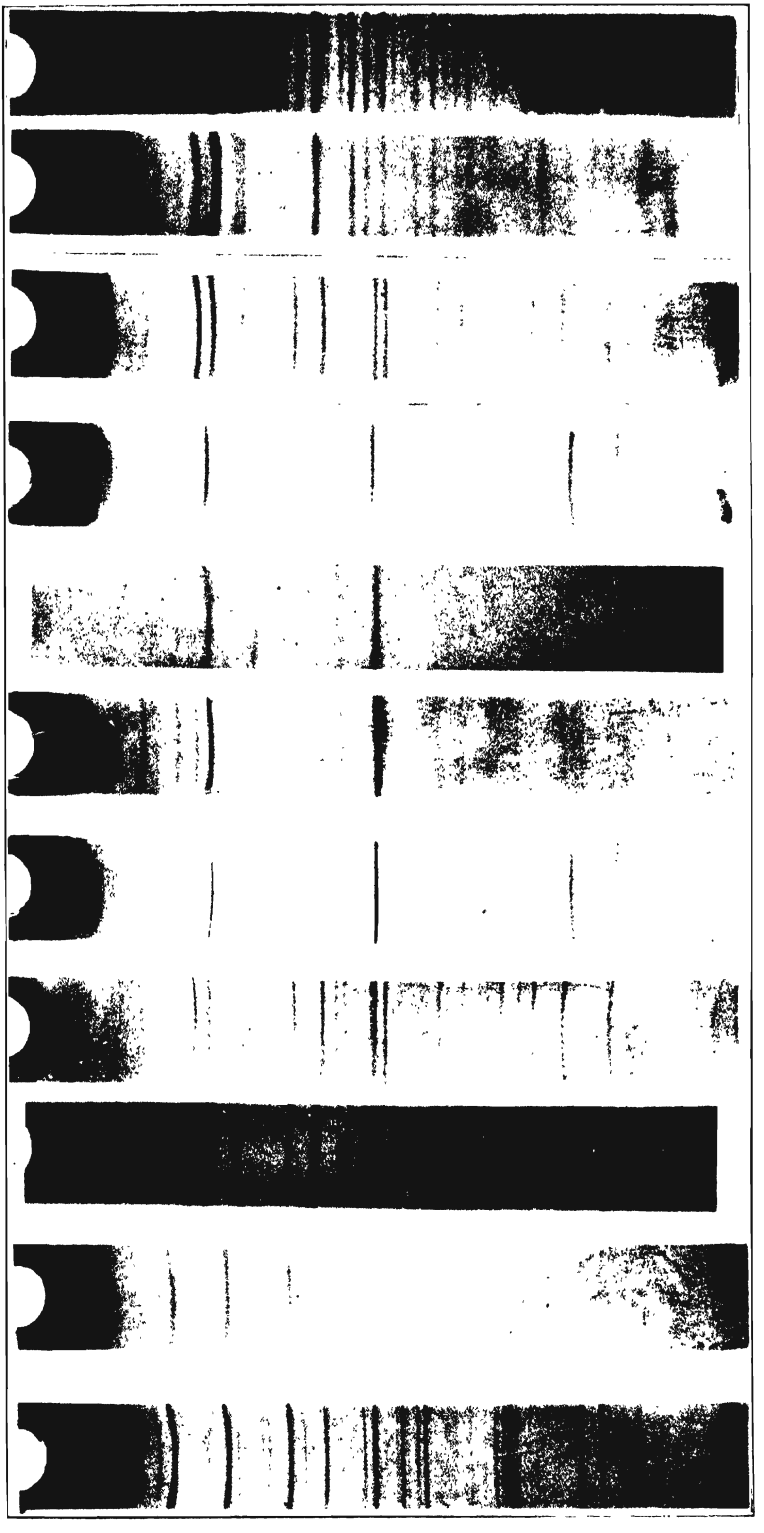
Die Temperatur, bei der eine Zersetzung der synthetischen Hydrate erfolgt, ist bedeutend niedriger als bei den natürlichen. Auch das Verhältnis H_2O: Fe_2O_3 ist nicht konstant; der Wasser-Gehalt ist meistens höher als der Theoretische. Während sowohl die analytische Bestimmung des Wasser-Gehaltes als auch die Pulver-Aufnahmen der Hydrate im „gealterten" Zustand die Beständigkeit derselben ergeben, zeigen die magnetischen Messungen eine deutliche Änderung mit der Alterung. Beim Altern unter Wasser sinkt bei beiden Hydraten die Susceptibilität, während beim Trocknen bis zu 100° im Luftstrom beim α-Hydrat die Susceptibilität sinkt, beim γ-Hydrat jedoch steil ansteigt. Im letzteren Falle ist die Erklärung gegeben, daß bereits bei dieser Temperatur eine teilweise Zersetzung der Hydrate eintritt. Der Abfall der Susceptibilität beim α-Hydrat ist nur gering, und das starke Ansteigen der Susceptibilität des γ-Hydrats kann durch geringe Mengen des ferromagnetischen γ-Oxyds bedingt sein. Bei der Alterung unter Wasser jedoch wird beim α-Hydrat die Susceptibilität um ca. 25% erniedrigt. Führt man diese Erniedrigung auf die Bildung des α-Oxyds zurück, so müßte das Präparat zu ca. 50% zersetzt sein, wofür jedoch in den Pulver-Aufnahmen keine Andeutung vorliegt. Anderseits haben die Messungen an dem natürlichen Hydrat in allen Fällen in guter Übereinstimmung mit Messungen anderer Autoren den höheren Wert von $\chi = 42.10^{-6}$ ergeben. Eine Erklärung der Erniedrigung der Susceptibilität scheint in diesem Falle im Augenblick nicht möglich. Beim γ-Hydrat führt die Alterung ebenfalls zu einer Erniedrigung der Susceptibilität. Man erhält einen Wert von $\chi = 42.10^{-6}$. Nimmt man diesen Wert als den richtigen für das γ-Hydrat an, so hat das γ-Hydrat dieselbe Susceptibilität wie das α-Hydrat, was um so wahrscheinlicher ist, als auch das natürliche γ-Hydrat ein $\chi = 42.10^{-6}$ hat.

Ein charakteristischer Unterschied zwischen den beiden beschriebenen Methoden zur Darstellung der verschiedenen Hydrate ist der Unterschied in der Wasserstoff-Ionen-Konzentration der beiden Reaktions-Systeme. Das α-Hydrat entsteht in einem sauren Medium, während das γ-Hydrat in einem neutralen Medium sich bildet.

Nun hat zwar J. Böhm (loc. cit.) gezeigt, daß das α-Hydrat auch aus stark alkalischem Medium sich bildet. Doch ist hier der Reaktionsverlauf prinzipiell anders, da, wie van Bemmelen (loc. cit.) gezeigt hat, bei diesem Vorgang sich primär Natriumferrit bildet, das hydrolytisch zum Hydrat umgesetzt wird. Man kann also diese Darstellungsmethode mit den beiden oben beschriebenen nicht vergleichen. Die Vermutung liegt nahe, daß je nach der Wasserstoff-Ionen-Konzentration entweder das α- oder das γ-Hydrat entsteht. Sowohl bei den Natur- als auch bei den synthetischen Hydraten ist das α-Hydrat beständiger als das γ-Hydrat. Die Entwässerung des α-Hydrates führt zum α-Oxyd, während die Entwässerung des γ-Hydrates zum γ-Oxyd führt, das sich dann bei höherer Temperatur in das α-Oxyd umwandelt. Bei den magnetischen Messungen von Pulvern in der magnetischen Wage erhält man die „scheinbare Dichte" der gemessenen Substanzen und damit eine Möglichkeit, etwas über die Packung der Teilchen, die wieder zum Teil von der Größe der Teilchen bedingt wird, auszusagen. Der Unterschied in der scheinbaren Dichte der natürlichen Hydrate von der der synthetischen ist bedeutend. Während von den fein gepulverten natürlichen Hydraten ca. 2 g auf einen ccm gehen, hat man bei den synthetischen Hydraten nur etwa den zehnten Teil, ca. 0.2 g pro ccm. Die Teilchen sind also viel kleiner als man sie durch Pulvern erreichen kann. Die geringe Größe der Teilchen kann man auch aus den stark verbreiterten Linien der Röntgen-Aufnahmen ableiten, während die Größe der Teilchen bei den natürlichen Hydraten für das gegenüber den synthetischen Hydraten geringere Wasser-Absorptionsvermögen zur Erklärung herangezogen werden kann. Auch das beim Entwässern des γ-Hydrates entstehende γ-Oxyd zeigt stark verbreiterte Linien, hat also kleine und wenig ausgebildete Krystalle.

Beim Entwässern des roten Eisenoxydhydrats entstehen Produkte, die ebenfalls Röntgen-Interferenzen zeigen, doch gleichen diese Interferenzen nicht denen der oben beschriebenen Hydrate. Sie sind ähnlich den Interferenzen des α-Oxydes und stellen wahrscheinlich eine Modifikation dieses Oxydes dar. Über sie soll an anderer Stelle gesprochen werden, ebenso über einen Versuch der Struktur-Bestimmung der beiden Hydrate.

Die Arbeit wurde begonnen im Laboratorium von Hrn. Prof. Dr. O. Baudisch im Rockefeller-Institut für medizinische Forschung, New York, und im Physikalischen Institut der Universität Manchester, Direktor Prof. W. L. Bragg, Abteil. Dr. Bradlay, zu Ende geführt. Den oben genannten Herren, besonders Hrn. Prof. Baudisch, bin ich zu großem Dank verpflichtet.



1 α-Hydrat (nach J. Böhm).

2. α Hydrat aus Ferro-bicarbonat-Lösung

3 α Oxyd (Hamatit).

4. γ-Oxyd (Magnetit).

5. γ-Hydrat (synthet.), 200°.

6. γ Hydrat, 350°, Lepidocrocit.

7 Mangan-ferrit, 1000°

8. Mangan-ferrit.

9 Rotes Hydrat, gealtert

10. γ-Hydrat (synthet.).

11. γ-Hydrat (Lepidoctocit)

[Reprinted from THE JOURNAL OF BIOLOGICAL CHEMISTRY, July, 1929,
Vol. 83, No. 1, pp. 177–183]

THE CONFIGURATIONAL RELATIONSHIP OF 2-METHYLHEPTANOL-(6) TO LACTIC ACID

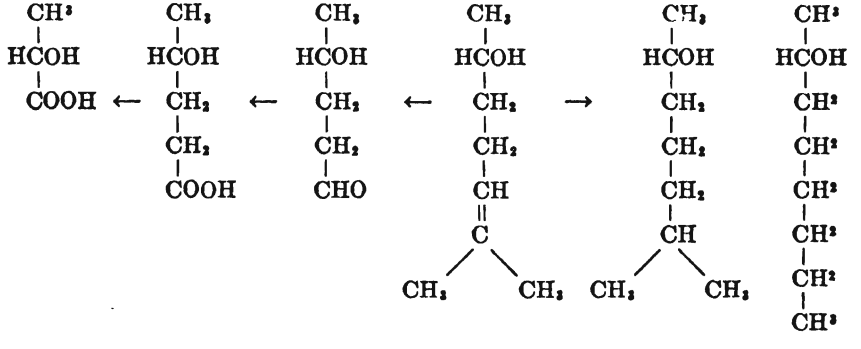
WITH A NOTE ON THE EFFECT OF UNSATURATION ON OPTICAL ACTIVITY

BY P. A. LEVENE AND H. L. HALLER

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(Received for publication, April 1, 1929)

The present communication is the first of a series which will deal with the configurational relationship of branched chain secondary carbinols with lactic acid. In the present case dextro-2-methylheptanol-(6) is correlated with dextro-lactic acid. The method employed in correlating the configurations of the two substances is one which has been used successfully on other occassions.[1] An unsaturated derivative, 2-methylhepten-(2)-ol-(6), is used as a connecting link between the substance of unknown configuration and one whose configuration is known. The details are given in Figures I to VI.



Dextro.	Dextro, +18.0 in water.	Levo, −7.7 in ether.	Dextro, +19.0 in ether, +16.1 homogeneous.	Dextro, +13.0 in ether, +10.3 homogeneous.	Dextro, +9.76 homogeneous.[2]
I.	II.	III.	IV.	V.	VI.

[1] Levene, P. A., and Haller, H. L., *J. Biol. Chem.*, **76**, 415 (1928); **79**, 475 (1928).

[2] Maximum rotation determined by Pickard and Kenyon. Pickard, R. H., and Kenyon, J., *J. Chem. Soc.*, **99**, 49 (1911).

From these figures it is seen that in the present case the substitution of a normal for a branched chain did not alter the direction of rotation of the carbinol. It is interesting in this connection to compare the rotation of the three related substances, namely, of the normal hexylmethyl carbinol, of the 2-methylheptanol-(6), and of the unsaturated 2-methylhepten-(2)-ol-(6). The respective molecular rotations are: $+12.7°$, $+13.4°$, and $+22.4°$.[3] In the case of these three substances, the substitution of a branched chain radical for a normal chain enhances the value little, if at all, of the optical rotation of the "iso" alcohol as compared with that of the normal alcohol; the introduction of a double bond in the heavier radical brings about an exaltation of the optical rotation. It would be unwise, however, to conclude that either one of these factors, the double bond or the branched chain, has a constant effect on the optical rotation. The data already available regarding the influence of the double bond bring out the importance of another contributing factor, namely, the distance of the double bond from the asymmetric carbon atom.

From Figures IV, V, and VII to XI, it seems that exaltation of the rotation by a double bond is produced when the double bond is situated between carbon atoms (3) and (4) from the asymmetric carbon atom. It had been previously accepted that the effect of a double bond generally is an exaltation of the rotation of the parent saturated substance. Thus we find the statement of Pickard and Kenyon,[4] "—the effect of unsaturation is exhibited in the exaltation of rotatory power." Again the same statement appears in the later article of Kenyon and Snellgrove.[5] Rupe,[6] who studied the effect of unsaturation in the series of menthyl esters of saturated and unsaturated acids observed that as a general rule unsaturation produced an exaltation, the only exception being in the case of the ester of γ,δ-hexenic acid. This ester showed a lower molecular rotation than the corresponding ester of hexonic acid. Thus neither the rule of Pickard and Ken-

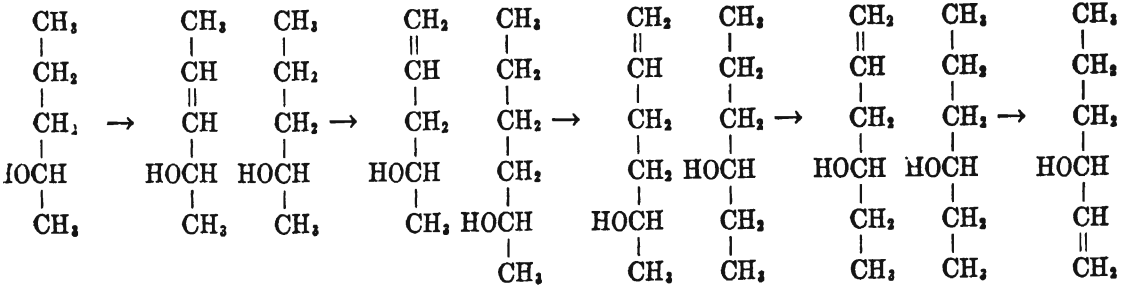
[3] It is not certain whether the last two values represent maximum rotations.

[4] Pickard, R. H., and Kenyon, J., *J. Chem. Soc.*, **99**, 47 (1911).

[5] Kenyon, J., and Snellgrove, D. R., *J. Chem. Soc.*, **127**, 1169 (1925).

[6] Rupe, H., *Ann. Chem.*, **327**, 157 (1903).

Influence of Double Bond.[7]



[7] All of the rotations are without solvent.

[8] Levene, P. A., and Haller, H. L., *J. Biol. Chem.*, **81**, 703 (1929).

[9] Levene, P. A., and Haller, H. L., *J. Biol. Chem.*, **81**, 425 (1929).

[10] Levene, P. A., and Haller, H. L., *J. Biol. Chem.*, **79**, 475 (1928).

[11] Levene, P. A., and Haller, H. L., *J. Biol. Chem.*, **76**, 415 (1928).

[12] Unpublished observations.

yon nor that of Rupe holds in our series. The difference between these three series and ours lies in the fact that in the former, the asymmetric carbon atom is connected to the unsaturated radical through an oxygen bridge, or the double bond in all members is at the same distance from the asymmetric carbon atom, whereas in our series, the asymmetric carbon atom and the double bond are located in the same carbon chain and the distance of the double bond from the asymmetric carbon atom is varied.

The general effect of the introduction of a branched chain will be discussed on another occasion when more experimental data will be available.

The general conclusion from our work up to date is that the optical rotation of a substance is the resultant of the simultaneous action of several factors, of which three have been definitely pointed out. These are: first, the respective masses of the radicals attached to the asymmetric carbon atom, second, the polarity of the groups, and third, the distances of the polar groups from the asymmetric carbon atom.

EXPERIMENTAL

2-Methylhepten-(2)-ol-(6).—The inactive carbinol was obtained on reduction of a commercial product of 2-methylhepten-(2)-one-(6) with sodium and absolute alcohol.

Resolution of 2-Methylhepten-(2)-ol-(6).—The acid phthalate of the carbinol was prepared by heating a solution of 128 gm. of the carbinol and 148 gm. of phthalic anhydride in 250 cc. of dry pyridine on the steam bath for 1 hour. The isolation and purification of the acid phthalate was carried out in the usual manner.[10] The acid phthalate remained a thick syrup in spite of repeated attempts to crystallize it.

50 gm. of the acid phthalate were dissolved in 400 cc. of acetone, the solution was heated to boiling on the steam bath, and 85 gm. of brucine were added. The hot solution was filtered and placed in the refrigerator. The brucine salt soon crystallized. It was filtered off and recrystallized several times from acetone. The rotation of the brucine salt in absolute alcohol then was

$$[\alpha]_D^{24} = \frac{-0.31^\circ \times 100}{1 \times 7.46} = -4.2^\circ.$$

The brucine salt, obtained as described above, was decomposed with dilute hydrochloric acid and the acid phthalate isolated by the usual procedure. In ether it had the following rotation.

$$[\alpha]_D^{24} = \frac{+ 11.82^\circ \times 100}{1 \times 19.6} = + 60.3^\circ.$$

The acid phthalate, obtained as described above, was dissolved in an aqueous solution of sodium hydroxide (3 mols) and the solution was steam-distilled. The carbinol was isolated in the usual way. It distilled at 60–61°, p = 4 mm. It analyzed as follows:

3.975 mg. substance: 10.945 mg. CO_2 and 4.470 mg. H_2O.
$C_8H_{16}O$. Calculated. C 75.00, H 12.50.
Found. " 75.08, " 12.58.

In ether the carbinol had the following rotation.

$$[\alpha]_D^{20} = \frac{+ 1.60^\circ \times 100}{1 \times 8.4} = + 19.0^\circ.$$

A carbinol obtained on decomposition of the mother liquors in the above resolution distilled at 87°, p = 22 mm. This carbinol without solvent had a rotation of $\alpha_D^{23} = -13.75^\circ$, $l = 1$. In ether the rotation was

$$[\alpha]_D^{23} = \frac{- 3.20^\circ \times 100}{1 \times 19.8} = - 16.2^\circ.$$

An aliquot part of the ether solution was diluted with an equal volume of ether; the rotation then was

$$[\alpha]_D^{23} = \frac{- 1.65^\circ \times 100}{1 \times 9.9} = - 16.7^\circ.$$

In absolute alcohol the rotation of the carbinol was

$$[\alpha]_D^{23} = \frac{- 7.50^\circ \times 100}{2 \times 24.9} = - 15.1^\circ.$$

α-Naphthylurethane of Dextro-2-Methylhepten-(2)-ol-(6).—The urethane was prepared from a 2-methylhepten-(2)-ol-(6) having $[\alpha]_D^{20} = + 19.0^\circ$ in ether, in the usual manner. Recrystallized from dilute alcohol it melted at 64–66°. It analyzed as follows:

0.1000 gm. substance: (Kjeldahl) 3.40 cc. 0.1 N HCl.
$C_{19}H_{23}O_2N$. Calculated. N 4.72.
Found. " 4.76.

In absolute alcohol the rotation of the urethane was

$$[\alpha]_D^{22} = \frac{+ 1.57° \times 100}{2 \times 2.39} = + 32.8°.$$

Levo-4-Hydroxyvaleric Aldehyde (*Pentanal-(1)-ol-(4)*).—The carbinol, obtained as described above, was ozonized in glacial acetic acid, the procedure being essentially the same as that described by Helferich[13] for the ozonization of the inactive carbinol.

Into 17.5 gm. of dextro-2-methylhepten-(2)-ol-(6) ($[\alpha]_D^{24}$ = +19.0° in ether) dissolved in 27 cc. of glacial acetic acid, a stream of ozonized oxygen was passed until the solution no longer decolorized bromine in glacial acetic acid. The remaining liquid was then diluted with ether. The decomposition of the ozonide and the isolation of the hydroxyaldehyde was then carried out in the same manner as described by Helferich. The hydroxyaldehyde distilled at 43–46°, p = 1 to 2 mm. It analyzed as follows:

4.870 mg. substance: 10.795 mg. CO_2 and 4.265 mg. H_2O.

$C_5H_{10}O_2$. Calculated. C 58.82, H 9.80.
Found. " 60.44, " 9.75.

In ether it had the following optical rotation.

$$[\alpha]_D^{22} = \frac{- 1.03° \times 100}{1 \times 13.2} = - 7.8°.$$

Dextro-4-Hydroxyvaleric Acid.—A mixture of 1.2 gm. of levo-4-hydroxyvaleric aldehyde ($[\alpha]_D^{23}$ = − 7.7° in ether), 2.5 gm. of silver oxide, and 40 cc. of water was heated to boiling under a reflux condenser for 20 minutes. The hot solution was filtered and concentrated under reduced pressure to a small volume. On addition of absolute alcohol the silver salt readily separated. It was filtered off and dried on a high vacuum pump. It analyzed as follows:

0.1008 gm. substance: 0.0490 gm. Ag.

$C_5H_9O_3 \cdot Ag$. Calculated. Ag 47.99.
Found. " 48.61.

0.15 gm. of silver salt was dissolved in water and the volume made up to 5.0 cc. The rotation was

$$[\alpha]_D^{1} = \frac{+ 0.32° \times 100}{2 \times 3.0} = + 5.4°.$$

[13] Helferich, B., *Ber. chem. Ges.*, **52**, 1123 (1919).

To 4.2 cc. of the silver salt solution employed above was added 0.7 cc. of 1.0 N HCl. The rotation was observed immediately. For the free acid,

$$[\alpha]_D^{22} = \frac{+ 0.50^\circ \times 100}{2 \times 1.33} = + 18.8^\circ.$$

Dextro-2-Methylheptanol-(6).—5 gm. of dextro-2-methylhepten-(2)-ol-(6) ($[\alpha]_D^{20} = +19.0^\circ$ in ether) were dissolved in ether and reduced with hydrogen in the presence of colloidal palladium as catalyst. The absorption of hydrogen was slow but constant. Reduction was complete in 30 hours. The ether extract was dried over anhydrous potassium carbonate, the ether was removed, and the carbinol was distilled under reduced pressure. It distilled at 61–63°, p = 4 mm. It analyzed as follows:

3.430 mg. substance: 9.315 mg. CO_2 and 4.390 mg. H_2O.
$C_8H_{16}O$. Calculated. C 73.85, H 13.85.
Found. " 74.05, " 14.32.

In ether it had the following rotation.

$$[\alpha]_D^{22} = \frac{+ 2.25^\circ \times 100}{1 \times 17.3} = + 13.0^\circ.$$

α-Naphthylurethane of Dextro-2-Methylheptanol-(6).—The urethane was prepared in the usual way. It was recrystallized from dilute alcohol. It melted at 75–77°. It analyzed as follows:

0.1000 gm. substance: (Kjeldahl) 3.35 cc. 0.1 N HCl.
$C_{19}H_{25}O_2N$. Calculated. N 4.68.
Found. " 4.69.

In absolute alcohol it had the following rotation.

$$[\alpha]_D^{22} = \frac{+ 0.82^\circ \times 100}{2 \times 2.50} = + 16.4^\circ.$$

[Reprinted from THE JOURNAL OF BIOLOGICAL CHEMISTRY, July, 1929, Vol. 83, No. 1, pp. 185–190]

ON THE CONFIGURATIONAL RELATIONSHIP OF CHLOROSUCCINIC ACID TO CHLOROPROPIONIC AND TO LACTIC ACIDS

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(Received for publication, May 13, 1929)

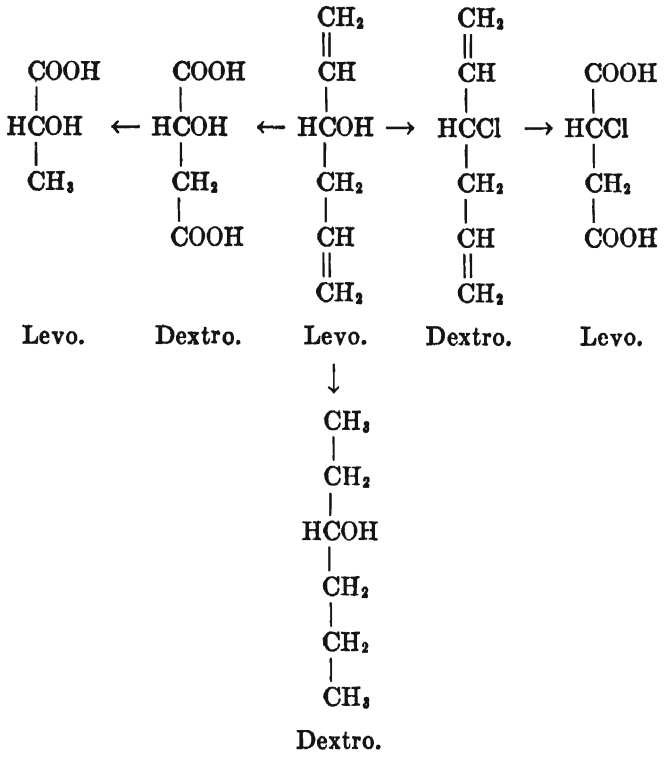
The considerations which led up to the present investigation have been discussed in an earlier publication.[1] The principal object was to test by a second method the conclusions which had been reached earlier by Levene and Mikeska[2] regarding the configurational relationships of hydroxy and halogeno acids. The conclusions formulated earlier were the following:—(1) Dextro-2-hydroxypropionic (lactic) acid is configurationally related to dextro-2-chloropropionic acid. (2) Dextro-3-hydroxybutyric acid is configurationally related to *levo*-3-chlorobutyric acid. (3) Dextro-malic acid is configurationally related to *levo*-chlorosuccinic acid.

The conclusions of Levene and Mikeska have been tested regarding the first two pairs of acids by Levene and Haller by a different method and the new method led to the original conclusion. The same method has now been applied for testing the conclusions regarding the configurations of malic and chlorosuccinic acids.

The new method is based on the assumption that in aliphatic alcohols the substitution of the hydroxyl by a halogen atom proceeds without Walden inversion. The set of reactions employed for the solution of our problem is the following.

[1] Levene, P. A., and Haller, H. L., *J. Biol. Chem.*, **81**, 425 (1929).

[2] Levene, P. A., and Mikeska, L. A., *J. Biol. Chem.*, **63**, 85 (1925); **70**, 365 (1926).



Thus, the new test substantiates the conclusion previously reached by Levene and Mikeska to the effect that dextro-malic acid is configurationally related to levo-chlorosuccinic acid.

Incidentally, it may be mentioned that the hexadienol which served as starting material for this investigation is a new substance. The method of its preparation is given in the experimental part.

EXPERIMENTAL

Hexadiene-(1,5)-ol-(3) (Vinylallyl Carbinol).—This carbinol[3] was obtained on condensation of acrolein and allyl bromide with zinc. It can also be prepared by the action of acrolein on allyl magnesium bromide. The crude product distilled at 125–135° at atmospheric pressure. It was purified through the acid

[3] We are indebted to Mr. R. E. Marker for assistance in the preparation of the carbinol.

phthalate. It then distilled at 133–134° at atmospheric pressure. $n_D^{25} = 1.4464$, $D_4^{25} = 0.8596$. It analyzed as follows:

4.305 mg. substance: 11.675 mg. CO_2 and 4.000 mg. H_2O.
$C_6H_{10}O$. Calculated. C 73.47, H 10.20.
Found. " 73.95, " 10.39.

Resolution of Hexadiene-(1,5)-ol-(3).—The acid phthalate of the carbinol was prepared by heating a solution of 107 gm. of the crude carbinol, 161 gm. of phthalic anhydride and 200 cc. of dry pyridine on the steam bath for 1 hour. After standing overnight at room temperature, the solution was cooled in an ice water bath, ice and ether were added, followed by 300 cc. of concentrated hydrochloric acid. The ether extract was washed with water and dried over sodium sulfate. The ether was removed under reduced pressure and the remaining syrup poured into a solution of 115 gm. of sodium carbonate in 1000 cc. of water. After standing for 1 hour at room temperature, the solution was extracted twice with ether, cooled, and acidified with 250 cc. of concentrated hydrochloric acid. The phthalate was extracted with chloroform, the chloroform extract was washed with water and dried over sodium sulfate. The chloroform was removed under reduced pressure and the remaining syrup was titrated and converted to the brucine salt.

236 gm. of the syrup, 60 per cent acid phthalate, were dissolved in 600 cc. of dry acetone, the solution heated to boiling and 290 gm. of brucine were added. The hot solution was filtered and placed in the refrigerator. On cooling with stirring, the brucine salt crystallized. It was filtered off and repeatedly recrystallized from dry acetone. The brucine salt was decomposed with dilute hydrochloric acid and the acid phthalate extracted with ether in the usual way. The phthalate was a thick syrup. In ether it had the following rotation.

$$[\alpha]_D^{24} = \frac{+ 8.75° \times 100}{2 \times 24.3} = + 18.0°.$$

The phthalate was dissolved in a solution of sodium hydroxide (3 mols) and the solution was steam-distilled. The carbinol was extracted with ether, and the ether extract was dried over anhydrous potassium carbonate. After removal of the ether,

the carbinol was distilled at atmospheric pressure. It distilled at 133–134°. It analyzed as follows:

5.105 mg. substance: 13.820 mg. CO_2 and 4.745 mg. H_2O.
$C_6H_{10}O$. Calculated. C 73.47, H 10.20.
Found. " 73.82, " 10.40.

The rotation without solvent was $\alpha_D^{24} = +16.0°$. $l = 1$ dm. In ether the rotation was

$$[\alpha]_D^{24} = \frac{+ 1.45° \times 100}{1 \times 11.2} = + 12.9°.$$

In absolute alcohol it had the following rotation.

$$[\alpha]_D^{24} = \frac{+ 2.25° \times 100}{1 \times 10.8} = + 20.8°.$$

Levo-3-Chloro-Hexadiene-(1, 5) (Vinylallyl Methyl Chloride).—Into 5 gm. of phosphorus trichloride cooled in an ice water bath was dropped a solution of 10 gm. of dextro-hexadiene-(1,5)-ol-(3) ($[\alpha]_D^{24} = +20.8°$ in alcohol) and 1.5 cc. of dry pyridine. The reaction mixture was constantly shaken and after addition of the carbinol was warmed gently. After standing for 1 hour at room temperature, the chloride was distilled under reduced pressure. It analyzed as follows:

0.1358 gm. substance: 0.1510 gm. AgCl.
No. 1271. C_6H_9Cl. Calculated. Cl, 30.47.
Found. " 29.69.

In a 1 dm. tube without solvent $\alpha_D^{24} = - 12.10°$.
In ether it had the following rotation.

$$[\alpha]_D^{24} = \frac{- 1.60° \times 100}{1 \times 12.1} = - 13.2°.$$

In absolute alcohol the rotation was

$$[\alpha]_D^{24} = \frac{- 1.95° \times 100}{2 \times 8.2} = - 11.9°.$$

Dextro-Chlorosuccinic Acid.—The chloride obtained as described above was ozonized in chloroform solution in 1 gm. lots. 1 gm. of the chloride (No. 1271) was dissolved in 40 cc. of chloroform and a stream of ozonized oxygen was passed into

the solution until it no longer decolorized bromine in glacial acetic acid. By this time a small quantity of a gelatinous precipitate adhered to the walls of the tube. The remaining chloroform was removed under reduced pressure and the ozonide poured into water and immediately treated with bromine. The reaction mixture was thoroughly shaken and then allowed to stand overnight with an excess of bromine. The remaining bromine was removed with a stream of air, the solution was thoroughly cooled and treated with silver sulfate until free of bromide ion, then with hydrogen sulfide to remove the excess silver. After saturating the solution with sodium sulfate, it was extracted with ether. The ether extract was dried over sodium sulfate. After removal of the ether, the remaining syrup was placed in the desiccator. After 2 days the crystals were filtered off. They were recrystallized from a mixture of ether and petroleum ether. The acid melted at 168–171°. It analyzed as follows:

7.235 mg. substance: 6.970 mg. AgCl.
$C_4H_5O_4Cl$. Calculated. Cl, 23.27.
Found. " 23.83.
12.078 mg. substance required 1.515 cc. 0.1 N NaOH.
Calculated. 1.580.

In ether containing 20 per cent alcohol the rotation was

$$[\alpha]_D^{24} = \frac{+ 5.25^\circ \times 100}{1 \times 11.0} = + 47.7^\circ.$$

In water it had the following rotation.

$$[\alpha]_D^{24} = \frac{+ 1.85^\circ \times 100}{2 \times 4.9} = + 18.9^\circ.$$

Dextro-Hexanol-(3) (*Ethylpropyl Carbinol*).—4 gm. of levo-hexadiene-(1,5)-ol-(3) ($[\alpha]_D^{23} = -6.2^\circ$ in ether) were reduced in ether solution with hydrogen in the presence of colloidal palladium as catalyst. Absorption of hydrogen was rapid and reduction was complete in 3 hours. The ether solution was dried over anhydrous potassium carbonate. After removal of the ether, the carbinol was distilled at atmospheric pressure.

It boiled at 128–130°. In a 1 dm. tube without solvent $\alpha_D^{23} = +1.20°$.

It analyzed as follows:

3.335 mg. substance: 9.155 mg. CO_2 and 3.795 mg. H_2O.

$C_6H_{14}O$. Calculated. C 70.59, H 13.72.
Found. " 70.58, " 13.21.

[Reprinted from THE JOURNAL OF BIOLOGICAL CHEMISTRY, May, 1929, Vol. 82, No. 2, pp. 403–409]

THE DIGITALIS GLUCOSIDES

III. GITOXIGENIN AND ISOGITOXIGENIN

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(Received for publication, April 2, 1929)

In a previous communication,[1] the conclusion was reached that gitoxigenin is a $\Delta^{\beta,\gamma}$-lactone like digitoxigenin[2] and the related cardiac aglucones. In certain respects, however, it exhibited an abnormal behavior which suggested a structural divergence from the latter. This dissimilarity became evident in the study of derivatives of isogitoxigenin which had been in turn obtained in rather poor yield by the isomerizing effect of alkali on gitoxigenin. Isogitoxigenin resembled the other iso compounds in that it no longer gave the nitroprusside reaction and resisted all attempts at catalytic hydrogenation. Its formation, therefore, must have involved the double bond of gitoxigenin. When saponified, isogitoxigenin yielded isogitoxigeninic acid. Contrary to the other iso acids, this substance did not appear to behave as a hydroxyaldehyde which may react in either the aldehydic or lactol form. As the methyl ester it did not yield a semicarbazone. When it was oxidized with chromic acid a neutral ester, the so called isogitoxigenonic methyl ester, was formed. Saponification experiments with this substance performed in the usual manner seemed to exclude the formation of a lactone group contrary to the experience with similar derivatives of isodigitoxigenin, etc. Finally, an acid was obtained on oxidation of isogitoxigeninic acid with hypobromite, which proved to be monobasic, and with this also no evidence of the formation of a lactone group was obtained by the customary procedure.

[1] Jacobs, W. A., and Gustus, E. L., *J. Biol. Chem.*, **79**, 553 (1928).
[2] Jacobs, W. A., and Gustus, E. L., *J. Biol. Chem.*, **78**, 573 (1928).

However, a reexamination of the evidence and further study have caused us to alter the previously published suggestion that degradation occurs during the hypobromite oxidation. The facts now available show definitely that isogitoxigenin, like the other iso compounds, is a lactone on the lactol form of a hydroxyaldehyde. Contrary to our former belief, isogitoxigenin when saponified displays great stability towards alkali. Renewed study has resulted in a great improvement in the yield of the iso compound which can be obtained from gitoxigenin. Reinvestigation of the preparation and composition of the so called isogitoxigenonic methyl ester mentioned above has confirmed the previously reported formulation, $C_{24}H_{34}O_6$. But its relationship to the parent substance was misinterpreted. This ester had been found to consume only 1 equivalent of 0.1 N alkali when saponified by the method which opened both ester and lactone group in the case of the analogous isodigitoxigenin isostrophanthidin,[3] etc., derivatives. However, more recently, by the use of stronger alkali and higher temperature, it has become possible to detect a relatively resistant lactone group in the isogitoxigenin derivative. This substance is, therefore, a ketolactone ester and in conformity with the analogous substances obtained from the other iso compounds should be called *isogitoxigonic methyl ester.*

More recently a similar experience has been encountered with the hypobromite oxidation product mentioned above. From the former analytical experience with this substance, the incorrect formula $C_{21}H_{30}O_6$, was derived. A renewed study of the preparation and purification of this acid has resulted in an unquestionably pure, anhydrous substance. The analytical figures obtained are now in agreement with the normal formulation $C_{23}H_{34}O_6$. As previously described, this substance neutralizes 1 equivalent of alkali on direct titration and when boiled with an excess of 0.1 N alkali extra consumption of the latter was negligible in contradistinction to the experience with similar derivatives of other iso compounds. However, with stronger alkali and higher tempera-

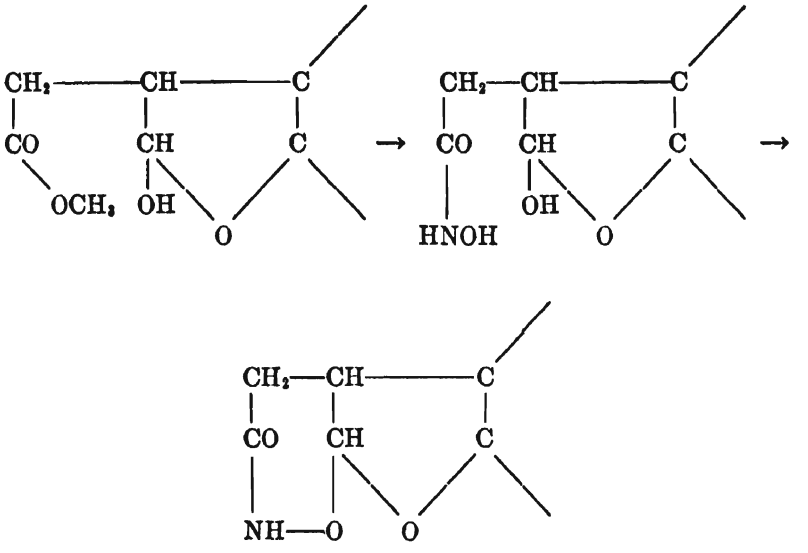
[3] Jacobs, W. A., and Collins, A. M., *J. Biol. Chem.*, **61**, 395 (1924). Jacobs, W. A., and Hoffmann, A., *J. Biol. Chem.*, **79**, 531 (1928). Jacobs, W. A., and Heidelberger, M., *J. Biol. Chem.*, **81**, 779 (1929).

ture an extra equivalent was consumed. This substance, *isogitoxigenic acid*, is therefore a lactone acid which is isomeric with isoperiplogenic acid[4] and isosarmentogenic acid.[5] It differs from the latter substances in the greater stability of its lactone group.

Gitoxigenin like digitoxigenin and the other well studied cardiac aglucones possesses one secondary alcoholic group and a tertiary hydroxyl which is presumably γ to the aldehydic carbon. The retention of the secondary hydroxyl in isogitoxigenin has been shown by its oxidation to the ketone *isogitoxigenon*. If one assumes the probable similarity in other respects of the general structural make-up of both gitoxigenin and digitoxigenin, it is possible that the difference in chemical properties noted above may be attributable to the position occupied by the extra tertiary hydroxyl of gitoxigenin. If this were so, the isogitoxigenin derivatives should present a normal behavior after removal of this hydroxyl. This suggestion was supported by the following observation.

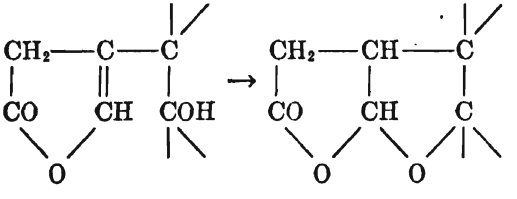
Isogitoxigenic acid was readily converted by strong hydrochloric acid into *anhydroisogitoxigenic acid*, due to the removal of the additional tertiary hydroxyl as water. This anhydro acid now presented the normal behavior in that its lactone group was much more readily opened by dilute alkali. It appears probable that the proximity of the extra tertiary hydroxyl group plays a rôle in the stability of the lactone group of isogitoxigenic acid. Probably, for similar reasons isogitoxigeninic acid exists only as the stable lactol and not as a hydroxyaldehyde. The previously reported failure of its ester to yield a semicarbazone would thus be explained. This has its parallel in our experience with pseudostrophanthidin[6] which was shown to be a γ-lactol. However, to submit isogitoxigeninic methyl ester to a further test, we have attempted its reaction with hydroxylamine. A reaction occurred, but instead of an oxime a substance was obtained which owed its origin apparently to the intermediate formation of a hydroxamic acid which then lost water with the lactol hydroxyl as shown in the following scheme:

[4] Jacobs, W. A., and Hoffmann, A., *J. Biol. Chem.*, **79**, 529 (1928).
[5] Jacobs, W. A., and Heidelberger, M., *J. Biol. Chem.*, **81**, 778 (1929).
[6] Jacobs, W. A., and Collins, A. M., *J. Biol. Chem.*, **65**, 495 (1925).



This substance gives a deep red purple color with ferric chloride which is characteristic of hydroxamic acids.

The present observations permit the definite conclusion that gitoxigenin like digitoxigenin is a tetracyclic $\Delta^{\beta,\gamma}$-lactone in which a carbon atom presumably γ to the lactone γ carbon atom carries a tertiary hydroxyl group. In the formation of isogitoxigenin this hydroxyl functions in an oxidic union between the two carbon atoms with a disappearance of the double bond as follows:



Additional work will shortly be presented on attempts at further structural correlation of gitoxigenin with digitoxigenin by means of the iso compounds.

We are especially indebted to both Merck and Company, Inc., of Rahway and E. Merck, Darmstadt for their generous gift of the "insoluble digitoxin by-product" which was the source of the gitoxigenin used in this investigation.

EXPERIMENTAL

Isogitoxigenin.—The following improvement in the method for the isomerization of gitoxigenin was developed, which departed from that previously employed by the use of stronger reagent.

1 gm. of isogitoxigenin was shaken at 25° in a solution of 1 gm. of potassium hydroxide in 10 cc. of dry methyl alcohol. Solution gradually occurred and at the end of 15 minutes the solution was well diluted. Acidification to Congo red caused an immediate precipitate which gradually crystallized. After standing 24 hours to insure complete relactonization, the mixture was repeatedly extracted with chloroform. After washing with dilute carbonate, the dried extract yielded on concentration a crystalline residue which was collected with a few cc. of dry chloroform and ether. The total yield was 0.70 gm. When recrystallized from alcohol, it softened above 220° and finally melted at 252°. In all other properties, it agreed with those already recorded. When heated in a sealed tube at 130° for 4 hours in an excess of N sodium hydroxide solution, unchanged isogitoxigenin was recovered after acidification to Congo red and relactonization.

Isogitoxigonic Methyl Ester.—Isogitoxigeninic methyl ester was oxidized as previously given with the slight modification that a mixture of 4 parts of acetic acid and 1 part of water was used as the solvent. When recrystallized by the cautious addition of dry ether to its concentrated methyl alcoholic solution, the keto-lactone ester slowly separated as broad, flat prisms which melted sharply at 174°.

5.135 mg. substance: 3.815 mg. H_2O, 12.970 mg. CO_2.
5.006 " " : 3.770 " " 12.614 " "
$C_{24}H_{34}O_6$. Calculated. C 68.86, H 8.19.
Found. " 68.87, " 8.32.
" 68.72, " 8.42.

That the substance is the ester of a lactone acid was shown as follows: 0.98794 gm. of substance was heated at 125–130° for 5 hours in a sealed tube with 1 cc. of alcohol and 2.190 cc. of N NaOH in an atmosphere of nitrogen and then titrated back against phenolphthalein. Found 0.412 cc. Calculated for 2 equivalents, 0.472 cc.

Isogitoxigenic Acid.—Isogitoxigeninic acid was oxidized with hypobromite as previously described. The acid, however, was

obtained from the reaction mixture by acidification with acetic acid instead of sulfuric acid. On rubbing, the clear solution deposited the crystalline lactone acid. For recrystallization the substance was dissolved in 50 per cent alcohol by the addition of ammonia. Reacidification with acetic acid after warming and careful dilution caused the acid to separate as microrosettes of leaflets which were anhydrous and melted at 260°.

$[\alpha]_D^{21} = -50°$ (c = 0.993 in 95 per cent alcohol.)

3.404 mg. substance: 2.622 mg. H_2O, 8.460 mg. CO_2.
3.333 " " : 2.560 " " 8.288 " "

$C_{23}H_{34}O_6$. Calculated. C 67.99, H 8.44.
Found. " 67.78, " 8.62.
" 67.81, " 8.60.

The lactone group of this acid was only incompletely saponified by boiling for 4 hours in N alkali. Correct results were obtained as follows: 0.10063 gm. of substance was sealed in a tube with 2 cc. of alcohol and 5.425 cc. of N NaOH in an atmosphere of nitrogen. After heating at 125–130° for 5 hours the mixture was titrated back against phenolphthalein. Found 0.480 cc. of N NaOH. Calculated for 2 equivalents, 0.495 cc.

To complete the data a direct titration was made with 0.1 N NaOH. 17.480 mg. of substance in 2 cc. of alcohol required 0.425 cc. of 0.1 N NaOH. Calculated for 1 equivalent, 0.430 cc.

Anhydroisogitoxigenic Acid.—0.1 gm. of isogitoxigenic acid was dissolved at 20° in 5 cc. of hydrochloric acid (1.19). After about 10 minutes crystallization readily occurred on rubbing. After several hours the substance was collected with hydrochloric acid followed by water.[7] The acid was recrystallized by dissolving a suspension in 50 per cent alcohol with the aid of ammonia and reacidification with acetic acid. It formed on standing aggregates of platelets which were anhydrous and melted at 215° after preliminary softening.

4.713 mg. substance: 3.500 mg. H_2O, 12.267 mg. CO_2.

$C_{23}H_{32}O_6$. Calculated. C 71.08, H 8.31.
Found. " 70.97, " 8.31.

[7] After this paper was sent to press it was found that this substance as directly obtained contains chemically bound chlorine and that during the recrystallization with the aid of ammonia the halogen has been removed with the formation of the anhydro acid described above. The nature of this intermediate chloro compound is under investigation.

Contrary to the parent substance, the anhydro acid was readily saponified by 0.1 N sodium hydroxide.

13.340 mg. of substance were dissolved in 1 cc. of alcohol and directly titrated with 0.1 N NaOH against phenolphthalein. Found 0.347 cc. Calculated for 1 equivalent, 0.344 cc.

2.6 cc. of 0.1 N NaOH were added to the above titration mixture which was then refluxed for 4 hours in an atmosphere of nitrogen and then titrated back. Found 0.341 cc. Calculated for 1 equivalent, 0.344 cc.

Isogitoxigeninic Methyl Ester and Hydroxylamine.—0.1 gm. of isogitoxigeninic methyl ester was refluxed with a solution made by mixing 0.1 gm. of hydroxylamine hydrochloride and 0.2 gm. of potassium acetate in 10 cc. of methyl alcohol and filtering. After 6 hours a crystalline substance was obtained after dilution. On concentration of its solution in methyl alcohol the substance formed aggregates of minute platelets which were anhydrous. It melted with decomposition at 287°. The alcoholic solution gave a deep purple red color with ferric chloride. The substance was neutral and contained no methoxyl.

3.730 mg. substance: 2.947 mg. H_2O, 9.285 mg. CO_2.
6.050 " " : 0.191 cc. N (24°, 752.5 mm.).
$C_{23}H_{35}O_5N$. Calculated. C 68.10, H 8.75, N 3.45.
Found. " 67.88, " 8.84.
N 3.59.

Isogitoxigenon.—A solution of 0.2 gm. of isogitoxigenin in a mixture of 4 cc. of acetic acid and 1 cc. of water was treated with an excess of Kiliani chromic acid solution, which caused a prompt reaction. After dilution, crystallization was induced by the addition of saturated ammonium sulfate solution. The ketone slowly separated from its concentrated alcoholic solution as sheaves of delicate needles which melted at 278–279° and were solvent-free. It proved to be easily soluble in chloroform, acetone, and less readily so in methyl and ethyl alcohol. It was but sparingly soluble in ether.

3.280 mg. substance: 2.496 mg. H_2O, 8.578 mg. CO_2.
$C_{23}H_{32}O_5$. Calculated. C 71.08, H 8.31.
Found. " 71.30, " 8.50.

[Reprinted from THE JOURNAL OF GENERAL PHYSIOLOGY, July 20, 1929,
Vol. 12, No. 6, pp. 761-781]

THE CONCENTRATION EFFECT IN NITELLA*

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(Accepted for publication, August 2, 1928)

Different concentrations of the same salt brought in contact with living tissue commonly show a P.D. which is known as the concentration effect. Since it can exist only when the surface is permeable to ions[1] it is obviously important for the study of permeability provided we can distinguish between effects due to the living protoplasm and those due to cell walls or other non-living structures. This viewpoint occasioned the present investigation. It early became apparent that in order to interpret the results a picture of conditions in the cell must be worked out which has necessitated additional experimentation.

Quantitative studies of concentration effects were first made by MacDonald,[2] experimenting on nerves but without distinguishing carefully between living and dead cells. The theoretical implications of such measurements were first pointed out by Loeb and Beutner.[3] They investigated plant organs with a cuticularized cell wall which may have been largely responsible for the observed effects;[4] their experiments on muscle,[5] like those of Matsuo[6] on frog liver, gave very little con-

* The authors desire to express their gratitude to the Carnegie Institution of Washington, D. C., which generously provided for the beginning of these investigations (1922-25).

[1] Osterhout, W. J. V., *J. Gen. Physiol.*, 1927-28, xi, 83.

[2] MacDonald, J. S., *Proc. Roy. Soc. London*, 1900, lxvii, 310; *Thompson Yates Laboratory, Liverpool*, 1902, iv, pt. 2.

[3] Loeb, J., and Beutner, R., *Biochem. Z.*, 1912, xli, 1; xliv, 303; 1913, li, 300. *Cf.* Beutner, R., Die Entstehung elektrischer Ströme in lebenden Geweben, Stuttgart, 1920.

[4] Beutner[3] states that the same results were obtained after the tissue was killed by chloroform. See also Fujita, A., *Biochem. Z.*, 1925, clviii, 11.

[5] The results of Loeb and Beutner on the finger nail and the skin are probably due in large part to non-living elements.

[6] Matsuo, T., *Arch. ges. Physiol.*, 1923, cc, 132.

centration effect. Recent experiments by Jost[7] have dealt with the concentration effect in *Nitella* without separating the effect due to the protoplasm from that of the cell wall: but in the experiments of Brooks[8] the effect of the cell wall seems to have been avoided.

Our experiments on *Nitella* have been carried on at various times from 1922 onward. The range of concentrations studied was as a rule from 0.001 M to 0.1 M. Above this plasmolysis and injury are apt to occur and in very dilute solutions there is danger of contamination. The experiments were arranged as in Fig. 1. The temperature averaged about 23°C. Details of technique are given in previous papers.[9]

In all cases the results were recorded photographically and the measurements were made from these records.

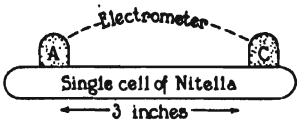


FIG. 1

FIG. 1. Diagram to show the arrangement of the experiments. Flowing junctions or wads of cotton soaked in solutions are applied at *A* and *C* or the ends are allowed to dip into cups containing solutions. Cotton soaked in distilled water is in some cases applied for a short stretch between *A* and *C* but a space is left on each side of the cotton to prevent short-circuiting.

It was first necessary to ascertain whether the observed concentration effect is entirely due to the protoplasm or whether the cell wall also plays a part.

In order to study the cell wall dead cells were emptied of sap by cutting off one end and squeezing out their contents. They were then allowed to fill with tap water, and again squeezed out and allowed to fill with tap water. This was repeated several times. Their contents were then squeezed out and they were then filled with air and used at once. In this way it is believed that we may approximate the condition of the cell wall in the living cell where there is probably not much ionic exchange between the cell wall and the protoplasm.

The ends of the dead cell walls (filled with air) were allowed to dip into solutions (in which calomel electrodes were immersed) in such fashion that the solutions did not creep into the lumen (if this occurs it may cause variations in the magnitude or even in the sign of the P.D.). On account of the high resistance the meas-

[7] Jost, L., *Sitzungsber. Heidelberger Akad. Wissensch., Abt. B*, 1927, Abhandl. 13, Nov.

[8] Brooks, S. C., and Gelfan, S., *Protoplasma*, 1928, v, 86. (This article was received after the completion of this paper.)

[9] Osterhout, W. J. V., and Harris, E. S., *J. Gen. Physiol.*, (*a*) 1927–28, xi, 673; (*b*) 1928–29, xii, 167.

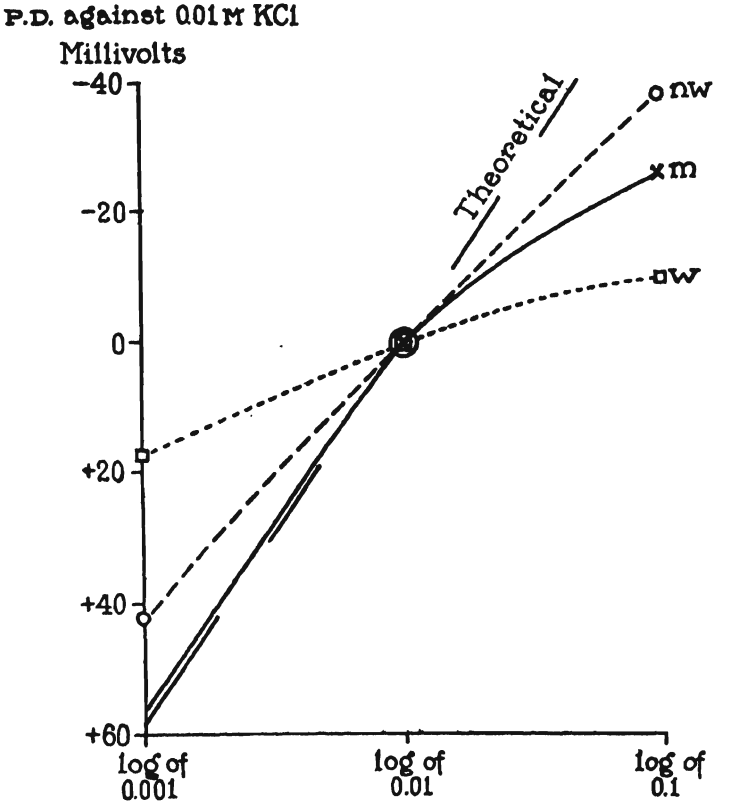


FIG. 2. Curves showing the concentration effect of KCl (each point is the average of 10 to 130 determinations, the probable error of the mean being in general less than 10 per cent of the mean). Curve *m*, concentration effect of the protoplasm alone (*i.e.*, on the *m* basis); Curve *nw*, that of the cell wall and protoplasm combined (*i.e.*, on the *nw* basis); Curve *w*, that of the cell wall alone. The potential difference across the protoplasm when in contact with 0.01 M KCl is taken as zero for purposes of comparison (thus if the curve shows 60 millivolts at 0.001 M KCl it means that this is the potential difference between 0.001 M KCl and 0.01 M KCl). The actual value (called the *AP* value) across the protoplasm in contact with 0.01 M KCl is −2.9 millivolts (average of 60 experiments).

For Curve *m* the values are +56.7 and −25.4; for Curve *nw* +42.2 and −37.9; for Curve *w* +16.8 and −10.

urements on the dead cell wall were made with a Compton electrometer (Cambridge Instrument Co.).

Flowing contacts must be used with caution (especially when the solutions are dilute) on account of "streaming potential" (a good criterion of this is the P.D. observed when identical solutions are placed at the opposite ends, one flowing and the other not, and unless this gave approximately zero the experiment was rejected: in all cases these results were checked by the employment of cups instead of flowing contacts).

Experiments of this kind on cell walls gave the results shown in Curve *w* (Fig. 2). It is therefore evident that the cell wall can give a concentration effect.[10]

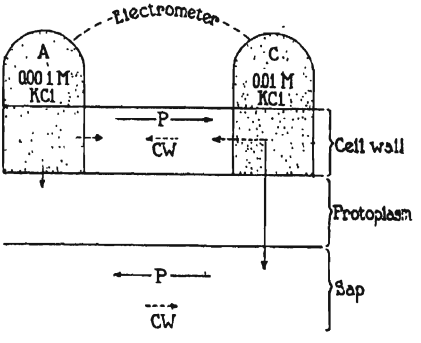


FIG. 3. Hypothetical diagram of conditions in the cell. The arrows show the direction in which the positive current tends to flow and their length the relative magnitude of the P.D. The vertical arrows represent the P.D. due to the protoplasm and the resulting P.D. is labelled *P*; the broken arrows represent the E.M.F. due to the cell wall, and the resulting P.D. labelled *CW*, is opposed to *P*. These relations are more simply represented in Fig. 4.

In order to have some picture of the conditions possibly existing in the cell we may perhaps employ the diagram in Fig. 3 (without by any means assuming that it is correct in all respects). The arrows show the direction in which the positive current tends to flow and their lengths the relative P.D. The vertical arrow at *C* represents the P.D.

[10] In *Nitella* the cell wall is much less cuticularized than the epidermis of the plants used by Loeb and Beutner and it gives a much lower concentration effect.

It may be added that when cells are killed by being placed for 10 minutes in pure chloroform (not in an aqueous solution), removed, and placed in the apparatus they may give a higher concentration effect than the dead cell wall treated as described above.

across the protoplasm: this is opposed by the vertical arrow at *A*. The arrow at *C* is longer than that at *A* since the higher concentration is at *C* (this would be the case theoretically if the cation has the greater tendency to enter or the greater mobility in the protoplasm) which is in accord with all our measurements.[9a] The resultant P.D. due to the protoplasm is shown by the arrow labelled *P* (*i.e.*, the current tends to flow from *C* through the protoplasm and sap to *A* and thence through the cell wall to *C*). (We neglect for the present any short circuit through the protoplasm.)

The relations are represented more simply in Fig. 4 where the resultant resistance of the protoplasm and sap is represented by R_P and that of the cell wall by R_O. The P.D. in the protoplasm is represented by E_{PA} and E_{PC}.

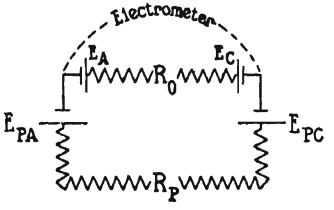


FIG. 4

FIG. 4. As in Fig. 3 except that the resultant resistance of the protoplasm and sap is represented by R_P (with one horizontal and two vertical components) and that of the cell wall by R_O (having only a horizontal component). The P.D. of the protoplasm is represented by E_{PA} and E_{PC}, that of the cell wall by E_A and E_C.

The relations in the cell wall may be inferred from the fact that when a strip of dead cell wall is used we find a positive current flowing through the electrometer from the dilute to the concentrated solution (which should be the case theoretically if the cation has a greater tendency to enter or a greater mobility than the anion). The P.D. is represented by arrows parallel to the surface (broken arrows) because the applied solution quickly penetrates through the wall (in a few seconds[11]) and then begins to diffuse along the wall, setting up the P.D. shown by the broken arrows in the figure: the resultant P.D. due to the cell wall is labelled *CW* and is opposite to that labelled *P*.

It is therefore evident that the concentration effect of the cell wall

[11] This is shown by the fact that substituting 0.01 M NaCl for 0.01 M KCl has practically no effect on the dead cell so that when such a change is made on the living cell we must regard the effect as due entirely to the protoplasm: the chief electrical disturbance due to such a change lasts only a few seconds during which time the solution must have penetrated through the wall to the protoplasm.

tends to increase the values due to the protoplasm. For example, in Fig. 4 (where the broken arrow at C is represented by E_C and that at A by E_A) we may write[12]

$$\text{Obs. conc. effect} = (E_{PC} - E_{PA}) \frac{R_O}{R_O + R_P} + (E_C - E_A) \frac{R_P}{R_O + R_P}$$

Here $E_{PC} - E_{PA}$ corresponds to the arrow labelled P in Fig. 3 and $E_C - E_A$ to that labelled CW. Since R_P is small[9a] compared to R_O the effect of the cell wall is probably small.

In order to eliminate the action of the cell wall and to ascertain the concentration effect due to the living protoplasm we may apply identical solutions to A and C, and kill C.[13] If, for example, we lead off at A and C with 0.01 M KCl we eliminate the effect of the cell wall but this gives no P.D. until we kill C (which reduces the E.M.F. of the protoplasm at C approximately to zero[14]). We make a photographic record on which we measure the P.D. immediately after the death of C to find the potential difference across the protoplasm at A (we shall call this the AP value).

In order to see what this means let us consider the factors which determine the P.D. We may assume that under normal conditions all the E.M.F. at A is balanced by corresponding E.M.F. at C so that if we lead off from A to C with the same solution the P.D. will be zero. When we change the salt solution at A it seems probable that its penetration is so slow that in our very brief experiments we make no change in the protoplasm except at the outer surface of X.

The electromotive force at the outer surface of X may be called E_{AX}, the corresponding E.M.F. at C being E_{CX} (the E.M.F. in the region lying between A and C, or outside these points, may be neglected for our present purpose). If the E.M.F. at the outer surface of X

[12] It is assumed that the resistance of the electrometer is sufficiently high to preclude any error in measurement and this is the case even when a grid leak is used to shunt the grid and filament (the resistance of the grid leak is 20 megohms).

[13] As shown in a former paper[9a] killing C usually produces no immediate effect at A, which can be detected by our present methods.

[14] The killing of C apparently produces no effect on the cell wall. There is, of course, some current flowing in the cell wall but the experiments of Dr. Blinks indicate that this produces no polarization.

in contact with 0.01 M KCl be called $E_{AX\ 0.01\ KCl}$ and all other E.M.F. at A be designated as E_{AZ} the total E.M.F. at A is $E_{AX\ 0.01\ KCl} + E_{AZ}$. What we measure is probably a certain fraction of this which we will call m,[15] so that the observed P.D. across the protoplasm at A is

$$\text{Observed P.D.} = m(E_{AX\ 0.01\ KCl} + E_{AZ}) = mE_{AX\ 0.01\ KCl} + mE_{AZ}$$

(The E.M.F. in the cell wall at A and C balances out since both spots are in contact with the same solution.[16])

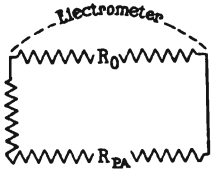


FIG. 5

FIG. 5. Hypothetical diagram of electrical resistances in the protoplasm when C is killed. R_O (with horizontal component only) represents the resistance of the cell wall and R_{PA} (with one vertical and one horizontal component) that of the protoplasm and sap.

When we substitute 0.001 M KCl for 0.01 M KCl at A and C we change $E_{AX\ 0.01\ KCl}$ to $E_{AX\ 0.001\ KCl}$ but in brief experiments we may assume that E_{AZ} will not change. If we subtract the observed P.D. across the protoplasm in contact with 0.01 M KCl from that across the protoplasm in contact with 0.001 M KCl we have

$$\text{Observed concentration effect} = mE_{AX\ 0.001\ KCl} - mE_{AX\ 0.01\ KCl}$$

The value of m depends on the resistance of the cell wall (R_O) between A and C and the resultant resistance (R_{PA}) of the protoplasm and sap in the circuit after C is killed. If we represent the resistances as shown in Fig. 5 we are measuring the P.D. across R_O and the value

[15] This has been discussed in a previous paper[9a] (where n was used in place of m).

[16] This would not be true if we did not employ a flowing contact at C for sap would come out through the dead protoplasm and change the concentration in the wall, unless the applied solution were sap or 0.05 M KCl or its equivalent. But the flowing contact appears to prevent any disturbance due to the coming out of sap as is shown in a former paper.[9a] Care must be taken to avoid any disturbances due to "flowing potential."

of m will therefore be $m = R_O \div (R_O + R_{PA})$. Measurements[17] made by Dr. Blinks indicate that when C is killed and the solution at A is 0.001 M KCl (or a higher concentration), with the cell wall between A and C imbibed with tap water, the values of R_O and R_{PA} are such that m is between 0.8 and 1.0.

In order to get some idea of the change in resistance due to killing C we apply 0.01 M NaCl to A and 0.01 M KCl to C (without killing A

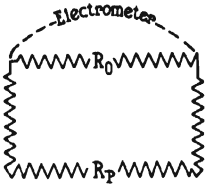


FIG. 6

FIG. 6. As in Fig. 5 but with both ends intact (R_P in place of R_{PA}).

or C). Since $E_{CX\ 0.01\ KCL} = E_{AX\ 0.01\ KCL}$ the observed effect (which will be called the chemical effect) may be regarded as

$$\text{Obs. chem. effect} = nE_{AX\ 0.01\ NaCl} - nE_{CX\ 0.01\ KCl} = nE_{AX\ 0.01\ NaCl} - nE_{AX\ 0.01\ KCl}$$

where $n = R_O \div (R_O + R_P)$ and R_P represents the resultant resistance of the protoplasm and sap in the circuit in the intact cell as indicated in Fig. 6. (The P.D. of the cell wall may be neglected since the cell wall alone gives no chemical effect.)

If we now kill C and ascertain the chemical effect (in the same manner as for the concentration effect, *i.e.*, by measuring the AP value at A of 0.01 M NaCl and then that of 0.01 M KCl) we shall have two measurements, *i.e.*, $m(E_{AX\ 0.01\ NaCl} - E_{AX\ 0.01\ KCl})$ and $n(E_{AX\ 0.01\ NaCl} - E_{AX\ 0.01\ KCl})$. From these measurements we find that the value of $m \div n$ is not far from 1 and direct measurements of resistance made by Dr. Blinks indicate that the value of n is not much

[17] We regard R_{PA} and R_O as resistances in parallel whose resultant resistance is equal to $\frac{(R_{PA})\,(R_O)}{R_{PA} + R_O}$ which is measured by determining the resistance of the circuit through A and C after C has been killed. We then measure an equal length of cell wall blown up with air (after the protoplasm has been squeezed out) to obtain R_O. From this we obtain R_{PA}.

below that of m. In other words we compare the values of $m = \frac{R_0}{R_0 + R_{PA}}$ and $n = \frac{R_0}{R_0 + R_P}$ and find that the difference between R_P (the resistance of the protoplasm and sap) and R_{PA} (the same resistance after killing C) is not enough to have any great effect on the values of m and n: this is, of course, due to the very high value of R_0 (according to the measurements of Dr. Blinks the resistance of the cell wall, imbibed with tap water, is considerably over 4 megohms per inch).

These results are checked by applying 0.001 M KCl at A and the same saturated with chloroform at B (Fig. 7): at the same time we

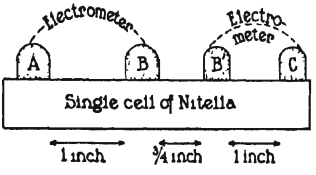


FIG. 7. Diagram to show arrangement of experiments

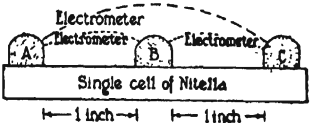


FIG. 8. Diagram to show arrangement of experiments

apply 0.01 M KCl at B' and the same saturated with chloroform at C (Fig. 7). We thus measure simultaneously the AP value of the two concentrations and by subtracting one from the other obtain the concentration effect. In this case we eliminate both the cell wall effect and errors due to the use of different cells but even so there may be considerable variation. The results agree with those previously described.

Still another method is to arrange the experiments as in Fig. 8 and to kill C with 0.01 M KCl saturated with chloroform. We apply 0.01 M KCl at B and 0.001 M KCl at A. Measuring from A to B gives us the concentration effect of the protoplasm on the n basis together with the cell wall effect: we may call this the nw basis. We

may get the *m* basis by measuring from *B* to *C* and subsequently replacing the 0.01 M KCl at *B* and *C* with 0.001 M KCl. We are thus able to measure a single cell on the *m* and on the *nw* basis and this may also be done for the chemical effect (*n* basis) to compare the values of *m* and *n*. Here too the results are similar to those already described.

It may be added that when the cell wall is imbibed with 0.001 M KCl and we place 0.01 M KCl at *C* and 0.001 M KCl at *A* the results

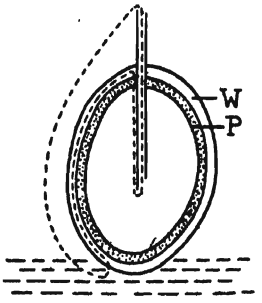


FIG. 9. Diagram to show the electrical leak when a capillary is inserted into *Valonia*

are practically the same as when the cell wall is imbibed with tap water.

The matter may be approached from another standpoint, *i.e.*, by inserting a capillary. It has been shown in a former paper[18] that when a capillary is inserted into *Valonia* (Fig. 9) we may have a

[18] Osterhout, W. J. V., Damon, E. B., and Jacques, A. G., *J. Gen. Physiol.*, 1927–28, xi, 193. The use of the capillary in *Valonia* was begun by one of us in the summer of 1923 (for the first reference to this see Osterhout, W. J. V., *J. Gen. Physiol.*, 1924–25, vii, 561). In the summer of 1925 Taylor and Whitaker (Taylor, C. V., and Whitaker, D. M., Carnegie Institution Year Book, 1925–26, No. 25, p. 248) introduced a microelectrode and recently Jost (Jost, L., *Sitzungsber. Heidelberger Akad. Wissensch.*, 1927, Abteilung 13, Nov.) has employed a capillary.

The use of the capillary in *Nitella* was begun by us in 1922 (cf. Osterhout, W. J. V., and Harris, E. S., *J. Gen. Physiol.*, 1927–28, xi, 391). Previous to this Miss Nichols (Nichols, S. P., *Bull. Torrey Bot. Club*, 1925, lii, 351) introduced needles to observe the effect upon structure and upon streaming. Recently Taylor and Whitaker (Taylor, C. V., and Whitaker, D. M., *Protoplasma*, 1927, iii, 1) and Brooks and Gelfan[8] have employed a microelectrode.

leak at the point of insertion but if we wait for a seal to form this leak disappears and we then may get the true value of the P.D. across the protoplasm. In the case of *Nitella* it is not necessary to wait for a seal to form since the resistance of the cell wall imbibed with tap water is so high that the leak is not important. Hence we may approximate the true values by measuring as soon as the capillary is inserted.

For this purpose the cell is placed on the paraffin block on the stage of a microscope and the capillary (filled with artificial sap) is held firmly in place near the end of the cell. We then seize with forceps a strip of dead cell wall adhering to the

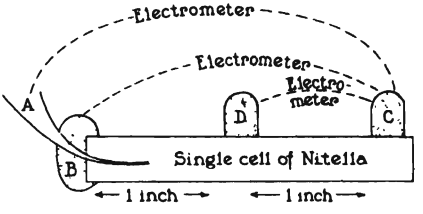


FIG. 10. Diagram to show the arrangement of the experiment when a capillary (*A*) is inserted in the cell

end of the living cell (this strip is a part of the neighboring cell whose end has been cut away) and pull the living cell along until it impales itself on the capillary. One can then observe with the microscope whether the capillary is projecting far enough into the vacuole to indicate that its point is not covered by a layer of protoplasm.

An experiment was arranged as shown in Fig. 10. By means of a rotary switch[19] we recorded the circuit from the interior of the capillary *A* to *C* (Fig. 11, Curve *A*, long dash): from the outside of the capillary where it pierced the cell wall, *B*, to *C* (Curve *B*, medium dash) and from *D* to *C* (Curve *D*, short dash). If such an experiment were performed on *Valonia* (Fig. 9) there would be a good deal of difference between *A* and *B* provided we had allowed the cell to form an electrical seal but in the case of *Nitella* no such seal is formed as the cell is used immediately after the insertion of the capillary and in consequence there is little difference between *A* and *B*.

[19] This was described in a previous paper (Osterhout, W. J. V., and Harris, E. S., *J. Gen. Physiol.*, 1927–28, xi, 391).

The values observed at *A* and *B* (Fig. 11) are a measure of the P.D. across the protoplasm at *C* (*AP* value) as would be the case if we killed with chloroform at *B*. (This *AP* value is negative but appears positive on the record because curves *A* and *B* record the inside of *C* instead of the outside.) The fact that *A* and *B* differ somewhat might be taken to indicate that the point of the capillary has gone through the protoplasm into the vacuole. If it had merely indented the protoplasm, making a deep pocket, without actually piercing it we might expect *A* and *B* to be identical.

The short dash, Curve *D*, records the P.D. of *D* against *C* (which is about 1 millivolt negative); the fact that there is so little differ-

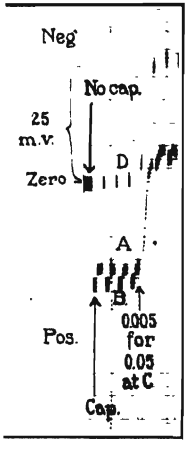


FIG. 11

FIG. 11. Photographic record showing potential differences. The experiment is arranged as shown in Fig. 10 with 0.05 M KCl at *B*, *D*, and *C*. Before the capillary is inserted there is only one curve (marked "No cap."). As soon as the capillary is inserted the record shows three curves: Curve *A* records the potential difference between the capillary and *C*; Curve *B* that between *B* and *C*; and Curve *D* that between *D* and *C*. When 0.005 M KCl is substituted for 0.05 M KCl at *C* the curves rise together. The vertical marks represent 5-second intervals.

ence between *D* and *C* indicates that injury has not spread from the point of entrance of the capillary to *D* (for when *D* is dead its apparent P.D. will be the same as that of the capillary).

When 0.005 M KCl is substituted for 0.05 M KCl at *C* both curves rise simultaneously (since *C* becomes more positive which makes both the capillary and *B* appear more negative) to a degree which indicates that *C* is not injured. The amount of this rise is the observed concentration effect which is measured on the equivalent of the *m* basis from *A* to *C* and on the *nw* basis from *D* to *C*. There is evidently no great difference between the two and this might be taken to indicate that the cell wall effect does not greatly alter the value due to the

protoplasm alone. For when we change from 0.05 to 0.005 M KCl at *C* we presumably have a cell wall effect when we measure from *D* to *C* but not when we measure from *A* to *C* or from *B* to *C*, yet the measurement (*i.e.*, the amount of rise) is practically the same in all cases.

Let us now consider certain other aspects of the experiments on *Nitella*. It might be thought that killing *C* tends to alter the concentration effect at *A* (as would be expected if *A* were injured). Hence experiments were made on intact cells having unlike concentrations at *A* and *C* (*i.e.*, on the *nw* basis). The average measurements of 130 such cells (Fig. 2, Curve *nw*) do not agree closely with those made on the *m* basis (58 cells, Fig. 2, Curve *m*), but this lack of agreement seems to have no theoretical significance. On the basis of Figs. 3 and 4 we might expect the values on the *m* basis to be approximately equal to those on the *nw* basis minus the cell wall effect. While this may be approximately true for the value of 0.01 *vs.* 0.1 M KCl it is just the reverse for the value of 0.01 *vs.* 0.001 M KCl. In view of this we are not inclined to attribute much quantitative significance to any of these figures.[20]

Experiments in which *C* was killed (with 0.01 M KCl plus chloroform) and kept in contact with 0.01 M KCl while the solution in contact with *A* was varied (which may be called the *mw* basis, since it includes the effect of the cell wall) gave results much like those in Curve *m*, indicating that the cell wall effect does not greatly affect the results (*i.e.*, curves on the *m* basis do not differ greatly from those on the *mw* basis).

