PREGNANCY DIAGNOSIS TESTS:
A REVIEW

by

ALFRED T. COWIE, B.Sc., M.R.C.V.S., Ph.D.
National Institute for Research in Dairying, University of Reading
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ALFRED T. COWIE.

National Institute for Research in Dairying,
Shinfield,
Reading.

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INTRODUCTION.

The importance of the early diagnosis of pregnancy in stock-breeding programmes and the success of the hormonal methods of pregnancy diagnosis in human medicine have stimulated, during the last twenty years, much research on the development of tests for pregnancy in farm animals.

In this field of investigation no comprehensive review of the literature has appeared, and the investigator must needs spend much time in searching the literature before an adequate picture of the work on this problem can be obtained. The object of this review is to provide the scientific worker with a brief summary of the more important researches leading to the development of pregnancy tests in the domestic animals and to provide an adequate bibliography of the widely scattered literature.

Although this review is primarily for those interested in the veterinary aspect of pregnancy diagnosis, the various tests with their numerous modifications have, in most cases, been developed and used initially for the diagnosis of pregnancy in women, and it has thus been necessary to summarize the development of each test in human medicine so that a more complete view of the test may be obtained.

A study of the clinical methods of pregnancy diagnosis in the domestic animals reveals that reliable tests for pregnancy would be of great supplementary value. Where such tests have already been developed, diverse opinions have been expressed on their relationship to the clinical methods. Some clinicians hold that the tests are unnecessary and a sign of incompetence in the clinician using them; the opposing view that in certain circumstances these tests have rendered the clinical method obsolete has been put forward by some research workers. Further elaboration of these arguments may be found in the polemical papers of Peters (1934), Zivotkov (1936, 1937), Zavadovskii (1936), and Oldenborger (1936). It is believed, however, that the two extreme views are equally wrong and that best results are obtained when the methods of the clinician and the laboratory are judiciously combined. The combined use of clinical and laboratory methods of pregnancy diagnosis, at present only possible in the case of the mare, has been little exploited as a research technique, but preliminary investigations on sterility in mares by Zavadovskii & Nesmejanova-Zavadovskaja (1945a,b) indicate that such methods may in the future prove of some value.

ARRANGEMENT OF THE REVIEW.

The tests for pregnancy have been divided into seven classes. It is admitted that in certain cases the class in which a test has
been placed may be open to criticism, but it is felt that further subdivision would be merely confusing. The following are the seven classes into which the tests have been placed:—

1. Tests based on clinical methods.
2. Tests based on hormonal investigations of body fluids.
3. Tests based on enzymic investigations of body fluids.
4. Tests based on other biochemical investigations of body fluids.
5. Tests based on physiological phenomena.
6. Tests based on immunological phenomena.
7. Tests based on physical investigations of body fluids.

The details of the tests in each class will be found in the table of contents (pages 4-6).

For the benefit of the practitioner, the stock owner and others who may be mainly interested in ascertaining the general applicability of the present tests for the diagnosis of pregnancy in domestic animals, a brief summary has been included which may relieve them of the tiresome necessity of reading through the relevant parts of the main review.

THE HISTORY OF PREGNANCY DIAGNOSIS TESTS.

This review is mainly concerned with the scientific researches during the last 25 years, but it may be noted that tests for pregnancy are not recent innovations.

In human medicine the problem of early diagnosis of pregnancy is one which has beset the physician throughout the ages—a fact delightfully illustrated by the drawings which accompany the review by Henriksen (1941). Readers interested in the history of pregnancy tests are advised to consult the scholarly review by Bayon (1939) in which are described pregnancy tests of ancient Egyptian medicine 3,000 to 4,000 years ago, of Hellenic medicine, and of medicine during the Middle Ages and the following centuries. There is evidence to suggest that certain tests passed from Egyptian medicine to Hellenic and thence to medieval medicine and, although undergoing many modifications, are essentially some thousands of years old.

In the 16th and 17th centuries, inspection of the urine (uroscopy) was a favourite method of pregnancy diagnosis, and has been featured in several paintings by 17th century painters (Frankel, 1934).

In the present century many primitive societies still lack an accurate scientific knowledge of the reproductive process, and it would be of interest to know how our primitive contemporaries deal with the problem of early pregnancy diagnosis and whether there is any similarity between their methods and tests and those employed in early Egyptian and European societies. Unfor-
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Fortunately, detailed information is apparently not available, for Ford (1945) has examined the literature on 64 societies representing Africa, Eurasia, Oceania and the Americas for all data relevant to the reproductive cycle, and has concluded that "little is known of the method by which primitive people determine the state of pregnancy. To judge from the meager information available, absence of one or more menstrual periods indicates that a woman is pregnant in primitive societies." He further states that a few societies consider other clinical signs, such as changes in the breasts, of importance in diagnosis, but no mention is made of any other type of test.

The veterinary aspect of this subject, unfortunately, does not appear to have been studied by the historian. Bayon, however, states that the ancient Egyptians were interested in the diagnosis of pregnancy in the domestic animals as well as in the human.

(A thesis by Harmisch (1924) may contain something of the history of pregnancy tests in animals, but it has not been possible to obtain a copy of this work.)

DEFINITIONS AND TERMINOLOGY.

Oestrogen: A collective term for all substances producing oestrous growth in the vagina, uterus and mammary glands, and female secondary sexual characters.

This term has been used to replace such terms as "follicular hormone," "folliculin," "oestrin," etc., which may have appeared in the original papers and where the exact chemical nature of the hormone is not known.

Progestogen: A collective term for substances responsible for progestational changes in the uterus, the most important of which is progesterone.

Gonadotrophin: A general term to describe hormones which govern the development and functional activity of the gonads.

This term has been used to replace terms such as "Prolan," "Anterior-Pituitary-Like Hormone," "Gonadokinetic Hormone," etc.

The spelling "gonadotrophin" is here used in preference to "gonadotropin" (see Parke, 1938; Corner, 1943; Sevringhaus, 1944).

Since their exact chemical structure is not yet known, the gonadotrophins are generally classified according to their physiological activity into two main classes: (1) Follicle-Stimulating Hormone (FSH), which in earlier literature is generally referred to as Prolan A and occasionally as the "oestrogenic hormone," a practice which is very confusing in view of the now accepted use of the word "oestrogenic" as the...
adjective derived from "oestrogen." (2) Luteinizing Hormone (LH), often termed Prolan B. The Luteinizing Hormone is probably identical with the so-called Interstitial-Cell Stimulating Hormone (ICSH) (see review by Pfeiffer, 1943).

**Phases of the Oestrous Cycle.**

In conformity with the recommendations of Asdell (1931, 1932), Tyson (1931), and Corner (1937), the word "oestrus" has been used in preference to the less correct word "oestrum," and the adjective "oestrous" in preference to "oestral" or "oestral." The now general practice of spelling the compound words "metoestrus," "dioestrus," "anoestrus," etc., with the ending "-us" has been adopted since it makes for uniformity with the basic word "oestrus," although on grounds of priority of usage the words "metoestrum," "dioestrum," "anoestrum," etc., would be more correct. The contention by Asdell (1931) that the ending "-um" is more correct, being the accusative case after the preposition, cannot be entirely accepted since one of the prefixes ("an-") is not a preposition but a negative particle and is not followed by the accusative case in the original language.
CHAPTER 1. CLINICAL METHODS OF PREGNANCY DIAGNOSIS

COW.

The early diagnosis of pregnancy is of fundamental importance in the control of infertility in the dairy cow and it is unnecessary to enlarge on this aspect of the subject (see also Veterinary Record, 1942).

It is not the purpose of this review to give working details of the technique of the clinical examination for pregnancy, but to indicate where the details of the various techniques may be found in the literature. For purposes of review it will be necessary to consider the problem of clinical diagnosis under various headings, but it must be kept in mind that the final diagnosis is based on a consideration of the total findings.

The stage at which pregnancy can be diagnosed accurately by clinical examination depends within limits on the experience of the veterinarian conducting the examination. An accurate diagnosis is generally possible from the end of the second and beginning of the third months of pregnancy. In heifers, it may be possible to make the diagnosis a week or two earlier than in cows. In this connection, the great mass of data on the accuracy of the various laboratory tests for pregnancy is in striking contrast to the almost complete lack of any data on the accuracy of the clinical methods. While the accuracy of clinical diagnosis is dependent on many subjective factors, and any percentage accuracy figures would be subject to a considerable individual variation, such data if available would be of great value in indicating where further research was necessary in improving the available methods of diagnosis and assessing more accurately the value of the available laboratory tests. Until such data are available, general statements on the accuracy of the clinical method must be accepted with some reserve.

RECTAL EXAMINATION.

Rectal examination for pregnancy involves the palpation through the wall of the rectum of the genitalia and the uterine arteries.

This clinical method of pregnancy diagnosis in the larger farm animals is a relatively new technique in animal husbandry and has been widely employed only in the last 50 years. In this country it received little attention until the beginning of the last decade.

A historical study of the development of this clinical method
would undoubtedly prove interesting, but is outside the scope of this review. It appears, however, that the method was employed by a few veterinarians during the last century. Calkovskii & Bondarenko (1933) mention that in 1815 a veterinarian by the name of lorg (not found) claimed that rectal examination was a useful method of early pregnancy diagnosis; unfortunately no indication is given as to where the original reference is to be found. Gerlach (1862), in his book on forensic veterinary medicine, discusses the diagnosis of pregnancy in the cow, but makes no reference to rectal examination although vaginal examination is mentioned. Fleming (1896), however, describes rectal examination for the diagnosis of pregnancy and states that it is a safe method and the risks have been greatly exaggerated. It appears that Broholm (1896) also found the method of value.

In reviewing the development of the method during this century, it is convenient to discuss separately the examination of the uterus and its contents by palpation, the palpation of the ovary and the palpation of the middle uterine artery, although in arriving at a diagnosis the complete findings are taken into consideration.

**Palpation of the Uterus.**—Numerous workers have from time to time stressed the innocuous nature of rectal palpation of the uterus as a routine measure in the diagnosis of pregnancy (Dennhardt, 1905; Zieger, 1908; Magnusson, 1921; Williams, 1921; Bahtov & Rumjancev, 1935; Richter, 1937). In this country, prejudice against the method was largely dispelled by the publicity it received in the veterinary press in the campaign against bovine infertility and sterility carried out during the war (1939-45). Additional proof of the safety of the method has been provided by Burgess (1942), who states that the cows in a dairy herd were examined regularly by numerous students, who were receiving instruction in the method, with no ill-effects.

Certain techniques of palpating the embryo have been described, but the safety of these has been seriously questioned, as will be noted later.

Apart from the belief that rectal examination was liable to produce abortion, there is good reason to believe that the method was unpopular because it was considered a "messy" objectionable procedure and, as certain of the opponents of the methods have observed, "unaesthetic." Such an attitude is inexcusable and failure to carry out rectal examination for such reasons is merely incompetence.

The routine procedures carried out in the palpation of the uterus for pregnancy will not be discussed here since there are many excellent descriptions; references to these will be given later. The points of particular importance are the enlargement of the
pregnant horn of the uterus and the accompanying changes in
the thickness of the uterine wall.

The palpation of the foetus and foetal membranes becomes
possible as pregnancy advances. There have been special tech­
niques described for the palpation of these at very early stages
and it is necessary to refer briefly to these methods.

Pissl (1923) claimed that it was sometimes possible to palpate
the embryo at the end of the first month of pregnancy. The horn
of the uterus, on the side corresponding to the corpus luteum,
is grasped between the thumb and finger and then by drawing
the thumb and finger backwards it is possible to detect small
quantities of fluid in the horn, and the foetus, about the size of
a lentil, may be felt as it is forced against the fingers.

Rütter (1923) observed that generally towards the end of the
4th week of pregnancy it is possible to detect the foetal membranes
and their folds by rubbing the walls of the uterine horn between
thumb and finger.

Eilmann (1924a) described the so-called "Aufrollen" method
in which the uterus is "folded up" so that the fluid wave and
membranes can be readily palpated by the 40th day of pregnancy.

Abelein (1928) described the "Ausschneigriffes" method with
which diagnosis was possible from the 5th week, although negative
results had to be regarded as inconclusive until the end of the
6th week. The uterine horn is turned backwards and sideways
and palpated between the thumb and index finger, when the foetal
fluids can be detected; the horn is then allowed to glide gently
between the thumb and finger when the foetal membranes can
be detected. Euler (1930) and Götte (1940b) have confirmed the
value of Abelein's method for diagnosing pregnancy from the
33th day.

Schmidt (1932), using methods similar to those of Pissl and
Rütter, claimed that in favourable cases it was possible to detect
the embryo in its sac from the 31st day, when the embryo is the
size of a lentil. After the 7th week, although the foetus is 1½ to
3 cm., owing to the increase in foetal fluids it is more difficult
to palpate and from the 7th-10th week diagnosis must depend
on the palpation of the foetal membranes.

Stoss (1928), Benesch (1932a), and Richter (1937) consider that
these methods, especially those of Pissl and Rütter, are unsafe
and liable to produce abortion. The "Aufrollen" and
"Ausschneigriffes" methods are considered less objectionable.
Schmidt (1932), however, considers that the methods are safe
provided that no attempt is made to palpate the foetus between
the 7th to the 10th week; she had carried out over 10,000
examinations using the methods described with no ill-effects.
Casidu, Barnett, & Lloyd (1946) have recently reported on diagnosing pregnancy from the 34th day after service by palpating the amniotic vesicle.

For the exact details of the above-mentioned techniques of palpation the original papers should be consulted. It must be remembered that although on paper the methods may appear simple, in practice they must be extremely difficult, and in view of the possible risks of injury to the foetus, such techniques are best left to the specialist.

**Palpation of the Ovary.**—Zieger (1922) and Zieger & Zschiesche (1922) stressed the importance of palpating the ovaries to obtain information on the corpus luteum which was said to be of value in the early diagnosis of pregnancy. While it is generally agreed that the absence of corpora lutea from both ovaries is strong evidence in favour of non-pregnancy, it is not generally considered possible to distinguish between a corpus luteum of pregnancy and one of non-pregnancy. Elder (1925) examined the ovaries of 62 pregnant cows and 89 non-pregnant cows at slaughtering and concluded that it was not possible to distinguish between the shape of the corpus luteum of a pregnant and a non-pregnant cow. Homm (1927) gave this question considerable study and he concluded that palpation of the corpus luteum from the 21st to the 24th day did not give reliable information for early pregnancy diagnosis. The diagnostic value of the corpus luteum is further considered by Conrad (1943) and Strerath (1944).

**Palpation of the Middle Uterine Artery.**—From the third month of pregnancy, palpation per rectum of the middle uterine artery provides information of considerable value in the diagnosis and differential diagnosis of pregnancy. The "thrill," "vibration" or "fremitus" which can be detected in the middle uterine artery supplying the gravid horn from about the 12th week of pregnancy was first investigated and described by Dennhardt (1905). On palpating the artery there can be felt a characteristic rhythmic "thrill" (Schwirren) of the artery wall which is synchronous with the maternal pulse. Dennhardt considered that this phenomenon in the cow was similar to the uterine souffle or murmur (Uteringeräusch) which can be detected in pregnant women by auscultation. According to Rapin (1872) the uterine souffle is produced as a result of the contraction of the pregnant uterus, set up by the stimulus of examination. This reduces the lumen of the artery in the wall of the uterus and produces eddies in the blood stream. Dennhardt considered that the phenomenon which he had observed by palpation in the pregnant cow arose in a similar manner from uterine contraction following the stimulus of manual examination; he referred to it as uterine
souffle and considered its presence as valuable evidence of pregnancy. The "fremitus" disappeared within 24 hours of calving.

Uterine souffle has also been detected in the cow by auscultation. Albrecht (1914) was able to detect the uterine murmur in pregnant cows by a stethoscope introduced into the vagina.

Bedel (1919) reported that in certain cows from about the 7th month of pregnancy a uterine souffle could be detected on auscultation, but owing to the advanced stage of gestation and difficulty in detecting the souffle he concluded it was of no diagnostic value. Zieger (1922) used a modified endophonescope to detect the uterine souffle in the cow.

It is generally agreed that "fremitus" can be detected by the 12th week of pregnancy; Richter (1937) has noted fremitus as early as the 50th day, but this would appear to be exceptional. Richter (1937) also points out that it may not always be detected after the 12th week, especially if rectal examination is carried out before the vaginal examination, when the stimulation from the rectal examination may not be sufficient to cause the necessary contractions of the uterus required for the production of fremitus.

Palpation of the Caudal Uterine Artery.

Andt (1905) also observed that fremitus could be detected caudal uterine artery when palpated per vaginam. This is less clearly defined and does not appear so early in gestation as the fremitus in the middle uterine artery (Richter, 1937).

Vaginotomy.

To overcome the disadvantages of having to palpate the reproductive organs through the wall of the rectum, Eilmann (1924b) recommends direct palpation through an incision in the vaginal wall, especially in cases where the diagnosis is uncertain. Eilmann claims that the vaginal wound heals rapidly and no ill-effects are observed.

Vaginal Examination.

Vaginal examination involves both the palpation and the visual inspection of the vaginal walls and cervix. The changes in the vagina associated with pregnancy become noticeable from the end of the second month, the walls of the vagina feel drier and the cervix becomes occluded with a plug of thick, tenacious mucus. These changes have been described in considerable detail by Bensches (1933) and other authors and it is unnecessary to describe them here. It must be noted that the clinical examination of the vagina is not by itself a reliable method of diagnosing pregnancy;
the results of the vaginal examination must be considered in conjunction with other methods of clinical examination.

Various laboratory tests which have been carried out on the secretions of the vagina and cervix will be discussed later (see page 173).

**Other Clinical Methods.**

Other methods such as external ballottement and palpation of the foetus *per vaginam* are described in any text-book of obstetrics. There is no need to discuss such methods since they are of no value in the early diagnosis of pregnancy.

**Differential Diagnosis of Pregnancy and Pyometra.**

In the clinical examination for pregnancy, pyometra may give rise to errors in diagnosis. The differential diagnosis depends mainly on the careful palpation of the uterus *per rectum*. In cases of pyometra the activity of the uterine wall is at a minimum, and response to the stimulus of palpation may be practically absent. These are highly subjective criteria and the information gained is dependent on the experience of the examiner. Certain workers have claimed that frèmitus is absent in cases of pyometra, but there is considerable evidence in the literature that this is not always true. The most certain evidence of pregnancy is the detection of the foetal membranes or the foetus by palpation. The difficulties and possible dangers of palpation of the foetus and foetal membranes during the early stages of pregnancy (the end of the first and beginning of the second month) have already been mentioned. By the end of the third month, however, these procedures may be carried out more easily and without danger to the foetus. Numerous continental workers have stressed the importance of palpating the foetal membranes. Richter (1937) considers that the detection of the foetus by ballottement *per rectum* is the most certain sign of pregnancy that can be obtained during the third month. The importance of detecting the foetal membranes as an aid in the differential diagnosis of pregnancy and pyometra has also been stressed by Rowson & Spriggs (1942), Rowson (1942), and Fincher (1943).

Further information on this undoubtedly difficult aspect of differential diagnosis will be found in the papers by Zieger (1922), Lamparter (1927), Richter (1937), Schubert (1938), Marbach (1938), Freise (1939), Gould, Hignett, & Steele-Bodger (1942), Gould (1942), Steele-Bodger (1942), and Strerath (1944).

Differential diagnosis may be still more difficult in cases of twins. This complication has been discussed at length by Williams (1921, 1939), Zieger (1922), Gallina (1926), and Richter (1937).
Differential diagnosis in the case of "white heifer" disease has been discussed by Spriggs (1946).

**FURTHER REFERENCES TO LITERATURE.**

One of the most detailed descriptions of the clinical findings at all stages of pregnancy in the cow is that of Richter (1937). Details of the clinical method of diagnosis of pregnancy in the cow are also given by Richter (1921a,b), Hart (1923), Cortes (1923), Gallina (1925a,b, 1927, 1942), Bastoni (1925), Richter (1926), Kiss (1926), Gerosa & Mirri (1929a), Stoss (1929), Kadletz (1931), Benesch (1932a), Kingman (1933), Duhaunt (1934), Lucas (1934), Rózsa (1935), Faletti (1936), Farinas, Gapuz, & Fernandez (1938), Wille (1938), Pirocchi (1939), Gerosa (1939), Rosenberger (1941), Runge (1942), Day (1942), Conradi (1943), and Streath (1944).

**MARE.**

As in the case of clinical diagnosis of pregnancy in the cow, great advances in the techniques of clinical diagnosis of early pregnancy in the mare have been made in the last few decades. It is again convenient for purposes of review to consider the various aspects of clinical diagnosis under separate headings.

**RECTAL EXAMINATION.**

Schumann (1921) considered that diagnosis of pregnancy in the mare by rectal examination was not accurate until the 4th to the 5th month of pregnancy. The technique of rectal examination is discussed by Holthöfer (1923-24) who considers that in some mares it is possible to make a diagnosis during the 5th-6th weeks, while diagnosis is generally possible by the 9th-12th weeks. Peters (1934, 1936) stressed the absence of risk associated with this method of examination and claimed that diagnosis was generally possible from the 8th week. Životkov (1936, 1937) reported that reliable results had been obtained from the 28th-35th day of pregnancy. Miller & Day (1939) and Day & Miller (1940), describing the technique of rectal examination in the mare, concluded that the best time for carrying out the examination is between the 90th and 100th day after service, considerable experience being required for diagnosis at earlier stages. By examination every third day it may be possible to obtain evidence of pregnancy at very early stages, as has been reported by Day (1940), who was able to detect the foetus 16-23 days after ovulation (service was generally 2 days previous to ovulation).

In 1940 two papers on the clinical diagnosis of pregnancy appeared, which are of special interest, since the authors give the
percentage of correct diagnoses obtained by them with the clinical
method (see page 11). Kerov (1940) reports an accuracy of
89.2% in a series of 1,528 mares examined by the rectal method.
He recommends that combined rectal and vaginal examination
be carried out at 20 to 30 days after service. Robles (1940)
examined 73 barren and 46 pregnant mares (40 to 213 days);
72 of the non-pregnant mares were correctly diagnosed and 43
of the pregnant mares. He considered that the rectal method
was most accurate from the 61st to 90th day.

In asses, Flegmatov (1940) considers that diagnosis by rectal
examination is possible from the 22nd-24th day.

**FREMITUS OF THE MIDDLE UTERINE ARTERY.**

Richter (1922) has described the occurrence of fremitus in the
mare from the 5th to the 9th month of pregnancy. Steenholdt
(1922) confirmed Richter's findings and considered fremitus a
reliable sign from the 4th month of pregnancy. Holthöfer
(1923-24) considers that fremitus in the mare is not definite until
the 5th month of pregnancy. Stoss (1923-24) also considered
that while fremitus might be detected earlier in some cases, it
was not constantly present until the 5th month of pregnancy;
he described the occurrence of fremitus in the caudal uterine
artery (palpated per vaginam) at similar stages of gestation.
Benesch (1932a) likewise places the definite appearance of fremitus
in the mare about the 5th month of pregnancy.

**VAGINAL EXAMINATION.**

Vaginal examination of the mare for pregnancy generally
includes macroscopic examination of the vagina and microscopic
examination of smears taken from the cervix. The microscopic
technique will be considered later and is generally referred to
as the Kurosawa method.

The macroscopic examination of the vagina in the mare was
shown to give valuable information for the diagnosis of pregnancy
by Benesch (1925, 1932a,b). (This method is generally referred
to as the "Benesch test"). Benesch observed that the changes
in the vaginal walls and mucous secretions were so characteristic
that an accurate diagnosis of pregnancy could generally be made
24-30 days after service. Mally (1926) confirmed the value of
the findings of Benesch, although in the first month of pregnancy
the error was about 14%. Spánul (1926) examined 186 mares
by the vaginal method between the 8th-30th day with an error
of 3.4-5%.

Kurosawa (1931) confirmed the findings of Benesch and sup-
plemented the method by introducing the microscopic examination
of smears taken from the cervix (see page 155). Kurosawa claimed that by macroscopic examination of the vagina an average accuracy of 95% was possible from the 21st to the 30th day. In subsequent literature the combined macroscopic examination of Benesch and the microscopic technique of Kurosawa is generally referred to as the "Benesch-Kurosawa" test. References to the literature on the use of the combined test will be found on page 155 in the section dealing with the Kurosawa test.

It is evident that in the mare the changes which occur in the vagina and cervix, both macroscopic and microscopic, appear very early in pregnancy, are much more specific and more readily recognised than the changes which occur in the cow, and are of great importance in the clinical diagnosis of pregnancy.

Further information on the clinical diagnosis of pregnancy in the mare will be found in the following papers: Grommelt (1922), Dimock & Fincher (1928), Gerosa & Mirri (1929b), Benesch (1932b), Patrusev (1933), Dimock (1934), Oldenberger (1936), Zavadovskii (1936), Farinas, Gapuz, & Fernandez (1938), Ewen & Sager (1939), Zalesskii (1939), Götze (1940a), Stoss (1941), Burg (1945), Skogen (1945), Markin (1946), Wagenaar (1946), Bareggi (1946).

OTHER DOMESTIC ANIMALS.

Ewe and Goat.—The diagnosis of pregnancy in the ewe and goat depends mainly on the detection of the foetus by external ballottement and is thus only possible in the later stages of pregnancy. The difficulties of diagnosis have been discussed by Benesch (1932a) who concluded that diagnosis with any certainty is possible only in the last third of pregnancy. Kust (1935b), and Kust & Vogt (1934b) consider that in certain cases diagnosis may be made from the beginning of the second half of pregnancy. The problem is further discussed by Altenkirch (1934a,b).

In certain cases in sheep and goats it is possible to detect, per vaginam, fremitus in the caudal uterine artery. Richter (1921a) reported that in 12 pregnant ewes fremitus was detected in two cases, but only 7-14 days before parturition, so it could not be considered of great diagnostic value. Kust & Vogt (1934a) mention that fremitus may be detected in the caudal uterine artery of pregnant ewes from about the 4th month of pregnancy.

Sow.—The difficulties of the clinical diagnosis of pregnancy in the sow are dealt with by Benesch (1932a) who concludes that even towards the end of gestation a diagnosis of "probably pregnant" is all that can be given.

Bitch and Cat.—Stoss (1929) and Benesch (1932a) consider that
diagnosis of pregnancy in the bitch by clinical methods is possible from the 4th week of pregnancy, provided that the bitch is not excessively fat. Diagnosis depends on palpatating through the abdominal wall the series of small distensions in the cornua of the uterus produced by the developing foetuses. A detailed description of the clinical diagnosis of pregnancy in the bitch has been given by Wright (1934) who claims it is possible in some bitches to give a diagnosis at the 18th to 21st day of pregnancy. By the 5th or 6th weeks of pregnancy the ovine distensions are less tense and less well defined since the entire horn of the uterus has enlarged, and palpation is then more difficult than at the earlier stages. By the 44th day it may be possible to palpate some of the foetuses. From the 50th day the diagnosis should present no difficulty. Whitney (1936) confirms in all essential details the technique and findings of Wright.

In primiparous bitches the teats become pink, enlarged and turgid at the 21st to 35th day of pregnancy (Wright, 1934). The technique of diagnosis in the cat is similar to that employed for the bitch (Benesch, 1932a; Kirk, 1945). Rabbit.—By palpating the uterus through the abdominal wall, Suitor (1946) considers that an accurate diagnosis of pregnancy can be made in the rabbit 14 to 16 days after mating. At this stage the embryos produce a series of marble-like distensions in the uterine horns which can be readily distinguished. With practice a diagnosis may be made as early as the 7th or 8th day. Suitor considers it unwise to attempt palpation after the 16th day as the risk of injury to the foetuses is then greater and diagnosis more difficult owing to the more even distension of the uterine horns.

Nutria.—Mörschel (1935) considers the best indication of pregnancy to be the changes observed in the teats.

**Diagnosis of Pregnancy by Radiography.**

It is convenient to refer in this section of the review to the use of radiography as an aid to clinical diagnosis.

Ewe and Goat.—Benesch (1932a) refers to the possibility of employing X-rays for the diagnosis of pregnancy in the small ruminants, but no reference to the actual use of this method has been found.

Bitch.—Nagel & Neumann (1926) observed that the foetal bones could be detected by radiography towards the end of pregnancy and considered the method promising. The technique was further investigated by Yryäneinen (1930) who found it possible to detect shadows of the foetal vertebrae and ribs at the end of the 6th week and beginning of the 7th. Approximately similar times were given by Balossier & Reydellet (1933) for the appearance
of the shadow of the vertebrae. These authors describe the uterine shadow which can be detected after the third week of pregnancy, but they point out that until the foetal bones can be detected it is practically impossible to distinguish between pregnancy, pyometra and hydrometra. In considering the question of pregnancy diagnosis in the bitch by radiography, Boddie (1937) concludes that, while it may be possible to demonstrate a foetus at 7 weeks, a negative result should not be accepted as conclusive and a second radiograph should be taken some days later.

Sartoris (1935) has claimed that much greater detail of the uterine shadow in the bitch can be obtained by starving the bitch for 12-16 hours and then, after emptying the bladder by catheter, introducing oxygen into the peritoneal cavity just before the radiograph is taken. The findings at the various stages of gestation are described.

**Electrocardiograph Methods.**

Nőrr (1921) showed that it was possible to diagnose pregnancy in the cow, goat, and mare by the presence of the foetal heart record which was found superimposed on the record of the mother's heart. The method was of little practical value since it was only reliable in the advanced stages of gestation. Falcoianu (1922) refers to further experiments with this method.

**Clinical Methods Commonly Used by Farmers and Stockmen.**

There are many tests in common use by farmers and stockmen for the diagnosis of pregnancy in farm animals. Some of these are undoubtedly of value in the later stages of pregnancy, others must be regarded as mere superstitions. Only a few of the more common of these tests will be noted here.

**Cow.**—The nature of the secretion from the udder has long been considered to provide useful information as to whether a heifer is or is not pregnant. There appear to be many variations of this test, but the general method of carrying it out is to milk a little of the mammary secretion into the palm of the hand and to note the colour and physical properties of the liquid. Hartmann (1939) investigated the accuracy of this test in a series of 125 non-pregnant and 75 pregnant heifers and concluded that pregnancy could be diagnosed with accuracy in about 70% of cases between the 4th and 8th months.

Further details of the characteristics of the mammary secretion of heifers during pregnancy are given by Hammond (1927) and Tagle (1940).

The appearance of the vulva is considered by some to be an
indication of pregnancy. In pregnant cows it becomes anaemic and wrinkled, whereas in non-pregnant animals it is generally hyperaemic, smooth, and somewhat oedematous (Hildén, 1942).

_Ewe._—Webb (1942) and Heckler (1942a) both describe tests for pregnancy in the ewe based on the behaviour of the mammary secretion when subjected to various “tests” in the palm of the hand.

_Mare._—A novel method of pregnancy diagnosis in the mare has been described by Heckler (1942b), who claims that when a mare becomes pregnant two small warts or teats in the mouth under the tongue (openings of the ducts of the submaxillary glands?) become red and enlarged 42-63 days after service. This “sign” is said to become more marked as gestation advances.
CHAPTER 2. TESTS BASED ON HORMONAL INVESTIGATIONS OF BODY FLUIDS. A. GONADOTROPHIC HORMONES.

WOMAN.

FEMALE MOUSE AS TEST ANIMAL.

Aschheim-Zondek Test. — The investigations into the interrelationship of the hypophysis and the reproductive organs which led to the evolution of the gonadotrophic hormone tests for pregnancy have already been extensively reviewed (Engle, 1932, 1939; Berg, 1938; Zondek, 1935; Courrier, 1945) and it is not necessary to consider them here.

Zondek (1926) and Aschheim (1926a) showed that a single implantation of either human or animal anterior-pituitary tissue into immature female mice produced precocious sexual maturity—the mice came into oestrus and showed the vaginal and uterine changes characteristic of this period of the reproductive cycle, the ovaries contained follicles, haemorrhagic follicles and corpora lutea. A few months later independent confirmation of these experiments was provided by the work of Smith (1926) and Smith & Engle (1927a,b).

Further details of the immature-mouse test for pituitary gonadotrophins were given by Zondek & Aschheim (1927a,b,c).

Aschheim & Zondek (1927) and Aschheim (1927) investigating the role of the pituitary hormones in pregnant women, showed by means of the immature-mouse test that gonadotrophic hormones were excreted in the urine as early as the 35th day after the last menstruation. Gonadotrophins were also found in the decidua of early pregnancy and in the blood of pregnant women (see page 30). Oestrogenic hormones were also present in the blood and urine during pregnancy (see page 68).

The changes in the reproductive organs of immature mice following implantation of pituitary tissue or injection of pregnancy urine were divided by Aschheim & Zondek (1929b) into three phases or reactions:—

Anterior-Pituitary Reaction I (APR I). — Follicle ripening and induction of vaginal oestrus.

Anterior-Pituitary Reaction II (APR II). — Hyperaemis, of ovaries and haemorrhagic follicles (blood dots or blood points).

Anterior-Pituitary Reaction III (APR III). — Luteinization and formation of corpora lutea atretica (corpora lutea formed from unruptured follicles containing the entrapped ova in the centre).

Zondek postulated the presence of two pituitary gonadotropic factors—Anterior-Pituitary Hormones A and B, Anterior-
Pituitary Hormone A being the follicle-stimulating hormone and responsible for APR I, and Anterior-Pituitary Hormone B being the luteinizing hormone responsible for APR III. [It is now customary to refer to these two factors as follicle-stimulating hormone (FSH), and luteinizing hormone (LH).] For further information regarding the nature of these two factors see the reviews by Fevold (1939) and Zondek & Sulman (1945b).