The curves in Fig. 2 are concave to the axis of the abscissæ. Somewhat the same sort of curve was found by Beutner[3] in studying organic substances immiscible with water,[21] although theoretical considerations (to be discussed a little later) lead us to expect in the ideal case the straight line labelled "theoretical" in Fig. 2.

[20] We do not know to what extent the values are cut down by short circuits and eddy currents: for example, the current due to the broken arrow at *C*, Fig. 3, might travel in the cell wall only a short distance toward *A* before passing into the protoplasm.

[21] This is discussed by Michaelis, L., Hydrogen ion concentration, Baltimore, 1926, 200.

In addition to the results shown in Fig. 2 there are a few anomalous cases which should be mentioned. Under certain circumstances the cell may produce a high P.D. (up to 120 millivolts) even with identical solutions at A and C. It is not surprising therefore that in certain kinds of material we find a concentration effect of 100 millivolts or more for a tenfold dilution (*e.g.*, 0.001 M *vs.* 0.01 M KCl). Since the theoretical limit for a tenfold dilution is about 60 millivolts it is natural to assume that in these anomalous cases the extra P.D. comes from the deeper layers, Y or W.

In view of such results as those of Michaelis and Fujita[22] on gelatin and of Amberson and Klein[23] on dead frog skin, which show a change of sign of the concentration effect with alteration of pH value, it may be well to state that no such change is found in living cells of *Nitella* if in the range studied (pH 5 to 9.5) the concentration of H^+ is small in comparison to that of other cations.

In view of the fact that organic substances immiscible with water containing organic acids show a concentration effect with KCl with the dilute solution positive while others containing alkalies show the dilute solution to be negative, it may be well to state that somewhat similar results may be obtained by means of a string soaked in acid or alkali, the result being easily accounted for as diffusion potential.

Let us now consider the interpretation or our results. If we regard the P.D. as due primarily to diffusion potential we shall conclude that the mobility of K^+ in the protoplasmic surface is greater than that of Cl^-. But if it is due to phase boundary potentials we may employ the usual formula, putting $R' = \dfrac{R}{0.4343\ n\ F}$ and $n = 1$

$$\text{P.D.} = R'T \log \left(\frac{C_{coh}}{C_{czh}}\right)\left(\frac{C_{czl}}{C_{col}}\right)$$

where C_{czh} and C_{czl} are the concentrations of the cations in X at the high and low concentrations respectively and C_{coh} and C_{col} are the corresponding concentrations in the external solution. The sign is that of the dilute solution.

[22] Michaelis, L., and Fujita, A., *Biochem. Z.*, 1925, clxii, 245.

[23] Amberson, W. R., and Klein, H., *J. Gen. Physiol.*, 1927–28, xi, 823.

If $C_{cxh} = C_{cxl}$ we have

$$\text{P.D.} = R'T \log \frac{C_{coh}}{C_{col}}$$

In metallic electrodes (and in some cases in glass electrodes[24]) this equation applies but it is a question to what extent this is true of protoplasm.[24]

In order to picture the relations involved it may be convenient to employ the treatment of Nernst, as discussed in a former paper,[1] where it is stated that if, for example, we apply equimolar solutions of RbCl and CsCl we may write

$$\text{P.D.} = \frac{R'T}{2} \log \frac{A_{Cs}}{A_{Rb}}$$

where A_{Cs} and A_{Rb} are the "true"[25] partition coefficients of Cs and Rb. The sign is that of the solution of Rb as shown by the electrometer. If we use two salts without a common ion, *e.g.*, equimolar solutions of RbCl and CsBr, we may take first the phase boundary potential at the spot in contact with RbCl: this is[26]

$$\text{P.D.} = \frac{R'T}{2} \log \frac{A_{Cl}}{A_{Rb}}$$

the positive sign meaning that the positive current tends to flow from Y to X in the protoplasm.[27] The corresponding value for CsBr would be

$$\text{P.D.} = \frac{R'T}{2} \log \frac{A_{Br}}{A_{Cs}}$$

---

[24] This has been discussed in a former paper.[1]

[25] The true partition coefficient of Rb is the partition coefficient (in this case the concentration at equilibrium in the protoplasmic layer X divided by the concentration in the external solution) it would have if it could enter unhindered by Cl, or in other words if Rb and Cl had the same "true" partition coefficient.

[26] For a discussion of this see[1].

[27] This is adopted as a convention.

Subtracting one from the other we have

$$\text{P.D.} = \frac{R'T}{2} \log \left(\frac{A_{\text{Cl}}}{A_{\text{Rb}}}\right)\left(\frac{A_{\text{Cs}}}{A_{\text{Br}}}\right)$$

The sign is that of the solution of RbCl, as shown by the electrometer.

We may calculate the concentration effect in similar fashion. If the "true" partition coefficient of the cation is A_{cl} at the low concentration and A_{ch} at the high concentration (the corresponding symbols for the anion being A_{al} and A_{ah}) we may for purposes of illustration make the following substitutions in the last equation: $A_{cl} = A_{\text{Rb}}$, $A_{ch} = A_{\text{Cs}}$, $A_{al} = A_{\text{Cl}}$, and $A_{ah} = A_{\text{Br}}$. We then have

$$\text{P.D.} = \frac{R'T}{2} \log \left(\frac{A_{al}}{A_{cl}}\right)\left(\frac{A_{ch}}{A_{ah}}\right)$$

The sign is that of the dilute solution as shown by the electrometer. It is evident from this equation that in order to get a concentration effect there must be a difference between the "true" partition coefficients of either the cations or of the anions at the two concentrations.

It may be of interest to see how variation of "true" partition coefficients will affect the sign of the dilute solution. If A_c and A_a, as well as $A_c \div A_a$, increase as the concentration increases the tendency of the cations to pass into X will always be greater at the higher concentration so that the positive current will tend to flow from the lower concentration through the electrometer to the higher (dilute solution positive) and at the same time relatively more of the salt will enter at the higher concentration. This would correspond to those cases described by Wosnessensky[28] in experiments on amyl alcohol (*e.g.*, the concentration effect of LiCl) where the dilute solution is positive.[29]

[28] Wosnessensky, S., *Z. physik. Chem.*, 1925, cxv, 405; cxvii, 457. Wosnessensky, S., and Astachow, K., *Z. physik. Chem.*, 1925, cxviii, 295; 1927, cxxviii, 362. Wosnessensky, S., Astachow, K., and Tschmutow, K., *Z. physik. Chem.*, 1926, cxxi, 143.

[29] In comparing two salts it must be borne in mind that if one is taken up more than the other it does not necessarily mean that in the former case there are more ions in X since the dissociation constants in X may differ. In our discussion we shall assume that the dissociation constants are not very different.

But if $A_c \div A_a$ and A_c increase with concentration while A_a decreases as the concentration increases the dilute solution will still be positive although relatively less salt will be taken up at the higher concentration and this may be true even if both A_c and A_a decrease as the concentration increases.

If on the other hand $A_c \div A_a$ decreases as the concentration increases the dilute solution will be negative even though A_a increases as the concentration increases or though both A_c and A_a increase as the concentration increases (in the latter case the penetration of salt increases as the concentration increases).

We therefore see that on this basis the sign of the dilute solution depends on the behavior of $A_c \div A_a$ and not on the relative amount of salt taken up.

According to recent researches[30] and theoretical discussions[31] little or no P.D. is to be expected with KCl at phase boundaries. On the other hand Michaelis and others[32] find fairly high values for the P.D. of KCl with collodion membranes. This might lead to the suspicion that the results found with *Nitella* are largely due to the cell wall but it is evident from what has been said that this is not the case. The concentration effect of the protoplasm is much greater than that of the cell wall and this difference is much more striking in the case of the chemical effect. It should be noted that collodion membranes give the same sort of chemical effect with KCl and NaCl as does protoplasm.

There are difficulties in regarding the P.D. as due to Donnan effect, since the cell cannot very well be in Donnan equilibrium with two different concentrations at the same time. We must assume that the indiffusible ion is positive in order to make the dilute solution positive. The numerical relations may be illustrated as follows: If we have inside an indiffusible anion $R^- = 21$ and outside KCl = 10 we have $(K^+_{outside})\ (Cl^-_{outside}) = 100$: inside the product of K^+ (= 25) and

[30] *Cf.* Holleman, L. W. J., and Werre, J. P., *Rec. trav. chim. Pays-Bas*, 1928, ser. 4, xlvii, 105.

[31] *Cf.* Cremer, M., in Bethe, A., *et al.*, Handbuch der normalen und pathologischen Physiologie, Berlin 1928, viii, 1034.

[32] *Cf.* Michaelis, L., and others. Various articles in *J. Gen. Physiol.*, 1925–29, ix–xii.

Cl^- (= 4) is also 100. If we now raise the outside concentration to 100 we have outside (K^+) (Cl^-) = 10,000 and inside the product of K^+ (= 111.1) and Cl^- (= 90.1) is also 10,000 (R^- being 21 as before). In the former case the value of $\frac{C_{co}}{C_{ci}}$ is $\frac{10}{25}$ and in the latter $\frac{100}{111.1}$. We then employ the usual formula

$$\text{P.D.} = R'T \log \frac{C_{co}}{C_{ci}}$$

(where C_{co} is the concentration of cations outside and C_{ci} the concentration inside) which would give for the lower concentration P.D. $= R'T \log \frac{25}{10}$ and for the higher concentration P.D. $= R'T \log \frac{111.1}{100}$ so that the concentration effect would be

$$\text{P.D.} = R'T \log \left(\frac{25}{10} \div \frac{111.1}{100} \right) = R'T \log 2.25$$

The dilute solution would be positive but the P.D. would be less than with a metallic electrode where the concentration effect would be $R'T$ log 10 (which at 18°C. would be 58 millivolts). Hence the assumption of a Donnan equilibrium as the explanation of the P.D. may not give values as high as occur in dilute solutions in the case of living cells (whose values approximate those found with metallic electrodes). In order to get values approaching those for metallic electrodes we should have to assume values for R^- more than 10 times as great as those given above.

It is evident that as the more dilute solution of KCl is positive with living protoplasm K^+ tends to enter and the value of $A_c \div A_a$ must increase with the concentration or else the mobility of K in the protoplasm must be greater than that of Cl^-.

There is no direct proof that Cl^- tends to penetrate[33] in ionic form.

Let us now consider the bearing of our results upon electrical conditions in the protoplasm. Adopting the hypotheses set forth in a former paper[1, 9a] that the protoplasm consists of layers with different

[33] It may reach the vacuole by passing through the non-aqueous layers of the protoplasm in the undissociated condition.

properties we might diagram the electrical conditions as shown in Fig. 12. The X and Y arrows marked A and C under "Net result" at the left have had their directions determined by considerations set forth in a former paper;[9a] and this is confirmed by the fact that when sap is applied to the outer surface of X (as at the right) the result shows that the X arrow (X^c) is longer than the Y arrow (Y_c).[9a] This might be regarded as indicating that X gives a greater P.D. with sap than does Y in which case we might make the arrow at the external surface of X longer than that at the inner surface of Y, as is done in the figure: we should in that case be inclined to suppose that X gives a greater P.D. against W than does Y and to make the arrows at the outer and inner surfaces of W of corresponding length (as in the figure). The arrows at b, c, and d will be of the same length at A as at C no matter what the external solutions may be, since in

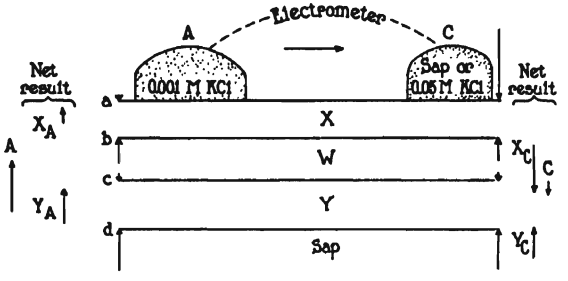


FIG. 12. Hypothetical diagram to show the condition of the protoplasm in contact with different concentrations of KCl. The direction of the arrows indicates how the positive current tends to flow and their length the relative P.D.

brief experiments such as we perform the arrow at a is the only one to change, except in case of injury.

If the electrolyte in W should happen to be mostly KCl the fact that with the protoplasm in contact with 0.001 M KCl the arrow at a is shorter than that at b would mean that the concentration of KCl in W was greater than 0.001 M. But if the electromotive forces at b and c are chiefly due to the diffusion potential, for example of an organic substance produced in the protoplasm, we cannot draw any conclusions regarding the concentration of inorganic salts in W.

If we suppose that X and Y are non-aqueous layers and that W is aqueous we may surmise that each surface is the seat of electromotive

forces as shown in Fig. 12. When we apply 0.001 M KCl at A and 0.1 M KCl at C it is probable that under normal conditions the E.M.F. at A and C is equal and opposite except those at the outer surface which are represented in the diagram by arrows of unequal length. If we regard the observed effects as due to diffusion potentials we should say that the cations appear to have a greater mobility in X than the anions and consequently we should expect the arrows at the outer surface of X to be directed inward, as shown in Fig. 12. The magnitude of the E.M.F. would increase with the concentration, hence the outermost arrow should be longer at C than at A and the current should flow through the electrometer from A to C (as shown by the horizontal arrow in Fig. 12). This is in accord with the experimental results which show that the dilute solution is positive not only with KCl but with many other salts. To judge from the literature this is the usual condition[34] (but it does not seem to be the case with *Valonia*).

It follows that if we lower the concentration of the solution at A we shorten the length of the arrow at a and if we could make it equal to that at b we should have $a - b = 0$ and we could then learn the value of $c - d$. Whether we can tell when such a state is reached must be decided by future investigation.

The study of the concentration effect may be of value as throwing light on the nature of protoplasm. According to some authors a salt may give no concentration effect with certain substances immiscible with water[28] but with others[35, 36] the dilute solution appears positive and with still others negative. If this be the case we may hope by such studies to gain some idea of the nature of the substance constituting the protoplasmic surface.

SUMMARY

A method distinguishing between the concentration effect due to the cell wall and that due to the protoplasm is described: the importance

[34] *Cf.* Höber, R., Physikalische Chemie der Zelle und der Gewebe, Leipsic, 6th edition, 1926.

[35] *Cf. e.g.* Beutner, R., Die Entstehung elektrischer Ströme in lebenden Geweben, Stuttgart, 1920.

[36] *Cf.* Cremer, M., in Bethe, A., *et al.*, Handbuch der normalen und pathologischen Physiologie, Berlin 1928, viii, 999.

of this lies in the fact that if the protoplasm shows a concentration effect one or both ions of the salt must tend to enter its outer surface.

Studies on the concentration effect of KCl with living protoplasm of *Nitella* show that when P.D. is plotted as ordinates and the logarithm of concentration as abscissæ the graph is not the straight line demanded in the ideal case by theory but has less slope and is somewhat concave to the axis of the abscissæ.

With a variety of salts the dilute solution is positive, which indicates that the cation has a greater mobility in the protoplasm than the anion or that the partition coefficient of the cation (A_c) increases faster than that of the anion (A_a) as the concentration increases. If the result depended on the partition coefficients we should say that when $A_c \div A_a$ increases with concentration the dilute solution is positive. When $A_c \div A_a$ decreases as the concentration increases the dilute solution is negative. In either case the increase in concentration may be accompanied by an increase or by a decrease in the relative amount of salt taken up. Theoretically therefore there need be no relation between the sign of the dilute solution and the relative amount of salt taken up with increasing concentration.

Hypothetical diagrams of the electrical conditions in the cell are given.

If we define the chemical effect as the P.D. observed in leading off at two points with equivalent concentrations of different salts we may say that the chemical effect of the protoplasm is very much greater than that of the cell wall.

[Reprinted from THE JOURNAL OF GENERAL PHYSIOLOGY, July 20, 1929,
Vol. 12, No. 6, pp. 805-811]

A GLASS ELECTRODE APPARATUS FOR MEASURING THE pH VALUES OF VERY SMALL VOLUMES OF SOLUTION

BY D. A. MACINNES AND MALCOLM DOLE

(From the Laboratories of The Rockefeller Institute for Medical Research)

(Accepted for publication, May 11, 1929)

In a recent article[1] the authors have described a new type of glass electrode, which is of convenient size, and at the same time of sufficiently low resistance for accurate work. Such an electrode is shown diagrammatically on the right-hand side of Fig. 1. The lower end of glass tube *A* supports the glass diaphragm *D* which in our experiments has been about 0.001 mm. thick. The tube *A* is partly filled with 0.1 N HCl into which is inserted the silver-silver chloride electrode *B*. More details concerning these electrodes are given in the article referred to above.[2]

Since glass electrodes of this type with glass diaphragms 4 mm. or less in diameter can be used, they seemed admirably adapted to measurements involving very small amounts of material. The need for such measurements arises frequently, especially in biological investigations. The apparatus shown diagrammatically in Fig. 1 was therefore designed to adapt the electrodes to this purpose. The vessel *C* holds a reference saturated calomel electrode. This electrode is connected, through a stopcock, with the reservoir *R* which contains

[1] MacInnes, D. A., and Dole, M., *J. Ind. Eng. Chem.* (Analytical Edition), 1929, i, 57. This paper contains references to the previous work with glass electrodes, with the exception of a recent article by Mirsky and Anson (Mirsky, A. E., and Anson, M. L., *J. Biol. Chem.*, 1929, lxxxi, 581.

[2] Through the kindness of Dr. Alexis Carrel we have tested the utility of these electrodes in connection with tissue culture work. The usual vessel for that work was provided with three outlet arms. One arm contained a tube through which the medium could be drawn to make contact with a saturated calomel electrode, and the other two arms held glass electrodes. One glass electrode was kept in the medium and the other in the immediate vicinity of the tissue studied. In this way it was possible to follow accurately the change of pH with time of the tissue and the medium separately.

a supply of saturated KCl solution. Another branch of the tubing connects with the tip *T*. An additional branch tube *F* is closed by a piece of rubber tubing (a so-called "policeman") on which a screw pinchcock is placed. The whole apparatus is mounted on adjustable screw clamps, as shown in Fig. 2, so that the glass electrode and the calomel electrode with its attachments can be independently raised and lowered. The clamp holding the glass electrode is insulated from the rest of the support with a piece of Bakelite.

To make a pH determination the following steps are necessary. The rubber tube on *F* is compressed by the pinchcock. The stopcock below the reservoir

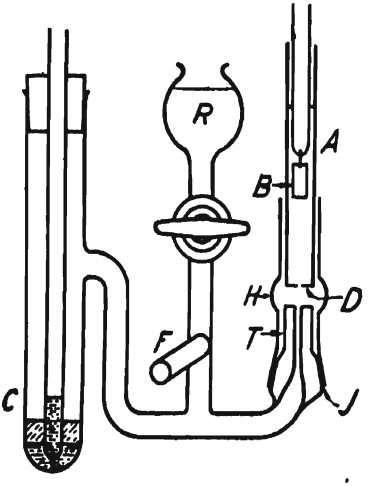


FIG. 1. Diagram of apparatus

R is then opened slightly and saturated KCl is run out on the tip *T*. With a piece of filter paper this solution is removed from the tip so that the solution lies in the capillary and flush with the surface. A drop of the solution whose pH is desired is then placed on the tip. By loosening the pinchcock on *F* slightly this drop is drawn into the capillary tube so that the liquid junction is lowered a few millimeters below the surface of the tip. The remainder of the drop is then removed with filter paper. This procedure has the effect of removing any KCl-bearing solution from the tip. Another drop of the solution under observation is then added, and the protecting tube *H* is put in place as shown. The glass electrode is next lowered, by means of the screw adjustment, until the glass diaphragm *D* comes in contact with the drop. An E.M.F. measurement may then be made with an electrometer and potentiometer.

It is necessary to put a thin coating of paraffin on the tubing up to the edge of

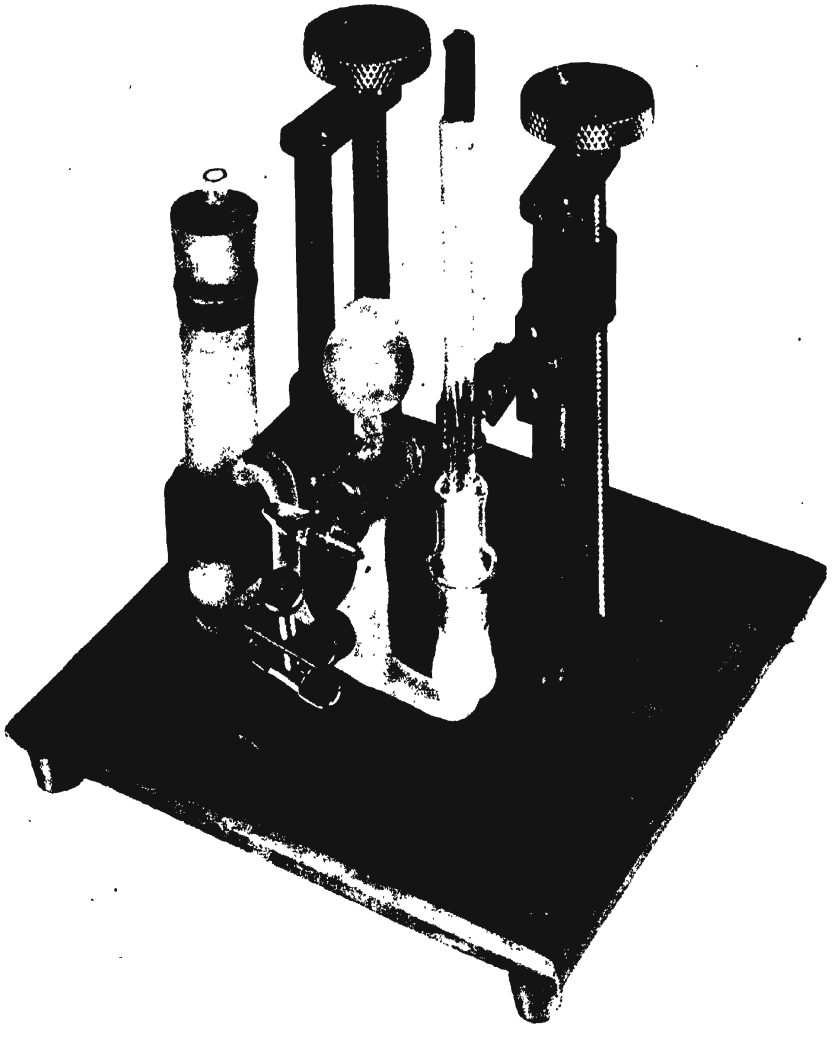


FIG. 2. Glass electrode apparatus for measuring the pH of very small volumes of solution

the tip T to prevent the drop of solution from spreading. A similar coating around the lower edge of the glass electrode is also desirable for the same reason. The protecting tube H was added to the apparatus after finding that the potentials observed were unsteady and drifting due to evaporation from the edge of the drop. It is important to have a close fitting ground joint at J so that rising air currents are prevented.

Our measurements have been made in a constant temperature room, at 25°, with a "Type K" Leeds and Northrup potentiometer, using a Compton electrometer, made by the Cambridge Scientific Instrument Co., as a null instrument. Readings could be made to about 0.2 millivolt. Due to the relatively low resistances of the glass electrodes (about 10 megohms in our most recent measurements) little screening of the electrical system was found necessary. For the same reason ordinary care in insulation was ample. The connections from the potentiometer to the electrometer were made with lead-screened wire.

The formula to be used in computing pH values from the measurements at 25°C. with the apparatus as described is

$$\text{pH} = \frac{E + G + 0.1066}{0.05915}$$

in which E is the measured potential, and G the potential (to be discussed below) which may be present in the glass. The constant 0.1066 can be obtained as follows. The potential of the system

$$\text{Hg} \mid \text{HgCl, sat'd KCl} \parallel 0.1\ \text{N HCl} \mid \text{glass} \mid 0.1\ \text{N HCl, AgCl} \mid \text{Ag} \qquad \text{(A)}$$

will be the same as the combination

$$\underset{\longleftarrow\text{B}\longrightarrow}{\text{Hg} \mid \text{HgCl, sat'd KCl} \parallel 0.1\ \text{N HCl} \mid \text{H}_2} \; - \; \underset{\longleftarrow\text{C}\longrightarrow}{\text{H}_2 \mid 0.1\ \text{N HCl, AgCl} \mid \text{Ag}}$$

if the glass acts as a hydrogen electrode and is the source of no other potential. The potential of the cell B is,[3] at 25°,

$$E = 0.2458 - 0.05915 \log a_H$$

in which a_H is the activity of the hydrogen ion in 0.1 N HCl. The cell C has the potential −0.3524.[4] The total potential is therefore

$$E = 0.2458 - 0.3524 - 0.05915 \log a_H \qquad \text{(D)}$$

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[3] Clark, W. M., The determination of hydrogen ions, Baltimore, 3rd edition, 1928, p. 672.

[4] From the work of Scatchard, G., *J. Am. Chem. Soc.*, 1925, xlvii, 641, involving a slight interpolation.

Now if we raise the hydrogen ion activity of the acid solution nearest the calomel electrode in the cell A to unity a potential will arise at the glass surface equal to

$$E = +0.05915 \log a_H \qquad (E)$$

so that the new potential will be equal to the sum of D and E or

$$E_o = 0.2458 - 0.3524 = -0.1066$$

which is the constant in the equation

$$E = E_o - 0.05915 \log a_H = E_o + 0.05915 \text{ pH}$$

or the potential of the cell A when the hydrogen ion activity between the glass and the saturated KCl solution is unity.

The potential G in the glass may be determined by placing the glass electrode (with the solution and silver-silver chloride electrode) in a beaker containing 0.1 N HCl and another silver-silver chloride electrode. In our more recent work we have used glass of a composition which gives membranes in which this potential is nearly zero. This and other information we have obtained concerning the relation of the composition of glass to its behavior when made into electrodes will be published elsewhere.

A number of tests were made to see whether the apparatus as described would yield correct pH values. For this purpose four glass electrodes were used with a buffer solution of pH 7.76, as determined by the hydrogen electrode. The results are shown in Table I.

TABLE I

Electrode	Date	pH
1	Apr. 24	7.84
2	" 24	7.74
3	" 24	7.77
3	" 24	7.75
3	" 25	7.76
4	" 25	7.76
1	" 25	7.76
1	May 2	7.74

Except for Electrode 1 (which initially gave an error of 0.08 pH unit) the measurements are all within 0.02 unit (about 1.2 millivolts).

Extensive tests (not made with this apparatus) have shown that

electrodes, made with the glass we have found most suitable, begin to deviate 0.02 pH unit from the correct values at pH 9.5 and show rapidly increasing deviations at higher pH values, if the solution measured is 0.1 N in sodium ion. The deviations begin at lower pH values if the sodium ion concentration is greater. In alkaline solutions the potentials may be dependent on the nature of the positive ions present and may vary with time. A more complete description of these tests will appear in another article.

An opportunity for testing the usefulness of this apparatus arose in connection with the work of Dr. Marian Irwin of this Institute. An important question to be investigated was whether the penetration of a basic dye changes the pH value of the vacuolar sap of living cells of *Nitella*. A few drops only of this sap can be conveniently obtained at one time. The question could not be settled by means of the hydrogen electrode since it is "poisoned" by the sap, and by the dye, brilliant cresyl blue, which was used. Such poisonings, which are, in many cases at least, due to irreversible oxidation-reduction potentials, do not appear to have any effect on the glass electrode.[5] Furthermore, the use of hydrogen gas would affect the concentration of CO_2 on which the pH value partly depends. The use of indicators with these dye solutions is obviously impossible. By employing the apparatus described above on samples of the sap prepared by Dr. Irwin it was shown that the entrance of the dye raises its pH value considerably. The results of these experiments will be fully described elsewhere.

[5] So far as we have been able to test the glass electrodes they seem, up at least to pH 9, in dilute salt solutions, to react only to changes in hydrogen ion activity. For instance, the paper referred to (1) gives data on the electrometric titration of sulfuric acid in the presence of potassium permanganate. The potentials followed the course to be expected from the change of hydrogen ion activity and were uninfluenced by the strong oxidation potential of the permanganate. On the other hand, the glass electrodes showed no change in potential when ferrous sulfate was titrated with potassium dichromate in the presence of an excess of sulfuric acid. In this case the hydrogen ion concentration remained substantially constant during the titration although there was a change in an oxidation-reduction potential of roughly 0.3 volt. We have other evidence, bearing on this matter, which cannot be conveniently summarized and will be published later.

SUMMARY

A glass electrode apparatus is described with which pH measurements can be made with as small volumes as 2 drops (about 0.14 cc.) of solution.

Using this apparatus the change of pH of the vacuolar sap of *Nitella*, due to the penetration of brilliant cresyl blue, has been readily followed. The sap and the dye have been found to poison the usual type of hydrogen electrode.

[Reprinted from THE JOURNAL OF THE OPTICAL SOCIETY OF AMERICA AND REVIEW OF SCIENTIFIC INSTRUMENTS, July, 1929, Vol. 19, No. 1, pp. 50–56]

AN IMPROVED CONSTANT CURRENT REGULATOR

BY LEWIS G. LONGSWORTH* AND DUNCAN A. MACINNES

(From the Laboratories of The Rockefeller Institute for Medical Research)

A constant current regulator for use in connection with the determination of transference numbers of ions of electrolytes by the moving boundary method has been described by MacInnes, Cowperthwaite, and Blanchard.[1] The moving boundary involved is at the junction of two solutions of electrolytes having a common ion. The leading solution contains ions having higher mobilities than those of the following solution. Such a boundary can, for instance, be formed between solutions of potassium chloride and lithium chloride. When current is passed the boundary moves and the resistance of the system steadily increases. The constant current regulator described in the paper referred to was limited to adjustments of the current for variations in only one direction, which met the requirements of the method. In constructing a new apparatus it was decided to remove this limitation in order to adapt the apparatus for general use. It was found that a simple additional device, to be described below, was sufficient to accomplish this result.

The apparatus is outlined diagrammatically in Fig. 1. The cell, through which the constant current is to pass, is connected to the leads at the point C. Current for the cell is furnished by the battery of storage cells $A-B$. Part of the cells are shunted by the resistance R which is provided with a screw adjustment for the contact T. This adjustable resistance may also be placed in series with the battery. The current through the cell is measured by determining the drop of potential across the constant resistance R' by means of the potentiometer P. The galvanometer G used in connection with the potentiometer is of the portable type, indicating with a needle. In our work this needle is provided with a vane V of aluminum foil. The light

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[1] MacInnes, Cowperthwaite, and Blanchard, J. Am. Chem. Soc., 48, p. 1909; 1926.

from the filament of the 6 cp lamp S is focussed on this vane by the lens L. If not shaded by the vane the light passes to the photoelectric cell K. In our recent work this cell is of the General Electric PJ-15 type. When the photoelectric cell is made conducting by illumination a potential is placed on the grid of the three element tube O, from the battery D, thus stopping the current, between the filament

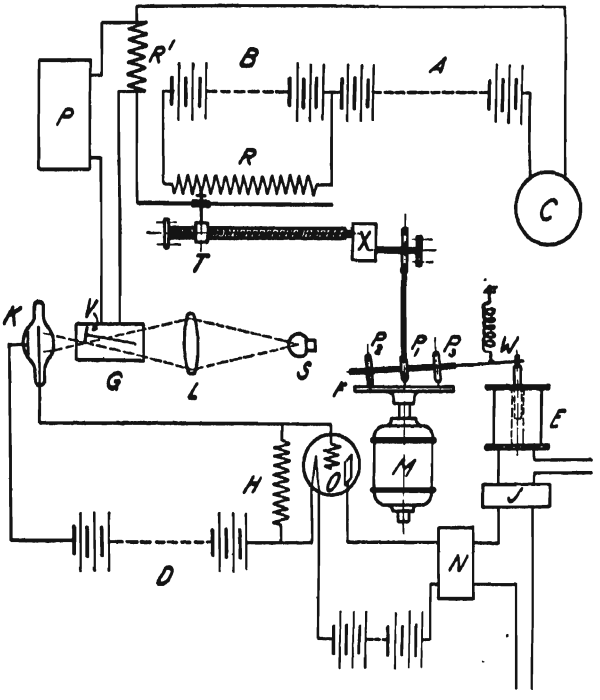


FIG. 1. Diagram of constant current regulator

and plate, which operates the sensitive relay N.[2] This relay in turn operates the relay J.

Up to this point the apparatus is, except for minor changes, the same as the one previously described. In order, however, to make corrections for both positive and negative deviations from the desired current strength the following scheme was adopted. A small motor M continuously turns a circular Bakelite disk F. A shaft holding the grooved pully P_1 and the rubber-tired pulleys P_2 and P_3 is pivoted with bearings above and below the pulley P_1 so that the shaft can

[2] Type Western Electric B 108 Telephone Relay.

swing around an axis at right angles to the axis of the disk F, and to the shaft itself. The pulley P_1 is connected by a light belt to the reducing gear X, which in turn operates the screw adjustment to the contact point T on the rheostat R. The shaft containing the pulleys P_1, P_2, and P_3 is normally held by a spring W so that pulley P_2 is in contact with the revolving disk F, thus driving the contact point T in a forward direction. If, however, the electromagnet E receives current through the operation of the relay J, pulley P_3 is brought in contact with F and the motion of the contact T is immediately reversed. The effect of this arrangement is as follows. If the potential across the resistance R' drops due to an increase of the resistance of the cell at C (or to running down of the batteries $A-B$) the vane V of the galvanometer swings so as to illuminate the photoelectric cell, thus stopping the current through the three electrode tube, the relays, and the magnet E. This permits a forward movement of the point T, which soon makes very slightly more than the required correction, whereupon the reverse movement of the vane V causes, through the operation of the relay system, the operation of the magnet E and the reversal of the adjustment of the contact T. The vane on the galvanometer needle is thus kept hovering closely around its zero position whether or not there are changes in the resistance of the cell at C or of the potentials of the battery $A-B$. The sensitiveness of the apparatus is shown by the fact that a reversal of the adjustment occurs if the potential across the terminals of R' differs by 0.1 millivolt from the reading of the potentiometer. The total potential across R' was usually about 1.5 volts. Thus a variation of less than 0.01 per cent from the correct potential will cause a reversal of the adjusting system. Still greater precision of adjustment may be obtained by increasing the resistance R', or by the use of a more sensitive galvanometer.

The motor, magnet, and pulleys are shown diagrammatically on a larger scale in Fig. 2. The pulleys P_2 and P_3 are constructed so as to be adjustable as to position along the shaft, so that if, for instance, greater positive than negative adjustments are expected the corresponding pulley can be placed further from the pivot U. The magnet E is constructed so as to have a nearly complete magnetic circuit and gives ample pull on 60 milliamperes.

In order to test the efficacy of this apparatus measurements were made with a silver coulometer under two different conditions. In the first series of experiments the current was derived from a six-volt storage cell, with the apparatus compensating for such changes of resistance and emf as normally occur in the cell and coulometer. In the second series an adjustable resistance which could be gradually increased or decreased was included in the circuit.

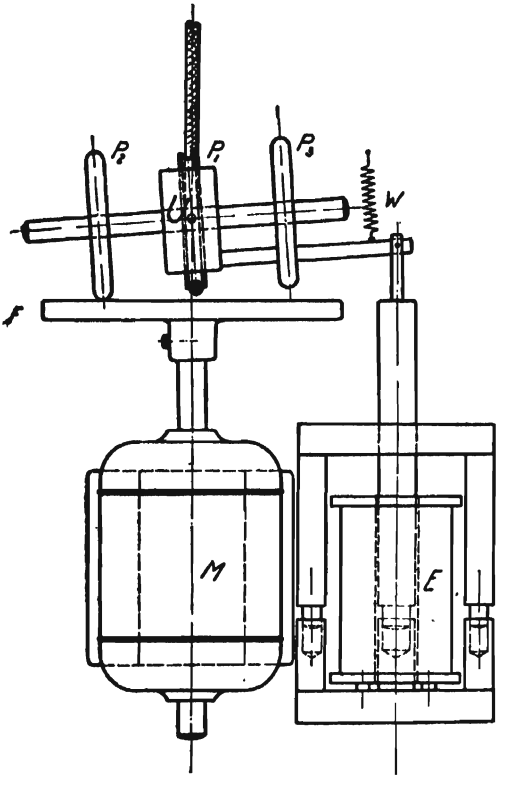


FIG. 2. Mechanism for reversal of adjustment

The silver coulometer used was of the usual Richards type consisting of a platinum dish of 75 cc. capacity containing 15% silver nitrate solution, in which was immersed a silver anode surrounded by an alundum porous cup. During the measurements the coulometer was kept in a large glass container. Most of the suggestions of the Bureau of Standards for accurate work with this instrument were followed. The silver nitrate solutions were prepared from the "*C. P.*" salt with

careful exclusion of organic matter. Weighings were carried out using weights which had been compared with a set calibrated by the Bureau of Standards. The silver deposits obtained consisted of uniformly distributed small crystals with no evidence of striations, the latter being, as is well known, evidence of contamination.

In the first series the connections were made as in Fig. 3 (a). The coulometer C was connected in series with the carefully calibrated oil immersed resistance R', of about 9 ohms (the terminals of which were connected to the potentiometer P of Fig. 1). In the same circuit was also a storage cell of 6 volts, and the resistance R_1 of about 40 ohms which was shunted by the variable resistance R of 1760 ohms.

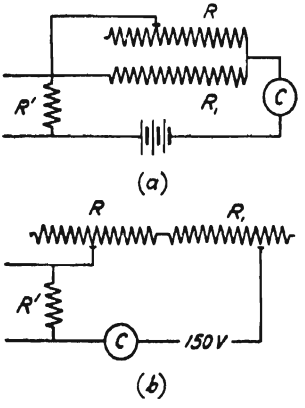


FIG. 3. Connections of coulometer circuits

The resistance R was automatically adjusted as described above. The results of this series of determinations are given in Table 1. During these runs the motion of the edge of the vane V of the galvanometer of Fig. 1 was about 0.2 millimeter. Since the galvanometer deflected 1 mm. by a deviation of 0.5 millivolt from adjustment and the total voltage reading of the potentiometer was 1.5 volts the current was always within 0.01% of the value given in the table. The reversals of adjustment took place about 35 times per minute. The number of reversals was increased from about 10 per minute by placing a tension (by means of cord, pulley, and weight) on the contact T, thus keeping the bearing surface on one edge of the screw thread. This avoided adjusting for the play between the threads on each reversal.

The time was obtained directly in seconds from a magnetic counter

operated from a pendulum clock. The calculated weights of silver given in the fifth column of the table were obtained by multiplying the number of coulombs by 0.00111800. This figure is the one agreed upon by the London conference (1908) as a secondary definition of the coulomb.

That accuracy of the kind shown in Table 1 depended upon the smooth functioning of the adjustment apparatus is shown by some

TABLE 1

Results of Coulometer Experiments. Small Changes in Resistance

Experiment	Current (amperes)	Time (seconds)	Weight of silver deposit, grams		Per cent error
			Observed	Calculated	
1	0.12842	10800	1.5506	1.5506	0.00
2	0.13794	18000	2.7763	2.7759	+0.01
3	0.16234	9000	1.6337	1.6335	+0.01

TABLE 2

Results of Coulometer Experiments. Large Changes in Resistance

Experiment	Current (amperes)	Time (seconds)	Weight of silver deposit, grams		Per cent error
			Observed	Calculated	
4	0.12787	8404	1.2013	1.2014	−0.01
5	0.14265	7200	1.1481	1.1482	−0.01
6	0.14216	9506	1.5108	1.5108	0.00
7	0.10958	9000	1.1024	1.1025	−0.01

Experiment	*Change of resistance R_1 during experiment*
4	Increased to full value when reduced to zero.
5	Decreased from full value to zero then returned to full value.
6	Twice increased to full value from zero and returned to zero.
7	Steadily decreased from about half value.

experiments in which some part of it failed to work for a few seconds at a time. The errors then increased, being, for instance, +0.02 and −0.03 on two occasions when relays failed to operate. On one occasion when the potential across R' was out of the range of the potentiometer for about 60 seconds, the error rose to 0.07%.

In the second series of determinations the connections were made as shown in Fig. 3 (b). The automatically adjusted resistance R was connected in series with another resistance R_1 of 960 ohms. These were in turn connected in series with a 150 volt storage battery, the coulometer, and the standard resistance R'. The resistance R_1 was of the screw adjustment type, and was so arranged that it could be made to throw a steadily increasing or decreasing resistance into the circuit through operation with a motor and reducing gear. The way this resistance was changed during each experiment is given, along with the other data, in Table 2. It will be seen that the accuracy obtained in the coulometer experiments is about the same with these arbitrary and relatively rapid changes of resistance in the circuit as with the slow changes encountered in the experiments outlined in Table 1. The fineness of the adjustments made by the apparatus was, however, limited to the potentials between adjacent turns of the rheostats R and R_1 of Fig. 3 (b). The deflections of the galvanometer were seldom if ever over 2 mm. from this cause. The momentary values of the current thus varied about 0.07% from those given in the table. It seems entirely probable that if rheostats with an indefinite number of steps were used the adjustment at each second would have been as good as in the series of determinations outlined in Table 1. Such rheostats are unfortunately not at present available. Adjustments for large changes of resistance in the circuit are, however, necessary in our work. It is evident from the coulometer experiments that the positive and negative errors due to irregularities in the rheostats cancelled out, on the average, within the experimental error.

In order to obtain the accuracy shown in the experiments described above it was necessary to obtain the best possible calibrations of the potentiometer P (Fig. 1) and of the resistance R'. The potentiometer P was of the Leeds and Northrup "Students" type. This was, however, calibrated against a "Type K" potentiometer made by the same firm. The individual coils of the latter were measured against a standard bridge, and the wire was calibrated by the internal method described by the makers. The resistance R', which had to carry larger currents than most coils are designed for, was made of No. 20 manganin wire and was immersed in oil. This coil needed frequent calibration since it had not had time to become properly aged. The Wheatstone

bridge on which these measurements were made had recently been very carefully standardized, by the Carey-Foster method, against Bureau of Standards resistances, by Dr. Theodore Shedlovsky of this laboratory. The authors are indebted to Mr. Irving A. Cowperthwaite for assistance in constructing the apparatus.

SUMMARY

An apparatus is described for automatically maintaining a constant current through a circuit in which gradual increases and decreases of resistance and emf are occurring. Observation of the galvanometer indicated that the regulation was within 0.01 per cent in a circuit in which only small changes of resistance and emf occurred. The operation of the system over considerable periods of time was demonstrated by silver coulometer experiments in which an accuracy of 0.01 per cent was obtained.

With comparatively large changes of resistance in the circuit the momentary accuracy of adjustment was limited by the number of steps in the rheostats used. Experiments with the silver coulometer showed agreement within 0.01 per cent even when there were large increases and decreases of resistance in the circuit, indicating that the momentary errors in adjustment (never over 0.07%) cancelled each other on the average.

[Reprinted from THE JOURNAL OF EXPERIMENTAL MEDICINE, May 1, 1929,
Vol. 49, No. 5, pp. 847–857]

A "SOLUBLE SPECIFIC SUBSTANCE" DERIVED FROM GUM ARABIC*

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(Received for publication, February 14, 1929)

The unforeseen identification of the so-called "soluble specific substances" (1) of Pneumococcus as polysaccharides (2) has led to an accumulation of evidence that analogous sugar derivatives play an important part in the immunological relationships of micro-organisms of the most diverse types (3). Thus, specifically reacting substances with the properties of carbohydrates have also been isolated from the Friedländer bacillus, the tubercle bacillus, the typhoid-colon group, and yeast; and evidence has been obtained of the existence of similar substances in Streptococcus, the anthrax bacillus, and other pathogenic microbes. This wide distribution of specifically reacting polysaccharides made it seem not improbable that there might occur among higher plant forms other sugar derivatives with specific properties. A number of water-soluble gums of plant origin were therefore tested against anti-pneumococcus sera of Types I, II, and III, and of these gums several were found to give the precipitin reaction. Since occasional samples of gum arabic (gum acacia) precipitated Type II (and Type III) antiserum at as high a dilution as 1:25,000 (*cf.* Table I, 47_2), but did not precipitate normal horse serum or Type I antiserum, this gum was chosen for further study.

It was soon found that the ordinary methods of fractional precipitation from neutral, acid, or alkaline solutions yielded products differing

* An abstract of this paper was presented at the Annual Meeting of the American Chemical Society in Philadelphia, in Sept., 1926. The paper itself was submitted for publication at the same time, but was withdrawn until more positive analytical data on the calcium aldobionate were available. In the meantime Cretcher and Butler have also published a note (*Science*, Aug. 3, 1928) indicating the presence of an aldobionic acid among the products of hydrolysis of gum arabic and have a paper in press giving further details.

little in their specific reactivity. Since it was known that the soluble specific substance of Pneumococcus was comparatively resistant to strong acid in the cold (*2b*, p. 305), fractional hydrolysis of this type was resorted to in the hope that the non-specific portions of the gum would prove the more easily hydrolyzed. This appeared to be the case, as the polysaccharide fraction recovered was found to possess a degree of specificity comparable with that of the bacterial specific substances (*cf.* Table I, 47_{10BIV}, 56). From the fact that 50 per cent of material 100 to 150 times as active as the original gum was recovered it is evident, however, that the process involves more than a mere hydrolysis of accompanying inert material. This point will be taken up more fully in the discussion.

The gum arabic purified in this way resembled in its physical properties the arabic acid so frequently described as the principal constituent of the gum (4). On hydrolysis, however, it yielded mainly galactose and an acid fraction consisting of at least 2 substances. Investigation of these acids has indicated that they are possibly disaccharide (aldobionic) acids of the type recently described as the principal product of hydrolysis of the soluble specific substance of Type III pneumococcus (*3a* and *3b*). Such a relationship would be of considerable theoretical interest in a study of the chemistry of bacterial specificity. The large quantities of starting material available should facilitate the investigation of aldobionic acids to an extent impossible in the case of the polysaccharides elaborated by pathogenic bacteria.

EXPERIMENTAL

1. Isolation of Reactive Material from the Original Gum

200 gm. of Squibb's powdered gum acacia were dusted slowly into 1 liter of chilled 1:1 hydrochloric acid. The mixture was stirred until solution was complete and allowed to stand at room temperature for 2 days. A small amount of dark, insoluble material was centrifuged off and the clear liquid precipitated with about 3 volumes of chilled alcohol. After the gum had settled the supernatant was poured off and the precipitate macerated with fresh alcohol. After several hours this was decanted and the gum was dissolved in water, centrifuged if necessary, and reprecipitated with redistilled acetone in the cold. After several hours the precipitate was stirred with fresh acetone, ground up under acetone when thoroughly hardened, filtered, washed with acetone until free from hydrochloric acid, and dried *in vacuo* over calcium chloride and crushed sodium hydroxide.

The yield was 70 to 80 gm. This product (Table I, 56) corresponded closely to that obtained in 97.5 gm. yield by a single acid treatment of 24 hours (47_{10}) and to preparation 47_{10BIV} obtained in 64 gm. yield by 2 single acid treatments of 24 hours each. It was sometimes necessary to redissolve the gum in water a second time and reprecipitate with acetone in order to remove all chlorine ion.

The product so obtained still contained about 0.3 per cent of nitrogen, or the entire amount in the original gum. 60 gm. of preparation 47_{10BIV} were therefore dissolved in 300 cc. of water and stirred ½ hour with 5 cc. of 30 per cent sodium nitrite solution and 25 cc. of acetic acid. About 1.5 volumes of acetic acid were then added, precipitating most of the gum. After 2 hours the deposit was drained, dissolved in about 200 cc. of water, and reprecipitated with acetic acid. It was finally treated with successive portions of redistilled alcohol and acetone, ground up, washed thoroughly with acetone, and dried as before. The yield was 42.3 gm. This product (Table I, 51E) contained less than 0.1 per cent of nitrogen and reacted with Type II anti-pneumococcus serum at a dilution of 1:5,000,000. It also precipitated Type III anti-pneumococcus serum. The fraction of the gum not thrown down by the acetic acid resembled the precipitated portion in all its properties, but it contained 0.5 per cent of nitrogen and was somewhat less reactive with Type II serum.

The purified gum is a white powder, readily soluble in water. It possesses marked acidic properties and rotates the plane of polarized light weakly to the left, somewhat more strongly on neutralization. It gives a positive naphthoresorcin test. When hydrolyzed it yields 68 per cent of reducing sugars, calculated as glucose, but since about one-third of the products of hydrolysis appear to be disaccharide or polysaccharide acids (see below), the actual yield of reducing sugars is higher. The pentose content, 19 per cent, calculated from the yield of furfural on distillation with hydrochloric acid,[1] is about one-half that of the original gum, so that much of the portion hydrolyzed in the method of preparation consisted of pentose or pentosan. Part of the remaining material which reacts as pentose is accounted for by the sugar acid fraction. The principal hexose component of the purified gum is galactose.

[1] A modification of Pervier and Gortner's method (5) was used. Instead of titrating at an acidity of 4 per cent and plotting the end-point with the aid of a bromine electrode, galvanometer, and stop-watch, it was found simpler to use an outside spot indicator of starch iodide solution. At an acidity of 3 per cent, the end-point is taken as the first burette reading at which a spot test is still obtained after 2 minutes. Large drops should be withdrawn for the test.

2. Attempts at Further Purification of the Specific Fraction

The specific fraction of the gum is incompletely precipitated by barium hydroxide in large excess. The precipitate soon turns yellow, and as will be seen from Table I the recovered gum (51B) shows practically the same properties as the original material.

Uranyl nitrate also precipitates the gum incompletely when the excess acid is neutralized, but in this case also no purification results.

Partial adsorption of specific gum on "Type C" aluminium hydroxide (6) resulted only in a product with the properties of the starting material (Table I, 54D).

Fractionation of the specific product with hydrochloric acid and alcohol gave 3 portions with practically identical properties.

3. Precipitation of the Specific Gum by Means of Type II Pneumococcus Antibodies

An attempt was made to determine whether the specific gum could be precipitated by Type II pneumococcus antiserum and recovered from the precipitate. This was of importance not only in establishing the polysaccharide as actually analogous or not to the specific polysaccharides of bacterial origin, but also in determining whether the reaction with Type III antiserum was caused by an accompanying impurity or was an inherent property of the specific polysaccharide itself. Thus, an accompanying substance which did not precipitate Type II serum, but yielded a precipitate with Type III serum, should be largely eliminated in effecting a specific precipitation with Type II serum.

3 liters of Type II pneumococcus antibody solution, prepared essentially by Felton's method (7), were precipitated by a slight excess of neutralized 1:1000 saline solution of preparation 47_{10}. The amount of precipitate was small, and only 0.07 gm. of specific gum was recovered by the method already described in detail in the case of the soluble specific substance of Type II pneumococcus (2*c*, p. 737).

Except for an unavoidably high nitrogen content (0.6 per cent) and a higher specific reactivity toward both sera the product (Table I, 47_{10}A) resembled the starting material in its analytical and physical properties. It still gave a precipitate with barium hydroxide in excess, gave the brown red color characteristic of galactans with orcin

TABLE I

Properties of Gum Arabic and Derived Specific Fractions

Preparation No.	$[\alpha]_D$		Acid equivalent	Nitrogen	Reducing sugars on hydrolysis		Highest dilution precipitating pneumococcus antiserum		Ash	
	Free acid	Na salt			Total	Pentose	Type II	Type III		
				per cent	*per cent*	*per cent*			*per cent*	
47_2		−31.0°		0.35	77.4	40	25,000	25,000	1.4	Squibb's gum acacia
47_{10}	−7.5°	−8.8°	1006			15	3,000,000*	1,000,000	0.3	Single acid treatment, 24 hours
$47_{10\text{-BIV}}$	−12.0°	−16.0°	906			19	4,000,000	+	0.03	Two acid treatments
47_{10}A		−20.0°	665	0.6	67.3		8,000,000	2,000,000	0.3	Recovered from immune precipitate
51B	−12.0°			0.15			4,000,000	+	0.1	From $Ba(OH)_2$ precipitate
51E	−10.7°	−14.3°	856	0.08	68.0	18	5,000,000	+	0.06	47_{10BIV}, treated with HNO_2 and precipitated with acetic acid
54D	−11.5°	−16.6°	723	0.2	68.5	18	4,000,000	+	0.03	Adsorbed on $Al(OH)_3$
56	−7.5°	−12.5°	866	0.33	68.0	19	3,000,000†	+	0.13	Single acid treatment, 48 hours

* There is apparently relatively little antibody to the specific gum in Type II and Type III antisera. In the former case the precipitate is a transparent jelly, and the tubes containing the higher dilutions must be centrifuged in order to render the deposit more compact and more easily visible. The Type III precipitate is loose and flocculent until centrifuged.

† Highest dilution tested.

and hydrochloric acid, and showed a positive naphthoresorcin test. Not only did it react at a dilution of 1:8,000,000 with Type II antipneumococcus serum, but also at a dilution of 1:2,000,000 with Type

III serum, indicating that it is actually the same substance which precipitates with both sera.

While this experiment seemed fairly conclusive in establishing the purified gum as a true "soluble specific substance," it remained possible that the polysaccharide reacted with some other constituent of the serum than the pneumococcus antibodies themselves.

Accordingly a portion of the Type II antiserum was absorbed by means of a saline suspension of heat-killed Type II pneumococci. Another portion of the serum was treated with small amounts of a 1:10,000 solution of the Type II soluble

TABLE II

Dilution of preparation 47_{10BIV}	Type II serum unabsorbed	Type II serum absorbed with Pneumococcus II	Type II serum absorbed with soluble specific substance II	Type II antibody solution absorbed with specific gum arabic
1:1,000	++++	−	−	
1:5,000	+++	−	−	
1:25,000	+++	−	−	
1:250,000	++	−	−	
1:1,000,000	−	−	−	
Saline	−	−	−	
Dilution of Type II soluble specific substance, 1:20,000			−	++++
Agglutination of Type II pneumococci			−	++++

Dilution of serum, 2:3. Tubes were not centrifuged. After centrifugation the 1:1,000,000 dilution reacted + in the unabsorbed serum and all the tubes containing serum absorbed by Pneumococcus II contained a trace of scaly precipitate. Otherwise the results were unchanged.

specific substance until no further precipitate could be obtained after 2 hours at 37° and standing over night in the ice-box. The reaction of the purified gum with these sera and untreated Type II serum was then tested, giving the results shown in Table II.

4. Hydrolysis of the Specific Polysaccharide

A. 41 gm. of preparation 51E were dissolved in water, treated with 85 cc. of concentrated sulfuric acid, diluted to 3 liters, and boiled for 4 hours, a preliminary experiment having shown a maximum reducing power after this time. The sulfuric acid was removed quantitatively with barium hydroxide, and to the filtrate, concentrated *in vacuo* to about 300 cc., basic lead acetate solution was

added in slight excess. The precipitate was suspended in water and treated with acetic acid in small amounts until only a small amount of yellow precipitate remained. The filtrate from this was again precipitated with an excess of basic lead acetate. The lead salt was decomposed with hydrogen sulfide and the solution concentrated repeatedly *in vacuo* to a syrup in order to eliminate acetic acid. The residue was taken up in hot water, boiled with acid-washed Norite, and concentrated to dryness, finally in a high vacuum. The yield of crude sugar acid was 5.5 gm. A determination of the reducing power by the Shaffer-Hartmann micromethod (8) gave a value of 44.6 per cent, calculated as glucose.

0.3329 gm., made up to 15 cc. with H_2O: α_D, 0.39°, $l = 2$. $[\alpha]_D = +8.8°$.

1 cc. of the same solution required 2.62 cc. 0.02 N NaOH for neutralization to phenolphthalein. Acid equivalent, 427. Calculated for $C_{12}H_{20}O_{12}$, 356.

The filtrates from the first and second precipitations of the lead salt were freed from lead, concentrated to small bulk, boiled with Norite, concentrated to a syrup, and, while still warm, were treated with about 2 volumes of glacial acetic acid and seeded with a few crystals of galactose. Crystallization took place rapidly, and the solid cake which formed over night in the ice-box was crushed, sucked off *in vacuo* on a Buchner funnel, and washed first with chilled 66 per cent acetic acid, then with the glacial acid, and finally with alcohol. The yield was 8.3 gm., with an initial $[\alpha]_D$ of +120.5°.

1 gm. of the crude sugar, oxidized with warm 1:1 nitric acid, began to deposit crystals within a few hours and ultimately yielded 0.46 gm. of mucic acid, melting at 214–215° with decomposition.

0.1009 gm. gave 0.1279 gm. CO_2 and 0.0458 gm. H_2O.

Calculated for $C_6H_{10}O_8$: C, 34.27 per cent; H, 4.80 per cent. Found: C, 34.57 per cent; H, 5.08 per cent.

6.8 gm. of the sugar itself were dissolved in water, boiled with Norite, and recrystallized as before, yielding 5.4 gm. of purified sugar, which from its analysis, rotation, and the isolation of mucic acid in good yield from the crude product, was chiefly galactose.

0.1007 gm. gave 0.1480 gm. CO_2, and 0.0620 gm. H_2O.

Calculated for $C_6H_{12}O_6$; C, 39.98 per cent; H, 6.72 per cent. Found: C 40.08 per cent; H, 6.89 per cent.

0.5513 gm., made up to 10 cc. with H_2O, gave an initial reading of 6.55° and a final value of 4.05°, $l = 1$. $[\alpha]_D$, initial, +118.8°; final, +73.5°.

The galactose isolated in this crop was not as pure as that recovered in later fractions (see below), but whether the impurity was the sugar acid still present in the mother-liquors, or some other sugar, has not been determined.

The filtrate from the initial crop of galactose was repeatedly diluted with water and concentrated *in vacuo* in order to remove acetic acid. It was then diluted to about 350 cc. and again treated with basic lead acetate solution, yielding a heavy

precipitate. This salt was worked up as was the first lead salt, and yielded 3.9 gm. of a crude sugar acid resembling the first product except in its rotation and somewhat lower reducing value, the latter being 39.2 per cent, calculated as glucose.

0.3064 gm., made up to 15 cc. with H_2O: α_D −0.17°, l = 2. $[\alpha]_D$ = −4.2°.

2 cc. of the same solution required 4.53 cc. 0.02 N NaOH for neutralization to phenol red. Acid equivalent, 450. Calculated for $C_{12}H_{20}O_{12}$, 356.

The filtrate from the above lead salt was freed from lead and acetic acid, taken up in a little water, neutralized with barium hydroxide, and again concentrated to a thick syrup. This was boiled with 3 successive 200 cc. portions of 90 per cent alcohol. The insoluble residue, purified over the lead salt, gave 2.0 gm. of a product which appeared to be a mixture of sugar and sugar acid, but was not further investigated. The alcoholic solutions after concentration to a syrup readily yielded 3.3 gm. of galactose, which melted at 161–163° after recrystallization.

0.5522 gm., made up to 10 cc. with H_2O, gave an initial reading of 7.52° and a final value, after addition of 0.5 cc. concentrated aqueous NH_3, of 4.11°, l = 1. $[\alpha]_D$, initial, +136.2°; final, +78.2°.

In Beilstein, 3rd edition, vol. i, p. 911, the rotations given for pure galactose are $[\alpha]_D^{20}$ +140°, 80.5°, respectively.

The mother liquors from the galactose contained but 1.7 gm. of sugar, calculated as glucose, and were not investigated.

(*B*).[2] 49 gm. of a product (active with Type II serum at a dilution of 1: 4,000,000) were hydrolyzed as in the previous instance. After removal of the sulfuric acid the concentrated solution was boiled with calcium carbonate and Norite, filtered, concentrated to small bulk, and fractionated with methyl alcohol as in the case of the calcium aldobionate derived from the Type III pneumococcus (3*b*). The partially purified salt thus obtained was further fractionated into three arbitrary portions with the aid of methyl alcohol and acetone.

Fractions	I	II	III	Theory
	per cent	*per cent*	*per cent*	*per cent*
Calcium	5.8	6.7	5.5	5.3
Reducing sugars (as glucose) (Schaffer-Hartmann)	44.8	44.3	34.4	48.0
Aldose (as glucose) (Willstätter-Schudel)	53.3	58.1	61.5	48.0

Fraction I[3] thus corresponds fairly closely to a calcium aldobionate, $(C_{12}H_{19}O_{12})_2Ca$, while the succeeding fractions show increasing contamination.

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[2] The experimental work in this section was carried out by one of us (M. H.) in the laboratories of the Mt. Sinai Hospital and the Presbyterian Hospital, New York. For the facilities offered by these institutions, and for the kindness of Dr. Forrest E. Kendall, of the Presbyterian Hospital, in carrying out the analyses, the writers wish to express their hearty thanks.

[3] A crystalline cinchonidine salt, and through this, the crystalline aldobionic acid have since been isolated, and will form the subject of a separate communication.

The analyses indicate that the chief impurity in Fraction II is possibly a salt of the type of calcium glucuronate, while Fraction III presumably contains galactose, especially as the mother-liquors from this deposited crystals of galactose on standing (melting point, 160–168°; yielded mucic acid, melting point, 206–207°, on oxidation).

DISCUSSION

That a polysaccharide with specific properties should occur among the higher plants without any apparent relation to the life processes of micro-organisms, is additional evidence of the wide-spread occurrence of carbohydrates with immunologically specific properties. Speculation as to their function and chemical and immunological relationships, while enticing, must be postponed until more information is at hand. There is evidence which has been interpreted by Beijerinck (9) as pointing to the elaboration of gum arabic as a result of the activities of molds, and by Greig Smith (10) as showing the gum to be a product of the metabolism of certain bacteria. As neither of these workers has proved, however, that contamination of the gum with the appropriate organism did not take place after its formation the hypothesis that gum arabic originates through the activities of micro-organisms, though attractive from the standpoint of bacterial specificity, must be considered as unproved.

In fact, certain chemical data obtained in the present investigation argue against this point of view. On partial hydrolysis about one-half of the original material is recovered with its specific activity increased 100 to 150-fold, showing that the specifically reacting gum does not exist as such in the original gum arabic, but is formed from it, probably by removal of a pentose grouping in glucosidic union, since the specific fraction contains less than one-half the pentose of the gum arabic itself. The low reactivity of the original gum would then be accounted for on the basis of traces of the specific material formed on exposure, in the process of refining, or by enzyme action. It is not excluded however, that the specific gum owes its presence to the synthetic activity of the strong hydrochloric acid on the hydrolytic products of the original gum (11), although this would seem less probable.

Another finding of chemical interest is the large proportion of complex sugar acids. It has recently been shown (3*a* and 3*b*) that the chief product of the hydrolysis of the soluble specific substance of

Type III pneumococcus is an aldobionic acid of which one component is glucose and the other glucuronic acid. A similar acid also forms one of the hydrolytic products of the soluble specific substance of the Type A Friedländer bacillus (12). At least one of the crude acid fractions from the hydrolysis of the specific gum arabic corresponds roughly to an aldobionic acid, while in the other the agreement is not so good. While both of these acid fractions may still be mixtures, their rough correspondence to important hydrolytic products of bacterial specific polysaccharides is of interest.

While the exact significance of these more or less complex sugar acids is still to be established, the finding of at least two of these acids as hydrolysis products of the specific gum arabic renders it evident that the specific gum, if actually a single substance in its present state of purity, is a more complex product than the bacterial specific polysaccharides hitherto investigated in detail. The recovery of the specific gum from its precipitate with Type II antipneumococcus serum with its reactivity for both Type II and Type III sera augmented (see experimental part) may be taken as evidence that it is a single constituent of the gum which precipitates both sera. This is in agreement with the relative complexity of the specific gum, since a substance containing the molecular groupings necessary for reaction with antibodies to both Type II and Type III pneumococci might be expected to yield more varied products on hydrolysis than one precipitating either serum alone. Whether any of these hydrolysis products of the specific gum arabic and the specific polysaccharides of Type II and Type III pneumococci are identical cannot be stated as yet. Thus far galactose has been found only in the first of the three, and this constitutes a marked difference.

From Table II it will be seen that Type II antipneumococcus serum which has been precipitated by the specific gum arabic still retains practically unimpaired its agglutinating power for the Type II pneumococcus and its precipitating power for the Type II soluble specific substance, whereas all of the antibodies are removed on absorption of Type II antiserum with Type II pneumococcus. This lack of reciprocal antibody absorption is suggestive of the relationship between Pneumococcus Type II and Type B Friedländer bacillus, in which the writers found (13) a certain chemical similarity between the specific polysaccharides of these bacteria and a corresponding immunological

relationship between the micro-organisms themselves. The serological and antigenic similarity of the otherwise unrelated bacteria was interpreted as an example of heterogenetic specificity; it is not unlikely that the reactions of specific gum arabic with Types II and III antipneumococcus sera may be accounted for in the same way.

SUMMARY

1. By partial acid hydrolysis a specific carbohydrate may be isolated from gum arabic (gum acacia). This carbohydrate is comparable in its precipitating activity for Type II (and Type III) antipneumococcus serum with the bacterial soluble specific substances themselves.

2. On hydrolysis this fraction yields galactose and two or more complex sugar acids, one of which appears to be a disaccharide acid comparable with those isolated from the specific polysaccharides of the Type III pneumococcus and the Type A Friedländer bacillus.

3. The significance of these findings is discussed.

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[Reprinted from THE AMERICAN JOURNAL OF ANATOMY, May 15, 1929, Vol. 43, No. 3, pp. 347–383]

STUDIES OF THE LYMPHATIC TISSUE

I. THE ANATOMY OF THE SECONDARY NODULES AND SOME REMARKS ON THE LYMPHATIC AND LYMPHOID TISSUE

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TWO TEXT FIGURES AND FOUR HELIOTYPE PLATES* (FOURTEEN FIGURES)

INTRODUCTION

By lymphatic tissue there may be understood, according to Aschoff (1), only a tissue characterized by the presence of germinal centers. Where no germinal centers are usually found, we should not speak of lymphatic tissue, but rather of lymphoid tissue, to which, for example, belong the thymus and the lymph nodules in the bone marrow.

Lymphatic tissue in this sense we find spread through the entire organism. According to its distribution in the fluid streams of the body as well as from its anatomical peculiarities, we must distinguish after Aschoff three groups, which appear to be of great importance on account of their regional significance and experimental criteria. These groups are:

1. The lymphatic tissue in the lymph nodes. It has afferent and efferent lymph vessels and is situated chiefly in the lymph stream.

2. The lymphatic tissue in the mucous membranes. It has only efferent lymph vessels and is situated in the fluid streams going from the mucous membranes into the interior of the organism.

3. The lymphatic tissue in the spleen. It has neither afferent nor efferent lymph vessels and is situated in the blood stream.

It should especially be pointed out that the bulk of the lymphatic tissue is arranged around the mucous membranes, the greatest number of cervical, thoracic, and mesenteric lymph nodes joining the subepithelial lymphatic apparatus. The tissue is grouped mainly around the pharynx, and in the intestine especially in those places where, ac-

* The heliotype plates will be found in the original article.

cording to Muthmann (2), a stasis of the faeces takes place, that is, in the ileocaecal valve, in the appendix, and in the flexures. In the appendix the most lymphatic tissue is found in the apex (Nagoya (3), Hellman (4)).

A much smaller quantity is situated regional to the skin and the smallest amount in the blood stream.

The germinal centers or secondary nodules are everywhere surrounded by lymphoid tissue, which in the mucous membranes usually forms only thin capsules around them. In the spleen it appears as the so-called outer zone. In the lymph nodes the lymphoid tissue forms the greatest part of the cortical substance and the medullary cords.

Great confusion has arisen in the literature, because the secondary nodules often have not been distinguished from lymphoid tissue, as for instance, in the lymph nodes where cortical substance also often has a rounded shape. It should be emphasized that Flemming (5), who, in 1885, for the first time described the germinal centers more closely, has already definitely defined them. He understands them as light centers with a dark shell, in which the reticulum is often arranged concentrically. Flemming called these formations morphologically secondary nodules, physiologically germinal centers or the places of origin of the lymphocytes.

In the present study an investigation has been made more especially of the secondary nodules, the significance of which as germinal centers has been doubted in recent years. It is the object of these studies to investigate whether they are germinal centers or whether they have certain relations to the defensive reactions of the organism against infectious or toxic irritations, as is supposed to be the case in the more recent literature. This investigation is all the more justified for, as Förster (6) has emphasized, the so-called germinal centers apparently represent the last and highest product of differentiation of the lymphatic tissue, observations in support of which will be shown here repeatedly.

In the following text Flemming's nomenclature will be used; the germinal centers will be spoken of as secondary nodules or Flemming's germinal centers, because their significance as germinal centers has not yet been proved.

Flemming (5) supported his theory that the secondary nodules are

the places of formation of lymphocytes by the observation that in the light centers more mitoses are usually found than in the other parts of the lymph nodes. The displacement of the previously regularly distributed reticulum by the growth of the secondary nodules, evident in the shell of the germinal centers, he thought an additional proof for the central growth. By a slow centrifugal pressure the daughter cells were compressed together toward the periphery and driven out through the spaces of the reticulum. Although no further proof has been brought forward, this conception became the general opinion and is presumably to-day still that of most authors.

Marchand (7) was the first to raise doubts about the theory of Flemming. He pointed out the frequent sharp circumscription of Flemming's germinal centers, which indeed allowed one only with difficulty to assume a formation of small lymphocytes from the germinal-center cells.

In the same year, Hellman (4, 8, 9), on the basis of a number of observations, thoroughly criticized this theory. He noted that the marginal zone does not grow along with the growth of Flemming's germinal centers and that the marginal zone of the small ones does not surpass that of large germinal centers. No relations between karyokinetic figures and the size of the marginal zone could be found. Transitions from the center cells to the small lymphocytes were never seen, neither in the center itself nor toward the marginal zone. There are often many nuclear fragments in the germinal centers. The germinal centers appear only some time after birth, although in the last months of embryonal life a considerable number of lymphocytes is produced. The germinal centers are missing in lymphatic leucaemia. Hellman (4, 10) made very thorough investigations, furthermore, of the development of the lymphatic tissue in rabbits and, later, in the human spleen. In rabbits he found two maxima of the weight curve of the lymphatic tissue, the first of which, at the time of puberty, coincided with the greatest number of lymphocytes in the blood, whereas at the second maximum, in the tenth month, the largest and most numerous Flemming's germinal centers were found, and these were double the quantity of those found at the time of puberty. All these observations led Hellman to the conclusion that the germinal centers of Flemming could not be the places of formation of lymphocytes.

Hellman (9, 11) placed special emphasis on his observation that mainly in infectious diseases there is a production of numerous Flemming's germinal centers, frequently without an increase of lymphocytes —an observation which Naegeli (12) had made already. Hellman even cites two cases in which germinal centers, caused by an infection, had already developed in utero. Hellman, on the ground of his investigations, arrived at the working-hypothesis that the secondary nodules are reaction centers against foreign irritations, entering into the lymphatic tissue. A small amount of toxic substance stimulates productive processes, large amounts lead to necrobiotic processes.

Since Hellman, quite a number of authors have worked on Flemming's germinal centers. Mostly they agree with Hellman, while in part they have held to the old theory. Few new points of view, however, have been raised. Latta (13, 14), who investigated the histogenesis of the lymphatic tissue of the appendix in rabbits, found that at first small lymphocytes are formed and only later so-called lymphoblasts and germinal centers. He concluded that the big lymphocytes are descendants of the small lymphocytes and under certain circumstances can again become small lymphocytes. He thought the germinal centers not to be centers of proliferation, but rather places of degeneration. Heiberg (15 to 19) especially emphasized the fact that most of the centers already show a certain degree of dissolution. Furthermore, he pointed out the existence of the regularly distributed phagocytes, always containing fragments of lymphocytes, and in which often from twelve to fifteen fragments can be seen. Heiberg concluded that the germinal centers are places of destruction of lymphocytes and that the so-called lymphoblasts are only substitutive cells for the phagocytes. Dietrich (20, 21), who studied Flemming's germinal centers more especially in chronic tonsillitis, concluded, on the ground of his own as well as of Hellman's investigations, that they are centers of resorption and not places of formation of small lymphocytes. The view of Hellman has been supported by Schlemmer (22), Förster (6), Pol (23), Heilmann (24 to 26), Bernheim (27), Wetzel (28), and Catania (29).

In view of the fact that, since the writings of Hellman until now, no cogent proofs have been brought forward against Flemming's theory that the germinal centers are places of formation of small

lymphocytes, it may be supposed that most authors to-day still hold the old theory. Their theory is best expressed in the conception of Aschoff (1). According to him, the germinal centers are neither an exclusive germinal tissue nor an exclusive protective tissue against infectious and toxic substances, but rather an especially young lymphatic tissue reacting to any irritation with lively proliferation. When lymphocytes are needed, there appear germinal centers rich in so-called lymphoblasts and karyokinetic figures, producing lymphocytes; whereas germinal centers rich in phagocytes have resorptive functions.

In the course of the present study it was soon found that the general conception of the normal anatomy of this tissue is very incomplete and in part also incorrect. The histology of the normal lymphatic tissue has therefore first been systematically examined. Those points which appeared to be incompatible with Flemming's theory to former investigators have received special attention. The lymphoid tissue has been considered, however, only in so far as seemed necessary in understanding the secondary nodules. Notice of pathological changes such as the formation of amyloid and hyaline degeneration or as the formation of so-called reticular and epithelioid centers has been omitted.

Material and Method

My anatomical studies include the consideration of lymphatic organs of several hundred rabbits and about 200 human beings. For comparison, a number of rats, mice, dogs, guinea-pigs, and cats have been included. In most of the cases all three groups of lymphatic tissue were examined: 1) axillary, popliteal, cervical; 2) mesenteric lymph nodes, tonsils, appendix; and, 3) spleen. In several cases the inguinal, retroperitoneal, portal, and bronchial lymph nodes and Peyer's patches were also examined. In addition, in rabbits the lymph nodules of the lungs were usually studied.

The material was fixed in Müller-formalin and in Zenker's fluids, and embedded in paraffin. The sections were usually cut 5μ in thickness. It is absolutely necessary to use only material fixed immediately after death, because post mortem even the secondary nodules decay very quickly. Human material is therefore less satisfactory for histological study as only in rare cases can the organs be had fresh enough.

The slides were stained by various methods, usually by Weigert's iron haematoxylin-eosin, by methylene blue-eosin (according to Mallory), by methyl green-pyronin (Pappenheim-Unna), and by silver impregnation (Bielschowsky-Maresch). In a number of the cases the stain of Altmann-Schridde (modified by Cowdry) was used and occasionally the connective-tissue stain of Mallory, fat-stains, iron-reactions, and others.

RESULTS

In the following paragraph the histology of normal lymph nodes is described. The conception of the normal anatomy of the lymph node which is presented here is pictured diagrammatically in figure A.

1. Secondary Nodules

The secondary nodules are, in principle, morphologically the same in human beings and in all the animals which have been examined. Nor could I find differences in the three groups of the lymphatic tissue, in the lymph nodes, mucous membranes, and in the spleen, at least, in omnivorous and carnivorous animals.

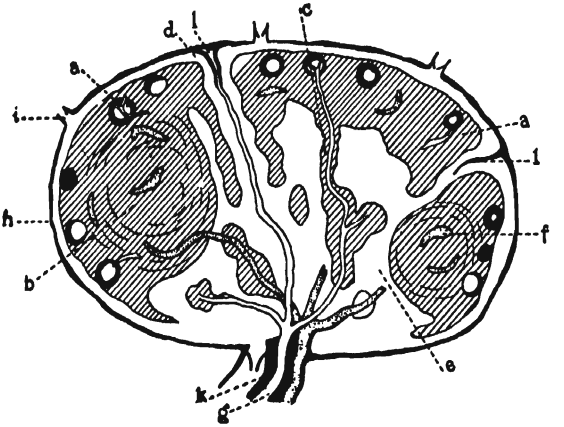
All secondary nodules have common characteristics distinguishing them sharply from the surrounding lymphoid tissue. They are always more or less globular and limited by a reticular net of variable density (figs. 1, 2) which has already been observed by Flemming (5), Hoyer (30), Bunting (31), Downey and Weidenreich (32). This net consists of fine and coarser bundles forming generally a net-like capsule with closely compressed interstices (Orsos (33)). As is well known, there is a displacement of the usually evenly distributed meshes of the reticulum by the central growth.

They differ, furthermore, from the lymphoid tissue by their vascular supply. Whereas the latter is rich in arteries and especially in peculiar veins, the secondary nodules always contain but one arterial precapillary and some arterial capillaries (fig. 3). Until now, this has apparently been observed only by Hueck (34) in the malpighian corpuscles, whereas the general opinion that the secondary nodules also contain veins could not be confirmed.