The concentration of gonadotrophic hormones in the urine of pregnant women was so great that the injection of 1 ml. of urine into immature mice was sufficient to produce the characteristic Anterior-Pituitary Reactions. The authors suggested that the presence of these gonadotrophins in the urine might serve as the basis of a test for pregnancy, and in the following year they published (Ascbheim & Zondek, 1928a,b) the results of tests on 276 samples (78 from pregnant and 198 from non-pregnant women); only 4 of the tests were incorrect.

The test is carried out by injecting 1.2-2.4 ml. of morning urine, subdivided into six doses, into five immature female mice (3-4 weeks old, 6-8 g. in weight). The mice are killed 100 hours after the first injection and the ovaries examined for signs of gonadotrophic stimulation, the presence of Anterior-Pituitary Reaction II (APR II) and/or Anterior Pituitary Reaction III (APR III) being the criterion for a positive test. For further details the original papers should be consulted.

In the majority of cases the test can be read by macroscopic examination of the ovaries, but in cases of doubt Zondek advised serial section of the ovaries. This tedious procedure was fortunately rendered unnecessary by mounting the ovaries whole in glycerine and examining under the low power lens (Kraus, 1929; Zondek, 1931e).

The reliability of this hormone test for pregnancy was soon established. Louria & Rosenzweig (1928) reported on 132 cases: 98% of the urine samples from pregnant women gave positive results, the earliest positive being from a woman seven days after the missed period; 91% of the samples from non-pregnant women gave negative results, 7% were doubtful, and 2% were positive. Brouha, Hinglais, & Simonnet (1928) were also satisfied with the preliminary trials of the method. Odescalchi (1928) reported only four errors in tests on 197 pregnant women, and four errors in tests on 258 non-pregnant control cases. In 169 tests on 109 women, Wermbter & Schulze (1929) encountered only two errors. Some 15 workers (Wagner, Hornung, Kriele, Schmidt, Pankow, Martius, Gragert, Futh, Siebke, Kehrer, Karg, Esch, Baisch, Mayer; and Hellmuth) contributed their experiences of the test in the Dtsch. med. Wschr. (1929), 55, No. 51. All were satisfied
with the accuracy of the test. In the following years the reports in the literature amply confirmed the great reliability of the test and its great practical value. There is no need to review in detail this literature except to give references to a proportion of it:—

Vogt (1929), Brühl (1929a), Kraus (1929), Ehrhardt (1929), Vozza (1929), Kraul & Rippel (1929), Mazer & Hoffman (1929b), Solms & Klopfstock (1929), Allan & Dickens (1930), Grover & Auer (1930), Brouha, Hinglais, & Simonnet (1930), Crew (1930), White & Severance (1931), Mathieu & McKenzie (1931), Ettinger, Smith, & McHenry (1931), Wiesner (1931), Mazer & Hoffman (1931), Frank & Felshin (1931), Hauptstein (1931), Stewart (1931), Brühl (1932a), Letulle (1932), Büttner (1932a), Fanz & Gault (1932), Cuboni (1932), Bland, First & Roeder (1932), Wiesner (1932), Rudd & Ingram (1932), Lassen (1932), Ehrhardt (1932a), Wiesner (1933), Eum (1933), Dary & Sevringshaus (1934), Mack & Agnew (1934), Dawson (1935), Figurnov (1935), Aschheim (1935), Woodhouse (1936), Crew (1936a,b, 1937a), Mekler (1938), Frank-Fahle (1939), Engelfried, Hawkinson, & Galandey (1945).

The average accuracy of the Aschheim-Zondek test is about 98%. Not all investigators have obtained so satisfactory results, but in these cases, generally, the original technique has not been closely followed, and too few mice have been used. The strain of mouse is also of considerable importance; some strains have been shown to be quite unreliable for the test (Hummel, 1942). The errors arising from the use of too few mice have also been discussed by Hansen & Gram (1935). With the strain of mouse used by them, in order to reduce the risk of an error of chance to less than 1:1,000, six immature mice were required of which five had to be alive at the time of reading the test.

The test is not absolutely specific for pregnancy as it depends on the presence of living chorionic epithelium within the body. For further details of the use of the test in pathological conditions, see page 46.

Despite the great accuracy of the test, there are several inherent technical drawbacks of considerable importance and much research has been aimed at simplifying the test.

Modifications involve detoxication and concentration of the urine; substitution of blood serum for urine; the use of test animals other than mice; and pre-treatment of the test animals to expedite the test.

Detoxication of Urine.—Zondek (1930e) found that 6.7% of urine samples were so toxic as to render the carrying out of the test impossible. He described two methods of treating such samples: (1) filtration of urine through a Berkefeld filter; (2)
extraction of urine with anaesthetic ether (ether method). The ether method was claimed to be very effective; it was thought that the ether acted mainly by removing toxic substances from the urine. Böhne (1931) considered that the efficacy of the ether treatment depended on its bactericidal action which rendered the urine sterile, but Zondek (1931b) did not accept this conclusion and reaffirmed that a toxic substance was extracted by the ether. Zondek & Black (1946), however, have now also reached the conclusion that ether acts on account of its bacteriostatic or bactericidal properties and not because it extracts a specific toxin; strange to say, these authors make no reference to the fact that Böhne reached this conclusion some 15 years earlier, although one of them (Zondek) at that time had criticized Böhne's conclusions.

Stewart (1931) confirmed that the ether treatment reduced the mortality of the test animals. This method, however, was found by Wiesner (1931) to be not entirely satisfactory; he found pre-treatment of the urine with sulphasalicylic acid more efficient (see also Wiesner & Marshall, 1931). As a result of observations made on urine from pregnant diabetic women, Zondek (1931b) further modified the ether method. He claimed that the addition of glucose to a concentration of 3%, after ether extraction of the urine, accelerated the development of Anterior-Pituitary Reaction III (APR III) as well as reducing the toxicity of the urine. Tests on 68 pregnancy urines and 32 non-pregnancy urines pre-treated by the ether-sugar method gave 100% accuracy and the test could be read at the 72nd hour. Ehrhardt (1932a), in a series of 400 cases, concluded that the ether-sugar method was more reliable than the ether method. Lassen (1932), on the contrary, failed to observe any diminution of toxicity or acceleration of the test after the addition of glucose.

Concentration of Gonadotrophins in Urine.—Biedl (1928) concentrated the gonadotrophin by precipitation from pregnancy urine with alcohol; this method was used by Zondek (1930e) in his "Precipitation-Rapid-Reaction," the precipitate being extracted with ether, dissolved in water and injected into mice. A similar method of concentration was used by Eberson (1931) and Eberson & Silverberg (1931) (see page 30-31).

Eratman & Doisy (1932) concentrated urinary gonadotrophins by adsorption on Norit and elution with phenol, or by adsorption on finely divided benzoic acid and elution by dissolving the acid in acetone. In 1934 the same investigators described a process of precipitation with tungstic acid. Hellbaum, Fovold & Hisaw (1935) precipitated the gonadotrophin with tannic acid and extracted with 50% aqueous pyridine. Fovold & Hisaw (1936)
extracted the urine with one-tenth its volume of cresol and precipitated the gonadotrophin from the cresol with acetone. The follicle-stimulating fraction is said not to be extracted by this method. In 1929, Reiss & Haurowitz showed that the gonadotrophin of pregnancy urine could be adsorbed on alumina and eluted with ammonia solution; a similar method—adsorption on kaolin and elution with 0.1N sodium hydroxide—was used by Scott (1940) for preparing extracts for biological tests. Katzman, Godfrid, Cain & Doisy (1942, 1943) adsorbed gonadotrophins from acidified pregnancy urine on a column of "Permutit" and eluted with ammonium acetate in aqueous ethanol. A similar method is described by Milton (1946). Precipitated gonadotrophins can be detoxified by dialysis (Heller & Chandler, 1942).

Courrier & Dognon (1939) obtained concentrates by an entirely different method. By bubbling nitrogen through a column of pregnancy urine they demonstrated that the froth so formed contained most of the gonadotrophic activity of the original sample. By removing the froth from 100 ml. of pregnancy urine treated by this method practically all the gonadotrophic activity could be obtained in a few ml. of liquid.

Rapid Tests.—Aschheim & Zondek (1928b) described a "Rapid Test"—read at the 48th hour—and a "Continuous Test"—read after the 60th hour. In both these methods a larger total volume of urine was injected than in the original method: In both methods only positive results were reliable. In 1930, Zondek modified his "Rapid Test" by concentrating the gonadotrophins from the urine by alcohol precipitation (see page 26). The mice were examined at the end of 51-57 hours. As in the former two methods, only positive results could be relied on and the test was only of value in cases of emergency. Crew (1930) also described how in cases of emergency large doses of urine could be injected and the mice examined at the 60th hour, a normal test being run concurrently. Latzka (1933) concluded that all the rapid modifications of the original Aschheim-Zondek test gave less accurate results than the original.

Hirsch-Hoffmann (1932a,b) utilized the early histological changes which take place in the ovary of immature, mature or pregnant mice following injections of pregnancy urine. From the appearance of the granulosa cells and the incipient luteinization of the follicle walls it was possible to read the test at the 36th hour. In a later paper, Hirsch-Hoffmann (1933) doubted whether these changes were caused by gonadotrophins since boiled pregnancy urine was active, but nevertheless the changes were claimed to be specific for pregnancy. Brühl & Hollstein (1933a,b) tested 79 cases with this method and found it satisfactory; only
two results proved incorrect. (See also Sar-Louis, 1935, page 29).

Eberson (1933) described a rapid test using urine concentrates (alcohol precipitation) dissolved in saline. Two immature mice were given a single subcutaneous injection of the extract and autopsied 16-18 hours later. Diagnosis was based on blood points and congestion of the ovaries; 361 tests by this method all proved correct (see also Kupperman & Greenblatt (1946) page 29).

More satisfactory rapid tests were evolved using the rat as a test animal (see page 31).

Special Treatment of Test Animal and Test Sample.—Ehrhardt (1929, 1932a) investigated many modifications of the test in an attempt to reduce the time interval before the final reading. These modifications included splenectomy of the test mice, unilateral ovariectomy, total hysterectomy, keeping the mice in higher temperatures (30-40°C.), simultaneous injections and implantations of other endocrine glands or gland extracts, irradiation of test animals with sunlight, irradiating the urine with radium and X-rays, addition of iodine to urine, preliminary concentration of urine, pre-treatment of test animals with haemophilic serum, intravenous injection of urine in mice, and feeding vitamin supplements to the test animals. None of these modifications had any real accelerating action. Kraul & Rippel (1923) observed that the effect of pregnancy urine in mice was inhibited when injections of adrenaline were given to the mice. Büttner (1931) suggested pre-treatment of the mice with follicle-stimulating hormone and claimed that mice so treated would show blood-dots (APR II) 26-36 hours after injection of pregnancy urine, but the difficulty of obtaining pure FSH preparations made the method impracticable. Künstner (1931a, b) claimed that by exposing mice to red light the Aschheim-Zondek test could be read in 55-60 hours and the mice were also more resistant to toxic urines. Mandelstamm & Kaplun (1934) also claimed that mature mice exposed to red light and injected intravenously with pregnancy urine showed characteristic blood-dots 48 hours later. Zondek (1935) failed to confirm Künstner’s conclusions on the effect of red light. Caffaratto & Bertini (1937) claimed that infra-red and X-rays increased the activity of pregnancy urine gonadotrophins while ultra-violet rays decreased the activity. Jongh & Woerd (1939) concluded that the action of pregnancy urine was independent of exposure to light, but that the production of gonadotrophic hormone from the test animal’s own pituitary might be dependent on light.

Attempts at speeding up the appearance of the reactions produced by gonadotrophin administration must be considered in the light of the conclusions reached by Zondek & Sulman (1945b) who
write: "The gonadotropic reactions appearing after gonadotropin treatment occur at different time intervals depending upon the species of animal used, the kind of hormone administered and the route of application. Experiments to shorten these intervals have been in vain. A study into the mechanism of gonadotropin reactions has shown that the delay encountered in their appearance is inherent chiefly in the target organ itself (which has to undergo a certain course of development) rather than in slow action of the gonadotropins."

Adult Mouse.—In the original test great care was taken to avoid using mice which were reaching sexual maturity lest spontaneous ovarian changes be considered as changes due to the administration of pregnancy urine. Several authors have shown, however, that with care adult and even pregnant mice may be used as test animals.

Adult female mice were used by Laffont & Chiapponi (1930). The injection of pregnancy urine (2-3 ml. daily for 3-4 days, commencing after oestrus) caused the reappearance of the oestrous smear and the production of numerous corpora lutea. Mandelstamm & Kaplun (1934) also utilized adult mice. Sar-Louis (1935), repeating Hirsch-Hoffmann's experiments (see page 27) with adult mice, found it possible to read the test 36-38 hours after the initial injection of pregnancy urine, the evidence for a positive test being luteinization of the interstitial cells of the ovary. Mice were also examined at the 72nd and 100th hours; in these mice there were characteristic changes due to increased rate and intensity of luteinization. These changes were typical and were not observed in adult mice injected with control urine at any stage of the oestrous cycle. Sar-Louis concluded that adult mice, even pregnant mice, were suitable for testing for gonadotrophins, the result being obtainable at the 38th hour, but more easily read at the 72nd-100th hour. Burdick & Whitney (1941), Burdick, Watson, Ciampa, & Ciampa (1943), and Burdick (1946) studied the effects of single subcutaneous injections of pregnancy urine in dioestrous and pregnant mice. Ovulation was observed within 24 hours. They considered this method very promising as an 18- to 24-hour test, the end-point being the presence of ova in the ampulla of the oviduct; these can be readily observed under the dissecting microscope. Hummel (1942) found that certain strains of mice were not suitable for the original Aschheim-Zondek test, but that the adults of these strains responded to pregnancy urine. He also had no difficulty in distinguishing control- and pregnancy-urine stimulated ovaries.

Kupperman & Greenblatt (1946) have observed that adult mice may be employed for the ovarian hyperaemia test (see page 31).
provided 15 hours are allowed to elapse between the injection and reading the test.

Tests Using Blood.—Gonadotrophins are present in the serum of women from the second month of pregnancy (Aschheim, 1926b; Zondek & Aschheim, 1927a; Aschheim & Zondek, 1927; Aschheim, 1927). Fels (1927a) demonstrated the presence of gonadotrophin in 30 of 38 samples of blood from pregnant women and he concluded that positive tests in immature female mice following injections of serum were diagnostic of pregnancy.

It is of interest to note that earlier investigators had studied the effect of human pregnancy serum on the reproductive organs of female mice. Polano (1923) and Binz (1924) observed that serum from pregnant women increased the weight of the sex organs of female mice. The increase in size of the uterus of the mouse following injection of pregnancy serum was used by Trovino (1926) as a test for pregnancy. He believed the active substance to be oestrogen. Although these workers failed to observe whether changes occurred in the ovaries, there can be little doubt that the changes observed by them were in the greater part due to the gonadotrophins and oestrogens present in the serum.

Siddall (1929a,b) weighed the uterus and ovaries of immature mice after injections of serum. The ratio, "weight of mouse (mg.)/weight of ovaries+uterus (mg.)", was used as an index for pregnancy diagnosis; ratios below 400 were indicative of pregnancy, those above 400 were negative. He had six errors in 139 cases. Vozza (1929) successfully substituted serum for urine in cases where the urine was toxic to the mice. Flühmann (1929a), Stewart (1931), Frank & Felshin (1931), László (1932), Kennedy (1933), Smith & Smith (1934, 1936), and Gutman & Dalsace (1935) used serum instead of urine for the immature-mouse test with satisfactory results. Smith & Smith (1944) claimed that serum was preferable to urine as it gave fewer false negative results.

Methods of preparing gonadotrophin concentrates from blood have been described by Frank, Goldberger, & Spielman (1931), Salmon & Frank (1935, 1936), and Freed (1936).

Female Rat as Test Animal.

Aschheim & Zondek (1926b) preferred to use immature mice for their test as they were less expensive than rats. Rats were used by Evans & Simpson (1930) who found them more resistant to toxic urines than mice. Bourg (1930a) observed that infantile rats did not show haemorrhagic follicles after injection of pregnancy urine, but Anterior-Pituitary Reactions I and III were present. Urine concentrates (alcohol precipitation) were used by
Eberson (1931) and Eberson & Silverberg (1931), the result being read on the 3rd or 4th day. Jones & Muggage (1931), Davis & Ferrill (1932), Davy & Nason (1934), and Mull & Underwood (1937) all obtained reliable results combined with a low mortality of the test animals by using immature rats. Aschheim (1942, 1946a,b) described a simplified test using two immature female rats. The rats received 0.5 ml. of urine subcutaneously, and vaginal smears were examined 72-84 hours later. This method depends on the presence of both gonadotrophins and oestrogens in the urine. Only cases of chorioepithelioma or teratoma are considered likely to give false positives with this method.

Kelly (1933) used the premature establishment of the vaginal orifice in the immature rat, following intraperitoneal injection of pregnancy urine, as a pregnancy test. This reaction is also based on the presence of both gonadotrophins and oestrogens in the urine. Results could be read in 72-84 hours. Hulpieu, Weatherby, & Culbertson (1934) concluded that this method entailed a high mortality in the test animals and the results were less reliable than those obtained by the Friedman test (see page 33).

Rapid Tests.—Eberson & Silverberg (1931), as a result of observations on 90 cases, concluded that the "tinctorial and structural changes in follicular cells seen in histological serial sections" were diagnostic of pregnancy 24-36 hours after the first injection of pregnancy urine concentrates. Correct results were obtained in all 90 cases (cf. Hirsch-Hoffmann (1932a,b, 1933) page 27).

There are, however, macroscopic signs present in the rat ovary soon after treatment with gonadotrophin which are much easier to read than these histological changes. The hyperaemia of the ovary (APR II) occurring after injection of gonadotrophin into immature rodents (Zondek, 1926) is very striking in the immature rat where the ovary is normally somewhat anaemic. In the immature mouse the ovary is normally rather hyperaemic. This sign was first utilized by Reiprich (1933, 1934) who injected immature rats subcutaneously with 10-14 ml. of urine in two or three doses during 6-9 hours and examined the ovaries at the 30th hour; with experience the test could be read at the 24th hour. In a series of 264 cases an accuracy similar to the slower Aschheim-Zondek test was obtained. Zondek (1935) pointed out that this method must be used with care since hyperaemia of the rat ovary depended to a great extent on the presence of follicle-stimulating hormone (FSH), so that urines which will give only APR I in the ordinary test (i.e., a negative result) will give a positive test with the hyperaemia method. (This view was later modified—see Zondek, Sulman & Black, 1945.) The modification by
Reiprich attracted little or no attention until the test was re-discovered by Walker & Walker (1938). The method aroused considerable interest and numerous reports on it have appeared. Kelso (1940) compared the results given by 130 urine samples on rats 24 and 72 hours after the initial injection. In 125 tests the conclusions were identical. The 24-hour readings gave an accuracy of 96%. Frank & Berman (1941) obtained an accuracy equal to the original mouse test in 233 tests. They found that transillumination of the ovary and examination at magnification of ×10 were aids in reading the test. In positive cases the follicles are surrounded by a brilliant red zone and covered with minute capillaries; positive results at the 8th hour were found to be reliable. Salmon, Geist, Salmon & Frank (1942) claimed that readings could be obtained at the 6th hour. 110 urine samples from 78 pregnant and 31 non-pregnant women gave practically 100% accuracy. Urine from ovariectomized and menopausal women gave negative results. The authors advise using concurrent 24-hour tests until experience is gained in reading the test at the 6th hour. It is thought that oestrogens as well as gonadotrophins play a part in these reactions. Salmon, Geist, Frank, Poole & Salmon (1942) found that the test could often be read as early as the 2nd hour. Kupperman, Greenblatt & Noback (1943) injected the urine intraperitoneally and read the test at the 2nd hour. For the 6-hour test these authors found both immature and adult rats satisfactory, provided that the vaginal smears of the adult rats are examined to ensure that the rats are in the di-oestrous or metoestrous stage of the cycle. Soman (1944), using the 24-hour test on 200 urine samples, considered that it was sufficiently accurate to replace the rabbit test (see page 33). Kaminester (1944) read the test at the 6th hour and reported two errors in 106 samples. Ramsey, Falkenstein & Sujkowski (1944), Kupperman & Greenblatt (1944), Kline (1944), Aschheim & Varangot (1945a,b), Sala, Orellana & Gonzalez (1945), and Hinglais & Hinglais (1947) all obtained satisfactory results with the hyperaemia test. Farris (1944), in a study of the 2-hour method, obtained false positive results from urine of non-pregnant women during mid-cycle and from men and women during sexual excitement. He considered that the test was not specific for pregnancy. The hyperaemia test was extensively investigated by Zondek, Salam & Black (1945) in some 300 cases. Three infantile rats were each given 4 ml. of urine subcutaneously in two injections at an interval of one hour. Readings at the 2nd hour were only reliable if positive. The 24-hour readings gave a 1% error in 300 cases. Zondek et al. concluded that the test was suitable for diagnosis of undisturbed pregnancy—in cases
of suspected ruptured extra-uterine pregnancy or incomplete abortion the original test should be used. The hyperaemia of the rat ovary is believed by Zondek et al. to be due to the luteinizing hormone (LH), the follicle-stimulating factor (FSH) playing an augmenting role.

Kupperman & Greenblatt (1946) have reported a 99.5% accuracy in 752 tests read at the 2nd hour. They were unable to confirm the non-specific positive reactions which were reported by Farris (1944), and consider the reaction is produced by the luteinizing hormone (LH).

Bunde (1947) has investigated the hyperaemia reaction, injecting the urine intraperitoneally and/or subcutaneously. Using two rats per test an accuracy of 84.5% was obtained in 108 tests and with three rats an accuracy of 90.5% in 84 tests. All errors were false negatives. The route by which the urine was injected had no significant effect on the result of the test. There was a marked variation between the individual responses of the test animals.

**Female Rabbit as Test Animal.**

_Friedman Test._—Heape (1905) demonstrated that ovulation in the rabbit occurs only after copulation. Bellerby (1929a,b) produced ovulation, haemorrhagic follicles and corpora lutea in the rabbit by injecting extracts of anterior pituitary. He postulated that the act of copulation stimulated the anterior pituitary to secrete into the blood-stream a hormone which produced the ovarian changes. It had been previously demonstrated by Hirose (1920), and Murata & Adachi (1927-28) that gonadotrophins (emulsions of human placenta) produced ovulation and formation of corpora lutea when injected into the rabbit, but the general significance of their findings was not realized.

Friedman (1929a,c) produced ovulation and fresh corpora lutea in the rabbit by a series of intraperitoneal injections of human pregnancy urine. He then found that a single intravenous injection of human pregnancy urine was sufficient to produce ovulation. 18 samples of pregnancy urine all produced ovulation, 16 non-pregnancy urine samples were all inactive. He concluded the method deserved further investigation as a pregnancy test. Reiss & Langendorff (1929) also observed ovulation, haemorrhagic follicles and corpora lutea in the virgin rabbit after injections of pituitary and pregnancy urine gonadotrophin. Jares (1930) confirmed Friedman's claim that single intravenous injections of pregnancy urine would cause ovulation in the rabbit, often within 10 hours. Shirai (1930), working in Japan, found the rabbit a reliable test animal, the test being read at the 20th hour, or preferably at
the 70th hour. Schneider (1930, 1931), employing immature rabbits (12-16 weeks old), injected 5-7 ml. of urine intravenously and examined the ovaries at 12-30 hours. He considered the test very simple, very rapid and of considerable practical value. Friedman & Lapham (1931), using mature rabbits, tested 92 samples with 100% accuracy; they preferred to read the test at the 48th hour, although positive results could be accepted at the 18th hour. Only haemorrhagic follicles and fresh corpora lutea are indicative of a positive test; clear unruptured follicles, whatever their size, by themselves must be regarded as negative.

The accuracy and reliability of this rapid and simple test have been confirmed by numerous workers:

Mettenleitner (1931), Magath & Randall (1931), Reinhart & Scott (1931a, b), Wilson & Corner (1931), Dodds (1931), Wiesner (1931), White & Severance (1931), Brouha (1931), Martins (1931), Parache (1932), Brindeau, Hinglais, & Hinglais (1932), Borrasses (1932), Ehrhardt (1932a, d), Rudd & Ingram (1932), West (1932), Büttner (1932b), Costa-Sacadura & Ross (1932), Schneider (1932), Grant, Zibel, & MacMahon (1932), Davis & Walker (1932), Ware & Main (1932), Schoeneck (1932), Clauberger (1932), Wood (1932), Stricker (1932), Nijima & Katahira (1933), Bishop (1933, 1934), Reinhart (1933), Hein (1933), Mann, Meranze, & Goub (1933), Ehrhardt (1933), Best & McHenry (1933), Wilson & Corner (1933), Morgan (1933), Veselk (1933), Kováts (1933), King (1934), Young (1934), Davy & Nason (1934), Gernez (1934), Hulpiue, Weatherby, & Culbertson (1934), Spielman (1934), Goldberger, Salmon, & Frank (1934), Lejwa & Frysberg (1934), Figurov (1935), Sharp (1935), Mills (1935), Woodhouse (1936), Morcos (1936), Wespì (1936), Bamforth (1936), Feresten (1937), Kelly & Woods (1937), Ankesaria (1937), Cuyler (1937), Heiberg (1937), Mekler (1938), Stadiem (1938), Impink & Mucklé (1938), Honda (1939), Randall, Magath & Pansch (1940), Scott (1940), Kup (1940), McCullagh & Cuyler (1940), Frank & Berman (1941), Kunz (1943), Buxton (1944).

The general overall accuracy of the rabbit test is similar to that of the original immature-mouse test.

Modification of the Rabbit Test.—As a precaution against a recent ovulation giving rise to false interpretations, Friedman (1929a) inspected the ovaries previous to injection, a procedure recommended by other workers (Priest, 1931; Lifvendahl, 1932; Hofmann, 1932; Schneider, 1932; Bishop, 1933, 1934). This precaution ought always to be taken if the rabbit has not been previously isolated for 3-4 weeks, since spontaneous ovulation may occur when female rabbits are housed together. Infantile
rabbits may ovulate spontaneously in sub-tropical climates (Zondek, 1935).

Mettenleitner (1931) and other workers advised reading the test by ovarian inspection under anaesthesia instead of killing the rabbit. Lifvendahl (1932) used the same rabbit for several tests, allowing a rest-interval of three weeks after a positive test, a practice followed by Hofmann (1933), Anklesaria (1937), and Stokes & Ortiz (1948).

For inspection of the ovaries under anaesthesia most workers have used the ventral approach to the ovaries. Goodale & Flanagan (1932) and Davy & Nason (1934) have claimed that the dorsal approach has much to recommend it by reducing the risk of infection.

Generally only one rabbit per test is used, but as a small percentage of rabbits are resistant to injections of pregnancy urine, the use of two rabbits per test has been recommended (Parache, 1932; Hein, 1933; Best & McHenry, 1933; Wilson & Corner, 1933; Spielman, 1934; Goldberger, Salmon & Frank, 1934; McCullagh & Cuyler, 1940). The use of an extra rabbit, however, increases considerably the cost of the test. The difficulty of the refractory rabbit can be overcome by the method described by Bishop (1933, 1934). He inspected the ovaries at the end of the 36th hour and, if negative, a sample of known pregnancy urine was injected and the ovaries inspected at the end of the next 36 hours, when the test should be positive if the rabbit is reacting normally to gonadotrophin. If positive the original negative test can be considered correct, if negative then the original urine must be tested on another rabbit.

Clauberg (1933) brought the ovaries into a subcutaneous position and kept them under observation by means of a "skin-window"—a watch glass being sutured in position over the ovaries. Campbell (1934) also brought the ovaries from the abdomen into a subcutaneous position in the rabbit's back.

Zweifel (1935) claimed that by unilateral removal of an ovary, Fallopian tube and uterine horn, a more rapid and delicate reaction could be obtained. Nikolaew (1935) thought that quicker results were obtained by exposing the urine to red light for five hours before injecting it.

Beohm (1941) inserted a "Zipp" fastener into the abdomen of the test rabbit to allow frequent inspection of the ovaries.

Suchet (1946) examined the ovaries through a small abdominal incision by means of an endoscope, thus reducing surgical interference to a minimum.

The rabbit, like the mouse, is not a suitable animal for the
study of early hyperaemia of the ovary following injection of pregnancy urine (Zondek, Sulman & Black, 1945).

**Ovarian Transplants.**—To allow frequent inspection of the ovary without the necessity of frequent laparotomies, several investigators have made ovarian transplants into the anterior chamber of the rabbit's eye. Allen & Priest (1932), in preliminary experiments with eight rabbits, obtained reliable results and considered the method worthy of further trial. Spirito (1933) and Geyer & Prister (1934) also carried out preliminary experiments with this method. Podleschka & Dworzak (1933) doubted if the method could be used for pregnancy diagnosis as the follicles which developed following the injection of pregnancy urine were not always in a visible position. Abramowicz & Zaleski (1935) obtained 20 positive results out of 22 tests with pregnancy urine and considered that the method would prove useful. Figurnov (1935) claimed that ovarian transplants into the anterior chamber gave as clear and rapid results as the original method. Chamorro (1936) described 12 tests carried out by this technique. Dworzak & Podleschka (1934, 1936) again warned against the use of this method as the transplants sometimes lost the property of ovulation and ovulation might occur behind the graft where it cannot be seen. They reported four errors in 25 tests. May (1944) has recently revived this method.

**Pregnant Rabbits as Test Animals.**—Shirai (1930), Martins & Fabiao (1930), Snyder & Wislocki (1931c), and Wolfe (1931) observed that pregnant rabbits ovulated in response to injections of pregnancy urine and could be used as test animals. Bishop (1933) concluded that only positive tests in pregnant rabbits could be relied on; negative tests must be repeated with a non-pregnant rabbit.

Friedman (1932) found that the ovary of the post-partum rabbit was highly sensitive to gonadotrophins.

**Tests Using Blood.**—Brown (1932), Brindeau & Hinglais (1932), and Hofmann (1932) demonstrated that blood serum gave as good results as urine in the rabbit test. The use of blood was further investigated by Schwarz (1933), Hofmann (1933), Segmund (1933), Kelly & Woods (1937), Di Gioia (1937), Rodriguez-Foo (1943), and Evans & Krajan (1944).

**Gonadotrophin Tests not Depending on Direct Ovarian Inspection.**—Markee (1933) described two tests for pregnancy depending on the changes observed in endometrial transplants on the iris of the rabbit's eye (see also Markee, 1932). One test depended on the presence of oestrogens in the urine and will be discussed later (page 69). The other test (indirect test) depended on the presence of gonadotrophins. The urine sample was injected intravenously
into the rabbit and within 7-9 hours characteristic modifications of the rhythmic vascular changes could be observed in the endometrial transplants as a result of oestrogen liberated into the blood stream from the rabbit's ovaries following stimulation by the gonadotrophins present in the urine sample. 147 pregnancy and 26 non-pregnancy urines were tested by this method and all the tests were correct.

Davis, Konikov & Walker (1934) described a pupillary reaction observed in rabbits used for the routine Friedman test. (It was based on the Bercovitz test, see page 159). The reaction of the rabbit's pupil immediately after the injection of pregnancy urine was noted. Contraction or dilatation of the pupil was considered a positive result. Of 148 tests with pregnancy urine 90.6% gave positive results; of 94 tests with non-pregnancy urine 82% gave negative results. The authors considered the method hopeful but requiring further investigation.

**FEMALE GUINEA-PIG AS TEST ANIMAL.**

Reports on the use of the guinea-pig as a test animal are somewhat contradictory. Maurizio (1930) claimed satisfactory results, but Jares (1931) failed to induce ovulation in adult virgin guinea-pigs with single intravenous injections of pregnancy urine administered during various stages of the oestrous cycle, and serial sections of the ovaries failed to reveal differences in structure when comparisons with ovaries from control guinea-pigs were made. Jares concluded that the guinea-pig was of no value as a test animal. Leeb (1932) concluded that pregnancy urine did not generally induce ovulation in the immature guinea-pig, although granulosa and theca interna lutein bodies were produced. Ehrhardt (1932a), and Megnin & Andreani-Constantin (1933) gave intracardiac injections of pregnancy urine to immature guinea-pigs, but obtained results of no practical value. King (1933b) also observed that pregnancy urine and, to a limited extent, blood from pregnant women, when injected into immature guinea-pigs, produced degeneration of the granulosa cells, luteinization of the theca interna and finally luteinization of the undifferentiated cells throughout the ovary. Enlargement of the clitoris was observed following injections of pregnancy urine in immature guinea-pigs by Papanicolaou & Falk (1934). Freed & Coppock (1936) investigated the gonadotrophic response in the immature guinea-pig and concluded that the response was similar to that observed in the rat, but the greater portion of the immature guinea-pig's life corresponded to the refractory period which in the rat is relatively very much shorter. Paddock (1941) described a test depending on the presence of gonadotrophins or oestrogens
in the blood. Young female guinea-pigs (30-45 days old) in which the vaginal membrane is closed are injected with 1.5 ml. of serum and inspected in 24-48 hours; if the vaginal membrane has opened, the test is positive. In a series of 254 tests 13 false negatives were recorded. (The mechanism of the opening and closing of the vaginal orifice in the guinea-pig has been studied by Kelly & Papanicolaou, 1927.) Young guinea-pigs were successfully used by Mello (1944). Three guinea-pigs, two in the dioestrous and one in the pro-oestrous stage of the cycle, were injected with urine concentrates prepared by the Scott method (page 27) and killed 24-48 hours after the first injection. Positive tests were characterized by enlargement of the ovaries and uterus, ovarian hyperaemia and haemorrhagic follicles and opening of the vagina. 63 urines tested by this method gave correct results. In a recent paper, Kupperman & Greenblatt (1946) have observed that guinea-pigs are unsatisfactory for rapid tests based on ovarian hyperaemia (see page 31) in addition, patency of the vaginal membrane was not observed 48 hours after injection of 10 ml. of pregnancy urine. (See also Kelly & Florence (1930), page 99).