Finally, it should be noted that, in contrast to the lymphoid tissue, the secondary nodules are always poor in lattice fibers.

Apart from pathological forms, three varieties of secondary nodules must be distinguished, as has been done before only by Barbacci (35). These forms are:

a. Solid secondary nodules. They consist nearly exclusively of small lymphocytes (fig. 4). Groll and Krampf (36) called them solid



Scheme of a Lymphnode

a. Secondary nodules with arterial precapillary and capillaries
b. Pseudo-secondary nodule
c. Medullary cord
d. Marginal sinus
e. Central sinus
f. Vein
g. Artery
h. Capsule
i. Afferent lymph vessel
k. Hilus
l. Septum

FIGURE A

secondary nodules. Some contain phagocytes evenly distributed, but these are rare. Their reticular limitation is usually distinct. They are supplied by arterial precapillaries or capillaries, around which the lymphocytes are often arranged concentrically (fig. 4). Although large solid secondary nodules can be found, their diameter does not often exceed 0.3 mm.

b. Flemming's secondary nodules. The second form of secondary nodules is that which Flemming (5) called germinal centers. These I will call Flemming's secondary nodules. They have a light center, as a rule, surrounded more or less by a dark marginal zone. The limitation against the lymphoid tissue is usually distinct. When they are larger, they are often encapsulated by a very dense and broad reticular net (fig. 1).

The dark marginal zone deserves special attention. It can surround evenly the entire light center, sit like a cap on the light center (fig. 3), or can be missing entirely (fig. 1). It consists nearly exclusively of small lymphocytes mixed with some reticulo-endothelial cells. When the marginal zone is narrow, the concentric fibers lie between the small lymphocytes in the marginal zone itself; when it is broad, they lie on its most distant margin. If there is a cap, the uncovered part of the secondary nodule is always distinctly limited by reticulum (fig. 3), whereas on the covered part the reticular fibers lie in the cap itself. The cap always rests on the side opposite the entrance of the vessel (fig. 3), in lymph nodes toward the marginal sinus and in the subepithelial organs toward the epithelium.

Here I must mention another observation that Hellman (8) has made and supported by measurements. Whether the light center is big or small, the marginal zone usually stays about the same in its total quantity—which means that large centers have a narrow zone and small ones a broad zone. I have seen very large centers which did not show any marginal zone (fig. 1).

The light center is built very evenly (fig. 5). It consists of dense cells of equal size, the diameter of which is about double the size of small lymphocytes. Their nuclei only are seldom round or oval according to the usual description, but instead are mostly irregular. They often have notches and a comparatively thick nuclear membrane as Schridde (37, 38) has already shown. The chromatin net consists of filaments of medium breadth irregularly arranged with irregularly distributed net nodes. Usually they have distinct nucleoli. The protoplasm is narrow and its basophilia only of medium intensity. It is stained pink by methyl green-pyronin. It contains Schridde's granules in typical arrangement. The delimitation of the area occupied by the cells is usually distinct.

Regularly distributed between these cells there are often large reticulo-endothelial cells, the number of which varies. Their nuclei are usually poor in chromatin and therefore easily distinguished from the other cells of the light centers. But often they are so similar that it is impossible to do so, especially because the reticulo-endothelial cells, as Wallgreen (39) has pointed out, also contain Schridde's granules.

The reticulo-endothelial cells often contain fats, pigments, and nuclear fragments as many as fifteen of which Heiberg (15) has seen in one optic plane of a phagocyte. These, according to Heineke's (40) observations, represent decayed lymphocytes. They are also found free between the large cells, as are some small lymphocytes, which show all transitions to the nuclear fragments (fig. 5). As Uchino (41) has pointed out, plasma cells are, however, always missing in the light centers.

The centers are characterized from most of the other tissues by the great number of karyokinetic figures (fig. 5). Several authors, as Ribbert and Baumgarten (42), have maintained that the karyokinetic figures belong to the reticular cells. But this could not be proved. The fact that in Flemming's secondary nodules with small or large numbers of reticular cells there is always found the same number of karyokinetic figures, that, on the other hand, the formation of the light centers always goes parallel with the number of karyokinetic figures, speaks rather in favor of Flemming's opinion that they belong to the specific center cells. As Heiberg (16) was able to demonstrate by measuring the equatorial plates, only cells of the same size originate by mitosis from the large cells.

The size of the centers varies a great deal. They range from quite small, often with broad wall of lymphocytes, up to large ones even with missing walls. The diameter seldom exceeds 0.5 mm. A transition of the large central cells to the small lymphocytes of the wall usually cannot be demonstrated. The light center is sharply discontinued at the marginal zone, though in some instances I have seen transitions.

Finally, some remarks about the vascular supply should be made. Flemming's secondary nodules also are supplied by arterial precapillaries and capillaries (fig 3). In their lumina, as in their walls, mostly lymphocytes are found which are very small and often regressively

changed. Any considerable number of lymphocytes, however, I have never seen in these vessels. It is perhaps remarkable that the mitoses are often accumulated mainly around the vessels.

c. Transition forms. Whereas Flemming's secondary nodules in apparently normal animals are fewer in number than are usually supposed, the transition form to be described now is by far the most frequent (compare also Schumacher (43) and Heiberg (15, 17)). These forms also consist of light centers and dark marginal zones (fig. 6). Their size is about the same as that of Flemming's secondary nodules. The wall of lymphocytes is of varying breadth. The limitation against the lymphoid tissue varies from a sharp to an indistinct one (fig. 2).

The light center is built very irregularly and is not sharply separated from the marginal zone (fig. 7). All transitions can be demonstrated. The centers are poorer in cells than those of Flemming's secondary nodules. The nuclei lie farther apart and are of varying size and often bizarre in shape. Their content of chromatin is usually greater. Often the cells show a distinct regressive character, hyperchromatosis of the nuclear wall, pyknosis, karyorrhexis. The content of phagocytes, nuclear fragments, and pigments varies. The protoplasm is more developed, and stained pale pink by methyl green-pyronin. Not infrequently, the protoplasm is abundant, so that then the nuclei seem to float in plenty of protoplasmic substance (fig. 7). These centers contain varying numbers of small lymphocytes. Plasma cells and young reticular cells are also found. The protoplasmic processes of the reticular cells are often very distinct. Karyokinetic figures are missing or rare. The vascular supply is similar to that of the other secondary nodules. Since these secondary nodules show all transitions from Flemming's secondary nodules to an entire dissoluton into lymphoid tissue, I have called them transition forms.

Here some remarks should be added on a pigment which, according to Lubarsch (44), is found regularly in the secondary nodules of the intestine of herbivorous animals. As in the mesenteric lymph nodes, a pigment is found here, which has been regarded as iron pigment by Simon (46) and as melanin by Lubarsch. Examinations showed that these pigment masses give the reaction for fatty acid. Apparently, it is a pigment dissolved in fatty-acid-glycerin-ester-cholesterol mixture, and therefore almost certainly a lipochrome. In the secondary

nodules of the intestine large cells, furthermore, are found which have been described by Hartmann (45). Their protoplasm is spongy and foamy and often contains round vacuoles of many sizes. The nuclei of these cells are partly like those of reticulo-endothelial cells and partly like those of lymphocytic cells. The protoplasm is difficult to stain. The vacuoles are stained yellowish by sudan, but do not give the fatty-acid reaction. From these cells all transitional stages to pigment cells are found.

2. Lymphoid Tissue

While the secondary nodules are sharply limited formations, lymphoid tissue usually is a more diffuse tissue, which in the lymph nodes, according to Heudorfer (47), covers the medulla like a coat. In principle, it is organized the same way everywhere. It consists of a more or less fine reticular network, in which are inserted small lymphocytes in varying numbers. They are mixed with reticular cells, which show longish nuclei very poor in chromatin and usually a little acidophilic protoplasm. These cells often reach numbers so large that they seem to predominate over the other elements of the tissue (fig. 8). Plasma cells are also found, in large numbers in the medullary cords. Finally, lymphoid tissue always contains very large cells, the protoplasm of which is wide and intensely basophilic. Their nuclei are round and contain a very fine chromatin net and very large nucleoli. Marchand (48) has pictured these cells and calls them proliferating endothelial (reticular) cells. What large numbers these cells can reach is shown in figure 9.

Especially characteristic of the lymphoid tissue are specifically formed veins that have until now hardly been given adequate attention. Barbacci (35) has described them as pathological changes in diphtheria, whereas Kuczynski (49) observed them in the intestine of rats and explained them as resulting from an attempt at increased nutrition. Weidenreich (50) and Hett (51) have also seen them, whereas Schulze (52) is the only one who has investigated them closely. When Schulze described them as lying nearly exclusively in the cortex of the secondary nodules, he does so apparently because he does not distinguish secondary nodules sharply from lymphoid tissue. I have never found such veins in a secondary nodule or in the

marginal zone. They always are found exclusively in lymphoid tissue. I have seen them very regularly also in the lymphoid tissue of the intestine, lungs, and tonsils. In the lymph nodes they lie mainly in the cortical substance.

The venous part of the capillaries and the veins themselves are formed of endothelium consisting of very high and crowded cells with large nuclei poor in chromatin (figs. 10, 11). Single endothelial cells and groups of them often project into the lumen. Between these cells deep fissures descend to the basal layer. In the basal layer Schulze believes that he was able to demonstrate stomata, the inner openings of which are directed toward the veins and the outer toward the capillaries. Between the endothelial cells in the basal layer and in the lumen numerous lymphocytes are often found, which, in contrast to the few lymphocytes of the arteries of the secondary nodules and cortex, are always well preserved and often appear very vigorous (compare artery and vein in fig. 12).

3. Pseudosecondary Nodules

Frequently, in the cortical substance of the lymph nodes large nodular formations are found (fig. 13) which are like the secondary nodules in that they are mostly distinctly limited by displaced reticulum and that they are usually of roundish shape. Their cellular composition, however, closely resembles that of lymphoid tissue, so that by this principally they are distinguishable from secondary nodules. They have a strong reticular net, and consist of small lymphocytes, reticular cells, and single plasma cells. Karyokinetic figures are found here as in all lymphoid tissues. The veins characteristic of lymphoid tissue are often well developed. On account of the similarity of these to secondary nodules, which often has led to confusion in the literature, I will call them pseudosecondary nodules.

The pseudosecondary nodules usually are much larger than secondary nodules and frequently have a diameter of 3 mm. and more. Often they not only fill an entire half of a lymph node, but project even farther into its interior, sometimes into the medulla of the opposite half. Surrounding these, toward the surface of the node, there are usually many smaller or larger genuine secondary nodules.

Their cellular composition varies. Sometimes they consist nearly

exclusively of small lymphocytes. Sometimes they look as if they had been emptied of lymphocytes (fig. 13) and contain mainly reticular cells, whereas the small lymphocytes sometimes accumulate only around the veins (fig. 11).

Transitional stages from secondary nodules to pseudosecondary nodules are hard to find. They usually are transitions from solid secondary nodules or transition forms to pseudosecondary nodules, while such from Flemming's secondary nodules could not be found.

4. Topography of the Secondary Nodules

Finally, I want to add some remarks on the topography of the secondary nodules. Most of them are grouped around the intestine. Here are also the most numerous and best examples of Flemming's secondary nodules. In the spleen they are only seldom found normally, and mostly transition forms are seen. In the lymph nodes their frequency and composition varies. While in those regional to the mucous membranes many Flemming's secondary nodules are found, as a rule, only transition forms and solid secondary nodules are found in the peripheral lymph nodes.

In lymph nodes secondary nodules always lie at the external margin of the cortex (fig. 6), and not, as usually described in the text-books, in its center. In the interior of the cortex, as in the medullary cords, they are found apart from the mesenteric lymph nodes only under pathological conditions. They lie mainly where the afferent lymph vessels enter into the marginal sinus. A direct relation between affluent lymph and the secondary nodules seems to exist. This inference is supported by the structure of the lymph nodes of pigs. In these animals afferent lymph vessels enter the lymph node in the inside, while the efferent lymph vessels leave the node at the periphery (Chievitz (53)). The secondary nodules are found, not at the periphery, as in other animals, but only in the inside of the node; that is to say, at the entrance of the lymph vessels.

DISCUSSION

It is the general opinion that the solid secondary nodules represent Flemming's secondary nodules replaced by small lymphocytes. Groll and Krampf (36), especially, have upheld this opinion. They looked

upon this change as a process of involution, accompanied by a reduction or suspension of the production of lymphocytes in the so-called germinal centers. They support their opinion by the fact that, in later life, after the thirtieth year, Flemming's secondary nodules usually are missing and only solid secondary nodules are found. Wätjen (54, 55) thought he had supported this theory by experiments. In rabbits in which he administered large doses of arsenic he found destruction of the secondary nodules similar to the one caused by x-rays, while in animals to which he gave very small doses solid secondary nodules were found. He believed, in short, that the so-called germinal centers were changed into solid secondary nodules by small doses of arsenic. Looking through his protocols, I find that those animals to which he gave small doses and in which he then found solid secondary nodules were all very old and weighed about 3000 grams. At this age we expect normally to find that solid secondary nodules are already present. All the animals which received large doses and showed destruction of the secondary nodules were very young animals, under 2000 grams in weight. His experiments prove therefore nothing but that with small doses of arsenic no destruction of secondary nodules appears.

For the theory of Groll and Krampf I can find no support; it should, moreover, be emphasized that transitions from Flemming's secondary nodules to solid ones have never been found. The fact, indeed, that the solid secondary nodules are usually much smaller than Flemming's secondary nodules speaks decidedly against this theory. In conformity with this observation, we have found solid secondary nodules beginning with few cells; only when they have reached a certain size do light centers appear in them, as has also been emphasized by Heiberg (17) and Pol (23). These observations can be explained only by a primary appearance of solid secondary nodules.

Flemming's secondary nodules probably arise in two ways: first, through the development of light centers in solid secondary nodules; secondly, in lymphoid tissue outside of secondary nodules; this is made probable by the presence of Flemming's secondary nodules without any marginal zone (fig. 1), or with marginal zones which are very narrow and in which the reticular net lies.

The disappearance of Flemming's secondary nodules takes place

by way of the transition form, in which all transitional stages are found, or in other words, by a diffuse dissolution into lymphoid tissue—a point which has been emphasized by Hellman (8, 9). The cells of the light centers partly degenerate and in part they seem to change into small lymphocytes, as has been shown earlier in this paper. Marchand's proliferating endothelial cells, as well as lattice fibers (fig. 2), appear in the centers, and plasma cells can be found also. Finally, we find lymphoid tissue. Late transition forms sometimes closely resemble solid secondary nodules, but by their finer histological structure are proved to be lymphoid tissue or pseudosecondary nodules.

This idea of the development and decay of the secondary nodules (fig. B) is supported, furthermore, by the fact that, not seldom, both kinds of light centers, those in Flemming's nodules and in the transition

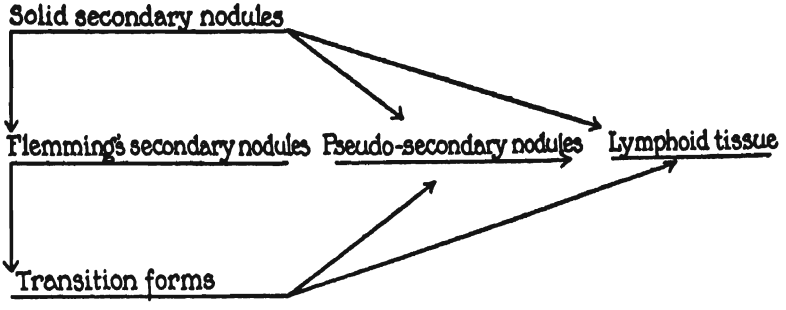


FIGURE B

forms, are found in one secondary nodule (fig. 14). At the place of the entrance of the vessel we find, first, a center of a Flemming secondary nodule (*b*) with regular large center cells and many karyokinetic figures. Toward the lymphoid tissue, these cells are distinctly limited. In the direction of the blood stream follows a transition center (*a*), distinctly separated from Flemming's center. Finally (*c*), a cap of small lymphocytes bounds the transition center. These forms show that the development of Flemming's secondary nodules need not cease with the change into a transition form (fig. 14), but that at the entrance of the precapillary a new Flemming's secondary nodule can be formed. This is the manner in which the confluent secondary nodules which are described in the literature probably arise.

It appears that in the change from solid secondary to pseudosecond-

ary nodules the development of the solid type need not always lead to the form of Flemming, but can lead directly to pseudosecondary nodules or to lymphoid tissue. These, on the other hand, can perhaps develop also from transition forms, as transition pictures indicate. Sometimes pseudosecondary nodules occupy so large a portion of the cortex, however, as to make it appear that they may also arise diffusely from lymphoid tissue. Whether they can arise from Flemming's secondary nodules as well, could not be decided.

That all transition forms change into pseudosecondary nodules is, however, improbable, because there are always in contrast to the greater number of transition forms only few pseudosecondary nodules in one lymph node. Usually the development of secondary nodules seems to be completed with the dissolution in lymphoid tissue.

It appears that pseudosecondary nodules grow under certain circumstances for a while, as is shown, 1) by the displacement of reticulum, 2) by the karyokinetic figures and, not least, 3) by the fact that the diameter of the secondary nodules seldom exceeds 0.5 mm., whereas that of the pseudosecondary nodules often amounts to 3 mm. and more. Then, after a while, the tissue becomes poor in small lymphocytes, and reticulum cells fill the tissue more and more, or the pseudosecondary nodules are dissolved into diffuse lymphoid tissue.

Furthermore, the veins of the pseudosecondary nodules or of the lymphoid tissue require mention. We often find in them numerous lymphocytes. They not only fill the lumina, but also are found in great numbers between the endothelial cells and in the basal layer. A comparison of arteries and veins (fig. 12) shows clearly that lymphocytes migrate into the blood stream and that they enter it through the walls of the veins. Whereas Schulze (52) looked upon the stomata as devices existing in the vessel walls through which injured cells and bacteria might escape into the lymphoid tissue, I look upon them, if they exist at all, only as passages which can be forced by lymphocytes, especially as their direction is oblique through walls and makes a smaller angle with the direction of the stream.

Maximow and Helly (56), Schridde (38), and Aschoff (1) have already shown that a part of the lymphocytes, when ripe to enter the blood, leave the lymphatic organs in the blood stream. It is improbable that this occurs also through the capillaries of the secondary nod-

ules, because I never have seen in them any number of lymphocytes worth mentioning.

Concerning the origin of the cells of the secondary nodules I cannot add much to what is known. Investigators have until now studied mainly the origin of the so-called lymphoblasts, because the general opinion has prevailed that the solid secondary nodules are Flemming's secondary nodules changed into small lymphocytes. The so-called lymphoblasts have been derivated from the lymphatic elements of the lymphoid tissue, from the reticular cells, and from the blood stream, whereas Marchand (7) partly believes that they originate from the adventitial cells.

The opinion that the so-called lymphoblasts originate from small lymphocytes is supported among others by the investigations of Bloom (57), from Maximow's institute, which were confirmed by Caffier (58), from Erdmann's institute. They have seen small lymphocytes in tissue cultures of lymph from the thoracic duct increase in size until, according to the descriptions, they are very similar to the so-called lymphoblasts. In addition, karyokinetic figures are found in them. The theory that they originate in the vascular wall is supported, however, by the fact that the secondary nodules are always developed in close relation to arterial precapillaries and that mitoses are often found along the vessel walls. On the other hand, the small lymphocytes in the vessels often seem regressively changed, and perhaps they emigrate rather from the blood into the secondary nodules, to be digested here by reticular cells, as Heiberg (19) supposes, than to become lymphoblasts.

Finally, I want to discuss to what extent these morphological studies of the secondary nodules throw light on Flemming's theory. Flemming's conception was that the small lymphocytes are formed in the central space of Flemming's secondary nodules from the so-called lymphoblasts and that they wander out through the marginal zone and the reticular net as the result of a slow centrifugal-pressure mechanism. Decidedly against this theory must be mentioned the following consideration, as recent authors have already contended in part. The Flemming secondary nodules are, for the most part, distinctly limited from the lymphoid tissue which may adjoin them by reticulum; the light centers are sharply discontinued at the marginal

zone, and a number of small lymphocytes worth mentioning is missing from their centers. Neither have other investigators been able to find a constant relation between the size of the marginal zone, the size of the center, and the number of karyokinetic figures. Large centers with many karyokinetic figures whose large marginal zones might be expected on the basis of Flemming's theory, often lack a marginal zone entirely. That the small lymphocytes formed in the center emigrate through the vessels of the secondary nodules, and in this way pass into the circulation, could not be confirmed. It was found, on the contrary, that the small lymphocytes outside of the secondary nodules in the lymphoid tissue migrate into the veins. It has by no means been proved, furthermore, that the nuclear fragments become free during mitosis; support can, however, be given for Heiberg's (19) theory, that they are lymphocytes, immigrating from the blood vessels into the light centers and perhaps decaying.

All these observations speak decidedly against Flemming's theory, but they can be brought into accord with it if the theory is modified so that the light centers of Flemming's secondary nodules are looked upon not as constantly producing lymphocytes, but as storing places or reserve depots for so-called lymphoblasts, which are accumulated here in order to be more or less suddenly changed into small lymphocytes under special conditions as, for example, when there is need of lymphocytes. Against the value of this modification some observations on the development and decay of secondary nodules, especially of the pseudosecondary nodules, lead, in the meantime, to doubt. In this connection two points require mention, namely, *a*) that relatively few secondary nodules only lead to pseudosecondary nodules, and, *b*) that no transitional stage from Flemming's secondary nodules to pseudosecondary nodules could be found. To this point will be given special attention in further studies.

SUMMARY

1. A distinct differentiation between secondary nodules and lymphoid tissue is made.

2. The secondary nodules are characterized by the fact that they are globular bodies more or less distinctly limited against the lympoid tissue by a reticular net, that they are very poor in lattice fibers, and contain only arterial precapillaries and capillaries.

3. The lymphoid tissue, however, is a more diffuse tissue, rich in lattice fibers, and characterized by peculiarly formed veins, through which small lymphocytes immigrate into the blood.

4. The secondary nodules are divided into:

a. Solid secondary nodules. These consist of small lymphocytes and single reticulum cells only.

b. Flemming's secondary nodules. These are Flemming's germinal centers. They have a light center of large even cells, poor in chromatin, with plenty of karyokinetic figures and ordinarily a dark marginal zone.

c. Transition forms. These are stages of dissolution of Flemming's secondary nodules and show all transitions from these to the lymphoid tissue.

5. The development of the secondary nodules usually starts with the development of a solid secondary nodule. By developing a light center, it can change into a Flemming secondary nodule. This latter dissolves diffusely by way of a transition form into lymphoid tissue.

6. Pseudosecondary nodules are called here roundish nodes, sometimes also of globular form, which are much larger than secondary nodules and by their histological structure are characterized as lymphoid tissue.

7. The pseudosecondary nodules probably originate from solid secondary nodules, late transition forms, and perhaps also from diffuse lymphoid tissue.

8. Flemming's theory in its well-known conception cannot be accepted. In the meantime doubts are raised against a modification in the sense that the light centers of Flemming's secondary nodules should be looked upon as reserve depots of so-called lymphoblasts which, when lymphocytes are needed, can be changed into small lymphocytes.

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[Reprinted from THE AMERICAN JOURNAL OF ANATOMY, May 15, 1929, Vol. 43, No. 3, pp. 385–401]

STUDIES OF THE LYMPHATIC TISSUE

II. THE FIRST APPEARANCE OF THE SECONDARY NODULES IN THE EMBRYOLOGY OF THE LYMPHATIC TISSUE

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TWO HELIOTYPE PLATES* (EIGHT FIGURES)

INTRODUCTION

In a previous paper (1) it has been shown that the development of secondary nodules in mature lymphatic tissue usually starts with the appearance of solid secondary nodules, that Flemming's secondary nodules can be formed from these by developing a light center, and that these are finally dissolved in lymphoid tissue by way of trasition forms. It has also been shown to be probable that the solid secondary nodules need not change into Flemming's secondary nodules, but that they can develop roundish formations of lymphoid tissue, and that these formations have some, but rather slight, similarity to secondary nodules. For this reason, I have called them pseudosecondary nodules.

If this conception of the development and decay of the secondary nodules is correct, it is to be expected that solid secondary nodules would appear first in the embryology of lymphatic tissue and that the pseudosecondary nodules, should such be found before the appearance of Flemming's secondary nodules, would originate here from solid secondary nodules.

This question has not been investigated before, probably because it was supposed that solid secondary nodules were stages of involution of Flemming's secondary nodules. This opinion was supported mainly by Groll and Krampf (2), but I can find no proof of it (compare 1). This is the reason why only the first appearance of Flemming's secondary nodules has been studied. But these, it is well known, do

* The heliotype plates will be found in the original article.

not appear under normal conditions until about three to six months after birth. This order of events has been given mainly by Hellman (3) as a proof that Flemming's secondary nodules are not the places of formation of lymphocytes, because, as a matter of fact, great numbers of lymphocytes are produced in the first months of life.

Baum and Hille (4) alone have described apparent secondary nodules as already present before birth in cattle, pigs, horses, and dogs, and have described them as indistinct germinal centers. These investigations have, however, of late passed unnoticed because the description is so vague.

Material and Method

The material which I have studied consists of lymphatic organs of forty-eight apparently normal human foetuses and newborn infants fourteen to forty weeks old. Death was due in all of them to intrauterine suffocation, immaturity, or trauma during birth, and, except for the presence of malformations in two cases, none showed any pathological changes. They died during or shortly after birth and only two foetuses lived longer than twenty-four hours.

In nine cases the whole body was examined, so that the lymphatic organs of various regions of the body could be studied. From the other thirty-nine only the axillary lymph nodes could be obtained, mostly of both sides.[1]

Beside material from these cases, there was examined also a foetus 34 cm. in length which showed toxic changes in the spleen, and two infants six and fourteen days old, the first having died of aspiration pneumonia and the other of septicemia.

No normal material during the first years of life was obtained, but the lymphatic organs of fourteen children $1\frac{1}{2}$ months to $2\frac{1}{4}$ years old who died of various infectious diseases were available for study.

The material was fixed, cut, and stained as described in the first paper of this series. The foetal material obtained from Doctor Plaut had already been stained with haematoxylineosin.

[1] I am indebted to the kindness of Doctor Plaut, of the Woman's Hospital, New York City, for the opportunity of examining this material.

RESULTS

In describing the results, I begin with the axillary lymph nodes of the apparently normal cases; these exhibit the largest and most complete material.

Already in intra-uterine life, at least in the axillary lymph nodes, solid secondary nodules appear (table 1) with regularity (figs. 1 to 3), as well as pseudosecondary nodules (fig. 4) and as well as veins characteristic of lymphoid tissue (fig. 5). Flemming's secondary nodules, as well as transition forms, were always missing.

The first solid secondary nodules, as well as pseudosecondary nodules, were found in a foetus of twenty-two weeks. Here, also, were the first veins characteristic of lymphoid tissue. Up to thirty weeks, only a few solid secondary nodules were ever found, whereas in some cases they were missing entirely. Beginning with the thirty-second week, they were more numerous (fig. 1), but they were found to be still missing in some older foetuses as well as in newborn. While they were usually small up to about the thirtieth week, they later reached considerable size (fig. 1) and were not much smaller than postfoetal solid secondary nodules.

The secondary nodules are typical solid secondary nodules. They consist mainly of small lymphocytes, which are often arranged concentrically around a central capillary or arterial precapillary (fig. 3). Among them a few reticuar cells are found. The reticular limitation is more or less distinct. All transitions are found from solid secondary nodules to pseudosecondary nodules, which consist principally of small lymphocytes together with reticular cells varying in numbers. Great numbers of reticular cells have not been seen.

The proportion of cortex and medulla, as well as the extent of the sinuses, varies. Lymph nodes were found with well-developed central sinuses, as well as others without them. According to Kling (5), central sinuses grow into lymph nodes first in the fifth month. In my cases central sinuses were already present in the axillary lymph nodes in a feotus of about fourteen weeks. According to Kling, the cortex up to the eighth month represents, furthermore, one single continuous peripheral mass. In the cases now studied the cortex in foetuses of twenty-two weeks was already divided by sinuses, which

TABLE 1

No.	Sex	Age in weeks	Lifetime in hours	Cause of death	Solid secondary nodules	Psuedo-secondary nodules	Characteristic veins
28623	Female	14	½	Immaturity	0	0	0
7	Male	20	0	Suffocation	0	0	0
8	Male	20	0	Suffocation	0	0	0
28803	Male	21	0	Immaturity	0	0	0
30392	Female	22	1	Immaturity	*	*	*
30569	Female	22	0	Immaturity	*	0	*
28216	Male	24	½	Immaturity	*	*	*
405	Male	24	24	Immaturity	0	0	0
29419	Female	25	24	Immaturity	*	0	*
28857a	Male	26	2	Immaturity	*	*	*
28857b	Male	26	2	Immaturity	*	*	*
29606	Female	26	2	Immaturity	0	*	*
28788	Female	26	15	Immaturity	*	0	*
28573	Female	27	7	Immaturity	*	0	0
28450	Male	28	17	Trauma	0	0	0
30206	Male	28	8	Immaturity	0	0	0
30621	Male	28	140	Immaturity	*	*	*
28886	Female	30	0	Trauma	*	0	*
29166	Female	30	1	Malformation	*	0	*
30700	Female	30	0	Immaturity	*	0	*
29840	Male	30	12	Immaturity	0	0	0
28856	Male	32	0	Trauma	**	*	*
28777	Male	32	0	Trauma	**	0	0
29418	Male	32	4	Immaturity	**	*	0
28653	Male	34	0	Malformation	**	*	0
28217	Female	36	0	Trauma	**	*	*
29550	Female	39	0	Suffocation	*	0	0
29453	Male	39	½	Immaturity	0	0	0
28539	Female	40	0	Trauma	*	*	*
28967a	Female	40	0	Trauma	*	*	*
28967b	Female	40	0	Trauma	*	*	*
28967c	Female	40	0	Trauma	*	*	*
29076	Male	40	0	Trauma	*	0	*
29097	Male	40	0	Trauma	**	*	*
1	Male	40	0	Suffocation	0	*	0
29451	Female	40	0	Trauma	*	0	0
30018	Female	40	0	Trauma	**	*	*
30298	Male	40	0	Trauma	**	0	*
30538	Male	40	0	Trauma	*	*	*
30787	Male	40	0	Trauma	0	0	0
30115	Male	40	¼	Trauma	**	*	*
30735	Female	40	½	Trauma	*	*	0
5	Female	40	24	Trauma	*	*	*
30019	Male	40	48	Trauma	**	*	*

Solid secondary nodules (*few, **many), pseudosecondary nodules, and veins characteristic for lymphoid tissue in axillary lymph nodes of foetuses and newborn.

joined the marginal sinus. As a rule, the cortex seemed to grow more and more narrow to the sixth to the seventh month (fig. 6) and, not seldom, to disappear entirely (fig. 7). It increases again in width with the appearance of secondary and pseudosecondary nodules.

Other lymphatic organs were examined only in nine apparently normal cases. The first solid secondary nodules in the inguinal lymph nodes were found in the sixth to the seventh month, in the mesenteric lymph nodes in the fifth to the sixth month, in the tonsils in the fifth to the sixth month, in the intestine only in the newborn, and in the spleen in the sixth to the seventh month.

Of special interest is a foetus, 34 cm. long, that was born of a mother suffering from lobar pneumonia. Whereas all its other organs showed neither macroscopical nor microscopical changes, distinct pathological changes were found in the spleen. The malpighian bodies, which consist usually of small lymphocytes, contained occasionally, in this case, polymorphonuclear leucocytes. In certain of them (i.e., malpighian bodies) central light areas were found which were very poor in cells and consisted mainly of reticular cells in continuity with which were associated a few polymorphonuclear leucocytes as well as degenerated lymphocytes (fig. 8). These are genuine reticular centers which have not been described before in foetal life.

In the two infants who died of pneumonia and septicemia, solid secondary nodules, as well as pseudosecondary nodules, were found in most of the lymphatic organs, while Flemming's secondary nodules and transition forms were missing.

Finally of the fourteen children, six who were $1\frac{1}{2}$ to $6\frac{1}{2}$ months old and who died of infectious diseases showed in most of the lymphatic organs, beside solid secondary nodules with pathological changes, so-called epithelioid secondary nodules in three instances and in three cases so-called reticular secondary nodules. Flemming's secondary nodules or transition forms, however, were always missing. The remaining eight children, $1\frac{1}{6}$ to $2\frac{1}{4}$ years old, showed epithelioid secondary nodules and transition forms beside solid secondary nodules.

DISCUSSION

As is shown by these studies, solid secondary nodules are already found in intra-uterine life with great regularity, while Flemming's

secondary nodules are normally always missing. That they are genuine solid secondary nodules is proved by their histological structure, which differs in no way from postfoetal ones. That they do not appear only after birth is shown by the fact that they are found also in stillborn children and that no relation can be found between the age of the premature infant and the number and size of the secondary nodules. It has been shown therefore that the first nodules to appear are solid secondary nodules. These are first to be found, judging from the axillary lymph nodes, about the middle of foetal life.

At about the same time, pseudosecondary nodules appear, too, which consist of typical lymphoid tissue and contain characteristic veins. All transitions from solid secondary nodules to pseudosecondary nodules are found, so that no doubt can exist that they arise in these cases from the solid ones. Later, they dissolve more diffusely into cortical substance, while new solid secondary nodules arise at the periphery.

These studies make it probable that the first appearance of secondary nodules takes place when the proliferating central sinuses have subdivided what was until then a compact lymphoid mass. The entire lymph node at this time consists more or less of medullary cords and sinuses only. That this is the arrangement is supported by the observation that, at the time when the first secondary nodules appear, many lymph nodes are found which have only a very narrow cortex or lack it entirely.

At birth the lymph nodes look as we are used to seeing them in later life. Only Flemming's secondary nodules and the transition forms are missing. In the cases examined the same picture was still present in infants up to fourteen days old who had died of infectious diseases.

On the first appearance of Flemming's secondary nodules, little can be added to what is already known. In intra-uterine life they have only been observed twice and then in the presence of intra-uterine infection (Hellman (3)). To these two cases can be added a very interesting one in which, in a foetus 34 cm. long, reticular centers were already found in the malpighian bodies. As Flemming's secondary nodules were nowhere found, not even in the spleen, and as all transitions from solid secondary nodules to those with reticular centers

were found, it is necessary to infer that they originated from solid secondary nodules. The fact that the reticular centers were found only in the spleen, the lymphatic tissue of which is that part of the lymphatic system that is to the whole blood stream what regional lymph nodes are to a part, makes it probable that 'toxins' are responsible for these centers and the 'toxins' having come by way of the blood from the mother who was suffering from pneumonia.

The first Flemming's secondary nodules were found, beginning in the second month, by Gundobin (6) and Nagoya (7), by Förster (8) in the third to the tenth month, whereas Wetzel (9) did not find them in children even several years old, if no infection had occurred. Normal material of the first years of life was not, as has been said, at our disposal. In the children of the present group who died of infectious diseases in the first year of life, beside solid secondary nodules only reticular and epithelioid secondary nodules were found, from a study of which it cannot be decided whether they represent pathologically changed Flemming's or solid secondary nodules. It is impossible to be certain whether epithelioid centers must necessarily be derived from Flemming's secondary nodules. Examination of the foetal case which is now reported decidedly supports the possibility that reticular centers also represent pathologically changed solid secondary nodules, especially since Flemming's secondary nodules as well as transition forms are missing.

In children in the second year of life, however, beside solid, reticular, and epithelioid secondary nodules, transition forms were also found. These have probably been derived from genuine Flemming's secondary nodules.

Some remarks should be added on the formation of new lymphatic tissue in extra-uterine life, because distinct similarity seems to exist between its development and that seen in foetal life. In new formation, also, according to various authors, lymphoid tissue appears first. Later, solid secondary nodules develop, and, as the stage of the last and highest differentiation, Flemming's secondary nodules finally arise. Heineke (10), for instance, has found that after the destruction of lymphatic tissue by x-rays regeneration starts with the formation of solid secondary nodules. Attention should, in addition, be called especially to the investigations of Christeller and his pupils (compare

Nishikawa (11)), who studied systematically the formation of new lymphatic tissue in chronic inflammation of the pelvis of the kidney, as well as in the appendix, and who came to the same conclusion. They found that at first within small groups of lymphocytes denser masses were formed, and that from these developed circumscribed lymph nodules without, or sometimes later, with light centers as well as entire lymph nodes. Christeller took pains to emphasize the fact that this lymphatic reaction appears only following a chronic inflammation and continues independently of it long after the inflammatory irritation has ceased. This reaction shows the highest development only when there is no longer need of a supply of lymphocytes. To the lymphatic reaction also pertains probably the formation of lymph nodules, as is sometimes found in the skin (compare Burckhardt (12)), or as it has been described by Orsos (13) in tertiary syphilis in the neighborhood of the thyroid gland, or as is so very characteristic of trachoma.

It is, finally, necessary to discuss to what extent these results concerning the development of secondary nodules agree with Flemming's theory. As is well known, Flemming's secondary nodules are found under normal conditions in human beings only from about the first to the twenty-fifth year (Ribbert (16), Gulland (17), Oberndorfer (18), Hellman (15)) and are said normally to be missing entirely after about the fortieth year. Therefore, Flemming's theory seems to be incorrect, because lymphocytes are produced not only before the first, but also after the fortieth year. Flemming's theory has therefore been modified as follows: According to this view, Flemming's secondary nodules are said to appear only during an increased need of lymphocytes. But this idea is decidedly contrary to the fact mentioned by Hellman (14), who was able to show that the greatest number of lymphocytes in the blood in rabbits (in the fifth month, compare Lindberg (19)) appears long before the greatest number and size of Flemming's secondary nodules have developed (in the tenth month). In human beings also, according to Benjamin (20), the greatest number of lymphocytes in the blood is found from infancy to the seventh year, while the greatest development of Flemming's secondary nodules is found at a later age.

The fact, however, that the first secondary nodules are solid

secondary nodules, as these investigations have shown, is opposed to Flemming's theory in its original form. In the second half of foetal life, as well as in the first months after birth, there is constant formation of solid secondary nodules which grow to be pseudosecondary nodules and become lymphoid tissue. At the same time, lymphocytes ready to migrate into the veins make their appearance. All these observations must signify the fact that in intra-uterine life as well as in the first months of life solid secondary nodules as well as pseudosecondary nodules are responsible for the formation of the small lymphocytes.

SUMMARY

1. Solid secondary nodules are the first secondary nodules to appear.

2. These are found with great regularity in the axillary lymph nodes of human beings beginning with the twenty-second foetal week. They seem to appear when the proliferating sinuses have divided completely the compact lymph nodes into medullary cords and sinuses.

3. Beginning at the same time, pseudosecondary nodules and the veins characteristic for lymphoid tissue are found.

4. In foetal life the pseudosecondary nodules originate from solid secondary nodules and are here the main places of formation of small lymphocytes.

5. In one foetus 34 cm. in length, the mother of which was suffering from pneumonia, reticular centers in the malpighian bodies are described which have probably arisen from solid secondary nodules.

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[Reprinted from THE JOURNAL OF EXPERIMENTAL MEDICINE, August 1, 1929, Vol. 50, No. 2, pp. 143–160]

THE RÔLE OF CARBOHYDRATES IN BIOLOGICAL OXIDATIONS AND REDUCTIONS. EXPERIMENTS WITH PNEUMOCOCCUS

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(Received for publication, June 4, 1929)

INTRODUCTION

When repeatedly washed in saline solution, living cells lose in a large measure their ability to oxidize and to reduce. This fact was established by studies of oxygen consumption and methylene blue reduction with muscle tissues and yeasts. It led to the concept that the oxidation-reduction properties of a tissue or a cell are really due to the oxidation-reduction properties of certain metabolites after they have become "activated" by the tissue or cell under consideration.

The analysis of phenomena of this nature in bacterial systems was apparently begun by Harden and Zilva (1) in 1915. They found that washed cells of *B. coli* which reduce methylene blue only very slowly, reduce the same dye very rapidly in the presence of various substances. This work was much extended by Quastel and his associates (2) who referred to the washed cells as "resting bacteria." "Resting bacteria are simply bacteria in a state of non-proliferation and may be investigated in a manner similar to enzyme or catalytic systems." Working with *B. coli*, *B. pyocyaneus* and *B. alkaligenes*, they found that these organisms can activate the reduction of methylene blue by certain sugars, amino acids, and fatty acids (especially formic, lactic and succinic). *B. coli* was found to have an even wider range of activating action than muscle, 56 substances out of 103 studied being "activated" in this manner. Quastel (3) suggested that the process of "activation" consists in a polarization of the substrate molecules at particular regions or centers on the surface structures (interfaces) of the cell.

In 1914, Cole (4) investigated the mechanism of the reaction whereby Pneumococci transform hemoglobin into methemoglobin. The reaction does not occur when hemoglobin is added to an emulsion of washed cells in salt solution. However, if minute traces of dextrose (or a number of other organic substances) be added to such a mixture, the reaction quickly occurs; it is always conditioned by the presence of oxygen. Avery and Neill (5) have described a large number of other oxidation-reduction processes exhibited by Pneumococcus cultures; amongst

which we shall consider in our studies the reduction of methylene blue and the formation of peroxide. The fact that these processes have the same optimum and limiting condition (temperature, pH, etc.,) suggests that they are brought about by one and the same system. They are exhibited not only by the whole intact cells, but also by sterile extracts of these cells prepared under proper conditions. They can also proceed under conditions of pH and temperature which do not permit active growth. As in the case of muscle, yeast and *B. coli*, the washed cells of Pneumococcus are unable to reduce, to consume oxygen, or to form peroxide. However, the oxidative-reductive properties can be restored by the addition to the washed cells of the cell washings, and of aqueous or alcoholic extracts of muscle, yeast and vegetable tissues. The oxidation-reduction system of the Pneumococcus cell therefore appears to consist of at least two components: 1) the heat stable component, just referred to, which can be readily washed out of the cell and is not necessarily of Pneumococcus origin. 2) a labile cellular component, not removed by washing, inactivated by 10 minutes heating at 65°C.

In the experiments to be presented here, we have attempted to analyze further the mechanism of bacterial oxidations and reductions and to establish new reactions of physiological significance. This report is limited to the influence of carbohydrates, especially glucose, as these substances appear to be of primary importance in the phenomena of growth. Although most of the work has been done with Pneumococcus cells, it is hoped that the facts established will prove to be of a more general significance in an understanding of cellular physiology.

EXPERIMENTAL

Experimental Methods

1. Bacteriological.—The experiments have been carried out with 3 strains of Pneumococcus: Type I (1/219/4), Type III (A/66/73) and an R cell derived from Type II (D/39/R). The results obtained apply to all 3 strains; for the sake of simplicity, all protocols refer to strain D/39/R.

Unless otherwise stated, very young cultures (6 hours old) were used in the tests; these cultures were obtained by seeding 2 cc. of a young culture into 150 cc. of meat infusion broth containing 1 per cent Witte's peptone and 0.03 per cent dextrose. The cells were separated from the medium and washed in physiological salt solution by centrifugalization (30 minutes—about 3000 rotations per minute).

By "sugar free meat infusion," we understand beef infusion prepared according to the standard method and from which the sugars have been removed by growing Pneumococcus in the infusion and filtering out the cells.

2. Chemical.—All the tests were performed in phosphate buffer solutions (0.03 molar) at pH 8.0.

The dye solutions were prepared as described in an earlier paper (6).

The following substances were used as source of oxidized thiol groups; a) glutathione (in the form of meat infusion); b) saturated cystine solution at pH 8.0; c) a product obtained by the auto-oxidation of thioglycollic acid (Eastman) in the presence of air, with sodium nitroprusside as a catalyst (in dilute ammonia solution); we shall refer to the last product as "oxidized thioglycollic acid" without attaching to this expression any real meaning concerning the exact nature of the compound.

The reduction experiments were carried out in Noguchi tubes under a vaseline seal of 2 inches thickness, at room temperature (about 20°C.). The nitroprusside test was used to detect the presence of reduced thiol groups. The formation of peroxide was tested by transferring 10 cc. of the systems under consideration to 150 cc. Erlenmeyer flasks which were incubated at room temperature; the titanium sulphate test was used for detecting the peroxide present.

TABLE I

Reduction of Methylene Blue by Pneumococcus Cultures at Different Periods of Growth

No. of hours after inoculation at which samples were taken	Time required for the reduction of 0.2 cc. of 0.0025 M methylene blue by 5 cc. culture	
	Plain broth culture	Glucose broth culture
1 hour	6 hours	6 hours
3 hours	4 "	3 "
4 " 30 minutes	2 " 20 minutes	1 " 5 minutes
6 "	2 " 10 "	0 " 30 "
7 " 30 minutes	4 "	0 " 25 "
12 "	8 "	0 " 30 "
24 "	18 "	2 " 10 "

The Reduction of Methylene Blue by Pneumococcus Cultures

When one attempts to determine the "reducing power" of a Pneumococcus culture, it is found that even under the same conditions of temperature and reaction, the rate of reduction of methylene blue varies a great deal, according to the composition of the medium and the age of the culture.

Experiment 1.—Two flasks, each containing 150 cc. of plain broth, were seeded with 2 cc. of a young culture of Pneumococcus. One of the flasks received in addition 0.1 per cent glucose. The two flasks were incubated at 37°C. and samples were removed after 1, 3, 4½, 6, 7½, 12 and 24 hours incubation. The reaction of these

samples was adjusted to pH 8.0 with NaOH and a reduction test was set up with 0.2 cc. of 0.025 M methylene blue. The times required for reduction are given in Table I.

Although no bacterial counts were made, the turbidity of the cultures indicated that the maximum growth was obtained after 6–8 hours incubation. The fact that during the early periods of incubation, the dye was more rapidly reduced in the glucose broth culture than in the plain broth culture may be accounted for by the more rapid multiplication of the organisms in the former medium. But the rapid decrease of the "reduction power" of the plain broth culture as compared with its constancy in the glucose broth culture cannot be attributed to the difference in the number of cells, for it is known that the number of R cells of Pneumococcus remain constant for at least 24 hours in plain broth cultures, while the same organisms begin to die off more rapidly in glucose broth. It appeared, therefore, that the constituents of the medium played an important rôle in the reducing power of the culture. This is shown by Experiment 2.

Experiment 2.—Flasks containing 150 cc. of plain broth were seeded with 1.25 cc. of a young D/39/R culture. 10 cc. samples were taken at different times after inoculation, and adjusted to pH 8.0. In each case, 2 samples received 0.1 cc. of a 1 per cent glucose solution and 2 were kept as control, one of the duplicate tubes in each series was placed in a bath of boiling water for 2 minutes. 2 cc. of 0.0025 M methylene blue was then added to each one of the tubes. The time required for reduction of the dye is given in Table II.

A number of conclusions can be drawn from this experiment: a) the addition of small amounts of glucose to Pneumococcus cultures brings about a more rapid reduction of the methylene blue; b) the decrease in the reduction power of a plain broth culture after the sixth hour of growth is probably due to the disappearance of the small amount of sugar originally present in the broth; c) the ability of the glucose to reduce methylene blue is probably due to some cellular factor, since it reaches its maximum at the time when the growth is maximum; d) this factor slowly loses its power as the culture ages.

It is known that oxidized methylene blue is bacteriostatic for Pneumococcus (7); this and the fact that the reduction of the dye occurs so rapidly (12 minutes) is an indication that the phenomenon of reduction

is not associated with a proliferation of the cells. The experiments with washed cells serve to demonstrate this point.

TABLE II

The Effect of Heating, Age of the Culture, and Addition of 0.1 cc. of 1 Per Cent Glucose on the Velocity of Reduction of 0.2 cc. of 0.0025 M Methylene Blue by Pneumococcus Cultures (10 cc.)

No. of hours after inoculation at which sample was taken	No. of cells per cc. of culture	Time required for reduction by:				
		Culture	Heated culture	Culture + dextrose	Heated culture + dextrose	0.1 cc. of 1 per cent dextrose
1 hour	10^7	12 hrs.	—*	12 hrs.	—	—
2 hours	10^8	9 hrs.	—	9 hrs.	—	
3 " 30 minutes	10^9	2 hrs. 15 min.	—	2 hrs. 15 min.	—	
5 "	10^9	55 min.	—	45 min.	—	
6 " 30 minutes	10^9	12 min.	—	10 min.	—	
8 "	10^{10}†	44 min.	—	13 min.	—	
10 "	10^{10}†	2 hrs. 30 min.	—	15 min.	—	
12 " 30 minutes	10^{10}†	6 hrs.	—	17 min.	—	
23 "	10^9	16 hrs.	—	27 min.	—	
26 "	10^9	—	—	45 min.	—	
30 "	10^8	—	—	48 min.	—	
47 "	10^6	—	—	6 hrs.	—	

* The sign — indicates that the dye was not reduced after 24 hours. The heated cultures, however, showed some reduction at that time. This reduction was of the same order as that exhibited by sterile media (6) and need not interest us here.

† The apparent increase in numbers observed after the 8th hour of incubation is probably due to the breaking up of the Pneumococcus chains

The Reduction of Methylene Blue by Washed Cells of Pneumococcus; Its Activation by Meat Infusion

Experiment 3.—A young culture of D/39/R in plain broth was centrifuglized, and the cells washed 7 times in saline. A sample corresponding to 15 cc. of culture was taken following each washing, and the ability of the washed cells to reduce methylene blue was tested in the absence and in the presence of glucose.

This experiment demonstrates that cells washed free of metabolites are unable to reduce methylene blue; however, they do reduce it rapidly

in the presence of glucose; the partial reduction obtained with the unwashed cells without further addition of sugar is to be traced to the metabolites associated with the cells and which are removed by repeated washings. It is also evident that, as the washings are repeated, the cells progressively lose their ability to reduce in the presence of

TABLE III

The Reduction of Methylene Blue (0.1 cc. of 0.005 M) by Washed Cells of Pneumococcus

No. of washings	Time required for complete reduction	
	No glucose	0.2 cc. of 0.005 M glucose
0†	—*	2 minutes
1†	—	5 "
2	—	9 "
3	—	16 "
4	—	29 "
5	—	40 "
6	—	—
7	—	—

* The sign — indicates that the dye was not reduced in 24 hours.

† The first and second samples of cells showed a partial reduction of the dye in 24 hours, even without glucose.

TABLE IV

Influence of Sugar-free Meat Infusion on the Velocity of Reduction of Methylene Blue by Washed Cells of Pneumococcus in the Presence of Glucose

Washed cells	Methylene blue 0.002 M	Glucose 0.002 M	Meat infusion	Time required for complete reduction
cc.	*cc.*	*cc.*	*cc.*	
10	1	1		90 minutes
10	1	1	0.5	40 "
10	1	0	0 5	Reduction only partial after 24 hours

glucose. Now, it had been observed in the course of previous experiments that the presence of sugar-free meat infusion increases the velocity of reduction of methylene blue by the Pneumococcus-glucose system, although a system containing washed cells and sugar-free meat infusion is unable by itself to bring about any *rapid* reduction. An example of such an action is given in Experiment 4.

Experiment 4.—A young plain broth culture of Pneumococcus was centrifugalized and the cells washed twice in saline. The washed cells were suspended in buffer pH 8.0 and their reducing power tested with 1 cc. of 0.002 M methylene blue and 1 cc. of 0.002 M glucose—with and without the addition of 0.5 cc. sugar-free meat infusion. A tube containing 10 cc. of cells + 0.5 cc. meat infusion + 1 cc. of 0.002 M methylene blue, but no glucose, was used as control (Table IV).

Since sugar-free meat infusion is found to increase the velocity of reduction of methylene blue by a system containing Pneumococcus + glucose, it appeared possible that the action of the cell on the glucose might depend on two factors, one which cannot be removed by washing and another present in meat infusion. Experiment 5 bears on this point.

TABLE V

Influence of Washing the Cells and of the Addition of Meat Infusion on the Reduction of Methylene Blue by the Pneumococcus-Glucose System

No. of washings	No meat infusion	0.5 cc. of meat infusion
1	+*	+
2	+	+
3	+	+
4	−*	+
5	−	+
6	−	+
7	−	−

* The signs + and − indicate that the methylene blue was, or was not completely reduced after 24 hours incubation.

Experiment 5.—Young cells of D/39/R were repeatedly washed in saline. At each one of the washings, experiments with amounts of cells corresponding to 10 cc. of culture were made to determine the reduction of 1 cc. of 0.001 M methylene blue by 1 cc. of 0.001 M glucose in the presence and in the absence of meat infusion (Table V).

The results of Experiment 5 confirm the view that in the mechanism responsible for the reduction of methylene blue in the presence of glucose there are at least two constituents involved; one present in meat infusion, readily washed out of the cells, and heat stable; the other persisting in the cell despite repeated washing but eventually lost as result of the process. It is not known as yet whether this sec-

ond factor is simply washed out, or destroyed following some injury caused to the cell in the process of washing. It has been found to be heat-labile and completely inactivated by 10 minutes exposure at 55°C.

Meat infusion is well known to contain reducing substances, and it is important to know to what extent methylene blue can be reduced by sugar-free meat infusion in the presence of Pneumococcus cells.

Experiment 6.—Young cells of D/39/R were washed four times in saline, and 5 cc. amounts were used to determine the comparative reduction of methylene blue in the presence of glucose, sugar-free meat infusion, and glucose + meat infusion. The details of the experiment are given in Table VI.

TABLE VI

The Activation of the Pneumococcus-Glucose System by Sugar-free Meat Infusion

Washed cells	Glucose 0.001 M	Meat infusion	Methylene blue (cc. of 0.001 M solutions)									
			0.1	0.2	0.3	0.4	0.5	0.6	0.7	0.8	0.9	1.0
cc.	cc.	cc.										
5	0	0	±	−	−	−	−	−	−	−	−	−
0	1	0	−	−	−	−	−	−	−	−	−	−
0	0	5	−	−	−	−	−	−	−	−	−	−
5	1	0	+*	±*	−	−	−	−	−	−	−	−
5	0	5	+	+	±	−	−	−	−	−	−	−
5	1	5	+	+	+	+	+	+	+	+	+	+

* In this table the signs + and − indicate that the dye had or had not been completely reduced in 3 hours. ± indicates that the reduction was complete only after 24 hours.

The results of Experiment 6 demonstrate that the rapid reduction of methylene blue by a system containing washed cells + meat infusion + glucose cannot be accounted for by the reducing power only of any one of the three components of this system or even by any combination of two of these components. A rapid reduction of the dye requires the presence of all three of them.

The Existence of a Definite Ratio Between the Amounts of Glucose Used and of Methylene Blue Reduced

In the course of the reduction which has just been described, the methylene blue is reduced and the glucose oxidized. An attempt has

been made to determine the relative amounts of the two substances involved in the reaction. However, great difficulties have been encountered in obtaining a constant ratio, although in all instances the reduction of 1 mol of methylene blue required from 0.7 to 1 mol of glucose. The following experiment is a typical example of the results obtained in such determinations.

Experiment 7.—Young cells of D/39/R were washed once in saline. This was found sufficient to rid the cell of practically all the metabolites they contained.

TABLE VII

The Quantitative Relation Between the Amounts of Glucose Used and of Methylene Reduced

Amount of cells	Methylene blue (0.001 M solutions)	Time in minutes required for complete reduction of the dye in the presence of the following amounts of 0.001 M glucose solution										
		0	0.1	0.2	0.3	0.4	0.5	0.6	0.7	0.8	0.9	1.0
cc.	*cc.*											
15.0	1	—*	—	—	—	—	—	—	—	360	45	60
10.0	1	—	—	—	—	—	—	—	240	—	330	120
5.0	1	—	—	—	—	—	—	—	—	—	—	120
2.5	1	—	—	—	—	—	—	—	—	—	—	145
15.0	0.1	—	60	x*	x	x	x	x	x	x	x	x
10.0	0.1	—	100	x	x	x	x	x	x	x	x	x
5.0	0.1	—	80	x	x	x	x	x	x	x	x	x
2.5	0.1	—	120	x	x	x	x	x	x	x	x	x

* The sign — indicates that the dye was not yet completely reduced after 12 hours incubation; x indicates that the test was not made.

Cells washed only once do not need meat infusion to be able to activate glucose. Varying amounts of these cells were used with varying concentration of glucose and of methylene blue for a reduction test. The details and results of the experiment are given in Table VII.

The results of this experiment demonstrate once again the reduction of methylene blue by Pneumococcus in the presence of glucose, and the lack of reduction in the absence of the sugar. It will be seen that, whereas 2.5 cc. of cells could reduce 1 cc. of the methylene blue solution in the presence of glucose, 15 cc. of the washed cells alone could not even reduce 0.1 cc. of the same dye solution. It seems true also that the ratio of glucose to methylene blue becomes narrower as larger

amounts of cells are used for the test; and this, as we have just seen, cannot be accounted for by the metabolites present in the cells. The change in the glucose-methylene blue ratio might conceivably be due to side-reactions which become manifest only when larger amounts of cells are used. In experiments not reported here, it has been established that the time required for the reduction of 1 cc. of 0.001 M methylene blue is not dependent upon the amount of glucose used, when this amount is larger than 1 cc. of 0.001 M solution. The fact that the reduction with 0.7, 0.8 and 0.9 cc. of the glucose solution takes

TABLE VIII

Time Required for the Reduction of rH Indicators by the Pneumococcus-Glucose System

Dye			Amount of cells			
Name	Concentration 0.01 M	rH	20 cc.	10 cc.	5 cc.	2.5 cc.
	cc.					
2 chloro indophenol..	0.1	21.8	Immediate reduction		6 minutes	30 minutes
1 naphthol-2 sulfonate indophenol...	0.1	18.1	"	"	10 "	30 "
Methylene blue.....	0.05	14.4	5 minutes	10 minutes	15 "	1 hour
Indigo tetrasulfonate.	0.1	12.5	16 hrs.	20 hrs.	2 days	2 days
Indigo trisulfonate...	0.1	11.3	2 days	8 days	Only partly reduced in 2 weeks	
Indigo disulfonate...	0.1	9.9	2 "	8 "	" "	" "

longer than with 1 cc. is another evidence that we are dealing with at least two different reactions.

In conclusion, it appears that, under the proper conditions, the reduction of one molecule of methylene blue requires one molecule of glucose.

The Reduction of the Indicators of Oxidation-reduction Potentials by the Pneumococcus-glucose-System

The work of the past few years has indicated that many biological systems develop reducing potentials much lower than that of reduced

methylene blue. Experiment 8 demonstrates that when glucose is placed in the presence of Pneumococcus cells, dyes with a very low rH are also reduced.

Experiment 8.—A young culture of D/39/R was centrifugalized and the cell suspended in buffer at pH 7.8. Amounts of the suspension corresponding to 20-, 10-, 5-, and 2.5 cc. of the original culture were added to solutions of the rH indicators for a reduction test. Each one of the tubes received also 0.2 cc. of a 1 per cent glucose solution. The time required for the reduction of the dyes is given in Table VIII.

Table VIII shows: 1) that the time of reduction increases as the amount of cell decreases; 2) that the dyes are decolorized in the order of the electromotive series; 3) that all dyes tested were reduced.

TABLE IX

The Reduction of Thiol Groups by Glucose (0.2 cc. of 1 Per Cent Solution) in the Presence of Cells of Pneumococcus

Nature of the oxidized thiol compounds	Incubation period (hours)			
	24	48	72	96
Glutathione (5 cc. of meat infusion).....	±*	±	+	+
5 cc. of saturated solution of cystine.....	−*	−	±	+
1 cc. of 0.05 per cent oxidized thioglycollic acid....	+*	++*	++	++

* In this table − indicates a negative test for −SH.
± indicates a doubtful test for −SH.
+ indicates a positive test for −SH.
++ indicates a strongly positive test for −SH.

The Reduction of Thiol Compounds by the Pneumococcus-Glucose System

In view of the importance of glutathione in cellular metabolism and of the fact that thiol compounds have been found to be an essential component of the media used for the growth of Pneumococcus,[1] an attempt has been made to find out whether oxidized thiol compounds can be reduced by a system consisting of Pneumococcus and glucose.

Experiment 9.—The set up of the experiment was the same as in Experiment 8, except that meat infusion, cystine and oxidized thioglycollic acid (see Experimen-

[1] Unpublished observation.

tal Methods) were used instead of the dyes. The systems were incubated for several days, and the presence of reduced thiol groups was tested for by the nitroprusside test.

Experiment 9 brings out the interesting fact that oxidized thiol compounds are reduced by glucose in the presence of Pneumococcus cells. This reduction could be obtained with as little as 2.5 cc. of cells.

The Formation of Peroxide by Washed Cells of Pneumococcus in the Presence of Glucose and Meat Infusion

Avery and Neill (5) have conclusively shown that Pneumococcus cells, when washed in saline, lose their ability to form peroxide, but regain it in the presence of yeast extract or meat infusion. Their results also indicate that the reduction of methylene blue and the formation of peroxide are controlled by the same mechanism. It was therefore tempting to see if peroxide formation could be obtained with the same system (washed cells + sugar-free meat infusion + glucose) which proved to be able to reduce methylene blue.

Experiment 10.—Young cells of D/39/R were repeatedly washed in saline and samples taken after the first, third, and fifth washings. Amounts of cells corresponding to 25 cc. of culture were suspended in 10 cc. of buffer at pH 7.8, and glucose and sugar-free meat infusion were added as described in Table X. The mixtures were transferred to 150 cc. Erlenmeyer flasks and incubated at room temperature. A test for peroxide was made after different periods of incubation, and the results corresponding to 18 hour periods are given in Table X.

It appears from the results of Experiment 10 that washed cells of Pneumococcus can form peroxide when placed in the presence of glucose and of sugar-free meat infusion under aerobic conditions. However, this property is completely lost after the cells have been washed 5 times. Peroxide formation and methylene blue reduction therefore appear as two similar reactions, in which oxygen and methylene blue respectively act as hydrogen acceptor.

The Activity of Carbon Compounds Other than Glucose in the Presence of Washed Cells of Pneumococcus

The reduction of the indophenols, of methylene blue and of the indigoes by washed cells of Pneumococcus, has been attempted in the

presence of inulin, lactose, saccharose, maltose, dextrose, levulose, mannose, arabinose, xylose, ribose, mannite and glycerine. It would be too long to report now the results of these studies. However, it may be said that the following substances have been found to be the most active (in order of velocity of reduction): levulose, dextrose, galactose, mannose, maltose, lactose. These same substances were also found to give rise to the formation of peroxide in the presence of washed cells of Pneumococcus under aerobic conditions; the test for peroxide in

TABLE X

The Formation of Peroxide by Washed Cells of Pneumococcus in the Presence of Glucose and Sugar-free Meat Infusion

Amount of cells	No. of washings	Glucose (20 per cent solution)	Meat infusion	Peroxide
cc.		*cc.*	*cc.*	
0		0.2	0.5	−
25	1	0	0	−
25	1	0.2	0	+
25	1	0	0.5	−
25	1	0.2	0.5	++
25	3	0	0	−
25	3	0.2	0	±
25	3	0	0.5	−
25	3	0.2	0.5	+
25	5	0	0	−
25	5	0.2	0	−
25	5	0	0.5	−
25	5	0.2	0.5	−

the same system was doubtful or negative when other compounds were used which bring about reduction of the methylene blue only slowly or not at all.

DISCUSSION

An interpretation of the facts which have just been presented requires an understanding of the nature of the interreaction between the glucose and the Pneumococcus cell. Is the glucose molecule split into reducing products, or does it itself become highly reactive owing to

some molecular rearrangement? The determination of the ratio between the amounts of glucose used and of methylene blue reduced is the first step in the elucidation of this problem.

As indicated above (Experiment 7), the value of this ratio seems to be affected by a number of side reactions, but a ratio very close to 1 can be obtained under proper conditions. It may be recalled here that Quastel has already attempted to determine the value of the same ratio. He found that, at pH 7.2, a single molecule of glucose donates at least 4 (and possibly 6) atoms of hydrogen to methylene blue in the presence of *B. coli*; galactose was less active and it was impossible to state whether this sugar donates more than 2 atoms of hydrogen per molecule. The least quantity of formic acid which could accomplish complete reduction of methylene blue was the amount to be expected if formic acid donated 2 atoms of hydrogen per molecule. Now, it is perhaps reasonable to assume that *B. coli* has a wider range of activation (in Quastel's terminology) than Pneumococcus, and that many of the products which result from the splitting of the glucose molecule by the cell become able to reduce methylene blue in the presence of *B. coli*, but not in the presence of Pneumococcus.[2] This would account for the narrow glucose- methylene blue ratio obtained with the organism first mentioned.

As a result of these observations, it appears likely that the first and fundamental reaction, in the reduction of methylene blue by the Pneumococcus-glucose system is one in which one molecule of the sugar becomes able to cause the reduction of one molecule of the dye, *i.e.*, to donate two atoms of hydrogen.

Nothing is known of the mechanism of the reaction. However, the following possibilities may be considered:

1. The glucose molecule is split into two fractions, each one donating one hydrogen atom.

2. The glucose molecule undergoes some rearrangement; two of its hydrogen atoms becoming "activiated." Examples of such "activation" are not unknown; succinic acid, for instance, although unable to reduce methylene blue by itself, does it in the presence of muscle tissue

[2] It has been established for instance that succinic acid, which is an excellent hydrogen donator in the presence of *B. coli* is inactive in the presence of Pneumococcus (unpublished observation).

and *B. coli* and is oxidized to fumaric acid in the course of the process. Concerning the hypothetical "activation" of glucose, it may be mentioned here that Aubel, Wurmser, Geloso, and Genevois (8) have observed the establishment of highly reducing potentials by glucose solutions at different electrodes. It would not be without bearing on this discussion to observe the potentials obtained with sterile glucose solutions compared with glucose in the presence of Pneumococcus cells.

3. Whatever may be the nature of the reducing substances formed from the glucose, another question remains to be answered. Do the glucose derivatives reduce the methylene blue directly, or do they act first on some of the cell components which in their turn reduce the hydrogen acceptor?

A knowledge of those constituents of the meat infusion which take part in the reaction would also greatly help in an understanding of its mechanism. Meat infusion contains autoxidizable substances which are able to reduce rH indicators by themselves (6); are these substances the ones which activate the system consisting of washed cells of Pneumococcus and glucose?

This is not a problem of theoretical interest only. The utilization of the nutrients in the process of growth is probably dependent upon what Quastel calls an "activation" of these nutrients by the cell under consideration (2). Is it not possible that the failure of Pneumococcus and other fastidious organisms to grow in synthetic media is due partly to the inability of these cells to "activate" the metabolites put at their disposal? It would be interesting to determine in what measure the ability to grow in synthetic media is related to the power of "activation" in the absence of meat infusion.

Experiment 8 indicates that glucose, in the presence of Pneumococcus cells, is able to reduce rapidly all the rH indicators, including indigo disulfonate. The reduction of glutathione by the Pneumococcus-glucose system is another evidence that this system can reach very negative potentials. Too little is known as yet to appreciate the significance of this reduction; however, its importance is suggested by the fact that thiol groups are essential for the growth of Pneumococcus[3] and that the addition of reduced cysteine to the medium always increases the rate of growth.

[3] Unpublished observations.

The formation of peroxide by glucose in the presence of Pneumococcus cells affords an explanation of Cole's (4) finding in 1914 that, although washed cells of Pneumococcus do not change hemoglobin to methemoglobin in the presence of air, they do so when traces of dextrose, or of certain other substances are added to the mixture. It is interesting that the substances which exhibit this property, also form peroxide in the presence of Pneumococcus cells. Ribose and inulin which fail in this particular are inactive in both instances. Since Avery and Neill (5) have shown that peroxide and methemoglobin formation are the expressions of the same mechanism, the relation between the presence of dextrose and the oxidation of hemoglobin appears evident.

Finally, it may be proper to point out briefly the significance of these observations for the characterization of bacterial cultures by their reducing properties. The bacteriological literature offers a great number of observations concerning the "reducing power" of different species of microorganisms. It is now known that sterile media themselves develop potentials corresponding to a great intensity of reduction (6, 9, 10); Coulter (11) has recently shown that typhoid bacilli, by using up the oxygen in solution in a medium, bring about the establishment of a potential similar to that which would develop after deaeration of the same sterile medium. The experiments reported here indicate that the amount and velocity of reduction of a dye in a culture depend largely on the presence of certain metabolites. What is, then, the "reducing power" of a tissue and of a culture? Is it only an expression of its ability to activate the molecules of the substrate, or does the protoplasm possess reducing properties of its own, determined by its physical and chemical constitution?

SUMMARY

The reducing power of plain broth cultures of Pneumococcus is largely dependent upon the presence in the medium at the time when the reduction test is performed of certain metabolites.

The washed cells of Pneumococcus are able to reduce the various indicators of oxidation-reduction potentials in the presence of glucose. The relative velocity of reduction of these indicators is determined by the number of cells used in the test, the concentration of the dyes, and their position in the oxidation reduction scale.

Oxidized thiol compounds (glutathione, cystine, oxidized thioglycollic acid) are likewise rapidly reduced by glucose in the presence of washed cells of Pneumococcus.

This Pneumococcus-glucose system is able to form peroxide under aerobic conditions. Those substances which form peroxide in the presence of Pneumococcus cells are also the ones which Cole found to be active in changing hemoglobin into methemoglobin under the same conditions.

The power of washed cells of Pneumococcus to reduce methylene blue in the presence of glucose is dependent on at least 2 constituents: one which can be readily removed from the cell by washing. Sugar-free meat infusion will function instead of it. The other is inactivated more slowly by the process of washing and is destroyed by 10 minutes heating at 55°C.

The interreaction between the glucose and the cell seems to result in a fundamental reaction in which one molecule of glucose becomes able to reduce rapidly one molecule of methylene blue. The existence of side-reactions often obscures this ratio.

The significance of these observations is considered in relation to the nature and mechanism of the "activation" of metabolites, the preparation of synthetic media, the phenomena of growth, and the meaning of the expression "reducing power of a bacterial culture."

ADDENDUM

There has just appeared in the *Compt. Rend Acad. Science* an article by R. Wurmser and J. Geloso (12) in which these authors present data indicating that, under anaerobic conditions, the glucose molecule changes to a new form developing a reducing intensity corresponding to rH = 8.2 at pH 7. This form seems to give rise to a reversible system of oxidation-reduction, with an rH = 15 corresponding to a mixture of 50 per cent oxidant-50 per cent reductant. Even in the presence of oxygen, the rH of this system does not go higher than 24.

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[Reprinted from THE JOURNAL OF EXPERIMENTAL MEDICINE, May 1, 1929,
Vol. 49, No. 5, pp. 883–897]

REACTIONS OF RABBITS TO NON-HEMOLYTIC STREPTOCOCCI

II. SKIN REACTIONS IN INTRAVENOUSLY IMMUNIZED ANIMALS

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In previous papers it has been shown (1, 2) that rabbits inoculated in practically any manner, except intravenously, with sufficiently large doses of certain non-hemolytic streptococci developed a condition of tissue hypersensitiveness. This was made evident by the occurrence of secondary reactions at the site of the primary inoculation, by the presence of corneal sensitivity after a certain period, by much larger reactions following intracutaneous re-inoculation than occur in normal animals inoculated with similar doses of culture; and finally by death of many of the animals following intravenous inoculation with amounts of culture well tolerated by normal rabbits. It was also shown that if the primary inoculation of the animals had been by the intravenous route, using amounts of culture and time intervals comparable with those employed in the hypersensitized (*i.e.*, intracutaneously inoculated) rabbits, these intravenously inoculated animals responded with none of these reactions of hypersensitiveness. From such observations one might have concluded that they reacted to re-inoculation in the same manner as normal animals; but on closer study it was found that their reactivity differed from that of either normal or hypersensitive animals. The object of this communication is to present the macroscopic evidence that these intravenously inoculated rabbits react differently than do normals to intracutaneous inoculation.

EXPERIMENTAL

The methods employed were comparatively simple: animals were inoculated intravenously with varying amounts of centrifugate of 18 to 24 hour blood broth cultures of non-hemolytic streptococci, and

after varying periods were inoculated into the previously depilated skin with decreasing amounts of similar cultures of homologous microorganisms. The test doses were usually 10^{-1} cc., 10^{-2} cc., 10^{-3} cc., and 10^{-4} cc., the first in 0.1 cc. volume, the other three in 0.05 cc. volumes, as it was found that with these amounts distinct differences in reaction of the various animals were made quite clear. At the time of the skin testing a group of normal control animals was inoculated in a similar manner; hence the strength of the culture used in any given experiment was determined.

Experiment 1.—Two rabbits, Q515 and Q516, were inoculated intravenously on Feb. 5 and Feb. 15, 1926, with the centrifugate of 5 cc. of culture of Streptococcus V92/0/11. On Feb. 19 they, together with 3 normal controls, Q531, Q532, Q533,

TABLE I

Average Size of Lesions following Intracutaneous Inoculation of Rabbits Previously Inoculated Intravenously, Compared with Controls

	No.	Size of inoculum		
		10^{-1} cc.	10^{-2} cc.	10^{-3} cc.
		mm.*	mm.	mm.
Intravenously inoculated	2	33	15	Negative
Normals	3	39	20	9

* Indicates average sum of 2 longest diameters of the lesions.

were inoculated intracutaneously with 10^{-1} cc., 10^{-2} cc., and 10^{-3} cc. of the homologous strain. The lesions were measured and described daily. 2 days later a striking difference in the reactions of the 2 groups of animals was evident: At the site of the 10^{-1} cc. inoculation of Rabbits Q515 and Q516 there were red, hard, shotty lesions; at the site of the 10^{-2} cc. lesions there were flat, barely palpable, pink, maculopapules, and where 10^{-3} cc. had been injected there was no macroscopic evidence of injury. The controls at this time showed larger and softer lesions resulting from inoculation of 10^{-1} cc. and 10^{-2} cc. doses and with one exception also distinct soft papules at the site of the 10^{-3} cc. inoculation.

Because all of the rabbits received intravenous inoculations 7 days later the subsequent development of the lesions was probably altered, hence the main differentiation between the 2 groups appears in the physical characteristics of the reactions and in the average size of comparable lesions in the 2 sets of animals, as shown in Table I.

The results of a different time interval between the immunizing and testing doses are shown in Experiment 2.

Experiment 2.—Each of 4 animals, Q380, Q381, Q385, and Q386, received intravenously the centrifugate of 10 cc. of blood broth culture, V92/0/10. 20 days later the reactivity of their skins, together with that of 4 controls, was tested with doses of 10^{-1} cc., 10^{-2} cc., and 10^{-3} cc. of homologous culture. The results are shown in Table II.

TABLE II

Comparison of Skin Reactivity of Rabbits Intravenously Immunized 20 Days Previously with That of Normal Animals

	Rabbit No.	Sum of diameter of lesions			Secondary reaction	Retested after 10 days; sum of diameter of lesions		
		Size of inoculum				Size of inoculum		
		10^{-1} cc.	10^{-2} cc.	10^{-3} cc.		10^{-1} cc.	10^{-2} cc.	10^{-3} cc.
		mm.	*mm.*	*mm.*		*mm.*	*mm.*	*mm.*
Immunized intravenously	Q380	55	22	16	0	44	23	16
	Q381	30	30	16	0	37	26	22
	Q385	46	26	24	0	52	24	16
	Q386	32	23	16	0	33	29	17
Average.....		41	25	18		41	25	18
Normal	Q415	36	22	16	+	76	30	24
	Q416	37	20	0	+	46	27	18
	Q417	49	25	27	0	47	25	16
	Q418	50	19	0	0	46	27	21
Average.....		43	21	11		54	27	20

It is at once obvious that the differences in the size of primary reactions between the intravenously inoculated group and normals was not so marked as in Experiment 1. Neither was the nodular character of the lesions of the immunized group so marked as in the first experiment. Nevertheless, 2 of the 4 normal rabbits showed secondary reactions at the sites of the 10^{-1} cc. and 10^{-2} cc. inoculations, while none of the immunized animals showed secondary reactions.

It thus appears that differences in the interval between immunizing inoculation and skin testing had a distinct influence on the character of the reaction following intracutaneous inoculation. When this period was short, as in Experiment 1, the differences between the

immune and normal animals were greater than in Experiment 2, where an interval of 20 days had elapsed. It is probable that the relatively slight immunity that followed the single intravenous injection of 10 cc. of streptococci was passing off by this time. Still it was sufficient to prevent the development of secondary reactions, and an accompanying general hyperergy; for the immunized group retested 10 days later showed practically the same sized lesions as when first tested, while the controls similarly retested showed distinctly larger lesions.