**FEMALE HAMSTER AS TEST ANIMAL.**

Kupperman & Greenblatt (1946) found the immature hamster a suitable animal for ovarian hyperaemia tests. The result could be read at the 15th hour.

**MALE RODENTS AS TEST ANIMALS.**

*Rat and Mouse.*—Smith & Engle (1927b) observed that daily implants of anterior-pituitary tissue into immature male rats and mice produced a marked increase in size of the accessory sex glands; the effect on the testes was more varied. Similar studies were made by Zondek & Aschheim (1928b). Fels (1927b) observed that serum from pregnant women when injected into male mice increased the size of the accessory sex glands; this reaction he considered to be due to the presence of gonadotrophins in the blood (see also Fels, 1928). A similar effect with pregnancy urine was noted by Engle (1929) in immature male rats.

Brouha & Simonnet (1929), and Brouha, Hinglais, & Simonnet (1930) used this reaction for the diagnosis of pregnancy. Immature or mature male mice were injected with 0.1-0.4 ml. of urine subcutaneously for 6-10 days and the seminal vesicles examined 24 hours after the last injection. In positive tests the vesicles were 2-5 times heavier than glands from control mice. 122 of these tests were all correct. Laurent (1930), on the contrary, concluded that individual variations in the size of the
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Semenal vesicles of mice were so great as to render the test unreliable. Kraus (1930, 1931a,b) decided that negative results on the 3rd day were reliable, but that positive results might be due to the presence of follicle-stimulating hormone (FSH) which is not a reliable criterion of pregnancy (see page 24) and that the test ought only to be used to supplement the immature female mouse test. However, with further work, Kraus (1932) came to the conclusion that with experience the test could equal the accuracy of the Aschheim-Zondek test. In a series of 200 tests the error was 3.8%. Bourg (1930b, 1930-31) used a male and a female immature rat, claiming that the male only reacted to luteinizing hormone (LH) and the female gave extra information regarding the presence of follicle-stimulating hormone and oestrogens. Dharmendra (1931) found it unnecessary to sacrifice the test animals, as reliable results were obtained by observing the descent of the testes into the scrotum which occurred after the injection of pregnancy urine into immature rats.

Brochu & Hinglais (1931) reported further results of tests on 251 urine samples in which only one error occurred. They now stressed the importance of using mice between the ages of 24-42 days, the weight of the mice being of less importance. With experience they found that the test could be read on the 8th day. Neumann (1931) found the method accurate but slow, requiring 12-14 days. Czyżak & Prochorow (1931), and Czyżak (1932), by using urine concentrates reduced the time required for the test to 100 hours. By injecting immature male rats intraperitoneally with urine, Davis & Ferrill (1932) obtained results in 48-78 hours; an accuracy of 98% in a series of 400 tests was obtained. White & Severance (1931), and Figurnov (1935) concluded that immature male mice gave just as reliable results as immature female mice. Oustiaevili (1935) claimed that the test could be read at 24-48 hours. Zaharesco-Karman, Porumbaru, & Fotin (1935) claimed that rats from 20 to 100 g. could be used if the injections were given over 7-10 days, and if the seminal vesicles were weighed at the end of the test and the "growth coefficient" (weight of seminal vesicles, mg./weight of rat, g.) calculated. Normal values lay between 0.2 and 0.4, values over 1.0 were regarded as indicating a positive test.

Aschheim (1942) found that his simplified test (see page 31) was not applicable to the male rat, doses of 1-2 ml. of pregnancy urine giving negative results.

Guinea-pig.—Maurizio (1930) claimed that immature, male guinea-pigs were suitable test animals. Megnin & Andreani-Constantin (1933) injected the urine intracardially and examined the testes and epididymides for hypertrophy. They obtained
correct results in 102 tests. Satisfactory results with this technique were obtained by Gismondi & Acevedo (1935).

Further references to the effects of gonadotrophins on the male reproductive organs will be found in the reviews by Zondek (1931e, 1935), Van Dyke (1936), Engle (1939), and Burrows (1945).

**BIRDS AS TEST ANIMALS.**

Sato & Kimura (1930) attempted to use hens to test for gonadotrophins in pregnancy urine, but without success. Roberto (1935) also obtained negative results when chorionic gonadotrophins were administered to pigeons.

It is now generally agreed that the gonads of birds are not stimulated by human pregnancy urine gonadotrophin (chorionic gonadotrophin). (Cf. gonadotrophin from pregnant mares' serum, page 54).

An interesting test for the luteinizing hormone has been reported by Witschi (1940) depending on the activation of the melanophores in the feather germs of African weaver finches (*Pyromelana afra* or *taha*). The material is given in two injections into the breast muscle on consecutive days. The appearance of a black dot or bar on an otherwise white abdominal feather indicates a positive reaction. Whether this method will prove of value for pregnancy diagnosis has yet to be investigated.

**AMPHIBIA AS TEST ANIMALS.**

*Rana.*—Zondek (1930a) considered that gonadotrophins were without effect on the ovaries when injected into frogs (*Rana esculenta* and *Rana pipiens*). Bellerby (1933b) demonstrated, however, that pituitary gonadotrophins when injected into the common frog (*Rana temporaria*) induced ovulation without oviposition, it being necessary to kill the frog to observe the reaction (cf. *Xenopus laevis*). Furthermore, *Rana* could not be used as a test animal during the months of March and April as it normally spawns in that season, and during the rest of the season the response showed considerable variations. Shapiro & Zwarenstein (1934b) also observed that oviposition did not occur in *Rana* after injection of pregnancy urine, but that a reaction was obtained in the oviducts which they proposed to investigate with a view to using it for pregnancy diagnosis.

*Female Xenopus.*—Hogben (1930), and Hogben, Charles, & Slome (1931) showed that injections of pituitary gonadotrophin could produce ovulation and oviposition in the South African clawed “toad” (*Xenopus laevis*). Spontaneous ovulation was never observed in *Xenopus* when kept in captivity, so it appeared to be a suitable test animal. These observations were confirmed
by Bellerby (1933a). At room temperatures oviposition generally occurred 18 hours after the injection of gonadotrophin, but by keeping the Xenopus at higher temperatures, 23°-31° C., the time interval between injection and oviposition could be reduced to nine hours, thus enhancing the value of the reaction for test purposes (Bellerby, 1933b).

Shapiro & Zwarenstein (1934a,b, 1935), and Bellerby (1934a) successfully used Xenopus to develop a rapid test for pregnancy diagnosis in the human. (No useful purpose would be served in discussing the vexed question as to who first employed Xenopus as a test animal for pregnancy diagnosis. See Gunn, 1939a,b; Hogben, 1939, 1946; Shapiro & Zwarenstein, 1944, 1946.) Urine or aqueous extracts of alcohol precipitates of urine were injected into the lymph sac of Xenopus; oviposition was observed in 5-18 hours if gonadotrophins were present. The reliability of the method was confirmed by Bosman (1937), and Crew (1937b) who obtained an accuracy of diagnosis similar to that obtained with the Aschheim-Zondek test. Further satisfactory reports came from Elkan (1938a,b; 1939a,b; 1946), Landgrebe (1939), Crew (1939), Laves (1940), Scott (1940), Sieckmann (1941, 1942), Petzold (1942), Landgrebe & Samson (1944), Herring (1944), Zwarenstein & Duncan (1944), Milton (1946), Foote & Jones (1946), and Wattenwyl (1946).

Geoghegan & McGath (1944) concluded that concentration of the urinary gonadotrophin was essential since 400 tests with native urine gave only 50% correct results; the errors were practically all false negatives.

Weisman & Coates (1941) introduced the test into America. Although at first somewhat dubious of the usefulness of the test they soon found it very quick and thoroughly reliable (Weisman, Hoenig, & Coates, 1941; Weisman, Snyder, & Coates, 1942a,b,c; Weisman & Coates, 1944b,d).

Weisman & Coates (1944c) observed that the oviposition response in Xenopus to gonadotrophins was more prompt if the animal was kept in the dark during the test.

Each toad can be used for numerous tests; Landgrebe & Samson (1944) reported that many of their toads had been used 24 times and were still in use as reliable test animals.

Sutherland & Zwarenstein (1939) showed that blood, especially plasma, could be substituted for urine with equally good results. This was confirmed by Rosenfeld & Rosenfeld (1944).

Roch & Amoudruz (1946) using serum instead of urine obtained results as accurate as those given by the Aschheim-Zondek and Friedman techniques.

Specificity of the Reaction.—Landgrebe (1939) noted that
menopause urine (i.e., follicle-stimulating hormone) would occasionally induce oviposition in *Xenopus*. Weisman, Snyder & Coates (1942c) believed that the luteinizing fraction in pregnancy urine was mainly responsible for oviposition in *Xenopus*. In a series of tests on the urine of 36 women in early menopause, negative results were obtained following the injection of urine concentrates (Weisman & Coates, 1944a). These authors considered *Xenopus* might prove a useful test animal for differentiating pregnancy and menopause urinary gonadotrophin.

Oviposition can be produced in *Xenopus* by numerous steroids including progesterone and testosterone. The activity of some 60 steroids was investigated by Shapiro (1936c, 1939). Since urine concentrates (alcohol or acetone precipitates) are generally used for the pregnancy test it is unlikely that any steroid would be present in sufficient quantity to interfere with the test.

Further information on the effects of gonadotrophins in amphibians can be found in the reviews by Van Dyke (1936, 1939).

**Breeding of Xenopus.**—As *Xenopus* has proved a most reliable and economical test animal for the detection of gonadotrophins, considerable attention has been focussed on the methods of breeding and keeping the "toad", which is a native of South and West Africa, under laboratory conditions.

Zwartenstein & Shapiro (1933) observed that when the female *Xenopus* was kept in captivity for six months the ovaries became atrophied and the animal became unsuitable for test purposes. This "captivity effect" appeared to be a considerable drawback to the use of the toad in countries other than South Africa. This effect was investigated by Alexander & Bellerby (1935, 1938), who clearly showed that the "captivity effect" was due to defective management—improper feeding and an insufficient volume of water. Under proper conditions the female *Xenopus* would remain normal and be a satisfactory test animal for indefinite periods (see also Weisman & Coates, 1944a).

To avoid the necessity of regularly importing stocks of the toad, attempts were made to breed the animal in captivity. Shapiro (1936a,b) demonstrated that by the injection of gonadotrophins, oviposition and coupling could be produced in animals in captivity and fertilized ova thus obtained. This observation was confirmed by Landgrebe & Purser (1941). A detailed description of a satisfactory method of breeding *Xenopus* in captivity was published by Gasche (1943). Further successful attempts at breeding *Xenopus* have been described by Landgrebe & Samson (1944), Aronson (1944), and Deanesly & Parkes (1945).

Further information on the management and breeding of *Xenopus* is given by Elkan (1947).
Bibliographies of the literature on all aspects of the *Xenopus* test have been prepared by Weisman & Coates (1944d), and Zwarenstein, Sapeika, & Shapiro (1946).

*Male Xenopus.*—It is probable that the male *Xenopus* will prove a useful test animal for urinary gonadotrophin. Robbins, Parker, & Bianco (1947) have recently demonstrated that following the injection of gonadotrophin into the male *Xenopus*, numerous actively motile spermatozoa appear in the cloacal fluid within 1½ to 2 hours. Comparative assays indicated that the male *Xenopus* was twice as sensitive as the rat to chorionic gonadotrophin and ten times as sensitive as the female *Xenopus*.

*Male Bufo.*—A test, which is apparently similar to the above except that the male toad (*Bufo arenarum* Hensel) is used, is being developed by Galli-Mainini (1947a). After the injection of pregnancy urine, spermatozoa are present in the cloacal fluid within two or three hours. Further details of the test developed by Galli-Mainini, (1947a,b,c) are now available. The toads should weigh about 100 g. and be injected with 10 ml. of urine. Of 177 tests made concurrently in toads and rabbits, 8 were discrepant. In at least four of these, the toad test proved to be correct. In all, 485 tests were carried out with 776 toads, only 7 toads dying because of the toxicity of the urine. Some evidence was obtained that toads kept for long in captivity were less suitable for test purposes. Injections of oestrogens, androgens, and progestogens gave negative tests.

A more recent report about this test in the *Journal of the American Medical Association* (1948) states that the results obtained by Galli-Mainini in 1354 tests on women in the first four months of pregnancy have been 99.5% correct. The serum and urine of the pregnant mare, cow, sheep, and sow are said to give negative results.

**Fish as Test Animals.**

Two types of fish have been relatively widely used as test-animals for pregnancy diagnosis—the bitterling (*Rhodeus amarus*) and the Japanese bitterling (*Acheilognathus intermedius*). The production of the nuptial coloration in the male and growth of the ovipositor in the female are the signs characteristic of a positive reaction.

It has now been established that these reactions are not due to the presence of gonadotrophic hormones in the urine, but for the sake of convenience these tests will be mentioned here. (For further information on the effect of gonadotrophins in fish, the reviews by Van Dyke (1936, 1939) may be consulted.)
Male Fish.—Tozawa (1929) demonstrated that the development of the nuptial coloration in the male Japanese bitterling was influenced by a hormone or hormones produced by the sex-glands. Wunder (1931) found that testicular extracts injected into the male bitterling affected the nuptial coloration whereas oestrogens and gonadotrophins were practically inactive. Saphir (1934), on the other hand, was able to develop the nuptial coloration in *Chromomus erythrogaster* by giving high doses of gonadotrophin, while oestrogen was ineffective. Studies by Owen (1936) led him to conclude that the male bitterling was responsive to gonadotrophins and oestrogens, but the response was too variable to use for pregnancy tests. (See also Chromatophore Hormones, page 108.)

Female Fish.—It has now been well established that gonadotrophins play no part in the elongation of the ovipositor in the female bitterling (Fleischmann & Kann, 1932; Ehrhardt & Kühn, 1933; Szusz, 1934; Kanter, Bauer, & Klawans, 1934, 1936; Baumann & Szusz, 1935; Kleiner, Weisman, & Mishkind, 1936c; Owen, 1936; Marcus, 1937; Stiasny, 1937; Duyvené de Wit, 1941b). (See also Oestrogen Section, page 70.)

**Distribution of Gonadotrophins.**

Quantitative Studies.—The first quantitative studies on the excretion of gonadotrophin in the urine during pregnancy were carried out by Aschheim & Zondek (1928a, b), and Zondek (1931e). The excretion of gonadotrophins during the last third of pregnancy was investigated by Runge, Hartmann, & Sievers (1932), and Runge & Clausnitzer (1932). Blood gonadotrophins were investigated by Kennedy (1933) and Hamburger (1933).

Much information on the quantitative determination of gonadotrophins in the blood and urine will be found in the review by Fosco (1943).

C. Ehrhardt (1936b), Browne & Venning (1936a,b), and Anklesaria (1937) found that the highest concentration of gonadotrophin in the urine occurred between the 6th and 10th week. Evans, Kohls, & Wonder (1937) made a careful study of six healthy pregnant women and found the highest urinary values between the 20th and 50th day, after which there was an abrupt decrease. Blood gonadotrophins ran a parallel course. The authors stress that this peak is a normal phenomenon and must be taken into consideration in studies attempting to relate high hormone levels with pathological conditions.

Boycott & Rowlands (1938) found the highest concentrations of gonadotrophins in the blood from the 8th to the 12th week, the factor present being chiefly luteinizing hormone. By their
method of assay they were unable to detect gonadotrophic activity in the blood of non-pregnant women.

Jones, Delfs, & Stran (1944) studied quantitatively the gonadotrophic level in the blood of 24 women throughout pregnancy (simultaneous studies were made on the pregnanediol excretion in the urine, see page 100). The peak level occurred between the 50th and 65th day when from 70,000-600,000 i.u. of gonadotrophin per litre were found; by the 100th day this had dropped to 10,000-12,000 i.u. per litre and by the 130th day the average level was 5,000-6,000 i.u. per litre. During the remainder of the period of gestation the level did not rise above 10,000 i.u. per litre.

Smith, Smith, Joslin, & White (1937) investigated the gonadotrophin content of the blood and urine of diabetic women during pregnancy and were unable to find any essential difference from the concentrations observed in healthy pregnant controls.

Further investigations on the serum gonadotrophin of pregnant diabetic women, with and without toxaemia, have been reported by Rubin, Dorfman & Miller (1946).

In Non-pregnant Women.—Frank, Goldberger, & Spielman (1931), using a special concentration method, were able to demonstrate the presence of gonadotrophins in the blood of non-pregnant women. By improved methods of concentration, Frank & Salmon (1935, 1936a) were able to show the presence of both follicle-stimulating hormone and luteinizing hormone in the blood during certain stages of the menstrual cycle.

Thomsen & Pedersen-Bjergaard (1935), by tannic acid concentration methods, found minute quantities of gonadotrophins in the urine of normal non-pregnant subjects. Levin (1941) also used tannic acid concentrates to study the gonadotrophic excretion throughout the menstrual cycle.

Owing to the presence of gonadotrophins in the non-pregnant state it is evident, as Aschheim (1935) stresses, that “the basis of the biological pregnancy test lies in the quantitative and not in the qualitative hormonal differentiation of the blood or urine of the pregnant woman as contrasted with the blood or urine of the non-pregnant woman. This differentiation is so exceptionally great that it may be considered the outstanding characteristic of the blood and urine in pregnancy.”

In Menopause and Castrate Women.—Brühl (1929a) and Fluhmann (1929a, b) observed that gonadotrophins were frequently present in the urine and blood of women following the menopause, and in the urine of women who had been ovariectomized. The gonadotrophin, in these cases, is practically entirely follicle-stimulating in nature and can be distinguished from pregnancy-
urine gonadotrophin by producing only Anterior Pituitary Reaction 1 in the Aschheim-Zondek test. (See also reviews by Zondek, 1931e, 1935; Engle, 1939).

In Pathological Conditions.—Certain neoplasms, especially hydatidiform mole, and chorioepithelioma, may secrete large quantities of gonadotrophin. Detailed consideration of these pathological conditions is beyond the scope of this review, but as these conditions may give rise to large quantities of follicle-stimulating hormone and luteinizing hormone which will produce reactions in test animals identical with those observed following injection of pregnancy urine, they must be kept in mind when interpreting pregnancy tests. Further information will be found in the reviews by Zondek (1931e, 1935, 1942), C. Ehrhardt (1936a), Randall, Magath, & Pansch (1940), Van Dyke (1939), Robson (1940), Schorlemer (1940), and Hamburger (1943).

It is of interest to note that gonadotrophins are present in the urine of non-pregnant women who have received blood transfusions from pregnant donors, positive Aschheim-Zondek tests being obtained up to 24 hours after the transfusion (Ehrhardt, 1930; Ehrhardt & Ruhl, 1933).

In Body Fluids other than Blood and Urine.—Traces of gonadotrophins were noted in the saliva of pregnant women by Zondek (1929c, 1932a). Trancu-Rainer (1931) concluded that the concentration of gonadotrophin in the saliva during pregnancy was a tenth of that present in the urine. On the other hand, Ofstad (1932) failed to obtain positive results with saliva, although some hyperaemia of the ovaries and uteri of the test-animals was noted. Heim (1932), Weisman & Yerbury (1936), Cameron & Guthrie (1941) all obtained negative results with the saliva of pregnant women.

Zondek (1932a) and Ehrhardt & Ruhl (1933) noted the occasional presence of follicle-stimulating hormone in sweat.

Follicle-stimulating hormone is rarely present in vaginal secretion (Zondek, 1932a).

There is some evidence that the colostrum contains small quantities of gonadotrophins immediately before and after parturition (Aschheim & Zondek, 1927; Brühl, 1929b; Heim, 1931a,b; Konsuloff, 1933).

No biological fluid other than blood or urine has proved satisfactory for use in pregnancy tests.

Origin of Gonadotrophin.—There were two main schools of thought concerning the origin of the gonadotrophins present in the blood and urine of the pregnant woman. One school, championed by Zondek, believed that the gonadotrophins were mainly produced by the anterior pituitary and simply stored in
the placenta; the other school, ardently sponsored by Philipp, maintained that the gonadotrophin represented an internal secretion of the placenta.

That the placenta can secrete gonadotrophin has now been proved beyond doubt by the tissue culture experiments of Nogayama (1937), Gey, Jones, & Hellman (1938), and Jones, Gey, & Gey (1943). Tissue cultures of the placenta retained their capacity to produce gonadotrophins for periods up to six months. Oestrogens were not detected in these cultures.

It is now generally accepted that the gonadotrophin in the blood and urine of pregnant women is chorionic in origin and is mainly luteinizing in action.

See also reviews by Van Dyke (1936), and W. H. Newton (1938).

DETECTION OF HORMONES BY PLANTS.

The effect of urine on the germination of seeds was used as a pregnancy test in ancient Egyptian, Hippocratic, and Pseudo-hippocratic medical practice (see review by Bayon, 1939). That this test was not entirely founded on superstition or magic has been shown by recent work.

Schoeller & Goebel (1931) demonstrated that crude oestrogen prepared from pregnancy urine had a stimulating effect on the growth and flowering of hyacinths and maize. Later experiments by these workers (1932) showed that pure oestrogen did not possess this activity. This was confirmed by Virtanen & Hausen (1933), and Virtanen, Hausen, & Saastamoinen (1934). Küstner (1931b,c, 1932) also showed that human pregnancy urine contained a substance which stimulated the germination and growth of barley. This stimulating effect could be increased by keeping the plants in red light. The activity of the urine was destroyed by boiling. Manger (1932, 1933) tested the urine of 100 pregnant women; 77 stimulated the growth of wheat or barley. He concluded that oestrogens inhibited the growth of wheat and stimulated the growth of barley, and that gonadotrophin was without effect on wheat and inhibited barley. Hoffmann (1934) considered it possible to diagnose pregnancy from the growth of cereals, but the time required was too long for practical purposes. He found pregnancy urine had an initial inhibitory effect on germination, but a stimulatory action on the vegetative development. Since boiled urine was equally effective, he considered that the effect was not due to gonadotrophins. Bak (1935a,b) tested 514 samples of pregnancy urine; 442 of these showed a growth stimulating effect on barley and wheat when compared with control urines from non-pregnant women and men. He concluded that the method was not sufficiently reliable and
that the active substance was neither oestrogen nor gonadotrophin. Virtanen (1935) claimed that pregnancy urine gonadotrophin (precipitated from urine with alcohol and extracted with ether) had a decided accelerating effect on the germination of seeds, the effect being evident in a few days. He proposed developing a test on these lines.

The scarcity of test animals in Hungary has led Orban (1947) to reinvestigate the possibility of testing for hormones in pregnancy urine by means of plants. He has claimed good results using the gladiolus as a test plant. Three gladioli are placed in the urine to be tested and three control stems are placed in non-pregnancy urine. Pregnancy urine causes an acceleration of flower and bud growth. The test is read in 16 to 24 hours by comparing the size of the blown buds. In 56 tests, 44 positive results (from women 3 weeks to 5 months pregnant) and 8 negative results were correct: in two cases the urine was stale and the gladioli failed to bloom, in the other cases the results were doubtful. Orban considers that gonadotrophins in the urine are responsible for the reaction.

CHEMICAL TESTS USING URINE.

Owing to the technical difficulties associated with biological tests, numerous chemical and biochemical tests for pregnancy have been tried. (These will be dealt with in a later section of this review.) Owing to the very complex chemical nature of the gonadotrophic hormones, it is only within the last few years that attempts at testing chemically for their presence in urine have been made.

Hartmann & Benz (1938) demonstrated that gonadotrophins in highly purified states contained considerable quantities of carbohydrate, and they believed that glycoproteins were present in the gonadotrophic factors. Chorionic gonadotrophin was thought to contain both mannose and galactose. Further studies on pregnancy urine gonadotrophin by Gurin, Bachman, & Wilson (1939a,b) revealed that it contained acetyl groups, hexosamine and a carbohydrate, and they considered it a carbohydrate-polypeptide complex which had the properties of a mucoid. The glucosamine content of the various gonadotrophins was studied by Evans, Fraenkel Conrat, Simpson, & Li (1939). Further studies by Gurin, Bachman, & Wilson (1940a,b), and Gurin (1942) led them to believe that the highly purified preparations they had obtained were practically homogeneous glycoproteins with a minimal molecular weight between 60,000 and 80,000. The non-hexosamine carbohydrate of chorionic gonadotrophin appeared to be galactose. For further details regarding the isolation and chemistry of the
GONADOTROPHIC HORMONES

Gonadotrophic hormones see Fraenkel-Conrat, Li, & Simpson (1943), Gurin (1944), Chow (1944), and Meyer (1945).

Attempts have been made to utilize the reducing properties of chorionic gonadotrophin as a test for its presence in pregnancy urine. The Visscher-Bowman test (see page 136) was an early attempt to diagnose pregnancy from the reducing properties of the urine, but many non-specific substances affected this test. Bowman, Visscher & Mull (1934, 1935) having noted that gonadotropic preparations would reduce the oxidation-reduction dye, o-chlorophenol-indophenol, attempted to use this reaction to detect gonadotrophic hormones in urine. The gonadotrophin was precipitated from the urine under test with tungstic acid and then further purified by extraction with pyridine and re-precipitation with acetone. The dye was added to the purified precipitate: with precipitates from non-pregnancy urine the dye turned a distinct blue, but when added to extracts from pregnancy urine it faded to an almost colourless solution. Of 35 samples tested by this method, 31 gave correct results.

In 1939, Bowman evolved a technique which, he claimed, eliminated the non-specific reducing substances from the specific substance related to pregnancy by fractional precipitation with acetone at a definite pH: the specific substance was considered closely associated with gonadotrophin. The reducing power of the substance was found by titrating with 0.0005N iodine solution. 303 cases were tested by this method with an accuracy of 98.4%. Notthdurft (1944) was only partially able to confirm the accuracy of Bowman's test, and he showed that gonadotropic preparations from pregnancy urine contained a factor exhibiting diastatic activity which was not identical with the gonadotropic hormone. He considered Bowman's method to be a measure of this diastatic factor. Bowman (1941) concluded that the oxidation-reduction properties of gonadotrophin preparations prepared by the benzoic acid method (Katzman & Doisy, 1932), with subsequent re-precipitation with acetone, could be attributed to the hormone itself, and he reported that he was working on a pregnancy test in which the reducing capacity of the hormone was used. Mello (1942), using a slightly modified form of the test proposed by Bowman (1939), obtained correct results on 23 pregnancy urines, in some cases as early as two weeks after the first missed period. In the following year (1943) Mello modified the test still further by adsorbing the gonadotrophin on kaolin (Scott, 1940) and eluting with 0.1N NaOH. The alkaline solution was treated with Somogyi's reagent and the excess of copper not reduced was determined isotometrically. 534 determinations were carried out on urine samples from 197 cases of pregnancy. 31 non-pregnancy
cases, and 10 males. Biological tests were also carried out and the deviation between the chemical and the biological tests did not exceed 4.5%. Bowman (1945) was unable to confirm the accuracy of Mello's (1943) method and showed that small percentages of sugar and other reducing substances in the urine would interfere with the test. Bowman concluded that "at present a suitable means for the chemical determination of pregnancy based upon the reducing properties of gonadotropin, if feasible, has not as yet been adequately defined."

Ultra-violet Absorption.—Huwer (1934) studied the ultra-violet absorption curves in an attempt to detect the presence of hormones in urine and serum of pregnant women. Uric acid, however, interfered with the method and it proved impossible to remove this without at the same time destroying or removing the hormones.

MARE.

FEMALE MOUSE AND RAT AS TEST ANIMALS.

Tests Using Urine.—Brühl (1929a), Schoop (1930), and Kunze (1930) applied the Aschheim-Zondek test to the urine of pregnant mares; all three investigators obtained negative results and concluded that gonadotrophins were not present. In more extensive investigations Zondek (1930g) demonstrated, by alcohol precipitation methods, small quantities of follicle-stimulating hormone. Zondek elaborated a test for pregnancy in the mare which he claimed depended on the presence of follicle-stimulating hormone (FSH) and oestrogen in the urine. As urine of mares was found to be very toxic to the immature mouse, Zondek employed immature rats as test animals. The urine was subjected to "ether-treatment" (see page 20) which was believed to detoxicate it and enhance the action of the follicle-stimulating hormone (FSH) and the oestrogen by removing interfering substances. Five immature rats were injected with a total of 0.3-0.6 ml of urine over a period of three days; vaginal smears were then examined for the appearance of oestrus. A positive test is recorded if one or more rats have shown an oestrous smear by the 5th day. In a series of 80 tests, Zondek had only two errors by this method. It must be noted that this method differed from the Aschheim-Zondek test in the woman in that it was based on the presence of follicle-stimulating hormone (FSH) producing Anterior-Pituitary Reaction I (APR I) (i.e., follicle ripening and vaginal oestrus) and oestrogens in the urine producing vaginal oestrus. Küst & Grawert (1930), Küst (1931a,b), and McKenzie (1931) failed to detect gonadotrophins in pregnant mares' urine. Goss & Cole (1931) found only traces of gonadotrophin in the
urine of two of eight pregnant mares between the 40th and 75th day of pregnancy, although during this time it was present in high concentration in the blood. Further studies on the presence of gonadotrophins in mare pregnancy urine (Hart & Cole, 1932; Catchpole, Cole, & Pearson, 1935) confirmed that only rarely do small amounts of the hormone appear in the urine. It must thus be concluded that the Zondek test depends practically entirely on the presence of oestrogens in the urine and further work on it will be considered under that heading. Gonadotrophins have been reported in pregnant mares' urine by Di Giano (1936) and München (1937).

Ehrhardt (1932b) reported the presence of follicle-stimulating hormone in the urine of pregnant asses.

Tests Using Blood.—The presence of large quantities of a gonadotrophic hormone in the blood of pregnant mares was first demonstrated by Cole & Hart (1930a,b). In studies on 62 mares they noted that when the blood serum from a pregnant mare was injected into immature rats it produced marked enlargement and extensive histological changes in the ovaries. The gonadotrophic activity of the serum appeared about the 37th-42nd day of pregnancy; it was most marked between the 43rd and 80th day. It then gradually diminished and disappeared and oestrogenic hormones appeared in its place. The authors indicated the practical importance of this discovery as a test for early pregnancy in the mare. Zondek (1930c) confirmed the presence of gonadotrophins in the blood of the mare during the early part of pregnancy, Küst & Zumbaum (1931) and Küst (1931b, 1932b,c,d) obtained reliable results using the blood serum from mares in the first 3½ months of pregnancy. Immature mice were found to be as satisfactory as immature rats, Anterior-Pituitary Reaction I (APR I) and often Reaction II and III being present. Further details of their technique were given by Hart & Cole in 1932. They recommend the use of at least three immature mice or rats. Two or three injections of serum were given, doses of 0.2 ml. to the mice and 2.0 ml. to the rats (or single injections of 0.5 ml. and 3.0 ml.), and the vaginal smear examined at the 96th hour; the animals were then killed and the ovaries and uterus examined and weighed, a control animal also being killed. For routine work they advised making tests between the 45th and the 150th day of pregnancy. Wolters, Sitterlin & Krampe (1932) carried out 108 tests on the serum from 80 different mares; in every case the diagnosis was correct. They considered that gonadotrophins were present from the 50th to 170th day. Catchpole (1934) found that routine testing of the mares on stud farms at the 42nd to the 45th day after service led to a much greater efficiency in
the management of breeding. This test aroused considerable interest and numerous reports on its use appeared in the literature. It is difficult to give a precise definition of its accuracy, but it would appear that when used with care and when only samples of serum from mares that have been served 45-110 days previously are tested an accuracy of 95-98% can be expected. The reasons for the poor results obtained by certain investigators include: insufficiently standardized procedure or test animals; applying the test too soon after service owing to incorrect service dates; the occurrence after the date of testing of unobserved abortion or resorption of the foetus. The above sources of error are discussed more fully by Cole & Hart (1942), Mayer (1944), and Zavadovskij & Nesmejanova-Zavadovskaja (1945).

For tests suitable for mares more than 110 days pregnant see Oestrogenic Hormones, page 76.

The following investigators have described their experiences with the test: Karmann & Wiethoff (1933), Stamler, Subgaler, & Fraermark (1933), Schätzl (1933), Glud, Pedersen-Bjergaard, & Portman (1933a,b), Küst (1933a), Catchpole (1934), Karmann (1934), Hamburger (1934), Stålfora & Hoflund (1934), Magnusson (1934a,b), Märtensson (1934, 1935), Becker (1934), Fritz (1934), Zavadovskiñ (1934), Richter & Gehring (1935), Stamler (1935), Barulin & Burčenko (1935a), Miller (1935a), Panin (1936), Burčenko (1936), Kaay, Jaarsma, & Groothuis (1936), Barulin (1956), Hennaux (1937), Svensson (1937), Hansen & Dybing (1937), Varley (1938), Schramm (1938), Miller & Day (1939), Kérov (1939), Samodelkin (1989), Svecin (1939), Day & Miller (1942), Cole & Hart (1942), Sachweh (1942, 1943), Mayer (1944), Zavadovskiñ (1945), Inglis & Robertson (1946). Rapid Tests with Rat.—Cole & Erway (1941) found it possible to read the immature rat test at the 48th hour by taking into consideration the increase in ovarian weight which proved to be as significant at the 48th hour as after longer intervals. This method was found to be as sensitive and accurate as the 96th-hour test (Cole & Hart, 1942). A single injection of 5 ml. of serum produced a 100% increase in uterine weight and/or a 50% increase in ovarian weight. Zondek & Sulman (1945a) used the hyperaemia of the immature rat ovary following injection of pregnant mares’ serum as a means of reading the test. In this way results can be obtained at the 24th hour. The test animals may be examined as early as 4-6 hours after the first injection, but then only positive results are reliable. Further investigations by Zondek and Sulman (1947) have shown that the most reliable period for reading the
The hyperaemia test is from the 11th to 24th hour. In view of the variability of the concentration of gonadotrophin in the blood of pregnant mares, the authors suggest that the diagnosis of pregnancy should be made on the basis of both the 11- to 24-hour hyperaemia test and the ordinary 96-hour test (see page 51).

Valle (1947) tested by the hyperaemia reaction blood samples from 38 mares and 4 female donkeys in the period from the 42nd to 138th day after service. Two infantile rats were used per test, one receiving 1 ml., the other 2 ml. of serum. The test was read at the 24th hour. The tests agreed with the clinical condition in 35 cases. Four pregnant mares gave negative tests 85, 75, 65, and 63 days after service, while three non-pregnant mares (aged 11, 13, and 18 years) gave false positive tests. Valle concludes that the ovarian hyperaemia test is not an exact method of pregnancy diagnosis in the mare.

**RABBITS AS TEST ANIMALS.**

*Tests Using Urine.*—Remlinger & Bailly (1934b) obtained negative results with urine from mares advanced in pregnancy.

Di Giano (1936) tested urine of ten mares, 2-6 months pregnant; seven gave a positive Friedman test.

Morcos (1940) reported that injections of urine from pregnant mares (2-3 weeks after service) produced thickening of the rabbit uterus, an effect which Morcos regarded as diagnostic although ovarian changes were absent.

*Tests Using Blood.*—Catchpole (1934) pointed out that the rabbit was less suitable than the immature rat as it was much less sensitive to gonadotrophin of pregnant mares' serum. Nevertheless, it has been used with success by several investigators. Magnusson (1934a,b) found the rabbit reliable in a series of 284 tests, provided only rabbits of 1,400 g. or over were used. Mårtensson (1934) found the rabbit test as accurate as the mouse test. Remlinger & Bailly (1934b) obtained negative results in a few tests on serum of pregnant asses and concluded that the test was valueless. As the mares were all in the advanced stages of pregnancy, when the gonadotrophin has disappeared from the blood, negative results were only to be expected. Cole & Hart (1942) conclude that the rabbit is satisfactory if samples are restricted to the 49th-84th day of pregnancy. The following workers have reported on the successful use of the rabbit as a test-animal: Grini (1935), Magnusson (1935a,b), Mårtensson (1935), Přibyl (1935a,b), Cuboni (1936a), Barulin & Burčenko (1936), Kerov (1939), Plum & Portman (1939), Vandevenne & Heuwerswijn (1940), Thomsen (1941), Berthelon (1944), Robin & Petrov (1945).
OTHER TEST ANIMALS

Male Mammals.—McKenzie (1931) observed that male mice were suitable test animals for detecting gonadotrophin in serum of mares. Hart & Cole (1932) carried out a few preliminary tests on the effect of pregnant mares' serum in male rats and mice. Increase in size of the testes and marked increase in size of the seminal vesicles were noted. Wolters, Sütterlin, & Krampe (1932) made a similar observation on the effect of the serum in male mice. More detailed observations on the immature male rat were recorded by Cole, Guilbert, & Goss (1932). Magnusson (1934a,b), Mårtensson (1934), and Kazarnovskaja (1937) considered that although male mice could be used, they were somewhat less reliable than female mice. Cole & Goss (1939) considered that approximately twice as much hormone was required to give a seminal-vesicle response as was required for an ovarian response.

Birds.—Hamburger (1934, 1936) and Martins (1934) noted that pregnant mares' serum gonadotrophin, like pituitary gonadotrophin, but unlike human pregnancy urine gonadotrophin, when injected into young cockerels stimulated the development of the testes and growth of the comb. Martins also observed some comb growth in female chickens, but this was not so marked as in the male. Similar results were obtained by Asmundson & Wolfe (1935). Lahr, Riddle, & Bates (1935) noted that injections of pregnant mares' serum increased the weight of the testes in the adult dove. Rocha e Silva & Trapp (1937) obtained negative results with chickens, but the sera tested appear to have been from mares that were at least five months pregnant. Zavadovskii & Nesmejanova-Zavadovskaja (1937) successfully used the growth of the comb in the male chick as a test for pregnancy in mares. Further experiments with this method (Zavadovskii, 1942a, 1945) confirmed its usefulness. White Leghorn male chicks (30-70 days old) are injected subcutaneously beneath the wing once a day with 2.5 ml. of serum for 3-5 days. When used between the 42nd and 90th day of pregnancy an accuracy of 98% is obtained with this method.

Xenopus.—Landgrebe (1930) failed to detect gonadotrophins in the urine of mares during the first three weeks of pregnancy by means of the Xenopus test.

Weisman, Snyder, & Coates (1942c) produced oviposition by injecting pregnant mare serum gonadotrophin, but, as egg extrusion in Xenopus appears to depend mainly on the luteinizing factor, relatively large doses of mare gonadotrophin were required and injections of less than 1,000 i.u. failed to react.
PREGNANCY.—Pregnant mare serum gonadotrophin is inactive in the bitterling test (Duyvene de Wit, 1941b).

**DISTRIBUTION OF GONADOTROPHINS.**

**Quantitative Studies.**—Cole & Hart (1930a,b) demonstrated that gonadotrophin could first be detected in the serum of the pregnant mare by the immature rat test from the 37th-42nd day, the greatest concentration being present between the 43rd and 80th day. Between the 80th and 180th day there was a gradual diminution. Subsequent work has not altered these original findings in any significant way. Comparison of the actual quantities of gonadotrophin present in the serum as observed by various authors is made very difficult owing to the different methods of defining a "unit" and this aspect will not be discussed in any detail. Experiments on four mares throughout pregnancy (Cole & Saunders, 1935) confirmed the presence of the hormone from the 50th-150th day with a peak concentration about the 70th day. Pedersen-Bjergaard & Glud (1936), using mice as test animals, found gonadotrophin present from the 40th-120th day. Cole (1938) studied the hormone concentration in pregnant Welsh ponies and found the concentration of gonadotrophin in the serum was about four times as great as found in draught horses and thoroughbreds. The peak occurred between the 60th and 75th day. Day & Rowlands (1940), in studies on six Welsh ponies, a New Forest pony and a Shetland pony, noted the appearance of gonadotrophins from the 32nd to 49th day of pregnancy; the peak concentration occurred between the 62nd and 75th day when quantities of 43,000-352,000 i.u. per litre were found. The gonadotrophins had almost completely disappeared from the blood by the 110th day. Aylward & Ottaway (1945) tested the blood from seven pregnant Welsh ponies from the 45th to 80th day of pregnancy; maximum values were obtained between the 54th and 64th day when 78,000-440,000 i.u. of gonadotrophin per litre were present. Further quantitative studies on the serum gonadotrophin in Welsh and Shetland ponies have been reported by Day & Rowlands (1947).

**In Non-pregnant Mares.**—Cole & Goss (1939) demonstrated that gonadotrophin is present in the serum of the non-pregnant mare, but to detect it by the immature rat test concentrates must be prepared from the serum (Cartland & Nelson, 1937) and the equivalent of 100-200 ml. of serum injected. There was some indication that the greatest hormone concentration was reached during metoestrus. The hormone could not be distinguished biologically from that present in the pregnant mare. Rimington & Rowlands (1944), however, failed to detect gonadotrophic
activity in highly concentrated preparations of serum from non-pregnant mares.

In Pathological Conditions.—Küst (1932c) observed that in certain pathological conditions in non-pregnant mares, such as ovarian cysts, where a positive oestrogen test is frequently obtained in the urine, the blood test for gonadotrophins was negative. Ståltors & Holfund (1934) reported negative blood tests on a mare suffering from pyometra.

In Body Fluids other than Blood and Urine.—Schäper (1931) was unable to detect gonadotrophic hormones in the allantoic fluid of the mare.

Trancu-Rainer and Vladutiu (1937) claimed to detect small quantities of gonadotrophin in the saliva of the pregnant mare.

Imreh (1940) failed to detect gonadotrophin in the colostrum of the mare.

Origin of the Gonadotrophin.—The pituitary of the horse is rich in gonadotrophic activity (Hellbaum, 1933; Hill, 1934). Catchpole & Lyons (1934) found that the pituitaries of mares in early pregnancy contained more gonadotrophin than those of mares in late pregnancy.

Its presence in the allanto-chorion and endometrium was demonstrated by Catchpole & Lyons (1933, 1934).

In 1933, Catchpole & Lyons considered that the evidence as to the origin of the hormone in the blood of the pregnant mare was equally balanced between the pituitary and the foetal and maternal placenta. In the following year they considered that the facts favoured the placental origin (chorionic epithelial cells).

In a recent paper by Cole & Goss (1943) some very interesting facts on the origin of the blood gonadotrophin in the pregnant mare have been established. Quantitative studies were made on the gonadotrophic concentration in the tissues of four pregnant mares killed between the 62nd and 105th day of pregnancy, coincident with histological studies of the endometrium and chorion. Distributed over a part of the endometrium in apposition to the chorion are specialized structures called “endometrial cups.” These cups were found to contain 4-12 i.u. of gonadotrophin per mg. of fresh tissue, and the secretion which they elaborate into the uterine space contained 50-314 i.u. of gonadotrophin per mg. in the fresh state. The authors conclude that these endometrial cups are the chief source of equine gonadotrophin. Considerable quantities were also present in the endometrium between the cups. These interesting observations of Cole & Goss have now been confirmed and amplified by Rowlands (1947).

Biological Properties.—The gonadotrophic hormone of pregnant
GONADOTROPHIC HORMONES

Mare serum is unquestionably follicle-stimulating in its effects (see review by Van Dyke, 1939).

Cole, Pencharz, & Goss (1940) concluded that the gonadotrophic activity of mare serum (exclusive of the augmenting effect of serum proteins on pituitary extracts) was dependent not on a mixture of follicle-stimulating hormone and luteinizing hormone, but upon a single gonadotrophin.

In the Stallion.—Küst (1932c,d) was unable to detect gonadotrophins in the urine and blood of the stallion (cf. Oestrogenic Hormones, page 80). Trancu-Rainer & Vălăduțiu (1937) claimed to have detected traces of gonadotrophin in the urine of stallions and geldings, as well as traces in the saliva.

Chemical Methods of Testing.

Chemistry of Mare Serum Gonadotrophin.—Evans, Gustus & Simpson (1933) prepared a concentrate of the gonadotrophin from pregnant mares' serum by adsorption on alumina. In 1937, Cartland & Nelson prepared purified extracts of the gonadotrophin by fractional precipitation with acetone or alcohol. Fleischer, Schwenk, & Mayer (1938) reported that pregnant mare serum gonadotrophins contained considerable quantities of reducing sugar, hexosamine and acetyl groups. Improved methods of concentration were described by Rinderknecht, Noble, & Williams (1939), and Rimington & Rowlands (1941, 1944), the latter workers obtaining preparations assaying 12,500 i.u. per mg.

Rimington & Rowlands (1941) also reported the presence of hexose in the active material. Gurin (1942) observed that the gonadotrophin of pregnant mare serum, like the gonadotrophin from human pregnancy urine, appeared to contain galactose rather than mannose which was present in pituitary gonadotrophin. Hexosamine was also present. Rimington & Rowlands (1944) pointed out that the carbohydrate content of serum concentrates was no index of their activity, since it was possible to prepare from non-pregnancy serum by their method of concentration a material devoid of biological activity, but containing the same amount of carbohydrate as their most active preparations. They conclude that active serum gonadotrophin must be only one of several glycoproteins of very similar composition. (See also review by Gurin, 1944.)

Chemical Tests.—Otte (1938a,b, 1939a,b) (see page 62) claimed to diagnose pregnancy in the mare by the presence of the so-called "Harn-Prolan" in the urine. This, he claimed, was regularly present in the urine of the non-lactating mare from the 42nd day of pregnancy. The urine sample had to be taken from rested mares, as muscular work caused the "Harn-Prolan" to disappear.
from the urine. This material from the pregnant mare appears to have been biologically inactive as was the material from the cow.

COW.

FEMALE MOUSE AND RAT AS TEST ANIMALS.

Tests Using Urine.—Aschheim & Zondek (1928b), Zondek & Aschheim (1928a), and Zondek (1931e) failed to find gonadotrophin in the urine of the pregnant cow. The success of the test in the woman, however, stimulated much interest and many attempts were made to apply the test to the cow. So far as the use of mice and rats as test animals is concerned, the results have been entirely negative. The following workers have failed to detect gonadotrophin in the urine of the pregnant cow: Bölcsész (1929a,b), Brühl (1929a), Plank (1929), Allan & Dickens (1930), Kunze (1930), Leonard (1931), Küst (1931b), Wiethoff (1931), Langlotz (1932), Collip (1932), Čalkovskii & Bondarenko (1933), Bruhn (1933), Asdell & Madsen (1933), Witzsch (1933), Chacun (1933), Küst (1934a,b), Přibyl (1935), Cicogna (1935), Di Giano (1936), Coronel (1936). As the conclusions of the above workers have all been in agreement, there is no need to review their work in any detail.

Zondek (1930g) and Di Giano (1936) attempted to detect gonadotrophin in pregnant cows' urine by injecting precipitates (alcoholic) into immature mice and rats, but the results were negative.

Tests Using Blood.—Zondek & Aschheim (1927a, 1928a) and Zondek (1929c, 1931a) failed to detect gonadotrophins in the serum of pregnant cows. Numerous investigators have examined bovine pregnancy serum and all have agreed with the findings of Zondek & Aschheim. Wiethoff (1931), Bertrams (1931), Collip (1932), Bruhn (1933), Asdell & Madsen (1933), Přibyl (1935a), Anderson (1936), and Cuboni (1939a,b) all obtained negative results.

RABBIT AS TEST ANIMAL.

Since, with the rabbit as a test animal, certain workers have claimed to detect gonadotrophins in the urine of pregnant cows it is necessary to deal with the literature on this subject more fully than in the case of other test animals.

Negative Results.—Costa-Sacadura & Rosa (1932) found no change in the ovaries or uterus of the rabbit following injections of 5-25 ml. of bovine pregnancy urine. Negative results were also obtained by Carbone (1933) who tested the urine from 22 pregnant cows (all stages of pregnancy) and eight non-pregnant
cows, the urine being injected in quantities totalling 50 and 70 ml. Urine concentrated in vacuo and blood serum (50 ml.) were also tested. A few unilaterally ovariectomized rabbits were included among the test animals. In every case the results were negative. Asdell & Madsen (1933) reported negative results with the blood and urine from pregnant cows. Large volumes of urine (up to 120 ml.) from pregnant cows were injected into rabbits by Remlinger & Bailly (1934a) with negative results. Rosa (1936) injected intravenously ether-extracted urine from a cow three months pregnant into rabbits in total doses of 50 and 100 ml. over a period of five days. No signs of gonadotrophic activity were observed, the uterus and vagina appearing anaemic. Coronel (1936) found the rabbit quite unresponsive to injections of urine from pregnant cows.

Extensive investigations were carried out by Cuboni (1939a,b,c, 1941). He prepared extracts of 1 and 2 litres of urine from pregnant cows by the Katzman and Doisy method, but these gave negative results. Further studies were made on the effect of injecting pregnant cows' serum intravenously and intraperitoneally into rabbits, some of which received simultaneous injections of oestrogen. Particular attention was given to the period of pregnancy from the 19th to the 96th day when a pregnancy test would be most useful in the cow. The tests were all negative, even with doses of serum as high as 60 ml. Bovine serum, when administered with human pregnancy serum, had no inhibitory action on the gonadotrophic activity of the human serum.

Positive Results.—Papi (1933) was the first to claim positive Friedman tests with the urine of pregnant cows. Injections of 15 ml. of urine from two heifers, 2 months and 7½ months pregnant, made subcutaneously into immature rabbits increased the size of the ovaries, produced swelling and congestion of the uterus and lengthening of the uterine horns, but did not produce haemorrhagic follicles. Urine from a non-pregnant heifer had no effect on the reproductive system. Intravenous injections of urine from cows one, two and five months pregnant, in doses of 10 ml. into adult rabbits, produced haemorrhagic follicles and swelling and congestion of the uterus. Although the number of tests was small, Papi concluded that the test might prove a valuable aid in the diagnosis of bovine pregnancy.

Results of a similar nature were obtained by Morini (1934) who injected immature and pregnant rabbits with 5 ml. of urine. All the rabbits injected with pregnancy urine showed congestion and enlargement of the uterus, the ovaries being enlarged or haemorrhagic or both. Pregnant rabbits appeared to be some-
what more sensitive than immature. Urine from non-pregnant cows gave entirely negative results. Morini concluded that his findings confirmed those of Papi. In a more extensive investigation, Menzani & Gentile (1934) tested the urine from 29 pregnant cows (29 to 150 days pregnant); in only two cases were positive results obtained (i.e., production of hemorrhagic follicles and corpora lutea atretica): these were animals 32 and 37 days pregnant. It is of interest to note, however, that although only two results were positive, a considerable number of rabbits injected with pregnancy urine showed marked congestion and swelling of the uterus, whereas in rabbits injected with control urine the uterus was only lightly hypaemic. They did not consider the test reliable in the cow. Di Giano (1936) tested urine concentrates (alcohol precipitation) from 10 pregnant cows (16 days to 7 months); all were negative but in three cases with urine from cows 6-7 months pregnant congestion of the uterus was noted.

Positive results were also reported by Pacheco Perez (1939). He injected rabbits with 5 ml. of urine intravenously and 15 ml. subcutaneously and examined the ovaries at the 48th hour. Urine from 5 cows 7-8 months pregnant, produced maturation of the follicles and corpora lutea formation, while urine from 5 non-pregnant cows gave negative results.

Morcos (1940) injected intraperitoneally urine from 15 pregnant cows and heifers and from several buffaloes into mature virgin rabbits. According to the usual criteria these tests gave negative results since no ovarian changes were observed, but in every case the uterus became greatly thickened and coiled. Morcos suggests that by relying on this uterine reaction, early pregnancy in the cow can be readily diagnosed.

If gonadotrophins exist in the blood and urine of the pregnant cow they are certainly not present in a form that can be detected by the usual biological methods, apart from a few sporadic instances. These positive cases have only been observed with rabbits as test animals and in relatively very few cases. There would appear, however, to be a substance frequently present which produces enlargement and congestion of the rabbit uterus, but whether this substance is present in all bovine pregnancy urine in the early stages of pregnancy, and is sufficiently specific to allow it to be used as a test for pregnancy, as suggested by Morcos, requires further research. Since thickening and congestion of the rabbit uterus has been observed with urine from the early stages of pregnancy (Menzani & Gentile, 1934; Morcos, 1946), it would seem unlikely that the substance producing this effect is an oestrogenic hormone (see page 87).
OTHER TEST ANIMALS.

Guinea-pig.—Papanicolaou (1931) injected immature guinea-pigs with urine from pregnant cows and observed only an increase in vascularization of the follicles. Coronel (1936) failed to obtain any gonadotropic response to injections of bovine pregnancy urine in guinea-pigs.

Male Mammals.—Cvrkovic (1932) injected urine from nine pregnant cows into immature male mice; the results were all negative. Negative results were also obtained by Costa-Sacadura & Rosa (1932) who injected bovine pregnancy urine into male rabbits and examined the testes and accessory reproductive glands. Sartoris (1937) injected immature male mice with pregnancy urine and urine concentrates (alcohol precipitation); the tests were all negative.

Xenopus.—Urine and serum from a cow 22 weeks pregnant gave negative results when tested on Xenopus (Greenbaum, 1943).

DISTRIBUTION OF GONADOTROPHINS.

The presence of gonadotropic hormones in the anterior pituitary of the cow was demonstrated by Zondek & Aschheim (1926, 1927a,b) by implanting pieces of bovine pituitary into immature mice. This observation was confirmed by Bölsházy (1929b), Ehrhardt & Mayes (1930), and Bacon (1930). Zondek (1931a) demonstrated that during pregnancy in the woman there is very little gonadotrophin in the anterior pituitary, whereas in the cow there is essentially the same amount as during the non-pregnant state. Magistris (1932), however, found a considerable increase in the gonadotropic content of the cow during pregnancy. Further studies in this field have been made by Bates, Riddle, & Lahr (1935), and Smelser (1944).

Cole & Earle failed to find gonadotrophins in extracts of blood and urine from non-pregnant cows (Cole & Goss, 1939).

Gutman (1930) failed to find gonadotrophins in the bovine placenta, endometrium or amniotic fluid. A similar finding was reported by Zondek (1931a).

Body Fluids other than Blood and Urine.—Wiethoff (1931) was unable to detect gonadotrophins in the milk of pregnant cows. Its absence was also noted by Heim (1932), Bruhn (1933), Halbfisch (1933), and Weisman, Kleiner, & Allen (1935).

Küst (1932a) and Bruhn (1933) failed to find gonadotrophins in the saliva of pregnant cows.

In Pathological Conditions.—Langlotz (1932) tested the urine from cows suffering from pyometra, cystic ovaries, and persistent corpus luteum, and in no case were gonadotrophins present.
Bruhn (1933) failed to find gonadotrophins in the urine, blood or saliva of cows in oestrus, cows with ovarian cysts or cows suffering from nymphomania.

Velasquez & Engel (1940) reported positive Friedman tests with the urine of five non-pregnant cows suffering from cancer of the eye.

**Chemical Methods of Testing.**

For the sake of convenience the researches of Otte (1938a,b,c, 1939a,b) will be mentioned here, although it is very doubtful if his tests are in any way specific for gonadotrophic hormones. Otte applied the chemical methods for preparing concentrated preparations of gonadotrophins from human pregnancy urine to the urine of the cow and mare. By dissolving the precipitate, obtained by the addition of acetone to the urine, in water and adding uranyl sodium bicarbonate, a fine precipitate of a protein material appears which Otte termed “Harn-Prolan” or “Trächtigkeitstoffes”. This precipitate did not appear with preparations from non-pregnancy urine. This “Harn-Prolan” be considered to be a compound of oestrogen and specific albumoses only found in pregnant animals. The albumoses differed from the substances obtained from human pregnancy urine in that they were biologically inactive when injected into mice. Otte claimed that this material was present in the urine of the pregnant heifer from the second month. In the cow, however, diagnosis had to be based on the presence of the oestrogen fraction (see page 89) as lactation is said to interfere with the excretion of “Harn-Prolan” and it can only be relied on after the 7th month. Otte points out that muscular exercise and the type of rations fed may influence considerably the excretion of “Harn-Prolan”.

**Other Animals.**

Ewe and Goat.

*Mouse and Rat as Test Animals.*—Ehrhardt (1932b) and Ehrhardt & Ruhl (1933) were unable to find gonadotrophins in the blood of pregnant sheep or in the urine of pregnant goats. Küst (1931b, 1932a) likewise failed to find gonadotrophins in the urine and blood of pregnant sheep and goats. In more extensive investigations, Küst & Vogt (1934a,b) were unable to detect gonadotrophin either in the blood or in the urine or urine concentrates (alcohol precipitation) of pregnant goats at any stage of pregnancy. Similar observations were recorded for pregnant sheep. The blood and urine of non-pregnant goats and sheep gave negative results. Negative results with untreated
and ether-sugar treated urine from goats and sheep during pregnancy were reported by Maurer (1934). Cole & Miller (1935) examined the blood of ewes at various stages of pregnancy, but obtained no sign of gonadotrophic activity. Blood from non-pregnant ewes was also negative.

_Rabbit as Test Animal._—Addis (1933) and Antonelli & Addis (1933) injected urine and blood from pregnant sheep into rabbits; in several cases with urine and blood from sheep more than 45 days pregnant, enlargement and congestion of the rabbit uterus was noted, but in no case were there any ovarian changes. Since a similar uterine reaction was produced by serum from a sheep in oestrus, it was not considered specific for pregnancy. Remlinger & Bailly (1934a) obtained negative results with the urine of two pregnant goats.

_Xenopus as Test Animal._—Landgrebe (1939) injected urine concentrates (in doses equivalent to 50 ml. of urine) from ewes in the first three weeks of pregnancy into Xenopus with negative results.

_Distribution of Gonadotrophins._—The pituitary of the sheep is very rich in gonadotrophic hormones (Van Dyke, 1936). From preliminary experiments, Cole & Miller (1935) concluded that the gonadotrophin content of the pituitary of the sheep does not change greatly with pregnancy.

Küst & Vogt (1934b) found gonadotrophin present in goat pituitary; in pregnancy the gonadotrophic content appeared to diminish.

Gonadotrophins have not been detected in the placenta, amniotic fluid or endometrium of the pregnant ewe (Gutman, 1930; Ehrhardt, 1932b; Ehrhardt & Ruhl, 1933; Küst & Vogt, 1934a).

_Sow._

_Mouse and Rat as Test Animals._—Aschheim & Zondek (1928b) were unable to detect gonadotrophic hormones in the urine of the pregnant sow. This has been confirmed by Plank (1929), Zondek (1931a,e), Küst (1931b), Faermark & Bigos (1933), Küst & Struck (1934), and Lozinski, Holden, & Macallum (1942).

Gonadotrophins have not been found in the blood of pregnant sows (Zondek & Aschheim, 1927a; Zondek, 1931a; Faermark & Bigos, 1933; Evans, Simpson, & Austin, 1933; Küst & Struck, 1934). Lozinski, Holden, & Macallum (1942), however, noted that the ovaries of immature rats injected with serum from sows in the last half of pregnancy were slightly heavier than, and showed some follicular growth in advance of the ovaries of control animals, indicating that the serum might possess some slight gonadotrophic activity.
Rabbit as Test Animal.—Hubmair (1932) tested the urine of 34 pregnant sows and 11 non-pregnant sows. Positive tests were obtained in three rabbits injected with pregnancy urine, but as ovulation was also observed in one of three rabbits which received no injections, it would appear that the isolation of the rabbits had not been sufficiently strict and that spontaneous ovulation had occurred. Carbone (1933) injected rabbits with urine from two sows in the 2nd and 3rd months of pregnancy; total doses of 40 ml. were given with negative results. Negative results were also reported on four sows by Remlinger & Bailly (1934a).

Distribution of Gonadotrophins.—Wolfe (1930) investigated the gonadotrophic content of the sow pituitary at different stages of the oestrous cycle. Zondek (1931a) found that during pregnancy the gonadotrophic content of the sow pituitary was essentially the same as in the non-pregnant animal. Magistris (1932), on the other hand, found a considerable increase in the gonadotrophin content of the sow pituitary during pregnancy.

Philipp (1929) found no gonadotrophin in the placenta of the sow. Gutman (1930) and Zondek (1931a) confirmed this.

Lozinski, Holden, & Macallum (1942) have claimed to detect gonadotrophin in the blood of sows during oestrus.

Bitch.

Mouse and Rat as Test Animals.—Philipp (1929) found no trace of gonadotrophin in urine from a pregnant bitch. In an extensive investigation with urine and urine concentrates from bitches at various stages of pregnancy and urine from non-pregnant bitches, Helm (1931) obtained entirely negative results. Similar negative findings have been reported by Kunze (1930), Ehrhardt (1932b), Küst (1931b), Heim (1931b, 1932), Evans, Simpson, & Austin (1933), Küst (1934b), Finck (1936), and Stiasny (1937).

Küst (1934b) was unable to demonstrate gonadotrophins in the blood of the pregnant bitch.

Rabbit as Test Animal.—Snyder & Wislocki (1931a,b) failed to produce ovulation in the rabbit by intravenous injection of urine from pregnant bitches. Lesbouyries & Bertholon (1936) tested the urine of three pregnant bitches every eight days from the beginning to the end of pregnancy with negative results. Negative results were also reported by Finck (1936). Morcos (1940), however, states that pregnancy can be diagnosed 2-3 weeks after service by injecting the urine into the mature virgin rabbit. In positive tests, although no ovarian changes are present, the uterus becomes greatly thickened and the horns coiled.
Distribution of Gonadotrophins.—Gonadotrophins are present in the pituitary of the dog (Magistriis, 1932; Hill, 1934). Philipp (1929) was unable to detect gonadotrophins in the foetal fluids of the bitch.

Heim (1931b, 1932) was also unable to find gonadotrophins in the amniotic fluids of the pregnant bitch or in the milk of the suckling bitch.

Cat.

Mouse and Rat as Test Animals.—Urines from three pregnant cats were investigated for the presence of gonadotrophic hormones by Allan & Dickens (1930); in two cases the urine proved highly toxic to the test animals and the third gave a negative result. Lubbers (1933) tested the urine of five cats at various times from the 8th to 56th day of pregnancy, as well as urine from non-pregnant cats; in every case negative results were obtained. Negative results were also obtained by Ehrhardt & Ruhl (1933), and Küst (1934b).

Gonadotrophins are absent from the blood of the pregnant cat (Lubbers, 1933; Ehrhardt & Ruhl, 1933; Küst, 1934b).

Rabbit as Test Animal.—Snyder & Wislocki (1931a,b) reported negative results with cat pregnancy urine. Results with the Friedman method have also been negative (Snyder & Wislocki, 1981a,b; Remlinger & Bailly, 1934a).

The presence of gonadotrophins in the rabbit pituitary has been demonstrated by Ehrhardt & Mayes (1930) and Hill (1934). Philipp (1929) found no trace of gonadotrophin in the placenta of the rabbit.

Allan & Dickens (1930) obtained negative results with Aschheim-Zondek tests on the urine of three ovariectomized rabbits; Jeffcoate (1932), however, found follicle-stimulating hormone in the urine of two of five ovariectomized rabbits; it was present as early as the 9th day after the operation.

Guinea-pig.—Evans, Simpson & Austin (1933) found no gonadotrophin in urine of pregnant guinea-pigs.

Remlinger & Bailly (1934a) injected urine from a pregnant
PREGNANCY DIAGNOSIS

Guinea-pig into rabbits with negative results. Negative Aschheim-Zondek tests were obtained with the urine from pregnant guinea-pigs by Stiasny (1937).

Gonadotrophins are present in the pituitary of the guinea-pig (Hill, 1934), but absent from the placenta (Gutman, 1930).

Rat and Mouse.—Aschheim & Zondek (1928b) and Zondek (1929c, 1931e) failed to find gonadotrophins in the blood or urine of pregnant mice and rats. Negative results were also reported by Snyder & Wislocki (1931a,b) following injections of rat pregnancy urine into rabbits.

Gonadotrophins have been demonstrated in the pituitary of the rat (Ehrhardt & Mayes, 1930; Magistris, 1932; Hill, 1934).

Gonadotrophins are present in the placenta of the mouse (Mirskaja, 1929-30).

Ferret.—Gonadotrophic hormones are absent from the urine of the pregnant ferret (Ehrhardt & Ruhl, 1933).

Wild Animals.

Felidae.—Ehrhardt (1932b) and Ehrhardt & Ruhl (1933) obtained negative Aschheim-Zondek tests on urine of pregnant lions and tigers. Stiasny (1937) obtained negative results with Aschheim-Zondek tests on urine from a pregnant lioness.

The pituitary of the leopard has been shown to possess gonadotrophic activity (Hill, 1934).

Elephant.—Zondek (1929c) and Miller (1934) concluded that gonadotrophic hormones are absent from the urine of the pregnant elephant.

Giraffe.—Wilkinson & Fremery (1940) reported the presence of gonadotrophic hormones in the urine of a pregnant giraffe. The giraffe was mated on 24/12/1937; the urine was tested on immature rats and mice on 24/8/1938 with negative results, but when re-tested on 11/11/1938 and 26/11/1938 positive results were obtained. A female calf was born on 8/4/1939.

Deer.—Unterberger (1932) reported that gonadotrophins were present in the blood of pregnant fallow deer a few months after mating. Blood from pregnant roe deer gave negative results. In this connection it is of interest to note that foetal development in the roe deer follows a rather unusual course. Oestrus and mating occur in July-August, but the segmented egg remains in the uterus until December without undergoing any change, after which time normal development takes place. The literature dealing with this phenomenon in the roe deer has recently been critically analysed by Prell (1938) and he concludes that this initial delay in the development of the egg is the normal course. In the fallow deer and red deer conception takes place in October
and development proceeds normally. Whether the blood of the
toe deer contains gonadotrophins when foetal development has
commenced does not appear to have been investigated.

Fischer & Zondek were unable to find gonadotrophins in the
blood or urine of pregnant roe deer during the time the ovum
was lying undeveloped in the uterus (Zondek, 1935).

Apes and Monkeys.—Aschheim & Zondek (1928b) reported
positive gonadotrophin tests on the urine of the pregnant ape,
orang-utan and rhesus monkey.

In the chimpanzee, Zuckerman (1935) obtained positive tests,
with immature rats and mice as test animals, as early as the
49th day after the last menstruation; there were no false positive
tests and the false negative tests all occurred with urine obtained
late in gestation. His results were too few, however, to give an
estimate of the reliability of the test. Schultz & Snyder (1935)
using rabbits as test animals obtained positive tests with the urine
of four chimpanzees from the 95th to 100th day; in tests made
later, two of the chimpanzees gave negative results. More
extensive experiments on the chimpanzee by Elder & Bruhn (1939)
showed that gonadotrophin tests (Friedman technique) were
reliable over a limited time during early pregnancy. The time
of appearance and duration of the presence of the hormone in
the urine varied with the individual; in most cases it was present
from the 30th to 120th day of pregnancy, but it was always absent
during the last two to three months of pregnancy.