The effect of more prolonged continuous intravenous inoculation is brought out better in Experiment 3.

Experiment 3.—A group of 7 animals, R651, R652, R653, R654, R655, R657, and R658, were selected for immunization with culture of Streptococcus V110A. Five of them were inoculated intravenously as follows: 1st day 1 cc., 3rd day 1 cc., 5th day 2 cc., 7th day 4 cc., 12th day 5 cc. Two received only 1 cc. each on the 11th and 14th days respectively. On the 16th day all were tested with intracutaneous inoculations of 10^{-1} cc. and 10^{-2} cc. of blood broth culture of homologous streptococci; 7 normal rabbits were tested with similar doses. On the 18th day the first 5 rabbits of the first group each received the centrifugate of 4 cc. of culture intravenously and the other 2 cc. On the 27th day all received 4 cc.; thus the immunization was continued during the period in which secondary reactions might have been expected to appear.

The results of these intracutaneous inoculations are shown graphically in Charts 1 and 2. In Chart 1 are given the curves formed from daily measurements of the sum of the 2 longest diameters of the lesions, while in Chart 2 the volumes of the lesions resulting from the 10^{-2} cc. inoculations are indicated.[1] A distinct difference is immediately evident. Only 3 out of the 7 rabbits inoculated intravenously showed secondary reactions, but 2 of these had received only 2 small inoculations before the skin testing; the third showed only a slight and late secondary reaction. Six out of the 7 normal animals, on the other hand, developed secondary reactions, which in most instances usually appeared about the 8th to the 10th day; none of the immune animals, on the other hand, showed secondary reactions before the 14th day. The primary reactions of the 2 groups of animals also showed similar differences in consistency and size to those previously noted. These differences in size are brought out more strikingly by comparing their

[1] The method of calculating these volumes is given in a previous paper (2).

volumes as shown in Chart 2, where not only the greater initial size of the primary lesions, but also that of the secondary reactions in the

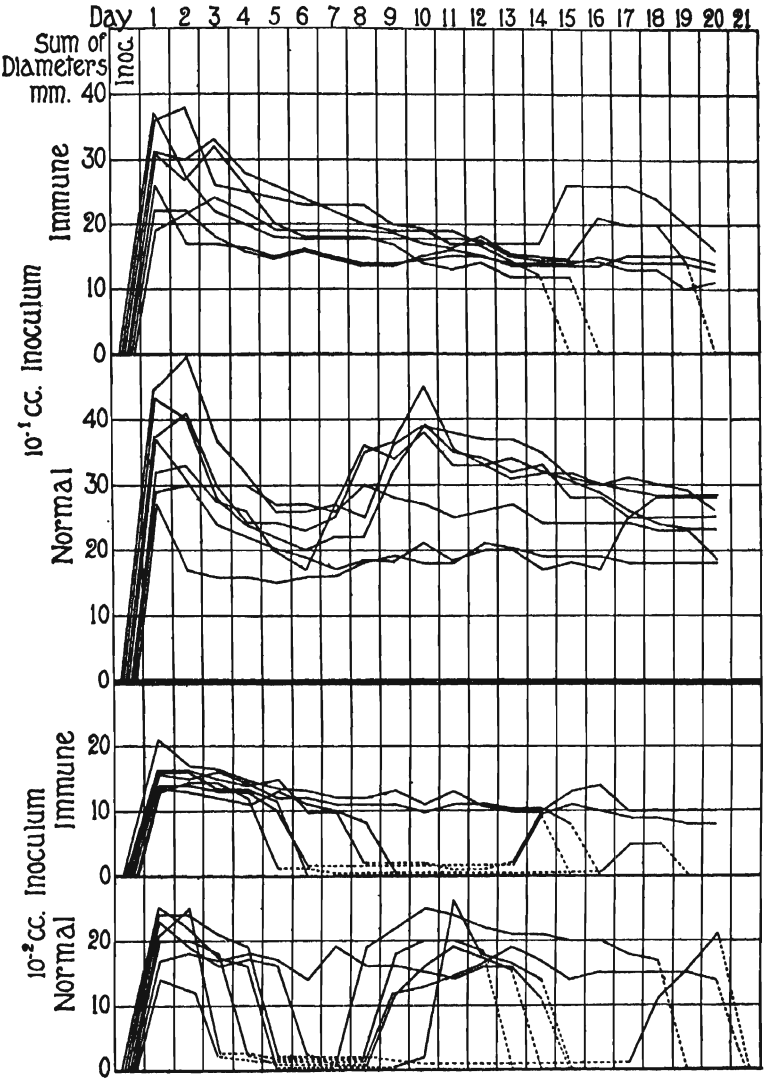


CHART 1. Comparison of sizes of lesions of immune and normal rabbits following intracutaneous inoculation with Streptococcus V110A

normal animals is made evident. The immunized animals showed only relatively small increases in their lesions at the time of their secondary reactions, while the normal animals showed very marked in-

creases. This experiment indicates how necessary it is not only to measure the diameters, but also to pay careful attention to the thickness of these lesions. The nodular character of the primary reactions in the immune group compared with softer lesions in the normal animals was observed here as in previous experiments.

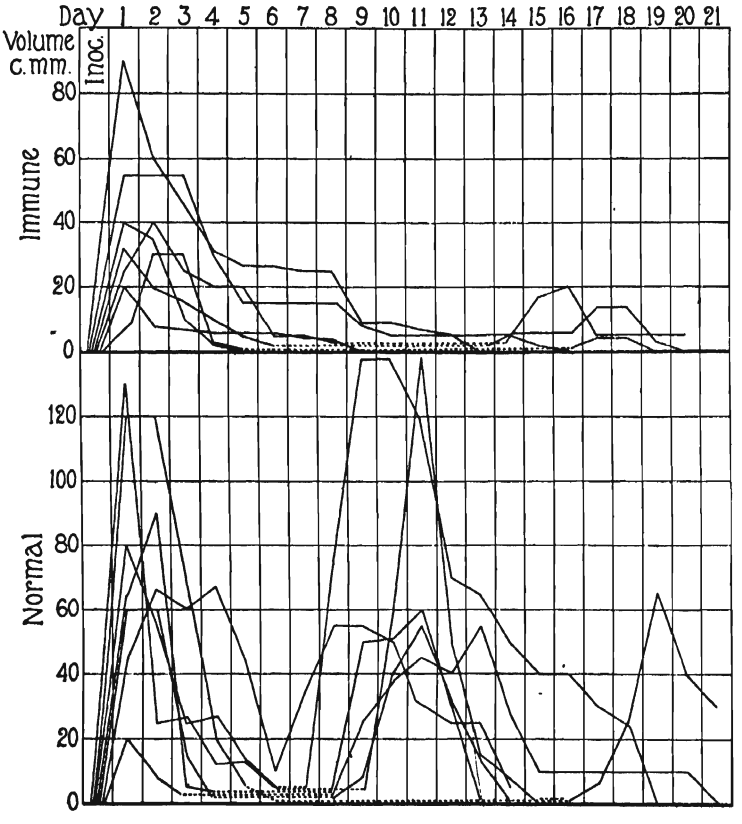


CHART 2. Comparison of volumes of lesions of immune and normal rabbits following intracutaneous inoculation with 10^{-2} cc. of blood broth culture of Streptococcus V110A

In view of the fact that such differences in the tissue response could be demonstrated when animals were previously inoculated intravenously with living organisms, it was decided to investigate whether or not a similar alteration in responsive capacity could be detected if a bacterial fraction were used as the immunizing agent. It had been previously demonstrated by Lancefield (3) that the nucleoprotein ex-

tract of green streptococci was the only fraction with a definite antigenic capacity. She, therefore, prepared a fairly large quantity of nucleoprotein of Streptococcus V92 with which a group of rabbits was immunized intravenously, as indicated in Experiment 4.

Experiment 4.—Eight rabbits were injected intravenously with a normal saline solution of green streptococcus nucleoprotein as follows: 1st, 2nd and 3rd days, 10 mg. each day; 9th, 10th, 11th and 12th days, 20 mg. each day; 17th, 18th, 19th and 20th days, 30 mg. each day. On the 26th day each rabbit received intracutaneous inoculations with the centrifugate of blood broth culture of Streptococcus V92 in the following amounts, 5 cc., 5 cc., 10^{-1} cc. and 10^{-2} cc.; 4 normal controls were similarly inoculated. The curves showing the reactions of 4 of the immunized animals compared with the 4 controls are shown in Charts 3 and 4. Only the curves of the 10^{-1} cc. and 10^{-2} cc. lesions are given because the intensity of the response to 5 cc. of culture was so marked in all rabbits that differences in the 2 groups were not made so evident as with the smaller inocula.

While differences in the diameters of the primary lesions in the 2 groups of animals were not very marked, it became apparent, as the evolution of the lesions was followed, that there was a distinct difference in the response of the animals. All of the normal controls showed well marked secondary reactions in all of their lesions, while only 3 of the 8 immunized animals developed secondary reactions in the smaller lesions; and in 2 instances these were slight and delayed. The striking difference in the character of these secondary reactions is brought out in Chart 4, where the volume of the 10^{-2} cc. lesions of 4 of the immunized rabbits is compared with similar lesions in the 4 controls.

While the average volume of the primary lesions was 69 c.mm. in the immunes compared with 94 c.mm. in the controls, that of the secondary reactions was 25 c.mm. in those developing these reactions compared with 230 c.mm. in the controls. It is evident, therefore, that immunization with nucleoprotein over a period of approximately 4 weeks had altered the rabbits' type of response towards intracutaneous inoculation in the direction of a diminished intensity of reaction. It must be realized that the amount of nucleoprotein used represented large bacterial growths, and that probably a similar period of immunization with a corresponding amount of vaccine would have been more efficacious. The main point to be gathered from this ex-

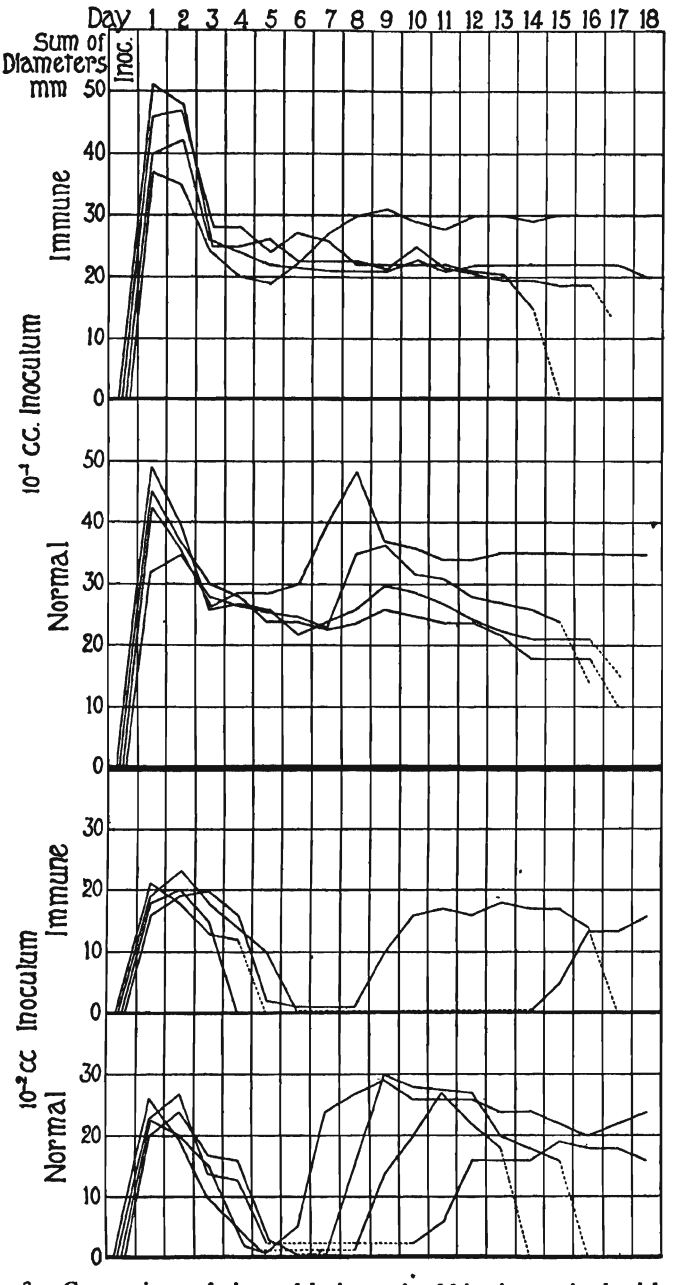


CHART 3. Comparison of sizes of lesions of rabbits immunized with nucleoprotein of green streptococci and normal rabbits following intracutaneous inoculation with Streptococcus V92

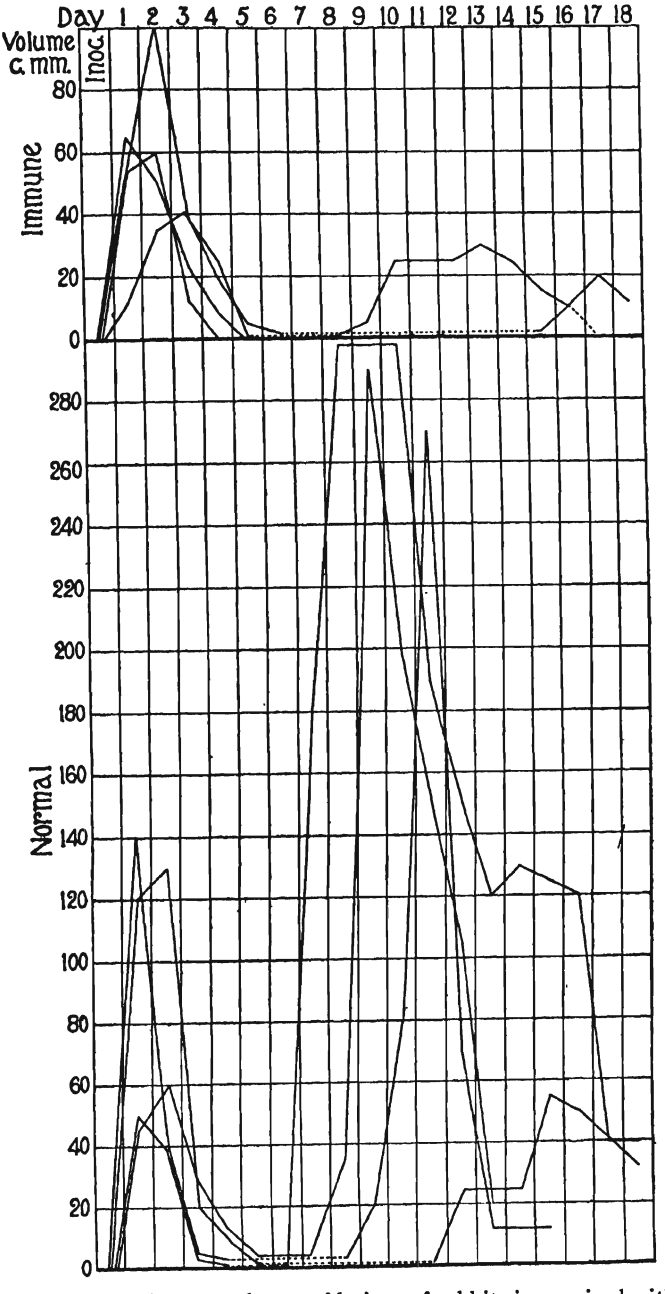


CHART 4. Comparison of volumes of lesions of rabbits immunized with nucleoprotein of green streptococci and normal rabbits following intracutaneous inoculation with 10^{-2} cc. of blood broth culture of Streptococcus V92

periment was that the nucleoprotein fraction carried the immunizing substance.

As a final experiment the effect of prolonged intravenous immunization was studied as shown in Experiment 5.

Experiment 5.—Twelve rabbits were inoculated intravenously over a period of 9 weeks. In the beginning 0.5 cc. of whole blood broth culture of Streptococcus V110A was employed. This was gradually increased to 2 cc. after 10 days; but the rabbits began to lose weight so rapidly that immunization was discontinued for a week, then recommenced with 1 cc. of heat killed culture. This, in turn, was gradually increased until 5 cc. doses were given; subsequently immunization with living culture was resumed without depressing the rabbits. During the succeeding period of 4 weeks 9 inoculations were given by gradually increasing the dose from 1 cc. to 4 cc. of living blood broth culture. At the end of this period the sera of all the rabbits showed a precipitin titer of 1:4000 to 1:8000 when tested against nucleoprotein prepared from Streptococcus V110A. The agglutinin titer of the sera could not be determined because of spontaneous agglutination of the cultures.

After this course of 9 weeks' immunization the skin reactivity of all the rabbits together with that of 4 normal controls was tested with Streptococcus V110A/0/5 in 6 doses of from 10^{-1} cc. to 10^{-6} cc. None of the animals showed reactions at the sites of the 10^{-4} cc., 10^{-5} cc., and 10^{-6} cc. inoculations. The maximum and average sizes of the lesions in the 3 larger lesions in all of the animals are shown in Table III; also the volumes of the 10^{-2} cc. and 10^{-3} cc. lesions are given.

While quantitative variations in the response of the different animals occurred, there was almost a uniform tendency for the immunized animals to show smaller lesions than the controls. The ratio of the averages in the immune group compared with the normals at the sites of the different inocula was 1^{-1} cc. 2:3; 10^{-2} cc. 1:2; 10^{-3} cc. 2:3. When volumes of lesions are compared, just as in previous experiments, the differences are even more striking. In the 10^{-2} cc. lesions the average ratio was 1:3, and in the 10^{-3} cc. lesions 4:9. The daily notes of the consistency of the lesions also brought out striking differences. The immune animals had very hard shotty lesions, particularly at the sites of 10^{-2} cc. inoculations, while the normal animals showed some edema with only moderately infiltrated lesions from the 1st to the 3rd days. These differences were so striking that the immune animals could be readily differentiated from the controls by simple comparison of the size and character of the corresponding lesions.

None of the immune animals showed secondary reactions in any of

their lesions, while 3 out of the 4 controls developed secondary reactions. It may be objected that the differences in the size of the 2 groups tend to invalidate the comparisons, but all of 4 other normal rabbits inoculated with a corresponding culture 2 days later developed primary lesions and secondary reactions similar to these 4 controls; so it may

TABLE III

Comparison of Skin Reactivity of Rabbits Immunized Intravenously Over a Long Period with Normal Controls

	Rabbit No.	Sum of diameters of lesions			Volume of lesions	
		Size of inoculum			Size of inoculum	
		10^{-1} cc.	10^{-2} cc.	10^{-3} cc.	10^{-2} cc.	10^{-3} cc.
		mm.	*mm.*	*mm.*	*c.mm.*	*c.mm.*
Immunized intravenously	1119	40	17	12	98	15
	1120	35	17	v. and p.*	90	v. and p.*
	1122	42	21	13	117	17
	1123	34	19	12	108	6
	1124	24	16	9	50	2
	1125	40	25	15	165	22
	1131	38	16	12	10	14
	1135	47	18	10	54	2
	1136	53	28	20	188	60
	1138	38	20	14	106	20
	1139	33	20	15	100	12
	1140	42	28	15	157	22
Average.....		39	20	12	110	16
Normal	1253	70	39	20	517	55
	1254	52	27	15	150	10
	1255	53	34	18	315	48
	1256	78	38	17	423	45
Average.....		63	37	17	334	37

* Non-measurable, visible and palpable.

be safely concluded that these controls represented the average response of normal animals at this period. This experiment, therefore, indicates that prolonged intravenous immunization causes a group of animals to react to subsequent intracutaneous inoculation in a more uniform manner than does short immunization such as was carried out with living streptococci in the earlier experiments, or with streptococcus nucleoprotein as in Experiment 4.

DISCUSSION

It is common knowledge that previous inoculation alters the reaction of an animal towards an infectious agent; in fact upon this knowledge are based our efforts towards the prevention and cure of infectious diseases where the various techniques of immunology are employed. In certain conditions the outstanding defensive agents are easily demonstrable in the body fluids in the form of antitoxins, opsonins or similar antibodies. In other diseases, where the invasive microorganisms have relatively low virulence, or elaborate comparatively little exotoxin, the defensive mechanism seems to reside in the cells rather than in the humors of the body. But in most infections both humoral and cellular factors apparently play a rôle; and in proportion as one or the other predominates, so, in part at least, may differences in the focal manifestations of infection and resistance be explained.

Following Pirquet's (4) observations and introduction of the term allergy, studies of the local manifestations of reinfection have assumed a constantly increasing importance, until at the present day an enormous literature has grown up about this word. It seems to be quite generally accepted that the phenomenon of allergy represents a very intense local effort on the part of the animal to limit the activity of infectious agents or foreign substances to the site where they gain entrance into the animal's body. It is also obvious that local reactions are quite different following the introduction of such agents as vaccinia, tubercle bacilli, egg albumin or primin into suitably allergized animals or men. Re-inoculation with vaccine virus results in smaller local injury than occurs at the time of first inoculation, while reinjection with egg albumin leads to more marked local reaction, which increases in intensity with increase of circulating antibodies (5). Re-inoculation with tubercle bacilli, on the other hand, is followed by destructive localized tissue reaction, the Koch phenomenon, without any corresponding increase of antibodies in the blood serum. Repeated treatment of the skin with primin, a nitrogen free ether extract of primrose results in a type of allergy made manifest by an eczematous inflammation of the skin (6). In these four examples of allergy the differences in local response may be explained in part by differences in the nature of the antigen or inciting agent. Following the introduction of tubercle bacilli into the body tubercles are formed

with an accompanying general state of tuberculin allergy regardless of the presence of demonstrable circulating antibodies. Egg albumin, on the other hand, may be introduced intravenously into an animal without producing gross focal injury; nevertheless with the development of antibodies a state of allergy ensues. It is therefore apparent, as pointed out by Zinsser (7), Doerr (8), Coca (9), and others, that the allergy induced by bacteria and by coagulable proteins is different. In practically all instances in which a tuberculin-like allergy is induced this follows some focal tissue reaction resulting from injury by the respective micro-organism.

But, in so far as we are aware, no comparison has previously been made of the type of tissue reaction which follows the introduction of bacteria into the body so that in the one instance gross lesions are produced and in the other no macroscopic lesions are formed. One difficulty attending such experimentation has been due to the fact that most bacteria, previously used in the study of allergy, induced focal lesions regardless of the route employed for inoculation. But with non-hemolytic streptococci, such as we have employed, these conditions could be more easily controlled. Strains with which it was possible to induce a condition of tuberculin-like allergy when they were injected into the tissues, could also be used to decrease the animal's tissue reactivity when they were injected intravenously in suitable doses. Ordinarily streptococci of this type quickly disappear from the blood stream without producing gross lesions; but in some instances, especially when large doses are employed, the animals develop endocarditis or arthritis. In one rabbit of Experiment 3, in which an arthritis of the wrist had followed the early intravenous inoculation, the skin test inoculation was followed by lesions of the hypersensitive rather than of the immune type. This isolated example taken in conjunction with other experiments previously reported (2) supports the viewpoint that focal lesions are probably necessary for the development of hypersensitiveness of infection.

Another point worthy of note is that intravenously immunized rabbits when inoculated intracutaneously with suitable doses show lesions of a different character than do either normal or hypersensitive animals, lesions that have little if any edema and are hard and firm after 24 to 48 hours. In fact these lesions show much less change in size

after 2 days than do the lesions in other types of animals. The larger inocula often are followed in 4 or 5 days by globular sac-like areas persisting for weeks, and smaller inocula produce small hard persistent nodules. In other words, the entire and complete reaction seems to take place much more quickly in the immune than in other states of allergy. The microscopic comparison of the different types of lesions will be reported in a later communication.

Finally, for purposes of completeness it should be noted that certain rabbits give reactions following intracutaneous inoculation which differ from any of those previously described by us. These reactions are usually less marked than those of normal animals, are soft, have very little color, fade rapidly, and do not show secondary reactions. They occur in rabbits which appear sick, either due to an overwhelming infection from streptococci, or from any other cause. These animals are usually emaciated and have a skin which is wrinkled, gray and lacking in tone. We have designated these reactions "cachectic," and after a little experience have learned to recognize them within 2 or 3 days following intracutaneous inoculation. Obviously the inclusion of such animals in any group is to be avoided for they vitiate statistical comparisons.

Within recent years it has become evident to many observers that different states of resistance towards a given bacterium could be indicated by differences in cutaneous reactivity. For example, a patient showing well marked positive reaction to intracutaneous injections of tuberculin may lose this skin reactivity during an attack of measles or other infectious disease, or if he becomes cachectic from a neoplasm or even from an overwhelming tuberculous infection. This has been designated as negative anergy, and corresponds with what we call cachectic reaction. It has also been observed that an animal may show decreasing skin reactivity to a certain fixed dose of a testing bacterial extract while it is becoming more resistant to a general infection with the same bacterium (10). This state has been designated as positive anergy, and probably corresponds with our designation, immune reaction. It seems probable, then, that the term allergy will have to be qualified in some manner, if it is to be continued as a means of expressing the idea of increased resistance accompanied by over-reaction of the tissues. For this reason we have at times used the term *hyperergy* which has been employed by a number of German

pathologists (11, 12) to indicate a hyper-reactivity of the tissues, and the terms immune or cachectic to indicate gross decreases in reactivity according to the manner in which this decreased tissue reactivity is induced.

SUMMARY

Rabbits immunized intravenously with living culture or nucleoproteins of non-hemolytic streptococci react to subsequent intracutaneous inoculations with homologous streptococci with smaller and harder lesions than are shown by normal animals similarly inoculated; and they do not develop the general manifestations of hypersensitiveness such as are shown by animals previously inoculated into the tissues with the same cultures.

A rabbit may react to intracutaneous inoculation with non-hemolytic streptococci in one of four ways, depending on whether it is normal, hypersensitive, immune or cachectic. Most normal animals show a secondary reaction about 10 days after inoculation with suitable strains of non-hemolytic streptococci; hypersensitive, allergic, or hyperergic animals show much larger lesions than do normals with the corresponding doses of the same streptococci, and practically never show secondary reactions; immune animals show smaller and harder early lesions and usually do not have secondary reactions if they are fairly well immunized. Cachectic animals show very soft and rapidly fading primary reactions and no secondary reactions.

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[Reprinted from THE JOURNAL OF EXPERIMENTAL MEDICINE, June 1, 1929,
Vol. 49, No. 6, pp. 899–906]

RELATION OF VARICELLA TO HERPES ZOSTER

I. STATISTICAL OBSERVATIONS

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(Received for publication, February 13, 1929)

Varicella, herpes simplex, and herpes zoster are diseases characterized by vesicular eruptions in the skin. Macroscopically and microscopically the individual lesions (1–4) in these maladies are frequently very similar. In spite of the striking similarity exhibited by the individual lesions, the picture as a whole, *i.e.*, distribution of the rash and immune reactions, is as a rule characteristic for each disease.

It is now generally believed that varicella and varioloid are caused by different viruses. Such a remark at present is trite, but a hundred years ago the difference between the diseases was questioned by many observers, and certain workers (5), even 30 years after Jenner's report on vaccination, still thought that chicken-pox and smallpox were produced by the same infectious agent.

Herpes simplex is so frequently associated with other diseases, *e.g.*, pneumonia, meningitis, malaria, that the name of symptomatic herpes is often used in referring to it. Through the recent work of Grüter (6), Löwenstein (7), and others, it is now known that this type of herpes, occurring alone or in connection with other diseases, is caused by a specific virus. As soon as it was admitted to the virus group, a discussion arose regarding its relation to herpes zoster. From Cole and Kuttner's (8) review of the literature and from their work concerning the identity of the diseases, it seems unlikely that the majority of cases of herpes simplex and herpes zoster are caused by the same virus.

Herpes zoster, because of its frequent association with injury of nerves and spinal ganglia, for a long time (1861) was considered to be a manifestation of trophic disturbances in the skin attendant upon altered innervation. This conception, however, of the etiology was not applicable to all cases of zoster, and soon (1878) certain cases came to be looked upon as being caused by an infectious agent. In 1900, Head and Campbell (9) reviewed the literature and presented their work upon the subject. They concluded, because of the changes observed in the spinal ganglia and nerves, in the skin, and in the lymph nodes, that many of their cases of zoster were produced by a specific virus. Other cases, however, were thought to be caused not by a virus but by a mechanical or chemical irritation of spinal ganglia.

In 1909 Bókay (10) set forth a hypothesis, based on clinical observations, that some cases of zoster (herpes zoster varicellosa) are caused by the virus of chicken-pox and that such cases are capable of giving rise to varicella in exposed susceptible individuals. Following Bókay's report, numerous observations confirming or refuting his idea have been recorded. Most of the reports are similar to those made a hundred years ago in regard to the relation of chicken-pox to varioloid and smallpox and consist in recording the occurrence of varicella in individuals exposed to zoster and *vice versa* or in relating the coexistence of the two diseases in the same person or in individuals in the same family. Chicken-pox is a common disease and zoster is not rare (11), consequently reports of the nature above mentioned are of little value, inasmuch as coincidence alone doubtless accounts for the apparent relation of the two diseases in certain instances. There are reports, however, of a different nature that must be considered seriously. These will be discussed in the second paper of this series.

The real problem regarding the diseases under discussion is not whether the virus of herpes simplex under rare conditions can cause a disease indistinguishable from zoster, or whether the virus of varicella under the proper circumstances is capable of producing a malady similar to zoster and *vice versa*, but whether there are 3 distinct viruses, though they be very much alike in certain respects, each of which as a rule causing a distinct clinical picture, herpes simplex, herpes zoster, and varicella. If the same virus is instrumental in producing the majority of the cases of any 2 of the diseases, certain types of statistical data should reveal this fact. The purpose of the present paper is to present such data that seems to bear upon the identity of the causal agents of the majority of cases of herpes zoster and varicella.

Methods and Materials

Herpes Zoster.—The records of cases of herpes zoster at the Bellevue and Vanderbilt clinics were examined and from them information concerning the monthly incidence and the age incidence of the disease was obtained. The selected clinics are large and their records undoubtedly are good indices of the prevalence of zoster at any given time throughout New York City.

Varicella.—The data concerning the monthly incidence of chicken-pox in New York City from Jan., 1914, through July, 1926, and from Aug., 1926, through Dec., 1926, were obtained respectively from the Monthly and the Weekly Bulletins of the Department of Health of the City of New York. The information regarding the age incidence of varicella was derived from the records of cases of chicken-pox observed at the Rockefeller Hospital 1922–28.

TABLE I

Tabulation of the Number of Cases of Varicella Reported Each Month for 13 Years in New York City

Year	Jan.	Feb.	Mar.	Apr.	May	June	July	Aug	Sept.	Oct.	Nov.	Dec.	Total
1914	1,121	1,156	1,222	1,217	1,451	1,104	319	85	56	315	574	1,112	9,732
1915	1,341	1,134	1,423	1,073	989	1,220	277	89	77	149	288	661	8,701
1916	956	874	1,439	1,623	1,747	1,331	430	70	44	303	325	631	9,779
1917	874	876	1,294	957	1,212	1,183	217	78	64	141	584	906	8,386
1918	785	598	559	441	542	363	175	95	56	136	174	272	3,776
1919	414	412	599	693	963	786	187	67	63	169	287	686	5,326
1920	726	492	536	467	629	680	227	80	74	230	485	908	5,534
1921	1,212	1,166	1,311	924	939	806	263	49	59	119	410	683	7,941
1922	841	656	663	540	673	490	131	48	67	207	475	947	5,738
1923	1,289	997	780	785	1,235	1,259	398	134	65	195	538	996	8,471
1924	1,155	1,048	1,200	1,000	857	767	230	108	83	318	636	858	8,260
1925	845	864	909	798	871	1,211	250	86	68	164	588	892	7,646
1926	1,091	874	645	456	542	731	343	111	57	241	650	963	6,704
Total.....	12,650	11,147	12,580	10,974	12,650	11,951	3,447	1,100	833	2,687	6,014	10,521	95,994
Mean 1914–26.	973	857	967	844	973	919	265	84	64	206	462	809	7,384
Mean 1922–26.	1,044	708	839	716	836	892	270	97	78	225	577	931	

TABLE II

Tabulation of the Number of Cases of Herpes Zoster Observed Each Month for 13 Years at the Bellevue Clinic

Year	Jan.	Feb.	Mar.	Apr.	May	June	July	Aug.	Sept.	Oct.	Nov.	Dec.	Male	Female
1914	2	2	1	5	1	3	2	3	1	2	1	2	14	11
1915	3	0	5	0	2	4	1	2	4	3	2	2	14	14
1916	0	1	2	2	2	0	3	1	1	1	2	4	11	8
1917	1	2	1	3	2	0	0	2	4	2	2	2	12	9
1918	6	0	2	0	2	4	2	1	3	2	2	0	15	9
1919	4	5	5	1	2	4	0	2	1	4	0	3	19	12
1920	2	3	3	2	3	3	0	4	3	3	2	3	19	12
1921	2	3	5	2	1	3	5	4	3	3	4	2	24	13
1922	8	5	2	3	7	2	4	2	4	3	7	1	35	13
1923	4	3	2	7	7	5	4	2	2	4	1	5	32	14
1924	2	2	3	3	4	4	3	2	3	6	4	3	30	9
1925	5	3	4	3	3	5	2	3	5	0	2	3	31	7
1926	4	5	0	2	5	3	4	6	4	0	1	4	24	14
Total.....	43	34	35	33	41	40	30	34	38	33	30	34	280	145
Mean 1914–26..	3.3	2.6	2.7	2.5	3.2	3.1	2.3	2.6	2.9	2.5	2.3	2.6		
Mean 1922–26.	4.6	3.6	2.2	3.6	5.2	3.8	3.4	3.0	3.6	2.6	3.0	3.2		

TABLE III

Tabulation of the Number of Cases of Herpes Zoster Observed Each Month for 5 Years at the Vanderbilt Clinic

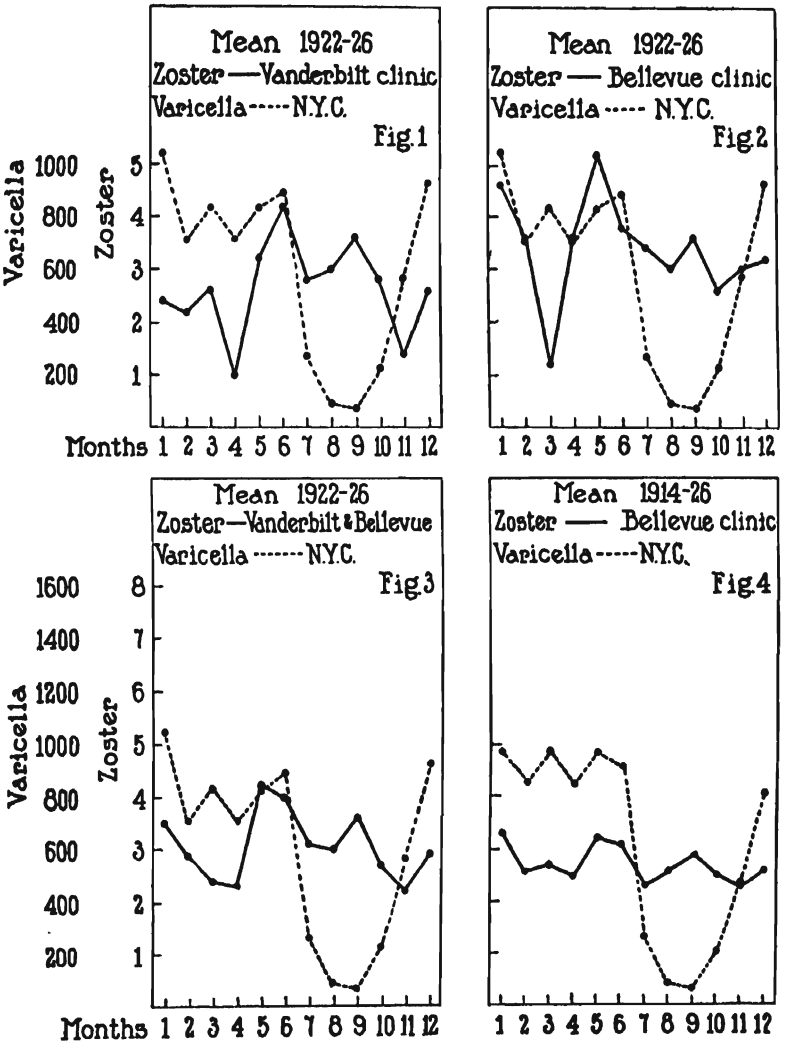
Year	Jan.	Feb.	Mar.	Apr.	May	June	July	Aug.	Sept.	Oct.	Nov.	Dec.
1922	3	2	3	0	3	5	1	4	1	1	3	2
1923	1	3	2	1	5	5	3	5	2	6	2	5
1924	4	2	2	0	2	5	5	5	5	1	0	0
1925	3	4	3	3	2	2	2	1	8	1	1	2
1926	1	0	3	1	4	4	3	0	2	5	1	4
Total...	12	11	13	5	16	21	14	15	18	14	7	13
Mean...	2.4	2.2	2.6	1	3.2	4.2	2.8	3.0	3.6	2.8	1.4	2.6

RESULTS

Monthly Incidence of Zoster and Varicella

In New York City during the 13 years, 1914–26, 95,994 cases of chicken-pox were reported. In Table I these cases have been set down

in a manner to indicate the incidence of the disease for each month during the 13 years. At the Bellevue clinic, 425 cases of herpes zoster were observed during the same period of 13 years, and 159



FIGS. 1–4. Curves showing the monthly incidence of varicella in New York City and herpes zoster in the Vanderbilt and Bellevue clinics

cases were registered at the Vanderbilt clinic during the 5 years, 1922–26. In Tables II and III these cases of zoster have been arranged according to their monthly incidence. The significant features of the

information in Tables I to III have been graphically portrayed in Figs. 1 to 4.

Examination of the tables and figures dealing with the monthly incidence of varicella and zoster reveals the fact that a remarkably constant seasonal variation occurs in the number of cases of chicken-pox reported, while such variation in the prevalence of zoster is not very obvious. In fact, when the mean of a large number of cases over a period of many years is plotted (Fig. 4), the curve indicating the monthly incidence of zoster approaches a straight line.

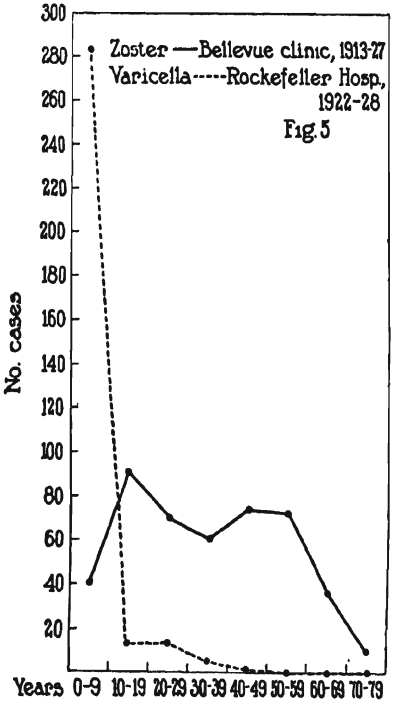


FIG. 5. Distribution curves illustrating the age incidence of varicella and herpes zoster

Age Incidence of Zoster and Varicella

In Table IV the age of 457 cases of zoster and of 318 cases of chicken-pox has been indicated. From the data in Table IV, distribution curves (Fig. 5) for the 2 diseases have been plotted. From the facts presented in Table IV and Fig. 5, it is quite evident that varicella occurs for the most part in individuals under 10 years of age, while zoster most frequently appears in the 2d, 3d, 4th, 5th and 6th decades of life. Obviously there is a striking difference in the age incidence of these diseases.

DISCUSSION

The difference in the age incidence of herpes zoster and varicella has been well recognized for a long time (11) and facts concerning it are again presented in this paper merely for emphasis. That there is a constant seasonal variation in the prevalence of chicken-pox is also well known, and certain authors (9, 12) have recorded observations concerning the monthly incidence of zoster. Up to the present time,

however, very few attempts have been made to compare the number of cases of these diseases occurring simultaneously in the same locality. Perutz (13) made such a comparison in 2 districts of Vienna for the years 1923–25 and found that the curves of monthly incidence of the 2 diseases did not parallel each other. Moreover, Cantor (14) reports that on Christmas Island,[1] Straits Settlement, where excellent vital

TABLE IV

A Tabulation of Varicella and Herpes Zoster Cases Showing the Difference in the Age Incidence of the 2 Diseases

Herpes Zoster, Bellevue Clinic, 1913–27, 457 Cases
Varicella, Rockefeller Hospital, 1922–28, 318 Cases

Age	Zoster	Varicella	Age	Zoster	Varicella	Age	Zoster	Varicella	Age	Zoster	Varicella
0	1	18	19	13	2	38	8	1	57	6	0
1	1	32	20	7	5	39	3	0	58	8	0
2	1	27	21	6	0	40	12	1	59	6	0
3	4	48	22	6	1	41	7	0	60	9	0
4	2	38	23	6	1	42	10	0	61	2	0
5	6	45	24	4	0	43	5	0	62	4	0
6	6	39	25	8	4	44	6	0	63	0	0
7	7	17	26	13	0	45	7	0	64	5	0
8	7	11	27	10	3	46	8	0	65	6	0
9	6	8	28	4	0	47	5	0	66	4	0
10	11	4	29	7	1	48	5	0	67	1	0
11	11	2	30	4	0	49	10	0	68	3	0
12	9	2	31	5	0	50	12	0	69	3	0
13	13	1	32	9	1	51	6	0	70	1	0
14	10	2	33	5	2	52	11	0	71	0	0
15	3	2	34	7	0	53	7	0	72	2	0
16	9	0	35	8	0	54	5	0	73	2	0
17	6	0	36	6	0	55	5	0	74	2	0
18	6	0	37	5	0	56	6	0	75	3	0

statistics have been kept for 20 years, chicken-pox is unknown, while the usual amount of herpes zoster is observed. The statistical observations recorded in the present paper are further evidence in favor

[1] This island was uninhabited before 1888. The population, 500 to 1500, consists of Europeans, Indians, Malays, and Chinese. Both sexes and all ages are represented.

of the idea that the majority of cases of herpes zoster is not caused by the virus of varicella.

SUMMARY

Varicella most frequently occurs in individuals under 10 years of age, while zoster as a rule is observed in persons beyond that age. The number of cases of varicella exhibits a markedly constant seasonal variation. The variations in the prevalence of herpes zoster are not regular and do not parallel those of varicella.

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[Reprinted from THE JOURNAL OF EXPERIMENTAL MEDICINE, June 1, 1929,
Vol. 49, No. 6, pp. 907–917]

RELATION OF VARICELLA TO HERPES ZOSTER

II. CLINICAL AND EXPERIMENTAL OBSERVATIONS

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(Received for publication, February 13, 1929)

In the preceding paper (1) the problem relating to the identity of the causal agents of varicella and herpes zoster was stated. The mere occurrence of varicella in an individual following exposure to herpes zoster might easily be explained upon coincidence. Certain other observations, however, dealing with complement fixation work, inoculation experiments, and protection tests conducted with convalescent sera deserve serious consideration.

Netter (2) and Netter and Urbain (3), de Lange (4), and others, because of their results with complement fixation tests, contend that the etiological agents of zoster and varicella are quite frequently identical. Lauda and Silberstern (5), however, were unable to confirm these observations. Kundratitz (6) and Lipschütz and Kundratitz (7) inoculated 28 children under 5 years of age with material from herpes zoster patients in the hope of immunizing them against chicken-pox. In 17 of the children a local reaction, characterized by papules and vesicles, was observed at the point of inoculation. The children were subsequently found to be immune to varicella. These writers also claim that injections of convalescent herpes zoster serum will protect exposed children against varicella. Lauda and Stöhr (8) inoculated 54 children with material from 17 cases of herpes zoster, and in none of them did they observe a local reaction at the site of the inoculation. Three of the inoculated children and 3 other uninoculated infants who came in contact with some of the zoster patients developed typical chicken-pox 2 or 3 weeks after exposure. These workers were unable to demonstrate that convalescent zoster serum protects against varicella and conclude from the results of their work that the majority of cases of zoster are not caused by the virus of chicken-pox.

Chicken-pox is a highly contagious disease, but for a long time doubt existed in regard to its inoculability. A great deal of this doubt arose from the fact that most of the experimental inoculations were made during epidemics of varicella and exposures to the disease under natural conditions could not absolutely be eliminated. Nevertheless, in spite of these difficulties, it has been shown (9) that

varicella is inoculable. Successful experiments, however, are not as constant as one might suppose when considering the extremely contagious nature of the disease. Evidence is accumulating in favor of the idea that zoster also is caused by a virus, but the question as to its inoculability in humans is still moot.

If it were possible without difficulty to produce varicella and zoster in animals, the relation of the two diseases would be settled quickly. It is very doubtful if the virus that causes the majority of cases of zoster has been propagated in experimental animals. With one exception this is also true in regard to varicella. Certain kinds of monkeys (*Cercopithecus sabæus* and *Cercopithecus lalandi*) are susceptible to the virus of chicken-pox. Even in these animals the results are irregular and the evidence of infection consists of the occurrence of acidophilic nuclear inclusions in affected cells of inoculated testicles. These inclusions, however, do not occur in controls and their appearance is regularly prevented by convalescent varicella serum (10, 11). In view of the regularity with which convalescent varicella serum prevents these inclusions in testicles inoculated with chicken-pox virus, it seemed likely that information regarding the identity of varicella and zoster might be obtained by conducting neutralization tests with varicella virus and convalescent zoster serum. It is with observations concerning these experiments that the present paper deals.

Methods and Materials

Monkeys Employed.—Young male green monkeys (*Cercopithecus sabæus*) were used. Animals in which spermatogenesis had been established were discarded.

Neutralization Tests.—Emulsified papules and vesicles collected from varicella patients, usually within the first 72 hours of the disease, were used as virus. Non-immune serum was secured from chicken-pox patients during the first 72 hours of disease. Immune varicella serum was obtained from convalescent patients 14 to 22 days after the appearance of the rash. Immune zoster serum was collected from convalescent patients 23 to 28 days after the appearance of the eruption.

The papules and vesicles were excised under aseptic conditions and emulsified by grinding in a mortar moistened with Locke's solution. Sand was not used. The emulsified material was taken up in 0.5 to 2.0 cc. of Locke's solution and portions of it were mixed as desired with equal amounts of non-immune serum, immune varicella serum, or immune herpes zoster serum. Measured amounts (0.25 cc.) of the mixtures were then injected into the testicles of monkeys. More than 45 minutes never elapsed between the collection of the varicella virus and its injection into the animals.

Removal and Examination of Testicles.—It was previously shown (10, 11) that nuclear inclusions are present in the testicles of green monkeys on the 5th and 6th days after inoculation with chicken-pox. Consequently, in the experiments reported at the present time, the monkeys were castrated[1] on the 5th or 6th day following inoculation. The testicles removed for histological studies were fixed in Zenker's fluid, sectioned, and stained with eosin and methylene blue. A careful search for eosin-staining nuclear inclusions was made in numerous sections of each testicle. Details concerning the tinctorial reactions of the inclusions are given by Tyzzer (12), Lipschütz (13), and Goodpasture (14).

Results.—Testicles in which nuclear inclusions were found were considered infected with varicella virus. If no inclusions were found, infection was considered not to have occurred. In this type of work, positive results are naturally much more significant than are negative ones.

EXPERIMENTAL

In the study of the relation of varicella to herpes zoster 4 neutralization experiments were performed.[2] A detailed account of each follows.

Experiment I

The first experiment was conducted in order to determine whether the serum of an adult, who had had chicken-pox in childhood, would neutralize varicella virus.

Serum was collected from J., 35 years of age, who had chicken-pox when 5 years old. Non-immune serum was obtained from W. R. during the first 48 hours after the appearance of the varicella rash. Virus in the form of 4 varicella vesicles was secured from B. The serum to be tested, from J., was mixed with an equal amount of virus, and then 0.25 cc. of the mixture was injected into each testicle of Monkey 1. Non-immune serum, W. R., was also mixed with virus, and 0.25 cc. of the mixture was injected into each testicle of Monkey 2. 5 days later the testicles were removed and examined in the usual manner for the presence of acidophilic nuclear inclusions. They were found in the testicles of both monkeys.

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[1] All operative procedures were conducted under anesthesia.

[2] Because of the scarcity of green monkeys, control tests with convalescent varicella serum were at times omitted. In fact, it is not essential to use this control with each experiment, inasmuch as convalescent varicella serum has never failed to prevent the appearance of inclusions in testicles inoculated with varicella virus (11).

The results of the above experiment indicated to us that the serum from an individual who had had varicella many years previously did not contain sufficient antibodies to prevent the appearance of inclusions in testicles inoculated with chicken-pox virus. Consequently it was deemed safe in this work to use convalescent zoster serum obtained from individuals who a number of years earlier in life had passed through an attack of varicella.

Experiment II

In this experiment an attempt was made to ascertain whether the serum from a case of so called idiopathic herpes zoster was capable of neutralizing varicella virus.

C. S., male, white, 16 years old. No history of having had chicken-pox or zoster previously. No exposure recently to either disease. Patient seen on the first day of the zoster eruption which was distributed over the right sacral and iliac regions; serum collected. Lesions healed slowly. Serum again collected 23 days after onset of the disease.

In the neutralization tests, 4 monkeys were used. Virus, 9 lesions, was collected from 3 varicella patients (G. K., G. M., H. B.). Non-immune serum (W. R.), immune serum (M. W.), and serum collected from C. S. on the 1st and 23d days after the appearance of the zoster eruption were employed. Virus and non-immune serum were injected into Monkey 3; virus and convalescent varicella serum into Monkey 4; virus and early zoster serum into Monkey 5; virus and convalescent zoster serum into Monkey 6. 6 days after the inoculations, the testicles were removed, fixed, sectioned, and stained. Upon examination no inclusions were found in any of the sections.

In view of the negative results in all the monkeys, the experiment was repeated. At this time, however, only non-immune serum (W. R.) and convalescent zoster serum (C. S.) were used. The virus, 6 varicella vesicles, was obtained from A. M. and W. McK. Virus and non-immune serum was injected into Monkey 7; virus and convalescent zoster serum into Monkey 8. 5 days later the testicles were removed and examined in the usual manner. In sections from the testicles of Monkey 7 numerous nuclear inclusions were found, while none were seen in those from Monkey 8.

From the results of this experiment one might conclude that the convalescent zoster serum neutralized the varicella virus. Monkeys, however, display a certain amount of irregularity in their susceptibility to chicken-pox virus. Consequently one set of negative findings is not highly significant.

Experiment III

The following test was made to determine whether the serum from a patient who developed herpes zoster following the administration of salvarsan would neutralize chicken-pox virus.

M. R., female, colored, age 36. No history of previous attacks of chicken-pox or herpes zoster. No known recent exposure to either disease. ++++ Wassermann. On Jan. 13, 1927, at the Vanderbilt clinic, the patient received her first intravenous injection of salvarsan. 24 hours later a burning sensation was felt over the left shoulder, and 48 hours following the treatment blisters were seen over the left side of neck and left shoulder. Patient was seen at the Rockefeller Hospital for the first time on Jan. 21 and was found to have typical herpes zoster. The lesions healed slowly. Convalescent serum was collected on Feb. 11.

Virus, 4 fresh vesicles, was collected from a varicella patient on the 3d day of eruption. Virus and non-immune serum (W. R.) were injected into Monkey 9; convalescent zoster serum (M. R.) and virus were inoculated into Monkey 10. 5 days after the injections the testicles were removed and examined for the presence of acidophilic nuclear inclusion. In the testicles of both animals many were found.

In this experiment it seems that the convalescent zoster serum did not neutralize the chicken-pox virus.

Experiment IV

The experiment described below is the most important one in the series. In it was used serum from a convalescent zoster patient who had had varicella $7\frac{1}{2}$ years previously and from whom a sister appeared to have contracted chicken-pox through exposure to her during the attack of zoster.

M. B., female, white, age 8. No history of previous attacks of zoster. Had chicken-pox when 6 months old. No recent exposure to varicella or zoster. Attends a public school. On Oct. 24, 1926, the patient developed blisters over the right side of the body. She was not quarantined and came in contact with relatives, classmates, and playmates. On Nov. 1, she was seen at the Vanderbilt clinic and referred to the Rockefeller Hospital. At that time the patient presented the picture of typical herpes zoster. The eruption was on the right side of the body over an area supplied by the 9th and 10th thoracic nerves. The child was not admitted to the hospital, but was seen from time to time in the clinic. Dr. E. visited the home on several occasions and a nurse observed the children in the patient's class at school. The lesions on the child healed slowly, and convalescent serum was obtained on Nov. 20, 26 days after the appearance of the eruption.

V. B., age 3, a sister who had never had varicella or zoster was constantly exposed to the patient. On Nov. 13, 19 days after the appearance of zoster in M. B., a chicken-pox eruption was observed on V. B. The child was admitted to the Rockefeller Hospital and after passing through a typical attack of varicella was discharged on Nov. 27. Just prior to discharge convalescent serum was obtained.

The parents said that V. B. had not been exposed to varicella, yet she developed the disease 19 days after exposure to herpes zoster in her sister. This, then, is an excellent example of chicken-pox developing in a child after exposure to herpes zoster. Many such examples have been reported and have been adduced as evidence of the identity of the two diseases. Upon pursuing the matter further, however, it was found that M. B.'s class at school consisted of 33 children, 21 of whom had never had varicella. All of them were exposed to M. B., yet not a case of chicken-pox developed as a result of the exposure.

Four monkeys were used in the neutralization tests. The virus consisted of 12 vesicles removed from 3 varicella patients (J. R., W. R., V. D.). Non-immune serum (W. R.), chicken-pox convalescent serum (M. W.), convalescent serum from M. B., and convalescent serum from V. B. were respectively mixed with equal amounts of virus. 0.25 cc. of the mixtures were then injected into the testicles of monkeys; virus and non-immune serum into Monkey 11, virus and convalescent varicella serum into Monkey 12, virus and convalescent serum from M. B. into Monkey 13, virus and convalescent serum from V. B. into Monkey 14. 5 days after the injections the testicles were removed and examined in the usual way for the presence of nuclear inclusions. Sections from Monkeys 11 and 13 showed numerous inclusions, while those from Monkeys 12 and 14 revealed none.

The results of the experiment indicate that the non-immune serum and the serum from the child (M. B.) convalescent after zoster did not neutralize varicella virus, while convalescent varicella serum of extraneous derivation and convalescent serum from the sister (V. B.) who had just had chicken-pox did inhibit its action. In view of these findings, together with the failure of varicella to develop in any of the classmates of the child (M. B.) who had zoster, one is justified in concluding that the relation of chicken-pox to zoster in the two sisters, V. B. and M. B., was apparent rather than real.

A summary of the experiments dealing with the neutralization of varicella virus by convalescent herpes zoster serum is given in Table I. An analysis of the results reveals that, under the conditions of the tests, sera from individuals who had had varicella $7\frac{1}{2}$ to 30 years previously did not neutralize varicella virus. This fact does not imply that these individuals were susceptible to chicken-pox and that their sera did not possess a certain amount of neutralizing power for varicella

virus. The experiments also indicate that 2 of 3 convalescent herpes zoster sera did not neutralize varicella virus while in each of 2 in-

TABLE I

Summary of Neutralization Experiments

Experiment	Monkey	Inoculum	Results
1	1	Varicella virus + non-immune serum	+
	2	" " + serum from adult who had varicella 30 years previously	+
2	3	" " + non-immune serum	−
	4	" " + convalescent varicella serum	−
	5	" " + serum from patient early in course of herpes zoster	−
	6	" " + serum from patient convalescent from herpes zoster	−
2	7	" " + non-immune serum	+
Repeat	8	" " + convalescent herpes zoster serum	−
3	9	" " + non-immune serum	+
	10	" " + convalescent herpes zoster serum	+
4	11	" " + non-immune serum	+
	12	" " + convalescent varicella serum	−
	13	" " + convalescent herpes zoster serum from an individual (M. B.) who had had varicella 7½ years previously	+
	14	" " + convalescent varicella serum (V. B.)	−

Monkeys inoculated in testicles.

+ indicates infection by virus, determined by presence of nuclear inclusions in sections of inoculated testicles.

− indicates no infection by virus, determined by absence of nuclear inclusions in sections of inoculated testicles.

stances[3] convalescent chicken-pox serum did neutralize it. An adequate explanation of why varicella virus appeared to be neutralized by one of the convalescent zoster sera is lacking. It is certainly not

[3] Convalescent varicella serum regularly prevents the occurrence of nuclear inclusions in monkeys' testicles inoculated with varicella virus (11).

safe to conclude, even though the experiment was repeated, that the herpes zoster in that case was caused by varicella virus. From the experiments reported in this paper and those previously recorded in regard to varicella in monkeys (10, 11), one is justified in concluding that herpes zoster is not always produced by varicella virus. Such is the case even in instances where clinical observations might lead one to suspect that the causal agents of the two diseases are identical.

DISCUSSION

Many investigators believe that certain cases of zoster are caused by a specific virus. Instances, however, of "recurring zoster," of zoster following mechanical or drug injuries to nerves, and of zoster occurring in patients with tabes, tuberculosis of the spine, and cord tumors have led numerous observers to consider that this type of the disease is due not to an infectious agent, but to trophic changes in the skin incident to nerve injury. Regardless of the conditions under which herpes zoster occurs, the character of the lesions has induced a number of workers familiar with virus diseases to entertain the idea that it is caused by some kind of virus.

The question as to whether zoster is caused by one or by several viruses naturally arises. The pathological changes observed in zoster lesions are strikingly similar to those seen in herpes simplex and varicella. In fact, lesions from these three diseases cannot be differentially diagnosed one from another by means of the microscope. As a group, however, they can be separated from other skin lesions occurring in man. This is made possible by the occurrence of acidophilic bodies in the nuclei of affected cells. From records of work dealing with the identity of herpes simplex and herpes zoster (15) it seems unlikely that the etiological agents concerned in the majority of the cases of these two diseases are identical.

It now remains to consider the relation of zoster to chicken-pox. The studies presented in the preceding paper (1), the experiments recorded in the present one, and the fact that an attack of herpes zoster as a rule does not protect against varicella and *vice versa* (16, 17, 18, 22) seem to indicate that the majority of the cases of the two diseases is not caused by the same virus. There is no good reason, however, why one should say that the virus of chicken-pox cannot under

certain conditions produce localized lesions clinically indistinguishable from herpes zoster. It probably does, but only rarely.

The conditions under which the virus of varicella causes a disease clinically similar to herpes zoster are probably dependent upon the rôle injury or irritation plays in the localization of many viruses. The viruses of measles (19) and varicella (20) localize in areas of irritated skin as is evidenced by a marked increase in the number of lesions appearing in such areas. Therefore, in hospitals where large numbers of tuberculous children and syphilic infants undergoing treatment with arsenicals are cared for, a few cases of clinical zoster may occur during an epidemic of varicella. In all probability, under these conditions, some if not all of the so called zoster is caused by chickenpox virus. Consequently it would be better to diagnose such cases as varicella with an abnormal localization of the rash.

It is not likely that trophic changes incident to nerve injury alone or to drug poisoning alone, *e.g.*, with arsenic, are capable of producing herpes zoster. If such were the case, the disease should be seen more often under these conditions. The fact that viruses tend to localize in irritated tissues may also account for the relation that has been observed to exist between certain cases of herpes zoster and nerve injury due to chemical, physical, or disease-producing agents. Whether the virus localizes primarily in ganglia and subsequently travels by way of nerves to the skin or whether the localization is only in the skin, the site being determined by circulatory and other changes incident to the injury of nerves supplying the region (21), is not definitely known. There is no reason why the primary localization of the virus may not occur in either place. The presence or absence of pain in zoster may in some instances be dependent upon the place at which the virus localizes.

Since in the majority of cases of herpes zoster the disease is caused neither by the virus of herpes simplex nor by the virus of varicella, is there any evidence at present that a special virus is concerned in its production? There is no direct proof that such a virus exists, yet the indirect evidence is suggestive. In the first place, the pathological picture presented by zoster lesions is one that has not been shown to occur in the absence of some kind of virus. Although zoster is not, as a rule, highly contagious, it breeds true when occur-

ring epidemically, *i.e.*, zoster gives rise to zoster (15, 22). Finally, in the majority of instances one attack of zoster confers an immunity against a second attack of the disease but not against an infection with herpes simplex and varicella viruses.

SUMMARY

Experiments and clinical observations dealing with the identity of the viruses of varicella and herpes zoster were presented. The results indicate that the etiological agents concerned with these two diseases are in the majority of instances not identical.

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[Reprinted from THE JOURNAL OF EXPERIMENTAL MEDICINE, August 1, 1929,
Vol. 50, No. 2, pp. 181–187]

OBSERVATIONS CONCERNING THE PERSISTENCE OF LIVING CELLS IN MAITLAND'S MEDIUM FOR THE CULTIVATION OF VACCINE VIRUS

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PLATE 7

(Received for publication, June 7, 1929)

The fact that vaccine virus is capable of multiplying in cultures of susceptible tissues is well established. Some investigators (1), however, have claimed that it is possible to obtain an increase of this infectious agent in the absence of living host cells. These claims have not been substantiated. Indeed, Harde (Steinhardt) (2, 3) and Nye and Parker (4) have reported that tissues killed by freezing and thawing and by hypotonic salt solutions did not support the survival or multiplication of vaccine virus.

Recently Maitland and Maitland (5) recorded observations concerning "cultivation of vaccinia virus without tissue culture." Their medium consisted of minced chicken kidney suspended in a mixture of chicken serum (1 part) and Tyrode's solution (2 parts). These workers found that the virus increased in the absence of any detectable growth of cells in the cultures. In fact, they state that "after 24 hours the small pieces of kidney had begun to disintegrate, and by the third day autolysis of the tissue was extensive." There is no reason to doubt that vaccine virus multiplied in Maitland's medium. Moreover, Eagles and McClean (6) and Andrewes (7) have shown that certain viruses are capable of increasing under such conditions. Inasmuch as most workers have been unable to cultivate vaccine virus in the absence of living cells, and since it is known that certain cells remain viable under many conditions (8, 9), there are reasons for ascertaining whether living cells can persist or whether growth of cells can occur in Maitland's medium. It is with this problem that the present communication is chiefly concerned.

EXPERIMENTAL

Methods and Materials

Vaccine Virus.—Levaditi's neurovaccine was injected in the testicles of a rabbit. 4 days later the testicles were removed[1] and ground in a mortar with sand and M/50 phosphate solution, pH 7.6. The emulsion was thoroughly centrifuged and the supernatant fluid was used as an inoculum. The titer of the virus was determined in rabbits by means of intradermal inoculations of 0.2 cc. of serial dilutions of virus emulsions.

Culture Medium.—Throughout this work Maitland's technique of preparing and testing cultures was followed.

"Blood was withdrawn from a hen and after it had clotted the serum was collected The hen was killed with chloroform and ether, and the kidneys, which were removed aseptically, were minced finely with scissors. Into a flask was put 0.66 c. cm. (approximately) of minced kidney and 1.33 c. cm. of inoculum diluted 1 in 6.6 with Tyrode's solution. The mixture was allowed to stand in the cold room for four hours. ' Then were added 12 c. cm. of Tyrode's solution and 6 c. cm. of hen's serum. The final dilution of the inoculum was thus 1 in 100. The mixture was distributed in 2 c. cm. into Carrel's tissue culture flasks, type D, which were incubated aerobically at 37°C., without caps. The cultures were tested after various periods of incubation by grinding the whole of the contents of each flask with sand and centrifugalising."

Controls.—In addition to the normal kidney tissue, controls with minced kidney tissue that had been frozen (CO_2 snow) and thawed 10 times were employed. To determine whether the tissues were living or dead at the beginning of each experiment, just prior to the distribution of the cultures in flasks, bits of the normal and of the frozen kidney tissue were placed in hen plasma and embryo extract on mica coverslips which were inverted and sealed over hollow ground slides. These preparations were then incubated at 37°C. After 5 or 6 days they were examined for evidences of cell growth.

To ascertain whether living cells persisted in the cultures prepared and handled according to Maitland's method, bits of tissue were removed from the Carrel flasks at 3, 4, and 5-day intervals, washed in Ringer's solution, and planted in plasma and embryo extract on mica coverslips These preparations were examined frequently for evidences of cell growth.

Inasmuch as it seemed possible that certain cells might at times multiply in a mixture of serum and Tyrode's solution, bits of normal kidney tissue were placed in such a mixture on mica coverslips over which hollow ground slides were placed and sealed. The slides were not inverted, since it is well known that in a liquid medium cells usually require a surface along which to grow. After incubation at 37°C., these preparations were examined for evidences of cell migration or cell growth.

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[1] All operations were performed under ether anesthesia.

To test the viability of bacteria one usually resorts to subcultures rather than to examinations of fresh and stained specimens. Consequently, in determining whether cells are able to remain alive in the medium employed by Maitland for the cultivation of vaccine virus, subcultures, as described above, of bits of tissue in a favorable medium were made. Under these conditions, it is appreciated that positive results are more significant than negative ones. In this work 4 experiments were performed, 3 of which will be described in detail. The fourth will be omitted, since the results were similar to those of the others.

Experiment I

Jan. 15, 1929.—Fresh hen kidney was minced. One portion was frozen (CO_2 snow) and thawed 10 times. Then both portions were placed in contact with vaccine virus in the ice box for 4 hours. After the exposure to virus, 10 pieces of unfrozen and frozen tissue respectively were cultured as controls in plasma and embryo extract on mica coverslips. The inoculated frozen and unfrozen tissues were then added to a mixture of serum and Tyrode's solution and distributed in Carrel flasks (2 cc. each)—3 with unfrozen and frozen tissue respectively. Titer of vaccine virus in the cultures at this time: frozen = 1:100; normal or unfrozen = 1:100.

Jan. 17.—Each of the 10 control cultures made on coverslips from the unfrozen bits of tissue showed growth of cells, while in those made from frozen tissue no evidence of cell growth was observed at this time or upon subsequent examinations.

Jan. 18.—The cultures were removed from the Carrel flasks. Bits of tissue from 2 of the flasks with unfrozen kidney and from 2 of the containers with frozen kidney were washed in Ringer's solution and subcultured in hen plasma and embryo extract. None of the subcultures of frozen tissue showed growth in 6 days (Fig. 2), while in all of those made from unfrozen tissue growth of cells (Fig.1) was evident. The predominant cells were fibroblasts, yet cells of the macrophage type evidencing phagocytosis were observed.

The titer of vaccine virus in the flasks with frozen (F) and unfrozen (N) tissues was as follows: N1 = 1:500, N2 = 0, N3 = 1:5,000; F1 = 0, F2 = 0, F3 = 1:50.

The results of the above experiment indicate that vaccine virus did not multiply in the presence of kidney tissue killed by freezing and thawing, while it did persist or increase in amount in cultures set up with normal kidney tissue in a mixture of serum and Tyrode's solution. Furthermore, it is quite obvious that living cells persisted for at least 3 days in Maitland's medium.

Experiment II

Feb. 13, 1929.—Cultures were prepared with frozen and unfrozen tissue as described in the previous experiment. From the respective mixtures 2 cc. were placed in each of 4 Carrel flasks. The titer of virus at this time: frozen = 1:1,000; normal or unfrozen = 1:1,000.

Feb. 16.—10 bits of tissue were removed respectively from each of 2 Carrel flasks containing unfrozen (N) tissue and from each of 2 flasks with frozen (F) kidney, washed in Ringer's solution, and subcultured in plasma and embryo extract. The results of the subcultures were as follows:

N1: 8 showed growth of cells, while 2 did not.
N2: 5 " " " " " 5 " "
F2: 10 " no growth of cells.
F3: 10 " " " " "

Feb. 18.—To test again the viability of cells, 20 subcultures were made from N3 and N4 respectively and 10 from F1 and F2 respectively. The results of the tests were as follows:

N3: 14 showed growth of cells, while 6 did not.
N4: 15 " " " " " 5 " "
F1: 10 " no growth of cells.
F2: 10 " " " " "

Titration of virus from 2 Carrel flasks containing unfrozen (N) tissue and from 2 containers with frozen (F) kidney resulted as follows: N1 = 1:1,000, N3 = 1:100,000, F3 = 0, F4 = 0.

From the results of Experiment II it is evident that certain cells are able to survive for at least 5 days in a mixture of serum and Tyrode's solution. It also appears that vaccine virus survived or multiplied in the presence of living cells while it ceased to be active in the cultures prepared with tissues killed by freezing and thawing.

Experiment III

Feb. 25, 1929.—The vaccine virus used in this experiment was obtained from N3 of Experiment II. It had been diluted ten times and stored on ice for 7 days. As previously described, cultures were prepared with frozen and unfrozen minced kidney tissue. Prior to distributing the cultures in flasks, the following controls were set up on mica coverslips:

10 cultures of frozen tissue in plasma and embryo extract.
10 " " " " " serum and Tyrode's solution.
10 " " unfrozen tissue in plasma and embryo extract.
10 " " " " " serum and Tyrode's solution.

Subsequent examinations of the above controls revealed the following facts: None of the frozen tissues showed growth; 9 of the unfrozen tissues in plasma and embryo extract presented signs of growth; 5 of the unfrozen tissues in serum and Tyrode's solution showed evidences either of definite cell growth (Fig. 3), of wandering out of surviving round cells, or of beginning giant cell formation (Fig. 4) through cell apposition.

March 2.—30 pieces of unfrozen tissue were taken from each of 2 Carrel flasks (N1 and N3) and 10 of frozen were removed from each of 2 containers (F1 and F2). To test the viability of cells, these bits of tissue were washed in Ringer's solutions and subcultured in plasma and embryo extract. The results of the tests were as follows:

N1: 10 cultures showed growth, while 20 did not.
N3: 29 " " " " 1 " "
F1: 10 " " no growth.
F2: 10 " " " "

At the beginning of the experiment, titration of the virus resulted in no vaccinal reactions in the rabbit. After incubation at 37°C. for 5 days the contents of the flasks were examined for the presence of vaccine virus. None was found.

The virus used in this experiment was culture virus from Experiment II. It had been diluted and stored on ice for 7 days. During that time it had ceased to be active. This experience coincides with the findings of Eagles and McClean (6) who have had difficulty in preserving culture virus. The results of Experiment III indicate that cells are not only able to survive but may at times multiply in a mixture of serum and Tyrode's solution.

DISCUSSION

The results of the experiments described above are in agreement with those obtained by other workers who found that tissues killed by freezing and thawing failed to support *in vitro* the multiplication of vaccine virus. No evidence was secured to cast doubt upon Maitland's observations concerning the increase of vaccine virus in a medium consisting of minced fresh normal kidney tissue suspended in a mixture of serum (1 part) and Tyrode's solution (2 parts). In this medium, however, which Maitland considered not to be a tissue culture and in which he thought autolysis of the cells to be extensive within 3 days, it was possible to show that many cells remain viable for at least 5 days. This was accomplished by subculturing bits of the

tissue in a favorable medium of plasma and embryo extract. Furthermore, it was found that a medium of serum and Tyrode's solution is capable at times of supporting multiplication of certain cells (Fig. 3).

From the work here presented it appears that the increase of vaccine virus obtained by Maitland did not occur in the absence of living cells. Nevertheless, Maitland has made a definite contribution to the study of viruses in that he has found a medium for the easy cultivation *in vitro* of vaccine virus and other infectious agents of a similar nature (7).

SUMMARY

Cells survive for at least 5 days and at times are capable of multiplying in a mixture of serum and Tyrode's solution used by Maitland for the cultivation *in vitro* of vaccine virus.

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EXPLANATION OF PLATE 7

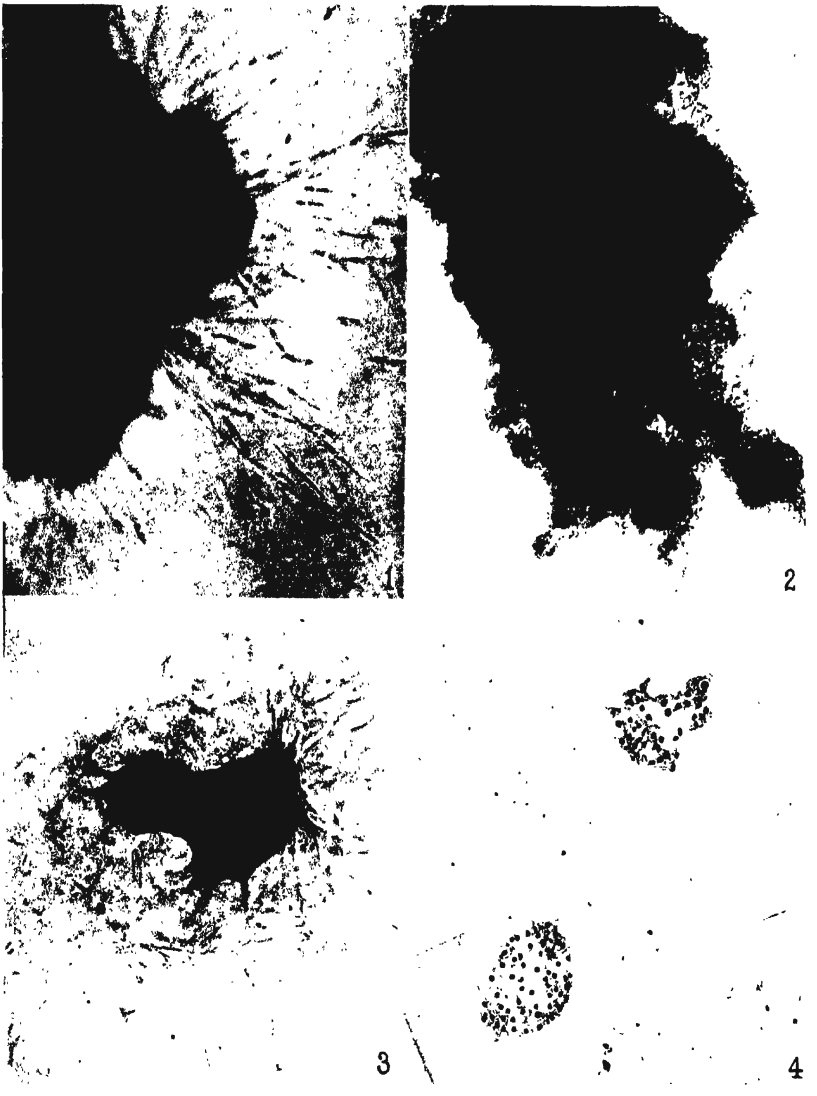
FIG. 1. A small piece of kidney tissue removed from a 3-day Maitland culture and subcultured in plasma and embryo extract. Note growth of cells. × about 95.

FIG. 2. Tissue treated similarly to that in Fig. 1 with the exception that it was

frozen and thawed before being used in a Maitland culture. Note absence of cell growth. × about 95.

FIG. 3. Fresh kidney tissue cultured 4 days in a mixture of serum and Tyrode's solution on a mica coverslip. Note growth of cells. Fixed preparation stained with Delafield's hematoxylin. × about 85.

FIG. 4. Giant cells formed by cell apposition in a medium of serum and Tyrode's solution. Fixed preparation stained with Delafield's hematoxylin. × about 85.



(Rivers *et al.*: Persistence of living cells in Maitland's medium)

[Reprinted from THE JOURNAL OF BIOLOGICAL CHEMISTRY, July, 1929, Vol. 83, No. 1, pp. 51–70]

GASOMETRIC DETERMINATION OF FERMENTABLE SUGAR IN BLOOD AND URINE

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(Received for publication, May 9, 1929)

INTRODUCTION

The method previously described by the authors (1928) for gasometric determination of total reducing substances in blood and urine is in this paper applied to the estimation of fermentable sugar in both fluids.

Approximate determination of fermentable sugar in urine by manometric determination of the CO_2 formed by yeast is also described.

Procedures for fermentative determination, in blood and urine, of glucose, or of sugars resembling it in their fermentability by yeast, have been refined by different investigators during the past few years. The chief source of error, undue prolongation of the time of fermentation to one or more days, was removed by Hiller, Linder, and Van Slyke (1925), who utilized conditions under which glucose could be completely removed from blood in a half hour. Folin and Svedberg (1926), Somogyi (1927, 1928), Benedict (1928), and Raymond and Blanco (1928) have utilized these conditions for blood analyses, and Eagle (1926–27) for urine. All the above authors estimated fermentable sugar by the decrease in reducing substances caused by fermentation with yeast. To the technique Somogyi added the improvement of using washed yeast cells, so that correction for reducing substances adherent to the yeast could be obviated. Lund and Wolfe (1926) used a short fermentation period for urine, with a pure culture of yeast, which permitted them to determine with a Barcroft apparatus the CO_2 formed as a measure of the sugar fermented.