Snyder & Wislocki (1931a,b) tested urines from the macaque
at various stages of pregnancy (Friedman test) with entirely
negative results. Negative results were obtained by Allen,
Maddux & Kennedy (1931) with the urine from pregnant rhesus
monkeys using immature rats as test animals. Kapeller-Adler &
Herrmann (1934) also noted negative Aschheim-Zondek tests in
the pregnant macaque.

Hamlett (1937) claimed that the pregnant macaque (Macaca
mulatta) excreted gonadotrophins in the urine only between the
19th and the 25th day of pregnancy (Friedman technique).

Makepeace (1941) prepared tannic acid concentrates of the
urine of two adult non-pregnant macaques and was able to detect
the presence of gonadotrophins at irregular intervals.
CHAPTER 3. TESTS BASED ON HORMONAL INVESTIGATIONS OF BODY FLUIDS. B. OESTROGENIC HORMONES.

WOMAN.

BIological TESTS.

*Allen-Doisy Test.*—The reliable identification and assay of oestrogenic hormones became possible with the discovery by Allen & Doisy (1923, 1924) that oestrogens produced in ovariectomized rats and mice typical oestrous growth changes in the vaginal walls as indicated by a study of smear preparations of the cells in the vaginal lumen.

*Tests Using Urine.*—The presence of large quantities of oestrogenic hormone in the urine of pregnant women was demonstrated by Aschheim & Zondek (1927), and Aschheim (1927). Oestrogens could be detected by the end of the 4th month of pregnancy by the injection of less than 1 ml. of urine into ovariectomized mice or rats. Aschheim & Zondek (1928a,b) concluded that although oestrogens could be detected in 1-2 ml. of urine at the 8th to 10th week of pregnancy, their presence was not sufficiently regular to serve as a means of diagnosing pregnancy and, moreover, oestrogens were frequently present in large quantities in the urine of women at certain stages of the menopause. Mazer & Hoffman (1929a) did not agree with the above conclusion, but claimed that oestrogens could be detected in the urine as early as a week after the first missed period. Oestrogens were found in 61 out of 67 urine samples from pregnant women, the samples giving negative tests being all from women in the first five weeks of pregnancy. 142 tests on non-pregnancy urine, including urine from women suffering from various pathological conditions, gave negative results with the exception of 15 samples. In later papers (1929b, 1931) these authors reported rather better results. Aschheim (1935) assayed the urine from 75 women in the first two months of pregnancy; only 17 gave positive Allen-Doisy tests with 4 ml. of urine, 25 gave doubtful results, and 33 were negative. There can be no doubt that the biological test for oestrogens in the urine is less satisfactory for the diagnosis of pregnancy than the test for gonadotrophic hormones.

Tests which depend on the presence of both oestrogenic and gonadotrophic hormones in the urine have already been mentioned in the section dealing with gonadotrophic hormones.

*Tests Using Blood.*—The experiments of Polano (1923), Binz (1924), and Triviño (1926) have already been mentioned in the
section on gonadotrophic hormones; although the effects described by these workers were in all probability partly due to oestrogens in the blood samples, they cannot be considered as conclusive proof of the presence of oestrogenic hormones in the blood of pregnant women. Using the Allen-Doisy technique, Aschheim (1926b) and Frank & Goldberger (1926a) demonstrated the presence of oestrogens in the blood of pregnant women. Similar observations were made by Fels (1926, 1927a) and Smith (1927).

Szego & Roberts (1946) have recently demonstrated that two-thirds of the total oestrogen in the blood is closely associated with the blood protein, the remaining one-third existing mainly in a hydrophilic non-protein-conjugated form (see also Mühlbock, 1939).

The presence of oestrogen in the blood during pregnancy has not proved of value for pregnancy diagnosis.

Other Tests.—Markee (1933) described a rapid test for pregnancy based on the modifications of the rhythmic vascular changes in endometrial transplants in the anterior chamber of the eye of the rabbit. The transplants can be made into either male or female rabbits and either gonadectomized or non-gonadectomized rabbits can be used. The oestrogen is extracted from 150 ml. of urine with ether, the ether is evaporated off and the residue dissolved in oil and injected subcutaneously into the rabbit. The test can be read in 40 minutes. The advantage claimed for this test was the speed with which the result could be obtained. The rabbit could be used for further tests if three days' rest between tests was allowed. 147 pregnancy urine samples and 26 non-pregnancy samples tested by this method gave correct results.

Other tests such as the premature opening of the vagina in the immature rat (Kelly, 1933) and in the guinea-pig (Paddock, 1941) have been considered in the previous chapter (Gonadotrophic Hormones). In neither case were ovariectomized test animals employed and a positive test could result from the presence of oestrogenic hormones in the urine or serum injected, or from the test animal's own ovaries as a result of stimulation by gonadotrophic hormones in the urine or serum.

The Foa-Kurloff Cell Test.—The presence of this type of lymphocyte in the blood of the guinea-pig was described independently by Foa and Kurloff in 1889. The history of the discovery and the references to the original literature have been discussed by Ledingham (1940). Ledingham also reviews in some detail the various theories which have been propounded to explain the nature and function of this cell. The relationship between this cell and the sex hormones, particularly the oestrogenic
hormones, is the only aspect of the subject which is of interest in connection with pregnancy diagnosis.

Wada (1928) and Alexeieff & Joukoff (1928) observed an increase in the number of these cells during pregnancy in the guinea-pig. Semenskaja (1930) noted that the injection of ovarian extracts into guinea-pigs increased the Foa-Kurloff cell counts, but she did not believe the effect to be specific. Fiorini (1933) observed that urine from pregnant women when injected into mature female guinea-pigs also increased the number of these cells, but since the effect was very gradual he did not consider the method of value for pregnancy diagnosis. Babudieri (1938) re-investigated the question and concluded that a test for pregnancy could be based on the increase in number of the Foa-Kurloff cells following the injection of pregnancy urine. Confirmation of Babudieri's findings was provided by Carola (1939). Further research on the relationship between the sex hormones and the Foa-Kurloff cell was carried out by Ledingham (1939, 1940), who showed that the injection of oestradiol could raise the Foa-Kurloff cell count to numbers representing 40% of the total leucocyte population. Testosterone had a similar but much less striking effect. Whether the effect of pregnancy urine is due entirely to oestrogens in the urine is, for the present, not entirely proved. Since in one experiment Ledingham was unable to obtain a marked rise in the Foa-Kurloff cell population in a young male guinea-pig with pregnancy urine although its female litter mate showed a marked response. Oestradiol, on the other hand, was equally effective in male and female guinea-pigs.

Tests with Fish.—Fleischmann & Kann (1932, 1935) obtained lengthening of the ovipositor of the bitterling in the non-breeding season by injection of oestrogens. Ehrhardt & Kühn (1933) observed a similar effect when human pregnancy urine was added to the water in which the bitterling were kept; occasional samples of non-pregnancy urine also gave positive results. These authors (1934a,b,c) doubted whether the ovipositor hormone was identical with oestrogen. Szűsz (1934) obtained positive results with urine from males and concluded the test was not specific for pregnancy. Kanter, Bauer, & Klawans (1934) asserted that if the bitterling were first “standardised” with pregnancy and non-pregnancy urines they could be used for pregnancy diagnosis, positive results being reliable at the 24th hour, negative results at the 72nd hour. They believed that the test depended on the presence of oestrogens in the urine. Kotz, Douglas, & Parker (1935), Kleiner, Weisman, & Barowsky (1935), and Gottlieb (1936) found that the “standardisation” of the fish was difficult, if not impossible, and the test unreliable for pregnancy diagnosis. Kanter, Bauer,
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Klawans (1936) maintained that although the test was not accurate in the early stages of pregnancy it was very useful in detecting the excretion of large quantities of oestrogen in the urine. Kleiner, Weisman, Mishkind, & Coates (1936) and Kleiner, Weisman, & Mishkind (1937a,c) concluded that the most important active substance in human urine was the male hormone, and the test was in no way suited for the diagnosis of pregnancy. On the other hand, Barnes, Kanter, & Klawans, (1936) were unable to obtain positive results with crystalline androsterone, but did obtain positive results with extracts of the adrenal cortex. Further investigations (Kanter, Klawans, & Barnes, 1938; Fleischmann & Kann, 1938) confirmed the activity of adrenal cortex extracts, and it was also shown that corticosterone and 11-dehydrocorticosterone gave positive tests, but 17-hydroxydehydrocorticosterone was inactive. Marcus (1937) obtained lengthening of the ovipositor with oestrogens, androgens, and progesterone. Duyvené de Wit (1939a, 1939, 1940, 1941a,b) confirmed the activity of oestrogens, androgens, progesterone and cortical steroids in producing growth of the ovipositor in the female bitterling. In a series of experiments he demonstrated that the rate of growth of the ovipositor following treatment with these substances was characteristic for each type of substance. He concluded that the bitterling test was not suitable for the qualitative or quantitative determination of androgenic hormones, but that it was suited for the qualitative determination of oestrogens provided progesterone was not also present. Duyvené de Wit concluded from a study of the ovipositor response to pure hormones and to human urine that there was present in urine a substance which was not identical with any of the known sex hormones. This substance (which he called "Luteidin") was not present in greater concentration during pregnancy than in non-pregnancy, and he considered that the bitterling was not suited as a test animal for the diagnosis of pregnancy irrespective of the nature of the substance causing the reaction.

Detection of Oestrogens by Plants.—See Gonadotrophin Section, page 47.

Distribution of Oestrogens.

Quantitative Estimation.—There is a gradual rise in the excretion of oestrogen in the urine throughout pregnancy to parturition when there is a rapid fall. The quantitative excretion of oestrogens in pregnancy has been extensively studied; for further details see Smith (1927), Runge, Hartmann, & Sievers (1932), Cohen, Marrian, & Watson (1935), Smith & Smith (1935a,b), Browne & Venning (1936a), Browne, Henry, & Venning
(1939), Hain (1938, 1939, 1940), Dingemanse, Laqueur, & Muhlbock (1939), Rubin, Dorfman, & Miller (1946). The excretion of oestrogen in five pregnant diabetic women was essentially similar to the excretion in normal pregnant women (Smith, Smith, Joslin, & White, 1937).

In Non-pregnant Women.—The presence of oestrogens in the blood and urine of healthy non-pregnant women has been demonstrated by numerous investigators. In general, the quantities present are very much smaller than those present during pregnancy and require special extraction methods for their detection. For further details see Loewe (1925, 1926), Frank & Goldberger (1926a,b), Loewe, Lange, & Faure (1926), Loewe & Lange (1926), Aschheim (1927), Frank & Goldberger (1928), Hirsch (1928), Wildebusch & McClendon (1929), Siebke & Schuschania (1930), Fluhmann (1934), Frank & Goldberger (1935), Jayle, Crépy, Vandel, & Judas (1946).

At the Menopause and in Pathological Conditions.—Aschheim & Zondek (1928a) and Zondek (1931e, 1935) showed that in certain stages of the menopause there occurred an increase in the urinary oestrogen. An increase in the oestrogen excretion, often very marked, was noted in patients with ovarian cysts.

Large quantities of oestrogen may also be excreted in the urine of women suffering from cancer of the adrenals (Frank, 1934).

In Body Fluids other than Blood and Urine.—The presence of oestrogens in the saliva of pregnant women was reported by Fraenkel (1928), but Edwards (1932) and Weisman & Yerbury (1936) have been unable to detect oestrogen in the saliva, even after concentrating ten times.

Origin of Oestrogens.—In a review on this subject, W. H. Newton (1938) concluded that “the balance of evidence at present indicates that the placenta can produce oestrin, but there is little to show that it is responsible directly for the enormous amounts found in human pregnancy.” In the same year, Corner came to the conclusion that “it is certain that an extra-ovarian source of the estrogen in placenta, blood and urine during pregnancy must be postulated. That this source is the placenta itself remains the best hypothesis.” Doisy, Thayer, & Bruggen (1942) concluded that the evidence indicated, but fall short of conclusive proof that, in the pregnant state, the placenta secretes oestrogens.

Chemical Nature.—Three oestrogens and the conjugation product of one of them have been isolated in pure form from human pregnancy urine (Doisy, 1942). These oestrogens are oestrone, \( \alpha \)-oestradiol, oestriol, and oestriol glucuronide. Oestrone is known to be present in human urine also in a conjugated form, but this has not yet been isolated. Doisy (1942) suggests that
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This form may possibly be identical with the oestrone sulphuric acid ester which has been isolated from mare urine (see page 80).

Cohen, Marrian, & Watson (1935) demonstrated that during pregnancy over 99% of the total oestrogen excreted was in the conjugated form.

Jayle, Crépy, Vandel, & Judas (1946) have obtained evidence that, in addition to the oestrogens already mentioned, another oestrogen is present in the urine during the 9th month of pregnancy.

Less is known of the nature of the oestrogens present during non-pregnancy; Marker, Rohrmann, Lawson, & Wittle (1938) obtained two oestradiols from non-pregnancy urine which they were unable to find in pregnancy urine.

For further information on the nature of these oestrogens, the reviews by Marrian (1938), Doisy (1942), and Pincus & Pearlman (1943) may be consulted.

CHEMICAL TESTS.

In attempts to overcome the necessity of employing biological tests for the diagnosis of pregnancy, and on account of the difficulty of obtaining a rapid reliable chemical test for the presence of the gonadotrophic hormones (see page 48), considerable attention has been given to the possibility of employing chemical tests for detecting and estimating the oestrogens in the urine during pregnancy.

Sulphuric Acid Test.—Wieland, Straub, & Dorfmüller (1929) observed that semi-crystalline oestrogenic concentrates from human pregnancy urine gave with chloroform and concentrated sulphuric acid a yellow coloration (Salkowski reaction) and with acetic anhydride and sulphuric acid a reddish-yellow colour with a green fluorescence (Liebermann-Burchardt reaction). These reactions are common to sterols. Marrian (1930a,b) confirmed these observations but also noted that crude crystalline preparations would give an orange colour with a green fluorescence merely on warming with sulphuric acid. Although this reaction is not specific for oestrogens (it is also given by crude bile acids), it has proved of considerable value for qualitative and quantitative determination of oestrogens. Kober (1931) modified this sulphuric acid reaction and developed a quantitative test. For quantitative analysis, Kober found a mixture of phenol and sulphuric acid more suitable than sulphuric acid alone. He also observed that the green-fluorescing orange-coloured solution obtained after warming the oestrogen with sulphuric acid was changed to green-fluorescing red on addition of water, whereas in the case of bile
acids, cholesterol, and many other steroids the addition of water discharged the colour.

Cuboni (1934a,b) applied the sulphuric acid reaction to detect oestrogens in the urine of the pregnant mare, a procedure which proved very successful (see page 81). Romaniello (1934) applied the Cuboni test to human urine but the results were unsatisfactory; of 60 pregnancy urines tested only two gave definitely positive results. Robecchi (1934a,b) obtained positive results with human pregnancy urine after the 5th month of pregnancy, but found the test valueless for early pregnancy diagnosis. Cuboni (1935a,b, 1946) likewise concluded that his method was not satisfactory for the diagnosis of pregnancy in the woman since clear-cut results were not obtained and interfering substances gave false positive tests. Similar conclusions were reached by Sala (1935), Josef (1936), Romano (1936), Argun (1936b), and Buhse (1938). (See also bibliography given by Cuboni, 1946).

Bierry & Gouzon (1936) claimed to detect oestrogens in human pregnancy urine by a modified sulphuric acid test; after preliminary hydrolysis of the urine the hormone was extracted by adsorption and elution to get rid of pigments, and the chloroform solution of the hormone was treated with sulphuric acid and examined by fluorescence spectrography.

Hasselmann-Kahlert (1930) asserted that human pregnancy urine, while not giving a well defined fluorescence with the Cuboni test, gave a clear colourless or light straw-coloured solution, whereas non-pregnancy urine gave a brown or dark brown colour, and by using these criteria she diagnosed 59 pregnancy urines correctly, but with 100 control urines there were 14 incorrect results.

It may be concluded that the Cuboni test has not proved satisfactory in human medicine.

The Kober test has given better results. Cohen & Marrian (1934) applied the Kober test to the quantitative estimation of oestrogens in human pregnancy urine with satisfactory results. The Kober test has been further developed by Carland, Meyer, Miller, & Rutz (1935), Pinous, Wheeler, Young, & Zahl (1936), Venning, Evelyn, Harkness, & Browne (1937), Kober (1938b), Bachman (1939), Szego & Samuels (1940), Jayle, Crépy, & Judas (1943), Stimmel (1946), and Jayle, Crépy, Vandel, & Judas (1946).

In general, the Kober test and its modifications have been used rather for quantitative estimations of oestrogens in the urine of women advanced in pregnancy than for diagnostic tests of early pregnancy. Patterson (1937), however, claimed that after hydrolysing the oestriol glucuronide present in the urine by bacterial means (incubation after inoculation with Bacterium coli) the
presence of a positive Kober reaction was indicative of pregnancy. This method of hydrolysis was preferable to acid hydrolysis which increased the pigmentation and interfered with the colour test. Urines from 65 cases were tested and all but one gave results in agreement with concurrent Friedman tests. The reactions are said to be rather weak in early cases when the menstrual period is only a few weeks overdue and the test may lag behind the Friedman test by a few days in giving positive results in early pregnancy.

Further details and references to the sulphuric acid test can be found in the reviews by Marrian (1938) and Zimmermann (1938).

**Phosphoric Acid Test.**—A sensitive fluorimetric method of assay of natural oestrogens has been described recently by Finkelstein, Hestrin, & Koch (1947) based on the fluorescence produced when these steroids are heated with phosphoric acid.

**Other Chemical Tests.**—A chemical reaction involving the coupling of oestrogens, by virtue of their phenolic properties, with diazotized aromatic amines to yield dyes, has been used by Schmulovitz & Wylie (1935). After extraction of the urine with ether and the removal of all volatile phenols from the carbonate-washed extract by steam distillation, the colour produced by the addition of diazotized p-nitroaniline was compared with the colour of a 33% solution of ferric chloride. An arbitrary scale of value for pregnancy and non-pregnancy samples was adopted; 89 samples of urine from 56 women were tested by this method. Of 58 pregnancy samples tested, 53 gave positive tests and three were doubtful; of 31 non-pregnancy samples, 24 were negative, six doubtful, and one positive. The authors also tested the extracts with the sulphuric acid test, but found that the colours developed were far less distinct and less sensitive than with the diazo reaction. In the following year (1936) the authors published a modification of this test using p-diazobenzenesulphonic acid with β-naphthol solution as a standard. This modification, which was originally said to be suitable for estimation of oestrogens in pure solution, was adopted for pregnancy diagnosis (Schmulovitz & Wylie, 1938); various other modifications regarding the pH of hydrolysis of the urine were also introduced (Savage & Wylie, 1937; Schmulowitcz & Wylie, 1937). 193 urine samples were tested, mostly from problem cases and 8.8% gave false positive results, 3.6% gave false negative results, and 9.9% were doubtful. Marrian (1938) criticizes this method on the ground that it is based on the entirely unjustified assumption that all non-volatile phenolic substances present in the extracts are oestrogens.

Zimmerman (1935, 1936) modified Jaffe’s reaction for
creatinine to detect and assay certain of the sex hormones. This
depends on the colour produced by the reaction of the hormone
with m-dinitrobenzene and potassium hydroxide. This reaction
is, however, only given by the ketonic sex hormones (oestrone,
progesterone, testosterone, etc.) and so is unlikely to prove of
value for the assay of oestrogens in human pregnancy urine (see
page 72). This method has, however, proved of great value in
the estimation of androsterone and other 17-ketosteroids (see
review by Pincus, 1943).

MARE.

BIOLOGICAL TESTS.

Allen-Doisy Test.—In certain reports on the use of biological
tests for pregnancy diagnosis in the mare, there exists some con­
fusion about the nature of the hormone responsible for the changes
observed in the reproductive system of the test animal. This
has arisen mainly through the use of immature rats and mice
where ovarioctomized animals ought to have been employed. It
must be noted that an oestrous vaginal smear following the
injection of a test solution into an immature rodent can indicate
the presence of gonadotrophic or oestrogenic hormones or com­
binations of both in the test solution. If, however, the ovaries
of the test animal giving the oestrous smears are examined and
none of the signs indicative of gonadotrophic activity are observed,
this is fairly good evidence that oestrogens in the test solution
are mainly responsible for the vaginal reaction. Certain authors
who have used immature rodents have unfortunately failed to
mention whether the ovaries of their test animals were examined,
while stating that the reactions were due to the presence of an
oestrogenic hormone. Fortunately, from the point of view of
pregnancy diagnosis, this confusion does not interfere with the
accuracy of the results, since in most cases the presence of either
gonadotrophins or oestrogens is indicative of pregnancy.

Tests Using Urine.—Zondek (1930g) found very large quantities
of oestrogen in the urine of pregnant mares. He developed a
test for pregnancy in the mare using immature rats on the
assumption that gonadotrophins were also present in the urine.
As has already been noted (page 51), the occurrence of gonado­
trophin in the urine of the pregnant mare is a rare occurrence
and the test depends on the presence of oestrogen.

Küst & Grawert (1930) and Grawert (1930) tested the urine of
18 mares for oestrogens and found the method satisfactory for
pregnancy diagnosis. Küst (1931a,b) reported the results of 243
tests on the urine of 187 mares, an accuracy of 98% being obtained;
the quantities of oestrogen excreted in the urine were small for the first five weeks of pregnancy, but increased markedly from the 7th week. Crew, Miller, & Anderson (1931) had only three errors in 390 samples tested. Schäper (1931) tested urine samples from 31 mares by the Zondek method; all were correct, the earliest positive being with urine from a mare 59 days pregnant.

The following investigators have reported on the use of the tests: Küst (1932a,b), Küst & Zumbaum (1932), Stamler & Faermark (1932), Becker (1932), Christiansen (1932), Ehrhardt & Ruhl (1933), Glud, Pedersen-Bjergaard, & Portman (1933a,b), Hespel (1933), Becker (1934), Miller (1934), Küst (1934a,b), Karmann (1934), Barulin & Burzenko (1935a), Miller (1935a,b), Príbyl (1935a), Sipov (1936), Karmann (1936a,b), Panin (1936), Anderson (1936), Rocha e Silva & Trapp (1937), Munich (1937), Miller & Day (1939), Day & Miller (1940), Anderson (1941), Mäyer (1944), Inglis & Robertson (1946).

The test has been found satisfactory for diagnosis of pregnancy in the ass (Samodelkin, 1939; Svecin, 1939). The test can be used from the 60th day of pregnancy, although positive results obtained before this are reliable. Some authors consider it advisable to re-test all negative samples from mares less than three months pregnant. There is ample evidence that the test is reliable, although 100% accuracy is not to be expected. Since oestrogens are present in small quantities in the urine of non-pregnant mares (see page 79) the test must be interpreted on a quantitative basis. This test is more likely to give false positives on account of pathological conditions than is the serum gonadotrophin test (see page 56). Errors may also arise for reasons already mentioned in connection with the serum gonadotrophin test (page 52). Errors liable to occur with the test are discussed by Miller (1935b).

Tests Using Blood.—Cole & Hart (1930a,b) observed that in addition to gonadotrophic hormones, oestrogenic hormones were also present in the blood of pregnant mares. These appeared in the blood from the 80th-180th day at the time when the gonadotrophin was disappearing. The presence of oestrogen in the blood of pregnant mares was also recorded by Zondek (1930g). Wolters, Stüttelin & Krampe (1932) noted that serum from mares advanced in pregnancy which failed to give a positive gonadotrophin reaction in immature mice would usually produce oestrous vaginal smears in the mice. Küst (1934a,b) observed that the oestrogen content of the blood of pregnant mares varied during pregnancy. Becker (1934) found the blood oestrogen to rise considerably from the 4th to the 9th month of pregnancy. Observations on the blood oestrogen were also made by Richter & Gehring (1935), Stamler
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(1935), Přibyl (1935a), Rocha e Silva & Trapp (1937), and Mühlbock (1937b).

Although the presence of oestrogen in the serum may be used for pregnancy diagnosis in the later stages of pregnancy when the gonadotrophic hormone has disappeared from the blood, it is preferable to test the urine for oestrogen as the quantities of oestrogen in the serum are rather variable.

Nipple Test.—Jadassohn, Uehlinger & Margot (1938) suggest the use of the "Nipple test" (increase in length of guinea-pig nipple after local application of oestrogen) to detect the oestrogen in urine of pregnant mares.

Birds as Test Animals.—Juhn & Gustavson (1930) observed that when oestrogens were injected into Brown Leghorn capons, the red or yellow pigment normally found in the breast feathers of the female was produced in the regenerating breast feathers of the capon. Greenwood & Blyth (1934) applied this reaction to the diagnosis of equine pregnancy. Seven days before the capon is required for test purposes about a dozen feathers are removed from the breast and the young feathers which grow in are used for the test. Detoxicated urine (sulphosalicylic acid method) is injected into the pectoral muscles on each of two consecutive days, and on the third day two or three of the young feathers are removed for examination. In positive tests there is an absence of black pigmentation along the growing base and up the lateral edges of the feather, and in its place is red or yellow pigment. The test was considered to be as sensitive as the mouse test.

Further information on the effect of oestrogens in birds can be found in the review by Parkes & Emmens (1944).

Bitterling Test.—Ehrhardt & Kühn (1934b,c) obtained negative or weak positive tests with urine from mares at various stages of pregnancy, with the female bitterling test (lengthening of the ovipositor).

DISTRIBUTION OF OESTROGENS.

Quantitative Estimation.—Owing to the different methods of assay it is not possible to compare directly the exact quantities of oestrogen found in the urine by the various investigators. There is, however, more or less general agreement that the increase in excretion commences during the second or third month of pregnancy and the maximum excretion is reached about the eighth or ninth month after which there is a decrease, the non-pregnancy level being reached a few days after parturition (Glud, Pedersen-Bjergaard & Portman, 1933a,b; Küst, 1934a,b; Cole & Saunders, 1935; Stamler, 1935; Pedersen-Bjergaard & Glud, 1936; Anderson,
Estimations by chemical methods were carried out by Kober (1935a,b, 1938a) and Beall & Edson (1936).

In Non-pregnant Mares.—Küst & Grawert (1930), having noted that oestrogens were present in the urine of non-pregnant mares, put the interpretation of their test on a quantitative basis. The quantity of oestrogen in the urine of non-pregnant mares is greatest at oestrus (Küst, 1931, 1932c, 1934a,b). The presence of oestrogen in the urine of the non-pregnant mare was confirmed by Crew, Miller & Anderson (1931). The following investigators have all noted the occurrence of oestrogen in the urine of non-pregnant mares especially at oestrus: Christiansen (1932), Glud, Pedersen-Bjergaard, & Portman (1933a,b), Zondek (1933a), Stamler (1935).

In Pathological Conditions.—Oestrogen was noted in relatively large quantities in the urine of certain mares suffering from ovarian cysts and chronic endometritis (Küst, 1932c, 1934a,b). Miller (1935b) and Anderson (1936) likewise found a high oestrogen excretion in cases of ovarian cysts and nymphomania.

In Body Fluids other than Blood and Urine.—Trancu-Rainer & Viadutiu (1937) found small quantities of oestrogen in the saliva of the pregnant mare. Imrech (1940) was unable to demonstrate oestrogen in the colostrum of the mare.

Origin of Oestrogens.—There is considerable evidence that the greater part of the oestrogen present in the blood and urine of the pregnant mare may be extra-ovarian in origin.

Gutman (1928), using the implantation technique, was unable to demonstrate oestrogen in the placenta of the mare. By using extraction methods, Zondek (1931a) was able to demonstrate large quantities of oestrogen in the placenta of the mare.

Cole, Hart, Lyons, & Catchpole (1933) and Catchpole & Cole (1934), as a result of hormonal studies on the foetus, postulated that, in addition to the secretion of oestrogen by the placenta, a large proportion of the oestrogen might arise from the foetus, especially the foetal gonads.

Very strong evidence in favour of a source of oestrogen other than the ovary was provided by Hart & Cole (1934), who ovariectomized a mare at about the 200th day of pregnancy. The oestrogen content of the urine was determined at frequent intervals for 150 days after the operation. There was an initial drop in the oestrogen excretion for the first few days after the operation, but the excretion subsequently rose and during the last 60 days was comparable to that found in a normal pregnant mare. The pregnancy proceeded normally; a normal foal was born and lactation was normal.
Chemical Nature.—Five ketonic oestrogens have been isolated and identified from the urine of pregnant mares, namely, oestrone, equilin, hippulin, equilenin, and \( \Delta 5,7,9 \)-estratrienol 3-one-17. In addition, the non-ketonic oestrogens \( \alpha \)-oestradiol, \( \beta \)-oestradiol and 17-dihydro-equilenin are also present.

Most of the oestrogen is present in a conjugated form. At parturition changes in the ratio of "free" to conjugated oestrogen, such as occur in the human, have not been observed in the mare (Schachter & Marrian, 1936). Schachter & Marrian (1936, 1938) have shown that the oestrogen is conjugated with sulphuric acid and not with glucuronic acid as in human pregnancy urine.

In all tests where oestrogen has to be extracted from the urine by immiscible fat solvents, it is essential to hydrolyse the watersoluble conjugated oestrogens before carrying out the extraction. In the Cuboni test (see page 81) this is carried out by heating the urine after the addition of hydrochloric acid.

Further references to the oestrogens in the urine of pregnant mares will be found in the reviews by Marrian (1938), Doisy, Thayer & Bruggen (1942), and Pincus & Pearlman (1943).

Part of the oestrogen present in the blood is in a conjugated form (Mühlbock, 1937b).

In Stallion Urine.—The presence of oestrogenic substances in the urine of stallions was first reported by Kist (1932b,c). Urine from 19 stallions gave positive pregnancy tests; the urine from geldings, and from two male foals under one year of age, gave negative results. This rather unexpected result with stallions' urine was further investigated by Zondek (1933a, 1934a,b) who found that stallions frequently excreted in the urine greater quantities of oestrogen than pregnant mares. The source of the hormone appeared to be the testes, which were shown to contain more oestrogen than any other tissue—much more than is found in the ovaries of the mare. In urine from geldings only very small quantities of oestrogen were found (Zondek, 1938a). Deulofeu & Ferrari (1934) and Häussler (1934) isolated oestrone from stallion urine. Jensen, Larivièere, & Elie (1945) have shown that at least part of the oestrone is conjugated with sulphuric acid. At least 60% of the total oestrogen present in stallions' urine is represented by oestrone (Cartland, Meyer, Miller, & Rutz, 1933). Recently, Levin (1945b) has isolated \( \alpha \)-oestradiol from urine of stallions.

Marrian (1938) considers it doubtful if the excretion of oestrogenic material by the stallion can be said to be higher than that of the pregnant mare as claimed by Zondek.
Cuboni Test.—The experimental work leading to the development of the chemical tests for oestrogens has been briefly mentioned on page 73. Cuboni was the first to use the sulphuric acid reaction for oestrogens as a means of diagnosing pregnancy in the mare (Cuboni, 1934a,b, 1935a,b). The test is very simple: after the addition of hydrochloric acid the sample of urine is heated to split up the conjugated oestrogens; a solvent, usually benzene, is then added and the mixture shaken; the "free" oestrogen passes into the solvent which, since it is immiscible with the urine, will form a layer on top of the urine. This layer is separated off, concentrated sulphuric acid is added and the mixture shaken and warmed. If oestrogens are present, the acid layer when examined by incident light shows a greenish fluorescence. Until experience is gained in reading the test, it is essential to carry out a control test with urine from a non-pregnant mare. This test for oestrogen is not so sensitive as the biological test and negative results are not reliable until the fifth month after service. Cuboni obtained correct results in tests on the urine from 60 pregnant mares and 110 non-pregnant mares. Although the test is only reliable in the second half of pregnancy, owing to its simplicity and the rapidity with which it can be performed, it has been widely used by veterinary practitioners. If its use is confined to urine from mares in the second half of pregnancy the accuracy is usually found to be over 95%.

There have been numerous minor modifications introduced by various investigators in the technique of carrying out the test; these are of little importance, except that of Cuboni (1946), see page 82, and will not be considered here.

The results of subsequent investigations with the test have in general confirmed the findings of Cuboni and it is unnecessary to review in detail these descriptions:—

Sala (1935), Dyekjaer (1935), Cuboni (1936a,b,c), Romano (1936), Argun (1936a,b), Karmann (1936a,b), Di Giano (1936), Sveusson (1936), Busch (1936), Runge (1937), Hennaux (1937), Rocha e Silva & Trapp (1937), Weber (1937), Schramm (1938), Cuboni (1938), Andersen & Pedersen-Bjergaard (1938), Buhse (1938), Christnach (1938), Jastrzebski (1938), Lentz (1938), O. M. Newton (1938), Lütje & Buhse (1939), Falcoianou (1939), Sholl & Derham (1939), Marta (1939), Kerov (1939), Dyekjaer (1939-40), Sardá & Sáinz-Sáinz Pardo (1940b), Mönnig (1941), Thomsen (1941), Sáinz-Sáinz Pardo (1942), Obrycht (1942), Frost (1943),
Mayer (1944), Racoules (1945), Tan (1945), Inglis & Robertson (1946). (See also bibliography given by Cuboni, 1946.)

Some investigators prefer to carry out the initial hydrolysis with sulphuric acid instead of hydrochloric acid, when heating on a water bath is said to be unnecessary (Babičev, 1938; Neves & Castro, 1939, 1941; Jensen, 1941, 1943).

Several attempts have been made to increase the sensitivity of the test to enable it to be used at earlier stages of pregnancy. Roussel & Gallot (1935) and Gallot & Roussel (1935) read the test by ultra-violet light, maintaining that this made doubtful results more easy to interpret. Buhse (1938) failed to obtain satisfactory results with ultra-violet light and observed no fluorescence at all with X-rays. Mönnig (1941), however, claimed that ultra-violet light was a great aid in reading the test and by its use obtained accurate results from the 50th day of pregnancy.

Voloskov (1939) claimed that preliminary hydrolysis of the urine was not necessary. Rautmann (1941) also dispensed with hydrolysis and read the test in ultra-violet light, a green fluorescence being regarded as positive, a blue fluorescence as negative. Cuboni (1942) (see also Cuboni, 1946) tested the urine from 207 mares by Rautmann’s method and found it quite unreliable as the errors reached about 30%. A similar conclusion was reached by Machens (1944).

Cuboni (1946) has recently given details of a modification of his test to be used for urine samples (from mares after the 120th day from service) which have not given clear-cut results with the original technique. The urine is hydrolysed with concentrated sulphuric acid and extracted with benzene. The benzene extract is washed twice with a 10% solution of sodium carbonate. The oestrogen is then extracted from the benzene by shaking with a 5% solution of sodium hydroxide. This alkaline extract is neutralized with sulphuric acid and the oestrogen extracted with benzene. Concentrated sulphuric acid is then added to the extract and the fluorescence developed by placing the extract in a boiling water bath. By this procedure, interfering substances are said to be removed and well-defined results obtained.

Cuboni (1934a,b) observed that oestrogen could be detected in the blood of mares during the second half of pregnancy by extracting 15 ml. of serum with benzene and testing the extract with sulphuric acid. Weber (1937) obtained negative results with serum, but the mares he tested were only 59 to 71 days pregnant. Christnach (1938) found that serum was less reliable than urine during the second half of pregnancy. Rautmann (1941) found his technique for urine unsatisfactory when applied to serum.
Kober Test.—The more complex Kober modifications of the sulphuric acid reaction (Kober, 1931, 1938b) have also been applied for pregnancy diagnosis in the mare. Although the test requires rather more time to perform, the results obtained are more quantitative and allow diagnosis to be made at an earlier date than the Cuboni modification.

Kober (1935a) tested 500 urine samples from pregnant and non-pregnant mares; negative results were reliable from the 140th day after service. Lapiner & Koševerova (1938) claimed reliable results from the 100th day. Kober (1940) tested urine samples from 3,537 mares at the 140th to the 190th day after service. The results were checked at foaling. Of 2,497 mares which foaled, only six had given a negative test (0.24%) and of 1,100 which did not foal, 16 had given a positive test (1.45%). Even without taking into consideration the possibility that some of the 16 mares which gave false positives may have aborted after the test had been made, and there was some evidence for this, these results are extraordinarily accurate. Mayer (1944) obtained good results with the Kober method from the 90th day of pregnancy.

In Non-pregnant Mares.—The Cuboni test gives negative results with the urine from oestrous mares (Cuboni, 1936c; Weber, 1937; Jastrzebski, 1938).

Lütje & Buhse (1939) reported positive Cuboni tests in two of five mares suffering from chronic genital disease.

In the Stallion.—Cuboni (1935c) obtained positive tests with urine from stallions. He considered that the fluorescence obtained was rather more blue-green than that obtained with pregnant mares' urine. Positive results were not obtained in every case since the oestrogen was not always present in sufficient quantity to give a positive chemical test, although Cuboni (1934a,b) was able to detect it by the biological test. The urine from geldings gave negative results. Weber (1937) also obtained positive results with urine from stallions and considered the colour was somewhat different from that obtained with mare's urine. Urine from geldings was negative. Similar findings were reported by Buhse (1938), Jastrzebski (1938), and Mönnig (1941). Bazilevskaja (1939) used the Kober method for estimating the excretion of oestrogens by stallions.

COW.

Biological Tests.

Allen-Doisy Test.—Aschheim & Zondek (1927, 1929b) observed that oestrogen was present in the urine of the pregnant cow, but in relatively much smaller quantities than in the urine of pregnant.
women, and extraction methods were required to demonstrate it. Bölcsázy (1929a,b), however, claimed that 3 to 4 ml. of urine from cows over five weeks pregnant would give a positive oestrogen test in ovariectomized mice and he believed the method would be useful in pregnancy diagnosis. Grawert (1930) was unable to confirm the practical value of the method.

The numerous investigations which followed can be divided into two main classes: (1) Tests with unextracted urine; (2) tests with urine extracts.

Unextracted Urine.—In general it may be stated that tests with unextracted urine have not given results of any practical value, since by this method dependable results have not been obtained until the second half of pregnancy. Positive tests, when obtained with unextracted urine in early pregnancy, may be regarded as reliable, but the general conclusions of the investigators listed below are that oestrogens cannot be regularly detected in the unextracted urine of pregnant cows until the 5th to 7th months of pregnancy. The test using the direct injection of urine into test animals is therefore of no practical value. This technique has been investigated by Wiethoff (1931), Langlotz (1932), Cvrkovic (1932), Brubn (1933), Witzsch (1933), Kust (1934a,b), Stålfor & Hoflund (1934), Anderson (1934), Miller (1935b), Cicogna (1935), Skvorcov (1936), Hennion (1937), Lesbouyries, Berthelon, & Hennion (1937), and O'Moore (1947).

Csiki (1942) claimed positive results with urine from buffaloes from the 100th day of pregnancy.

Urine Extracts.—Tests in which the oestrogen has been extracted from the urine by shaking with various solvents have given more promising results. To obtain satisfactory extracts, the urine should be heated with strong mineral acids to hydrolyse the conjugated oestrogens before extraction.

Čalkovskij & Bondarenko (1933), by using ether and benzene extracts of cows' urine, obtained positive results with a few cows in early pregnancy (1½-2 months); they also obtained positive results with three of 28 non-pregnant cows. These false positives they considered to be due to the extraction of too large quantities of urine (2-3 litres).

Ocáriz & Gilsanz (1934) [see also Ocáriz, 1940] hydrolysed 200 ml. of urine with hydrochloric acid, extracted the oestrogen by shaking with olive oil, separated off the oil and injected it into ovariectomized rats. 121 urine samples were tested by this method: 24 from non-pregnant cows were all negative. Of 18 samples from cows in the first month of pregnancy nine were positive; of 14 in the second month 11 were positive; and of 12 in the third month 11 were positive. The samples from the more advanced
cases were all positive. It must be noted that these investigators considered pro-oestrous and metoestrous smears positive in addition to oestrous smears. Tests using a somewhat similar technique by Zavadovskaja & Stamler (1935) gave less promising results. Josef (1936) was unable to confirm the good results of Ocáriz & Gilsanz and introduced several modifications into their method. The urine sample was evaporated to one-tenth its volume in the presence of 2% hydrochloric acid and extracted with ether; the residue after evaporation of the ether was taken up in solution of sodium hydroxide and again extracted with ether; the ether was evaporated and the residue was dissolved in dilute alkali solution and injected into immature mice. Josef applied this test to urine samples from 58 pregnant and eight non-pregnant cows. In the urine of cows from 21-112 days pregnant there was no evidence of oestrogen being present; from 112-140 days oestrogen was sometimes present, and after 140 days the test was always positive. Lesbouryries, Berthelon, & Hennion (1937) tested the urine from a few cows by the Ocáriz & Gilsanz method with unsatisfactory results. Cuboni (1939a,b,c,d) carried out an extensive investigation on both the Ocáriz & Gilsanz method and the Josef method. Urine samples from 124 pregnant cows (up to seven months pregnant) were tested by the Ocáriz & Gilsanz method, but up to the sixth month there were numerous false negative tests; of 15 non-pregnant cows three gave a positive result. Cuboni slightly modified the Josef test by hydrolysing and evaporating the urine in the presence of 10% hydrochloric acid. The final residue was taken up in olive oil instead of dilute alkali solution and injected into adult ovariectomized mice. 108 pregnant cows and 57 non-pregnant cows were tested by this method (Josef-Cuboni test) and of 75 cows over 4½ months pregnant 73 gave positive tests. Two of the non-pregnant cows gave wrong results. Cuboni concludes that the test is of no practical value unless the owner does not wish clinical examination of the animal, when it may be used in pregnancies of five months and over.

Sardá & Sáinz-Sáinz Pardo (1940a) and Sáinz-Sáinz Pardo (1942) have suggested that useful results may be obtained by the intravaginal technique for detecting oestrogens. The urine is hydrolysed and extracted with ether. A small quantity of the residue, obtained after evaporating off the ether, is inserted into the vagina of an ovariectomized rat, and the vaginal smear examined 48 hours later. No indication of the reliability of the method is given.

Blood.—Aschheim (1926b) was unable to detect oestrogens in the blood of cows in the second half of pregnancy by the direct
injection of serum into castrated mice. Bolshazy (1929b) claimed to detect oestrogen in the blood of three of 12 cows in the third month of pregnancy. Morrell, McHenry & Powers (1930), by extraction methods, were able to detect the presence of oestrogen in the blood of cows, but it is not clear whether these animals were pregnant or not. Wiethoff (1931) reported positive tests with the serum from two pregnant cows. Bertrams (1931) was unable to find oestrogen in the blood of 43 pregnant cows. Negative results on serum were also reported by Bruhn (1933). Cuboni (1939a,b,c, 1941) also reported negative results with serum when injected into ovariectomized rats and mice. Extracts from 150 ml. samples of serum from cows 19 to 96 days pregnant also gave negative results. Szego & Roberts (1946) found 0.38 µg. and 0.36 µg. oestrogen per 100 ml. (as µg. α-oestradiol) in the blood of two pregnant cows (six months). They showed that two-thirds of the total oestrogen in the blood was associated with protein, the remaining one-third was mainly in a hydrophilic non-protein conjugated form (i.e., only one-third of the total oestrogen is present in the acetone extracts of fractions from which the protein has been removed without de-naturation). From the experimental evidence it can be concluded that oestrogens may occasionally be present in the blood of pregnant cows, but blood tests are of no value for pregnancy diagnosis.

**Bitterling Test.**—Stiasny (1937) obtained negative results when the urine of four cows, all at different stages of pregnancy, were tested by the bitterling test.

**Distribution of Oestrogens.**

**Quantitative Estimation.**—The first quantitative studies of urinary oestrogen in pregnant cows were carried out by Hisaw & Meyer (1929). Twenty-four-hour samples of urine were collected, extracted with ether and the residue taken up in oil and injected into ovariectomized rats. Considerable individual variations were observed, but in general the excretion of oestrogen rose gradually from the 44th day of pregnancy. At parturition there was a sudden drop and little or no oestrogen was present 24 hours after calving. Lipschütz, Veshnjakov, & Wilckens (1939) and Lipschütz & Veshnjakov (1930) were unable to detect an increase in oestrogen excretion during pregnancy in the cow, the highest result being obtained from a cow in early pregnancy.

A very extensive investigation on the oestrogen excretion in pregnant dairy cows and beef cows was carried out by Nibler & Turner (1929) and Turner, Frank, Lomas, & Nibler (1930). Twenty-four-hour samples of urine were collected and extracted by shaking with olive oil, adult castrated rats being used as test
animals. Urines from all stages of pregnancy were collected from 45 dairy cows and 32 beef cows; in all, some 275 individual 24-hour samples were assayed. There was considerable individual variation in the daily oestrogen excretion, especially in the later stages of pregnancy, but the general trend of the results shows that until the 100th day the increase in excretion of oestrogen is very slight, but after this the excretion curve rises rapidly till the end of gestation. There appeared to be slight differences in the excretion level in the various breeds of dairy cows studied, but when the dairy cows were compared with the beef cows the excretion levels after the 100th day of pregnancy in the former were considerably higher than in the latter. Anderson (1934) concentrated the urine from pregnant cows by boiling and assayed the concentrates on ovariectomized mice. He also observed a rise in excretion level as pregnancy advanced.

Owing to the different methods of extraction and techniques of assay it is not possible to compare the actual quantities of oestrogen found in the urine by the investigators mentioned above. Barrie, Patterson & Underhill (1935) carried out a small-scale investigation on three cows at different stages of pregnancy, expressing the results in international units. The urine was acidified to a standard pH and concentrated in vacuo; ether extraction was then carried out and the extract washed till free from acid. The residue was dissolved in castor oil for assay. Twelve samples were assayed. It was concluded that during the first 21 weeks of pregnancy the oestrogen excreted was less than 50 i.u. per litre; by the 23rd week it had reached 100 i.u.; 700 i.u. per litre were obtained at the 30th week, 1,000 i.u. at the 32nd week, 4,000 i.u. at the 34th week, and 17,000 i.u. per litre at the 37th week.

In Non-pregnant Cows.—By direct injection of urine into test animals, oestrogen has not been detected in the urine of non-pregnant cows (Wiethoff, 1931; Langlotz, 1932; Witzsch, 1933, etc.). By extraction methods, however, the presence of oestrogen has been noted by Lipschütz & Veshnjakov (1930), Turner, Frank, Lomas, & Nibler (1930), Čalkovskij & Bondarenko (1933), Zavadovskaja & Stamler (1935), and Cuboni (1939a).

Traces of oestrogen were reported by Loewe (1925) to be present in the blood of cows at various stages of the oestrous cycle, but it required extraction methods to demonstrate this. Wiethoff (1931) failed to find any indication of oestrogen by direct injection of blood into mice.

It is obvious from the presence of oestrogen in the urine of non-pregnant cows that any pregnancy test must be founded on a quantitative and not a qualitative basis. It is now fairly well
established that the excretion of oestrogen in pregnancy does not become significantly greater than the excretion in non-pregnant animals until well on in the gestation period, and it would thus seem improbable that any test based on the excretion of oestrogen will give reliable results in the early stages of pregnancy when a test would be most helpful.

In Pathological Conditions. — Oestrogens have not been found in the blood and urine of cows with cystic ovaries, persistent corpora lutea or in cases of nymphomania (Bertrams, 1931; Langlotz, 1932; Bruhn, 1933; Lesbouyries, Berthelon, & Hennion, 1937).

An interesting observation was made by Skvorcov (1936), who noted that cows infected with Brucella abortus frequently excreted oestrogens in the urine.

In Biological Materials other than Blood and Urine. — Oestrogen has not been found in the milk or saliva of pregnant and non-pregnant cows ( Bölösházy, 1929a, b; Wiethoff, 1931; Bruhn, 1933; Küst, 1934a, b).

Bruhn (1933) was unable to detect oestrogens in the feces of pregnant cows, but more recent experiments by Levin (1945a) have shown that during the last two weeks of pregnancy at least, oestrogen is present in the feces, apparently in the form of oestradiol.

Supperer (1942) reported that oestrogens were present in the vaginal secretion of estrous cows.

Origin of Oestrogen. — Little is known concerning the origin of the oestrogen present in the urine of pregnant cows. Apart from secretion from the ovary it is possible that oestrogen may be produced by the placenta. Its presence in the placenta and amniotic fluid has been described by Parkes & Bellerby (1927), Allen (1927), Morrell, Powers, & Varley (1930), Küst (1934a, b), and Scheibe (1936).

Chemical Nature. — The oestrogens present in the urine of pregnant cows do not appear to have been isolated or identified.

In the Bull. — Frank, Frank, Gustavson, & Weyerts (1925), Bertrams (1931), and Küst (1934a, b) were unable to detect oestrogen in the blood or urine of the bull. Zondek (1934a), however, was able to detect small quantities in the urine of bulls. This is in striking contrast to the very large quantities excreted by the stallion (see page 80). Oestrone has been isolated from bulls' urine by Marker (1939a).

Chemical Tests.

Câboni (1935a, b) pointed out that his test for oestrogen (see page 81) was not satisfactory for testing for oestrogen.
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in the urine of pregnant cows, since samples of urine from non-pregnant animals gave a slight fluorescence and various attempts to overcome this by concentration of the urine and adsorption of the hormone had not been successful. The test was investigated by several other workers, and they likewise came to the conclusion that, owing to similarity of the colour and fluorescence obtained with samples of pregnancy and non-pregnancy urine, the test was unreliable in the cow (Josef, 1936; Di Giano, 1936; Argun, 1936a,b; Busch, 1936; Frullini, 1936; Liess & Dickenschied, 1937; Weber, 1937; Dickenschied, 1939; Mönnig, 1941; Olbrycht, 1942; Frost, 1943; O’Moore, 1947).

Romano (1936) increased the quantity of urine extracted and claimed that reliable tests could be obtained, but not until the 8th-9th month; a result of no practical value. Liess & Dickenschied (1937) used a quartz lamp for reading the test, but found it of no benefit. Galambos (1937) made an extensive investigation on the use of the test on 64 pregnant cows and heifers and 46 non-pregnant animals using various quantities and concentrations of urine. He was unable to improve the results and concluded the test was valueless in the cow. Weber (1937) also used large quantities of urine for the test, but results were not satisfactory. He also obtained negative results with nine tests on the blood of pregnant cows.

Cuboni (1939a,c) reported numerous experiments which he had carried out in an attempt to remove the non-specific substance which gave rise to the fluorescence in the urine of non-pregnant animals, and in the urine of bulls, calves, and steers. The modifications tried included the use of ether instead of benzene, phenol-sulphuric acid instead of sulphuric acid, and extraction of the residue with alkali. None of the methods resulted in the removal of the non-specific fluorescence. Sarda and Sáinz-Sáinz Pardo (1940c) and Sáinz-Sáinz Pardo (1942) stated that by extracting the urine with ether, reliable results could be obtained from the 5th month of pregnancy, but no data on the accuracy of the method were provided. Mönnig (1941) claimed that by reading the test with ultra-violet light the urine from non-pregnant cows always gave negative results, but as positive results were not obtained with urine until the 7th month of pregnancy, the test was still of no practical value.

Cski (1942) obtained positive results from the Cuboni test with the urine from buffaloes after the 100th day of pregnancy.

Otte (1939a,b,c, 1939a,b) claimed that the presence of "oestrogen" in the urine of cows four weeks after service was a reliable indication of pregnancy. His test for the so-called "oestrogen" was based on a colour reaction with a solution of
ammonium molybdate. The test was in two parts—Molybdenum Reactions I and II. In interpreting the results of the tests the type of food the cow was receiving had to be taken into consideration. Otte claimed good results with this method despite the influence of food and muscular activity on the test.

OTHER ANIMALS.

SHEEP AND GOAT.

_Sheep._—Küst & Vogt (1934a) investigated the blood and urine of two pregnant sheep throughout pregnancy, but failed to detect any oestrogenic hormone from the 38th day of pregnancy to three days after lambing. No trace of oestrogen was found in the blood or urine of non-pregnant ewes. Negative results were also reported by Hayston (1939). In a more recent study on two pregnant ewes, Whitten (1943) was able to detect oestrogens in the urine during the last few weeks of pregnancy. He also carried out estimations on the excretion of keto-steroids, the excretion of which rose as pregnancy progressed.

Oestrogens have been detected by extraction methods in the placenta and amniotic fluids of the sheep by Parkes & Bellerby (1927). Küst & Vogt (1934a) were unable to detect oestrogens in the amniotic fluid by direct injection into test animals.

Küst & Vogt (1934a) found no trace of oestrogen in the blood or urine of non-pregnant sheep.

Küst (1934a,b) reported negative results with oestrogen tests on the blood and urine of rams.

_Goat._—Küst & Vogt (1934b) were unable to detect oestrogen in the blood of pregnant goats. Oestrogens were, however, present in the urine in small amounts after the 44th to 75th day, the amount had doubled by the 100th day and rose steadily till parturition; only small quantities were present one day after kidding. It is concluded that testing for oestrogen would give moderately reliable results for the diagnosis of pregnancy from the 100th day of pregnancy, but by this time clinical methods can be used.

Küst & Vogt (1934b) were unable to detect oestrogen in the blood or urine of non-pregnant goats.

Oestrogen is absent from the blood and urine of male goats (Küst, 1934a,b).

_Sow._

_Biological Tests._—Zondek (1929c) reported negative results with tests for oestrogens on the urine of pregnant sows. Further investigations by Küst (1931b) and Struck (1931) demonstrated
that oestrogens were present in the urine of pregnant sows, but only at certain stages of gestation. By direct injection of urine it was possible to demonstrate the presence of oestrogen in the urine from the 23rd to the 31st day of pregnancy; oestrogen then disappeared from the urine and could not be detected until the 12th week of gestation. The amount present in the urine then increased until parturition, when it fell suddenly and was absent the day after parturition. Its presence was sometimes noted for a brief period about a fortnight after parturition (Küst, 1934a,b; Küst & Struck, 1934; Struck, 1935). Heisig (1932) confirmed these interesting findings of Küst and Struck. In addition, by using extraction methods he was able to show that very small amounts of oestrogen were present in the urine before the 10th week. A distinct excretion of oestrogen was also noted in some sows 2-6 weeks after parturition. Similar results were reported by Hauer (1933). Faermark & Bigos (1933) and Faermark (1935a) obtained results in complete agreement with those of Küst and Struck and they also noted that between the 4th and 11th weeks oestrogens could be demonstrated if extraction methods were employed. This method of detecting pregnancy was further investigated by Kazarnovskaja & Vakusevič (1935), Kazarnovskaja & Tatarko (1936), and Šipov (1936) and all agreed that for accurate results in early pregnancy the urine had to be examined between the 20th and 30th days of pregnancy.

Stiasny (1937) obtained positive results when he tested urine from sows in advanced pregnancy with the female bitterling test. Aschheim (1926b) was unable to demonstrate the presence of oestrogens by direct injection of the blood from a sow at mid-pregnancy into test animals. Negative results were also reported by Zondek (1929c). Küst & Struck (1934) tested the blood from sows at various stages of pregnancy, also with negative results.

**Distribution of Oestrogens.**—Using extraction methods, Frank, Frank, Gustavson & Weyerts (1925) tested blood from five sows in oestrus and five sows not in oestrus. Four of the oestrous sows gave positive results when an extract of 300 ml. of blood was injected into castrated rats. The results with the other six sows were negative. (This was one of the earliest reports of the detection of oestrogens in the blood of mammals.) The occasional occurrence of oestrogens in the urine of the post-parturient sow has been noted above.

Heisig (1932) found small amounts of oestrogen in the urine of a sow in oestrus.

Allen (1927) and Gutman (1928) were unable to detect oestrogen in the placenta of the sow. Philipp (1920) and Küst (1934a,b), however, claimed that it was present. Their claim was confirmed
by Faermark (1935b) who tested placentae from 17 sows and found that the curve of oestrogen content ran parallel to the curve of urinary excretion of oestrogen; he considered it likely that the placenta secreted at least some of the oestrogen present in the urine of the pregnant sow.

The oestrogens present in the urine of the sow do not appear to have been identified.

Hauer (1933) and Küst (1934a,b) found large quantities of oestrogen in the urine of adult boars. It was not present in the blood (Küst, 1934a,b).

Chemical Tests.—Heller (1940) investigated the possibility of using the sulphuric acid reaction for the detection of pregnancy in sows. Of four non-pregnant sows tested, three gave a positive test; of 15 pregnant sows (from one to 16 weeks pregnant) three gave positive tests, five were doubtful, and seven negative; of five sows from 12-16 weeks pregnant only one gave a positive test. Heller concluded the test was of no value in sows.

Roth, Mayer, & Bogart (1941) obtained much more promising results. The urine from six pregnant sows was tested at regular intervals throughout pregnancy along with control samples from 13 non-pregnant sows. It was found helpful to treat the urine before hydrolysis with zinc sulphate and sodium hydroxide to remove certain pigments which were apt to interfere with the test. The authors consider it advisable when positive tests are obtained to add water to the fluorescent solution; non-specific fluorescence will then disappear (Kober, 1931). All the tests from pregnant sows obtained between the 20th and 33rd days of gestation and after the 72nd day of gestation were positive. Samples from pregnant sows during the interval from the 33rd to the 72nd day were negative, as were all samples from non-pregnant sows. These results confirm the findings of the investigators who used biological methods of assay (see page 91), concerning the periods of pregnancy when oestrogens are excreted in the urine in quantities sufficiently great to be readily detected. The authors believe that this test ought to give reliable results in swine, provided that accurate records of breeding dates are kept so that the urine can be tested during the period from the 21st-32nd day of pregnancy.

Lozinski, Holden, & Macallum (1942), using a chemical test, detected small quantities of oestrogen in the urine of sows at the 8th week of pregnancy; oestrogen was not observed again by them until the 11th week.

Bitch.

Helm (1931), by direct injection of urine into ovariectomized
and immature mice, was unable to demonstrate the presence of oestrogens in the urine of 28 bitches at various stages of pregnancy. Negative results were also obtained with urine from non-pregnant bitches, bitches in oestrus, and post-parturient bitches. Küst (1931b) also reported negative tests with urine from pregnant bitches. Lesbouyries & Berthelon (1936) tested the urine of three pregnant bitches every eighth day throughout pregnancy. Negative results were obtained up to the 15th to the 18th day; after the 18th day oestrogen was present in sufficient quantities to produce partial oestrus in the test rats. Finck (1936) also found small amounts of oestrogen in the urine of the pregnant bitch.

The oestrogen content of the blood of the bitch during pregnancy does not appear to have been investigated.

Urine from two bitches in advanced pregnancy gave positive results with the female bitterling test (Stiasny, 1937).

Allen (1927) and Gutman (1928) were unable to detect oestrogens in the placenta of the bitch.

**Cat.**

Lübbers (1933), using the Allen-Doisy reaction, tested the blood and urine of 5 cats at various stages of pregnancy with negative results. Urines from non-pregnant cats, a cat in oestrus, and a male cat were also devoid of oestrogenic activity.

Allen (1927) and Gutman (1928) found no oestrogens in the cat placenta.

**Laboratory Animals.**

- **Rat and Mouse.** Zondek (1929c) was unable to detect oestrogen in either the blood or urine of pregnant mice.

- D'Amour, Funk, & Glendenning (1936) reported small but demonstrable amounts of oestrogen in the urine of pregnant rats; none was detected in the placenta.

- Beerstecher (1941) was able to demonstrate small quantities of oestrogen in the urine of non-pregnant rats; in pregnancy the excretion rose from about the 5th day. No oestrogen was detected in the urine of male rats.

- **Rabbit.** Zondek (1929c) was unable to detect oestrogens in the urine of pregnant rabbits. Negative results were also obtained with blood (Aschheim, 1926b; Zondek, 1929c). Philipp (1929), however, found oestrogen in the blood of the rabbit at the end of pregnancy. Beerstecher (1942) found small quantities in the urine of non-pregnant rabbits. In pregnancy there was a marked rise in excretion which reached a maximum at mid-term. Pseudo-pregnancy also produced a rise in excretion, but only
about a tenth of that observed in pregnancy. No oestrogen was found in the urine of the male rabbit.

Gutman (1928) was unable to detect oestrogen in the rabbit placenta.

Urine from two pregnant rabbits gave negative results with the female bitterling test (Stiasny, 1937).

Guinea-pig.—Stiasny (1937) obtained positive bitterling tests with urine from two pregnant guinea-pigs. Oestrogen is absent from the guinea-pig placenta (Gutman, 1928).

WILD ANIMALS.

Lioness.—Urine from a pregnant lioness gave a positive bitterling test (Stiasny, 1937).

Elephant.—Zondek (1929c) was unable to detect oestrogenic hormones in the blood or urine of the pregnant elephant. Negative tests on the urine of pregnant elephants were also reported by Miller (1934).

Deer.—Fischer & Zondek (cited by Zondek, 1935) were unable to find oestrogens in the blood or urine of roe deer at the time when the fertilized ovum was lying free in the uterus.

Male Equidae.—Zondek (1934a) found large quantities of oestrogen in the urine of the male zebra, Grévy's zebra, ass. and kiang.

Dromedary.—Only traces of oestrogen are found in the urine of the male dromedary (Zondek, 1934a).

Apes and Monkeys.—Considerable quantities of oestrogens were recovered from the urine of chimpanzees at various stages of pregnancy (Allen, Diddle, & Elder, 1935).

In non-pregnant chimpanzees, the greatest urinary excretion of oestrogen was noted between the onset of two menstrual periods (Allen, Diddle, Burford, & Elder, 1936).

Oestrogen has been found in the full-term placenta of the chimpanzee by Allen, Diddle, & Elder (1935).

Allen, Maddux, & Kennedy (1931) tested the urine of a gravid macaque monkey at various stages of pregnancy; they concluded that oestrogen was present, but in smaller quantities than in human pregnancy urine. Dorfman & Van Wagenen (1941) also noted an increase in oestrogen excretion during pregnancy in the macaque.

Oestrogen is present in the full-term placentae of the macaque (Allen, Maddux, & Kennedy, 1931).
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TESTS PROBABLY BASED ON GONADOTROPHIC AND OESTROGENIC HORMONES.

HYPERCHOLESTEROLAEMIA REACTION.

Woman.—Masciottra & Martínez de Hoz (1932, 1933) claimed that when urine from a pregnant woman was injected into a guinea-pig it caused a marked rise in the blood cholesterol, and that the method had considerable advantages over the Aschheim-Zondek test, as test animals of either sex could be used and results were obtained in 24 hours. A 25% increase in the blood cholesterol level was regarded as a positive result. Subsequent investigators have failed to obtain accurate results by this method. Potter (1933) found that the effect of pregnancy urine on the blood cholesterol was so inconsistent that the test was valueless. Similar conclusions were reached by Eiras & Rojas (1934), Savona (1934), Gavioli (1934), and Lépine & Mélas-Joannides (1934).

Although this test has not been found to be sufficiently accurate for practical purposes, the reaction would appear to depend on the presence of oestrogenic and gonadotrophic hormones in the urine. Mori & Reiss (1928) demonstrated that injection of oestrogens increased the blood cholesterol. Further work by Reiss & Langendorff (1929) demonstrated that gonadotrophic hormones injected into rabbits and dogs had a similar effect except in the case of ovariectomized test animals, and it was concluded that gonadotrophin acted by liberating oestrogen from the ovaries. Perhon & Cahane (1930) observed a hypercholesterolaemia in rabbits following injection of pregnancy urine. Cioglia & Tore (1934), contrary to the findings of Reiss & Langendorff, did obtain an increase in blood cholesterol in ovariectomized rabbits following injections of gonadotrophin. Further investigations on the effect of oestrogens and pregnancy urine on the blood cholesterol have been carried out by Artom & Cioglia (1934), Artom, Cioglia, & Tore (1934, 1936), Cioglia & Tore (1934, 1935), Cioglia (1935, 1936), and Cioglia & Tore (1936a,b), and it is clear that the effect depends both on the amounts and the proportion of the hormones present in the urine.

Further details will be found in the reviews by Tramontana (1936) and Sanchez Cozar (1941).

Mare.—Brocq-Rousseu, Roussel, & Gallo (1933b) observed that injections of urine from non-pregnant mares into rabbits had little effect on the blood cholesterol. Of 24 samples of mare pregnancy urine, 18 caused a rise and six a fall in the blood cholesterol of the rabbit. The authors conclude that the test by
itself cannot be considered reliable for the diagnosis of pregnancy in the mare.

(Changes in the blood cholesterol during pregnancy will be considered in the section on Biochemical Methods of Diagnosis, page 143.)

**Tests Involving Unicellular Organisms.**

It is by no means certain that the tests to be described are based on the presence of hormones, but owing to the difficulties of classification they will be considered in the Hormone Section.

*Woman.*—Popoff & Dimitrova (1933) observed that when cysts of *Euglena gracilis* were placed in diluted pregnancy urine, or in urine from a woman a few days prior to menstruation, a marked growth stimulation took place. This stimulation was believed to be due to the presence of gonadotrophins in the urine and it was considered that this method gave results as reliable as those obtained with the Aschheim-Zondek test, provided it was borne in mind that urine from women just prior to the onset of menstruation gave positive tests. The test was claimed to give results within 24 hours. The above workers also studied the effect of pregnancy urine on the fermentation rate of yeasts. Although some stimulation was observed, the results were not sufficiently reliable for test purposes. The use of yeast was further investigated by Kalledey (1937), who studied the rate of carbon dioxide production by yeast in solutions of sugar with and without the addition of pregnancy urine. Tests on 144 samples of pregnancy urine gave 94.5% positive results; 176 samples of non-pregnancy urine gave 90% negative results. Kalledey considered that the method might prove to be of value.

Kustallow (1937) studied the effect of pregnancy urine on *Paramecia*, obtained by making an infusion of hay, by adding a drop of pregnancy urine to a drop of the infusion and examining under the microscope. It was claimed that the *Paramecia* would stop moving and clump together within a minute. If non-pregnancy urine was added to the infusion, motility would still be present at the end of five minutes.

Unfortunately this very simple and rapid test has not proved reliable in the hands of later investigators. Uhlich (1937) tested 38 samples of pregnancy urine and 44 samples of non-pregnancy urine; in every case the protozoa ceased to show motility within a minute. Uhlich concluded that the test was quite non-specific and appeared to depend on the pH and specific gravity of the urine; the higher the specific gravity the more toxic the urine. Similar conclusions were reached by Hinck (1938) and Wattenwyl (1938).
Cow.—Gettkandt (1937) applied the Kustallow reaction to the cow. Forty-seven of 49 tests on pregnancy urine and 20 tests on non-pregnancy urine were correct. He considered the test depended on the presence of oestrogens in the urine and concluded that it was suitable for early diagnosis of pregnancy in the cow. Krauss (1938) obtained much less favourable results in tests on 10 non-pregnant cows; in all but one case the protozoa were dead in one-half to two minutes. It was observed that the specific gravity of the urine had a marked effect on the survival time of the Paramecia. Solutions of oestrogens or gonadotrophins had no effect on the protozoa. 343 cows were tested by Kühn (1938), who concluded that the test was valueless. Radoff (1941) found that Paramecia were killed equally quickly in urine from pregnant and non-pregnant cows and buffaloes.