By all these authors except Lund and Wolfe the reducing sugars have been estimated by difference, according to the equation:

$$F = T - N \tag{1}$$

where F = fermentable sugar, T = total reducing substances, and N = non-fermentable reducing substances.

A factor of minor but measurable influence on the precision of results in most of the procedures used is the permeability of the yeast cells to the non-fermentable reducing substances of blood and urine. The existence or non-existence of such permeability must be considered in calculating results when the latter are based on analyses of aliquot parts of the yeast-blood or yeast-urine filtrate. Hiller, Linder, and Van Slyke (1925) avoided the necessity of considering this factor, because they used the Hagedorn-Jensen (1918, 1923) blood sugar method. This method is unique in that the coagulated blood protein precipitate is quantitatively extracted with several portions of hot water, so that the filtrate analyzed represents the entire blood sample, not an aliquot part. Some authors (Eagle, 1926–27; Folin and Svedberg, 1926; Somogyi, 1927; Benedict, 1928; Raymond and Blanco, 1928) have diluted blood or urine plus yeast to a measured volume, and analyzed an aliquot part of the filtrate, with the apparent assumption that the non-fermentable reducing substances diffused through the yeast in the same concentration as through the solution in which the cells were suspended. According to the results of Somogyi (1928), however, which we have confirmed, the non-fermentable reducing substances of blood do not diffuse into the yeast. These substances therefore are more concentrated in the filtrate of the blood + yeast mixture than in the filtrate of blood that has been diluted to the same volume without yeast. The fermentable sugar calculated by difference is diminished by the error. In the technique of Raymond and Blanco (1928) and the earlier procedure of Somogyi (1927) the yeast cells constituted 6 and 7 volumes per cent of the final mixture respectively, and their bulk introduced a corresponding positive percentage error into the N of Equation 1. The effect on the value found for F, the fermentable sugar, caused by a 6 per cent error in N is about 2 per cent of F, if, as in normal blood, N is about one-fourth of T.

In urine we have found that yeast, although not entirely impermeable to the non-fermentable substances, absorbs them in relatively small amounts. The amount taken up per cc. of yeast cells averages only about one-fifth the amount left in solution per cc. of fluid. We have governed accordingly our technique, outlined below. Eagle (1926–27) made no correction for the volume occupied by yeast cells (about 6 per cent of the volume of the yeast-urine mixture) in his urine fermentations. Such correction would increase his nearly negative fermentable sugar values, but in no case apparently to as high as 0.01 per cent.

DETERMINATION OF FERMENTABLE SUGAR IN BLOOD FROM DECREASE IN REDUCING POWER CAUSED BY YEAST TREATMENT

Removal of Blood Proteins.—The blood proteins are removed with the modification of Folin and Wu's (1919) tungstic acid procedure described on p. 741 of Van Slyke and Hawkins (1928).

Removal of Fermentable Sugar from Blood Filtrate.[1]—From one portion, conveniently about 10 cc., of the filtrate the fermentable sugar is removed according to Somogyi (1928). We have applied the procedure as follows:

A portion of Fleischmann's yeast cake is pulverized and suspended in 4 times its weight of water. Of the suspension a volume, approximately equal to that of the blood filtrate sample to be fermented, is placed in a centrifuge tube and washed five times by repeated centrifugation and decantation. After the last centrifugation the water is decanted as completely as possible, and the water film adherent to the walls of the tube above the packed cells is removed with a roll of filter paper. Adherent water remaining between the cells is not sufficient to dilute significantly the blood filtrate. The blood filtrate is added to the yeast packed in the centrifuge tube. Filtrate and yeast are mixed and permitted to stand at room temperature for 15 minutes. The mixture is then centrifuged.

Determination of Total and Unfermentable Blood Sugar.—3 cc. portions of the supernatant fluid obtained by the above yeast treatment and 3 cc. portions of untreated filtrate are analyzed as described by Van Slyke and Hawkins (1928) for blood sugar.

The p_0 value, for the analyses of the yeast-treated portions, is determined by blank analysis of the supernatant fluid obtained from a centrifuged mixture of 1 volume of washed yeast cells and 4 volumes of water.

[1] Folin and Svedberg (1926) have shown that yeast acts well in Folin-Wu blood filtrates. Somogyi states furthermore that fermentation of the filtrate instead of whole blood obviates one source of occasional error. He found that some pathological bloods when mixed with yeast reacted with it to *produce* reducing substances even in the few minutes required for the short fermentation technique. These substances in part replaced the glucose removed by the yeast, and caused erroneously low values to be calculated for fermentable sugar. Such error was obviated when the yeast acted on the blood filtrate instead of the blood itself.

Until these observations of Somogyi (1928) appeared, we had used yeast treatment of the whole blood to determine fermentable sugar, since the technique involves one filtration less than that for fermentation of the filtrate. In our analyses, limited chiefly to normal and nephritic subjects, we did not encounter any pathological blood specimens of the type mentioned by Somogyi (1928). Nevertheless in view of his experience with such specimens it appears desirable to abandon treatment of whole blood with yeast, and to treat only the blood filtrate.

It is essential in determining this blank to treat the yeast with water rather than with tungstic acid solution. When water is mixed with washed yeast we have found that it extracts the same minimal amount, if any, of reducing material from the cells that a sample of glucose-free (previously fermented) Folin-Wu blood filtrate extracts from them. Hence it appears that the water extract gives the correct blank. If yeast is mixed with tungstic acid solution, the latter extracts measurably more reducing material, enough to be equivalent to 5 or 10 mg. per cent of blood sugar,—sometimes more. Presumably the reason for this phenomenon is that filtrate from tungstic acid-yeast mixture has a much greater acidity and contains more titratable acid than filtrate from tungstic acid-blood mixture; in the latter case the blood proteins remove the acid almost completely. The effect of using a tungstic acid extract of yeast cells for the blank determination would be to lower erroneously the p_0 value, and hence to lower the value, calculated as $(p_0 - p_1) \times$ factor, obtained for non-fermentable reducing material in blood.

Calculation of Fermentable Blood Sugar[2]

For calculating both total sugar from analysis of the untreated filtrate, and unfermentable sugar from analysis of the yeast-treated filtrate, the $p_0 - p_1$ values are multiplied by the usual calculation factors in Table II of our former paper (1928).

If a temperature change occurs during the interval between the p_0 and p_1 observations, p_0 is to be corrected as indicated in their Table I.

From the total and unfermentable reducing material the fermentable sugar is found by difference.

Fermentable sugar = (total sugar) − (unfermentable sugar)

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[2] If *only* fermentable sugar is desired, only the p_1 values of the two analyses need be determined. Fermentable sugar is then calculated as $(p_{1T} - p_{1N}) \times$ *factor*, where p_{1T} is the p_1 reading for the total sugar determination, p_{1N} is the p_1 for the non-fermentable sugar analysis, and the factor is from Table II of our former paper (1928). This procedure eliminates the two p_0 determinations. It can be used, however, only if the yeast employed has been washed quite free of reducing substances.

DETERMINATION OF FERMENTABLE SUGAR IN BLOOD FROM DECREASE IN REDUCING POWER CAUSED BY SPONTANEOUS GLYCOLYSIS

Spontaneous disappearance of reducing sugar from blood was a phenomenon known to Claude Bernard and studied by many later investigators (see Tolstoi, 1924). The sugar is transformed into lactic acid, as shown by Evans (1922). Hiller, Linder, and Van Slyke (1925) and Folin and Svedberg (1926) have found that the same amount of reducing substance is removed by spontaneous glycolysis at 38° for 20 or more hours that is removable by short fermentation with yeast.

Procedure.—Two analyses, designated as *A* and *B* are required.

A.—In one sample of fresh blood the total reducing material is determined at once.

B.—Another sample of the whole blood in a stoppered tube is incubated at 38° for 20 to 24 hours, and the non-glucose reducing material left in it is then determined.

Calculation. $A - B$ = fermentable sugar.

DETERMINATION OF FERMENTABLE SUGAR IN URINE BY MEASURING DECREASE IN REDUCING SUBSTANCES CAUSED BY YEAST TREATMENT

The procedure outlined below, with minimum dilution of the urine, is designed primarily for urine with amounts of reducing substances of the order of magnitude found in non-diabetic cases. It is usually in urines of slight reducing power that one needs to determine the fermentability of the material, either for diagnostic or experimental purposes. The method as given is designed for urines with reducing powers not exceeding that of a 0.5 per cent glucose solution.

To determine the fermentable sugar in urines more heavily loaded with reducing substances, such urines are diluted sufficiently to bring the total reducing power below that of a 0.5 per cent glucose solution.

Reagents for Urine Analysis

Ferricyanide Solution.—This contains 14 grams of $K_3Fe(CN)_6$, 75 grams of K_2CO_3, and 75 grams of $KHCO_3$ per liter. It is identical with the reagent described for urine in our previous paper (1928), except that here only half as much ferricyanide is used, because of the smaller amounts of reducing material encountered.

The solution is to be prepared in the manner directed in our previous paper.

Oxalic Acid.—0.1 N solution.

Hydrazine Solution.—Same as previously used (1928).

Lloyd's Reagent.—The preparation of fullers' earth known by the above name.

Procedure for Urine Analysis

Preparation of Urine for Analysis.—Creatinine, uric acid, and other non-glucose materials exert reducing effects which combined usually exceed that of the fermentable sugar in non-diabetic urine. The amount of such substances present is diminished by treatment of the urine with Lloyd's reagent, as described by Folin and Berglund (1922). The substitution of oxalic acid for the sulfuric acid used by them obviates formation of a calcium salt precipitate when the urine filtrate is later mixed with ferricyanide-carbonate solution.

To 10 cc. of urine add 5 cc. of 0.1 N oxalic acid, 5 cc. of water, and 1.5 gm. of Lloyd's reagent. Shake gently for 2 minutes and filter.

A control filtrate is also made at the same time: Add 5 cc. of 0.1 N oxalic acid and 1.5 gm. of Lloyd's reagent to 15 cc. of water, shake for 2 minutes, and filter.

Determination of Total Reducing Material in Urine Filtrate.—2 cc. portions of the filtrate are mixed with 2 cc. portions of the ferricyanide reagent in test-tubes, and the analysis is carried out as described for diluted urine on p. 751 of our previous paper (1928), in all respects save the difference in ferricyanide reagent. The p_0 value is determined by like analysis of the control filtrate.

Determination of Non-Fermentable Reducing Material in Urine Filtrate

A second portion of urine is treated as follows: To 10 cc. of urine are added 7.5 cc. of a 40 per cent yeast suspension (20 gm. of a Fleischmann's compressed yeast cake in 50 cc. of water. The mixture contains, as shown by centrifuging, about 40 per cent by volume of moist yeast). The mixture is allowed to stand for 15 minutes. 5 cc. of 0.1 N oxalic acid and 1.5 gm. of Lloyd's reagent are added. The mixture is shaken for 2 minutes and fil-

tered. Under these conditions, the yeast will remove glucose in amounts up to 0.5 per cent in the original urine.

2 cc. portions of the filtrate are analyzed as in the determination

TABLE I

Correction to p_0 for Temperature Change

Temperature range.	Increase of vapor tension of water per 1° temperature rise.	Increase of N_2 pressure of control per 1° temperature rise.	Total p_0 correction per 1 temperature rise.*
°C.	mm.	mm.	mm.
15–20	0.7	0.6	1.3
20–25	1.2	0.6	1.8
25–30	1.6	0.6	2.2

* Add correction to p_0 when temperature is higher at p_1 reading; subtract the correction when temperature is lower.

TABLE II

Factors by Which N_2 Pressure Fall, $p_0 - p_1$ in Millimeters, Is Multiplied to Calculate Reducing Sugar of Urine in Terms of Grams Glucose per 100 Cc.

Temperature of gas chamber.	Factor.*	Temperature of gas chamber.	Factor.*
°C.		°C.	
15	0.00143	25	0.00138
16	2	26	8
17	2	27	7
18	1	28	7
19	1	29	6
20	0	30	6
21	0	31	5
22	0.00139	32	5
23	9	33	4
24	8	34	4

* These factors hold when, as in the procedure described, undiluted urine is used, and the final 3 cc. of ferricyanide-urine mixture used for the gasometric determination represent 0.75 cc. of urine. If, by reason of high sugar content, the urine is diluted before analysis, the above factors are multiplied as many times as the urine is diluted.

of the total reducing material. The p_0 value is determined by similar analysis of filtrate from a control suspension of yeast and Lloyd's reagent, in which water replaces the urine.

The proportions of yeast and fluid are designed to give the same

concentration of non-fermentable reducing material in the fluid that is occasioned, in the preceding total reducing material determination, by diluting the 10 cc. of urine to 20 cc. with water solutions. In the present yeast-fluid mixture, 10 cc. of urine are mixed with 9.5 cc. of water solutions and 3 cc. of yeast cells. The latter, as will be shown, take up about as much non-fermentable reducing material as would the 0.5 cc. of water required to make the fluid volume up to 20 cc.

Calculation of Fermentable Urine Sugar.—The results of the two analyses are calculated by means of the factors in Table II, which is constructed from data given in the authors' previous paper (1928). If temperature change occurs between p_0 and p_1 readings, p_0 is corrected according to Table I. The fermentable sugar is calculated as

Fermentable sugar = (total sugar) − (non-fermentable sugar)

TABLE III

Impermeability of Yeast to Non-Fermentable Reducing Substances of Blood Filtrate

Filtrate No.	Mg. non-fermentable reducing substances calculated as glucose per 100 cc. blood.	
	Blood filtrate treated with one portion of yeast.	Blood filtrate treated with two successive portions of yeast.
1	19.5	20.3
2	19.9	19.2
3	20.8	22.0
4	25.6	28.1

EXPERIMENTAL

Test of Permeability of Yeast for Non-Fermentable Reducing Substances of Blood Filtrate

To ascertain whether measurable amounts of the non-fermentable reducing material in blood filtrate diffuse into yeast cells the following experiment was tried. 1 volume of blood was precipitated with 1 volume of $\frac{2}{3}$ N sulfuric acid, 1 volume of 10 per cent sodium tungstate, and 2 volumes of distilled water, making, in order to obtain a higher concentration of the substances determined, a 5-fold dilution of the blood instead of the usual 10-fold dilution. This filtrate, after treatment with washed, centrifuged

yeast as described above, to remove the fermentable sugar, was divided into two portions. One portion was analyzed to determine the amount of non-fermentable reducing material. The second portion was added to an equal volume of washed packed yeast in a centrifuge tube, was mixed with the yeast, and was allowed to stand for 15 minutes. The yeast was then thrown down by centrifugation and the supernatant solution was analyzed for reducing substances. It is seen from Table III that the amounts of non-fermentable reducing substances in the two filtrates were the same. Consequently there was not sufficient water adherent to the centrifuged yeast to dilute measurably the blood filtrate, nor did the yeast absorb non-fermentable reducing substances from the blood filtrate. Either occurrence would have lowered the concentration of reducing substances in the filtrate.

Permeability of Yeast for Non-Fermentable Substances of Urine

Normal urines were treated with yeast according to the method described above, under conditions assuring complete removal of glucose. The filtrates were then mixed with equal volumes of yeast which had been washed free of reducing substances and centrifuged free of adherent water. The mixtures were centrifuged, and the reducing substances were determined in the supernatant solutions. If the non-glucose urinary reducing substances had been, like those of the blood, not measurably diffusible into the yeast, this second treatment would not have altered the content of reducing substances. If, on the other hand, these substances had distributed themselves uniformly, volume for volume, between yeast and urine filtrate, the second yeast treatment would have lowered by 50 per cent the concentration of reducing material in the urine filtrate. From Table IV it is apparent that neither extreme is realized: the concentration of reducing material in the filtrate was lowered by an average of about 16 per cent, from which there were some wide deviations. According to the average result, non-fermentable reducing materials distributed themselves in about the ratio:

$$\frac{\text{Non-fermentable urinary reducing substances per cc. yeast}}{\text{Non-fermentable urinary reducing substances per cc. urine filtrates}} = \frac{1}{5}$$

Estimation of Fermentable Sugar in Blood Filtrates from Amount of Reducing Material Removed by Yeast. Comparison of Results Obtained with Different Reagents Used for Determining the Reducing Material

Comparison of results obtained by the Benedict copper-colorimetric (1928), Folin ferricyanide-colorimetric (1928), Shaffer-

TABLE IV

Permeability of Yeast to Non-Fermentable Substances of Urine Filtrate

Sample No.	Non-fermentable reducing substance in glucose equivalents.			
	Determined after first yeast treatment.	Determined after treating filtrate from yeast-treated urine with equal volume of moist yeast.	Removed by second yeast treatment.	Proportion of reducing material removed by second yeast treatment.
	gm. per 100 cc.	*gm. per 100 cc.*	*gm. per 100 cc.*	*per cent*
1	0.112	0.101	0.011	9.8
2	0.096	0.078	0.018	18.7
3	0.117	0.096	0.021	18.0
4	0.053	0.042	0.011	20.7
5	0.032	0.032		0.0
6	0.128	0.120	0.008	6.2
7	0.034	0.033	0.001	2.9
8	0.075	0.057	0.018	24.0
9	0.177	0.138	0.039	22.0
10	0.020	0.020	0.000	0.0
11	0.042	0.036	0.006	14.3
12	0.170	0.134	0.036	21.2
13	0.129	0.102	0.027	20.9
14	0.077	0.062	0.015	19.5
15	0.114	0.091	0.023	20.2
16	0.107	0.086	0.021	19.6
17	0.051	0.044	0.007	13.7
18	0.096	0.081	0.015	15.6
19	0.109	0.082	0.027	24.8
20	0.049	0.039	0.010	20.4

Hartmann-Somogyi copper-titration (Somogyi, 1926), and the Van Slyke-Hawkins (1928) ferricyanide-gasometric blood sugar methods in analyses of fourteen bloods, normal and pathological, is shown in Fig. 1. In the case of each blood aliquot parts of one portion of Folin-Wu filtrate were analyzed by the respective

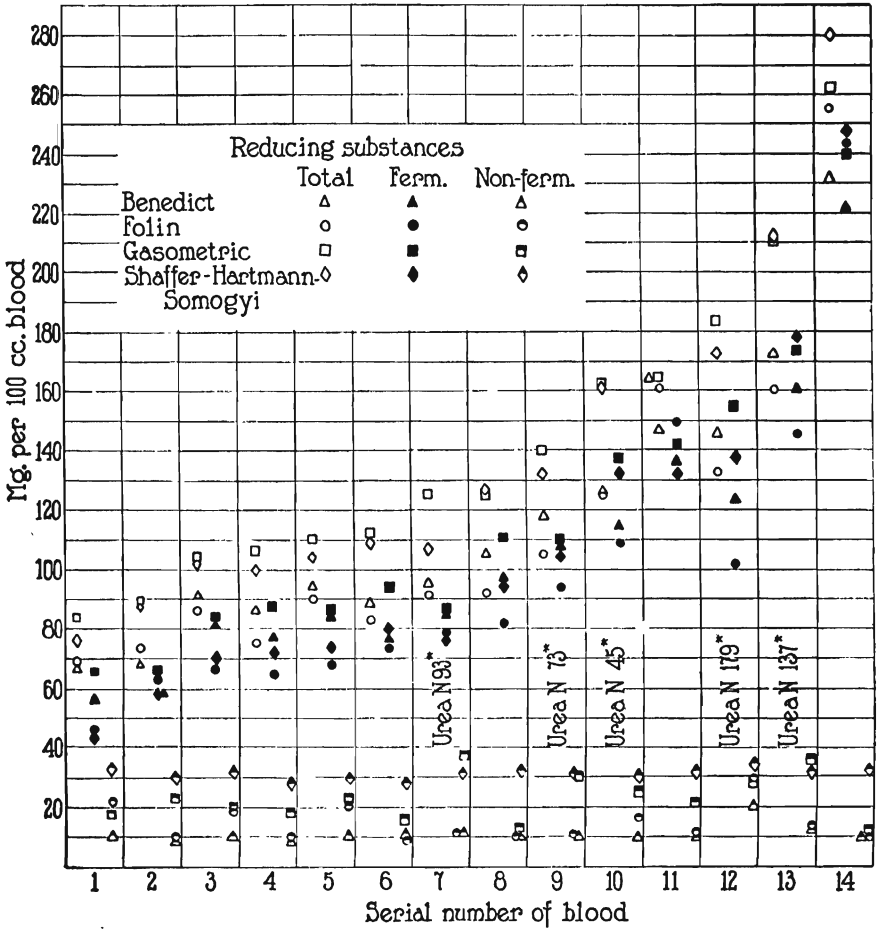


FIG. 1. Comparison of total, fermentable, and non-fermentable sugar found in blood by the Benedict copper-colorimetric, Folin ferricyanide-colorimetric, Shaffer-Hartmann-Somogyi copper-titration, and Van Slyke-Hawkins ferricyanide-gasometric methods. Ordinates represent mg. of sugar per 100 cc. of blood. Each symbol represents an average of duplicate determinations.

methods. The fermentable sugar was then removed from another portion of filtrate by treatment with washed yeast as described above, and aliquot parts of the fermented filtrate were analyzed by the same methods.

The values for non-fermentable reducing material varied with the reagent used. Benedict's method gave the lowest and most constant value, averaging about 10 mg. per 100 cc. of blood. The Shaffer-Hartmann-Somogyi method gave about 30 mg. Folin's method gave values varying from 10 to 30 mg. The gasometric method gave values of about 20 mg. per 100 cc. except in three bloods with advanced urea retention. In these cases the non-fermentable reducing materials determinable by the gasometric ferricyanide reagent were increased to over 30 mg., presumably because of retained substances such as creatinine and uric acid, which were shown by Hagedorn and Jensen (1923) to reduce ferricyanide appreciably. The maximum non-fermentable reduction, in a blood with 193 mg. of urea N per 100 cc., was equivalent to 38 mg. per cent of glucose.

The fermentable blood sugar values, if they represented exactly glucose, would be expected to be independent of the reduction method used, as Somogyi and Kramer (1928) have recently found, in fact, with two different copper methods and one ferricyanide method. We have not obtained quite such concordant results. Both the Benedict (1928) and Shaffer-Hartmann-Somogyi (Somogyi, 1926) methods indicated in our hands about 10 mg. per cent less of fermentable sugar than the gasometric ferricyanide method, and the Folin (1928) ferricyanide method averaged still lower.

If one could assume entire absence of technical error, such results would indicate that yeast removes from blood filtrate small amounts of *non-glucose* reducing substances, towards which the reagents of the above respective methods react differently. On the amount of work presented, we hesitate to consider that conclusion as established.

Determination of Fermentable Sugar in Blood by Spontaneous Glycolysis

Comparison of results obtained by Somogyi's (1926) modification of the Shaffer-Hartmann (1920–21) copper reduction method,

with those yielded by the gasometric blood sugar method in analyses of four bloods, normal and pathological, is shown in Table V.

Fermentable and Non-Fermentable Reducing Substances in Urine of Normal Men, Determined by Gasometric Ferricyanide Method

In Table VI are given a number of determinations by the gasometric reduction method described above, of the total and fermentable reducing substances in urine from normal men.

Our values for fermentable reducing sugar in the urines of normal men are mostly between 0.009 and 0.023 per cent, one out of twenty-seven being below and three above this range. Eagle's

TABLE V

Determination by Glycolysis of Fermentable and Non-Fermentable Reducing Substances in Blood with the Shaffer-Hartmann-Somogyi and Gasometric Methods

Mg. reducing substances per 100 cc. blood.

Shaffer-Hartmann-Somogyi method.			Gasometric method.		
Total.	Fermentable.	Non-fermentable.	Total.	Fermentable.	Non-fermentable.
122	85	37	116	85	31
110	86	24	104	82	22
140	112	28	135	114	21
122	86	36	126	88	38

(1926–27) values are all below 0.01 per cent, even when corrected for the effect of yeast volume, discussed above. Eagle determined reducing material with the Benedict copper reagent, while we used a ferricyanide reagent. Whether this difference in reagents is the cause of the apparent difference in results is uncertain. In any case, it appears that the fermentable sugar in normal urine is usually less than 0.025 per cent.

Fermentable Sugar in Urine Determined by Measurement of CO_2 Formed by Yeast

Fermentable sugar in the urine was also determined by the measurement of the carbon dioxide formed in urine incubated with

yeast. A technique was used similar to that employed by one of us (Van Slyke, 1927) for determining urea by measurement of the CO_2 yielded by the action of urease.

TABLE VI

Content of Fermentable and Non-Fermentable Sugar in Urine of Normal Subjects about 2 Hours after Mixed Breakfast

Subject No.	Fermentable sugar determined by CO_2 formation.	Sugar concentration by gasometric reduction method.		
		Total determined. (*a*)	Non-fermentable, determined after yeast treatment. (*b*)	Fermentable, calculated by difference. (*a*) − (*b*)
	gm. per 100 cc.	*gm. per 100 cc.*	*gm. per 100 cc.*	*gm. per 100 cc.*
1	0.077	0.270	0.242	0.028
2	0.003	0.058	0.054	0.004
3	0.026	0.140	0.128	0.012
4	0.099	0.199	0.136	0.063
5	0.026	0.171	0.155	0.016
6	0.066	0.199	0.185	0.014
7	0.024	0.160	0.149	0.011
8	0.027	0.155	0.136	0.019
9		0.185	0.178	0.007
10		0.079	0.075	0.004
11		0.050	0.041	0.009
12		0.133	0.120	0.013
13		0.135	0.120	0.015
14		0.150	0.131	0.019
15		0.140	0.127	0.013
16		0.189	0.150	0.039
17		0.126	0.115	0.011
18		0.108	0.098	0.010
19		0.115	0.094	0.021
20		0.195	0.175	0.020
21		0.116	0.104	0.012
22		0.181	0.166	0.015
23		0.162	0.141	0.021
24		0.064	0.045	0.019
25		0.159	0.138	0.021
26		0.300	0.277	0.023
27		0.080	0.071	0.009

10 cc. of urine were added to 2 cc. of a 30 per cent suspension of washed Fleischmann's yeast in a 20 cc. volumetric flask. (The proportion of yeast was kept smaller than in the fermentation-

reduction method, in order to minimize the correction due to respiratory CO_2 formation in the yeast.) The flask was closed with a 1-hole rubber stopper, the hole of which was filled with a vaselined glass rod. The contents of the flask were mixed, and allowed to stand 1 hour for the yeast to act. The glass rod was withdrawn from the stopper and 8 drops of approximately CO_2-free N NaOH were added through the hole by means of a finely drawn out capillary delivery tip. The contents of the flask were made up to the mark and shaken to mix the alkali with the solution and to absorb any CO_2 that might have escaped into the gas space beneath the stopper. 5 cc. of the yeast-urine suspension, equivalent to 2.5 cc. of urine, were transferred to the Van Slyke-Neill blood gas apparatus. The suspension was acidified with 2 cc. of 5 N H_2SO_4, and the CO_2 was determined as described by Van Slyke and Neill (1924). The amount of CO_2 in the sample was calculated in mg. by the factors of Van Slyke and Sendroy (1927).

From the total CO_2 found in the above analysis, two corrections were determined and subtracted: (*a*) for the CO_2 preformed in the urine, yeast, and alkali used, (*b*) for the CO_2 formed by the yeast from its own substance during the period of fermentation. (*a*) was determined by CO_2 analysis of a mixture of urine + alkali + yeast put together in the order given so that the alkali inhibited action of the yeast on the urine. (*b*) was determined by mixing yeast with water in place of urine, and determining, by analyses at once and after an hour, the amount of CO_2 formed. This amount was relatively small. We assumed that yeast mixed with urine formed from yeast material the same amount of CO_2 as yeast mixed with water. If this assumption was erroneous, because of retarding or accelerating effect of urinary substances on the yeast's inner metabolism, it is not probable that the error introduced was important, because the entire value of the (*b*) correction was small.

With corrections (*a*) and (*b*) fermentable sugar was calculated as:

$$\text{Mg. fermentable sugar per 100 cc. urine} = 40\,K\,(\text{total mg. } CO_2 - a - b)$$

where K represents mg. of glucose yielding 1 mg. of CO_2.

The factor 40 is introduced because the samples analyzed each represent 2.5 cc., or $\frac{1}{40}$ of 100 cc., of urine.

The value of K was determined by incubating in the above manner with a portion of the same yeast 10 cc. of a 0.1 per cent glucose solution. The only blank required was the determination of the CO_2 in a control yeast suspension in water incubated for the same period. The CO_2 in the blank represented preformed CO_2 in the yeast + CO_2 formed from yeast substance during the time used for fermentation. The CO_2 found in the blank was subtracted from that found in the yeast-glucose mixture. The difference represented CO_2 formed from 2.5 mg. of glucose. Hence K was calculated as

$$K = \text{mg. glucose yielding 1 mg. } CO_2 = \frac{2.5}{\text{mg. } CO_2 \text{ formed from 2.5 mg. glucose}}$$

TABLE VII

Example of Calculation of Fermentable Urine Sugar from CO_2 Formed by Yeast

	P_{CO_2} at 2.0 cc. volume.	Temperature.	CO_2 from sample analyzed, equivalent to 2.5 cc. urine.
	mm.	*°C.*	*mg.*
Total CO_2	176.9	25.0	0.968
Blank (*a*)	37.4	25.0	0.205
" (*b*)	4.5	25.0	0.025

Mg. fermentable sugar per 100 cc. urine = 40 K (total mg. $CO_2 - a - b$)
= 105.6 (0.968 − 0.205 − 0.025)
= 77.9

Per cent fermentable sugar in urine = 0.078

K was 2.64 for the yeast used.

The value of K varied, for Fleischmann's yeast from different cakes, from 2.88 to 2.58. If the fermentation ran entirely in accordance with the equation

$$C_2H_{12}O_6 = 2\ CO_2 + 2\ C_2H_6O$$

the glucose:CO_2 ratio would be 180:88 = 2.045. The glucose per mg. of CO_2 in our fermentations was found to be from 1.41 to 1.26 greater than this amount. In other words, the CO_2 yield was 71 to 79 per cent of the theoretical corresponding to the above equation. An example of the calculation is given in Table VII.

The amount of fermentable sugar in normal urine determined in this manner is seen in Table VI to be from 0.00 to 0.05 grams per 100 cc. greater than that determined by difference in reducing value before and after yeast treatment. In seven of these eight normal urines CO_2 was formed in measurable amounts from materials other than glucose. Possibly these materials are amino acids and aliphatic α-keto acids, which have been shown (Harden, 1923) to be decomposed by the carboxylase of yeast with evolution of CO_2. The amounts of CO_2 formed from non-glucose, and

TABLE VIII

Content of Fermentable Sugar Determined by CO_2 Formation and by Gasometric Reduction Method in Urine of Patients with Definite Glycosuria

Fermentable sugar determined gasometrically by CO_2 formation.	Sugar concentration by gasometric ferricyanide method.		
	Total. (*a*)	Non-fermentable, determined after yeast treatment. (*b*)	Fermentable, calculated by difference. (*a*) − (*b*)
gm. per 100 cc.	*gm. per 100 cc.*	*gm. per 100 cc.*	*gm. per 100 cc.*
0.263	0.391	0.117	0.274
0.282	0.355	0.065	0.290
0.354	0.476	0.127	0.349
0.640	0.794	0.089	0.705
0.682	0.835	0.152	0.683
1.302	1.261	0.068	1.193
1.318	1.223	0.118	1.105
1.450	1.608	0.118	1.490

probably non-sugar, substances are obviously great enough to invalidate measurement of CO_2 formed by commercial yeast as a precise method for determining the slight amounts of glucose in normal urine.

In cases of definite glycosuria (urines with positive qualitative Benedict sugar test) the CO_2 method yields, as shown in Table VIII, results approximating those of the reduction method. In such urines the ratio of glucose to interfering substances is so large that the effect of the latter becomes relatively unimportant.

Effect of Ingestion of Glucose upon Urine Sugar Excretion of Normal Men

The subjects of this study were given no food or fluids for a 12 hour period preceding the determinations. Each subject was given 200 cc. of water at 7.00 a.m. and the urine was collected for

TABLE IX

Excretion of Fermentable and Non-Fermentable Reducing Substances after Glucose Ingestion by Normal Men

Subject No.	Time after feeding 1 gm. glucose per kilo body weight.	Urine volume.	Concentration of reducing substances in urine.			Excretion rate of reducing substances.		
			Total. (*a*)	Non-fermentable, determined after yeast treatment. (*b*)	Fermentable, calculated by difference. (*a*) − (*b*)	Total. (*c*)	Non-fermentable, determined after yeast treatment. (*d*)	Fermentable, calculated by difference. (*c*) − (*d*)
	hrs.	*cc. per hr.*	*gm. per 100 cc.*	*gm. per 100 cc.*	*gm. per 100 cc.*	*mg. per hr.*	*mg. per hr.*	*mg. per hr.*
1	−1 to 0	64	0.079	0.075	0.004	51	48	3
	0 " 1	26	0.103	0.101	0.002	27	26	1
	1 " 2	94	0.090	0.084	0.006	85	79	6
	2 " 3	218	0.025	0.025	0.000	55	55	0
	3 " 4	198	0.027	0.025	0.002	53	50	3
2	−1 " 0	28	0.185	0.178	0.007	52	50	2
	0 " 1	27	0.203	0.187	0.016	55	51	4
	1 " 2	32	0.168	0.153	0.015	54	49	5
	2 " 3	25	0.154	0.144	0.010	39	36	3
	3 " 4	26	0.171	0.158	0.013	44	41	3
3	−1 " 0	31	0.123	0.114	0.009	38	35	3
	0 " 1	31	0.110	0.093	0.017	34	29	5
	1 " 2	39	0.101	0.088	0.013	39	34	5
	2 " 3	24	0.126	0.114	0.012	30	27	3
	3 " 4	16	0.169	0.154	0.015	27	25	2

the period from 7.00 to 8.00 a.m. Glucose was given in 200 cc. of water at 8.00 a.m. Urine specimens were collected after 1, 2, 3, and 4 hours. The amount of glucose administered was 1 gm. for each kilo of ideal weight, estimated for the subject's height by Fig. 1 of McIntosh, Möller, and Van Slyke (1928). The glucose used was Merck's C.P.

The fermentable sugar in the urines of these subjects (Table IX) remained below 0.02 per cent throughout the test period. The data on excretion of urine sugar after the ingestion of glucose confirm the findings of Eagle (1926–27) that fermentable sugar does not usually increase in the urine after ingestion of such quantities of glucose by the normal individual.

SUMMARY

Procedures are described for applying the authors' gasometric ferricyanide reduction method to determination of fermentable sugar in blood and urine. Fermentable sugar is measured by the decrease in reducing material caused by brief contact with yeast under conditions producing complete removal of glucose.

The usual normal urine contains about 0.15 ± 0.10 per cent of total reducing substances, of which on the average only about one-tenth is fermentable. Only three urines out of twenty-seven showed more than 0.023 per cent of fermentable sugar. Our normal fermentable urine sugar values are somewhat higher than Eagle's (1926–27), which were below 0.01 per cent, but confirm him in showing that only a relatively slight proportion of the reducing material usually encountered in normal urine can be glucose.

The ingestion of 1 gram of glucose per kilo by three normal men did not in any of them increase significantly the concentration or output rate of fermentable sugar in the urine. This result again confirms Eagle.

Manometric measurement of the CO_2 formed by yeast indicated in normal urine fermentable sugar contents averaging 0.03 per cent higher than those estimated from the decrease in reducing power. The extra CO_2 is attributable to non-glucose urine constituents, of which amino acids and α-keto acids have previously been shown to yield CO_2 under influence of yeast carboxylase.

In glycosuric urines the amount of extra CO_2 formed by yeast from non-glucose substances is relatively unimportant, and in such urines results by the CO_2 method do not deviate significantly from those by the fermentation-reduction method.

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[Reprinted from THE JOURNAL OF BIOLOGICAL CHEMISTRY, August, 1929, Vol. 83, No. 2, pp. 415–423]

THE DETERMINATION OF ACETONE BODIES IN BLOOD AND URINE

REPLY TO CRITICISMS BY E. C. SMITH

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(Received for publication, June 8, 1929)

Methods for determination of the "acetone bodies," acetone, acetoacetic acid, and β-hydroxybutyric acid, have been published by the writer (Van Slyke, 1917), based upon Deniges' procedure for precipitating acetone as a basic mercuric sulfate compound weighing approximately 20 times as much as the acetone it contains. Hydroxybutyric acid was oxidized to acetone by the chromate oxidation introduced by Shaffer (1908–09). Oxidation and precipitation were carried out simultaneously, so that the technique for determining the total acetone bodies consisted merely in boiling properly prepared urine or blood filtrate (Van Slyke and Fitz, 1917, 1919) with a specified mixture of sulfuric acid, mercuric sulfate, and dichromate under a reflux condenser, the resultant precipitate being either weighed or redissolved and determined by titration of its mercury according to Personne's method. Acetone and acetoacetic acid, without the hydroxybutyric acid, could be determined separately by omitting the chromate from the reaction mixture.

Glucose and most interfering substances occurring in normal or pathological urine were removed by a preliminary precipitation with cupric sulfate and calcium hydroxide. A trace was found to remain of non-acetone substances which precipitate with the mercuric sulfate. For these substances a correction was described; the acetone was distilled off from the clarified urine and the residual solution was boiled with mercuric sulfate without chromate. In a series of twenty-three normal urines the total acetone bodies determined without subtracting the correction for these interfering substances was in all cases below 0.05 per cent, calculated as acetone. Corrected for the non-acetone precipitate the total acetone bodies in all cases were below 0.03 per cent. Ketone-free diabetic urines yielded similar values. In a series of ketone-containing urines the method for hydroxybutyric acid was compared with that of Black (1908–09), in which the acidified urine is mixed with plaster of Paris, the hydroxybutyric acid is extracted with ether, and estimated polarimetrically by its levorotation. Black's method gave 85 to 100 per cent as much hydroxybutyric

acid as the writer's. Considering that, as previously pointed out by Shaffer and Marriott (1913–14), the polarimetric results are likely to be somewhat low, because of adsorption of hydroxybutyric acid by the charcoal used in preliminary clearing of the urine, because of slight racemization of the acid during extraction, and because of incomplete extraction, it was considered that the agreement was satisfactory. From the comparison with Black's method in ketone-containing urines and from the nearly negative results with ketone-free urines, it appeared that the new method could be trusted to give complete yields of the acetone bodies present, and to be free from significant error due to interference by non-ketone urinary constituents. Since its appearance the method has received fairly extensive use over periods of years in a number of laboratories, and has apparently met these requirements.

Smith (1926), however, has recently criticized the procedure, for both blood and urine, on the following grounds.

1. In blood used for perfusion experiments he found that the amount of lactic acid present (of the order of magnitude of 100 mg. per 100 cc.) could increase significantly the yield of acetone-mercuric-sulfate precipitate obtained in determination of the total acetone bodies. Smith ignores the fact that the behavior of lactic acid was pointed out with quantitative data, on p. 486 of the writer's original paper (1917). It was there shown that lactic acid when boiled with chromate under the conditions of the analysis yields some product which precipitates with mercuric sulfate. The amount of precipitate formed per mg. of lactic acid was found, however, to be only about one-twentieth of that formed per mg. of acetone. Blood freshly drawn from the circulation has not enough lactic acid to cause a significant error, except in comparatively rare specimens, such as are drawn immediately after severe exercise or asphyxia. As shown by Evans (1922), however, if drawn blood is allowed to incubate for some hours its glucose is in large part converted into lactic acid. This conversion appears to be especially rapid with dog blood, compared with human and horse bloods. The effect of the amount of lactic acid in the blood used in Smith's perfusion experiments could have been foretold from data in the writer's original paper.

In ordinary blood analyses the effect is insignificant, as shown by the fact that only 1 or 2 mg. of precipitate are obtained from the filtrate of 5 cc. of normal blood (Van Slyke and Fitz, 1917). To yield an amount of precipitate equal to that from acetone bodies

in a concentration of 1 millimol per liter of blood, about 110 mg. of lactic acid per 100 cc. of blood would be required. Normal, diabetic, and most pathological blood drawn during rest or ordinary activity contains only 10 to 40 mg. per 100 cc. (Clausen, 1922; Ronzoni and Wallen-Lawrence, 1927). After severe exercise it may rise to over 100 mg., likewise in cardiac decompensation sufficient to result in severe anoxemia (Meakins and Long, 1927). In these conditions the lactic acid may cause 5 to 10 mg. of precipitate in the total acetone bodies determination in blood, enough to simulate a slight ketosis, sufficient to be detectable, but not sufficient to be important in the acid-base balance of the blood.

2. Smith states that with diabetic urine the writer's method for total acetone bodies (acetone plus acetoacetic acid plus β-hydroxybutyric acid) yields results which are markedly too high. The basis for this conclusion was that when the precipitate was redissolved in 10 per cent hydrochloric acid, and the acetone was distilled therefrom and titrated with iodine, only 72 per cent as much acetone was indicated by the titration as was expected on the assumption that the precipitate consisted of 5 per cent by weight of acetone. When Smitl. applied the same procedure to the mercury precipitate from a pure acetone solution, the titration indicated the amount of acetone estimated from the weight of the precipitate. In reporting the low yield of acetone obtained by distillation from the urine precipitate Smith gives no details: it is impossible to tell whether he gives the result of a single observation or the mean of a number, whether the urine analyzed contained much or little acetone, and whether correction was made, as directed in the writer's original paper, for the slight weight of precipitate formed by the mercuric sulfate with non-acetone urinary constituents. The precipitate from such substances is only 1 or 2 per cent of the total precipitate obtained in analyses of urines from subjects with severe or moderate ketosis, but is more important if the ketosis is slight, and in normal urine may exceed the precipitate yielded by the acetone bodies themselves. These facts are illustrated by the series of analyses of diabetic and normal urines published in the original paper (Van Slyke, 1917). Smith limited his control analyses to pure acetone solutions. He reports none on pure β-hydroxybutyric acid, although in diabetic

ketosis that substance constitutes about four-fifths of the "total acetone bodies."

We have repeated Smith's distillation procedure with mercury precipitates obtained in determinations of acetone plus acetoacetic acid and of total acetone bodies in a number of diabetic urines, and have controlled the results by performing similar determinations with solutions containing known amounts of both acetone and β-hydroxybutyric acid. Proper correction was made, as described in our original paper, for the mercury precipitate yielded by non-volatile substances in the urine filtrates. The results fail to confirm Smith's criticisms; in fact the data constitute additional proof that the writer's method is extremely specific for acetone, acetoacetic acid, and β-hydroxybutyric acid among the constituents of human urine.

When pure acetone solutions were precipitated and the acetone from the redissolved precipitate was distilled and titrated, the yield per gram of precipitate depended somewhat on the amount of precipitate. When the latter was in the neighborhood of 500 mg., (representing 25 mg. of acetone) distillation yielded 97 to 98 per cent of the expected 5 per cent of the precipitate's weight of acetone. When the precipitate was a third as great the yield of titrated acetone from it was only 90 per cent of the expected (see Table I).

Precipitates from the acetone plus acetoacetic acid of diabetic urines, when the prescribed correction was deducted for the small portion due to non-acetone substances, yielded, within 1 or 2 per cent, the same proportion of distilled acetone as precipitates of similar weight formed from solutions of pure acetone (compare Tables I and III).

Precipitates from pure hydroxybutyric acid yielded, as shown by Table II, about 10 per cent less distilled acetone than precipitates of the same weight from acetone solutions. It appears accordingly that when β-hydroxybutyric acid is boiled with sulfuric acid, chromate, and mercuric sulfate, under the conditions prescribed for determining β-hydroxybutyric acid or total acetone bodies, about 10 per cent of the precipitate formed is from an oxidation product or products other than acetone. As seen by comparison of Tables II and III, *precipitates from diabetic urines obtained in determination of the total acetone bodies yielded the*

same proportion of distilled acetone as precipitates of the same weight from pure hydroxybutyric acid. This fact adds to the evidence, quoted above from the writer's original paper (1917), that no significant part of the precipitate obtained in the determination of hydroxybutyric acid or total acetone bodies in ketone-containing urines originates from substances other than the acetone bodies sought.

The factors, by which hydroxybutyric acid or total acetone bodies are calculated from the weight of the precipitate obtained by the writer's method, were determined empirically by analyses of known solutions of pure acetone and β-hydroxybutyric acid (Van Slyke, 1917). These factors express the directly determined relation between the weight of precipitate and the amount of acetone or hydroxybutyric acid in the fluid analyzed, and involve no assumption concerning the amount of acetone recoverable from the precipitate. Hence the results in the present paper do not necessitate changing any of the factors used in calculating blood or urine acetone bodies content from the amount of precipitate obtained.

EXPERIMENTAL

The acetone used in the experiments recorded in Table I was prepared by redistilling Kahlbaum's preparation made from the sulfite compound. Stock solutions containing approximately 1 mg. per cc. were made by pipetting portions of the acetone into weighed measuring flasks partly filled with water, the acetone being measured by the increase in weight of the flask. These stock solutions when titrated for acetone with 0.1 N iodine solution and thiosulfate gave theoretical values.

The calcium-zinc salt of hydroxybutyric acid used for the experiments recorded in Table II was from the same lot of which the preparation (by the method of Shaffer and Marriott), analysis, and rotation are described in the original paper (Van Slyke, 1917).

The precipitates were all formed under conditions with regard to volume of solution, reagents, time of boiling, etc., which conform to those prescribed in the original paper (Van Slyke, 1917), for determination of acetone plus acetoacetic acid or of total acetone bodies.

The following procedure was used for distilling, and titrating by Messinger's method, the acetone from the mercury precipitates.

Each precipitate was collected, dried, and weighed in a Gooch crucible. It was then moistened with water, and the precipitate, together with the asbestos in the crucible, was transferred with the aid of a rod and wash water to a 500 cc. Kjeldahl flask. The volume of water in the flask was brought up to about 300 cc., and

TABLE I

Results with Solutions of Acetone

Acetone present. (*a*)	Weight of precipitate obtained. (*b*)	0.1 N iodine to titrate acetone distilled from precipitate. (*c*)	Acetone calculated from weight of precipitate = $\frac{(b)}{20}$. (*d*)	Acetone calculated from titration of distillate = 0.967 (*c*). (*e*)	Ratio of distilled acetone to acetone calculated from precipitate = $\frac{(e)}{(d)}$.
mg.	*mg.*	*cc.*	*mg.*	*mg.*	
2	37.3	1.59	1.86	1.54	0.830
	38.1	1.61	1.91	1.56	0.820
4	76.9	3.49	3.85	3.37	0.876
	79.8	3.57	3.99	3.45	0.865
8	156.3	7.29	7.82	7.05	0.902
	161.5	7.53	8.07	7.28	0.902
10.63	214.4	10.13	10.72	9.80	0.915
	207.8	9.93	10.37	9.60	0.926
	207.0	9.93	10.35	9.60	0.927
21.26	421.3	20.82	21.06	20.13	0.956
	409.0	20.64	20.45	19.96	0.976
	422.0	21.22	21.10	20.52	0.972

15 cc. of concentrated hydrochloric acid were added, dissolving the precipitate. The flask was immediately closed with a stopper bearing a Kjeldahl trap which was connected with a glass condenser. The outlet tube at the bottom of the condenser dipped below the surface of 150 cc. of water in a 500 cc. flask, which was cooled in an ice bath. The distillation was continued for 25 to 30 minutes. Longer distillation did not increase the yield.

To the distillate 10 cc. of 40 per cent KOH were added, and an excess of 0.1 N iodine solution. The flask was covered with a watch-glass and permitted to stand at room temperature for 20 minutes. Then sufficient concentrated hydrochloric acid (5.5 cc.) was added to neutralize the KOH and provide an excess of 0.2 cc. of acid. The solution was gently shaken to mix the reagents and the excess iodine was titrated with thiosulfate until the brown color had nearly disappeared. Then a few cc. of 5 per cent starch

TABLE II

Results with Solutions of β-Hydroxybutyric Acid

Hydroxybutyric acid present.		Weight of precipitate.	0.1 N iodine to titrate acetone distilled from precipitate.	Acetone calculated from weight of precipitate $= \frac{(b)}{20}$.	Acetone calculated from titration of distillate = 0.967 (c).	Ratio of distilled acetone to acetone calculated from precipitate $= \frac{(e)}{(d)}$.
Calcium-zinc salt added.	Amounts of free acid equivalent to Ca-Zn salt. (a)	(b)	(c)	(d)	(e)	
mg.	*mg.*	*mg.*	*cc.*	*mg.*	*mg.*	
12.0	19.6	83.2	3.50	4.16	3.38	0.813
		80.8	3.39	4.04	3.28	0.812
24.0	19.3	161.4	6.93	8.07	6.70	0.830
		161.6	6.95	8.08	6.72	0.832
56.0	45.0	379.8	16.44	18.99	15.90	0.837
		381.0	16.64	19.05	16.12	0.842
		382.2	16.85	19.11	16.30	0.853
112.0	90.0	765.6	32.59	38.28	32.59	0.850
		772.2	32.77	38.61	32.77	0.848
		779.2	32.80	38.96	32.80	0.842

solution were added and the titration was continued until the blue color disappeared.

Blank determinations were performed in which asbestos mats from control Gooch crucibles were washed into Kjeldahl flasks, acidified, distilled, and the distillates were titrated in the same manner. The iodine used in the blank, about 0.4 cc. of 0.1 N solution, was subtracted from that used in the titration of the acetone distillates.

TABLE III

Results with Ketone-Containing Urines

Urine No.	Determination.	Precipitate. (a)	Blank precipitate due to non-acetone substances. (b)	Precipitate corrected for blank = (a) − (b). (c)	0.1 N iodine to titrate acetone distilled from precipitate. (d)	Acetone calculated from corrected weight of precipitate. (e)	Acetone calculated from titration of distillate = 0.967 (d). (f)	Ratio of distilled acetone calculated from precipitate = $\frac{(f)}{(e)}$.
		mg.	*mg.*	*mg.*	*cc.*	*mg.*	*mg.*	
1	Acetone + aceto-acetic acid.	123.6	4.6	119.0	5.24	5.95	5.07	0.853
		123.4	4.6	119.0	5.19	5.95	5.02	0.843
	Total acetone bodies.	40.34	4.6	398.8	17.28	19.94	16.70	0.837
		40.46	4.6	400.0	17.25	20.00	16.69	0.834
2	Acetone + aceto-acetic acid.	164.2	2.6	161.6	7.50	8.08	7.25	0.897
		166.0	2.6	163.4	7.67	8.17	7.42	0.908
	Total acetone bodies.	564.2	2.6	561.6	24.04	28.08	23.25	0.828
		565.6	2.6	563.0	24.38	28.15	23.60	0.838
3	Acetone + aceto-acetic acid.	158.4	4.0	154.0	7.21	6.97	7.70	0.905
		160.8	4.0	156.8	7.29	7.05	7.84	0.897
	Total acetone bodies.	428.2	4.0	424.2	19.12	18.5	21.21	0.872
4	Acetone + aceto-acetic acid.	122.0	3.2	118.8	5.36	5.94	5.18	0.872
		121.2	3.2	118.0	5.34	5.90	5.16	0.874
	Total acetone bodies.	405.6	3.2	402.4	17.90	20.12	17.32	0.861
		408.6	3.2	405.4	18.05	20.27	17.41	0.860
5	Acetone + aceto-acetic acid.	51.8	1.2	50.6	2.17	2.53	2.10	0.830
		51.2	1.2	50.0	2.14	2.50	2.07	0.828
	Total acetone bodies.	151.5	1.2	150.3	6.44	7.52	6.22	0.827
		150.2	1.2	149.0	6.40	7.49	6.19	0.826
6	Acetone + aceto-acetic acid.	108.2	1.4	106.8	4.96	5.34	4.80	0.899
		109.0	1.4	107.6	4.92	5.38	4.76	0.884
	Total acetone bodies.	322.4	1.4	321.0	14.28	16.05	13.80	0.860
		317.4	1.4	316.0	14.03	15.80	13.56	0.859

Stock solutions of acetone distilled and titrated in this manner gave theoretical results.

SUMMARY

The basic mercuric salt precipitates, obtained by applying to diabetic urines the writer's methods for determination of acetone plus acetoacetic acid and of total acetone bodies (acetone plus acetoacetic acid plus β-hydroxybutyric acid) yield when dissolved and distilled, the same proportions of acetone as precipitates of equal weight obtained from solutions of pure acetone and β-hydroxybutyric acid respectively. This fact affords added evidence that no significant amount of the precipitate obtained from diabetic urines originates from substances other than the acetone bodies. Smith's contrary conclusion is attributable to his failure to carry out control analyses with pure hydroxybutyric acid.

The analyses reported above were performed by Mr. John Plazin.

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[Reprinted from THE JOURNAL OF BIOLOGICAL CHEMISTRY, August, 1929,
Vol. 83, No. 2, pp. 425–447]

MANOMETRIC DETERMINATION OF PRIMARY AMINO NITROGEN AND ITS APPLICATION TO BLOOD ANALYSIS

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(Received for publication, June 8, 1929)

The writer has previously published (Van Slyke, 1910, 1911) a procedure for determination of aliphatic amino nitrogen by measurement of the N_2 gas evolved by the reaction with nitrous acid.

$$RNH_2 + HNO_2 = ROH + 2\,H_2O + N_2$$

A special apparatus was devised, which was later improved (1912) and developed for micro analyses (1913–14, 1915).

The more generally useful Van Slyke-Neill manometric apparatus, however, gives equally rapid and accurate amino nitrogen determinations, and makes possible micro analyses with smaller amounts of material than even the micro type of the former apparatus. This advantage in favor of the manometric apparatus is due partly to the greater accuracy with which it permits measurement of small gas amounts, partly to its permitting one to use larger volumes of amine solution. In consequence, whereas the micro form of the previous apparatus permitted measurement of amino nitrogen concentration in 2 cc. of solution to ± 0.001 mg. per cc., the manometric apparatus permits measurement in a 5 cc. sample to ± 0.0001 mg. per cc., this amount causing a change in the manometer reading of 1.3 mm. With the manometric apparatus one can accordingly perform a blood amino nitrogen determination directly on 5 cc. of the Folin-Wu tungstic acid blood filtrate, without the concentration to smaller volume which was formerly a necessary preliminary to analysis.

For discussion of the principle of the reaction and the manner

in which varying types of aliphatic amines react with nitrous acid under the conditions employed, the reader is referred to the original paper (Van Slyke, 1911). The most significant facts are that the NH_2 groups in the α-amino acids react quantitatively in 3 to 4 minutes at room temperature; while NH_2 groups in other types of substances react much more slowly. Of ammonia about 25 per cent reacts in the time required for complete reaction of α-amino acids (Van Slyke, 1912), and of urea only 6 to 7 per cent (Levene and Van Slyke, 1912).

The reaction is carried out by mixing three solutions; *viz.*, of sodium nitrite, acetic acid, and amine. In the original special apparatus (Van Slyke, 1910, 1911, 1912) the order of addition was obligatory: the nitrite and acetic acid had to be added first, and shaken until the NO gas evolved by spontaneous decomposition of HNO_2 had washed all the air out of the reaction chamber, before the amine solution was added. In the manometric apparatus any two of the reagents, AcOH and $NaNO_2$, $NaNO_2$ and RNH_2 (in neutral or alkaline solution), or AcOH and RNH_2, may be mixed and freed of air in the extraction chamber, and the third reagent then added. The last of the above three orders proved in general to be preferable. The amine solution and acetic acid are mixed and freed of air in the chamber, and the $NaNO_2$ is then added in saturated solution. The saturated nitrite solution need not be freed of dissolved air before it is used. Because of its high salt content (60 grams per 100 cc.), this solution when saturated with air at room temperature dissolves only 0.2 volume per cent of the atmospheric gases (one-tenth as much as water), of which one-third is O_2 and disappears by combination with NO during the reaction. The amount of dissolved atmospheric N_2 carried into the apparatus by the 2 cc. of nitrite solution exerts only 4 mm. of pressure when the gas is measured at 0.5 cc. volume, 1 mm. when at 2 cc. The corrections for these small amounts of dissolved air are too small to vary measurably with room temperature or barometric pressure, and are automatically included in the blank analysis on the reagents.

The entire procedure requires 12 to 15 minutes. The maximum amount of amino nitrogen that can be determined in a sample is about 0.6 mg., which at 2 cc. volume yields nitrogen gas giving a little over 400 mm. of pressure. The minimum amount meas-

urable in micro determinations is about 0.0004 mg., which yields nitrogen gas giving 1 mm. of pressure at 0.5 cc. volume. Since samples of 5 cc., and, if desired, greater volume can be analyzed, a concentration of 0.01 mg. of amino nitrogen per cc. suffices for an analysis capable of 1 per cent accuracy.

DESCRIPTION OF METHOD

Reagents

Sodium Nitrite Solution.—800 grams of $NaNO_2$ dissolved with the aid of warming in 1 liter of water.

Glacial Acetic Acid.

Alkaline Permanganate.—50 grams of $KMnO_4$ are shaken with 1 liter of 10 per cent NaOH solution until the latter is saturated with the permanganate.

Caprylic Alcohol.—This is used when necessary to prevent foaming of viscous solutions.

Procedure

The analysis consists of the following steps.

1. The amine solution and acetic acid are freed of air in the apparatus.

2. Sodium nitrite solution is added and the resultant nitrous acid is permitted to react for the necessary time, 3 to 4 minutes in the case of α-amino acids at room temperatures of 25–20°.

3. The mixture of N_2 and NO (the latter formed by spontaneous decomposition of HNO_2) is transferred to a Hempel pipette of the type described by Van Slyke and Hiller (1928), where the NO is absorbed by permanganate.[1]

4. The chamber of the Van Slyke-Neill apparatus is washed

[1] It is possible by using the Harington-Van Slyke (1924) type of extraction chamber to avoid the use of the Hempel absorption pipette. The nitrous acid solution is drawn off and ejected through the trap at the bottom of the Harington-Van Slyke chamber, which is then washed with absolutely air-free 20 per cent NaOH solution, followed by air-free permanganate, over which the residual N_2 is finally measured. However, in practice this procedure has proved less convenient and certain than that described in the text. Introduction of the slightest trace of air with the alkali or permanganate suffices to invalidate a micro amino determination. And the permanganate each time fouls the mercury, although the latter is instantly cleaned by contact with the next nitrous acid solution.

free of nitrous acid, and the purified N_2 gas is returned from the pipette.

5. The amount of N_2 gas is measured by the pressure it exerts at either 0.5 or 2.0 cc. volume.

The details of the successive steps follow.

1. Removal of Air from Mixed Solution of Amine and Acetic Acid.—The sample of amine solution may vary from 1 to 8 cc. in volume. 5 cc. is usually a convenient size. The amine solution is run into the chamber of the Van Slyke-Neill apparatus, followed by 1 cc. of glacial acetic acid. The most convenient way to add the amine solution is to run it directly into the chamber from a rubber-tipped, stop-cock pipette, as shown in Fig. 3 of the paper on the portable manometric apparatus (Van Slyke, 1927). However, one may run the solution from an ordinary pipette into the cup of the apparatus and wash into the chamber with small amounts of water, or with the 1 cc. of acetic acid divided into three or four portions. The total volume of fluid added should be known, because the time required for the subsequent reaction is proportional to the dilution of the reagents. If protein or other content of the amine solution makes the latter likely to form troublesome foam, a drop of caprylic alcohol is added with the acetic acid. The amine solution and acetic acid being in the chamber, the cock of the latter is sealed with a drop of mercury, and the chamber is evacuated and shaken for 2 minutes at the usual tempo of 250 to 300 times per minute.

The air extracted from the solution is then ejected from the chamber as follows: The leveling bulb is placed in its uppermost ring, slightly above the chamber. The cock admitting mercury from the leveling bulb into the chamber is opened, so that the extracted air is compressed into a bubble at the top of the chamber. The cock from the leveling bulb is now closed, and the cock at the top of the chamber (see Fig. 1) is opened. Part of the air escapes by its own pressure. The rest is expelled by admitting mercury slowly from the leveling bulb, until the solution in the chamber has risen just high enough to expel the gas and fill the capillary above Cock *b*.

2. Decomposition of Amino Groups.—2 cc. of the nitrite solution are measured into the chamber. Evolution of N_2 and NO begins at once. The cock is sealed with a drop of mercury, and the

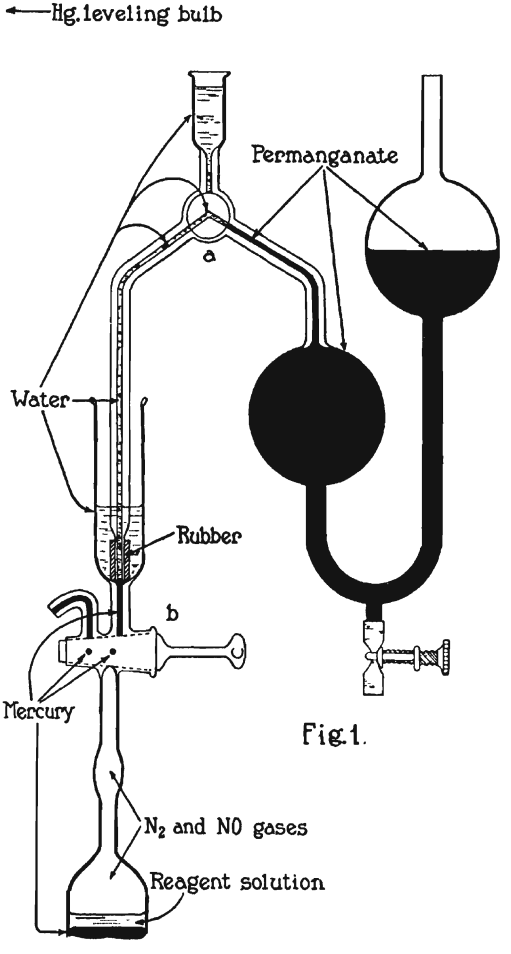


FIG. 1. Apparatus arranged for transfer of N_2 + NO gas mixture to Hempel pipette by turning Cock *b*

chamber is evacuated until the mercury in it has fallen to a level 1 or 2 cm. above the 50 cc. mark. The reaction mixture is permitted to stand in this position until within 1 minute of the end of

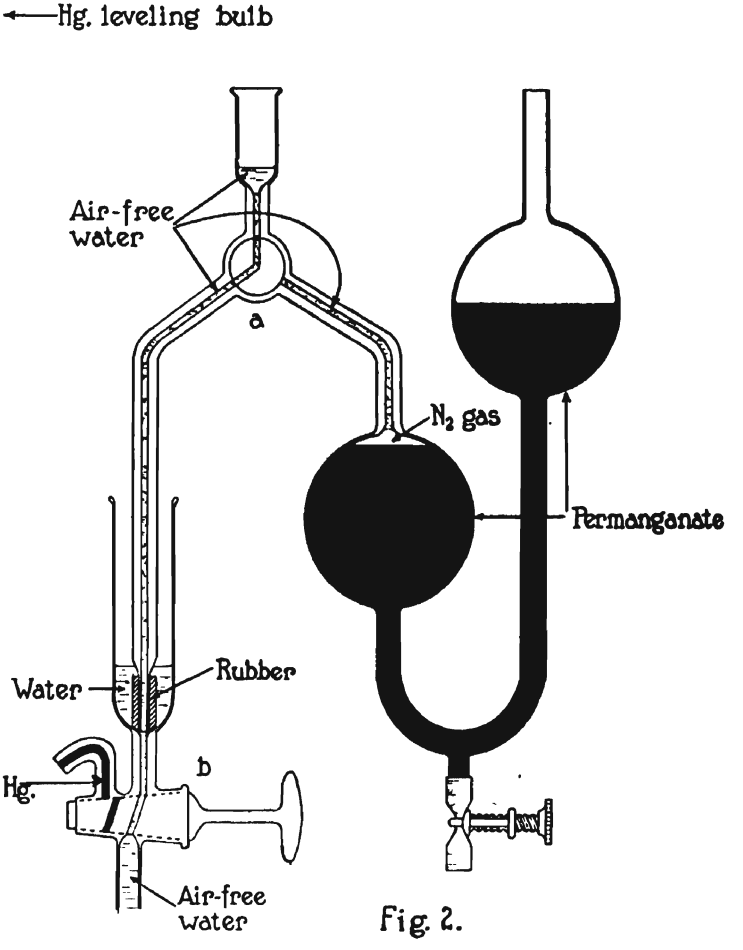


FIG. 2. After absorption of NO by permanganate in Hempel pipette, and replacement of reagents by air-free water in extraction chamber, the Hempel pipette is placed again in connection with the chamber, and water from the chamber is forced up through the capillary into the cup of the pipette.

the reaction time, given below. During the last minute the mixture is shaken to complete the evolution of the N_2 formed. The relatively large amount of NO gas evolved with the N_2 by spon-

taneous decomposition of the nitrous acid tends to press the mercury meniscus down into the tube below the chamber. To prevent this, one admits mercury from the leveling bulb once or twice during the shaking, so that the mercury meniscus in the chamber remains within a centimeter of the 50 cc. mark.

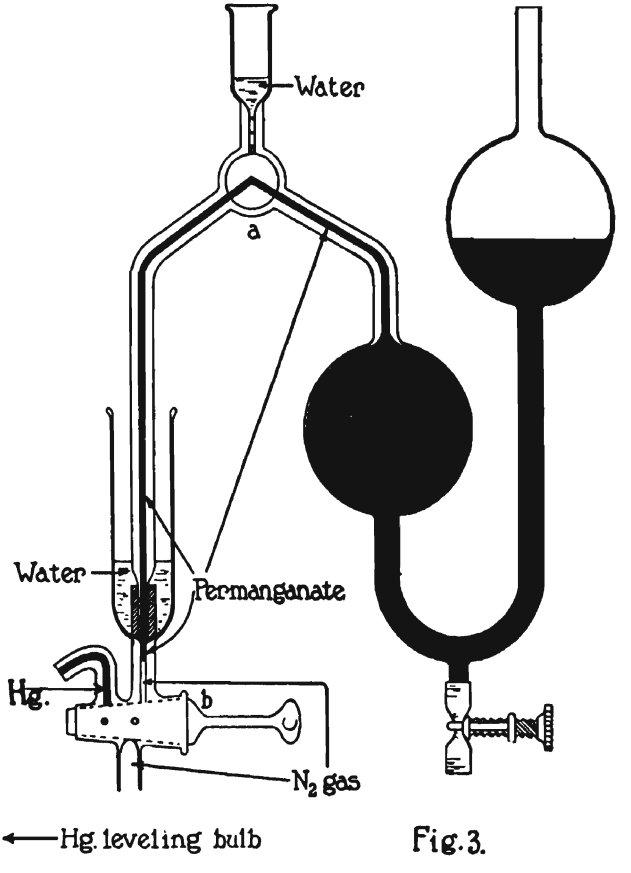


FIG. 3. Mercury-leveling bulb has been lowered and N_2 gas drawn back to chamber, followed by permanganate solution as far as point indicated.

The time required for complete decomposition of the alpha NH_2 groups of amino acids, measured from the moment when the nitrite is run into the chamber till the end of the minute of shaking, varies with the temperature, and at a given temperature it is proportional to the volume to which the reagents are diluted. When the amine solution plus the water added with it is 5 cc., so

that the total volume of solution in the chamber is 8 cc., the time required for quantitative reaction of α-amino acids is 3 minutes at 25°, 4 minutes at 20°, and 6 minutes at 15°, as indicated by Fig. 4. If the volume of the mixed solutions is greater or less than 8 cc., the reaction time is proportionally increased or diminished.

3. Transfer of NO + N_2 Gas Mixture to Permanganate Pipette, and Absorption of NO.—After the reaction between amine and nitrous acid is completed, the mercury leveling bulb of the Van Slyke-Neill apparatus is raised to the level indicated in Fig. 1, and the cock (not shown in the figure) connecting the leveling bulb with the gas chamber is opened. The mercury rises in the chamber, and the gases in it collect at the top under positive pressure, as shown in Fig. 1. 2 or 3 cc. of water are placed in the cup above the chamber, and the Hempel pipette, with its capillaries filled with water, is pressed firmly into the position shown in Fig. 1. Cock *a* is turned into position as shown in Fig. 1'. Then by opening Cock *b* the gases are forced over into the pipette. When the nitrous acid solution, following the gases, has travelled up the pipette capillary nearly as far as Cock *a*, both Cocks *b* and *a* are closed. It is preferable not to let any of the nitrous acid solution pass over into the permanganate, because it exhausts the latter unnecessarily.

With Cock *a* in a position intermediate between those shown in Figs. 1 and 2, the Hempel pipette is disconnected from the extraction chamber. The gas in the capillary between Cock *a* and the permanganate bulb is forced down into the latter by water from the cup. The remainder of the water in the cup is driven out through the left hand capillary, to wash the nitrous acid solution out of it.

The disconnected Hempel pipette is gently shaken horizontally by hand to absorb the NO gas. The time required is 20 to 40 seconds, depending on whether the amount of N_2 approximates the minimum or maximum determinable by the method. The pipette is then set aside. (It may be conveniently suspended from a hook at the right of Cock *a*.)

4. Return of Purified N_2 Gas to Manometric Apparatus.—Before the N_2 is returned to the gas chamber, the nitrous acid solution is ejected from the latter, which is then washed twice by the following technique. The mercury leveling bulb is placed in its ring

at the low level, where it evacuates the chamber. As the mercury in the latter falls, 10 or 15 cc. of water, but no air, are admitted to the chamber from the cup at its top. The bulb is then raised, and the water is ejected. 30 seconds suffice for each washing.[2] After the second washing 10 cc. of water, measured in two portions from the cup, are admitted into the chamber, which is evacuated and shaken for 1 minute to remove the greater part of the air from the water. The extracted air is ejected, and 1 cc. of the water is forced up into the cup above the chamber.

The N_2 gas from the Hempel pipette is now returned to the chamber by connecting as shown in Fig. 3. The capillary between Cocks *a* and *b* is filled with water from the chamber, and a little water is forced up into the cup above Cock *a*. The mercury leveling bulb is now lowered to its middle position, level with the bottom of the extraction chamber and the N_2 is admitted from the pipette to the chamber. The flow of gas to the chamber can be regulated either by Cock *b* or by the cock (not shown in the figures) which connects the chamber to the mercury bulb. The writer prefers the mercury cock because of the nicety with which the flow of the mercury, and thereby that of the other fluids, can be regulated. The flow is stopped and Cock *b* is closed when the column of permanganate has reached the position shown in Fig. 3, in the capillary above Cock *b*. It is preferable to get as little permanganate into the chamber below as possible, in order to have a clear water meniscus there for reading.

After removing the Hempel pipette the cup of the gas chamber is washed with water to remove permanganate that may have escaped into it, and about 1 cc. of mercury is run underneath water into the cup. As much of this mercury is run into the chamber as may be necessary to clear of permanganate the bore of Cock *b* and the constricted top of the chamber below the cock.

5. Measuring the Nitrogen Gas.—The level of the water in the chamber is lowered until the water meniscus is at either the 0.5 cc.

[2] In case large amounts of protein are present in the solution analyzed, deaminized protein is precipitated and floccules of it are likely to adhere to the walls of the chamber when the nitrous acid is ejected. Such particles are dissolved by running in a few cc. of 5 or 10 per cent NaOH solution, and raising and lowering the mercury and the alkali solution in the chamber. The latter is then washed twice with water, as above described.

or the 2.0 cc. mark, according to the amount of gas present. If the latter at the 2.0 cc. mark exerts less than 100 mm. pressure it is preferable to use the 0.5 cc. mark, employing a reading glass to locate the meniscus exactly on the line. The manometer reading, p_1, is taken. The gas is ejected from the chamber by the technique described for the first step of the analysis, and the manometer reading p_0 is taken with the water meniscus in the gas-free chamber at the same level used for the p_1 reading.

Blank Analysis of Reagents.—A control analysis is carried out in the same manner, except that an equal volume of water replaces the amine solution. The difference $p_1 - p_0$ obtained in the control is the c correction used in the calculation below. For some days or weeks after the sodium nitrite solution has been prepared the c correction appears to diminish, and finally to become constant. It is convenient to set aside a considerable amount of nitrite solution and acetic acid on which the c correction has been determined, in order to avoid necessity of frequent redetermination on new solutions. With the Merck's "Reagent" nitrite used by us the c correction has been 20 to 30 mm. measured with the gas at 0.5 cc. volume, and one-fourth as much at 2.0 cc. volume. When there is sufficient amino nitrogen to give over 100 mm. pressure at 2 cc. volume, variations of the c correction with ordinary variation in room temperature may be neglected. For micro analyses, however, with gas measurements at the 0.5 cc. mark the c correction must be determined at a temperature near that of the analysis.

Calculation.—The pressure of N_2 gas from the amine analyzed is calculated as

$$P_{N_2} = p_1 - p_0 - c$$

whence the weight of amino nitrogen in the sample is calculated as

$$\text{Mg. amino N} = P_{N_2} \times \text{factor}$$

The values of the factor are found in Table I.

These values are calculated from Equation 5 of Van Slyke and Neill (1924); *viz.*,

$$\text{mm gas} = P \times \frac{i\,a\,\cdot}{17{,}024\,(1 + 0.00384\,t)}\left(1 + \frac{S}{A - S}\,\alpha'\right)$$

where P is the observed mm. pressure of gas at a cc. volume, S the cc. of solution present in the extraction chamber of A cc. capacity, and α' is the Ostwald solubility coefficient of the gas in the solution. The millimols obtained by the equation are multi-

TABLE I

Factors for Calculation of Amino Nitrogen

Temperature.	Factors by which mm. P_{N_2} are multiplied to give mg. amino N in sample analyzed.		Factors by which mm. P_{N_2} are multiplied to give mg. amino N per 100 cc. blood when filtrate sample is equivalent to 0.5 cc. blood.
	a = 0.5 cc.	a = 2.0 cc.	a = 0.5 cc.
°C.			
15	0.000390	0.001561	0.0780
16	389	55	777
17	387	49	774
18	386	44	772
19	385	38	769
20	383	33	766
21	382	27	763
22	380	22	761
23	379	16	758
24	378	11	756
25	376	06	753
26	375	00	750
27	374	0.001495	748
28	372	90	745
29	371	85	743
30	370	80	740
31	368	74	737
32	367	69	734
33	366	64	732
34	365	59	730

plied by $\frac{28.02}{2} = 14.01$ to obtain mg. of amino nitrogen, since of each millimol (28.02 mg.) of N_2 yielded by the reaction with nitrous acid only half comes from the amine. The value in the equation for the reabsorption factor, i, in the case of N_2 is 1,

because no measurable reabsorption of N_2 occurs under the conditions of the analysis.

The factor $(1 + \frac{S}{A - S}\alpha')$, correcting for the solubility of N_2, affects in this analysis the results by only about 1 part in 700, which is within the limit of error. We have, however, included it in the calculations. It proved impracticable to determine the solubility of N_2 directly in the reaction mixture, because of the continuous effervescence of NO gas. The value for α_{N_2} in water containing the concentration of sodium nitrite present in the 8 cc. of reacting solution, however, we have found to be 50 per cent of α_{N_2} in pure water. Acetic acid in the concentration present does not alter the solubility of N_2 from that in pure water to an extent significant in these calculations. The acid dissociation constant of HNO_2 is about 25-fold that of AcOH (see Landolt-Börnstein's "Talbellen"). Hence, with equimolar proportions of $NaNO_2$, and AcOH present, about 0.96 of the nitrite keeps the form of the sodium salt, and about 0.96 of the AcOH the form of free acetic acid. One appears justified in assuming that the effects on N_2 solubility in the mixed solution are approximately the added effects of the $NaNO_2$ and AcOH.

Equation 5 of Van Slyke and Neill becomes therefore, for our present case,

$$\text{Mg. amino N} = P_{N_2} \times \frac{14.01\ a}{17{,}024\ (1 + 0.00384\ t)} \left(1 + \frac{8}{42} \times \frac{\alpha'_{N_2}}{2}\right)$$

$$= P_{N_2} \times \frac{0.0008230\ a}{1 + 0.000384\ t} (1 + 0.095\ \alpha'_{N_2})$$

$$= P_{N_2} \times \text{factor}$$

where α'_{N_2} indicates the solubility of N_2 in pure water, as given in Table I of Van Slyke and Neill.

In the case of blood analyses, the 5 cc. of filtrate represent 0.5 cc. of blood. Consequently P_{N_2} is multiplied by 200 times the above factor in order to obtain mg. of amino nitrogen per 100 cc. of blood.

When less than 5 cc. of water are added with the amine solution, so that total solution volume, S, is less than 8 cc., the solubility

correction will be even less than that calculated by the factor $(1 + 0.095\ \alpha'_{N_2})$. Since the difference, however, would affect results by less than 1 part in 1000, the same factors, in Table I of this paper, may be used when the volume of the reaction mixture varies by 3 cc. on either side of the 8 cc. assumed in the calculation.

Shortened Procedure for Series of Analyses

When a number of determinations are performed in succession, it is convenient to change the procedure to the following.

The above described p_0 reading is omitted in the amine analysis, the latter being concluded after p_1 is noted. The p_1 reading of the blank analysis is taken as p_0 for the amine analysis. Then

$$P_{N_2} = p_1 - p_0$$

The p_0 thus determined in the blank analysis includes the correction for impurities in the reagents, so that there is no c correction to subtract. The advantage of this procedure is that it obviates one reading with each analysis. The disadvantage is that the p_0 varies with the temperature, due chiefly to effect on vapor tension in the chamber. If the temperature rises between the time of the blank analysis and the subsequent amine analysis, 1.5 mm. may be added to p_0 for each degree rise, subtracted for each degree fall. If the temperature change exceeds 2°, however, it is well to redetermine p_0.

Use of Modified Hempel Pipette

One's ability to perform easily and without the loss of 0.001 cc. of gas the transfers to and from the modified Hempel pipette depends upon proper construction of the latter. The rubber tip, made from soft tubing of 7 mm. outer diameter and 2 mm. bore, should project a hair's breadth below the glass tip of the pipette, and should be so shaped that it fits snugly into the bottom of the cup of the Van Slyke-Neill chamber. The bore of the capillaries of the pipette should be between 0.9 and 1.1 mm. In particular the 3-way cock must be accurately ground, and the bore through the stopper must maintain its diameter sharply to the surface of the stopper. If the bore is widened out funnel-like at the ends, traps are formed in which small gas bubbles are likely to be held

back, sufficient to affect significantly the results of a micro analysis, such as is performed on blood filtrates. The 120° angle in the bore of the cock must be exact, so that in all of its three positions the cock will unite the connected capillaries into smoothly continuous tubes.

It is desirable for each day's analyses to fill the pipette with fresh permanganate solution saturated with air at room temperature. One makes certain that the solution is at room temperature, and then whirls 75 cc. of it about the walls of an open 1 liter flask for 1 or 2 minutes, in order to bring it into equilibrium with air at atmospheric pressure before the solution is put into the pipette.

During the course of a series of analyses a film of manganese dioxide forms on the wall of the pipette near the capillary outlet. When the permanganate is renewed the film is as a rule readily detached by shaking water in the pipette. Film which becomes adherent is removed by washing with a saturated solution of oxalic acid in normal sulfuric acid.

Theoretically one would anticipate some error from contact of the pure N_2 gas, left after absorption of NO, with the permanganate solution in the Hempel pipette. The permanganate solution is saturated, not with pure N_2, but with the N_2-O_2 mixture of the atmosphere. The water of the permanganate solution must give off some O_2 to the nitrogen bubble, and absorb some of the N_2. In control analyses we have found,. however, that the amount of such exchange which occurs decreases the volume of gas, returned as N_2 to the gas chamber, by only about 0.0012 cc., sufficient to lower the p_1 value 2 mm. when measured at 0.5 cc. volume, or 0.5 mm. when measured at 2.0 cc. Error even of this small extent is, however, avoided by using a c correction determined by the same technique, with the same slight loss of N_2.

Determination of Amino Acid Nitrogen in Blood

Of the nitrogenous constituents of protein-free blood filtrates not only the amino acids, but also the urea reacts measurably with nitrous acid under the conditions of the analysis. In the time used for complete reaction of the α-amino acids about 7 per cent of urea nitrogen is decomposed. In human blood without pathological urea retention the urea nitrogen is ordinarily about twice,

at most three times, the amino acid nitrogen content. Under these conditions the amino nitrogen can be determined without preliminary removal of the urea, a correction of 0.07 of the urea nitrogen being subtracted from the total nitrogen obtained by the nitrous acid reaction.

When, however, there is gross urea retention, with blood urea nitrogen above 50 mg. per 100 cc., it is desirable for exact results to remove the urea. The removal is easily accomplished with urease, the resultant ammonia being boiled off before the amino nitrogen is determined.

Accordingly two procedures are described for blood analysis.

Method A. For Blood of Normal or Moderately Increased Urea Content

5 cc. of blood filtrate, prepared by the tungstic acid method of Folin and Wu (1919), and representing 0.5 cc. of blood, are pipetted into the chamber of the gas apparatus and analyzed as above described. The only difference in detail is that in the present analysis the time of reaction, measured *from the moment the sodium nitrite solution is run into the chamber to the end of the 1 minute shaking*, must be regulated with regard to the temperature somewhat more carefully than is ordinarily necessary, in order that the proportion of urea decomposed shall approximate the constant value of 0.07 allowed for. The reaction periods used for different room temperatures are indicated on the scale of Fig. 4. With a stop-watch or interval timer one can readily control the reaction time within 10 seconds.

The urea content of the blood is determined independently.

From the amino nitrogen content of the blood calculated by Table I, 0.07 of the urea nitrogen content is subtracted, to correct for N_2 evolved from that proportion of the urea.

Fig. 4 is constructed in accordance with the assumption that the speed of reaction between urea and nitrous acid obeys the usual temperature rule of time reactions at room temperature, which double in speed with each 10° temperature increase in accordance with the exponential formula

$$\frac{\theta_2}{\theta_1} = 10^{0.03\,(t_1 - t_2)}$$

where θ_1 and θ_2 are the periods required at temperatures t_1 and t_2 for the reaction to proceed to a given point. That, for the reaction between urea and nitrous acid under the conditions of the analysis, the above temperature rule is followed with a sufficiently close degree of approximation is indicated by the results of Levene

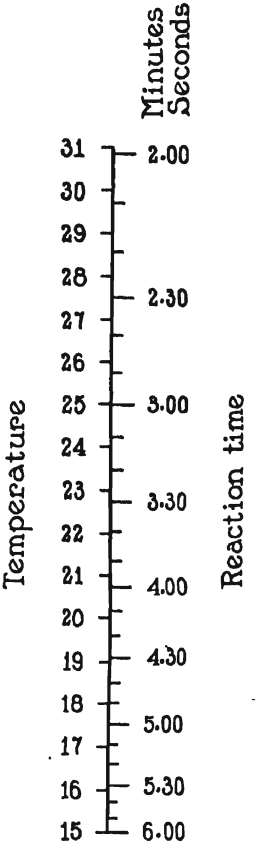


FIG. 4. Scale indicating reaction period required for complete decomposition of α-amino acids, and 0.07 decomposition of urea, when total volume of reacting solution is 8 cc.

and Van Slyke (1912), and confirmed by additional analyses with the present technique.

Method B. For Blood of Either Normal or High Urea Content

In this procedure the urea is destroyed with urease. It is desirable to use a relatively small proportion of urease in order to keep down the correction for amino acids in the urease preparation.

The blood sample, 1 to 5 cc., is placed in a flask calibrated to hold 10-fold the volume of the sample. For each cc. of blood 1 cc. of a 0.6 per cent KH_2PO_4 solution and 0.02 cc. of a 10 per cent aqueous solution of Squibb's jack bean urease are added. The mixture is permitted to stand an hour at a room temperature of 20° or over.[3]

The proteins are precipitated with 10 per cent sodium tungstate and 2/3 N sulfuric acid as described by Folin and Wu (1919), with the modification that *in uremic blood enough extra 2/3 N sulfuric acid is added to neutralize the ammonia* formed from the urea. If the blood urea nitrogen is 100 mg. per 100 cc., 0.1 cc. of 2/3 N sulfuric acid is added per cc. of blood in addition to the 1.0 cc. ordinarily used. Otherwise some protein may come through into the filtrate. The precipitated blood mixture is brought to 10-fold the volume of the blood and filtered.

To boil off the ammonia formed from the urea, 5, 10, 15, or 20 cc. of filtrate are measured into a Pyrex Erlenmeyer flask of 25 to 150 cc. capacity and a few drops of magnesium hydroxide suspension are added, sufficient to make the entire solution turn white. The mixture is boiled for 5 or 10 minutes in the open flask until the volume has been reduced about one-half. Glacial acetic acid is then added, a drop at a time, until the solution turns acid and the magnesium hydroxide dissolves.[4]

[3] The enzyme is prepared from jack beans by the acetone precipitation method of Van Slyke and Cullen (1914). With Squibb's urease of the quality at present provided, capable of decomposing per minute nearly 0.1 mg. of urea per mg. of dry urease, 0.02 cc. of 10 per cent urease solution per cc. of blood suffices, even in uremic cases. If an enzyme preparation is used which, when standardized as previously described (Van Slyke, 1927, pp. 714–716), proves to be much weaker, one must either use more or let it act longer. On the other hand half as much enzyme can be used if the digestion time is doubled, or the temperature raised to 30°.

[4] Boiling with magnesium hydroxide is used to remove ammonia because Osborne, Leavenworth, and Brautlecht (1908) found in analysis of hydrolyzed proteins that this treatment removed ammonia quantitatively without appreciably affecting the amino acids. Boiling with more powerful alkalies, even dilute alkali carbonates, splits off ammonia from some of the amino acids. We have used Phillips' milk of magnesia which by titration is equivalent to an alkali solution of 2.7 N concentration. Magnesium hydroxide suspension has over magnesium oxide the advantage that it forms an even suspension, and dissolves instantly as soon as an excess of acetic

If 10 cc. or more of filtrate have been used the contents of the Erlenmeyer flask are poured into a volumetric flask and the Erlenmeyer flask is washed with small portions of water until the sample has been brought back to its original 10, 15, or 20 cc. volume. 5 cc. portions are used for amino nitrogen determination.

If only 5 cc. of filtrate were boiled down, the Erlenmeyer flask is drained directly into the cup of the gas apparatus, and the volume noted, *e.g.*, 2.7 cc. Then enough water to make this up to 5 cc. is drawn into a graduated pipette, and is used in successive portions to wash the flask, whence the washings are poured into the cup of the gas apparatus, and from that passed into the chamber.

The *blank analysis* in this case includes some amino nitrogen from the urease (the latter is ordinarily free of ammonia), and is performed as follows: 1 cc. of the 10 per cent urease solution is placed in a 10 cc. flask. 0.5 cc. each of 10 per cent sodium tungstate and 2/3 N sulfuric acid is added, the mixture is diluted to the mark, shaken, and, after a half hour's standing to flocculate the proteins, is filtered. 1 cc. of the filtrate is diluted to 50 cc. with water.

The blank analysis is performed with 5 cc. of the diluted filtrate instead of 5 cc. of water.[5]

Calculation.—The pressure of N_2 from amino acids in blood is calculated as

$$P_{N_2} = p_1 - p_0 - c$$

where c is the $p_1 - p_0$ value determined in the above blank analysis. With the urease we have used, the c value, measured at 0.5 cc. gas volume, is increased several mm. by the non-protein urease constituents. The effect could be diminished by using less

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acid is added.

The present method gives with normal blood values for amino nitrogen about 1 mg. per 100 cc. higher than those obtained with the nitrous acid reaction by Bock (1917) presumably because Bock used strong alkali, KOH, to boil off the ammonia.

[5] The 50-fold dilution is used only when the amount of 10 per cent urease employed has been 0.02 cc. per cc. of blood. In this case each cc. of blood-urease filtrate contains the non-protein constituents of 0.2 mg. of urease preparation, whereas the undiluted blank filtrate contains per cc. the non-

urease and longer or warmer digestion, or by using especially purified urease, but the correction is sufficiently small and constant to make such refinements appear unnecessary.

The amino nitrogen values by Method B are usually 0.1 to 0.3 mg. per cent lower than by Method A (*e.g.*, see Table IV). Apparently boiling with MgO decomposes a slight amount of some amino compound in the blood filtrate, although such treatment has been observed to split no ammonia from the amino acids yielded by protein hydrolysis.[5]

EXPERIMENTAL

Analyses of Leucine Solutions

Of leucine, prepared from hydrolyzed casein by the ester method and purified by precipitation as the lead salt, as described by Levene and Van Slyke (1909), 100.0 mg. were dissolved in 100 cc. of water. With 5 cc. portions the first five analyses of Table II were carried out as described above. A portion of solution was then diluted 10-fold, and used for the last five analyses of Table II. Since leucine contains 10.69 per cent of nitrogen, the theoretically calculated nitrogen contents of 5 cc. portions of the two solutions analyzed were 0.534 and 0.0534 mg., respectively. The fact that 99.5 instead of 100 per cent of these amounts was obtained may be due either to traces of impurity in the leucine preparation or to analytical error within the limit of accuracy of the determinations. Comparison of the results obtained with different reaction periods indicates that the time required for complete reaction, as indicated by maximum N_2 yield, is the same as in the former special amino apparatus, approximately 3 minutes at 25°, 4 minutes at 20°. That the time

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protein constituents of 10.0 mg. of urease, or 50 times as much. In case more or less than 0.02 cc. of 10 per cent urease per cc. of blood is used, the extent to which the blank filtrate is to be diluted will vary accordingly, being represented by the denominator of the fraction of a cc. of urease solution used per cc. of blood.

The reason, in the blank analysis, for precipitating a relatively concentrated urease solution and diluting the filtrate instead of precipitating an already diluted urease solution, is that the latter would be so extremely dilute that the proteins could not be made to coagulate within a practicable time.

requirement would be nearly the same was to be expected from the fact that the concentrations of sodium nitrite and acetic acid in the reacting mixture are but slightly different from those used in the former apparatus. Under the conditions used in the latter, for each analysis, 1 cc. of glacial acetic acid and 0.96 gm. of $NaNO_2$ were diluted to a total volume of 7.5 cc. In the reaction

TABLE II

Analyses of 5 Cc. Samples of Leucine Solutions

Concentration of leucine solution.	Duration of reaction.	Temperature.	P_{N_2}	N_2 volume at P_{N_2} reading.	Factor.*	Amino N found.	
per cent	*min.*	*°C.*	*mm.*	*cc.*		*mg.*	*per cent of theoretical*
0.1000	1	25.0	322.0	2.004	0.001509	0.4972	91.0
0.1000	2	25.0	344.2	2.004	0.001509	0.5194	97.2
0.1000	3	25.0	352.1	2.004	0.001509	0.5313	99.5
0.1000	4	25.0	351.5	2.004	0.001509	0.5304	99.3
0.1000	5	25.0	352.5	2.004	0.001509	0.5319	99.6
0.0100	1	21.5	127.0	0.502	0.000382	0.0485	90.8
0.0100	2	21.5	134.5	0.502	0.000382	0.0514	96.3
0.0100	3	21.5	137.5	0.502	0.000382	0.0526	98.5
0.0100	4	21.5	139.0	0.502	0.000382	0.0531	99.5
0.0100	5	21.5	139.3	0.502	0.000382	0.0532	99.6

* Since the *a* volumes of the apparatus were 2.004 and 0.502 instead of exactly 2.000 and 0.500 cc., the factors from Table I are multiplied by $\frac{2.004}{2.000}$ and $\frac{0.502}{0.500}$.

mixture used in the analyses of Table II, the same amounts of acetic acid and $NaNO_2$ are diluted to 8 cc.

Analysis of Blood Plus Amounts of Amino Acid and Urea

Four portions of 10 cc. each of the same blood were placed each in a 100 cc. measuring flask and treated as follows:

Sample 1.—The blood was diluted to about 70 cc. 10 cc. each of 10 per cent sodium tungstate and 2/3 N sulfuric acid were added. The volume was made up to 100 cc., and the mixture was filtered after a half-hour.

Sample 2.—0.2 cc. of 10 per cent Squibb's urease was added, let stand 1 hour at 22°, then diluted and precipitated with tungstic acid as above.

Sample 3.—2 cc. of 1 per cent urea solution (200 mg. of urea = 89.3 mg. of urea N, per 100 cc. of blood) were added; then

TABLE III

Analysis of Blood, Blood Plus Urea, and Blood Plus Leucine

Urea N = 13 mg. per 100 cc.

Sample No.	Treatment of blood other than precipitation of proteins.	P_{N_2} at 0.5 cc. volume.	Temperature.	Amino N per 100 cc. blood.
		mm.	*°C.*	*mg.*
1	None. Analyzed by Method A.	113.7	21.5	8.67
		112.7	21.5	8.59
		Average minus 0.07 × urea N.....		7.72
2	Removed urea with urease and magnesia. Analyzed by Method B.	99.3	21.5	7.57
		97.6	21.5	7.44
		Average.....		7.51
3	Added 89.3 mg. urea N per 100 cc. Analyzed by Method B.	100.8	21.5	7.68
		99.8	21.5	7.51
		Average.....		7.60
4	Added 21.4 mg. amino N per 100 cc. as leucine. Analyzed by Method B.	380.3	21.5	28.98
		379.5	21.5	28.92
		Average.....		28.95
		Preformed amino N from Sample 2.....		7.51
		Added amino N recovered.....		21.44

treated as was Sample 2, except that in precipitating the proteins 11 instead of 10 cc. of 2/3 N sulfuric acid were used to neutralize the ammonia from the added urea.

Sample 4.—2 cc. of 1 per cent leucine solution (21.4 mg. of amino N per 100 cc. of blood) were added; then treated as was Sample 2.

Of filtrates of Samples 2, 3, and 4, 20 cc. portions were freed of ammonia by boiling with magnesia, and brought back to 20 cc. volume, as described above, under "Method B" for blood analysis.

The urea content was determined on a separate portion of the blood, and found to be 13.0 mg. of urea N for 100 cc.

In the amino nitrogen determinations of Filtrates 2, 3, and 4, the reaction period was 4 minutes. In the analysis of Filtrate 1,

TABLE IV

Comparison of Methods A and B in Analyses of Normal and Uremic Blood

Blood.	Method A. Correction made for urea.				Method B. Urea removed.		
	P_{N_2} at 0.5 cc. volume.	Temperature.	Amino N per 100 cc. blood.		P_{N_2} at 0.5 cc. volume.	Temperature.	Amino N per 100 cc. blood.
			Uncorrected.	Corrected by subtracting 0.07 urea N.			
	mm.	*°C.*	*mg.*	*mg.*	*mm.*	*°C.*	*mg.*
1. Normal. Urea N = 12.4 mg. per cent.	128.8	22.0	9.80	8.93	111.3	22.0	8.47
	127.9	22.0	9.73	8.86	113.0	22.0	8.60
2. Normal. Urea N = 6.5 mg. per cent.	124.0	20.5	9.49	9.03	115.2	23.8	8.72
	122.4	20.5	9.36	8.90	119.0		9.01
3. Uremic. Urea N = 121.7 mg. per cent.	205.5	24.0	15.74	7.21	93.1	21.5	7.09
	213.0	24.0	16.22	7.69	96.2	21.5	7.33
	205.0	24.0	15.70	7.17	95.1	21.5	7.25
					94.9	21.5	7.23
4. Uremic.* Urea N = 122.0 mg. per cent.	256.0	22.0	17.96	9.42	123.2	21.2	9.39
	256.0	22.0	17.96	9.42	122.0	21.2	9.30

* Blood drawn 2 days before death in uremia.

where urea was present, the reaction period was made as exactly as possible 3 minutes and 45 seconds, as indicated for 21.5° by the scale of Fig. 4.

It is evident from the results in Table III that the urease treatment of the blood followed by boiling of the filtrate with magnesia satisfactorily removed the urea nitrogen, and that the added amino acid nitrogen was completely recovered.

The margin by which the 7.72 mg. per cent of amino N found

by Method A exceed the 7.5 mg. per cent by Method B (see Sample 2) is about the difference usually noted, as stated above in the description of Method B.

Comparison of Methods A and B in Analyses of Uremic and Normal Blood

The analyses in Table IV illustrate the nature of the results in normal and in uremic blood. Method A is not recommended for uremic blood. However, even in such blood, as shown by Samples 3 and 4, Method A is capable of giving approximate results.

SUMMARY

Gasometric determination of primary aliphatic amino nitrogen by the nitrous acid reaction in the manometric apparatus of Van Slyke and Neill is described. The procedure requires the same time, 12 to 15 minutes per analysis in the case of α-amino acids, as in the original amino nitrogen apparatus of the writer, and permits measurement of amino nitrogen concentration to 0.0001 mg. per cc., or one-tenth the former limit. In consequence, amino acid nitrogen can be manometrically determined directly in 5 cc. portions of Folin-Wu blood filtrate.

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[Reprinted from THE JOURNAL OF BIOLOGICAL CHEMISTRY, August, 1929, Vol. 83, No. 2, pp. 449–461]

THE MANOMETRIC DETERMINATION OF UREA IN BLOOD AND URINE BY THE HYPOBROMITE REACTION

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(Received for publication, June 8, 1929)

The writer has already published (11) gasometric methods for exact estimation of urea in blood and urine by determination of the CO_2 formed when urea is converted into ammonium carbonate by the action of urease. This enzyme is probably the most specific reagent we possess for determination of urea, and there is every reason to believe that the results obtained with it are exact within the limits of the methods of measurement used to determine the NH_3 or CO_2 formed, when the conditions for the action of the urease are properly chosen.[1]

Hypobromite is a much less specific reagent for urea. According to the conditions of the reaction it evolves varying proportions of nitrogen gas from other nitrogenous products, such as uric acid and creatinine. From ammonia it drives off the nitrogen as completely as from urea. With urea itself the reaction does not

[1] Addis (2) has shown that when extract of jack beans, used as "urease," is mixed with liver tissue, the arginase of the tissue splits urea from some constituent of the extract, and that the yield of ammonia obtained in consequence is manyfold that attributable to the urea content of the liver. He finds that when whole blood is treated for an hour at 38° with a great excess of jack bean extract the same phenomenon occurs to an appreciable extent. Under the conditions of analysis defined by Van Slyke and Cullen (13) and by Van Slyke (11), with action of the urease at room temperature and not unnecessarily prolonged, the reaction discovered by Addis does not enter as a source of error, even when whole blood is treated with the enzyme. As shown recently by the writer (11) the results are the same, under these conditions, as when the enzyme acts on the Folin-Wu filtrate.

yield quite the theoretical amount of nitrogen gas indicated by the equation,

$$CO(NH_2)_2 + 6\ Br + 6\ NaOH = Na_2CO_3 + 6\ H_2O + 6\ NaBr + N_2$$

Marie Krogh (5) found that, depending upon the concentrations of NaOH and Br present, the proportion of nitrogen evolved as N_2 from urea varied from 86 to 100 per cent, the highest N_2 yields being obtained when the least excess of Br was used. The writer has never been able to obtain quite 100 per cent.

Despite the lack of specificity and stoiciometrical precision, the great speed and convenience of the hypobromite reaction and the simplicity of the reagents have caused its survival and have induced modern investigators (6, 8) to seek conditions under which the drawbacks could be minimized. Thus Marie Krogh (5) precipitated the interfering urinary substances with phosphotungstic acid, and Stehle (8) removed the chief offender, ammonia, with the permutit reagent introduced into analytical chemistry by Folin and Bell (3).

In the procedures for analysis of urine and blood outlined below we have utilized the hypobromite solution previously adapted to gasometric ammonia determination in micro Kjeldahl analyses (9). This reagent with pure urea solutions yields up to 99 per cent of the theoretical amount of nitrogen gas, the yield being higher when the hypobromite is more dilute.

For urine analyses we have followed Stehle (8) in removing ammonia with permutit. When the resultant filtrate is allowed to react with hypobromite for 1 to 2 minutes in the manometric apparatus of Van Slyke and Neill (14) the amount of non-urea nitrogen evolved approximately compensates for the deficit of N_2 from urea, as exemplified in Table V. In urines with relatively low percentage of total nitrogen in the form of urea, the N_2 from the non-urea substances will somewhat overcompensate for the 5 per cent deficit in the nitrogen gas yield from urea itself, while in urines, such as are obtained with very high protein diets, with relatively high percentages of the total nitrogen in the form of urea, the non-urea substances will fall somewhat short of compensating. A plus or minus error amounting to 4 per cent of the urea present may thus occur. The hypobromite method is not

to be used when such an error would invalidate the interpretation of the results.

In blood filtrates the ammonia is negligible, but the relative proportion of other non-urea nitrogenous substances, not so simply removed, is much greater than in urine. Consequently hypobromite with blood filtrates evolves more nitrogen than is contained in the urea present. Under the conditions outlined below for use of the hypobromite reaction with the Folin-Wu filtrate, the yield of N_2 usually indicates in human blood from 1 to 4 mg. more of urea nitrogen per 100 cc. of blood than is present, according to exact analysis with urease. If a correction of 2 mg. is subtracted, the hypobromite nitrogen figure thus corrected falls usually within 1 or 2 mg. of the correct value. Because of the margin of error the hypobromite method is not recommended when the blood urea content is to be compared with the urea excretion rate (1,7), in order to ascertain whether there is a moderate diminution of renal function. The 2 mg. error which may occur may exceed 20 per cent of the blood urea nitrogen, and lead to a corresponding error in the interpretation of the results.

However, the hypobromite blood urea determination is adequate when one wishes only to ascertain whether sufficient urea has been retained to raise the blood level above the maximum normal limit, which MacKay and MacKay (6) put at 23 mg. of urea nitrogen per 100 cc. The hypobromite urea nitrogen is the quickest and simplest of all blood nitrogen determinations, duplicate analyses are easy to repeat with constancy extraordinary for micro analyses, and the determination may well replace that of non-protein nitrogen in laboratories where the latter is a routine procedure for detection of nitrogen retention.

Reagents

Hypobromite Solution.—To 50 cc. of an alkali solution containing 40 grams of NaOH per 100 cc. add 1 cc. of bromine. The solution is prepared in a 250 cc. Erlenmeyer flask. Before portions are withdrawn for analyses the solution is rotated about the walls of the flask for a half-minute in order to permit the escape into the air of slight amounts of oxygen which form when the reagent

stands. The hypobromite solution should be used the day it is prepared.

Permutit.—See Folin and Bell (3).

Tungstic Acid.—The sodium tungstate, sulfuric acid, and water, with which Folin and Wu (4) mix blood in order to obtain a protein-free filtrate, can be conveniently combined into a single solution. To 7 volumes of water add 1 volume of 10 per cent sodium tungstate and 1 volume of 2/3 N sulfuric acid. This solution may be used for about 2 weeks. Eventually too much of the tungstic acid settles out as a precipitate, and a fresh solution must be prepared.

Procedure for Urine Analysis

1 cc. of concentrated urine (sp. gr. over 1.030), or 2 cc. of more dilute urine, are placed in a 100 cc. Erlenmeyer flask, and either 19 or 18 cc. of water from a burette are added, to make the volume up to 20 cc. 3 grams of permutit are added and the mixture is shaken 4 minutes to remove ammonia. The fluid is then filtered through a dry filter.

1 cc. of water is placed in the cup of the Van Slyke-Neill apparatus. 2 cc. of the urine filtrate are either layered under the water, or are pipetted through it into the chamber of the Van Slyke-Neill apparatus, as shown in Fig. 4 of Van Slyke and Neill's paper (14). After the pipette has been withdrawn, the water is run into the chamber after the urine filtrate and is followed by 1 cc. of the hypobromite solution. The cock is sealed with a drop of mercury. The mercury in the chamber is at once lowered to the 50 cc. mark, and the chamber is shaken, according to the temperature, for 1.5 minutes at 25°, 2 minutes at 20°, or 3 minutes at 15°. The volume of gas is then reduced to 2 cc. and the pressure p_1 is read on the manometer.

A blank analysis is run, in which 2 cc. of water, previously shaken with permutit, replace the urine filtrate. The manometer reading is taken as p_0.

The chamber of the apparatus need not be washed between the successive analyses of a series. Consequently analyses can be run off at the rate of about one every 4 minutes.

One blank analysis, run at the beginning, serves for an entire series of analyses. If the temperature in the water jacket of the

apparatus rises between the time at which the blank was run and the time of the urine analysis, 1.3 mm. are added to p_0 for each degree of temperature increase, to correct for rise in vapor tension; and a similar correction is subtracted from p_0 if the temperature falls.

Calculation of Urine Urea.—The pressure of N_2 is

$$P_{N_2} = p_1 - p_0$$

The urea content of the urine is calculated as:

$$\text{Per cent of urea or urea nitrogen} = P_{N_2} \times \text{factor}$$

The values of the factor are given in Table II.

Procedure for Blood Urea

The proteins are precipitated by diluting 1 volume of blood to 10 volumes with the tungstic acid solution. The filtrate is passed through a dry filter.

Of the filtrate 5 cc., equivalent to 0.5 cc. of blood, are pipetted through a mercury seal into the chamber of the Van Slyke-Neill apparatus, in the manner shown in Fig. 3 of a former paper (10). 1 cc. of hypobromite solution is then passed into the chamber in the same manner. The mercury in the cup is then run down into the chamber, only enough remaining above the cock to fill the capillary. The mercury level in the chamber is lowered to the 50 cc. mark, and the chamber is shaken for 1.5 minutes at 25°, 2 at 20°, or 3 at 15°. The reaction must not be allowed to continue longer; slow decomposition of non-urea nitrogenous substances would occasion too high results.

The meniscus of the solution is brought to the 0.5 cc. mark in the chamber in analyses of bloods of urea nitrogen content up to 40 or 50 mg. per 100 cc. For uremic blood the 2.0 cc. mark is used. The reading on the manometer is taken as p_1.

A blank analysis is performed with a 5 cc. portion of 0.9 per cent NaCl solution replacing the blood filtrate. The manometer reading is recorded as p_0. It is taken with the gas volume at both the 0.5 and 2.0 cc. marks, in order that p_0 values shall be available for bloods of either normal or high urea content. The 0.9 per cent NaCl solution has the same solubility for air as the Folin-Wu

filtrate. Hence the blank analysis corrects for dissolved air in the sample of filtrate. In order to make the correction exact, the temperature of the filtrate should differ by not more than 0.2° from that of the 0.9 per cent NaCl. It is advisable to put a 10 or 20 cc. portion of the latter into a 100 or 200 cc. flask, similar to those used for receiving the blood filtrate, before beginning the analyses of a series of filtrates. One ascertains that the temperatures of all are alike, and then rotates them about the walls of their flasks for about a minute, to make certain that they are all saturated with air at the same temperature.

As in the urine analyses, the chamber of the gas apparatus need not be washed out between analyses, so that the latter in series can be run off rapidly.

If the temperature rises during the interval between the blank analysis and the analysis of the blood filtrate, 2.0 mm. are added to p_0 for each degree of temperature increase, or subtracted for each degree of temperature fall, when the manometer readings are taken with the gas at 0.5 cc. volume. Of this correction 1.3 mm. are for the change in vapor tension of water in the chamber, and 0.7 mm. for pressure change in the amount of air extracted from the 5 cc. of solution analyzed.

When the manometer readings are taken with the gas at 2.0 cc. volume, the correction to p_0 for temperature change after the blank analysis is only 1.5 mm. per degree, the pressure change of the admixed air at the larger volume being only 0.2 mm. per degree of temperature change.

Calculation of Blood Urea.—The pressure P_{N_2} of nitrogen gas is

$$P_{N_2} = p_1 - p_0$$

$$\text{Mg. urea nitrogen per 100 cc. blood} = (P_{N_2} \times \text{factor}) - 2.0$$

$$\text{Mg. urea per 100 cc. blood} = (P_{N_2} \times \text{factor}) - 4.0$$

The values of the factors for different room temperatures are given in Table II. The subtraction of 2 mg. of urea N, or 4 mg. of urea, per 100 cc. of blood, from the amount indicated by the nitrogen gas evolved, is to correct for the N_2 yielded by non-urea substances of the blood filtrate (see Table VI).

The factors in Tables I and II are calculated as described in the preceding paper on amino nitrogen determination (12), except

that the millimols of N_2 gas are multiplied by 28.02 instead of 14.01 to obtain mg. of nitrogen in the sample. In the factors of Tables I and II no correction is made for the fact that the hypobromite reaction yields only 0.95 mol of N_2 per mol of urea under

TABLE I

Factors by Which Millimeters P_{N_2} Are Multiplied to Give Urea Nitrogen and Urea Contents of Sample Analyzed

Temperature.	Factors giving mg. urea N.		Factors giving mg. urea.	
	a = 0.5 cc.*	a = 2.0 cc.	a = 0.5 cc.	a = 2.0 cc.
°C.				
15	0.000780	0.003121	0.001671	0.00669
16	77	10	66	67
17	75	0.003099	60	64
18	72	87	54	62
19	69	76	48	60
20	67	65	42	57
21	64	54	36	55
22	61	43	30	53
23	59	33	24	50
24	56	22	19	48
25	53	11	13	46
26	50	01	07	44
27	48	0.002990	01	41
28	45	79	0.001596	39
29	43	69	90	37
30	40	59	85	35
31	37	49	80	32
32	35	39	74	30
33	32	29	69	28
34	30	18	63	26

* a indicates the volume at which gas pressure in the Van Slyke-Neill apparatus was read.

the conditions of urine analysis, and 0.98 mol under the conditions of blood analysis. The deficit of N_2 yield from the urea is compensated by N_2 from other substances in the urine, and more than compensated in the blood.

TABLE II

Blood and Urine Analyses

Factors by Which Millimeters P_{N_2} Are Multiplied to Give Urea Nitrogen and Urea Content

Temperature.	Factors for urine analyses.				Factors for blood analyses.*			
	Giving gm. urea N per 100 cc.		Giving gm. urea per 100 cc.		Giving mg. urea N per 100 cc.		Giving mg. urea per 100 cc.	
	Sample = 0.1 cc. urine. (a=2.0 cc.)	Sample = 0.2 cc. urine. (a=2.0 cc.)	Sample = 0.1 cc. urine. (a=2.0 cc.)	Sample = 0.2 cc. urine. (a=2.0 cc.)	Sample = 0.5 cc. blood.		Sample = 0.5 cc. blood.	
					a = 0.5 cc.	a = 2.0 cc.	a = 0.5 cc.	a = 2.0 cc.
°C.								
15	0.00312	0.001561	0.00669	0.00335	0.1561	0.624	0.335	1.336
16	11	55	67	34	55	22	34	31
17	10	49	64	32	49	20	32	26
18	09	44	62	31	44	18	31	22
19	08	38	60	30	38	15	30	17
20	07	33	57	29	33	13	29	13
21	05	27	55	28	27	11	28	08
22	04	22	53	27	22	09	27	03
23	03	16	50	25	16	06	25	1.298
24	02	11	48	24	11	04	24	94
25	01	06	46	23	06	02	23	90
26	00	00	44	22	00	00	22	85
27	0.00299	0.001495	41	21	0.1495	0.598	21	80
28	98	90	39	20	90	96	20	76
29	97	85	37	19	85	94	19	72
30	96	80	35	18	80	92	18	67
31	95	74	32	16	74	90	16	62
32	94	69	30	15	69	88	15	58
33	93	64	28	14	64	86	14	54
34	92	59	26	13	59	84	13	50

* To approximate the true urea content of blood, subtract from the blood urea values calculated by the above factors, 2 mg. of urea N or 4 mg. of urea per 100 cc. of blood, as correction for the N_2 yielded by non-urea substances in blood filtrate. No correction is required for urine urea values calculated by the above factors.

EXPERIMENTAL

Reaction of Urea with Hypobromite under Conditions of Urine Analysis

Urea solutions of 1 and 3 per cent concentration were analyzed as above outlined for urines, with the exception that in some of the determinations the treatment with permutit was omitted, in order to ascertain whether the reagent has any effect on urea. The results in Table III show that the N_2 yield approximated 95 per cent of theoretical, and that permutit has no effect whatever.

TABLE III

Hypobromite Reaction with Urea under Conditions of Urine Analysis

1 cc. hypobromite + 2 cc. 20-fold diluted solution + 1 cc. water.

Concentration of urea solutions before dilution.	Permutit used or not.	Time of reaction with hypobromite.	P_{N_2} a = 2 cc.	Temperature.	Urea found.	Per cent of urea present found by analysis.
gm. per 100 cc.		*min.*		*°C.*	*gm. per 100 cc.*	
2.97	0	1	433.2	23.5	2.818	94.8
	0	2	436.0	23.5	2.830	95.3
2.97	+	1	434.5	23.0	2.824	95.1
	+	2	435.0	23.0	2.828	95.2
0.99	0	1	144.4	23.5	0.937	94.6
	0	2	145.2	23.5	0.942	95.2
0.99	+	1	143.5	23.0	0.933	94.2
	+	2	144.5	23.0	0.939	94.8

Reaction of Urea with Hypobromite under Conditions of Blood Analysis

Solutions of urea were prepared simulating in concentration those observed in normal and nephritic human blood. These solutions were diluted 10-fold, and were analyzed as described above for Folin-Wu blood filtrate, in all details except one; the blank analysis for determination of the p_0 value was performed with water instead of 0.9 per cent NaCl solution, since in these analyses the urea solutions were free from salts. The results in

Table IV indicate that approximately 98 per cent of the theoretical amount of N_2 is evolved. The 1 cc. of hypobromite solution in this case is diluted with 5 cc. of urea solution, instead of the 3 cc. added in the urine analyses. The greater dilution of the alkaline hypobromite apparently increases the yield of N_2 from the urea.

TABLE IV

Hypobromite Reaction with Urea under Conditions of Blood Analysis

5 cc. of 10-fold diluted urea solution + 1 cc. hypobromite. Reaction for 2 minutes.

Urea added to solution.	Concentration of urea N in solution,* before dilution.	P_{N_2}	Volume of gas when P_{N_2} was measured.	Temperature.	Urea N found.	Proportion of urea N present found by the analysis.
mg. per 100 cc.	*mg. per 100 cc.*	*mm.*	*cc.*	*°C.*	*mg. per 100 cc.*	*per cent*
20	9.24	60.0	0.5	22.2	9.13	98.9
		59.6	0.5	22.2	9.07	98.2
50	2.31	149.9	0.5	22.2	22.8	98.7
		147.6	0.5	22.2	22.5	97.4
		148.2	0.5	22.2	22.5	97.4
100	4.62	297.3	0.5	22.2	45.2	97.8
		295.0	0.5	22.2	44.9	97.2
		299.0	0.5	22.2	45.5	98.5
200	9.24	147.5	2.0	22.2	89.7	97.1
		148.2	2.0	22.2	90.1	97.6
		148.5	2.0	22.2	90.3	97.8

* The N content of the urea used was found by Kjeldahl analyses to be 46.20 instead of the theoretical 46.62 per cent.

Urine Analyses.—The results obtained in urine analyses are exemplified in Table V. It is evident that the non-urea substances continue reacting after the 1 minute which suffices for the maximum yield of N_2 from urea. The rate, however, at which N_2 is evolved from these substances after the first minute is relatively so slow that the increase per minute is less than 1 per cent of the N_2 evolved in the first minute. In the time that we have used for the reaction the amount of nitrogen from the non-urea substances approximately compensates for the deficit of 5 per cent in the nitrogen evolved from the urea itself.

Blood Analyses.—The results recorded in Table VI are from human blood, partly normal, partly from nephritic subjects with nitrogen retention. The hypobromite analyses were performed

TABLE V

Urine Analyses

Urea N by Hypobromite Method

Urine No.	Urine sample represented in portion of filtrate analyzed.	Time of reaction with hypobromite.	Temperature.	P_{N_2} (a = 2 cc.)	Urea N content of urine found by hypobromite.	Urea N content by urease method.
	cc.	*min.*	*°C.*	*mm.*	*per cent*	*per cent*
1	0.1	1	24.0	179.4	0.542	
	0.1	2	24.0	180.9	0.546	0.524
	0.1	3	24.0	182.5	0.551	
2	0.1	1	24.5	160.2	0.483	0.492
	0.1	2	24.5	162.0	0.489	
	0.1	3	24.5	163.8	0.494	
3	0.1	1	25.0	397.5	1.197	1.208
	0.1	2	25.0	400.5	1.206	
	0.1	3	25.0	403.5	1.215	
4	0.1	1	25.0	285.0	0.858	0.869
	0.1	2	25.0	286.0	0.861	
	0.1	3	25.0	287.0	0.864	
5	1.0	1	25.0	314.0	0.095	0.098
	1.0	2	25.0	321.0	0.097	
6	0.2	1	25.0	234.0	0.352	0.366
	0.2	2	25.0	235.0	0.354	
7	0.2	1	25.0	559.0	0.842	0.860
	0.1	1	25.0	279.8	0.842	
	0.2	2	25.0	561.0	0.845	
	0.1	2	25.0	281.8	0.848	

as outlined above, the urease determinations by the gasometric determination of the CO_2 of the ammonium carbonate formed, as described in a previous paper (11).

TABLE VI

Blood Analyses

Blood No.	Urea N by urease method. (*a*)	Hypobromite N, total uncorrected. (*b*)	Excess hypobromite N over urea N. (*b*) − (*a*)	Difference between corrected hypobromite N* and urea N. ((*b*) − (*a*) − 2)
	mg. per 100 cc.	*mg. per 100 cc.*	*mg. per 100 cc.*	*mg. per 100 cc.*
1	6.8	9.0	2.2	+0.2
2	8.5	11.0	2.5	+0.5
3	8.7	11.0	2.3	+0.3
4	8.8	9.0	0.2	−1.8
5	9.3	9.7	0.4	−1.6
6	9.6	12.1	2.5	+0.5
7	11.1	13.1	2.0	0.0
8	11.3	13.7	2.4	+0.4
9	11.4	13.9	2.5	+0.5
10	12.6	16.0	3.4	+1.4
11	12.7	13.9	1.2	−0.8
12	15.0	14.6	0.4	−2.4
13	15.3	15.8	0.5	−1.5
14	15.6	19.5	3.9	+1.9
15	17.0	20.2	3.2	+1.2
16	18.4	20.7	2.3	+0.3
17	18.9	20.0	1.1	−0.9
18	19.0	20.9	1.9	−0.1
19	19.3	20.9	1.6	−0.4
20	19.5	23.3	3.8	+1.8
21	22.5	26.1	3.6	+1.6
22	26.1	26.6	0.5	−1.5
23	27.2	29.5	2.3	+0.3
24	31.4	35.1	3.7	+1.7
25	38.7	42.3	3.6	+1.6
26	57.3	58.0	0.7	−1.3
27	57.7	61.6	3.9	+1.9
28	58.7	57.9	0.8	−2.8
29	71.1	69.5	1.6	−3.6

* Corrected hypobromite N = (total observed) − (2 mg. per 100 cc.).

SUMMARY

Procedures are described for the approximate determination of urea in urine and in the Folin-Wu blood filtrate by the hypobromite reaction with the Van Slyke-Neill manometric gas apparatus. 3 minutes suffice for the gasometric determination. The results can be utilized when a maximum error is permissible of 1 part in 25 of urine urea, or of 2 mg. of blood urea nitrogen per 100 cc. In blood analyses the method is adequate to ascertain whether the urea concentration is within the limits of normal variation, and affords a rapid and simple substitute for nonprotein nitrogen estimation as an indicator of nitrogen retention.

The analyses presented above were in part performed by Mr. John Plazin.

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[Reprinted from the ARCHIVES OF PATHOLOGY, May, 1929, Vol. 7, No. 5, pp. 767–786]

A STUDY OF CERTAIN EFFECTS OCCASIONED IN DOGS BY DIPHTHERIA TOXIN

II. ANALYSIS OF THE MECHANISM POSSIBLY RESPONSIBLE FOR THE ALTERATIONS OF THE HEART

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In a preceding paper[1] were reported certain results that followed the injection of diphtheria toxin into dogs. Among the results reported were changes in the size of the heart. An analysis of these changes in size forms the subject of this paper.

Following the intravenous injection into dogs of 0.00135 cc. or more of diphtheria toxin per kilogram of body weight, the animals became ill, lost weight, presented jaundice, showed urinary changes indicating irritation of the kidneys and died from two to nineteen days after the injection. There was, in most of these dogs, progressive decrease in the amplitude of the R_2 and R_3 waves of the electrocardiogram. The ratio of the weight of the left ventricle of the heart to that of the right ventricle (called hereafter the L/R ratio) was below the average for the hearts of normal dogs in all except one animal (no. 109). The ratio of the combined left and right ventricular weights to the body weight (called hereafter the $\frac{L + R}{B W}$ ratio) in twelve dogs was below the normal average. In the remaining eight dogs, it was equal to the normal average.

The method of injecting the diphtheria toxin into the dog was described in the first paper.[1] Briefly, diphtheria toxin having a minimal lethal dose for guinea-pigs of 0.00125 cc. was injected intravenously through the marginal ear veins. The toxin was diluted with sterile physiologic sodium chloride solution, and the dose was calculated in cubic centimeters per kilogram of body weight. Electrocardiograms and roentgenograms of the heart were made, and the body weight was taken

1. Stewart, H. J.: A Study of Certain Effects Occasioned in Dogs by Diphtheria Toxin: I. A Report of the Visceral Lesions, Arch. Path., 7: 601 (April) 1929.

immediately before the injection of the toxin. These observations were repeated daily thereafter until the animals succumbed to the intoxication, or until further changes in those animals that survived were not observable. The roentgenograms of the heart were made at a distance of 2 meters, and the cardiac area was measured by the technic devised by Levy[2] and modified by Stewart[3] for use in dogs. The dogs were divided into two groups according to the amount of toxin injected per kilogram of body weight.

Four dogs (nos. 81, 82, 83 and 84 of group 1, table 1) were given from 0.00161 to 0.00232 cc. of diphtheria toxin per kilogram of body weight intravenously, and from one to four days later a second injection of from 0.00105 to 0.00168 cc. per kilogram of body weight. These dogs exhibited a remarkable decrease in the size of the heart shadow, ranging from 20 to 42 per cent (fig. 1). The decrease was seen as early as twenty-four hours after the first injection. In dogs 81 and 83, it was at its maximum then, while in dogs 82 and 84 the area of the heart continued to decrease until the death of the animals, none of the animals surviving longer than five days after the first injection. This decrease in size is well seen in the roentgenograms of dog 82 (fig. 2).

The question arose: To what mechanism was this decrease in size of the heart due? A number of possibilities presented themselves. The decrease might have been due (1) to the decrease in body weight that also took place or (2) to the toxin injected. If due to the latter, there should be a dose that would fail to cause this change. The toxin could bring about the decrease in several ways: (1) by decreasing the total amount of the circulating blood, evidence of which might be found in a study of the number of red cells and the hemoglobin content of the blood, the blood volume and the microscopic sections of the organs after death; (2) by producing capillary dilatation, which would allow a redistribution of the blood in the body; (3) by destruction of the substance of the heart muscle, and (4) by an injury of the fibers of the heart muscle allowing a change in the water equilibrium, so

2. Levy, R. L.: The Size of the Heart in Pneumonia: A Teleroentgenographic Study, with Observations on the Effect of Digitalis Therapy, Arch. Int. Med. **32**: 359 (Sept.) 1923.

3. Stewart, H. J.: A Technique for Measuring X-Ray Photographs of the Cardiac Areas of Dogs, J. Clin. Investigation **3**: 475, 1927.

TABLE 1

The Effect of Diphtheria Toxin on the Cardiac Area and the Body Weight in Dogs of Group 1

Dog	Time of observation	Amount of toxin injected per kg., Cc		Died	Weight		Area of heart		Per cent change in	
					Kg.	Per cent of first weight	Sq cm.	Per cent of first area	Weight	Area of heart
81	Before injection	.....			10.75	100.0	41.40	100.0		
	After injection									
		0.00232								
	1 day.....		0.00337		9.80	91.2	29.63	71.3		
	2 days	0.00105		3d day	9.55	88 8	29.35	70.9	11.2	29.1
82	Before injection	.....			12.45	100.0	53.03	100.0		
	After injection									
		0.00161								
	1 day.....				11.75	94.4				
	2 days		0.00327		11 55	92.7	39.20	73.9		
	3 days.....				11.50	92.3	37.57	70 8		
	4 days.....	0.00166			10.85	87.1	37.50	70.7		
	5 days	.....		5th day	10.20	81.9	30.63	57.7	18.1	42.3
83	Before injection	.....			7.95	100.0	42.70	100.0		
	After injection									
		0.00166								
	1 day		0 00332		8.00	100.6	34.50	80.7		
	2 days.....	0.00166			7 80	98.1	34.20	80 0		
	3 days	.....		3d day	7.55	94.9	34.05	79.7	5.1	20.3
84	Before injection	.....			8.90	100.0	38.40	100.0		
	After injection									
		0 00168								
	1 day.....		0.00336		8 67	97.4	27.73	72.2		
	2 days.....	0 00168			8 37	94.1	27.55	71.7		
	3 days	.....		3d day	7.85	88.2	22 75	59.2	11.8	40.8

that water would pass out of the cells and the cells be decreased in size. Each one of these possibilities was examined and the results are now reported.

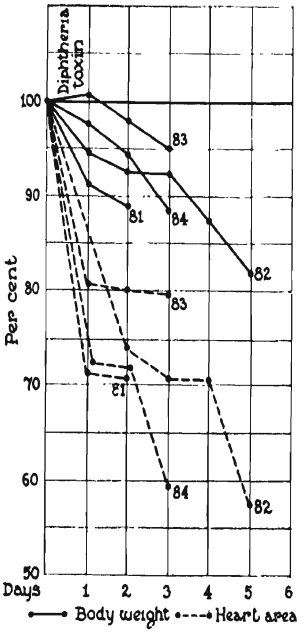


Fig. 1.—Graph showing the effect of diphtheria toxin on the cardiac area and the body weight in the dogs of group 1 (table 1). In this and in the succeeding figures, the lapse of days after the injection of the toxin is plotted on the abscissae. The numbers at the ends of the curves refer to the dogs.



Fig. 2.—Roentgenogram of the heart of dog 82. *A* was taken Jan. 4, 1923, before, and *B* was taken Jan. 10, 1923, after a total of 0.00327 cc. of diphtheria toxin per kilogram of body weight had been given.

OBSERVATIONS

Effect of a Decrease in Body Weight on the Area of the Heart.— In the dogs of group 1 there was a loss in body weight of from 11 to 18 per cent, and a decrease in the size of the heart of from 20 to 42 per cent (fig. 1). That the decrease in the area of the heart did not parallel and was not dependent on the loss in body weight is shown by the following experiments:

Seven dogs (nos. 85, 86, 87, 103, 104, 105 and 106) were not given food for from three to four days. They had water as desired. Roentgenograms were made before the period of fasting began and were repeated frequently throughout

TABLE 2

The Effect of Fasting for Four Days on the Body Weight and the Cardiac Area in Normal Dogs

Dog	Per cent change in body weight after fasting four days	Per cent change in area of heart after fasting four days
85 ♂	−12.0*	−7.6*
86 ♀	− 7.7	+5.5
87 ♀	− 6.5	−3.6
103 ♂	−10.3	−2.8
104 ♂	− 9.0	+8.1
105 ♂	−14.4	+6.9
106 ♀	−10.7	−3.6

* The negative sign indicates a decrease and the positive sign an increase.

the period of fasting. The loss of body weight amounted to from 6 to 14 per cent (as recorded in table 2 and fig. 3 A), while the cardiac area varied between a decrease of 7 per cent and an increase of 8 per cent (table 2, fig. 3 B). These figures are within the limits of a variation of 10 per cent, which represents the error involved in the method. Although the fasting dogs showed approximately as great a decrease in body weight as did the dogs suffering from diptheria intoxication, there was not a parallel decrease in the size of the heart. The loss in the dogs suffering from diphtheria intoxication, in all probability, was therefore due to the toxin and was not an aspect of the accompanying loss of body weight. The fasting dogs were later fed until they regained their former weight. They were then given diphtheria toxin. Promptly they showed a decrease in the size of the heart (fig. 2). Further evidence exhibiting the absence of parallelism between the loss of body weight and the decrease of cardiac area was furnished by the dogs in group 2 C (nos. 88, 89, 90 and 101 of table 3 and fig. 4). They received

only 0.001 cc. per kilogram of body weight, which was a dose too small to produce death. They showed as great a loss of weight as the dogs given the larger dose, yet a decrease in cardiac area did not accompany this loss in weight. In addition, dog 108 (table 4, fig. 5) which received 0.00135 cc. of toxin per kilogram of body weight did not show changes in body weight, but did show a decrease in the size of the heart.

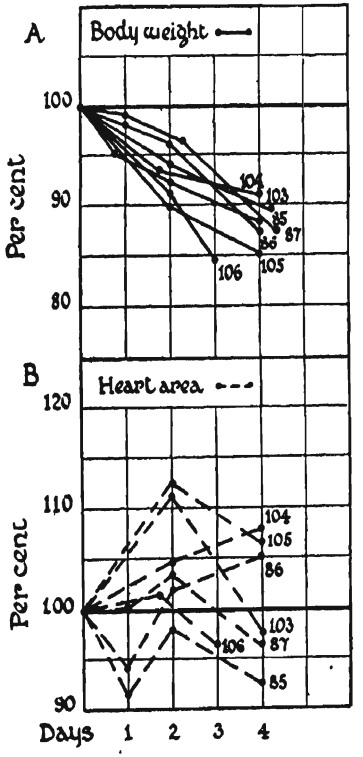


Fig. 3.—Graph *A* shows the effect of fasting on the body weight of dogs; graph *B*, the effect on the cardiac area.

Effect of Diphtheria Toxin on the Area of the Heart.—Since the dogs of group 1 that received a total of from 0.00327 to 0.00337 cc. of diphtheria toxin per kilogram of body weight showed striking decreases in cardiac area, it was important to learn how much toxin was necessary to bring about this condition. To acquire data on this point, I gave decreasing dose of toxin to a second group of dogs, group 2 A being given 0.00168 cc., group 2 B, 0.00135 cc. and group 2 C, 0.001 cc. per kilogram of body weight.

TABLE 3

The Effect of Diphtheria Toxin on the Cardiac Area and the Body Weight in Dogs of Group 2 C

Dog	Time of observation	Amount of toxin injected per kg., Cc.	Died	Weight		Cardiac area	
				Kg.	Per cent of first weight	Sq. cm.	Per cent of first area
88 ♂	Before injection	.....	.....	7.92	100.0	25.70	100.0
		0.001					
	After injection						
	1 day	.....		7.46	94.1	27.35	106.4
	2 days	.....		7.20	90.9	26.78	104.2
	3 days	.....		7.20	90.9	27.75	107.9
	4 days	.....		7.05	89.0	26.40	102.7
	6 days	.....		7.20	90.9	28.18	109.7
	7 days	.....		6.85	86.4	26.65	103.7
	9 days	.....		6.80	85.8	27.95	108.7
	11 days	.....		6.65	83.9	25.20	98.0
	14 days	.....	Lived	6.80	90.9	27.30	106.2
89 ♀	Before injection	.....	.....	6.70	100.0	27.35	100.0
		0.001					
	After injection						
	1 day	.....		6.43	95.9	26.18	95.7
	2 days	.....		6.40	95.5	26.50	96.9
	3 days	.....		6.50	97.0	26.40	96.5
	4 days	.....		6.35	94.7	28.68	104.8
	6 days	.....		6.15	91.7	27.80	101.6
	7 days	.....		5.85	87.3	26.85	98.1
	9 days	.....		5.90	88.0	25.35	92.6
	11 days	.....		5.75	85.8	28.60	104.5
	14 days	.....	Lived	6.10	91.0	25.65	93.7
90 ♂	Before injection	.....	.....	11.20	100.0	39.55	100.0
		0.001					
	After injection						
	1 day	.....		11.15	99.5	37.30	94.5
	2 days	.....		11.05	98.6	39.70	100.3
	3 days	.....		10.95	97.7	41.30	104.4
	4 days	.....		10.70	95.5	40.45	102.2
	6 days	.....		10.20	91.0	37.60	95.0
	7 days	.....		10.05	89.7	37.15	93.9
	9 days	.....		9.70	86.6	36.75	92.9
	11 days	.....		9.95	88.8	41.30	104.4
	14 days	.....	Lived	10.20	91.0	38.35	96.9

TABLE 3—*Concluded*

Dog	Time of observation	Amount of toxin injected per kg., Cc.	Died	Weight		Cardiac area	
				Kg.	Per cent of first weight	Sq. cm.	Per cent of first area
101 ♀	Before injection	.....	.....	12.30	100.0	39.40	100.0
		0.001					
	After injection						
	1 day	.....		12.00	97.5	39.80	101.0
	2 days	.....		11.90	96.7	37.60	95.4
	3 days	.....		12.25	99.6	43.45	109.7
	4 days	.....		11.65	94.7	42.80	108.6
	5 days	.....		11.90	96.7	45.55	118.1
	7 days	.....		11.15	90.6	43.65	110.7
	10 days	.....		10.43	84.7	38.20	96.9
	15 days	.....	Lived	11.82	96.1	39.10	99.2
106 ♀	Before injection	.....	.....	11.57	100.0	39.35	100.0
		0.001					
	After injection						
	1 day	.....		11.50	99.4	38.30	97.3
	2 days	.....		11.30	97.6	36.75	93.4
	3 days	.....		10.81	93.4	34.75	88.3
	4 days	.....		11.11	96.0	32.40	82.3
	6 days	.....		10.39	89.8	34.75	88.3
	7 days	.....		9.98	86.2	35.40	89.9
	8 days	.....		9.67	83.5	31.80	80.8
	9 days	.....		9.40	81.2	33.40	84.8
	14 days	.....		7.10	61.3	33.70	85.6
	20 days	.....		7.50	64.8	41.75	106.0
	23 days	.....	23d day	7.05	60.9	37.75	94.9

In group 2 A, in which nine dogs (nos. 85, 86, 87, 97, 99, 102, 103, 104 and 105 of table 5) received, in a single injection, 0.00168 cc. per kilogram of diphtheria toxin, decrease in the area of the hearts promptly took place to the extent of from 19 to 39 per cent. The decrease in body weight was from 4 to 17 per cent (fig. 6). The animals died from two and a half to five days after the first injection. These changes were as great as those observed in the dogs receiving two injections (group 1).

In group 2 B, in which six dogs (nos. 107, 108, 109, 110, 111 and 112 of table 4) received 0.00135 cc. per kilogram of toxin, dog 108 became acutely ill and died on the fourth day after the injection; the other five dogs gradually became ill and died from twelve to nineteen days after the injection. The decrease in cardiac area was, on the average, as great as in those receiving the larger doses. In

TABLE 4

The Effect of Diphtheria Toxin on the Cardiac Area and the Body Weight in the Dogs of Group 2 B

Dog	Time of observation	Amount of toxin injected per kg., Cc.	Died	Weight		Cardiac area		Per cent change in	
				Kg.	Per cent of first weight	Sq. cm.	Per cent of first area	Weight	Cardiac area
107 ♂	Before injection	.....	.....	17.18	100.0	66.50	100.0		
		0.00135							
	After injection								
	1 day	.....		17.41	101.3	51.10	76.8		
	2 days	.....		15.80	91.9	50.45	75.8		
	3 days	.....		15.83	92.1	56.45	84.8		
	5 days	.....		15.96	92.3	55.85	83.9		
	6 days	.....		16.00	93.1	51.10	76.8		
	8 days	.....		15.30	89.0	50.70	76.3		
	9 days	.....		14.90	86.7	47.70	71.7		
	10 days	.....		14.51	84.4	42.80	64.3		
	12 days	.....		14.10	82.0	40.25	60.5		
	13 days	.....		13.90	80.9	43.30	65.1		
	14 days	.....		13.85	80.6	42.65	64.1	19.4	35.9
	15 days	.....	16th day	13.50	78.5	*			
108 ♂	Before injection	.....	.....	19.15	100.0	57.80	100.0		
		0.00135							
	After injection								
	1 day	.....		19.22	100.3	59.70	103.2		
	2 days	.....		19.55	102.0	55.60	96.1		
	3 days	.....		19.40	101.3	48.60	84.0	+1.3	16.0
	4 days	.....	4th day						
109 ♂	Before injection	.....	.....	15.38	100.0	51.85	100.0		
		0.00135							
	After injection								
	1 day	.....		15.25	99.1	49.90	96.2		
	2 days	.....		15.35	99.8	43.80	84.4		
	3 days	.....		14.93	97.0	45.55	87.8		
	5 days	.....		14.24	92.5	41.80	80.6		
	6 days	.....		14.65	95.2	47.60	91.8		
	8 days	.....		14.30	92.9	49.45	95.3		
	9 days	.....		13.85	90.1	51.60	99.5		
	10 days	.....		13.50	87.7	47.70	91.9		

* The heart shadow was not distinct enough to be outlined.

TABLE 4—*Continued*

Dog	Time of observation	Amount of toxin injected per kg., Cc.	Died	Weight		Cardiac area		Per cent change in	
				Kg.	Per cent of first weight	Sq. cm.	Per cent of first area	Weight	Cardiac area
109 ♂	After injection								
	12 days			13.27	86.3	45.55	87.8		
	13 days			12.90	83.8	43.00	82.9		
	14 days			12.85	83.5	38.50	74.2		
	15 days			12.55	81.6	46.70	90.2		
	16 days			12.40	80.6	42.20	81.3		
	17 days		18th day	12.03	78.2	43.20	83.3	21.8	16.7
110 ♂	Before injection	..... 0.00135	.....	13.85	100.0	48.80	100.0		
	After injection								
	1 day			14.15	102.1	41.35	84.7		
	2 days			13.88	100.2	42.25	86.5		
	3 days			13.45	97.1	45.30	92.8		
	5 days			12.90	93.1	43.65	89.3		
	6 days			12.85	92.7	41.25	84.5		
	8 days			12.10	87.4	42.40	86.8		
	9 days			11.80	85.1	43.70	89.5		
	10 days			11.40	82.3	32.90	67.4		
	11 days			10.97	79.9	33.15	67.9		
	13 days			10.85	78.3	31.00	63.5	21.7	36.5
	14 days			10.75	77.6	32.90	67.4		
	15 days			10.53	76.0	33.45	68.5		
	16 days			10.38	74.9	35.55	72.8		
	17 days			10.25	74.0	34.15	69.9	26.0	30.1
	19 days			9.96	71.8	*			
	20 days		20th day	9.65	69.6	*			
111 ♂	Before injection	..... 0.00135	.....	16.45	100.0	49.70	100.0		
	After injection								
	1 day			16.08	97.7	44.50	89.5		
	2 days			15.85	96.3	41.85	84.3		
	3 days			15.70	95.2	42.20	84.9		
	5 days			15.55	94.5	43.35	87.2		
	6 days			15.18	91.6	37.90	76.3	8.4	23.7
	8 days			14.80	89.9	41.80	84.1		
	9 days			14.05	85.4	40.50	81.0		
	10 days		11th day	13.64	82.9	41.90	84.3	17.1	15.7

TABLE 4—*Concluded*

Dog	Time of observation	Amount of toxin injected per kg., Cc.	Died	Weight		Cardiac area		Per cent change in	
				Kg.	Per cent of first weight	Sq. cm.	Per cent of first area	Weight	Cardiac area
112 ♂	Before injection	.....	.....	14.70	100.0	57.95	100.0		
		0.00135							
	After injection								
	1 day.....	.....		15.00	102.0	50.15	86.5		
	2 days.....	.....		15.12	102.8	50.85	87.7		
	3 days.....	.....		14.88	101.2	57.90	99.9		
	5 days.....	.....		15.15	103.0	53.60	92.4		
	6 days.....	.....		14.62	99.4	49.40	85.2		
	8 days.....	.....		14.20	96.5	50.70	87.4		
	9 days.....	.....		13.65	92.8	50.38	86.7		
	10 days.....	.....		13.02	88.5	43.80	75 5		
	12 days.....	.....	13th day	12.55	85.3	43.90	75.7	14.7	24.3

four dogs (nos. 107, 110, 111 and 112 of fig. 5B) the decrease was shown on the day following the injection, and in two dogs (nos. 108 and 109) a definite decrease in cardiac size did not take place until the second or third days after the injection. The size of the heart did not decrease as steadily as in the dogs receiving 0.00168 cc. per kilogram of body weight; the decrease was more gradual and fluctuated (as in dog 109). The loss of body weight observed in these dogs was greater than that observed in the other groups, presumably because the animals survived for a longer time (fig. 5A).

In group 2C, in which five dogs (nos. 88, 89, 90, 101 and 106 of table 3) received 0.001 cc. of toxin per kilogram of body weight, the clinical course of dog 106, which survived the injection only twenty-three days, was somewhat similar to that of the animals in group 2B; the other four dogs did not show decreases in cardiac size (fig. 4B), although they lost as much weight immediately following the toxin injections as did the dogs given the larger dosages (fig. 4A). These dogs were living and well twelve months after receiving the toxin.

Summary: From these experiments, it is clear that the decrease in the size of the heart was dependent on the diphtheria toxin. Doses of 0.00135 cc. or more per kilogram of body weight resulted in decreases in the area of the heart, while doses of 0.001 cc. per kilogram of body weight were without effect.

Effect of Diphtheria Toxin on the Amount of the Circulating Blood.—I next studied the animals with a view to learning whether a decrease in the amount of the circulating blood took place. These studies were made in dogs into which had been injected 0.001 and 0.00168 cc. of toxin per kilogram of body weight.

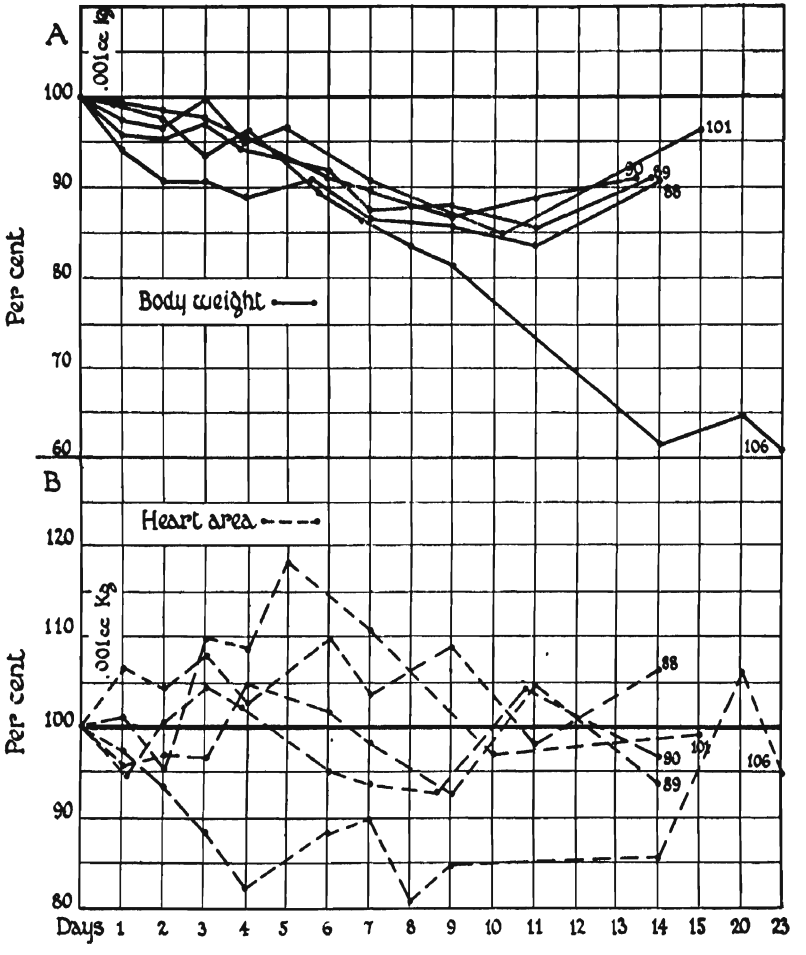


Fig. 4.—Graph *A* shows the effect of diphtheria toxin on the body weight and graph *B* the effect on the cardiac area in the dogs of group 2 C (table 3).

Method: To ascertain whether blood destruction occurred after the injection of the diphtheria toxin, I counted the red blood cells and made estimations of the hemoglobin (expressed as oxygen capacities). As these do not give any indication of the volume of whole blood in circulation, estimations of blood volume were

TABLE 5

The Effect of Diptheria Toxin on the Cardiac Area and the Body Weight in the Dogs of Group 2 A

Dog	Time of observation	Amount of toxin injected per kg., Cc.	Died	Weight		Cardiac area		Per cent change in	
				Kg.	Per cent of first weight	Sq. cm.	Per cent of first area	Weight	Cardiac area
85 ♂	Before injection	.....	.....	7.00	100.0	27.70	100.0		
		0.00168*							
	After injection								
	1 day	.....		6.30	90.0	28.50	102.8		
	2 days	.....		6.30	90.0	24.25	87.5		
	3 days	.....		6.05	86.4	23.63	85.3		
	5 days	.....		5.80	82.8	19.55	70.5	17.2	29.5
	6 days	.....		5.62	80.3	20.20	72.8		
	7 days	.....		5.62	80.3	25.80	93.1		
	8 days	.....	8th day	5.50	78.5	27.50	99.2	21.5	0.8
86 ♀	Before injection	.....	.....	9.77	100.0	35.40	100.0		
		0.00168							
	After injection								
	1 day	.....		9.00	93.1	29.70	83.8		
	2 days	.....		8.80	90.0	24.40	68.9	10.0	31.1
	3 days	.....	3d day	8.55	87.5	25.65	72.4	12.5	27.6
87 ♀	Before injection	.....	.....	16.10	100.0	51.75	100.0		
		0.00168							
	After injection								
	1 day	.....		15.15	94.0	45.40	87.7		
	2 days	.....		14.45	89.7	33.50	64.7		
	3 days	.....	3d day	13.90	86.3	33.35	64.4	13.7	35.6
97 ♀	Before injection	.....	.....	16.20	100.0	51.65	100.0		
		0.00168							
	After injection								
	1 day	.....		16.00	98.7	43.15	83.5		
	2 days	.....		15.60	96.3	38.50	74.5		
	3 days	.....	3d day	15.50	95.7	34.70	67.2	4.3	32.8

* The dog moved during the injection, so that about one half was given subcutaneously.

TABLE 5—*Concluded*

Dog	Time of observation	Amount of toxin injected per kg., Cc.	Died	Weight		Cardiac area		Per cent change in	
				Kg.	Per cent of first weight	Sq. cm.	Per cent of first area	Weight	Cardiac area
99 ♂	Before injection	.....	.....	14.00	100.0	57.15	100.0		
		0.00168							
	After injection								
	1 day	.....		13.40	95.7	50.48	88.3		
	2 days	.....		13.25	94.6	43.80	76.6		
	3 days	.....		13.30	95.0	39.75	69.5	5.0	30.5
	4 days	.....		13.20	94.3	42.05	73.5		
	5 days	.....	5th day	13.20	94.3	40.60	71.0	5.7	29.0
102 ♂	Before injection	.....	.....	14.00	100.0	52.35	100.0		
		0.00168							
	After injection								
	1 day	.....		13.70	97.9	32.75	62.5		
	2 days	.....		13.50	96.4	39.60	75.6		
	3 days	.....		13.10	93.6	34.80	66.4		
	4 days	.....	4th day	12.30	87.9	31.70	60.5	12.1	39.5
103 ♂	Before injection	.....	.....	11.20	100.0	38.45	100.0		
		0.00168							
	After injection								
	1 day	.....		10.85	96.8	31.50	81.9		
	2 days	.....		10 95	97.7	32.70	80.4		
	3 days	.....		10.75	95.9	35.60	92.5		
	4 days	.....	4th day	10.32	92.1	25.75	66.9	7.9	33.1
104 ♂	Before injection	.....	.....	9.02	100.0	36.45	100.0		
		0.00168							
	After injection								
	1 day	.....		8.76	97.1	34.10	93.5		
	2 days	.....	2d day	8.70	95.7	28.40	77.9	4.3	22.1
105 ♂	Before injection	.....	.....	11.12	100.0	43.85	100.0		
		0.00168							
	After injection								
	1 day	.....		10.80	97.1	36.10	82.3		
	2 days	.....		11.40	102.5	37.35	85.1		
	3 days	.....		10.95	98.4	38.10	86.8		
	4 days	.....		10.92	98.2	34.00	77.5	1.8	20.5
	5 days	.....	5th day	10.60	95.3	35.70	81.4	4.7	18.6

also carried out. The red cells were counted in blood obtained from the ear by a needle. The blood used for the estimations of oxygen capacity was taken from that drawn from a femoral artery in the course of the measurements of the blood volume. The analyses for oxygen capacity were made according to a method described by Van Slyke and Neill,[4] the Van Slyke manometric apparatus being

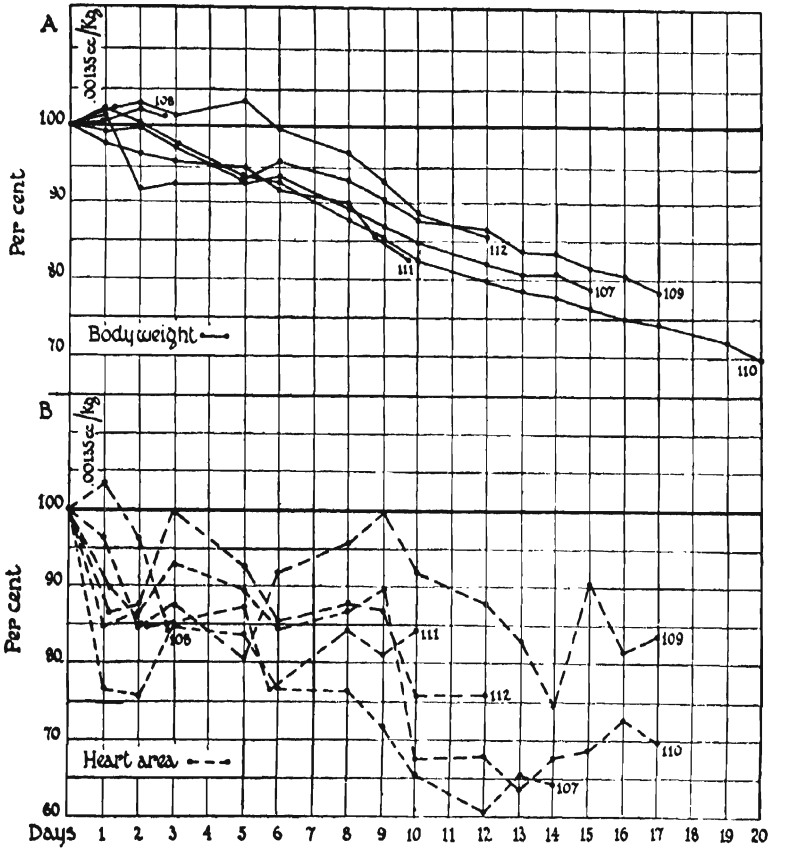


Fig. 5.—Graph *A* shows the effect of diphtheria toxin on the body weight, and graph *B* that on the cardiac area in the dogs of group 2 B (table 4).

used. The estimations of the blood volume were made by the vital red method introduced by Keith, Rowntree and Geraghty.[5]

4. Van Slyke, D. D., and Neill, J. M.: The Determination of Gases in Blood and Other Solutions by Vacuum Extraction and Manometric Measurement. I, J. Biol. Chem. **61**: 523, 1924.

5. Keith, N. M., Rowntree, L. G., and Geraghty, J. T.: A Method for the Determination of Plasma and Blood Volume, Arch. Int. Med. **16**: 547 (Oct.) 1915.

Vital red was used in 1.5 per cent solution in freshly distilled water. Of this solution, 1 cc. was injected for each 5 Kg. of body weight. The standard was made of one part of dye diluted 1:200, one part control plasma (that is, plasma of the blood taken before the injection of the dye) and two parts of 0.9 per cent sodium chloride. The blood used as control was drawn from a femoral artery; the dye was then injected into a superficial vein of that same leg; in from four to five minutes, the second sample of blood was drawn from the opposite femoral artery. The femoral artery was used for obtaining the sample because it is superficial in dogs; it is easily punctured with a needle and as large a sample may be obtained as is necessary. Stasis is thereby avoided. Pressure was applied for a few minutes over the artery after the removal of the needle to prevent extravasation of blood into the tissues. Potassium oxalate was used as an anticoagulant. Hematocrit readings were made after three Epstein tubes had been filled, one with each specimen of blood, and centrifuged until further centrifugation did not give any change in the packing of the cells.