Other Animals.—The test does not appear to have been applied to other domestic animals apart from a single positive test obtained by Krauss (1938) with the urine of a 36-week-pregnant mare.

The Chauchard Chronaxie Test.

Chauchard & Chauchard (1944) and Chauchard (1945) suggested a test for pregnancy based on the effect which pregnancy urine has on the excitability of the uterus of the non-pregnant guinea-pig. Chronaxie measurements are made on a horn of the guinea-pig uterus in situ; the urine under test is then swabbed on the uterus in the neighbourhood of the cathode and further measurements are made. If the urine is from a woman one or more months pregnant the chronaxie will be increased at least thirty-fold. Non-pregnancy urine or male urine has no effect.

The rat and rabbit uterus show a similar reaction.

It was demonstrated that solutions of gonadotrophins also produced an increase in chronaxie and the reaction is believed to be based on the presence of hormones in the urine of pregnant women.

Blood serum can be used instead of urine and it is suggested that it may give more accurate results (Chauchard & Lecoq, 1946).
Progesterone, the principal progestogenic hormone of the corpus luteum, has not in itself proved to be a suitable hormone on which to base tests for pregnancy, but a related steroid—pregnanediol—has proved of value. This compound is in all probability the form in which progesterone is excreted.

Test for Progestogens.—Before reviewing briefly the literature dealing with the presence of progestogens in the blood and urine, it is necessary to refer briefly to some of the tests for detecting the presence of the hormone.

There is at present no chemical test specific for progesterone and other progestogens, and biological tests must be used. The earliest of these is the Corner-Allen method. Sexually mature rabbits are mated and approximately 18 hours later the ovaries are removed and the material to be tested is injected subcutaneously over a period of five days. On the sixth day the uterus is removed and examined histologically. The response is fully positive when a degree of progestational proliferation is produced equal to that observed at the height of pseudopregnancy. To obtain full proliferation by this method about 1 mg. of progesterone is required. This method was modified by Clauberg who found that if immature rabbits were given preliminary treatment with oestrogen over a period of eight days they could then be used for progesterone assay. This method avoids the necessity of ovariecnomizing the rabbits. The results with this method have not proved quite so uniform as those obtained by the original method. Further references to these methods will be found in the reviews by Allen (1939) and Courrier (1945).

A method for detecting smaller quantities of progestogens has been described by McGinty, Anderson, & McCullough (1939). Immature rabbits are pre-treated with oestrogens and the horns of the uterus are then surgically divided into isolated portions each with its blood supply. The material to be assayed is introduced into one of the isolated portions of uterus. This portion is later examined histologically for proliferation, the untreated portions serving as control preparations. By this method it is claimed that 0.5-5.0 μg. of progesterone can be detected.

Shapiro (1936c) reported that ovulation and oviposition could be induced in Xenopus laevis by the injection of 0.25 mg. of progesterone. Since a large number of other steroids will also induce oviposition (Shapiro, 1936c, 1939), as well as gonadotrophic
hormones, this reaction is much less specific than the rabbit tests.

Duyvène de Wit (1938a, 1941a) has claimed that the female litterling is an excellent test animal for detecting and assaying progesterone. By noting the rate of elongation of the ovipositor it is possible to differentiate within certain limits the steroids responsible for the reaction. By this test, 10 μg. of progesterone can be readily detected. If these claims are substantiated, the method ought to be of considerable value in endocrine research.

WOMAN.

Progestogens.

Urine.—Progestogens are not excreted in demonstrable quantities in the urine of pregnant women (Clauberg, Thiel, & Ziecker, 1933; Ehrhardt, 1934; Loewe & Voss, 1934; Ehrhardt & Hagena, 1935). Marker, Kamm, & McGrew (1937) were unable to isolate progesterone from the steroids extracted from 10,000 gallons (37,850 litres) of human pregnancy urine.

Blood.—Kelly & Florence (1930) described a pregnancy test based on the prolongation of the oestrous cycle of the guinea-pig following injections of serum from pregnant women. By injecting the animals from the 9th day of the cycle for four days, the onset of the next oestrus, in positive cases, was postponed for about 6 days. At that time, the authors postulated that this effect might in part be due to progesterone in the serum having an inhibitory or antagonistic action on the production of oestrogen from the test animal's ovaries. In the light of more recent work it may be safely assumed that progesterone played no part in the test, the delay being caused in all probability by the luteinizing effect of the gonadotrophin present in the serum (see page 30).

Clauberg (1933) and Clauberg, Thiel, & Ziecker (1933) were unable to detect the presence of progestogens in extracts of up to 300 ml. of blood from women in the early stages of gestation. Extracts of 70 ml. of blood from cases of advanced pregnancy also gave negative results. Extracts of non-pregnancy blood were all negative. Negative results were also reported by Bloch (1936) who tested extracts of 150 to 500 ml. of blood from women 2 to 6 months pregnant.

Sutherland & Zwarenstein (1939) tested ether extracts of plasma from pregnant women on Xenopus for the presence of progesterone, or other steroids causing oviposition in Xenopus, with negative results.

The presence of progestogens in human pregnancy blood has been reported by Haskins (1941) who tested extracts by the McGinty, Anderson, & McCullough (1939) method. Twenty of 21 samples
from women 2-7 months pregnant gave positive results. Similar results appear to have been obtained by Hoffmann & Läm (1942). Duyvené de Wit (1941a) obtained a weak positive test with the bitterling reaction with 50 to 60 ml of pregnancy blood. He also detected progestational activity in the blood of a patient with a persistent corpus luteum.

While it would appear that progestogens can be detected in the blood during pregnancy by the more sensitive methods of assay, it would seem that, in the human subject at least, in view of the reliability of the gonadotrophin tests, the method offers no advantages as a means of diagnosing pregnancy.

Source of Progestogens in Pregnancy.—Apart from the production of progesterone by the corpus luteum, there is strong evidence in favour of the production of progestogens by the placenta. They have also been found in the adrenal glands (see review by Burrows, 1945).

Pregnanediol.

Pregnanediol [pregnanediol-3(\(\alpha\)), 20(\(\alpha\))] was first isolated from human pregnancy urine by Marrian (1929). This steroid, a reduction product of progesterone, received little attention until Venning & Browne (1936) showed that it could be readily extracted quantitatively from pregnancy urine in a conjugated form (see also Odell & Marrian, 1936). Numerous studies have since been made on the significance of its presence in urine.

Biological Tests.—In mammals pregnanediol is practically devoid of any biological activity, although Freud (1937) found that it would produce some mucification in the vagina of ovariectomized mice.

In the female bitterling, pregnanediol and several other steroid derivatives of progesterone which are inactive when tested in mammals, will readily produce elongation of the ovipositor (Duyvené de Wit, 1941a).

In view of the relative simplicity of the chemical methods of assay it is unlikely that a biological method would present any practical advantages.

Chemical Tests.—Venning & Browne (1936) described a method for isolating from pregnancy urine sodium pregnanediol glucuronidate (see, however, Marrian & Gough, page 102). The urine is extracted several times with butyl alcohol, the extract evaporated to dryness and taken up in 0.5N sodium hydroxide and re-extracted with butyl alcohol. The butyl alcohol is washed with water and evaporated to dryness. The residue is dissolved in water and the substance precipitated with acetone. Purification is carried out by repeated crystallization from water and ethyl alcohol. The
The final product is then weighed. Further technical details will be found in the papers by Venning, Henry, & Browne (1937) and Venning (1938). The presence of this steroid is not confined to pregnancy; it is found in small quantities in the urine during the luteal phase of the menstrual cycle (Venning & Browne, 1937). If its presence is to be used as a test for pregnancy, the test must therefore be of a quantitative nature. From the results of Venning (1938) and workers since that date it has now been established that the excretion of sodium pregnanediol glucuronidate increases rapidly after the first two months of pregnancy when the quantity of conjugated pregnanediol excreted is equivalent to approximately 10 mg. of pregnanediol per 24 hours. By the end of pregnancy this has risen to an equivalent of 80 mg. of pregnanediol per day. In the non-pregnant woman the total quantity excreted during the whole of the luteal phase of the cycle varies from 30-55 mg. of pregnanediol. Where facilities do not exist for carrying out the biological tests for pregnancy, much helpful information can be obtained from pregnanediol estimations, provided that the findings are interpreted in conjunction with the history of the case. Where repeated determinations are carried out on the same patient it may be possible to make a diagnosis at a very early stage (Hain & Robertson, 1939).

Further information about the reliability and value of the test may be found in the following papers:

Wilson & Randall (1938, 1939), Hamblen, Ashley, & Baptist (1939), Wilson, Randall, & Osterberg (1939), Stover & Pratt (1939), Buxton (1940), Shinowara & Reinhart (1940), Hain (1940), Tien (1941), Bachman, Leekley, & Winter (1941), Hamblen, Cuyler, & Baptist (1942), Jones, Defts, & Stran (1944).

Various modifications of the technique of Venning and Browne have been reported. Allen & Viergiver (1941) precipitated the final compound as a lead salt and determined the pregnanediol by estimating the amount of glucuronic acid liberated after acid hydrolysis by measuring its reducing capacity with the Shaffer-Hartmann-Somogyi reagent. Crismer (1939a, b) had previously described a method of estimation of sodium pregnanediol glucuronidate based on the estimation of glucuronic acid by a colorimetric method (Tollens reaction); his method of obtaining the conjugated compound was however essentially different from that of Venning and Browne and appears to have been less specific as other glucuronides interfered to some extent.

As a result of studies on the distribution of sodium pregnanediol glucuronidate between butyl alcohol and urine, Woolf, Viergiver, & Allen (1942) were able to reduce the number of extractions, the
Jayle, Crépy, & Wolf (1943) also reduced the number of extrac­
tions and estimated glucuronic acid by a colorimetric method based
on the Tollens reaction. Further work by Jayle & Libert (1946)
indicates that this method, or modifications of it, may be of value
in the early diagnosis of pregnancy.

Astwood and Jones Method.—The method of isolating the preg­
nanediol as the glucuronidate complex is open to certain errors.
In warm weather, if suitable precautions are not taken, sponta­
aneous hydrolysis of the complex to free pregnanediol may occur
before analysis has commenced and, since only conjugated preg­
nanediol is measured by the Venning and Browne method, a low
result will be obtained. In addition the calculation used in deter­
mining the free pregnanediol equivalent of the glucuronidate
complex is considered by certain workers to be subject to error
owing to the presence of water of crystallization in the final product.
These difficulties are reviewed more fully by Astwood & Jones
(1941). To overcome the error due to free pregnanediol in the
urine, Bucher & Geschickter (1940) modified the Venning and
Browne method to recover the free as well as the conjugated preg­
nanediol. Beall (1937) has described a method for the isolation of
both free and combined pregnanediol from pregnancy urine by
adsorption on benzoic acid, but this technique does not appear to
have been widely used. Astwood & Jones (1941) developed a simpler
procedure of determining the total pregnanediol content of the
urine. They extracted the pregnanediol in the free form by acid
hydrolysis of the urine in the presence of toluene, most of the im­
purities in the toluene extract were separated by precipitation with
alcoholic alkali, and the final quantitative crystallization of the
pure pregnanediol was made from aqueous alcohol. These workers
observed that when the product obtained by the Venning and Browne
method was hydrolysed in aqueous solution the free pregnanediol
recovered was generally about 20% less than the theoretical quan­
tity, assuming that the original complex was sodium pregnanediol
glucuronidate. This loss they were unable to explain. It has now,
however, been demonstrated by Marrian & Gough (1946a,b) that all
samples of sodium pregnanediol glucuronidate prepared from
pregnancy urine contain up to 20% of another steroid, pregnane­
3(α:)-ol-20-one, presumably in the form of sodium pregnane-3(α:)
ol-20-one glucuronidate. (This steroid has been isolated in the free
form from pregnancy urine by Marker & Kamm, 1937.) Marrian &
Gough found that this steroid is completely eliminated in the
Astwood and Jones method for determining pregnanediol.

Modifications of Astwood and Jones Method.—Kober (1931)
observed that concentrated sulphuric acid, added to pregnanediol, produced an orange colour. This colour reaction was employed by Talbot, Berman, MacLachlan, & Wolfe (1941) to estimate pregnanediol after purification by the Astwood and Jones technique. This colour reaction was also utilized by Guterman (1944, 1945) to develop a semi-quantitative test for pregnanediol in urine. 100 ml. of morning urine is hydrolysed and extracted as in the Astwood and Jones method and a similar purification method is employed. Sulphuric acid is added to the final solution and the colour noted. An orange to deep orange-brown colour is considered as indicating pregnanediol in quantities characteristic of pregnancy, colourless to light yellow solutions are regarded as negative. Guterman considered this a rapid and reliable method provided it was not employed until the patient had missed at least one menstrual period. Tests on 248 patients involving obstetrical diagnostic problems gave an accuracy of 92%, whereas the Friedman test in the same series gave an accuracy of 87%. McCormack (1946) using Guterman’s method tested urines from 304 patients and obtained an accuracy equivalent to that obtained with concurrent Friedman tests. Reinhard & Barnes (1946), however, in a study of the method in 180 cases, concluded that the test was of little value. The errors, ranging from 16 to 42%, were considered to be due to the individual variations in the progesterone metabolism and not due to errors of technique. Morrow and Benua (1946) have also reported a high percentage of errors.

Using a modification of the Guterman method, Davis and Fugo (1947) have carried out serial determinations of pregnanediol excretion in normal and complicated pregnancy in over 100 patients. Mack & Parks (1947) have developed a rapid test for pregnancy which depends on the presence of a macroscopic precipitate of pregnanediol at a certain stage in the Astwood and Jones procedure. This precipitate is said only to occur with pregnancy urine or with urine from non-pregnant women during the luteal phase of the cycle. Correct positive results were obtained with samples from 65 of 67 patients. Samples from 51 of 54 patients gave correct negative results. The authors believe that their method will be of considerable clinical value.

An important observation concerning the technique of the Astwood & Jones procedure and the methods derived from it has been made by Somerville, Gough, & Marrian (1947). They have shown that slow controlled cooling is essential for exact quantitative recoveries of pregnanediol after the hot-precipitation purification from ethanolic solution with alkali or water.

Pathological Conditions.—The excretion of pregnanediol during toxæmias and other pathological conditions which may be associa-
ted with pregnancy have been studied by several investigators including Weil (1938a), Browne, Henry, & Venning (1939), Hamblen, Ashley, & Baptist (1939), Cope (1940a,b), Guterman (1944, 1946), and Rubin, Dorfman, & Miller (1946).

Metabolism of Progesterone.—It is now generally agreed that pregnanediol is largely derived from progesterone although there is some evidence that a part may arise from deoxycorticosterone. Further information about the intermediate metabolism of progesterone will be found in the review by Pincus & Pearlman (1943).

Other Reduction Products of Progesterone.

Numerous other steroids which are in all probability reduction products of progesterone have been isolated from pregnancy and non-pregnancy urine. These include two isomers of pregnanediol—allopregnanediol-3(α),20(α) and allopregnanediol-3(β),20(α). (See review by Pincus & Pearlman, 1943) None of these steroids has so far proved of value for pregnancy diagnosis tests.

Domestic Animals.

Mare.

Progestogens.—Clauberg (1933) extracted pregnant mares' urine in quantities of 3-5 litres but was unable to detect progestogen. Using the Clauberg method, Hetzel (1936) found small quantities of progestogen in the urine of the mare at the tenth month of gestation. Positive results with the urine from mares advanced in pregnancy were also obtained by Münich (1937). Trancu-Rainer & Vlăduțiu (1937) were unable to detect progestogen in the urine or saliva of the pregnant mare but their method of assay is obscure. Negative results were reported by Kimura & Lyons (1937).

Kimura & Lyons (1937) were unable to detect progestogen in the blood of pregnant mares.

Kimura & Lyons (1937) did not find progestogen in the chorion or endometrium of pregnant mares, although it was present in the corpora lutea. Ehrhardt & Hardt (1937), however, considered that the placenta of the mare contained traces of progestogenic substances.

Pregnanediol.—Pregnanediol has been isolated from the urine of pregnant mares (Marker, Kamm, Crooks, Oakwood, Lawson, & Wittle, 1937.) A simple method of isolating pregnanediol from pregnant mares' urine was described by Weil (1938b) which involves enzyme hydrolysis by incubating at 37° C. for four days, with purification of the pregnanediol by precipitation from alkaline acetone. Weil states that a litre of urine from a mare 6 months' pregnant was used but gives no indication of the amount
of pregnanediol obtained. Further studies by Marker & Rohrmann (1939a) revealed that the pregnancy urine of the mare differed from other urines in the proportion of the three pregnanediols present, allopregnanediol-3(\(\beta\)),20(\(\alpha\)) being present in considerable quantity (approximately 25 mg. per gallon (3.8 litres)) whereas pregnanediol (i.e. pregnanediol-3(\(\alpha\)),20(\(\alpha\)) and allopregnanediol-3(\(\alpha\)),20(\(\alpha\)) were present respectively in quantities of 3 mg. and 2 mg. per gallon.

The Venning and Browne method does not appear to have been applied to the urine of the pregnant mare and the nature of the conjugation product is not known. Stevenson (1947), using both the Astwood & Jones, and the Guterman techniques (see pages 102-103), failed to detect pregnanediol in the urine of mares four or more months' pregnant.

Other Reduction Products of Progesterone.—The following ketonic steroids have been isolated from the urine of pregnant mares—allopregnanol-3(\(\beta\))-one-20, pregnanedione-3,20, and allopregnanedione-3,20. In addition there are various other steroids which may or may not be derived from progesterone; these include allopregnanetriol 3(\(\alpha\)),16,20, uranediol-3(\(\beta\)),11, and uranol-11-one-3. Most of this work has been carried out by Marker and his co-workers and further details and references will be found in the review by Pincus & Pearlman (1943).

The three isomeric pregnanediols are absent from or present only in traces in the urine of the stallion (Marker, Wittle, & Lawson, 1938).

**Cow.**

Progestogens.—Zsemyle (1937) tested the urine from 36 cows at various stages of gestation for progestogens by a modification of the Clauberg test. He claimed that by using extracts of urine he was able to detect the presence of progestogens in the urine during the second half of pregnancy. This claim, however, has still to be confirmed.

Traces of progestogens were found in the blood of a pregnant cow by Duyvené de Wit (1938b) using the female bitterling as a test animal.

Apart from their presence in the corpora lutea (Bretschneider, Duyvené de Wit, & Kaay, 1942), progestogens have been detected in the placenta of the cow (Adler, Fremery, & Tausk, 1934; Kimura, 1935; Ehrhardt & Hardt, 1937).

Pregnanediol.—Marker (1938) isolated from urine of pregnant cows pregnanediol and its isomers in approximately half the quantities present in human pregnancy urine. The form in which it is excreted is not known; it does not appear to be excreted conjugated.
with glucuronic acid, since no sodium pregnanediol glucuronidate was obtained from cow pregnancy urine by the Venning and Browne method (Cowie & Greenbaum, 1943) (cf. excretion in the bull). O’Moore (1947) assayed the urine of 72 cows (pregnant and non-pregnant), 4 bulls, 3 bullocks and one heifer-calf by the Astwood and Jones and Guterman techniques (see pages 102-103). Small quantities of impure sterols (in the case of the cow 0.8 mg. to 8.0 mg. per litre) which were neither cholesterol nor oestrogens were obtained. The amount of these residues was not related to pregnancy. A few samples of urine from pregnant cows tested by the Venning & Browne method (see page 100) and the Weil method (see page 104) gave negative results. O’Moore concludes that none of the above tests can be used for the diagnosis of pregnancy in the cow. Stevenson (1947) also reports negative results with tests on urine samples from cows 2-8 months pregnant using both the Astwood and Jones, and the Guterman methods.

Greenbaum (1944) found no pregnanediol in the milk or in the butterfat of cows.

Marker, Wittle, & Lawson (1938) found that bulls’ urine was one of the richest sources of pregnanediol. They were able to isolate pregnanediol and its isomers in quantities twice or more that found in the urine of pregnant women (pregnanediol-3(\(\alpha\)),20(\(\alpha\))-100 mg. per gallon (3.8 litres); allopregnanediol-3(\(\alpha\)),20(\(\alpha\))-50 mg. per gallon; allopregnanediol-3(\(\beta\)),20(\(\beta\))-12 mg. per gallon). No pregnanediols were found in the urine of steers (Marker, 1939b). The pregnanediol does not appear to be conjugated with glucuronic acid, for Strickler, Walton, & Wilson (1941) were unable to isolate sodium pregnanediol glucuronidate from bull urine by the Venning and Browne method.

**Other Animals.**

*Goat and Ewe.*—Ehrhardt & Hardt (1937) detected traces of progestogen in the placenta of the goat. Negative results were obtained with 2 placentae from ewes.

*Sow.*—The urine of the pregnant sow does not appear to have been investigated for the presence of progestogens.

Bloch (1936) was able to detect progestogen in extracts of 8 litres of sows’ blood using the Corner-Allen method of assay. Duyvené de Wit (1941a) using the bitterling test estimated that there was approximately 40 \(\mu\)g. of progestogen per litre of blood in the pregnant sow.

Apart from its presence in the corpora lutea, progestogen has been detected in the placenta of the sow by Duyvené de Wit (1938b).

The three common isomeric pregnanediols are absent from the urine of the pregnant sow, but two pregnanolones (pregnanol-3
(\text{\textsuperscript{\alpha}})-one-20 and allopregnanol \text{3(\beta)}-one-20) were isolated by Marker & Rohrmann (1939b).

Bitch.—Ehrhardt & Hardt (1937) were unable to detect progestogens in the placentae of bitches. Knoppers (1940) was unable to find sodium pregnanediol glucuronidate in the urine of pregnant bitches.

Cat.—Westphal & Buxton (1939) were unable to isolate sodium pregnanediol glucuronidate from the urine of pregnant and non-pregnant cats.

Rabbit.—Bloch (1936) tested the blood of the pregnant rabbit for progestogens and concluded that in the whole of the circulating blood there was less than 1 rabbit unit of progestogen (Corner-Allen method).

Westphal & Buxton (1939) and Knoppers (1940) were unable to find sodium pregnanediol glucuronidate in the urine of pregnant and non-pregnant rabbits. Traces of the complex were found in the urine of two post-parturient rabbits (Westphal, 1942a).

It is of interest to note that the rabbit is the only animal, apart from man, known to excrete pregnanediol conjugated with glucuronic acid. Following the administration of progesterone to rabbits, Heard, Bauld, & Hoffman (1941) were able to isolate pregnanediol from the urine; at first they considered that it was not conjugated with glucuronic acid, but Hoffman & Browne (1942) and Hoffman (1942) showed that this was so. This was confirmed by Westphal (1942a). It has also been shown in the rabbit that when deoxycorticosterone is administered, part of it is excreted in the form of sodium pregnanediol glucuronidate (Westphal, 1942b; Hoffman, Kazmin, & Browne, 1943).

Guinea-pig.—Westphal (1942a) was unable to find free or conjugated pregnanediol in the urine of guinea-pigs either before or after injection of progesterone.

Apes and Monkeys.—Westphal & Buxton (1939) were unable to isolate free or conjugated pregnanediol from the urine of pregnant and non-pregnant monkeys. Marker & Hartman (1940) confirmed these findings, failing to find even a trace of pregnanediols.

Elder (1941) found no trace of sodium pregnanediol glucuronidate in the urine of two chimpanzees during pregnancy. Fish, Dorfman, & Young (1942) isolated small amounts (2 mg. per day) of pregnanediol from the urine of two chimpanzees during the 5th and 6th month of pregnancy. Fish, Horwitt, & Dorfman (1943) isolated pregnanediol from the urine of an ovariectomized chimpanzee which had received deoxycorticosterone acetate.
CHAPTER 5. TESTS BASED ON HORMONAL INVESTIGATIONS OF BODY FLUIDS. D. OTHER HORMONES

Chromatophore Hormone.

A secretion from the pars intermedia of the pituitary, or in certain cases from the pars glandularis, is of great importance in regulating the distribution of the pigment granules in the chromatophores in the skin of cold-blooded animals. The chromatophores of interest in connection with pregnancy tests are the melanophores of fishes and amphibia, and erythrophores of fishes. The degree to which the chromatophores are controlled by a hormone from the pituitary varies with the species; in some cases the hormone plays a negligible part and the regulation is practically entirely nervous. Further information on this subject can be obtained in the excellent reviews by Van Dyke (1936, 1939).

This hormone is also found in the pituitary of mammals, including man, it can be detected in the blood and urine of normal men and women and, since it appears to be present in greater quantities than normal in the urine of pregnant women, tests for its presence have been used for pregnancy diagnosis. Van Dyke concludes that if the hormone has a function of importance to mammals this has not yet been demonstrated. It has been suggested that it may be concerned in visual adaptation in mammals to which further reference will be made (page 110).

Woman.—Ehrhardt (1927a,b) observed that the blood of pregnant women when injected into frogs (Rana temporaria) produced dispersion of the pigment granules in the dermal melanophores the skin as a result becoming much darker in colour. Biehl (1927) obtained negative results with serum but she observed that when pregnancy serum was given along with pituitary extract it had a synergic action over pituitary extract alone. Jores & Helbron (1933) were unable to demonstrate any increase in the quantity of melanophore hormone in the blood of women during pregnancy nor was there any substance which intensified the action of the melanophore hormone.

Drouet & Florentin (1933) injected urine from pregnant women into frogs but melanophore activity was only intermittently observed.

Konsuloff (1934a) attempted to use hypophysectomized fish (Gobius melanostomus) for detecting melanophore hormone in the urine. In a later publication (1934b) he claimed more satisfactory results with hypophysectomized frogs (Rana esculenta). Urine from non-pregnant women was said scarcely to alter the colour of the frog whereas pregnancy urine produced a chocolat
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brown colour in 1/2-3/4 hours with a maximum effect at 1½ hours.

He compared this test with the Aschheim-Zondek test and claimed that while the results were just as accurate, they were obtained very much more quickly and the frogs could be used every second day for several months. Brühl & Rieckhoff (1935-36) repeating Konsuloff's work were unable to confirm the reliability of the test. False positive results were obtained with urine from cases of cancer, tuberculosis, and venereal diseases. Somewhat more accurate results were obtained by concentrating the urine before testing, but it was concluded that the method could not be considered as an accurate substitute for the Aschheim-Zondek test. Jores (1936) and Bruckmann (1937) also found the test unreliable. Recently, however, Bourgraaf & Dingemanse (1946) have obtained good results with Konsuloff's method. In 400 samples of urine only one incorrect result was observed. Raza &Spurrell (1937), using intact frogs and Ringer-perfused frog limbs as indicators of the presence of the hormone, tested 46 pregnancy and 52 non-pregnancy urines. Forty-five of the pregnancy samples and 20 of the non-pregnancy samples gave positive results. They concluded that the high percentage of positive results in the non-pregnant group completely invalidated the use of the reaction for diagnostic purposes. Dychno (1936), using a dermatoscope for reading the skin reaction, is reported to have obtained reliable results with the Konsuloff hypophysectomized frog technique.

Binet, Verne, & Luxembourg (1934) suggested a simpler method of detecting the hormone. Scales from the Prussian carp (Carassius vulgaris) were placed in a saline solution of the precipitate obtained by adding acetone to urine. If melanophore hormone was present in the test sample, dispersion of the pigment in the melanophores was observed in 2-3 minutes. A similar method was proposed by Konsuloff (1936).

Mandelstamm (1935) claimed that an accurate and simple way of diagnosing pregnancy from the presence of melanophore hormone in the urine was to place sticklebacks (Gasterosteus aculeatus) in the undiluted urine. In positive tests the fish showed a darkening within an hour. No indication of the accuracy of the method is given.

Simola & Rivas (1936) used isolated pieces of frog skin placed in the urine as test objects. The interpretation was considered easier with this technique than with the hypophysectomized frog. Positive results were obtained in only 50 to 65% of pregnant women.

There appears to be conclusive evidence that the presence of chromatophore hormones in the urine is not sufficiently charac-
teristic of pregnancy to be of diagnostic value. (See also Richter, 1944.)

There is some slight evidence that the chromatophore hormone in mammals is in some way connected with pupillary dilatation. Jores (1933) noted that when melanophore hormone was instilled into the human eye the adaptation time was shortened. Konsuloff (1934b) observed that when urine from pregnant women was injected into normal frogs, an unmistakable and characteristic change in pupil size occurred within half an hour. Whether there is any connection between these observations and the pupillary tests of Bercovitz (page 159) and the pupillary reflex tests of Mintscheff (page 160) is an interesting speculation (see Mintscheff, 1937). [Compare also tests of Davis et al., 1934 (see page 37); and Takakusu, 1931 (see page 161).]

An account of the chemistry of the chromatophore hormone is given in the reviews by Van Dyke (1936, 1939).

Animals.—Russeff (1937) reported the presence of melanophore hormone in the urine of the pregnant cow, the quantity excreted being greatest in early pregnancy.

Simola & Rivas (1936), using isolated pieces of frog skin, tested the urine of non-pregnant and pregnant mares, and stallions. The urines from non-pregnant mares and stallions gave negative or doubtful results; with pregnant mares' urine only one sample gave a positive result. Mintscheff (1937) employing Konsuloff's carp-scale technique (see page 109) tested the urine of 60 mares. An increase in the excretion of hormone was observed during gestation; this was most marked in early pregnancy. Mintscheff concludes that the reaction is not sufficiently accurate for pregnancy diagnosis. Mintscheff also described certain interesting changes in the pupillary reflexes during pregnancy and discusses their relationship to the melanophore hormone and the Bercovitz test (page 159).

Traneu-Rainer & Vălăutiu (1937) have claimed that chromatophore hormone is present in the saliva of the stallion. They carried out the test by placing the fish Phoxinus lacus in saliva. Simola & Rivas (1936) were unable to detect chromatophore hormone in the urine of rats.

Mammogenic Hormones.

It appears unlikely that mammogenic hormones will prove of value in pregnancy diagnosis tests.

In a few experiments Chamorro (1943) was unable to detect mammogens in the serum of a pregnant woman or of a pregnant mare.

Kawano & Nakano (1938) claimed that urine from pregnant
cows injected subcutaneously into ovariectomized rabbits produced mammary development and lactation.

Injections of urine from two pregnant cows into ovariectomized rats were without effect on the development of the mammary glands (Cowie, 1943).

Relaxin.

Hisaw (1929) demonstrated that the separation of the pelvic bones in the guinea-pig during pregnancy is under hormonal control. He postulated that the hormone was produced by the corpus luteum and that the animal had to be under the influence of oestrogenic hormones before this hormone—"relaxin"—would produce relaxation of the ligaments. Hisaw was able to demonstrate the presence of the hormone in the blood of the pregnant mare, sow, dog, cat, rabbit, and guinea-pig. He was unable to detect it in the blood of the pregnant cow or in the blood of women during the last three months of pregnancy. The hormone was found in the rabbit placenta but was not present in human or bovine placentae.

Pommerenke (1934) showed that this hormone was present in the serum of pregnant women in the early stages of pregnancy, the quantity becoming less as pregnancy advanced. As it could only be detected in 75% of cases he did not consider its presence offered a reliable means of diagnosing pregnancy. Abramson, Hurwitt, & Lesnick (1937) further investigated the possibilities of developing a pregnancy test based on the presence of relaxin. From preliminary experiments on 57 women they concluded that reliable results might be obtained in early pregnancy and that the test could be read in 12 hours after injecting the guinea-pig with the first dose of serum. The disadvantage lay in the pre-treatment required—ovariectomy and induction of oestrus with oestrogen in the test animal—which for routine work made the test troublesome and expensive.

There have been doubts cast on the existence of relaxin and it has been suggested that the effects are due to the combined action of oestrogens and progesterone, but recent work by Abramowitz, Money, Zarrow, Talmage, Kleinholtz, & Hisaw (1944) and Hall & Newton (1946) has confirmed the separate identity of relaxin. (See also Brit. med. J., 1945.)
Further information and references to the hormonal tests for pregnancy in the woman and domestic animals will be found in the following reviews:

Zondek & Aschheim (1927a), Simonnet (1931), Stålfors (1931), Siegert (1931), Aschheim (1931), Vozza (1931), Cuboni (1932), Ehrhardt (1932b), Parvey (1932), Aschheim (1933), Bourg (1933), Robson (1934, 1936), Vogt (1935), Millar (1936), Zavadovskii, Kazarnovskaja, Stamler, & Faermark (1936), Szepeshelyi (1937), Fluhmann (1937), Crew (1937a), Barulin (1937), Berthelon (1937), Weisman (1938), Zondek (1941), Resser (1941), Held (1941), Taperhoux (1942), Iyer (1942), Wattenwyl (1942), Joël (1945).

Further information will also be found in the recent books by Cameron (1947), Robson (1947) and Samuels (1947). Samuels deals at some length with the gonadotrophic and oestrogenic hormones in domestic animals during pregnancy. His theories of endocrine function are, however, somewhat unorthodox.
CHAPTER 6. TESTS BASED ON ENZYMIC INVESTIGATIONS OF BODY FLUIDS

PROTECTIVE ENZYMES—THE ABDERHALDEN REACTION.

The experimental evidence on which Abderhalden based his reaction has been reviewed by him (1913, 1922) and only the outline of the theoretical basis of the test need be given here.