Effect of Diphtheria Toxin on the Number of Red Cells and Hemoglobin: Five animals in group 2A that exhibited decrease in size of the heart did not show any change in the red blood cell count following the injection of 0.00168 cc. of diphtheria toxin per kilogram of body weight (dogs 97, 99, 102, 103 and 105 of table 6 and fig. 7). One of the dogs (no. 97) did not show any change in the oxygen capacity of the blood, the other four showed a slight increase. Of the two animals that received 0.001 cc. per kilogram of body weight, a dose that was without effect on the size on the heart, one (dog 101) showed a 33 per cent decrease in the red blood cell count with a smaller decrease of 6 per cent in the oxygen capacity. The other animal (dog 106) did not show any change in the red blood cell count and the capacity for oxygen. Dogs 99, 102 and 103, which were given 0.00168 cc. toxin per kilogram of body weight exhibited an increase in the proportion of cells to plasma, according to the hematocrit readings; dogs 97 and 105, which received the same amount of toxin per kilogram of body weight, and dogs 101 and 106, which received 0.001 cc. per kilogram, did not show any deviation in the hematocrit readings. The hematocrit readings agree with the estimations of capacity for oxygen because they were made from arterial blood; in those instances in which there is a divergence of the red cell counts from the figures that one would expect from the oxygen capacity and the hematocrit readings, it is likely that the discrepancy is due to the fact that the blood for the red blood cell counts was taken from the superficial vessels of the ear, where the concentration of cells was possibly different from the concentration in the arterial blood.

Summary: There was, then, no decrease in the number of red blood cells, in the amount of hemoglobin or in the relative proportion of cells to plasma in five dogs in which there was a decrease in cardiac area. One of two dogs that received a dose of toxin too small to affect the area of the heart, showed a slight decrease in the number of red blood cells and in the amount of hemoglobin, while

the other dog did not show any change. These data indicate that destruction of red blood cells was not a factor in the mechanism responsible for the decrease in the cardiac area.

Effect of Diphtheria Toxin on the Blood Volume: Estimations of blood volume were made on five dogs before and after injections of diphtheria toxin. The total blood volume of dog 101 (table 6, fig. 7) was 1,282 cc. before the injection of toxin; three days after the injection of toxin, it was 1,041 cc. This dog, which had been given 0.001 cc. per kilogram of body weight and which showed a decrease of 18 per cent in blood volume, did not present any significant change in the size of the heart. Before the injection of toxin into dog 102, the blood volume was

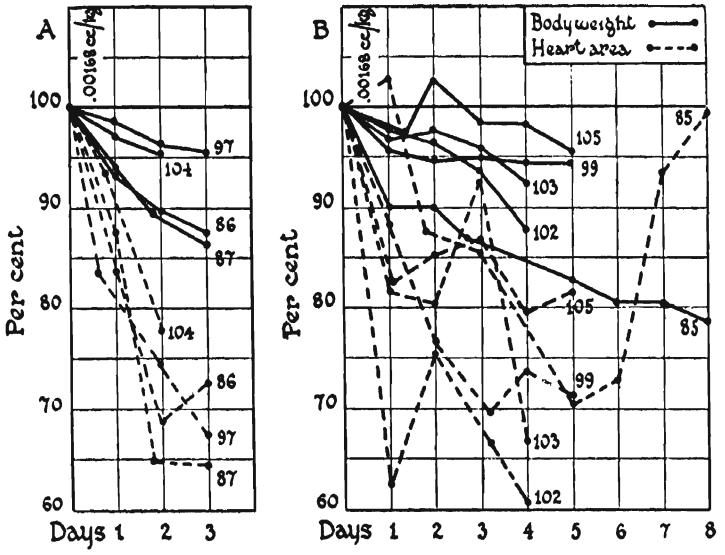


Fig. 6.—Graph *A* shows the effect of diphtheria toxin on the cardiac area, and graph *B* the effect on the body weight in the dogs of group 2A (table 5).

1,864 cc. It was 1,739 cc. three days after the injection, a decrease of 6 per cent (this is within the limits of error of the method) at a time when the cardiac area was decreased 33 per cent. The estimations for dog 103 revealed an increase in the total blood volume of 26 per cent on the third day after the injection of toxin, while the cardiac area showed a decrease of 19 per cent. There was an increase of 6 per cent in the blood volume of dog 105, while the area of the heart was decreased 13 per cent from that before the injection. Dog 106 showed a decrease of 12 per cent in the total blood volume accompanying a decrease of 11 per cent in the area of the heart.

Summary: Following the injection of diphtheria toxin, one dog showed a decrease of blood volume and not any change in the size of the heart; two did not show any change in blood volume and did show decreases in the cardiac area;

TABLE 6

The Effect of Diptheria Toxin on the Blood Volume, the Number of Red Blood Cells, the Hemoglobin and the Size of the Heart in Dogs

Dog	Time of observation	Hematocrit reading, per cent cells	Red cell count, millions	Hemoglobin of oxygen capacity per cent by volume	Plasma, Cc.	Whole blood, Cc.	Cardiac area, Sq. Cm.	Amount of toxin injected per kg., Cc.
97	Before injection.....	41.6	8.9	21.52	.....	.....	51.65	
	Third day after.....	43.5	9.2	21.66	.....	.....	34.70	0.00168
	Percentage change*....	+1.9	+3.3	+0.6	.....	.....	−32.8	
99	Before injection.....	43.8	8.7	23.59	.....	.....	57.15	
	Third day after.....	55.5	8.5	28.62	.....	.....	39.75	0.00168
	Percentage change.....	+11.7	−2.3	+21.2	.....	.....	−30.5	
102	Before injection.....	39.2	10.1	21.06	1,136	1,864	52.35	
	Third day after.....	50.9	9.7	22.96	854	1,739	34.80	0.00168
	Percentage change.....	+11.7	−4.0	+9.4	−24.9	−6.7	−33.6	
103	Before injection.....	43.3	8.3	20.98	608	1,074	38.45	
	Second day after.....	51.5	8.3	24.40	658	1,357	32.70	0.00168
	Percentage change.....	+8.2	0.0	+16.3	+8.2	+26.3	−19.6	
104	Before injection.....	41.4	7.7	21.72	657	1,121	36.45	
	Second day after.....	.....	.....	.....	.....	.....	28.40	0.00168
	Percentage change.....	.....	.....	.....	.....	.....	−22.1	
105	Before injection.....	37.3	6.8	17.01	793	1,265	43.85	
	Third day after.....	39.0	7.0	18.40	823	1,349	38.10	0.00168
	Percentage change.....	+1.7	+2.9	+8.1	+3.7	+6.6	−13.2	
106	Before injection.....	48.3	8.5	22.78	1,032	1,996	39.35	
	Third day after.....	49.7	8.7	22.16	871	1,731	34.75	0.001
	Percentage change.....	+1.4	+2.3	−2.7	−15.6	−12.8	−11.7	
101	Before injection.....	24.9	7.1	10.18	964	1,282	39.40	
	Third day after.....	23.6	4.7	9.57	795	1,041	43.45	0.001
	Percentage change.....	−1.3	−33.8	−6.0	−17.6	−18.8	+9.7	

* The positive sign indicates an increase; the negative sign a decrease.

one showed an increase in blood volume and a decrease in cardiac area, and one, a slight decrease in blood volume accompanying a slight decrease in cardiac area (table 7).

Of the four dogs showing a decrease in cardiac area, only one showed a decrease

in total blood volume. From these few experiments, the limits of error of the method of estimating blood volume being taken into consideration, the volume does not appear to have changed and therefore could not have been a factor in bringing about the decrease in cardiac size.

Effect of a Change in the Blood Volume on the Area of the Heart: On account of the lack of relationship between the cardiac size and the blood volume in these experiments, it seemed important to learn precisely what the effect on the size of the heart is when the blood volume is altered. The following experiment was devised to show the effect of a change in blood volume on the size of the heart. A known amount of blood was taken from a dog, and the effect on the size of the heart was observed. The blood withdrawn from this dog (no. 1) was transfused into another dog (no. 2), and the effect of the increase in blood volume was ob-

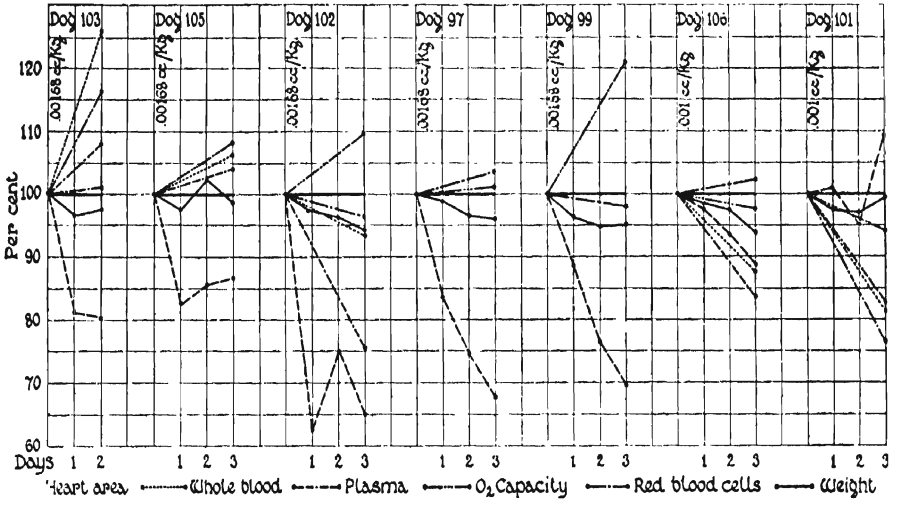


Fig. 7.—A graph showing the effect of diphtheria toxin on the hemoglobin, the number of red blood cells and blood volume in dogs (table 6). The cardiac areas and the body weights of the corresponding dogs are also plotted.

served. Dog 116, weighing 13.44 Kg., presumably had a total blood volume of approximately 12,200 cc. After a roentgenogram of the heart had been taken, 500 cc. of blood was removed from the left femoral artery. The dog was in place on the roentgen-ray table during this procedure. Roentgenograms were made immediately after the blood had been withdrawn, at short intervals during that day, and then daily. Counts of the red blood cells were made at the same time that the roentgenograms were made. There was a sharp fall of 20 per cent (table 8, fig. 8A) in the cardiac area immediately after the blood had been removed, and this fell further to 25 per cent in two hours and forty minutes. The count of the red blood cells did not show any change until the day after the bleeding, then the count was found to have decreased 17 per cent and the following day 30

per cent, probably owing to the dilution of the blood in the body's attempt to restore the blood volume to normal. The cardiac area showed a decrease of from 20 to 30 per cent for six days; it then returned to 88 per cent of the area first observed, and remained at this figure for one month. At the time of the return of the cardiac area to 88 per cent of its initial size, the count of the red blood cells had still further decreased, indicating a further dilution of the blood.

The 500 cc. of blood removed from dog 116 was collected under sterile procedure. Clotting was prevented by the addition of 3 per cent sodium citrate to a final dilution of 0.3 per cent. This blood was transfused into dog 115, weighing 11.96 Kg. The blood of dog 115 had beforehand been cross-agglutinated against the blood of dog 116 by the rapid method of Rous and Turner.[6] Dog 115 presumably had a blood volume of approximately 1,000 cc.; by the addition of 500 cc. of blood, the total blood volume was increased about 50 per cent. Roentgenograms and counts of the red blood cells were made immediately after the transfusion and at

TABLE 7

Summary of the Changes in Blood Volume with the Corresponding Changes in Cardiac Area in Dogs Following the Injection of Diphtheria Toxin

Dog	Cardiac area	Blood volume
101	No change	Decrease
102	Decrease	No change
103	Decrease	Increase
105	Decrease	No change
106	Decrease	Decrease

short intervals later. Immediately after the transfusion was completed, the cardiac area increased 8 per cent (table 9; fig. 8B). The next day it was only 2 per cent greater than it had been in the beginning. Since the position of the dog was not changed between the taking of the first roentgenogram and that immediately after the transfusion, this 8 per cent increase in size is significant. Three days after the transfusion, the area had decreased to 91 per cent of its original size, and on the fifth day to 83 per cent. The cause of the secondary decrease is not clear. The count of the red blood cells increased 25 per cent immediately after the transfusion and, with slight fluctuations, continued to rise until the eighth day, when the increase reached 42 per cent. The amount of the urine of this dog was large during the first two days after the transfusion.

Summary: It may be seen, then, that a decrease in blood volume decreased the size of the heart, and a corresponding increase in blood volume increased the size

6. Rous, P., and Turner, J. R.: A Rapid and Simple Method of Testing Donors for Transfusion, J. A. M. A. 64: 1980 (June 12) 1915.

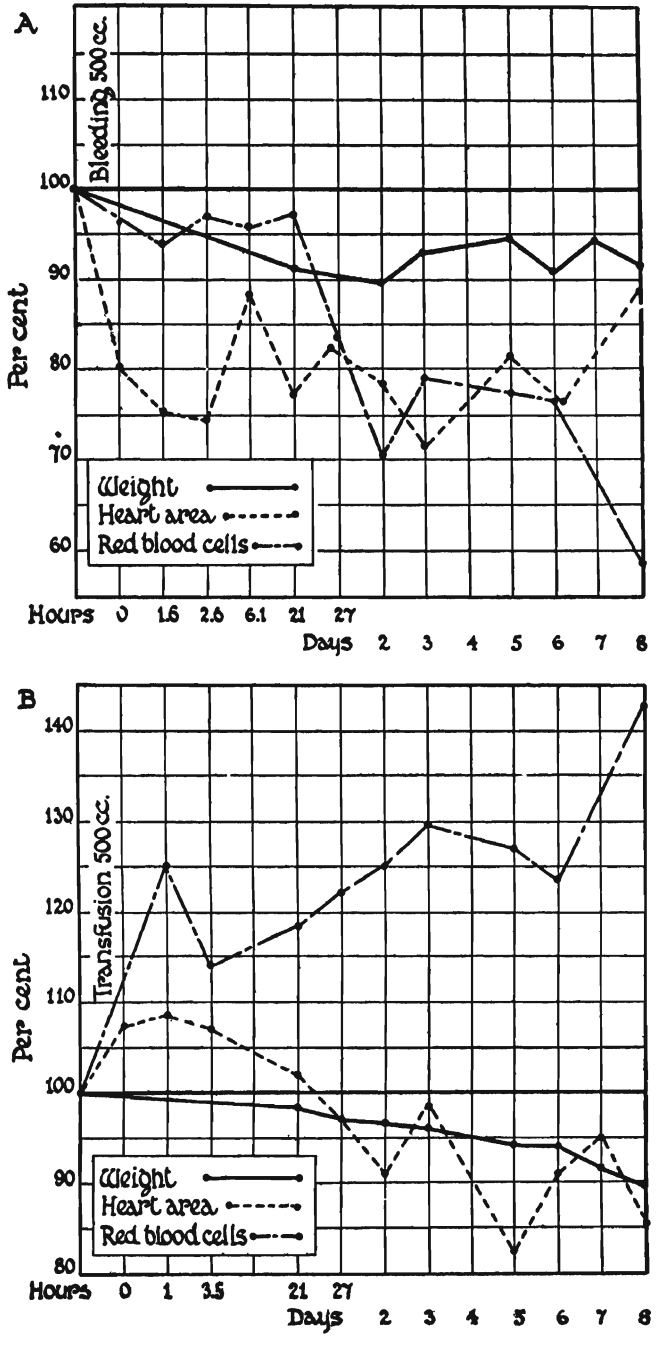


Fig. 8.—Graph *A* shows the effect of a decrease in blood volume on the cardiac area and the number of the red blood cells; graph *B*, the effect of an increase in the volume of the blood.

of the heart slightly. That the increase in the latter case was not as great as one might have expected was probably due to the elasticity and distensibility of the vascular bed, which can take care of the increased blood volume without great dilatation of the heart.

Effect of Diphtheria Toxin on the Regeneration of Red Blood Cells: Although counts of the red blood cells and studies of the blood volume do not point to destruction of blood as the cause of the decrease in cardiac size, jaundice seen in many of the dogs suggested that it might have occurred. In sections from some of the livers there were nests of cells[1] suggestive of foci of regenerating red blood

TABLE 8

The Effect of Decreasing the Blood Volume by 500 Cc. on the Cardiac Area and the Number of Red Blood Cells (Dog 116)

Time of observation	Weight		Cardiac area		Red cells	
	Kg.	Per cent of first weight	Sq. cm.	Per cent of first area	Millions	Per cent of first count
Before bleeding	13.44	100.0	47.80	100.0	7.2	100.0
Immediately after bleeding	.....	.....	38.60	80.7	...	.....
Hours after bleeding						
1.6	.....	.....	36.20	75.7	6.8	94.4
2.6	.....	.....	35.45	74.1	7.0	97.2
6.1	.....	.....	42.35	88.5	6.9	95.9
21	12.27	91.3	37.20	77.8	7.0	97.2
27	.....	.....	39.60	82.8	6.0	83.3
Days after						
2	12.02	89.4	37.45	78.3	5.1	70.8
3	12.40	93.0	34.00	71.1	5.7	79.1
5	12.75	94.8	39.10	81.5	5.6	77.7
6	12.15	90.4	36.70	76.6	5.5	76.3
7	12.75	94.8	39.60	82.8	...	.....
8	12.25	91.1	42.20	88.2	4.2	58.3

cells, and, in some of these, cells resembling nucleated red blood cells were seen. It is unusual that signs of regeneration of red blood cells should appear so early after the injury, and, in addition, the liver is not commonly supposed to take on the function of formation of red blood cells until considerable anemia is present, or until damage has been suffered by the other blood-forming organs. The sections of the liver occasionally showed bile thrombi, but they were not present in the livers of all the animals exhibiting jaundice; therefore, this could not be the factor that caused jaundice. It is possible that jaundice was caused by destruction of red blood cells by the toxin; indeed, the destruction of a small amount of blood might readily have given rise to sufficient pigment to cause jaundice, although the

decrease in the total number of red blood cells and the total blood volume was too small to be detected by the methods used.

Summary: Studies of the blood did not give evidence, therefore, that blood destruction was the factor concerned in the decrease in size of the heart in these animals. The presence of jaundice clinically, and of foci of cells in the microscopic sections of the organs after death which may have been regenerating red blood cells, however, suggests that a certain degree of blood destruction may have occurred.

TABLE 9

The Effect of Increasing the Blood Volume by 500 Cc. on the Cardiac Area and the Number of Red Blood Cells (Dog 115)

Time of observation	Weight		Cardiac area		Red cells	
	Kg.	Per cent of first weight	Sq. cm.	Per cent of first area	Millions	Per cent of first count
Before transfusion	11.96	100.0	47.55	100.0	6.4	100.0
Immediately after transfusion	.....	.....	51.35	107.9	...	.....
Hours after						
1	.....	.....	51.50	108.3	8.0	125.0
3.5	.....	.....	51.00	107.2	7.3	114.0
21	11.75	98.2	48.53	102.0	7.5	118.7
27	.....	.....	46.30	97.3	7.8	121.8
Days after						
2	11.55	96.5	43.45	91.3	8.0	125.0
3	11.45	95.7	46.68	98.1	8.3	129.6
5	11.25	94.0	39.35	82.7	8.1	126.5
6	11.25	94.0	43.30	91.0	7.9	123.4
7	10.95	91.5	45.25	95.1	...	.....
8	10.75	89.8	40.60	85.4	9.1	142.5

Effect of Diphtheria Toxin on the Capillaries.—The possibility that diphtheria toxin is a poison to the capillaries was mentioned. The toxin may cause dilatation of the capillaries similar to that caused by histamine, and the total increase in the vascular bed so brought about would result in a redistribution of the blood in the body—in a sense, draining it away from the heart. The heart would then become smaller, although the amount of blood in the circulation was the same. That there was a change in the capillaries is suggested by the frequent occurrence of ecchymoses at autopsy.

Effect of Diphtheria Toxin on the Structure of the Heart Muscle.—

The gross and microscopic examinations of the hearts of the dogs that died of intoxication with diphtheria toxin have already been reported.[1] Lesions of the fibers of the heart muscle or of the interstitial tissues that could be attributed to the diphtheria toxin were not found. Therefore, the decrease in cardiac area could not have been due to the actual destruction of muscle tissue.

Effect of Diphtheria Toxin on the Weight of the Heart Muscle.—There remains the possibility that the decrease in the size of the heart resulted from a loss of weight by the heart muscle itself. This was definitely exhibited in twelve dogs.

In dogs 81, 84, 86, 87, 97, 102, 103, 105, 107, 108, 110 and 112, there was a decrease in the ratio of the combined ventricular weights to the body weight (fig. 9B);[7] in these the heart muscle must have lost weight more rapidly than the body. If loss in both had proceeded at an equal pace, the ratio would have been undisturbed. In the other eight dogs, there might have been a decrease in heart weight, but in these the loss of weight by the body proceeded at a greater rate, so that the $\frac{L + R}{B W}$ ratio appeared greater than normal. This, however, did not preclude an absolute loss of weight by the heart muscle, although relative to the body weight this was not apparent. For this reason, the figures are misleading. This objection may be obviated in the following way: It may be assumed that, before the injection of the toxin, the weights of the hearts were such that $\frac{L + R}{B W}$ would have a value approximating the average for normal dogs. If the body weight observed just before the injection of the toxin is used to calculate $\frac{L + R}{B W}$, the weight of the heart at the time of death is seen to have decreased, so that in all animals, except four (dogs 82, 83, 99 and 104), the heart did not weigh as much as it should have for a dog as large as the animals were before the injection. There was a tendency for the $\frac{L + R}{B W}$ ratio to be lowest in those animals in which there was the greatest decrease in cardiac size (fig. 9B). The $\frac{L + R}{B W}$ ratios for all except the four dogs mentioned fell, then, below the average figure. These four dogs, however, showed large decreases in cardiac size and this mechanism cannot be called into play to explain them. The decrease in the L/R ratio (fig. 9A) in all dogs, except dog 109, showed that there was some dis-

7. The data from which figure 10 was constructed are contained in the first paper of this series.[1]

turbance in the heart muscle that caused the left side of the heart to lose weight more rapidly than the right side, resulting in a change in the ratio of the two sides. The decrease in the amplitude of the R_2 and R_3 waves (Stewart[1]) in the electrocardiogram also pointed to some disturbance in the muscle itself.

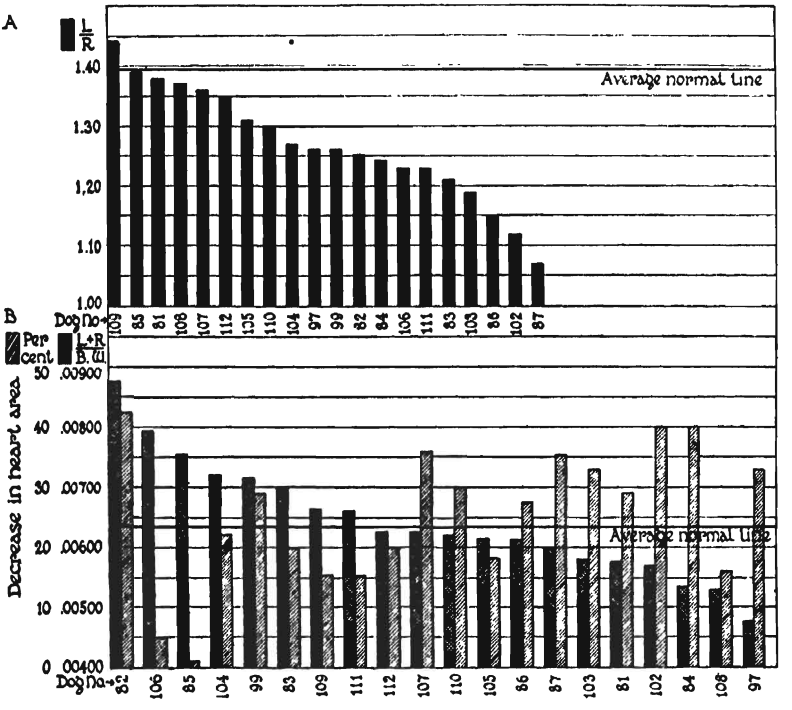


Fig. 9.—Graph *A* shows the grouping of the L/R ratios in dogs suffering from diphtheria intoxication with reference to the average L/R ratio in normal dogs. The height of the solid column represents the L/R ratio. Graph *B* shows the association between the change in the $\frac{L + R}{B W}$ ratio and the decrease in cardiac area in dogs suffering from diphtheria intoxication. The total height of the left hand column represents the $\frac{L + R}{B W}$ ratio when the weight of the dog at autopsy is used in calculating the ratio. The height of the solid column represents this ratio when the weight of the dog before the injection of diphtheria toxin is used in calculating this ratio. The height of the diagonally ruled area represents the decrease in cardiac area.

Summary: There was a decrease in the ratio of the combined ventricular weights to the body weight, which is most easily explained on the basis of a decrease in the weight of the heart. Parallel with this

decrease in the weight of the heart, there was a decrease in the size of the heart. This relationship is still more striking, and the decrease in the weight of the heart can explain the decrease in size of the heart in all but four instances, if the ratio is calculated from the weight of the animal just before the injection of the toxin.

By what mechanism, then, does the heart muscle lose weight? Nothing in the microscopic sections of the heart muscle indicated that an actual destruction of heart muscle cells had taken place. One is forced to look for some other mechanism by which the heart muscle could lose weight. Since there was not any destruction of muscle cells, it is possible that a loss of substances, such, perhaps, as fluid and salts, might account for the decrease in its weight and for the decrease in its size. Whether a change such as this actually occurred I cannot say; I am without evidence on this point.

COMMENT

Following the injection of 0.00168 cc. or more diphtheria toxin per kilogram of body weight into dogs, the cardiac area in the dogs, as measured in roentgenograms, decreased appreciably. It was found that 0.00135 cc. of toxin per kilogram of body weight caused a similar change, but that 0.001 cc. per kilogram did not. There was approximately the same decrease in body weight in all the dogs, an observation that precluded the possibility that the decrease in cardiac size was due to the decrease in body weight. Moreover, in dogs that had fasted for from three to four days, and in which there was a loss of body weight comparable to the loss of weight following diphtheria intoxication, there was no change in the size of the heart. This indicated that the decrease in cardiac size was due to the toxin. Counts of the red blood cells and estimations of the hemoglobin did not show evidence of marked destruction of blood. In these dogs, there was no consistent change in the total blood volume. In this connection, experiments showed that changes in blood volume are reflected in alterations in the size of the heart, in the following manner: A decrease in blood volume is accompanied by a decrease in the size of the heart and an increase in blood volume by a small increase in the size of the heart. The possibility that diphtheria toxin injures the capillaries was mentioned. A

study of the $\frac{L + R}{B W}$ ratios, however, pointed to the conclusion that there was a loss of weight by the heart muscle which could account for a part of, if not for all, the decrease in the size of the heart. This mechanism failed to explain the phenomenon in four instances. Histologic study of the muscle of the heart did not reveal an actual destruction of the cells of this muscle to account for the decrease in weight. There is a possibility that the latter was due to a disturbance in some mechanism involved in the maintenance of the water balance of the heart muscle cells. It may be that the decrease in the weight of the heart is not the only factor causing the decrease in size of the heart; the other possibilities mentioned may also play a rôle. Whatever the mechanism it must take place rapidly, for the decrease in cardiac size was always well developed within twenty-four hours and was often at a maximum at that time.

CONCLUSION

The injection of diphtheria toxin into a dog in a sufficiently large dose is followed by a decrease in the size of the heart. Analysis of the factors that may be involved in this alteration indicates that it is due to loss of weight by the heart, although other factors possibly play a part.

[Reprinted from THE AMERICAN HEART JOURNAL, June, 1929, Vol. 4, No. 5, pp. 512-520]

THE ORAL ADMINISTRATION OF CALCIUM CHLORIDE IN CONGESTIVE HEART FAILURE

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In the treatment of patients suffering from heart failure there are often present edema and collections of fluid in the serous cavities which are refractory to removal by drugs, such as digitalis, novasurol, and the theobromine diuretics which are commonly in use. For this reason it is desirable to test thoroughly measures which promise to be of benefit in these cases.

In 1921 and 1922 Blum[1] and his coworkers described a new group of diuretics. According to their conception edema is caused by the retention of sodium ions; sodium ions in being retained hold water which accumulates in the tissues and serous cavities. They found that diuresis was induced in cases of edema of nephritic origin, in cirrhosis of the liver with ascites and in inflammation of the liver with fluid accumulations by the oral administration of salts of calcium, potassium and strontium. They thought that the sodium ions in the tissues were replaced by calcium, potassium or strontium ions; that they were excreted as sodium chloride and in being excreted carried water with them. In this way water was removed from the tissues. They were of the opinion that diuresis occurred by the "replacement of ions." Though they believe that this group of diuretics was effective in the conditions just mentioned, they stated that they were usually ineffective in edema due to heart failure and gave warning that the use of them in these cases was dangerous.

At the time this series of papers appeared we had under observation a patient whom we were unable to free of edema by limitation of fluids, by the use of a diet free of salt or by the administration of digitalis and the usual diuretics. This patient we succeeded in making free of edema by giving calcium chloride by mouth. From this experience we were led to give the salt to a small number of patients; a preliminary report has already appeared.[2]

Since the appearance of Blum's papers, Keith, Barrier and Whelan[3] have reported the occurrence of satisfactory diuresis in cases in which there was edema due to nephritis, following the report of Atchley, Loeb and Benedict[4] concerning a patient who suffered from edema occurring in the course of diabetes. Recently, Segall and White,[5] after giving calcium chloride to a number of patients, concluded that it may be employed as a diuretic "in cases of cardiac failure with edema in which constant rest in bed, digitalization and administration of various diuretics have not resulted in satisfactory diuresis."

Method of Investigation

Calcium chloride was administered orally to six patients suffering from heart failure in whom edema was present. All patients were subjected to a preliminary period of rest in bed. They were given a fixed amount of fluid per day. These conditions were maintained during the administration of calcium chloride. It was given as a concentrated solution. Divided into two doses, 15 gm. were usually given a day. It was followed by a small amount of orange juice for relief of the bitter taste. After taking calcium chloride patients complained occasionally of a burning sensation in the epigastrium or of abdominal cramps. These symptoms were, however, not severe. One patient (Case 3) vomited on one occasion and another (Case 2) on two occasions. Other untoward symptoms were not encountered. In some observations calcium chloride was given alone; in others it was given to patients who had first received a sufficient amount of digitalis to affect the T-wave of the electrocardiogram or to slow the ventricular rate in auricular fibrillation. In still further observations, the administration of calcium chloride was followed by that of digitalis. A few patients were the subjects of the three sets of observations. Most of the patients were those to whom other diuretics had been given without effect.

OBSERVATIONS

CASE 1. S. F. (See Case 18, Stewart[6]) was a woman, 24 years old. She was under treatment in the hospital from September 13, 1922, until July 29, 1924. The diagnosis* was: *Etiological:* acute rheumatic fever (inactive); *anatomical:*

* The diagnoses conform to the nomenclature for cardiac diagnosis approved by the American Heart Association. AM. HEART J. 2: 202, 1926–1927.

cardiac hypertrophy, mitral stenosis and insufficiency, aortic insufficiency, left ventricular preponderance; *physiological:* auricular fibrillation, congestive heart failure. She was admitted to hospital because of shortness of breath and edema of the legs. She had suffered from numerous attacks of acute rheumatic fever between the ages of five and eighteen years. Cardiac involvement occurred at the time of the first attack. The heart was very much enlarged. There were signs of mitral stenosis and insufficiency and of aortic insufficiency. The systolic blood pressure measured 110 to 115 mm. of mercury and the diastolic 70 mm. Auricular fibrillation was present. From September, 1922, until June, 1923, the patient was given a diet free of salt, and the fluid-intake was restricted to 1500 c.c. a day.

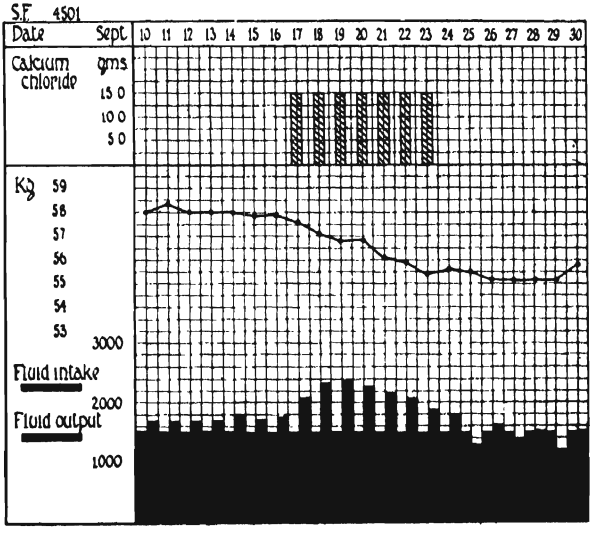


Fig. 1.—Shows the effect of the administration of calcium chloride upon the volume of urine in Case 1.

She was given digitalis either alone or combined with diuretin or theocin. By these means sporadic increases in volume of urine were obtained, but only rarely did the increase continue long enough to make the patient free of edema for even a day or two. Finally, the patient was given calcium chloride 15 gm. a day by mouth, on four days. Up to this time the volume of urine had been 1300 c.c. a day when she was taking 1500 c.c. of fluid a day. On the first day that calcium chloride was given the volume of urine was 1265 c.c., 673 c.c. on the second, 1575 c.c. on the third, 1970 c.c. on the fourth, and 2210 c.c., 1706 c.c. and 1769 c.c. on the three days following, although during these calcium chloride was not given. The patient lost 0.8 kg. in weight, and the edema disappeared. Edema reappeared as soon as diuresis stopped, and on the third day after the end of diuresis, calcium chloride was accordingly given again on five days. One day only the output rose

to 1800 c.c. Edema decreased. After an interval of three days (during which the volume of urine decreased to 400 c.c. per day, and edema increased) calcium chloride was given again. The day the administration of calcium chloride was begun, digitan 0.9 gm. was also given. The output rose to 1730 c.c., 1750 c.c., 1666 c.c., 1325 c.c., 800 c.c., and 1805 c.c. respectively on the six days that it was given and remained 1853 c.c., 1880 c.c., 1438 c.c., and 1790 c.c., on the four days afterward; it fell then to 1200 c.c. The patient lost 1.5 kg. in weight and became free of edema. She was given no drugs from June 27 to July 20. At the end of this time edema recurred and she gained 3.0 kg. in weight; the output of urine was approximately 1000 c.c. a day. Calcium chloride was then given on four days. On the first three days the output of urine was 705 c.c., 750 c.c., and 785 c.c., respectively. Diuresis did not begin until the fourth day when the output rose to 1595 c.c., and was 1695 c.c., and 2065 c.c. on the next two days. The patient lost 1 kg. in weight. Edema again decreased, and a few days later when digitan 0.7 gm. was given the output increased for a day or two and she became free of edema. She was given maintenance doses of digitalis. She was allowed to sit up; edema recurred, however, and she remained in bed. Beginning September 17 calcium chloride was given on seven days (Fig. 1). The output had been, on the average, 1700 c.c. a day before calcium chloride was given. During the seven days that calcium chloride was given, the volume of urine rose to 2060 c.c., 2362 c.c., 2178 c.c., 2284 c.c., 2190 c.c., 2085 c.c., and 1865 c.c. respectively; it was 1818 c.c. the next day and then fell to 1291 c.c. During the period of diuresis the patient lost 2.5 kg. in weight, and she became free of edema. She received no drugs from September 23 to November 12. Edema recurred. Calcium chloride was again given during five days. The output rose from 1000 c.c. to 1200 c.c. a day, to 1425 c.c., 1475 c.c., 2043 c.c., 1890 c.c., and 1890 c.c. respectively, and the patient lost 2 kg. At the end of this time there was only a slight trace of edema of one ankle. The patient was then given digitalis; a slight increase in output of urine occurred. In spite of this, edema persisted and she gained weight. Beginning December 3, calcium chloride was given on five days. The output rose from 1400 c.c. to 1600 c.c., and remained at that amount for seven days.

Summary.—This patient with edema due to heart failure was given calcium chloride by mouth on seven occasions, four times alone and three times combined with full therapeutic doses of digitalis (0.7 gm. to 0.9 gm.). Diuresis followed on each occasion and on four of them she became free of edema. Calcium chloride appeared to be equally effective whether the patient was or was not under the influence of digitalis; the effect which could be ascribed to digitalis did not appear, however, to be greater when this drug was given in combination with calcium chloride than when given alone, that is to say a synergistic

action between these two drugs was not demonstrated in the case of this patient.

CASE 2. S. C. (See Case 14, Stewart[6]). This patient was a man, 66 years old. He was admitted to hospital complaining of shortness of breath of five years duration. The cardiac diagnosis of this patient was: *Etiological:* arteriosclerosis; *anatomical:* cardiac hypertrophy, mitral insufficiency, left ventricular preponder-

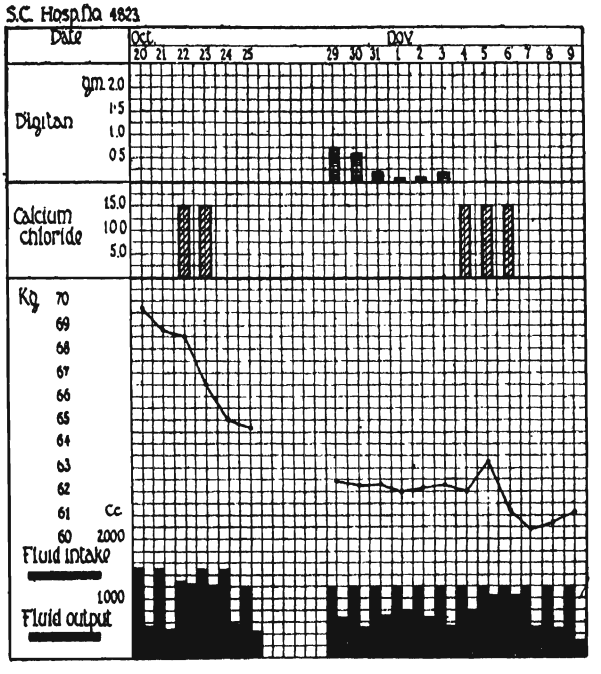


Fig. 2.—Shows the effect of the administration of calcium chloride upon the volume of urine in Case 2, first when given alone, and second when given after the administration of digitalis.

ance; *physiological:* auricular fibrillation, congestive heart failure. There was no history of acute rheumatic fever. The heart was enlarged. Auricular fibrillation was present. A soft systolic murmur was heard at the apex. The systolic blood pressure was 150 mm. of mercury and the diastolic 80 mm. Cheyne-Stokes respirations were present. There were moist râles at the bases of the lungs posteriorly. There was marked edema of the legs. The Wassermann reaction of the blood was negative.

The fluid intake was limited to 1500 c.c. a day. After he had been in bed for three days he was given, on October 22, 1923, calcium chloride 15 gm. (Fig. 2). The output of urine, which had been 497 c.c. the day before rose now to 1210 c.c.

The following day, when the same amount of calcium chloride was given it was 1226 c.c.; the edema disappeared. The next day calcium chloride was not given; the output of urine fell to 525 c.c. On October 25 the fluid intake was reduced to 1200 c.c. per day. The volume of urine remained low until October 26 and 27. On each of these days calcium chloride 15 gm. was given and the output rose to 621 c.c. and 1215 c.c. respectively; a decrease then occurred. The patient was given digitalis until the ventricular rate was slow and the electrocardiogram showed changes in the T-wave. There was one day (November 1) a slight increase in output to 770 c.c. He was then given calcium chloride 15 gm. a day on three days. The output amounted to 805 c.c. on the first, 1122 c.c. on the second and 1136 c.c. on the third day, but fell to 590 c.c. the next day when calcium chloride was not given. The patient was without medication for the next eleven days; the output of urine varied between 250 c.c. and 500 c.c. a day. A slight degree of edema recurred. Beginning November 18 he was given calcium chloride again, 15 gm. a day on 4 days. There was a slight but definite increase in output of urine amounting to 835 c.c. a day. During this time he lost weight and edema disappeared. He was then given digitalis from November 23 until December 10. Toward the end of this period the volume of urine was slightly increased, and he lost 2 kg. in weight. One day the volume of urine was 1100 c.c.; it then decreased. On December 12 he was given calcium chloride 15 gm.; the output rose from 770 c.c. to 1224 c.c. The next two days, when given the same amount of calcium chloride, it was 1105 c.c. and 1103 c.c. respectively; on the fourth day when he received calcium chloride 7.5 gm., the output fell to 516 c.c. and remained at that level. During the four days it was given he lost 2 kg. in weight. He was now given a sufficiently large amount of digitalis daily to keep the ventricular rate slow. About six weeks later acute dilatation of the heart occurred, and the patient died. An autopsy was performed. The diagnosis was as follows: General arteriosclerosis; arteriosclerotic involvement of the cusps of the aortic valves; cardiac hypertrophy; aneurysm of the abdominal aorta*; infarcts of the kidneys; arteriosclerosis of the kidneys; terminal broncho-pneumonia; venous stasis of the organs.

Summary.—Calcium chloride was given then on six occasions to this patient, on three without digitalis, on two, following the administration of digitalis, and on one the use of digitalis was followed by calcium chloride. The administration of calcium chloride was always accompanied by increase in volume of urine; it was often more than doubled on the days the drug was given. The resulting diuresis was sufficient to free the patient of edema. It appeared to be effective

* The patient frequently complained of abdominal pain. The abdomen presented no abnormality on physical examination.

whether it was given when the patient was or was not under the influence of digitalis. Vomiting occurred twice during its administration.

CASE 3. I. K. This patient was a man 37 years old. He complained of shortness of breath, palpitation and edema. The cardiac diagnosis was: *Etiological:* unknown; *anatomical:* cardiac hypertrophy, mitral stenosis and insufficiency, right ventricular preponderance; *physiological:* auricular fibrillation, congestive heart failure. Cyanosis was present. The heart was enlarged. There were signs of mitral stenosis and insufficiency. The systolic blood pressure was 115 mm. of mercury and the diastolic 75 mm. There was free fluid in the right pleural cavity. Moist râles were heard in both lungs. The liver was enlarged; ascites was present. There was edema of the lower extremities.

The patient was so ill that digitalis was given at once. The ventricular rate became slow. While taking 1200 c.c. of fluid a day the ouput of urine remained between 295 c.c. and 519 c.c. per day. On the seventh day after admission to the hospital (the patient was still under the influence of digitalis) he was given calcium chloride 15 gm. The volume of urine on that day was 489 c.c. The following day, when calcium chloride was given again, the output was 618 c.c. On continuing its administration on the third and fourth days the output was 810 c.c. and 655 c.c., respectively. On the fifth day it was not given, and it fell to 524 c.c. Then it gradually decreased to 300 c.c. per day. Later, satisfactory diuresis occurred when novasurol was given.

Summary.—This patient was under the influence of digitalis when calcium chloride was given. There occurred a very slight increase in the output of urine. It was not sufficient, however, to have an effect on the accumulation of fluid in the tissues and serous cavities. Vomiting occurred once during administration of the salt.

CASE 4. G. B. (See Case 17, Stewart[6]). This patient was a man, 69 years old. He complained of shortness of breath and edema. The cardiac diagnosis was: *Etiological:* arteriosclerosis; *anatomical:* cardiac hypertrophy, aortic stenosis and insufficiency, mitral insufficiency, left ventricular preponderance; *physiological:* auricular fibrillation, congestive heart failure. There was no history of acute rheumatic fever. The heart was enlarged; auricular fibrillation was present. Over the base there was a rough systolic and a soft diastolic murmur. Soft systolic and diastolic murmurs were also heard at the apex. The systolic blood pressure was 120 mm. of mercury and the diastolic 90 mm. There was free fluid in the right pleural cavity. The liver was enlarged; ascites was present. There was marked edema of the legs. The Wassermann reaction of the blood was negative.

The patient was given a large amount of digitalis. Diuresis did not occur. The output of urine remained 500 c.c. when he was taking 1000 c.c. of fluid a

day. At a time when he was not under the influence of digitalis he was given calcium chloride 15 gm. a day on four days. On these days the output was 680 c.c., 755 c.c., 610 c.c., and 788 c.c., respectively. During this time there was no loss of weight, and the edema did not decrease. Later, after giving diuretin the volume of urine increased to 1588 c.c. on one day. About ten days later the patient died suddenly. An autopsy was performed. The diagnosis was: chronic cardiac valvular disease (aortic); general arteriosclerosis; perforation of the interventricular septum; contraction scar in the conus of the pulmonary artery; hypertrophy and dilatation of the right and the left ventricles, chronic myocarditis; thrombosis of the pulmonary artery; edema, ascites, hydropericardium; hydrothorax, venous congestion of the organs; arteriosclerosis of the kidneys; cysts of the kidneys.

Summary.—Calcium chloride was given to this patient when he was not under the influence of digitalis. The slight increase in output of urine which occurred was not sufficient to influence the degree of congestive heart failure.

CASE 5. A. B. This patient was a female, 44 years old. She complained of swelling of the abdomen, shortness of breath and edema. The diagnosis was: *Etiological:* acute rheumatic fever (inactive); *anatomical:* mitral stenosis and insufficiency, cardiac hypertrophy, right ventricular preponderance; *physiological:* normal sinus rhythm, congestive heart failure. She suffered from an attack of acute rheumatic fever 7 years before. Involvement of the heart occurred at that time. Fluid had been removed from the abdominal cavity by paracentesis every three weeks during the last 20 months. The heart was enlarged. The rhythm was normal. There were signs of mitral stenosis and insufficiency. The systolic blood pressure was 100 mm. of mercury and the diastolic 80 mm. The lungs were clear. There was marked ascites. The liver was enlarged. There was marked edema.

During the first 5 days in hospital the patient gained 2.5 kg. in weight. The volume of urine was not more than 319 c.c. per day. Calcium chloride 10 gm. a day was given on 5 days. The output on these days was 315 c.c., 333 c.c., 474 c.c., 565 c.c., and 515 c.c., respectively. The next day, when it was not given, the output was 510 c.c. and then fell to 300 c.c. per day. Digitalis and novasurol were given also without diuretic effect. Ascites increased so rapidly that abdominal paracentesis was performed and 13 liters of fluid were removed. The patient died suddenly 4 days later. An autopsy was performed. The diagnosis at autopsy was as follows: chronic cardiac valvular disease (mitral stenosis); ascites; hydropericardium; chronic passive congestion of the liver, spleen and pancreas; chronic peritonitis, perihepatitis, perisplenitis; cirrhosis of the liver.

Summary.—The oral administration of calcium chloride to this patient was followed by an increase of only a few hundred cubic

centimeters in the volume of urine. In this instance its use was not combined with the administration of digitalis.

CASE 6. E. A. This patient was a male, 72 years old. He complained of shortness of breath and swelling of the legs. The cardiac diagnosis was: *Etiological:* arteriosclerosis; *anatomical:* cardiac hypertrophy, chronic myocarditis, left ventricular preponderance; *physiological:* normal sinus rhythm, congestive heart failure. The heart was enlarged. The rhythm was regular. The sounds were clear both at the apex and over the base. The systolic blood pressure was 150 mm. of mercury and the diastolic 110 mm. There was fluid in the right pleural cavity. The liver was enlarged. Marked edema of the legs was present. The Wassermann reaction of the blood was negative with alcoholic antigen and positive with cholesterin antigen.

TABLE I

Summary of Results Following the Administration of Calcium Chloride

Case No.	Calcium chloride				Number of observations on each patient
	Without digitalis		With digitalis		
	Diuresis	No diuresis	Diuresis	No diuresis	
1	4*		3*		7
2	3*		3*		6
3				1	1
4				1	1
5		1			1
6	2*		1		3
Total.....	9	1	7	2	19

* Refers to number of separate observations.

The patient was given 1200 c.c. of fluid a day. Calcium chloride, 15 gm. a day, was administered on 2 days. Increase in output of urine did not occur. It became necessary to remove fluid (1000 c.c.) from the right pleural cavity by paracentesis. Administration of digitalis, 1.3 gm. in 3 days, was followed by only a slight increase in output of urine. Fluid reaccumulated in the right pleural cavity, and 1200 c.c. of it were removed. Two weeks later calcium chloride was given again on 4 days; the administration of digitalis was continued, however. On the 4 days preceding the administration of calcium chloride the output of urine was 570 c.c., 782 c.c., 853 c.c., and 610 c.c., respectively. On the days it was given, the output rose to 886 c.c., 868 c.c., 1200 c.c., and 915 c.c. respectively. Diuretin and later theocin were given; no greater increase in output occurred than had resulted from the administration of calcium chloride. Calcium chloride was given again on 4 days. On this occasion increase in output did not occur. Although we were not able to induce diuresis by the usual measures, edema gradually

diminished. Paroxysmal auricular fibrillation was often present. A hemorrhage, the origin of which was not discovered, occurred from the gastrointestinal tract. Later, strangulation of an inguinal hernia occurred, and the patient was removed to another hospital for operation. The patient died a few days later. An autopsy was not performed.

Summary.—In this patient there was severe cardiac decompensation. On two occasions the use of calcium chloride was without effect on the volume of urine. On another occasion, when combined with the use of digitalis, a slight increase occurred.

SUMMARY

Calcium chloride was given orally to six patients suffering from edema due to heart failure (Table I). Slight increase in the output of urine occurred, but marked diuresis was not observed. The administration of digitalis simultaneously with the oral administration of calcium chloride did not appear to be more effective than its use alone. The observations were repeated on several occasions on each patient. In two patients only was diuresis sufficient to free them of edema.

DISCUSSION

We have observed an increase in the output of urine following the oral administration of calcium chloride. Diuresis was never very marked, and in only two patients was it effective in decreasing edema. The results in even these two patients are contrary, however, to Blum's statement that the salt was without diuretic effect in edema due to heart failure. Nor did we observe deleterious effects following its administration. It may be recalled that Blum was of the opinion that cardiac patients do not tolerate the drug.

The mechanism by which calcium chloride acts as a diuretic is not known. Hjort[7] and Salvesen, Hastings and McIntosh[8] and others[4,9] have found that the administration of it by mouth produces a severe uncompensated acidosis in dogs and in human subjects. This is due to a replacement of the HCO_3 radicle by Cl in the blood, as the result of absorption from the alimentary tract of the Cl of calcium chloride without Ca. There is an actual loss of base from the blood and a failure to adjust the carbon dioxide tension to the lowered bicarbonate. The calcium content of the blood serum may increase or may be unchanged. On the other hand the intravenous administration of calcium

chloride has no effect on the acid-base equilibrium of the blood; there occurs, however, a moderate rise in the phosphates. The injected calcium leaves the blood in from three to six hours. Whether the diuretic effect is dependent on the presence of the anion or upon the acidosis or upon some other mechanism is at present unsettled.

CONCLUSIONS

1. The administration of calcium chloride to cardiac patients with edema increases the volume of urine.

2. The increase in output which results is only occasionally effective in decreasing edema.

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[Reprinted from THE JOURNAL OF CLINICAL INVESTIGATION, August 20, 1929,
Vol. 7, No. 3, pp. 339–351]

THE EFFECT OF EXERCISE ON THE SIZE OF NORMAL HEARTS AND OF ENLARGED HEARTS OF DOGS

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(Received for publication, February 6, 1929)

Although the changes in the heart produced by exercise have long been made the subject of investigation by clinicians and physiologists, the question has not yet been settled. The earliest view, formulated from clinical experience, was that when the heart was subjected to exertion it dilated. This opinion was derived from estimations of changes in the borders of the heart on percussion (T. Schott (1890) and Williams and Arnold (1899)). T. Schott (1897) was the first investigator to employ x-ray examination in studying this problem. The data he collected appeared to support his view that dilatation of the heart occurred. Moritz (1908) in 1908 first demonstrated by means of orthodiagrams that acute cardiac enlargement did not follow over-exertion. Indeed, on the contrary, he found that in certain instances both in normal and pathologic hearts contraction took place. Since then, studies have been made of the effect of many varieties of exertion (swimming, rowing, running in marathon races) on the heart of man, from the point of view not only of discovering whether the heart reacts to acute exertion by dilating or contracting, but also of learning whether hypertrophy is necessarily a consequence of athletic training. More and more, the evidence which has been accumulated seems to indicate that dilatation does not occur as a consequence of acute exertion, but that on the contrary contraction in size takes place. Recently Gordon and Strong (1923) have studied in rabbits the effect of vigorous exercise on the size of the normal and abnormal heart. The hearts designated as abnormal were those in which enlargement resulted from repeated injections of spartein sulphate. According to these authors myocarditis was the resulting pathologic lesion. They found that the effect of exercise was the



1a 1b 1c

FIG. 1. In this figure are reproduced x-ray photographs showing the effect of exercise on the size of the normal heart. Photograph 1a was taken before and 1b after running. 1c was taken after the dog had rested for 1 hour.

same in both instances: a decrease in size of the hearts always occurred.

We have had an opportunity also to investigate this subject. For several years we have been engaged in attempting to find a method which would establish a state in dogs comparable to heart failure in man. One of the methods has consisted in the attempt to bring about this state by rendering the mitral valve insufficient. We have for this reason in our possession a number of dogs in which defects of the mitral valve have been made by operation and in which enlarged hearts have in consequence developed. They show no signs however of heart failure. It has been in connection with the study of the circulation of these dogs that we have investigated the response of the hearts to exercise. In them we are able to estimate the influence that valvular defect alone (presumably without disease of the muscle) exerts on the reaction of the heart to exertion. The form of exercise which we chose was running on a treadmill. We took the precaution of studying the effect of the same form of exertion on the hearts of normal dogs. These experiments form the subject of this paper.

MATERIAL

The subjects of certain experiments, as has been stated, were normal intact dogs. The dogs which were the subjects of other experiments were those which had been operated on 2 to 3½ years ago. Evidence of the lesions which were then created still existed at the time of the present experiments (table 1). Complete data concerning the operations will be published later (Stewart). A brief description only of the method used in operating on the valves need be given. Under ether anesthesia and under aseptic conditions the left auricular appendage was exposed and incised. A cardioscope[1] was then inserted through this opening. By manipulation of the knife attached to the cardioscope the leaflets of the

[1] The cardioscope which we used was designed with the assistance of Mr. R. Wappler, and was made for us by the Wappler Electric Company, Long Island City, New York. The idea of cutting the valves of the heart under direct vision was suggested to us by the preliminary report of Allen and Graham (1922). As complete data for the construction of their instrument was not available at the time, we devised this new instrument. The optical system is similar to that used in cystoscopes. We are much indebted to Doctors Graham and Allen for valuable aid in learning their methods and desire to express our thanks to them for their courtesy.

TABLE 1

Effect of exercise on the size of the heart of normal dogs

Dog number	Date	Weight	Cardiac area	Cardiac area per cent of initial	Decrease in cardiac area	Heart rate	Duration of exercise	Distance run	Time with reference to running
		kgm.	*sq. cm.*	*per cent*	*per cent*	*per minute*	*minutes*	*miles*	
235	March 22, 1927	15.2	60.4	100.0		96			Before
			57.3	94.8	5.2	120	45	0.9	Immediately after
			56.1	92.8	7.2	128			One hour after
250	October 26, 1927	10.5	35.8	100.0		120			Before
			34.4	96.1	3.9	110	60	0.8	Immediately after
			34.9	97.5	2.5	130			One hour after
	October 27, 1927	11.0	40.8	100.0		120			Before
			38.9	95.4	4.6	90	60	1.1	Immediately after
			37.2	91.2	8.8	120			One hour after
	October 28, 1927	11.6	36.3	100.0		120			Before
			35.6	98.0	2.0	90	60	1.3	Immediately after
			34.0	93.7	6.3	120			One hour after
	October 29, 1927	10.3	37.9	100.0		85			Before
			35.7	94.2	5.8	85	45	*	Immediately after
			35.6	94.0	6.0	85			One hour after
251	October 27, 1927	12.7	48.8	100.0		110			Before
			46.1	94.5	5.5	90	35	0.4	Immediately after
			46.1	94.5	5.5	115			One hour after

* Speedometer not working.

TABLE 2

Enlargement of the heart following induction of artificial mitral insufficiency in dogs

Dog number	Area of heart before operation	Area of heart after operation	Time since operation	Increase in area of heart	Presence of murmur at time of running
	sq. cm.	*sq. cm.*	*years*	*per cent*	
158	46.4	84.4	3⅝	81.8	+
161	46.0	72.3	2½	57.2	+
171	50.3	68.9	2	37.7	+
153	56.2	68.2	2⅛	21.3	0
90	43.0	42.6	3⅛	0.0	0

mitral valve were brought into view. The leaflets could then be cut under direct vision. Development of a marked systolic thrill was regarded as evidence that the operation had succeeded. The dogs completely recovered within 10 days to 2 weeks. The hearts began to increase in size after varying intervals of time. A loud systolic murmur persisted in each of the dogs (see exceptions, table 2). The dogs studied for the present purpose were in good health. They were trained to laboratory procedures and were not disturbed by them.

METHODS

The dogs were first trained to run on a treadmill. Certain dogs ran readily; others could not be induced to run and were not studied. After the preliminary training, the effect of running on the size of the heart was investigated by means of x-ray photographs of the heart. The x-ray photographs were made and measured according to the method described by Stewart (1927) for obtaining photographs of the hearts of dogs under uniform conditions. The anticathode was placed at a distance of 2 meters from the photographic film. A suture was inserted in the skin in the mid-line of the anterior chest wall at the level of the heart. The anti-cathode was always centered on this point before plates were exposed. Three x-ray photographs were made during each session: the first, before the dog began running; the second, immediately after the dog had stopped running; and the third, after the dog had rested for one hour. Since the treadmill was not driven by a motor, but by the dogs themselves, they ran only as long as they did so voluntarily. They ran quite steadily for 25 to 60 minutes. The second photographs were made as soon as it was evident that the dogs did not wish to run longer. The tread was placed at an angle of 19 degrees with the horizontal and a speedometer recorded the number of revolutions from which the distance was calculated. Since the treadmill was in the x-ray room only a few seconds were required to transfer the dog from it to the dog board under the x-ray tube. The x-ray photographs were taken within 2 to 3 minutes after the dogs had stopped running. The rate of the heart was counted at the apex for one minute immediately after the photographs were taken. The observations were usually repeated several times in each dog.

The exposures were sufficiently long to photograph the diastolic heart shadow; in those photographs in which both systolic and diastolic positions of the heart could be identified, the diastolic area was the one measured.

OBSERVATIONS

Effect of exercise on the size of the heart in normal dogs. In dog 235 the initial cardiac area was 60.4 sq. cm. (table 2, fig. 1). It decreased to 57.3 sq. cm. after the dog had been running for 45 minutes. After resting 1 hour it decreased still further to 56.1 sq. cm. There was

TABLE 3

The effect of exercise on the size of enlarged hearts in which systolic murmurs were still present

Dog number	Date	Weight	Cardiac area	Cardiac area per cent of initial	Decrease in cardiac area	Heart rate	Duration of exercise	Distance run	Time with reference to running
		kgm.	*sq. cm.*	*per cent*	*per cent*	*per minute*	*minutes*	*miles*	
158	February 10, 1927	20.6	82.1	100.0		130			Before
			77.2	94.0	6.0	148	60	0.6	Immediately after
			81.9	99.7	0.3	136			One hour after
	March 4, 1927	22.3	87.5	100.0		148			Before
			80.4	91.8	8.2	132	38	0.4	Immediately after
			82.8	94.7	5.3	150			One hour after
	October 13, 1927	19.9	84.4	100.0		90			Before
			79.1	93.7	6.3	120	40	0.5	Immediately after
			80.9	95.8	4.2	114			One hour after
	October 15, 1927	20.1	87.2	100.0		130			Before
			79.9	91.6	8.4	110	57	0.6	Immediately after
			83.7	96.0	4.0	130			One hour after
161	January 28, 1927	16.0	72.3	100.0		160			Before
			65.9	91.1	8.9	142	38	0.3	Immediately after
			71.0	98.2	1.8	144			One hour after
	February 9, 1927	16.8	73.6	100.0		122			Before
			68.3	94.1	5.9	142	25		Immediately after
			68.5	94.4	5.6	134	31	0.2	Immediately after
			74.1	100.6	0.6				One hour after
	October 13, 1927	14.5	72.0	100.0		104			Before
			68.3	94.8	5.2	104	30	0.3	Immediately after
			71.2	98.8	1.2	120			One hour after
171	March 14, 1927	20.5	68.9	100.0		140			Before
			66.9	97.1	2.9	130	60	0.8	Immediately after
			62.8	91.2	8.8	148			One hour after
	March 15, 1927	21.3	64.5	100.0		140			Before
			60.5	93.8	6.2	142	44	0.8	Immediately after
			59.0	91.5	8.5	140			One hour after



2a 2b 2c

FIG. 2. In this figure are reproduced x-ray photographs which show the effect of exercise on the size of the enlarged heart. Photograph 2a was taken before and 2b after running. 2c was made after the dog rested for 1 hour.

accordingly a fall to 94.8 per cent of the initial size, followed by a further decrease to 92.8 per cent of the initial size one hour after exercise.

There are 4 observations on dog 250 and one on dog 251 (Table 2). The results in all instances are similar to the one just reported. The size of the hearts decreased 3.9 to 5.8 per cent immediately after running, but decreased still further 6.0 to 8.8 per cent 1 hour later. In not a single instance did the size of the heart become greater than it had been in the beginning.

Effect of exercise on the size of enlarged hearts in which systolic murmurs are present. Mitral insufficiency was created in dog 158 on December 2, 1924, $3\frac{5}{6}$ years ago. During this time the area of the heart increased 81.8 per cent from 46.4 sq. cm. to 84.4 sq. cm. (table 2). This was the area on October 13, 1927 (table 3, fig. 2). After running 40 minutes on this day it decreased to 79.1 sq. cm., 93.7 per cent of the value before exercise. One hour later it was 80.9 sq. cm., 95.8 per cent of what it was before the start. There was then on this occasion a decrease of 6.3 per cent in the size of the heart after exercise. The dog ran on three other occasions. On these also the cardiac area decreased, the maximum decrease varying between 6.0 and 8.4 per cent.

Mitral insufficiency was created in dog 161 on December 11, 1924, $2\frac{1}{6}$ years ago. During this time the area of the heart increased 57.2 per cent from 46.0 sq. cm. to 72.3 sq. cm. (table 2). This was the cardiac area on January 28, 1927 (table 3). After running 38 minutes on this day it was 65.9 sq. cm., that is to say, it had diminished 8.9 per cent. One hour later it was 71.0 sq. cm., approximating the size it was before the start. The dog ran on two other occasions. On these also a decrease in size of the heart occurred. The maximum decrease amounted to 5.9 per cent and 5.2 per cent respectively.

Mitral insufficiency was created in dog 171 on April 22, 1925, 2 years ago. During this time the heart had enlarged 37.7 per cent from 50.3 sq. cm. to 68.9 sq. cm. (table 2). He ran on the treadmill on two occasions. The decreases immediately after running were 2.9 and 6.2 per cent respectively and were 8.8 and 8.5 per cent respectively one hour later (table 3).

There are observations therefore on 3 dogs in which the heart was

enlarged and in which there was still evidence that defects of the mitral valve (murmurs) were still present. In every instance the size of the heart decreased following the exercise of running on a treadmill.

Effect of exercise on the size of the heart in dogs subjected to operation but in which there was no longer evidence that valvular defect was present. Mitral insufficiency was created in dog 153 on November 20, 1924,

TABLE 4

Effect of exercise on the size of the hearts of dogs subjected to operation but in which systolic murmurs were no longer present

Dog number	Date	Weight	Cardiac area	Cardiac area per cent of initial	Decrease in cardiac area	Heart rate	Duration of exercise	Distance run	Time with reference to running
		kgm.	*sq. cm.*	*per cent*	*per cent*	*per minute*	*minutes*	*miles*	
153	January 18, 1927	23.0	69.6	100.0					Before
			66.7	95.8	4.2		42	0.6	Immediately after
			62.9	90.4	9.6				1 hour after
	January 22, 1927	23.0	67.8	100.0		182			Before
			60.9	89.0	11.0	145	55	0.6	Immediately after
			67.9	100.0		170			1 hour after
	January 25, 1927	23.0	68.2	100.0		165			Before
			62.5	91.5	8.5	170	50	0.4	Immediately after
			67.5	99.0	1.0	155			1 hour after
90	April 15, 1927	11.5	42.6	100.0		130			Before
			40.2	94.3	5.7	120	38	0.8	Immediately after
			42.6	100.0		112			1 hour after

$2\frac{1}{6}$ years ago. During this time the size of the heart increased 21.3 per cent, from 56.2 sq. cm. to 68.2 sq. cm. (table 2). A systolic murmur was heard for some months after operation but later it disappeared. It was not heard at the time of these experiments. The dog ran on three occasions. On each occasion the heart was smaller after running than it had been in the beginning (table 4). It decreased 8.5 per cent on the first, 11 per cent on the second and 4.2 per cent on the third occasion. One hour later it had nearly regained its

initial size on the first, it had returned to its initial size on the second, and decreased still further (9.6 per cent) on the third occasion.

Mitral insufficiency was created in dog 90 on February 28, 1924. A soft systolic murmur was heard for some months after operation but later it disappeared. Three and one sixth years after operation the area of the heart was approximately the same as before operation (0.8 per cent decrease) (table 2). The area of the heart was 42.6 sq. cm. on April 15, 1927 (table 4). After running on the treadmill for 38 minutes it was 40.2 sq. cm., that is to say, a decrease of 5.7 per cent had occurred. The initial size was regained one hour later.

In one dog (3 occasions) then the subject of an artificially enlarged heart and in a second in which there was no enlargement, the hearts decreased in size after running on a treadmill. The maximum decrease was 11 per cent. A systolic murmur was not heard in either case.

Effect of exercise on the heart rate. The heart rate decreased after running in 2 normal dogs (dog 250, 3 occasions and dog 251, once) (table 2), unchanged once (dog 250) and increased once (dog 235). In the case of the dogs in which the hearts were enlarged, the rate sometimes decreased (dog 158 twice, dog 161 once, dog 171 once, dog 90 once, and dog 153 once), sometimes increased (dog 158 twice, and dog 161 once) and was sometimes unchanged (dog 161 once, dog 171 once, and dog 153 once). It is clear that the same effect was not always observed even in the same dog.

DISCUSSION

We have presented data showing that the hearts of normal dogs decrease in size following exercise. Decrease in size also occurred in hearts that were enlarged, whether the valve defect (murmur) was or was not still present. The effect in dogs in which the hearts were enlarged was the same as in the normal hearts; the dogs were all in good health and exhibited no evidence of heart failure. Although the mitral valves had been subjected to damage and the hearts had enlarged in consequence of this injury, there had occurred no disease of the heart muscle so far as is known. Although the decrease in size was small, 3.9 to 11 per cent, it is nevertheless significant and it occurred consistently. Enlargement never was observed. In the hour after exercise the hearts sometimes decreased still further in size

(if the decrease in the first instance was small), sometimes remained unchanged and at other times regained their initial size. We have no explanation to offer for this difference. The changes which we observed in dogs were not as great as those observed in rabbits by Gordon and Strong (1923). The difference in the results obtained is probably due to the fact that the rabbits ran until completely exhausted, while our dogs ran only as long as they did so voluntarily. The distance which the dogs ran may appear to be small. The average distance was 0.6 mile, but the inclination of the tread (19°) necessitated a vertical ascent of 1000 ft. in this distance.

We cannot be certain of the factors responsible for the occurrence of the decrease in size of the hearts of these dogs after exercise. It was observed also to accompany regular tachycardia (Stewart and Crawford, 1927). Decreased filling of the heart in the shortened diastole may be one of the factors. In the case of these experiments however tachycardia was not a factor, since the ventricular rate exhibited no consistent change, remaining unchanged or becoming either slower or faster than the initial rate. Increase in size of the vascular bed may of course have taken place due to the opening of channels which were hitherto closed, for exercise may be believed to be the occasion for the opening of a greater number of capillaries in the muscles and skin than when the animals are in a resting state. The blood would then be drained away from the heart.

Both Meek and Eyster (1922) and Stewart (Stewart, a) have shown that the size of the heart decreases when the volume of circulating blood is diminished. But decrease in volume of blood was in all probability not a factor in these cases since Hastings (1921) has shown an increase following exercise in the hemoglobin content (expressed as oxygen capacity) of the blood and Broun (1922) has demonstrated an actual increase in the total blood volume.

In another connection also there is similarity in behavior of large and small hearts. The same dogs which served in these experiments were utilized also in studying the effect of digitalis. In both groups decrease in cardiac size, decrease in cardiac output and increase in ventricular excursion followed the administration of this drug (Cohn and Stewart, 1928a and 1928b).

It is not certain how far an application can be made of the data of

these experiments to cases of heart disease in man. The analogy is not close enough to warrant the conclusion that acute exercise is not harmful to patients suffering from valvular disease when they exhibit no signs of heart failure. It is infrequent that one finds in patients the conditions which were present in these experiments, that is to say, valvular insufficiency and cardiac enlargement without concomitant disease of the myocardium.

SUMMARY

The effect of running on a treadmill on the size of the hearts of dogs has been studied. It was found that the size of both normal and enlarged hearts always decreased.

CONCLUSIONS

Dilatation of the heart does not occur in normal dogs following voluntary exercise. On the contrary, the size of the heart decreases. When dogs in which the heart is enlarged in consequence of artificially created valvular defects, but in which there is presumably no myocardial disease, and in which there are no signs of heart failure, are subjected to exercise, the size of the heart likewise decreases.

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[Reprinted from THE JOURNAL OF PHYSICAL CHEMISTRY, May, 1929, Vol. 33, No. 5, pp. 745–754]

THE EFFECT OF SALTS ON WEAK ELECTROLYTES

III. INTERACTION OF CERTAIN WEAK ELECTROLYTES

BY HENRY S. SIMMS

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I. INTRODUCTION

In the two previous papers[1] it was shown that the ionic activity of weak electrolytes in dilute solutions is markedly affected by the presence of strong electrolytes. NaCl was found in general to give effects on weak acids in agreement with the limiting Debye-Hückel equation (providing we modify it to allow for the distance between charges in divalent acids). The anomalous effects produced by Mg^{++} and $SO_4^{=}$ were studied. Amines and ampholytes were found to be abnormal with all salts.

It has also been shown[2] that in the case of oxalic acid the large effect of $MgCl_2$ is antagonized by the addition of NaCl or KCl. Likewise[3] the effect (in the opposite direction) of Na_2SO_4 is antagonized by NaCl or KCl. In either case, large additions of NaCl or KCl tend to produce the effect of these salts alone, drowning out the effects of the anomalous salts. These antagonisms follow equations indicating a mechanism involving a mass action effect.

Similarly NaCl, KCl and $MgCl_2$ each lower the pH of gelatin solution (original pH 7.367) when present alone; but the addition of a small amount of a second salt raises the pH; more of the second salt lowers the pH; and still more again raises the pH of the solution. For details one must consult the original paper.[4]

[1] Simms: J. Phys. Chem., **32**, 1121, 1495 (1928).
[2] Simms: J. Gen. Physiol., **12**, 241 (1928).
[3] Simms: J. Gen. Physiol., **12**, 259 (1928).
[4] Simms: J. Gen. Physiol., **12**, 511 (1929).

Exactly the same phenomena are produced by the addition of salts to glycine solutions[5] showing that this salt antagonism is unquestionably noncolloidal in character.

On the assumption that such anomalies are due to inactivation of a weak electrolyte by a strong electrolyte, it seems advisable to determine whether one weak electrolyte may be inactivated by another weak electrolyte.

II. EFFECT OF GLYCINE ON H_3PO_4 TITRATION WITH SALTS

In the present paper we find, first, that the presence of glycine causes a drop in P_{K_2}' of H_3PO_4. This drop increases with addition of salt, being greatest at zero ionic strength (extrapolated). (See Fig. 1.)

III. EFFECT OF VARIOUS AMOUNTS OF GLYCINE ON $HPO_4^=$ ACTIVITY

This indicates that the glycine inactivates the phosphate diion ($HPO_4^=$) perhaps by means of a loose combination. In order to study this we prepared solutions of H_3PO_4 with 1.4 equivalents of NaOH containing varying amounts of glycine. The results are plotted in Fig. 2 where it is evident that the effect on the P_K' reaches a maximum and then decreases per increment of glycine added. This is, however, only an indirect measure of the effect on the activity.

If we calculate the remaining activity f of $HPO_4^=$ ion (see Table II) the fraction inactivated by glycine is $(1 - f)$. Plotting f against *log* c_G (*i.e.*, log of the glycine concentration) gives a symmetrical S-shaped curve indicating a mass action mechanism. (Fig. 3.)

We may moreover calculate a mass action constant k from the equation:

$$k = \frac{[\text{Glycine}] \times [HPO_4^=]}{[\text{Combined}]} = c_G \frac{f}{1 - f} = 0.40$$

where the free $HPO_4^=$ equals $f\ \alpha_p c_p$ and the combined $HPO_4^=$ equals $(1 - f)\ \alpha_p c_p$, see Table II. It will be observed that it is the neutral (zwitterion) form of glycine which does the inactivating. Correction was made for the degree of ionization of glycine in each solution.

[5] Simms: (Unpublished).

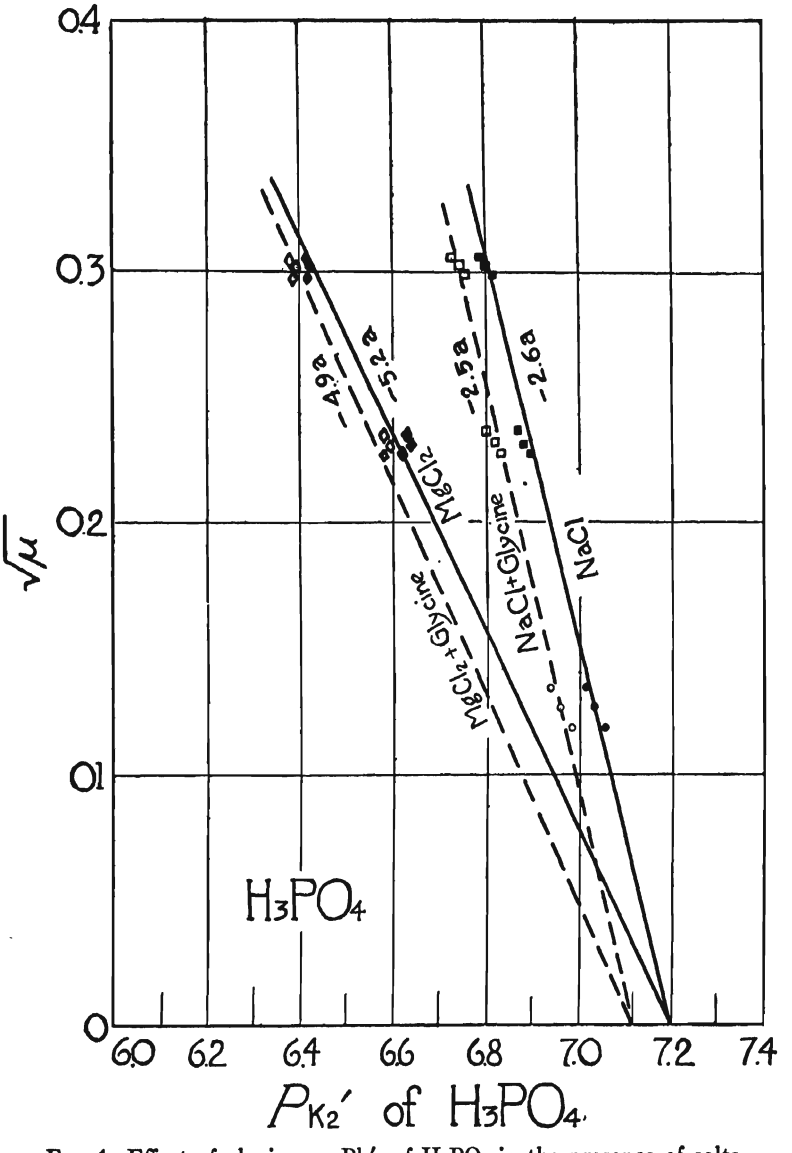


FIG. 1. Effect of glycine on Pk'_2 of H_3PO_4 in the presence of salts

IV. EFFECT OF PHOSPHATE AND OTHER POLYVALENT ANIONS ON GLYCINE

One would expect from the above that if HPO_4^- inactivates neutral glycine, then the reverse should be true and the presence of phosphate should affect the ionization of glycine. This is found to be so. In Fig. 4, if we compare the $NaHPO_4$ curve with that of NaCl, it will

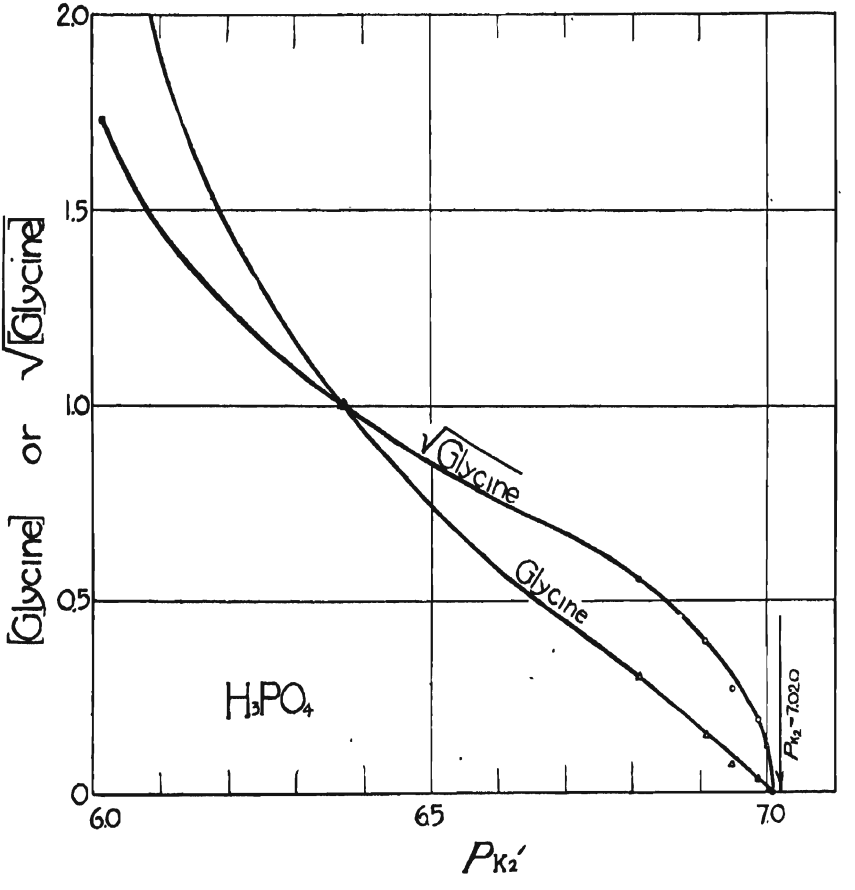


FIG. 2. Effect of glycine on pk'_2 of H_3PO_4 (where $pk'_2 = \log f + 7.620$)

be seen that Na_2HPO_4 *raises* P_{K_2}' of glycine to an extent depending on the amount of phosphate. The direction of the effect agrees with the above assumption that HPO_4^- and *neutral* glycine inactivate each other.

To determine if this effect is specific for phosphates, we substituted Na_2SO_4 (since H_2SO_4 is the only other suitable inorganic divalent acid

and since it is known to produce anomalous effects). The curve for Na_2SO_4 in Fig. 4 shows that SO_4^- ion produces the same result as HPO_4^- ion. It therefore appears that SO_4^- also inactivates neutral glycine.

If this effect is dependent upon the electrostatic charge on the sulfate and phosphate ions it is to be expected that the polyanions of organic acids should also produce the same effect. A study of

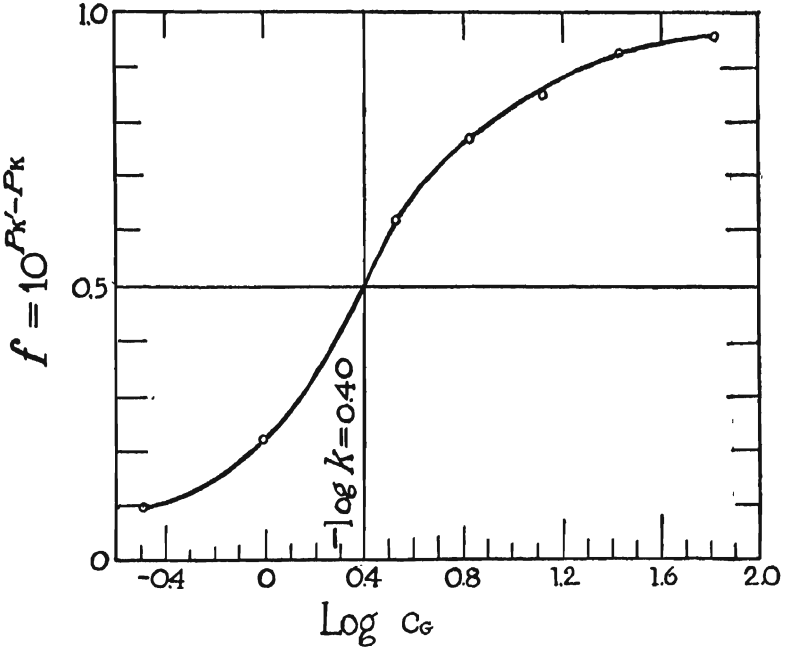


FIG. 3. Plot of HPO_4^- activity against log of glycine concentration

glycine in the presence of the sodium salts of oxalic, succinic and citric acids shows that these salts in very dilute solution (0.01μ) behave *oppositely*, but in higher concentration (0.1μ) behave the *same* as SO_4^- and HPO_4^- ions.

A more careful examination of the Na_2HPO_4 curve shows that in very dilute solution it too tends to have the opposite effect to that in higher concentration. Apparently all these salts have the same effect (of raising P_{K_2}' of glycine) in the higher concentrations (studied up to 0.2μ) but that they differ in their tendency to produce the opposite

effect at lower concentration (about 0.01μ). The order of the latter effect is:

oxalate > succinate > citrate > phosphate > sulfate.

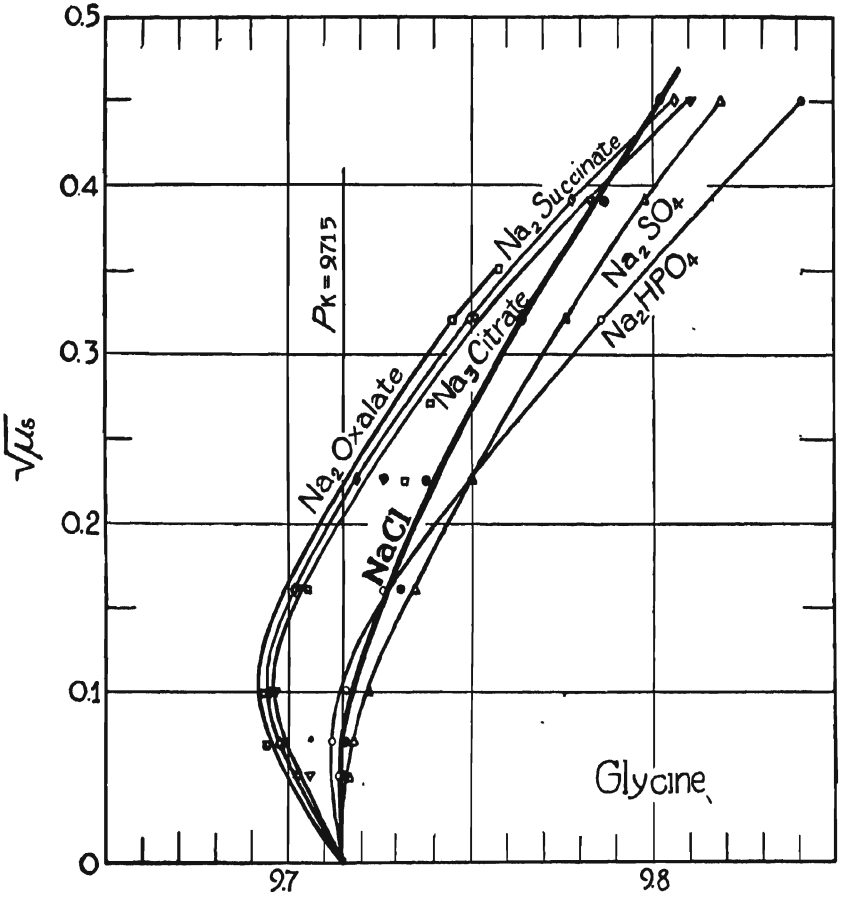


FIG. 4. Effect of salts on Pk'_2 of glycine. μ_s is the ionic strength due to added salts. The abscissas are Pk' values corrected for the normal deviation expressed by the limiting Debye-Hückel equation.

V. EFFECT OF POLYVALENT ANIONS ON SUCCINIMIDE

Succinimide is a very weak acid ($P_K = 9.560$) ionizing in the P_H range suitable for studying its effect with $HPO_4^=$. We have already seen that $SO_4^=$ produces anomalous effects on both P_{K_1}' and P_{K_2}'

of divalent weak acids (such as malonic[1] and oxalic[2]). With succinimide we may study a monovalent weak acid not only with sulfate but also with phosphate diions, and furthermore with the diions of weak acids.

The results are shown in Fig. 5. The curves of Na_2HPO_4 and Na_2SO_4 show that their effect on succinimide is very similar to their

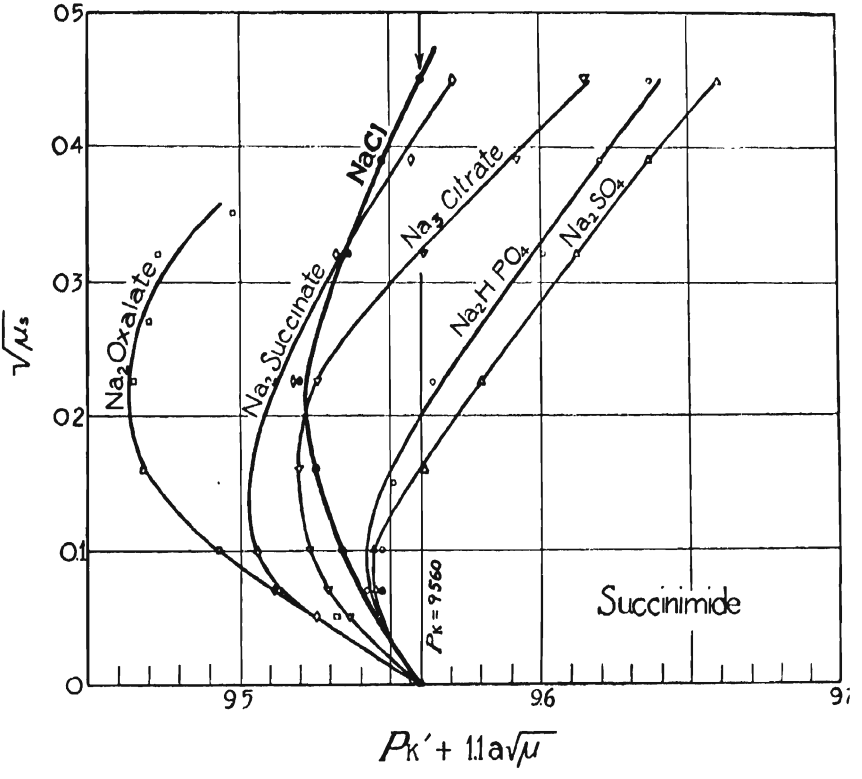


FIG. 5. Effect of salts on Pk′ of succinimide

effect on glycine (Fig. 4). Furthermore, the organic salts have a similar, but more marked, effect on succinimide.

There is a gradual transition in the shape of the curves as we pass from sulfate, to phosphate, to citrate, to succinate, and to oxalate. The differences are quantitative rather than qualitative. It would appear that the same effect is produced by all these ions in less dilute solutions but that they differ in their behavior in more dilute solutions (0.01μ).

VI. CONCLUSIONS

The observations do not warrant any definite explanation. Glycine causes a drop in P_{K_2}' of H_3PO_4 (the effect being decreased by addition of salts). Na_2HPO_4 causes a rise in P_{K_2}' of glycine. These results suggest that neutral glycine and the phosphate diion ($HPO_4^=$) inactivate each other. On this basis a consistent mass action constant can be calculated.

The assumption of such a mechanism necessitates that we also assume that the diions of sulfates, succinates, and oxalates and the triion of citrates, all inactivate neutral glycine and nonionized succinimide (and other weak acids; and, to a less extent, monoions of divalent weak acids).

These observations may find their explanation in the work of LaMer and his collaborators[6] who explain some anomalies of polyvalent ions on the basis of neglected terms in the Debye-Hückel equation.

In more dilute solution (0.01μ) the salts of divalent anions tend to produce an effect opposite to the above effect at 0.2μ. The order of this effect is the same with either glycine or succinide, namely: oxalate > succinate > citrate > phosphate > sulfate. This order is rather strange since the size of the ion and the distance between the charges of oxalate diion are about the same as with phosphate and sulfate diions; while succinate and citrate ions are larger ions and have more remote charges;[1] furthermore the citrate ion has a higher charge.

VII. EXPERIMENTAL

The experimental methods are fully described in previous papers. The data are given in Tables I to IV.

The degree of dissociation into $HPO_4^=$ is given by

$$\alpha_p = b' - 1 - \frac{c_G}{c_P}\alpha_G$$

where b' is the corrected equivalents of base (1.400); c_G and c_P are the concentrations of glycine and phosphate, respectively; and α_G is the degree of dissociation of glycine (P_{K_2}' taken as 9.68).

[6] LaMer, King and Mason: J. Am. Chem. Soc., **49**, 363; LaMer and Mason: 410 (1927).

TABLE I

Effect of Glycine on P_{K_2}' of Phosphoric Acid in the Presence of Salts (0.0100 M NaH_2PO_4 plus NaOH, and 0.0750μ (C.—) or 0.0375μ (D.—) of NaCl or $MgCl_2$; each solution being measured both without glycine and in the presence of 0.075 molar glycine)

Salt	$\frac{b-a}{c}$	$\sqrt{\mu}$	Without glycine		With glycine	
			P_H	P_{K_2}'	P_H	P_{K_2}'
C.—$MgCl_2$	1.196	0.298	5.809	6.421	5.783	6.395
D.—$MgCl_2$	1.196	.227	6.012	6.624	5 974	6.586
No salt	1.196	.118	6.445	7 057	6 370	6.982
D.—NaCl	1.196	.227	8.287	6.899	6.223	6 835
C.—NaCl	1.196	.298	6.206	6.818	6.147	6 759
C.—$MgCl_2$	1.294	.302	6.045	6.427	6 017	6.399
D.—$MgCl_2$	1.294	.231	6.259	6.641	6.216	6.608
No salt	1.294	.126	6.651	7.033	6.577	6.959
D.—NaCl	1.294	.231	6.501	6 883	6 440	6.822
C.—NaCl	1.294	.302	6.423	6.805	6.367	6.749
C.—$MgCl_2$	1.392	.305	6.228	6 419	6.193	6.384
D.—$MgCl_2$	1.392	.236	6.440	6.631	6 396	6 587
No salt	1.392	.134	6.824	7.015	6.749	6 940
D.—NaCl	1.392	.236	6.682	6.873	6.611	6.802
C.—NaCl	1.392	.305	6.600	6.791	6.541	6.732

TABLE II

Effect of Glycine on $HPO_4^=$ Activity as Shown by P_{K_2}' Values of Phosphoric Acid,—Calculation of Inactivation Constant k. (0.0100 M NaH_2PO_4 plus 0.400 equivalents of NaOH plus various amounts of glycine)

Glycine concentration	P_H	α_P	P_{K_2}'	f	k
c_G					
0	6.832	0.400	7.008		
0.0150	6.822	.398	7.002	0.959	0.37
.0375	6.802	.395	6.987	.927	.48
.075	6.756	.391	6.948	.85	.42
.150	6.705	.385	6.908	.77	.45
.300	6.580	.370	6.811	.62	.49
1.00	6.137	.369	6.369	.22	.29
3.00	5.77	.361	6.02	.10	.33
		Average			0.40

TABLE III

Effect of the Sodium Salts of Four Dianions and One Trianion on P_{K_2}' of Glycine

The effect of NaCl is given for comparison (0.0100 *M* glycine with 0.315 equivalent of NaOH (for NaCl, Na_2SO_4 or Na_2HPO_4, or 0.300 equivalent with the other salts) and various amounts of respective salts; $P_{K_2} = 9.715$).

μ_s*	$a\sqrt{\mu}$**	With NaCl		With Na_2SO_4		With Na_2HPO_4		Na_2 oxalate		Na_2 succinate		Na_3 citrate	
		P_H	P_K'	P_H	P_K'	P_H	P_K'	P_H	P_K'	P_H	P_K'	P_H	P_K'
0	0.027	9.350	9.688	9.350	9.688	9.350	9.688	9.316	9.686	9.316	9.686	9.316	9.686
0.0025	.037	9.340	9.678	9.343	9.680	9.367	9.678	9.296	9.666	9.296	9.666	9.299	9.669
.0050	.045	9.333	9.671	9.335	9.673	9.347	9.667	9.279	9.649	9.283	9.653	9.284	9.654
.0100	.056			9.323	9.666	9.323	9.660	9.267	9.637	9.269	9.639	9.270	9.640
.025	.083	9.311	9.649	9.315	9.653	9.306	9.644	9.258	9.623	9.250	9.620	9.252	9.622
.050	.114	9.286	9.624	9.298	9.636	9.298	9.636	9.238	9.608	9.235	9.605	9.242	9.612
.075	.138							9.221	9.591				
.100	.159	9.267	9.605	9.279	9.617	9.289	9.627	9.216	9.586	9.221	9.591	9.221	9.591
.125	.177							9.211	9.581				
.150	.194	9.255	9.593	9.266	9.604	9.286	9.264			9.214	9.584	9.221	9.591
.200	.224	9.240	9.578	9.257	9.595	9.279	9.617			9.212	9.582	9.217	9.587

* μ_s is the *ionic strength* due to added salt. The *concentration* of Na_2SO_4 or Na_2HPO_4 equals $\mu_s/3.00$; of sodium oxalate, $\mu_s/2.75$; of sodium succinate $\mu_s/2.65$; and of sodium citrate $\mu_s/4.8$.

** $a\sqrt{\mu} = 0.495\sqrt{\mu_o + \mu_s}$ where $\mu_o = 0.0031$ is the ionic strength without added salt.

TABLE IV

Effect of Salts of Dianions and Trianions on P_K' of Succinimide

(0.0100 *M* succinimide with 0.250 equivalent of NaOH (with NaCl 0.255 equivalents were added) and various amounts of salts; $P_K = 9.560$).

μ_S*	$a\sqrt{\mu}$**	With NaCl		With Na_2SO_4		Na_2HPO_4		Na_3 oxalate		Na_3 succinate		Na_3 citrate	
		P_H	P_K'	P_H	P_K'	P_H	P_K'	P_H	P_K'	P_H	P_K'	P_H	P_K'
0	0.025	9.055	9.521	9.055	9.531	9.055	9.531	9.057	9.533	9.056	9.532	9.054	9.530
0.0025	.035	9.052	9.518	9.032	9.508	9.032	9.508	9.018	9.494	9.011	9.476	9.022	9.498
.005	.043	9.035	9.501	9.020	9.496	9.023	9.499	8.991	9.467	8.989	9.465	9.007	9.483
.010	.055	9.007	9.473	9.010	9.486	9.007	9.483	8.956	9.432	8.969	9.445	8.986	9.462
.025	.082	8.969	9.440	8.985	9.461	8.995	9.471	8.902	9.378	8.937	9.413	8.954	9.430
.050	.114	8.929	9.395	8.963	9 439	8 979	9.455	8.864	9.340	8.917	9.393	8.925	9.401
.075	.138							8.842	9.318				
.100	.159	8.895	9.361	8.951	9.427	8.961	9.437	8 822	9 298	8 881	9.357	8.910	9.386
.125	.177							8.827	9.303				
.150	.195	8.868	9.334	8.931	9.427	8.947	9.423			8.868	9.344	8.903	9.379
.200	.223	8.848	9.314	8.915	9.391	8 938	9.414			8.849	9 325	8.893	9.364

* μ_s is the ionic strength due to added salt. The concentration of Na_2SO_4 or Na_2HPO_4 equals $\mu_s/3.00$; of sodium oxalate, $\mu_s/2.75$; of sodium succinate, $\mu_s/2.65$; and of sodium citrate, $\mu_s/4.8$.

** $a\sqrt{\mu} = 0.495\sqrt{\mu_o + \mu_s}$ where $\mu_o = 0.0025$ is the ionic strength without added salt.

$$P_{K_2}' = P_H - \log \frac{\alpha_P}{1 - \alpha_P}$$

f is calculated from: $f = 10^{P_K' - P_K}$ where $P_K = 7.020$

k is calculated from: $k = c_G \frac{f}{1 - f}$ where $1 - f$ is the fraction of $HPO_4^=$ inactivated by glycine.

VIII. SUMMARY

1. The presence of glycine *lowers* the value of P_{K_2}' of H_3PO_4, this effect being antagonized by increasing concentrations of salts.

2. With increasing amounts of glycine the drop in $HPO_4^=$ actvity per increment of glycine added is found to reach and pass a maximum, indicating that neutral glycine inactivates $HPO_4^=$ ion. This obeys a mass action formula: $k = C_G \frac{f}{1 - f}$ where $1\text{-}f$ is the fraction of HPO_4 = inactivated by glycine, and C_G is the concentration of glycine.

3. Na_2HPO_4 was correspondingly found to *increase* P_{K_2}' of glycine (as would be expected). A similar effect on glycine is produced by Na_2SO_4.

4. In concentrations approaching 0.2μ sodium oxalate, sodium succinate and sodium citrate give curves parallel to that of Na_2HPO_4, in their tendency to increase P_{K_2}' of glycine. However 0.01μ of these salts tends to decrease P_{K_2}' of glycine in the order:

oxalate > succinate > citrate > phosphate > sulfate

This order does not agree with the size of the respective ions or with the distance between the charges.

5. The above salts produce the same effects on P_K' of succinimide (a weak acid) as they do on P_{K_2}' of glycine. The curves are qualitatively the same; are in the same sequence; but are more separated.

[Reprinted from THE JOURNAL OF GENERAL PHYSIOLOGY, July 20, 1929,
Vol. 12, No. 6, pp. 783–792]

CHEMICAL ANTAGONISM OF IONS

IV. EFFECT OF SALT MIXTURES ON GLYCINE ACTIVITY

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(Accepted for publication, April 15, 1929)

I

INTRODUCTION

The three previous papers of this series[1] demonstrated that Na^+ ions counteract the anomalous effect of Mg^{++} ions on the activity of oxalate di-ions ($C_2O_4^-$); and also that Cl^- ions counteract the effect of SO_4^- ions on oxalate di-ions. Furthermore, NaCl, KCl, or $MgCl_2$ each affect gelatin activity in the same manner when only one of these salts is present. Each causes a *lowering* of the pH. However, the addition of a small amount of NaCl or KCl to a gelatin solution already containing another of the above salts causes a very marked *rise* in pH (an effect opposite to that produced by one salt alone). This occurs up to 0.10 molar Na^+ or K^+ ion concentration. Further additions cause a sharp lowering of pH up to 0.15 molar Na^+ or K^+. Beyond that there is little effect.

The analogy of these observations to physiological antagonisms is obvious. In order to investigate the mechanism it is desirable to see if still simpler substances than gelatin behave in the same way. In the present paper it will be shown that NaCl, KCl, $MgCl_2$, and $CaCl_2$, singly and in mixtures, affect the activity of glycine (a simple amino acid) in practically the same manner that they affect gelatin activity.

II

RESULTS

The results obtained in these experiments are obvious from the accompanying figures. Fig. 1 shows the influence of salts on the pH

[1] Simms, H. S., *J. Gen. Physiol.*, 1928, xii, 241, 259; 1929, xii, 511.

of a 0.0100 molar solution of glycine containing one-half equivalent of NaOH. NaCl alone or KCl alone *lower* the pH in the manner shown. However, if we start with 0.00416 molar KCl and add increasing amounts of NaCl, we get first a *rise* in pH until 0.007 M NaCl is present. Then there is a sharp break and further additions *lower* the pH until 0.035 M NaCl is present. Further additions of NaCl *increase* the pH as shown.

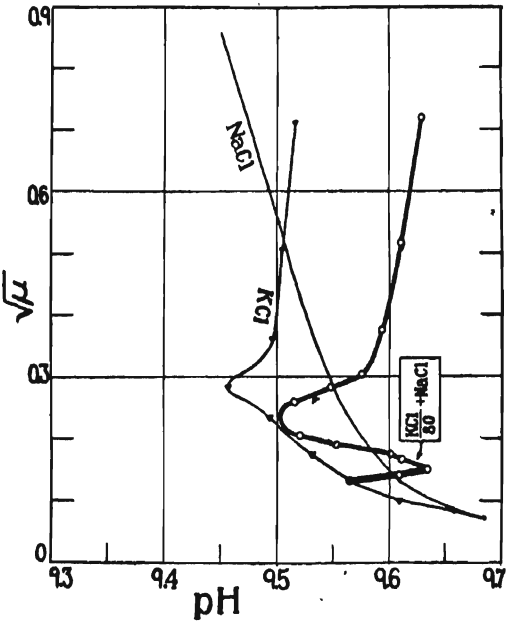


FIG. 1. Antagonistic effects of KCl + NaCl mixtures compared with the effects of KCl or NaCl alone on the pH of glycine solutions (containing 0.5 equivalent of NaOH). Note that both cations are monovalent.

Fig. 2 shows that the same phenomena take place when NaCl is added to glycine solutions containing $MgCl_2$. The breaks come at 0.015 molar and 0.085 molar NaCl concentration *regardless of the amount of $MgCl_2$ present.* This is the same result that was observed with gelatin.

Fig. 3 shows that $CaCl_2$ + NaCl mixtures behave essentially the same as $MgCl_2$ + NaCl mixtures and KCl + NaCl mixtures.

Fig. 4 shows the effect of adding $CaCl_2$ to glycine solution containing $MgCl_2$. The same sort of curve is produced as is found with the other

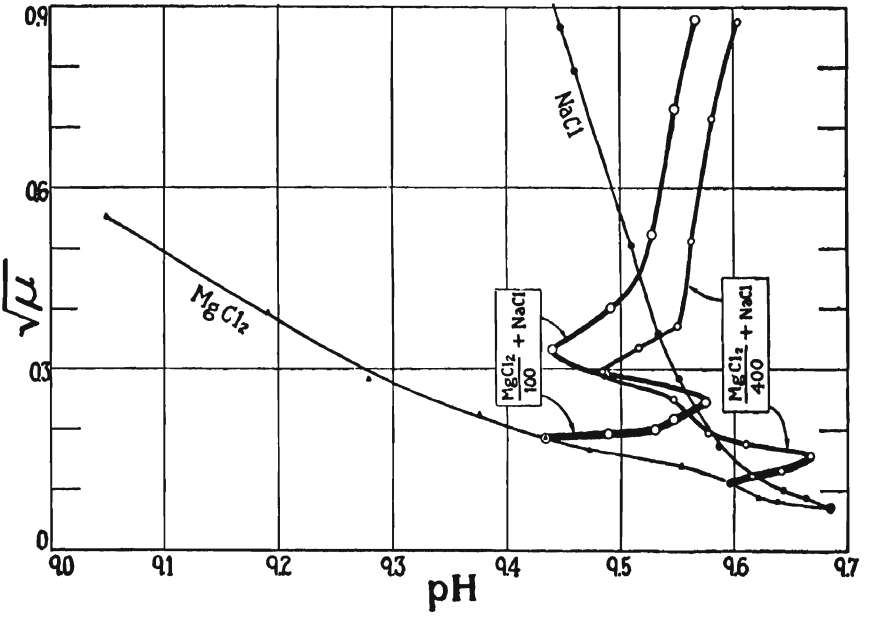


FIG. 2. Effect of $MgCl_2$ + NaCl mixtures on the pH of glycine solutions, compared with the effect of each salt alone. One divalent and one monovalent cation.

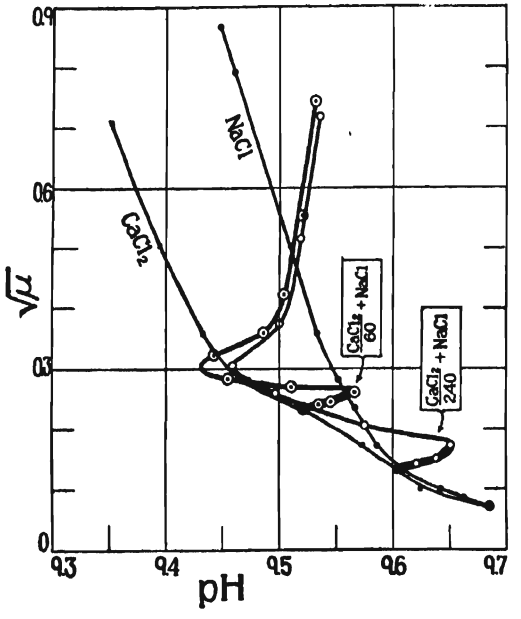


FIG. 3. Effect of $CaCl_2$ + NaCl mixtures on the pH of glycine solutions, compared with the effect of each salt alone. One divalent and one monovalent cation.

salt mixtures. In Fig. 4 it is noteworthy that after the first break the curve for the mixture of $MgCl_2$ and $CaCl_2$ becomes almost identical with that for $MgCl_2$ alone. After the second break it swings to the right and there is a third break near where it crosses the curve for $CaCl_2$ alone. There appears to be a tendency toward the same phenomena in Figs. 1, 2, and 3.

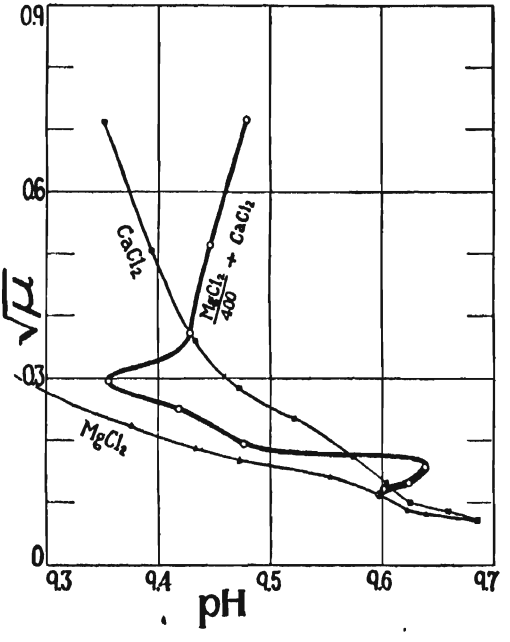


FIG. 4. Effect of $MgCl_2 + CaCl_2$ mixtures on the pH of glycine solutions. Both cations are divalent.

In Fig. 5 the curves for the salt mixtures KCl + NaCl; $MgCl_2$ + NaCl; and $CaCl_2$ + NaCl are plotted in another manner. The ordinates are the square root of the Na^+ ion concentration (including the NaOH added to the mother solution). The abscissas are the difference between the observed pH and that obtained with the same ionic strength with NaCl alone. In other words the curve for NaCl is taken as a standard, the deviations from which are plotted in Fig. 5.

It will be observed that the first break of each curve (except the KCl + NaCl curve) comes at 0.015 M NaCl concentration *regardless of the amount of the other salt.*

The second break for the $MgCl_2$ curves is at 0.085 M NaCl and is a little lower for the $CaCl_2$ curves.

The KCl + NaCl curve has its breaks at about one-half the NaCl concentrations at which they occur with $MgCl_2$ + NaCl, or $CaCl_2$ + NaCl.

The $MgCl_2$ + $CaCl_2$ curve (of Fig. 4) was not plotted in the manner of Fig. 5. However, the first break is at 0.005 M Ca^{++} and the second

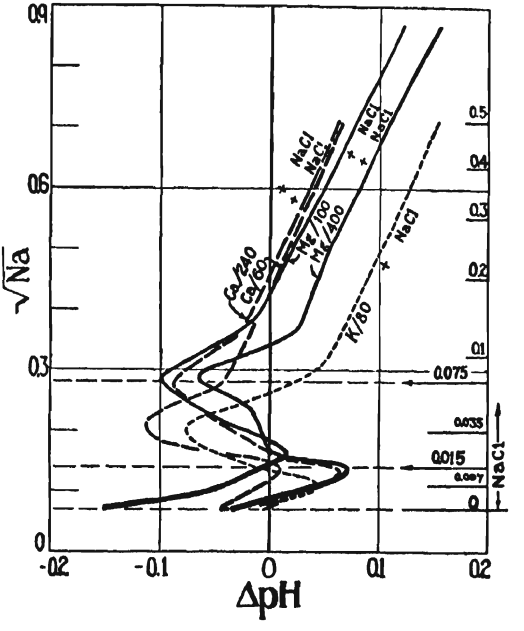


FIG. 5. Effect of NaCl in the presence of another salt, relative to the effect of NaCl alone. The breaks are independent of the amounts of the other salts.

break is at 0.025 M Ca^{++}. It was stated above that the breaks with $MgCl_2$ + $NaCl_2$ were at 0.015 M and 0.085 M NaCl, respectively. These two sets of values do not agree.

If, however, we convert the ion concentrations into "ionic strength" values we have the breaks with $MgCl_2$ + NaCl coming at 0.015 μNaCl and 0.085 μNaCl, while with $MgCl_2$ + $CaCl_2$ they come at 0.015 μCaCl and 0.075 $\mu CaCl_2$. Apparently the position of the breaks is a function of the ionic strength of the added salt rather than of its concentration.

These results with glycine cannot be explained on the basis of colloidal phenomena. They tend to support the mechanism suggested

in the previous paper of the series, namely, that cations in mixtures selectively inactivate the two ionic species[2] to degrees not proportional to the inactivation by one cation alone.

III

EXPERIMENTAL

0.02 molar glycine plus 0.500 equivalent of NaOH was made up in 100 cc. amounts. These were used as mother solutions. For each observation 5.00 cc. of mother solution was placed in a 10 cc. volumetric flask, together with desired amounts of salt solutions, and made up to 10 cc. (*i.e.*, 0.0100 molar with respect to glycine). The salts were added from stock solutions of three different ionic strengths (0.025μ, 0.25μ, and 2.50μ). The pH of each solution was measured at 25°C. in a water-jacketed hydrogen electrode of the bubbling type.

Solutions free from salt had a pH of 9.685.

The data are given in Tables I to VIII.

[2] The two ionic species in the case of glycine in this pH range are, first the *neutral* (or zwitterion) form which predominates at neutral pH values, and second the *anion* form which predominates in alkaline solutions. The lowering of the pH on the addition of a single salt presumably "inactivates" the glycine anion more than the zwitterion. We do not measure the actual inactivation, but rather the ratio of inactivation of the two ionic species. It is reasonable to suppose that this ratio may be disturbed in a mixture of salts.

(Summary on page 578.)

TABLE I

Effect of NaCl on Na Glycinate

($\mu = C_{NaCl} + 0.005$)

C_{NaCl}	pH	C_{NaCl}	pH	C_{NaCl}	pH
0	9.685	0.050	9.567	0.625	9.460
0.0025	9.663	0.075	9.551	0.750	9.448
0.0050	9.643	0.125	9.533	1.000	9.416
0.0250	9.587	0.250	9.510		

TABLE II

Effect of KCl on Na Glycinate

($\mu = C_{KCl} + 0.005$)

C_{KCl}	pH	C_{KCl}	pH	C_{KCl}	pH
0	9.685	0.025	9.526	0.250	9.504
0.0025	9.558	0.050	9.494	0.500	9.516
0.0050	9.609	0.075	9.457		
0.0125	9.565	0.125	9.497		

TABLE III

Effect of $MgCl_2$ on Na Glycinate

($\mu = 3\ C_{MgCl_2} + 0.005$)

C_{MgCl_2}	pH	C_{MgCl_2}	pH	C_{MgCl_2}	pH
0	9.685	0.0050	9.553	0.0250	9.279
0.00050	9.638	0.0075	9.472	0.0500	9.191
0.00100	9.621	0.0100	9.433	0.100	9.049
0.00250	9.596	0.0150	9.376		

TABLE IV

Effect of $CaCl_2$ on Na Glycinate

($\mu = 3\ C_{CaCl_2} + 0.005$)

C_{CaCl_2}	pH	C_{CaCl_2}	pH	C_{CaCl_2}	pH
0	9.685	0.0083	9.574	0.083	9.394
0.00083	9.658	0.0167	9.521	0.167	9.352
0.00167	9.624	0.0250	9.472		
0.00417	9.604	0.0417	9.433		

TABLE V

Effect of NaCl on a Glycine Solution Containing 0.0125 M KCl

(pH without salt = 9.685)

($\mu = C_{NaCl} + 0.0175$)

C_{NaCl}	pH	ΔpH	C_{NaCl}	pH	ΔpH
0	9.565	−0.045	0.050	9.516	−0.042
0.0025	9.609	+0.005	0.075	9.577	+0.031
0.0050	9.634	+0.034	0.125	9.594	0.064
0.0125	9.601	+0.014	0.250	9.611	0.104
0.0250	9.521	−0.056	0.500	9.628	0.155

TABLE VI

Effect of NaCl on Glycine Solutions Containing $MgCl_2$

(pH without salt = 9.685)

C_{NaCl}	pH	ΔpH	C_{NaCl}	pH	ΔpH
A. 0.00250 M $MgCl_2$ plus various amounts of NaCl ($\mu = C_{NaCl} + 0.0125$)					
0	9.596	−0.034	0.075	9.484	−0.064
0.0025	9.616	−0.003	0.100	9.516	+0.021
0.0050	9.641	+0.029	0.125	9.550	+0.020
0.0125	9.667	+0.070	0.250	9.563	+0.056
0.0187	9.610	+0.023	0.500	9.582	+0.110
0.0250	9.577	−0.003	0.750	9.604	+0.157
0.0500	9.546	−0.016			
B. 0.0100 M $MgCl_2$ plus various amounts of NaCl ($\mu = C_{NaCl} + 0.035$)					
0	9.433	−0.151	0.075	9.440	−0.099
0.0025	9.489	−0.093	0.125	9.491	−0.034
0.0050	9.530	−0.048	0.250	9.528	+0.022
0.0125	9.547	−0.025	0.500	9.548	+0.078
0.0250	9.575	+0.012	0.750	9.567	+0.122
0.0500	9.486	−0.062			

TABLE VII

Effect of NaCl on Glycine Solutions Containing $CaCl_2$

(pH without salt = 9.685)

C_{NaCl}	pH	ΔpH	C_{NaCl}	pH	ΔpH
A. 0.00416 M $CaCl_2$ plus various amounts of NaCl ($\mu = C_{NaCl} + 0.0175$)					
0	9.604	−0.006	0.050	9.497	−0.061
0.0025	9.621	+0.017	0.075	9.458	−0.088
0.0050	9.638	+0.038	0.125	9.500	−0.030
0.0125	9.651	+0.064	0.250	9.518	+0.011
0.0250	9.575	−0.002	0.500	9.536	+0.063
B. 0.0167 M $CaCl_2$ plus various amounts of NaCl ($\mu = C_{NaCl} + 0.050$)					
0	9.521	−0.046	0.050	9.443	−0.097
0.0025	9.535	−0.030	0.075	9.486	−0.047
0.0050	9.546	−0.017	0.125	9.504	−0.018
0.0125	9.567	+0.010	0.250	9.520	+0.019
0.0187	9.511	−0.021	0.500	9.532	+0.064
0.0250	9.455	−0.096			

TABLE VIII

Effect of $CaCl_2$ on Na Glycinate Solutions Containing 0.0025 M $MgCl_2$

(pH without salt = 9.685)

($\mu = 3\ C_{CaCl_2} + 0.0125$)

C_{CaCl_2}	pH	C_{CaCl_2}	pH	C_{CaCl_2}	pH
0	9.596	0.0083	9.476	0.083	9.447
0.00083	9.602	0.0167	9.418	0.167	9.479
0.00167	9.624	0.025	9.355		
0.00417	9.639	0.0417	9.428		

SUMMARY

The pH of a 0.01 molar solution of glycine, half neutralized with NaOH, is 9.685. Addition of only one of the salts NaCl, KCl, $MgCl_2$, or $CaCl_2$ will *lower* the pH of the solution (at least up to 1 μ).

If a given amount of KCl is added to a glycine solution, the subsequent addition of increasing amounts of NaCl will first *raise* the pH (up to 0.007 M NaCl). Further addition of NaCl (up to 0.035 M NaCl) will *lower* the pH, and further additions slightly raise the pH.

The same type of curve is obtained by adding NaCl to glycine solution containing $MgCl_2$ or $CaCl_2$ except that the first and second breaks occur at 0.015 M and 0.085 M NaCl, respectively.

Addition of $CaCl_2$ to a glycine solution containing $MgCl_2$ gives the same phenomena with breaks at 0.005 M and 0.025 M CaCl; or at *ionic strengths* of 0.015 $\mu CaCl_2$ and 0.075 $\mu CaCl_2$. This indicates that the effect is a function of the ionic strength of the added salt.

These effects are sharp and unmistakable. They are almost identical with the effects produced by the same salt mixtures on the pH of gelatin solutions. They are very suggestive of physiological antagonisms, and at the same time cannot be attributed to colloidal phenomena.

[Reprinted from MEDICINE, May, 1929, Vol. 8, No. 2, pp. 193–209]

UNDULANT FEVER

ITS RELATION TO NEW PROBLEMS IN BACTERIOLOGY AND PUBLIC HEALTH[1]

BY THEOBALD SMITH

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It is somewhat hazardous to deliver a formal lecture on a subject in which we are just beginning to realize the importance of accurate data and in which a fair number of questions can be asked and problems formulated, but no definite conclusions drawn. As in nearly all somewhat unexpected developments in science, the new problem of undulant fever in populations not using goat's milk seemed at first simple. The cow was regarded as the source of the infection owing to the wide dissemination of the bovine disease. The problem has grown more obscure and complex with the publication of fresh cases and the more thorough study of the organisms obtainable from them. Hasty conclusions in this as in other fields of public health may do no damage as long as the problem remains in the stage of discussion. The results are more serious when hasty legislation results or new rulings under existing law are promptly invoked. Moreover such action may tend to discourage or stifle further necessary research. In order that the lecture maintain a certain unity I shall view the subject solely from the standpoint of one who is endeavoring to find out from existing data whether or not the bovine type of *Bacillus abortus* produces undulant fever in man, primarily, and not as a secondary invader or graft on some preëxisting pathological state. This formulation of the subject is still sufficiently comprehensive to bring to the surface what is most significant.

There are three known, widely diffused animal sources of the *Brucella* group, the cow, the pig, and the goat. In some European

[1] De Lamar lecture delivered October 30, 1928, before the Johns Hopkins University School of Hygiene.

countries sheep are hosts. A few horses have been found infected and other host species may be discovered. I assume that the goat and sheep races of *B. melitensis* are the same and that those found in the horse are accidental and aberrant parasites. Hence a brief description of the three major races may well precede a discussion of the human strains.[2]

The bovine disease and its affiliated microbe, *Bacillus abortus*, may be regarded as highly specialized, established types. Both have rather unique characters. In the cow the infectious agent is restricted primarily to the pregnant uterus, more particularly to the fetal membranes (chorion), and secondarily to the udder ducts. There is no clinical disturbance manifested by the pregnant cow and the infected udder presents no signs of mastitis. The special characteristic of the chorionic disease is the invasion of the epithelium by the bacillus and its multiplication therein.[3] In this situation the organism is entitled to be called a Rickettsia. From the chorion the invasion of the epithelial covering passes to the cotyledons and here there develops a gradual blocking of the vascular villi. The fetus suffers not from any localized disease due to *B. abortus* but from a gradual interference with its circulation in contact with the maternal vessels. Death of the fetus is associated with extensive subcutaneous edema, with serous effusions tinged with blood coloring matter into the large serous cavities—conditions referable to a more or less acute interference with the circulation. *B. abortus* may be absent or restricted to the digestive tract of the fetus, or it may very rarely appear in cultures from the viscera. In the cow's udder the bacteria, escaping into it from the circulation, are probably retained in the residual milk from day to day and multiply in it. The calves which drink this milk or which were born at full term in spite of a late uterine infection with *B. abortus* fail

[2] As might be anticipated in a rapidly developing subject, the nomenclature is in a more or less fluid state. The Malta fever organism first described by Bruce in 1889 and subsequently traced to goats was at first named *Micrococcus melitensis*. The bovine organism first isolated by B. Bang in 1897 was called *Bacillus abortus*. The bringing together of these organisms by Alice Evans in 1918 gave impulse to a new terminology. The genus, including pig, goat, and cow strains, became *Brucella* in honor of Bruce, and later another genus designated *Alcaligenes* has been suggested.

[3] Smith, T., J. Exp. Med., 1919, xxix, 451.

to contract any disease probably because there is no pregnant uterus. Very rarely, however, the mucoid fluid of the ducts of the undeveloped udder may harbor *B. abortus*. Such animals will grow up with the udder permanently infected. They may also thereby become immune to the uterine disease and remain as carriers in the herd.

In 1911, M. Fabyan and the writer[4] called attention to a peculiar disease induced in guinea pigs by pathological material containing *B. abortus* as well as by pure cultures of the same. Before this no small animal had been recognized as susceptible to this agent. The study of *B. abortus* was greatly assisted by this new fact, for passage through guinea pigs made it possible, as in tuberculosis, to obtain pure cultures when other methods failed. Since then various species of monkeys have been used by K. F. Meyer and others in attempts to differentiate the bovine from other races of this species. Inoculation of fetal membranes, contents of fetal stomachs and rectum into guinea pigs, or of pure cultures leads to a disease which is non-fatal and self-limited. The infection may be by way of the subcutis or the peritoneal cavity, the dose may be large or small, the condition of the animal, say at the end of eight weeks, is about the same. There is no local lesion or if some swelling occurs it disappears and the subcutis is normal where the material was deposited. The regional and other subcutaneous nodes may be swollen to twice their size or but little. Necrotic foci are absent unless other bacteria were introduced in pathological material. The only other changes are a large, highly congested spleen with dimensions one and one-half to three times the original, with or without numerous minute gray foci, and an infiltration followed by suppuration of the epididymis of one or both testicles. The body of the testicle is not visibly involved. Rarely swelling of the carpal joints is present. Although *B. abortus* may be obtained from most organs if sufficiently large pieces are cultured, with the exception of the abscessed epididymis, the spleen contains the larger number. The bacilli slowly multiply in the guinea pig up to the fourth week and then decline. At the same time the gross lesions become more pronounced. While the prolonged disease gives rise to more conspicuous lesions, the shorter period yields a larger crop of colonies.

[4] Smith, T., and Fabyan, M., Centr. Bakt., 1st Abt., Orig., 1912, lxi, 549.

The lesions produced are of interest. So far as I have been able to study tissues of inoculated guinea pigs no invasion and multiplication within epithelial cells has been observed. This unique locus seems to be limited to the bovine chorionic membrane. The lesions wherever found consist of a diffuse multiplication of local reticulo-endothelial cells or of an infiltration of mobile cells or both combined. A section of a lymph node showing no abnormality excepting a slightly larger size brings out this diffuse general replacement of the normal lymphocytes by the larger monocytic type of cell. This change goes on in lymph nodes, spleen, and the interstitial tissue of the epididymis. In the liver minute depressions indicate the prompt arrest of a process probably due to emboli from the large spleen. The sometimes enor-

TABLE 1

Cows with Moderately High Agglutinin Titer

UNDER QUARTERS	CULTURES OBTAINED THROUGH GUINEA PIGS (+)	
	No. 696	No. 1486
Right fore	−	−*
Left fore	−	−*
Right hind	+	+
Left hind	+	−*

* Another organism simulating *B. abortus* obtained.

mous size of the latter organ is due to interference with the circulation through the formation of epithelioid foci. I have gone into this phase of the subject somewhat in detail because, in view of the probable failure of other methods, we may be compelled to depend upon the induced disease in guinea pigs, and perhaps in other small animals still to be discovered, for guidance in differentiating the races of this species.

We have no reason to assume that *B. abortus* is shed in any large numbers from the infected udder. As an illustration I quote the results of two recent tests for the presence of *B. abortus*. Eight guinea pigs received into the abdomen either cream or mixed cream and sediment or whole milk in 5 cc. amounts. The result is shown in table 1.

It will be noted that milk from the fore quarters failed to infect

guinea pigs. Milk from both hind quarters of one cow and only the right hind of the other produced the typical lesions in guinea pigs from which *B. abortus* was isolated. In the guinea pigs from one of these cows killed five weeks after the injection, a minute bacillus morphologically like *B. abortus* was isolated from the spleen of three animals. Evidently this organism originated in the udder and was able to maintain itself in the guinea pig for a long period. A hasty microscopic examination of the cultures might lead one to assume the presence of *B. abortus*. The organism actively ferments various sugars and is thereby readily differentiated from *B. abortus*. Following inoculation of the bacillus in pure culture into guinea pigs it was not recovered a second time.

The number of cows, aborting or presenting a high agglutinin titer of the blood serum, which shed *B. abortus* in the milk has been found quite variable. Some writers have reported between 80 and 90 per cent, others down to 34 per cent. An important aspect of the public health problem lies embedded here, for it might be claimed that in view of the general immunity of workers with infected material from fetuses and of veterinarians handling adherent diseased fetal membranes so frequently in their practice, the placental strain is harmless to man whereas the prolonged multiplication in the udder might cause a change favorable to invasion of the human subject. In the udder ducts we have also to deal with association of *B. abortus* with a variety of other bacteria, such as streptococci, staphylococci, *B. pyogenes* and others, and some modification due to such contacts might take place. Changes bearing on cultural and pathogenic characters have not been noted however.

In recent years vaccines have been used quite extensively both here and abroad. These consist of living cultures, either virulent or attenuated under cultivation. Such vaccinal strains may enter the udder and continue to multiply in the ducts and acini for we have isolated them from the milk of vaccinated animals. We may also enquire whether the lowered virulence of the cultures may not favor infection of man.

The porcine variety of *B. abortus* has been under observation since 1914 when J. Traum first isolated it from swine. Since then it has been encountered in various states of the Middle West and the Pacific

Coast. Reports of tests on guinea pigs from various sources have been more or less in agreement. It has been more virulent; that is, the lesions have been more conspicuous, subject to softening and abscess formation, and rather widely distributed in the lymph nodes as well as in spleen, testicles, and limbs.[5] The writer received from the late Dr. E. C. Schroeder of the United States Bureau of Animal Industry a number of swine strains which he himself had studied on guinea pigs and found more virulent than the bovine strain. Although these cultures were somewhat old their behavior in guinea pigs could be distinguished by the larger foci with tendency to softening in lymph nodes and a greater involvement of the liver with such foci. In 1927 the writer was able to isolate a strain during the prevalence of porcine abortion in a herd in New Jersey. The still unpublished data may be briefly summarized. The organism was readily recovered from the viscera of the fetuses, even from the heart's blood. It multiplied readily in unsealed tubes of agar. Its effect on guinea pigs was the same as that produced by bovine strains plus larger foci in liver and lymph nodes. The strain was less virulent than those porcine strains already studied but more so than bovine strains.

Of the three animal races of *B. abortus* I assume that the bovine and the caprine races have been adapted to their respective hosts through long series of passages. The porcine race may be a more recent adaptation from the bovine originating in the Middle West where the opportunity for the mingling of cattle and swine on a large scale is given. This supposition is supported by the fact that it has not thus far been encountered in Denmark or in Germany where animal diseases are very closely watched. The strain isolated by me may be a more recent adaptation from the bovine than the earlier western strains. The gradual adaptation and modification in swine may be associated with an increasing invasiveness towards man and virulence for the guinea pig.

The caprine race of *B. abortus* has been much less thoroughly studied in some of its phases than the bovine disease, although longer under observation. We know nothing definite concerning its behavior in the guinea pig, nor of its relation to disease or abortion in

[5] For a review of the literature, see J. Exp. Med., 1926, xliii, 215.

the goat beyond casual statements. Its frequent presence in the goat's udder was determined early together with many other data by an English Commission working in Malta but facts bearing on differential characters were not gathered, because at that time nothing was known of its relation to the bovine disease and for the time being the bacillus, then called micrococcus, stood out unique in the known microbic world. The occurrence of Malta fever in this country traced to goat's milk has been recorded by various writers during the present century. The foci of the human disease are some of the southwestern states, more particularly Arizona, New Mexico, Texas and Mississippi. Goats carrying the infectious agent were imported from Mexico into the United States for many years. In this published material we miss the data necessary for a comparative study of the caprine and the bovine strains.

The writer has examined one caprine strain isolated in 1921 and received from the Pasteur Institute in Tunis. This strain was entirely innocuous to guinea pigs and not even recoverable from the spleen. This absence of virulence may be ascribed to prolonged artificial cultivation. A second culture was isolated in 1924 by Ten-Broeck in China from a man living on goat's milk. A recent preliminary test on guinea pigs shows this four year old strain to possess pathogenic capacities very like those of the bovine race. *Bona fide* caprine races should, however, be isolated directly from goats if they are to be used in any comparative study.

Among the factors to be used for distinguishing between the races of *B. abortus*, are morphological, cultural or physiological, pathogenic, and serological. Concerning morphological distinctions little can be stated. The organism is very small and slight differences in size not readily detected. It is significant, however, that the caprine race was originally regarded as a micrococcus and is still so denominated by recent writers. Whether a difference in form actually exists here among the races should be more carefully investigated.

The most impressive physiological distinction among the animal races is the relation to CO_2. The use of CO_2 in cultures of various bacteria to stimulate growth began in 1918, but Huddleson[6] appears

[6] Huddleson, I. F., Cornell Veter., 1921, xi, 210.

to have been the first to use it with the bovine race in 1921, interpreting the favoring action as an adjustment of the pH of the medium. The refusal of *B. abortus* to multiply in ordinary cotton-plugged culture tubes was noticed by B. Bang in 1897. He used deep serum-agar cultures. Later Nowack found that a culture of *B. subtilis* placed with the medium inoculated with *B. abortus* in a closed receptable would start and promote multiplication. This was at first regarded as an indication that a reduced oxygen tension was necessary. Hence in 1911, Fabyan and the writer sealed the culture tubes with sealing wax and obtained growth. This method is not quite so universally successful as the use of CO_2. Owing to the wide variation in the amount of this gas which favors growth as well as a fairly wide pH zone of the medium within which *B. abortus* multiplies, the simple explanation of an adjustment of pH by CO_2 is not tenable. Freshly isolated bovine races have been encountered now and then without the CO_2 requirements, but the use of saprophytized strains as vaccines may explain such occurrences. After prolonged artificial cultivation for at least six months, it may be possible to obtain a feeble growth in the usual way. After variable periods of months and years all strains multiply without seal or CO_2. In this stage, sealing actually interferes with the most vigorous growth. Concerning freshly isolated caprine strains no statements are at hand. The inference to be made is that the goat strain is cultured from the start without the devices mentioned. All reports on the isolation of porcine strains from swine agree that CO_2 sealing is unnecessary from the start.

Coming to serological procedures, we may state in general that there is no distinction to be made among these races or among the strains from undulant fever by means of direct and cross agglutination. On the basis of absorption tests Miss Evans has formulated several groups. To be demonstrative the agglutinin absorption procedure must be carried out in many combinations. Miss Orcutt[7] working under my directions was unable, for instance, to note any differences between two human strains and two bovine strains, although, as will be shown farther on, the pathogenic activities of the human strains departed widely from those of the bovine. Further

[7] Smith, T., J. Exp. Med., 1926, xliii, 207; and Orcutt, M. L., ibid., 225.

tests are under way in which certain irregularities have been observed which may explain some of the serological results on record. These pertain chiefly to spontaneous clumping of old strains.

The pathogenic properties of the bovine and porcine strains have been briefly referred to above. It was stated that porcine strains produce more marked lesions in guinea pigs which tend to suppurate. The greater virulence of strains from swine has been observed by earlier workers. Of the caprine races we have no definite information which can be used comparatively in evaluating relative virulence. In general it may be said that cultures should be tested as fresh as possible, perhaps within a year of the date of isolation. The comparison of old strains with those freshly isolated may lead to false issues, since a strain highly virulent at the start may appear like a freshly isolated strain of low virulence after prolonged artificial cultivation.

Coming now to the subject of undulant fever in man we have a considerable background of studies on the human disease as produced by the ingestion of goat's milk. Since 1913 various publications have appeared dealing with the goat disease in man in Arizona, New Mexico, Mississippi, and Texas. So far as I know no thorough investigation of this disease and the associated organism in goats has been made very recently. In view of the potentialities of this group of bacteria this is highly desirable. It was not until 1922 that a case of undulant fever in man outside this endemic area was brought to light in the Johns Hopkins Hospital and described by Keefer.[8] This was the starting point for a number of discoveries of similar character. Probably more than a hundred cases arising within the United States are now on record. A large number of human cases have recently been uncovered by the agglutinin test in Denmark. Scattering reports are coming from Germany. It is not my purpose to go into any analysis of this material. Drawn into it by what appeared too hasty conclusions concerning the relation of the bovine race to the prevailing disease, I have been content to point out gaps and discrepancies in the evidence presented and emphasize the need of further study both of the cases clinically and of the associated organ-

[8] Keefer, C. S., Bull. Johns Hopkins Hosp., 1924, xxxv, 6.

isms. Owing to the numbers now engaged in this field we may expect in due time a more precise allocation of the human cases to the respective animal races of *B. abortus*. The inferences drawn in many of the publications on the sources of undulant fever in man are based on the identity of agglutinins in the strains from animal and human sources. Although this identification of agglutinins with the strains giving rise to them has been a basic assumption in serology, it is subject to limitations like all cultural and pathogenic likenesses and must be applied with discretion. There is no doubt that the agglutinins appearing as a result of infection with bovine, porcine, and human strains fail as a rule to discriminate among the latter. The direct and cross agglutinations agree closely. The absorption process has also been found to show no differences in the few strains to which we have applied it. However, certain groupings have been created by Miss Evans on the basis of absorption differences which allocate certain human cases to the bovine type.

About 23 strains of human origin have passed through my hands. They have come from various observers who, knowing my interest in the animal strains, kindly sent them to me for comparative study. Of these, 18 were isolated from patients in the United States. The remainder came from other countries. Some of the American strains have undoubtedly been studied by others. It would be going beyond the limits of a single lecture to speak in detail of these strains. I shall give only a brief review of our own studies.

There were no CO_2 inhibitions in any of the American cultures when they were received. Some cultures were perhaps too old to manifest this peculiarity. Some were not. In view of the fact that the swine types have not shown this growth requirement from the start this point is significant.

The pathogenic activities as developed in guinea pigs showed much variation. None of the strains were old enough to have lost such activities in any large degree. A rough grouping only can be attempted as follows:

a. No effect whatever on guinea pigs and no recovery of the injected bacilli from the entire spleen. Three strains.

b. The usual more or less variable enlargement (congestion) of the spleen, slight enlargement of lymph nodes, atrophy of testicles. Here we must remain somewhat in doubt whether attenuation had taken place. One strain.

c. The usual lesions accompanied by small necrotic foci in spleen and lymph nodes. Four strains.

d. The usual lesions accompanied by relatively large or numerous small necrotic-suppurative foci. Ten strains.

The entirely negative outcome of inoculation into guinea pigs with infected tissues or cultures even in high dilutions has not occurred in hundreds of bovine cases in my experience. Even when the lesions, such as spleen swelling, are slight, the organism is recoverable from the spleen two to three months after inoculation. Strains three to four years under cultivation still respond to these tests. The nature, therefore, of the three strains with entirely negative pathogenic characters is left in obscurity. The strain under (*b*) may or may not be a bovine strain. The groups (*c*) and (*d*) I should regard as either not bovine or as bovine modified by passage through swine or perhaps other species. They differ markedly among themselves. Two of these were studied rather carefully in close association with two bovine strains and the results have been published.[7] The lesions were strikingly different from those due to bovine strains. The lymph nodes were relatively very large and completely softened. Necrotic-suppurative foci occurred in the spleen. In fact, the gross appearances of the guinea pig lesions were such as to give me the impression that I had a wholly different disease entity to deal with. Both patients had been in contact with swine material.

One group of five cultures coming from the same locality and from cases occurring at short intervals are of special interest in so far as the guinea pig lesions all presented certain peculiarities. Besides the focal lesions in spleen, lymph nodes, and testicles, the thymus lobes were either permeated with small abscesses or else these had fused and produced a single large abscess about 1 cm. in diameter. Although presumably the result of drinking raw milk these cases are explainable as not truly bovine.

Another strain from a midwestern state presented lesions, which, encountered a generation ago, I should have regarded as due to the glanders bacillus. In fact such diagnoses may have been actually made in the past owing to the negative cultural characters of both species of bacteria. The guinea pigs became emaciated and one died in two months. The other at this time was much emaciated and

was killed. In both the testicles were converted into large abscesses. The fore and hind feet were swollen and covered with small ulcers. Of the many hundreds of guinea pigs which we have inoculated with bovine material or cultures none have died.

Five human strains from European sources came into my hands, one from the Institut Pasteur at Tunis; one from Dr. Duncan of the London School of Tropical Medicine, which had been isolated from a patient infected in Rhodesia; and three from Dr. Kristiansen of Denmark. The two first mentioned strains were about two years old. In both the lesions approximated closely to the bovine standard.

TABLE 2

Strains from Undulant Fever (Denmark)

CULTURE METHOD	DAYS OF INCUBATION AT 37°C.		
	No. 18	No. 19	No. 20
a. Cotton plug + some paraffin	Growth in 3 days	No growth in 12 days*	No growth in 12 days*
b. Sealed with sealing wax	Growth in 4 days	No growth in 12 days*	Growth in 12 days
c. In 10 per cent CO_2	Growth in 3 days	Growth in 3 days	Growth in 3 days

* Developed in CO_2 atmosphere after 12 days.

The three strains from Denmark are of special interest. Although probably one and a half years old, two of these still showed a marked demand for CO_2 while the third grew equally well in the ordinary cotton-plugged tube, as is indicated in table 2.

After twelve days' incubation, the three still sterile tubes were placed in an atmosphere of 10 per cent CO_2. The originally sealed tube developed a film of growth in three days; the others after six or seven days in CO_2. Probably some bacteria had survived in the condensation water of these latter partly dry tubes. The table furthermore makes it clear that while CO_2 universally favors growth the method does not make as sharp distinctions as the sealing. In fact, no distinction could have been made between the partially saprophytized (No. 18) and the other strains if CO_2 had been exclusively

used. In general a distinction between the lesions produced by two of the Danish cultures subject to CO_2 requirements could not be differentiated from those of the bovine type. The third strain, however, produced no lesions in guinea pigs and could not be recovered from the spleen. It may have been a highly saprophytized, perhaps vaccinal, strain of *B. abortus*.

These illustrations must suffice to point out the want of uniformity in the pathogenic capacities of the strains derived from human patients and emphasize the need for more work in locating the sources. The inferences to be drawn from the labors of bacteriologists up to the present may be briefly presented.

1. Bovine strains or strains not distinguishable from them have been cultured from human patients in a small per cent of the cases studied.

2. The caprine strains isolated directly from goat's milk need more detailed study before we can use this type in comparative studies or allocate them to human cases of undulant fever in man in territories ostensibly free from milk goats or the fresh products of goat's milk.

3. The cultures isolated from man and presumably ingested in cow's milk but not fitting the bovine type, may have been swine strains introduced into that receptive organ, the cow's udder, just as hemolytic and scarlatinal streptococci may gain a foothold in it under certain unknown conditions and cause localized epidemics in man. This view is supported by the rather remarkable drift of the infecting organism of five human strains from one locality into the thymus of inoculated guinea pigs. The characters of strains from such local groups of cases should be more thoroughly studied since it may be possible to locate the infecting cow and isolate the organism directly from the milk.

4. The partly saprophytized cultures which have been used on a large scale in vaccinating cows against infectious abortion may be another possible source of bovine strains. It is conceivable that artificial cultivation in a certain stage may prepare an organism otherwise incapable of multiplication in a new host. It is also possible that some porcine strain may have been distributed as a vaccine in cows. The third saprophytic, non-virulent Danish strain described may be a vaccinal strain. Some slight change may have in some way

favored the infection of human beings in laboratories with glanders during the wide prevalence of this disease in horses towards the close of the nineteenth century, for these cases seemed to be out of proportion to the number of people who became infected while tending glandered horses.

The two sites of *B. abortus* in the cow are the fetal membranes and udder ducts and acini. The question will naturally be asked if the sojourn of *B. abortus* in the udder modifies the uterine type and perhaps makes it more invasive for man. This problem already referred to has been kept in mind during our work with this race. No modification of virulence or CO_2 requirements has been noticed except in a few instances readily referable to a vaccinal strain used at certain periods. Further careful studies are desirable, however, but only in herds not subject to vaccination with living cultures.

5. The swine type of *B. abortus* may have been developed in the mid-western states in recent years, for here the enormous development of the swine industry, the feeding of by-products of the dairy, as well as association between the two species in feed lots, have given Nature numerous opportunities to form new varieties. The relationship is further emphasized by the serological classification of bovine and porcine races together by Miss Evans. The varying virulence of the porcine races may be due to varieties not yet fully adjusted to the new host. Another reason for proposing this genetic source of the porcine type is the reported absence of this type from European countries, where the live-stock conditions mentioned are realized only to a very slight degree. It may be stated in passing that the great increase in swine tuberculosis in the Middle West in the recent past was definitely traced to the intimate association of cattle and swine.

6. The further internal differentiation of the non-bovine types found in man suggests other possible forms of aberrant parasitism of a more or less permanent character developing locally in rats, mice, wild rabbits, ground squirrels, and the like, some of which species may be more receptive to the porcine than to the original bovine races.

7. The gradual development of the milch-goat industry in northern states needs supervision as a possible source of human disease.

In the further unraveling of the sources and significance of this

new disease, declared by Etienne Burnet to be the disease of the future, the clinician can be of great service in obtaining as accurately and fully as possible a history of the patient's environment and habits with reference to foods and contact with domestic animals. From the clinical standpoint it is furthermore desirable to obtain cultures of the hypothetical organism, which has been done in only a small per cent of cases, and to standardize serological methods. It is also highly important to determine if possible how far undulant fever is a primary disease and how far merely grafted on other favoring pathological states. A detailed search of hospital records during the past thirty years may perhaps assist in explaining why undulant fever has not been recognized until recently.

I have purposely refrained thus far from bringing in so-called epidemiological data. It should however be mentioned that the bovine disease has been widespread in the United States and that until recently very few if any dairy herds were free from it. I have evidence of its existence from laboratory tests on guinea pigs as far back as 1893. The ingestion of the bovine type by man must have been nearly universal since this early date. How can we explain the scarcity of the human affection even today when attention has been thoroughly focused on it and when it is being diagnosed and perhaps overdiagnosed? How can we explain the freedom from disease of laboratory workers and tenders of dairy cattle, of veterinarians handling diseased tissues and cultures without more than the usual precaution of cleanliness? How can we explain the freedom from disease of groups of adults and children drinking raw milk from herds in which nearly every first pregnancy terminated in abortion?[9] The explanation nearest at hand is that the bovine type is so slightly invasive for man that it fails to produce appreciable disturbances but that as a by-effect it may immunize towards the more virulent types of swine and caprine origin. The evidence brought together points first to the stability of the bovine race of *B. abortus* as isolated directly from bovines, and second to a number of divergent pathogenic characters isolated from undulant fever patients, only a few of which have distinctive bovine attributes.

[9] In 1916 Cooledge reported an experiment on 7 human beings each of whom voluntarily drank daily 1½ pints of milk from infected cows over a period of 8 weeks. No febrile states were observed. (J. Med. Research, 1916, xxxiv, 459.)

Knowing so little of the life of microörganisms outside the narrow confines of the laboratory we are at a loss to explain the situation except by hypotheses which might serve as a formulation of problems to be attacked rather than as a final explanation of the precise sources of undulant fever. The recent history of this disease stresses again the significance of animal life in the development and maintenance of infectious agents capable of starting disease in man. It is of interest to note that nearly all human diseases traceable to animals are septic in type, with a definite invasion of the blood, and some of them highly fatal.[10] May it not be possible that the highly virulent pandemics of the human race, coming as they do from regions where man lives in close association with animals and hunts them for food, are due to organisms reinvigorated by passages through animal species. We know so little of the flora and protozoan fauna of domestic and free-living species that a study in this field may be revolutionary in its results.

In the bovine disease we have touched upon today there is some evidence that, say within a generation, a decline in virulence has taken place. The first case in guinea pigs seen by me in 1893 and some cases in 1911 presented lesions which indicated a much greater invasiveness than any seen in recent years. In these earlier cases the lesions extended to the kidneys and the bones. The kidneys were in an advanced stage of interstitial sclerosis and appeared like two large white tumors. The bones of the limbs and the ribs were the seat of destructive changes. It should be remembered that bovine *B. abortus* may pass from animal to animal as often as the fetal membranes are formed—about once a year. In other words, the passages are more frequent than the life span of the cow. There has been, without doubt, an increasing resistance developed in bovines going parallel with a certain decline in virulence of *B. abortus*. Whether any rise in invasiveness towards man has been associated with this decline may be worth consideration. A general decline in virulence of organisms parasitizing the same host species may be considered as deducible from available evidence. To reactivate this virulence some other host may be needed, or more likely Nature from time to time

[10] Smith, T., Bull. N. Y. Acad. Med., 1928, iv, Ser. 2, 476.

may tap the original source of virulent material in certain animal hosts. In the numerous attempts for increasing virulence or modifying essential functions of pathogenic bacteria by passages through animals in the laboratory a few rather artificial methods are used, whereas in nature a great many different combinations may occur, some one of which may open the path to new physiological races. In this way there may have been formed a number of races of the *Brucella* group for some of which the immediate animal sources are still to be discovered.

As to active measures against undulant fever, if it should be made fairly clear that the bovine race of *B. abortus* is the real source of the other races then the time would be ripe for an active campaign against it. The same should be true for goats if the parent race was harbored by this host species.

[Reprinted from SCIENCE, May 31, 1929, Vol. 69, No. 1796, pp. 577-579]

TWO COMMON FLY SPECIES EASILY REARED IN THE LABORATORY

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Interest attaches to forms easily reared throughout their life cycles in the laboratory, because of their possible value both in the classroom and in research work. During a study of certain insects found about sheep manure, the ease was noted with which two species of *Leptocera* (*Limosina*) were carried through from generation to generation in milk bottles or shell vials, when sheep dung was used as food. The two species studied, *Leptocera longicosta* and *Leptocera ordinaria*,[1] belong to the family Borboridae, formerly in the old family Muscidae, and are not distantly related to the Drosophilidae. In size also they approximate the smaller fruit flies, *L. longicosta* being 2.0–2.5 mm. in length, and *L. ordinaria* 1.5–1.8 mm. At Princeton summer temperatures the former completes a life cycle in eleven to fourteen days, the modal period being twelve days, while the latter is shorter at nine to ten days. They are handled in transferring after the manner familiar with fruit flies, being positively phototropic and withstanding etherization well. It is probable from our observations on nearly a dozen generations that they may be maintained indefinitely by successive transfers. While only two species are here discussed, additional species of the same family were encountered in our catches out-of-doors, *viz.*, *Leptocera frontenalis*, *Borborus equinus* and *Sphaerocerus subsultans*, and they are probably susceptible to similar handling.

These small flies of the genus *Leptocera* are numerous about dung, especially sheep dung, during apparently the whole of the summer

[1] We are indebted to C. W. Johnson, of the Boston Society of Natural History, for species classifications, and to C. Jaynes, D. V. M., for certain preliminary observations on the breeding of one of these species.

season. They are easily captured in the field with a sweeping net, or at the windows of barns, where they gather in large numbers at the top of the window-panes, and may be collected by taking advantage of their positive phototropism. *Leptocera* spp. are distinguished from the other members of the family Borboridae[2] by the fact that the

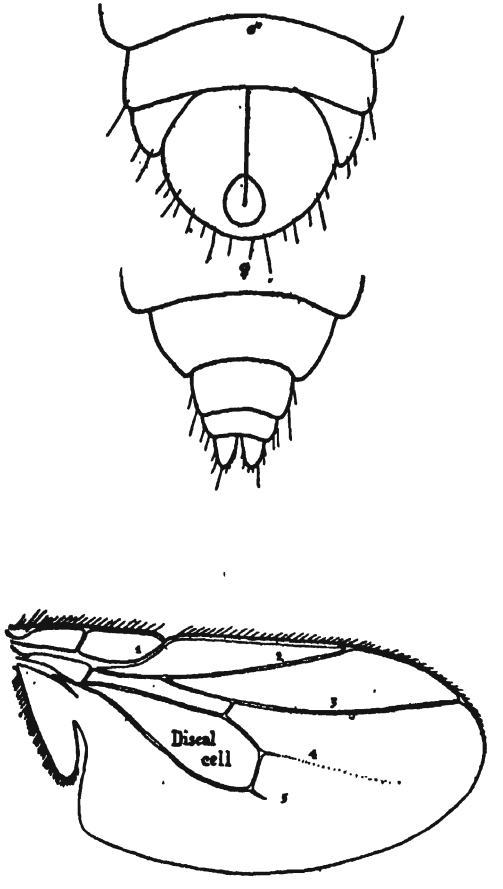


FIG. 1. Above: Posterior abdominal segments of male and female *Leptocera* spp. Below: Wing venation in *Leptocera* spp.

fourth and fifth wing veins are incomplete or obsolescent beyond the discal cell, as shown in the accompanying text figure. *L. longicosta* may be readily separated from *L. ordinaria* by the shape and vestiture of the scutellum. The scutellum of *L. longicosta*, which is truncated at the rear giving it a trapezoidal outline, bears four long scutellar

[2] Williston, "North American Diptera," 1908 (3rd ed.), p. 316.

bristles and many dorso-scutellar bristles. That of *L. ordinaria* is shorter and rounded at the rear, and while possessing the four rather long scutellar bristles, lacks entirely any dorso-scutellar bristles. Additional characters include the slightly smaller size of *L. ordinaria*, as already mentioned, and the fact that individuals of this species retain a lighter coloration on the lower part of the head and thorax up to several days after emergence, whereas in *L. longicosta* a nearly uniform dark coloration may appear within an hour. Eye color in the former species is red; in the latter nearly black. Some variation has been noted in these characteristics, however.

Sexes are easily determined with the aid of a hand lens or dissecting microscope in accordance with the characters illustrated in the text figure. Occasionally the anal plates of the female are not visible. Slight pressure on the abdomen of an etherized individual with a camel's-hair brush will force the anal plates beyond the edge of the last segment, if the individual be a female.

In breeding the flies we have used sheep dung, although it appears probable that other food materials ("decomposing organic matter"[2]) may be used. Our method was to collect sheep pellets, preferably fresh samples, which were first crushed in water and then boiled. This resulted in sterilizing the dung to a large extent as well as permitting it to be brought to a certain desirable consistency. After cooling, pint milk bottles were about one fourth filled with the cooked dung and plugged with cotton, after which newly emerged flies were transferred to them. Flies for breeding were allowed to remain in the bottles seven or eight days, by which time the females have laid most of their eggs.

In an experiment, one male and one female *L. longicosta*, newly emerged, were placed in each of twenty-four shell vials (length 7.5 cm., diameter 2.0 cm.), upon the boiled dung food. Four females produced no progeny, but in the other twenty vials twelve days after the parents had been transferred to them the new generation began to emerge and was removed daily. The parent flies were placed in fresh vials after eight days in order to keep them separate from the the offspring. Parent females lived up to twenty-three days, males up to twenty-four days. The number of progeny from the twenty fertile females ranged from seven to 393, average 146.5 $\pm$ 14.7, with a

ratio of 116 males to 100 females, the total count being 1,573 males to 1,356 females. Apparently virgin females produce no progeny, following the usual rule among the Muscidae.

It seems to us possible that *Leptocera* spp. as representatives of a fly family, the Borboridae, which are widespread if not cosmopolitan in nature upon the dung of mammalia,[3] with their small convenient size, short life cycle, easily satisfied food conditions, capability of continuing their life histories in the now familiar laboratory milk bottle, and apparent hardihood in withstanding repeated etherization, combine a group of characteristics which might well make the mutilizable material for investigations in insect physiology, genetics, etc. It may be mentioned in addition that members of the Borboridae, both larvae and adults, are reported as hosts of herpetomonads.[4]

[3] Howard, "A Contribution to the Study of the Insect Fauna of Human Excrement," *Proc. Wash. Acad. Sciences*, 1900, **2**: 541–604.

[4] Patton and Cragg, "A Text-book of Medical Entomology," 1913, p. 311.

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ZOSTER. *See* Herpes.