The test is based on the assumption that when foreign proteins gain access to the blood stream of mammals without having undergone degradation in the alimentary tract, the cells of the body elaborate specific ferments or enzymes which are capable of breaking down these foreign proteins into simpler products such as amino-acids. The protective enzymes (Abwehrfermente) thus elaborated are set free into the blood plasma and according to Abderhalden their presence there can be detected by noting the changes which occur when such plasma is allowed to act in vitro on the specific substrate, i.e., on the appropriate foreign protein. That changes occur under such circumstances has been amply confirmed, but whether a specific enzyme has produced the changes has been the subject of great controversy. Abderhalden further postulated that during pregnancy cells from the chorion entered the maternal circulation and acted as foreign proteins by evoking the production of specific proteolytic enzymes; the presence of these enzymes specifically proteolytic to chorionic tissue was the basis of the Abderhalden test for pregnancy. Since numerous proteolytic enzymes exist in the body, it is obvious that the specificity of the reaction is all-important.

Abderhalden claimed that by the use of suitable substrates the reaction could be used to diagnose cancer and certain chronic bacterial diseases; this aspect is, however, outside the scope of this review.

Before giving a brief outline of the multiplicity of methods and modifications described for detecting the protective enzymes, the history of the application of the reaction to pregnancy diagnosis will be summarized.

Abderhalden described his test for pregnancy in 1912. It was given much publicity and great accuracy was claimed for it. Reports on its use rapidly appeared and for the first two years these were mainly favourable. The interest aroused by the Abderhalden reaction may be gauged from the number of publications which appeared concerning it—by 1915 over 300 papers had already appeared (Pfeiler, Standfuss, & Roepke, 1915) and
by 1922 the task of giving a complete bibliography was not attempted even by Abderhalden in his book *Die Abderhaldensche Reaktion* on the grounds that a complete list of references would have occupied most of the small book (it contains some 350 pages). He gave, however, a selection of references which cover the first decade of the test's life-history.

The accuracy of the test, the most important factor from the clinical aspect, depended on the specificity of the reaction which, according to Abderhalden and his most ardent supporters, was beyond dispute. The test *if properly carried out* was considered to be infallible. Adverse results were encountered, but these were readily excused on the grounds of faulty technique or failure to take into consideration certain fallacies which had been postulated rather than proved by Abderhalden. The few investigators who failed to reach the optimistic conclusions of the majority and who doubted the specificity of the reaction were criticized and even scorned. To their claims that they had carefully followed the technique laid down by Abderhalden, it has been reported that Abderhalden replied "I treat such assertions with the scepticism born of a rich experience." While the techniques of enzyme chemistry are exacting, it is unlikely that those who failed to confirm Abderhalden's results were all careless and unskilled workers; it is also unlikely that those who found the test infallible were all beyond reproach in matters of technique.

The critics, however, were not to be silenced. Michaelis & Lagermarck (1926) after careful experimentation, questioned the reliability of the test and severely criticized the theory on which the reaction was based. A yet more vigorous attack was made by Leitch (1914) who ridiculed the Delphic claim of Abderhalden that, properly read, the result is never wrong, and condemned the flood of uncritical communications which had hailed the reaction as infallible and had blindly overlooked and excused adverse results. In a series of tests in 100 pregnant and non-pregnant women (in which, in the author's own words, "he had done his best to follow Abderhalden's directions without actually having made the customary pilgrimage to Halle to receive the direct apostolic revelations from the master") there were 17 "most glaring mistakes" even after reading the test in a most generous manner and making allowances for all hypothetical fallacies. Leitch concluded that there probably exists in serum, more frequently but not exclusively in pregnancy, a general proteolytic and peptolytic enzyme which can be demonstrated by adding a suitable though not necessarily a specific protein or peptone. Since the enzymes were present in serum apart from pregnancy, Leitch considered the method quite unreliable for pregnancy diagnosis.
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workers confirmed in general the conclusions of Leitch (Pfeiler, Standfuss, & Roepke, 1915; Oppler, 1916; Rivas & Buckley, 1916).

Despite the gradual realization that the Abderhalden reaction did not possess the reliability claimed for it, interest in the test was sustained by the frequent publication of modifications, all claiming to eliminate certain of the fallacies associated with the original method, but none of these modifications was successful in converting the Abderhalden reaction into a reliable test for pregnancy. Claims that a test had been developed from the Abderhalden reaction but differing fundamentally in that it was not based on enzyme activity, were made by Lütting and Mertz (see page 117). Not only was it said to be a reliable pregnancy test but by a slight modification, the sex of the foetus could be correctly established in 99% of cases. Unfortunately, this test, like its predecessors failed to fulfil the extravagant claims of its originators.

General interest in the Abderhalden reaction came to an end with the advent of the hormonal tests for pregnancy, and references to it disappeared from gynaecological literature although a few unsuccessful attempts were made to relate it to the hormonal tests, by claims that the protective enzyme was probably identical with the gonadotrophic hormones (Lütting, 1931a). Abderhalden remained undaunted. He demonstrated (1930a) that the protective enzyme was excreted by the kidney and was present in the urine of pregnant women (a similar claim had been made by Kafka, 1914a,b) and maintained that by substituting urine for serum, his reaction could be simplified. This modification aroused little interest, but there has been confirmatory evidence that non-specific proteolytic enzymes may occur more frequently in pregnancy than in non-pregnancy urine (Cadness & Wolf, 1931). Numerous further modifications of the urine test have been published by Abderhalden and his co-workers in his journal Fermentforschung from 1930 onwards; the latest so far seen appeared in 1942.

The methods employed for the detection of protective enzymes can be divided roughly into three main classes.

1. Dialysis methods.
2. Optical methods.
3. Direct methods.

1. Dialysis Methods.

The serum under examination for the presence or absence of specific enzymes is placed in a special dialysis thimble along with a small portion of specially prepared placental substrate. The thimble is placed in a flask of distilled water and incubated at 37° C. for 16 hours. Where a specific
enzyme is present, part of the placental tissue will be split up into peptones and amino-acids which diffuse through the dialysis thimble into the distilled water. The presence of amino-acids in the dialysate can be detected by the colour reaction given with ninhydrin. The biuret reaction was originally used but was not entirely satisfactory. The dialysate can also be tested for nitrogen by the Kjeldahl method. Eigenberger (1934) tested the dialysate with Esbach’s reagent. For the details of the techniques the original papers of Abderhalden must be consulted (for references see Abderhalden, 1922). All stages of the test must be carefully controlled, the dialysis thimbles must not be permeable to proteins, and the entire test must be carried out with chemically clean and sterile apparatus and all precautions taken against bacterial contamination.

2. Optical Methods.

To overcome various technical difficulties inherent in the dialysis method various optical methods were employed to detect proteolytic changes in the serum-substrate mixture.

(a) Polarimetric methods were employed instead of, or to supplement, the dialysis method (see Abderhalden, 1922).

(b) Changes in the refractive index of the serum-substrate mixture were also used to detect enzyme activity.

Hirsch employed the interferometer for this purpose. For references see Abderhalden (1922), Hirsch (1925), Küster (1925), Kleesatel (1926), Kaufmann (1926, 1927), Hellmuth (1926), Haynes & Wolf (1927), Eiger, Grosmann, & Kleczynski (1927), Küster & Koulén (1927), and Zimmer, Lendel, & Fehlow (1928-29).

Pregl & Crinis (1919) used the refractometer. See also Kupelwieser (1922, 1924), Serejski (1924), and Schnupp (1925).

(c) The use of the spectrophotometer was described by Abderhalden & Haas (1926-28).

(d) Zangemeister & Krieger (1928) and Zangemeister (1929) employed a photometer to study the turbidity of serum-substrate mixtures. It was claimed that this turbidity reaction was probably fundamentally different from the Abderhalden reaction. Rabau (1930), however, found it unreliable.

3. Direct Methods.

The methods to be described in this class are varied and unrelated but are classed together for convenience.

(a) Macroscopic Examination. The serum-substrate mixture is incubated in a test-tube. If the serum is from a pregnant woman it will become turbid and milky, otherwise it will remain clear (Abderhalden, 1921).

(b) Microscopic Examination. The action of the serum on the
substrate may be observed under the microscope (Abderhalden, 1922).

(c) Dyes and Other Indicator Substances. Attempts were made to combine with the placental substrate some material which would be liberated by the action of the specific enzyme and could be readily detected. Certain dyes were first used. A placenta-iron preparation was then described by Kottmann who claimed that the iron combined in the substrate was only liberated by the action of pregnancy serum, when it could be readily detected with potassium thiocyanate. For references see Abderhalden (1922).

(d) Filtration Methods. Sdrodowsky (1920) filtered the serum-substrate mixture and tested the filtrate at various dilutions with ninhydrin, using filtrates of serum alone for controls.

(e) Protein Precipitation. Various methods have been described for detecting proteolysis by the presence of non-coagulable nitrogenous substances in the filtrate of serum-substrate mixture (Abderhalden, 1922).

This method was further investigated and developed by Lütte & Mertz (1924) and Sellheim (1925). After incubation of the serum-substrate mixture, alcohol was added and the mixture boiled and centrifuged. The supernatant fluid was then tested with ninhydrin or by one of the optical methods.

Sellheim (1925) described a further modification developed by Lütte, Mertz, & Berger, who prepared a dry powder from defatted placenta which they claimed was superior to the placental substrate described by Abderhalden. After precipitation of proteins by alcohol the filtrate was tested as above with ninhydrin. It was claimed that by the use of dry extract the incubation time could be reduced to half-an-hour. This method is generally referred to in the literature as the “Alcohol-Substrate Reaction” (ASR). Sellheim also described a “turbidity-phenomenon” which occurred when dilute hydrochloric acid was added to the filtrate after alcoholic precipitation of the proteins.

In 1926 Lütte & Mertz described a further development of their test. This is generally referred to as the “Alcohol-Extract Reaction” (AER). 1 ml. of the serum to be tested was added to 1 ml. of a special fat-free placental extract, the mixture shaken and 10 ml. of absolute alcohol added. No incubation was necessary. After filtration, 0.2 ml. of 1% alcoholic ninhydrin was added to the filtrate which was then boiled for 1 minute and a drop of dilute hydrochloric acid added. In positive tests a blue colour was present. This reaction was claimed to be fundamentally different from the Abderhalden reaction in that enzymes played no part but the mechanism is by no means clear, although it is claimed to be a purely physico-chemical process.
Lütte and Mertz also described a modification of the "Alcohol-Substrate Reaction," which is generally referred to as "ASR 2," and a "Saline-Extract Reaction" (KER). Details of all these tests can be found in the book by Lütte & Mertz (1927).

Abderhalden Reaction Using Urine.—Reference has already been made to this method (page 115). Further details will be found in the work of Kafka (1914a,b), Cadness & Wolf (1931), and Tanabe (1938) and in numerous papers by Abderhalden and co-workers published in Fermentforschung from 1929 onwards.

Further references on the Abderhalden reaction will be found in reviews by Oppenheimer (1926, 1936a,b), Honda & Yanagi (1928), Pincussen (1929), Abderhalden (1937), Ammon & Chytrek (1939), and Abderhalden (1941).

Mare.

Dialysis Methods.—Claims that the Abderhalden reaction gave reliable results in mares were made by Schlimpert & Issel (1913), Rehbock (1914), Kahn (1915), and Wecke (1915, 1916). In more comprehensive experiments by Raebiger, Wiegert, Siebold, Roecke, & Rautmann (1915), Pfeiler, Standfuss, & Roepeke (1915), and Bernhardt & Hofherr (1915) these claims were not substantiated and it must be concluded that the method is of no value in the mare.

Optical Methods.—Using the interferometer method, Hirsch (1923-24) carried out 110 tests on pregnant and non-pregnant mares and claimed an accuracy of 96%. This figure was, however, obtained after discarding 14 errors which were considered to be due to faults in technique. Knauer (1923) also believed the test to be very useful; he had one error in 50 tests. Miessner (1923) and Wellmann & Manninger (1927) found the method completely unreliable.

Direct Methods.—Denker (1920) obtained good results with the Kottmann modification for pregnancy diagnosis in the mare. Kreibohm (1921) obtained, however, only 50 correct results in 60 tests.

Barulin (1933-34) using a simplification of the Abderhalden reaction (he gives no technical details) obtained an accuracy of 92.4% in tests on 400 mares, but as the mares had to be starved for 24 hours before testing he considers the test of limited value.

Dahmen & Wollersheim (1927) in a few tests on mares obtained accurate results with their "Hormone-Method" (page 120). These results were not confirmed by Helm & Zuhdi (1938) who also found that the results obtained by the "Alcohol-Extract Reaction" were not sufficiently reliable for practical purposes.

Further references will be found in the reviews of Abderhalden (1922), Hirsch (1925), and Wernery (1927).
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Dialysis Methods.—Abderhalden & Weil (1912) described the use of the Abderhalden reaction for pregnancy diagnosis in the cow. Tests on 18 sera from non-pregnant animals were negative while 18 of 20 sera from pregnant cows were positive. The two pregnancy sera which gave negative results were considered not quite fresh. Further reports on the use of the dialysis method for pregnancy diagnosis soon appeared. Fauser (1912), Schattke (1913), Rehbock (1914a,b), and Kalm (1915) claimed practically 100% accuracy with the method; in most cases the number of animals tested was small and few details were given. Roos (1913) tested 8 pregnancy sera and 86 non-pregnancy sera; 2 of the pregnancy sera were negative and 12 of the control sera gave positive results. Richter & Schwarz (1913) obtained better results, having three errors in 63 tests. From tests on sera of 37 cows, Behne (1914) concluded that the test was not accurate for early pregnancy diagnosis. Wecke (1915, 1916) had one error in tests on 23 pregnant cows and 2 doubtful results with 7 non-pregnant cows. He observed that the reaction was positive for about three weeks post-partum and could be used to establish whether a cow was newly calved or otherwise (see also Falk, 1913). Raebiger, Wiegert, Siebold, Roecke, & Rautmann (1915) in a series of tests on 47 cows obtained an average accuracy of about 84 per cent. Even less satisfactory results were obtained by Pfeiler, Standfuss, & Roepke (1915) who in tests on 54 pregnancy sera and 96 non-pregnancy sera obtained only 32 and 44 correct results respectively. Weise (1916) considered that if the test was applied only to cows over three months' pregnant it was reliable and was a useful supplement to clinical examination. Zell (1917-18) on the basis of a few confirmed results considered the test very reliable provided it was carried out with great care and the animals were "in an absolute stage of hunger" when the sample of blood was taken—which in the ruminant is an impossible stipulation under practical conditions. Giuliani (1917) and Nebelung (1929) obtained an average accuracy of 88-90%.

Optical Methods.—Miessner (1923) failed to find the interferometer method reliable in the diagnosis of bovine pregnancy. Andersson (1930) using Zangemeister's photometric technique (see page 116) failed to note any changes in bovine serum-substrate mixtures indicative of pregnancy.

Direct Methods.—Campus (1919) considered that the Kottmann placenta-iron method was more satisfactory in the cow than the original Abderhalden reaction, a conclusion which appears to have
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been based on a small number of tests. Boye (1921) tested the sera of 82 cows by this method; 68 gave correct results.

Busch (1936) tested the sera of 32 cows by the Sdrodowsky modification (page 117). He concluded that it was of no value in the cow since there were 9 errors in 16 pregnancy sera and 1 error in 16 non-pregnancy sera.

Dahmen & Wollersheim (1927) applied the "Alcohol-Substrate Reaction" the "Alcohol-Extract Reaction" and the turbidity-phenomenon of Lütgge and Mertz (page 117) to bovine serum but found all three methods unreliable. From these methods Dahmen and Wollersheim developed what they chose to call a "Hormone-Method" of pregnancy diagnosis, based on the postulate that during pregnancy there must be present in the blood of the cow an anti-hormone to oestrogen or an enzyme which destroys oestrogen since the cow shows no signs of oestrus although oestrogens have been shown to be present in the blood. The test described by these authors is similar to the "Alcohol-Substrate Reaction" except that a preparation of ovarian hormone is substituted for the placenta substrate. This test gave 36 correct and 2 incorrect results with sera from pregnant cows; tests with non-pregnant animals were all negative.

Helm & Zühdi (1928) were unable to obtain reliable results in cows with either the "Alcohol-Extract Reaction" or the so-called "Hormone-Method" of Dahmen & Wollersheim.

Nebelung (1929) considered the "Alcohol-Substrate Reaction" less reliable in cows than the original dialysis method.

It can be concluded that the Abderhalden reaction has not proved a practical test for the diagnosis of early pregnancy in the cow. In general, negative results have been found more reliable than positive, and it would appear that although proteolytic enzymes are more frequently present in the serum during pregnancy they are also present in the blood at other times.

Further references and information may be found in the reviews by Abderhalden (1922), Hirsch (1925), Wernery (1927), and Becker (1927).

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Ewe.—Richter & Schwarz (1913) found the dialysis method quite unreliable in sheep. On the other hand Schlimpert & Issel (1913) and Weise (1916) believed the method to be of some value.

Müller (1923) obtained very variable results with the interferometer technique.

Helm & Zühdi (1928) found that neither the "Alcohol-Extract Reaction" nor the "Hormone-Method" was reliable in sheep.

Goat.—Richter & Schwarz (1913) considered the dialysis method
unreliable in the goat. Rehbock (1914a,b) and Giuliani (1917) believed it might prove useful for pregnancy diagnosis in the goat.

Reliable results were not obtained with either the interferometer or refractometer technique (Miessner, 1923; Turcan, 1924-26).

Sow.—Pfeiler, Standfuss, & Roepke (1915) and Weise (1916) agreed that the Abderhalden reaction was of no value for pregnancy diagnosis in the sow.

Bitch.—Lux (1923, 1923-24) investigated the possibility of diagnosing pregnancy in the bitch with the Abderhalden reaction using the refractometer technique. He concluded that provided the bitch was starved for 24 hours previous to taking the blood sample the results were reliable from the second week of pregnancy.

Guinea-pig.—Fauser (1912) claimed that the dialysis method gave reliable results in the guinea-pig.

**DIAMINO-OXIDASE, HISTAMINASE.**

**Woman.**

The presence of histaminase or diamino-oxidase in the blood is proving of considerable value as a means of diagnosing pregnancy in the human.

An increase in the histaminase content of the blood during pregnancy was first postulated by Marcou & Atanasiu-Vergu (1937) from a consideration of the results of experimental work on blood histamine. Later experimental work by Marcou (1938, 1939) confirmed this supposition. The possibility of utilizing this phenomenon for the diagnosis of pregnancy was investigated by Zeller (1940a) and Werle & Effkeman (1940a,b). Preliminary tests gave encouraging results and further tests were reported by Zeller and Birkhäuser (1940), Anrep, Barsoum, Ibrahim, & Amin (1941), Zeller (1941a,b,c, 1942), Llabhardt (1941a,b), Wenner & Wattenswyl (1941), Kapeller-Adler (1944), Ahlmark (1944a,b), Kolosynski (1945), and Mundwyler (1945). There is complete agreement among the various workers that histaminase or diamino-oxidase occurs in the blood of non-pregnant women only in very small quantities. From the 8th to the 12th week of pregnancy there is a marked increase. According to Ahlmark (1944a,b) the enzyme activity of the blood serum increases a hundred-fold by the 3rd-4th month of pregnancy and during the later part of pregnancy the blood attains an histaminolytic activity 500-1,000 times the normal.

There seems to be little doubt that this method of pregnancy diagnosis gives reliable results provided the test is not applied to patients less than three months pregnant.
Methods of Estimation.—According to Zeller the diamino-oxidase reaction is an oxidative one,

\[ R-\text{CH}_2\text{NH}_2 + \text{O}_2 + \text{H}_2\text{O} = R-\text{CHO} + \text{NH}_3 + \text{H}_2\text{O}_2 \]

and it will be obvious from the equation that there are several possible methods of measuring diamino-oxidase activity.

1. The oxygen consumption of the enzyme system can be measured in the Warburg manometer.

2. The ammonia produced as a result of the enzyme activity can be measured by various methods; the two more commonly used are the Conway diffusion technique and a Warburg manometric method.

3. The hydrogen peroxide produced can be estimated by its capacity for decolorizing certain dyes such as indigo disulphonate. This is the method commonly used in pregnancy tests.

4. When the substrate has biological activity (e.g. histamine) the biological activity remaining after the enzyme has been allowed to act for a certain time can be measured and compared with the original activity. In the case of histamine, the biological activity is measured by its action on the blood pressure of an anaesthetized cat or its effect on isolated portions of guinea-pig small intestine.

References to all these methods will be found in the reviews by Zeller (1942) and Ahlmark (1944a).

Identity.—As a result of a series of experiments, Zeller concluded that the diamino-oxidase which he described in 1938 was identical with the histaminase discovered by Best in 1929 (see review by Zeller, 1942). Zeller has shown that diamino-oxidase is capable of destroying not only histamine but also other naturally occurring diamines such as cadaverine, putrescine, spermidine, spermine, etc. In the techniques used in pregnancy diagnosis, Zeller advocates the use of cadaverine as a substrate rather than histamine since histamine is liable to inhibit its own oxidation if present in super-optimal amounts.

Kapeller-Adler (1944) has recently questioned Zeller’s claim that diamino-oxidase and histaminase are identical. Kapeller-Adler carried out parallel tests on the sera and placenta of pregnant women using two different substrates—histamine and cadaverine. In normal pregnancy the tests were found to be positive irrespective of the substrate used, but the sera from women suffering from toxaemia of pregnancy generally gave negative results with histamine as substrate, but positive results when cadaverine was used. The problem of the identity of the two enzymes is further discussed by Koloszynski (1945).

Pathological Conditions.—Apart from its value as a pregnancy test, the level of histaminase in the blood of pregnant women may
ENZYMIC TESTS

prove to be of value in the diagnosis of certain pathological conditions associated with pregnancy. It is outside the scope of this review to discuss this aspect; further information may be found in the papers by Effkemann & Werle (1940a), Ahlmark (1944a), Kapeller-Adler (1944), and Koloszynski (1945).

Distribution.—Effkemann & Werle (1940b) considered that diamino-oxidase was not present in urine. Later experiments (Werle, 1942) demonstrated that it was present in an inactivated form and if the urine was dialysed against running water for 16 hours the enzyme could be demonstrated. The nature of the interfering substance was not discovered. Werle was unable to demonstrate an increase in diamino-oxidase content of the urine in pregnancy.

Danforth & Gorham (1937) showed that the human placenta contained histaminase, the amount present showing some correlation with the efficiency of the uterine contractions. The presence of histaminase in the placenta was confirmed by Zeller, Schär, & Staehlin (1939) and Ahlmark (1944a,b). Kapeller-Adler (1944) obtained strong evidence that the quantity of histaminase in the placenta was inversely proportional to the uterine efficiency in labour.

Details of the presence of diamino-oxidase in other organs of the body will be found in the review by Zeller (1942).

DOMESTIC ANIMALS.

Werle & Effkemann (1942) reported that an increase in the blood histaminase did not occur during pregnancy in the cow, sheep, mare, sow, bitch or rabbit.

Ahlmark (1944a) tested the blood of 6 cows (3 were pregnant) and 2 bulls. All the samples showed considerable histaminolytic power. There was a tendency for higher values to occur in the samples from pregnant animals but this was not significant. The blood from a non-pregnant ewe and a non-pregnant sow showed histaminolytic activity.

Ahlmark (1944a,b) found no increase in the blood histaminase of the cat and rabbit during pregnancy, but an increase was observed in the blood of the pregnant rat and guinea-pig. Ahlmark noted that the histaminase content of the placenta of the rat was high whereas in the rabbit, guinea-pig, and cat it was low. The increase in the blood histaminase in the rat during pregnancy was also observed by Mundwyler (1945) who noted a rise in activity about the 7th day of pregnancy.

Details on the occurrence of diamino-oxidase in the organs of animals will be found in the review by Zeller (1942).
OTHER ENZYMES.

Pitocinase.—Page (1946b) observed that the ability of human pregnancy plasma to inactivate pitocin increased over a thousand-fold from the time of conception until term. The enzyme, pitocinase, which is considered to be responsible for the inactivation, has not been fully characterized but is thought to be a peptidase. Further studies by Page (1947) have shown that the concentration of the enzyme in the blood increases in a regular fashion for the first 16 weeks after conception. The mean value for plasma pitocinase in 22 normal non-pregnant women was 0.023 units (S.E. ± 0.0024) per ml. plasma, the pitocinase concentration fluctuating in the menstrual cycle. In healthy women a value of 0.07 units per ml. plasma is considered to be diagnostic of pregnancy, this level being reached four weeks after conception. By plotting the pitocinase values (on a logarithmic scale) against time after conception (on an arithmetic scale), the points were found to lie on a straight line for the first 16 weeks after conception, the week of pregnancy being equal to $6 \times (\log_{10} \text{plasma pitocinase} + 1.7) \pm$ a standard deviation of 0.7 weeks.

The author points out that, for the present, the method of assay of pitocinase requires more time and skill than a biological test for urinary gonadotrophin and he considers that the method will find usefulness when quantitative information on the duration of early pregnancy is required rather than as a purely qualitative indication of pregnancy.

[Added in Proof.—That the blood of pregnant women contains an enzyme capable of destroying oxytocin was first observed by Fekete (1930, 1932). The presence of this enzyme in the blood of pregnant women was further investigated by Werle & Effkemann (1941) and Werle, Hevelke, & Buthmann (1941), who were able to detect its presence in the blood from the beginning of the second month of pregnancy. The oxytocin-destroying capacity of the blood increased rapidly during the second month, then remained steady until the last month when there was a further increase in activity. These workers termed the enzyme "oxytocinase." It was not present in the blood of non-pregnant women.

Oxytocinase was sometimes present in the urine of pregnant and non-pregnant women.

No trace of oxytocinase was found in the blood of non-pregnant or pregnant mares, cows, sows, bitches, rats, or guinea pigs.]

Arginase.—Wehefritz & Gierhake (1931) suggested that the presence of arginase in the blood might be used as a test for pregnancy. They claimed that arginase was present in the blood of women during the early stages of pregnancy whereas it was
absent from the blood of non-pregnant women. No figures or experimental results were produced to support their claim.

Catalase.—Briihl (1932b) found no significant difference in the catalase content of the blood from non-pregnant and pregnant women. Further studies on the catalase system of the blood of pregnant and non-pregnant women were made by Gotsman (1940). There is no evidence that this enzyme would be of value for pregnancy diagnosis tests.

Diastase or Amylase.—Herrmann & Hornfeld (1926) studying the serum diastase in pregnant and non-pregnant women, observed a sharp rise in early pregnancy followed by a gradual decline. Somewhat similar results were obtained by Piano (1927). Pall (1927) in a study of the urinary diastase of pregnant and non-pregnant women found that the diastase content of the urine increased to three times its normal value in pregnancy, and he suggested that since the increase appeared to be characteristic of pregnancy the estimation of urinary diastase might serve as a simple pregnancy test. Schmidt (1928) found considerable variations in the amount of diastase present in the urine during pregnancy; in 18 of 17 pregnant women the values were normal; in the others the values were either above or below the figures for non-pregnant women. Ussolzew (1932) studied the urinary diastase in 54 pregnant women and completely failed to confirm Pall's claims; in only one case was a value obtained which was higher than that found in non-pregnant women. Further studies by Arneson & Morrin (1932) showed that normal pregnancy had no effect on the blood diastase, while wide variations in the amount of diastase in the urine were observed. From the results of further investigations by Spitzer (1932), Goldschmidt-Fürstner (1933), and Schiller & Smith (1943) it is clear that the estimation of urinary or serum diastase is unlikely to serve as a method of pregnancy diagnosis. (It is of interest to note that preparations of gonadotrophin from pregnancy urine have a factor which exerts marked diastatic activity associated but not identical with the active gonadotrophin (Nothdurft, 1944), see page 49.)

Lipase.—Herrmann & Kornfeld (1926) observed a sharp fall in the blood lipase in early pregnancy; later in pregnancy the activity increased but did not reach the non-pregnant value. Ussolzew (1932) investigated the blood lipase during pregnancy in 10 women to ascertain whether a pregnancy test could be evolved. He concluded that the method was quite unreliable.

Phosphatase.—The plasma phosphatase levels observed by Kay (1930) in a few cases of advanced pregnancy tend to be somewhat higher than the normal non-pregnancy level.

Meranze, Meranze, & Rothman (1937) carried out 347 phospha-
PREGNANCY DIAGNOSIS

tase determinations on the blood of 201 pregnant women. They
found that the values during the first six months of pregnancy lay
within normal limits, while the values for the 8th and 9th months
were definitely elevated. Quinto (1939) and Vermehren (1939)
also observed a gradual increase in blood phosphatase during
gestation with maximum values during the last month.

In view of the above findings it appears unlikely that a test for
early pregnancy can be based on the blood phosphatase.

The plasma phosphatase in the pregnant cow has been studied
by Wilson & Hart (1932). The results were somewhat variable
and inconclusive. Auchinaeie & Emslie (1933) studied the plasma
phosphatase in both cows and ewes during pregnancy and observed
a fall in the plasma phosphatase during pregnancy. Folley & Kay
(1936) reviewing these experiments consider that they are incon-
cclusive and that further work is desirable to establish the behaviour
of the plasma phosphatase in the cow and sheep during pregnancy.
Extensive investigations by Allcroft & Folley (1941) have shown
that in the cow pregnancy per se may be accompanied by a slightly
raised serum phosphatase although the level of phosphatase did
not appear to be correlated with the stage of pregnancy.

It does not seem likely that it will be possible to develop a test
for pregnancy based on the plasma phosphatase in the cow or sheep.

Antitryptic Action of Blood Serum.—It has been known for some
considerable time that during pregnancy the blood serum of women
becomes markedly antitryptic in action. A review of the earlier
work on this subject is given by Grafenberg (1909) who confirmed
the increase in antitryptic activity. Rosenthal (1911) investigat-
ged the possibility of developing a pregnancy test based on the
above observations. He investigated 120 cases and observed that
the antitryptic activity of the serum increased from the first
month onwards. The rise in the antitryptic activity, however, was
not specific for pregnancy but also occurred in certain pathological
conditions and he concluded that these had to be excluded before a
positive test could be considered indicative of pregnancy. Franz
(1914) and Adachi (1915) considered the method not entirely
specific as a test for pregnancy, but worthy of further investiga-
tion. Bar & Ecalle (1919), after testing 80 sera, were of the opinion
that the method was not sufficiently specific and of no practical
value. In 1929 the method was again studied by Flexner, Berkson,
Winters, & Wolman, in a series of tests on 159 sera from pregnant
women; 149 of these sera showed a higher antitryptic titre than
normal. They considered that there was a gradual increase in
the titre from conception until the 3rd-4th month of pregnancy
after which time there was a gradual fall. They concluded that
"whether this fact can be utilized for the diagnosis of pregnancy is yet to be determined ".

Further references to this test will be found in the papers by Rosenthal (1911), Murray (1913), and Franz (1914).

Berrrár & Raitsits (1913) examined the blood of a few cows, mares, bitches, cats, and rabbits for an increase in antitryptic activity during pregnancy. An increase was observed only in two cases in the bitch; in other animals no significant alteration took place. In the rabbit, Shinoda (1924) noted a rise in antitryptic activity of the serum a few days before parturition.
CHAPTER 7. TESTS BASED ON OTHER BIOCHEMICAL INVESTIGATIONS OF BODY FLUIDS AND TISSUES

HISTIDINE TEST.

Woman.—The tests used for the detection or estimation of histidine in urine are practically all modifications and developments of the Knoop test for histidine. Only the major modifications and developments of the technique will be referred to in this review. It must be noted that in many cases the individual worker has introduced some slight modification into the technique being used and for the exact details the original papers must be consulted.

Voge (1929, 1930), using the Knoop test for histidine, showed that there was a marked correlation between the presence of histidine in the urine and the presence of gonadotrophic hormones as determined by the Aschheim-Zondek test. Of 326 tests on urine, 91% were in agreement with the biological test; of 100 non-pregnancy urines tested, 98 gave negative results. He suggested that the method was worthy of further investigation as a pregnancy test, since if it proved reliable it would possess great advantages over the biological tests on account of the simplicity and rapidity of the method. Burt-White (1930) obtained 91% positive tests with 245 samples from pregnant women, but with samples of non-pregnancy urine, many of which were from pathological cases, numerous positive results were obtained. Dodds (1930) reported on the results of tests on 380 patients, with errors varying from 13 to 26%. She concluded that the method was not sufficiently reliable for practical diagnostic purposes. Siddall, Hui, Ha, & Wong (1931) after carrying out some 260 tests considered that negative were more reliable than positive results and that the test might have a limited value. They noted that fever was a common cause of false positive tests. The following workers have investigated the Voge test—Young (1933), Pellizzari (1934), Simola & Mantylä (1936). There is general agreement that although positive tests are generally obtained with urine from pregnant women the reaction is not sufficiently specific for practical purposes.

Neuhardt (1937-8) claimed that the accuracy of the method could be improved if the specific gravity of the urine was taken into account. By this means he obtained an accuracy of 97.5% in tests on 448 samples.

Tschopp & Tschopp (1938) studied the histidine excretion in 300 patients in both health and disease, and concluded that the test was of no diagnostic value in pregnancy since histidine was frequently found in the urine in certain diseases, especially diseases of the liver.
Quantitative Tests—Kapeller-Adler (1933, 1934) developed a quantitative test based on the Knoop reaction for histidine. In the urine of pregnant women there was generally present 6 to 74 mg. of histidine per cent., whereas in non-pregnant women it was absent or present only in traces. From the results of 300 tests, Kapeller-Adler considered that the method showed promise as a pregnancy test. Louros (1934) tested the urine of 200 patients but obtained an accuracy of under 70%; he considered that the method was unreliable and that urinary pigments interfered with the test. Rather better results were obtained by Alders (1934) with an error of 4.2% in 500 tests and by Hecksteden (1934-5) who had only 5 errors in 101 tests; 11 tests, however, gave doubtful results. Weiss (1934), Renton (1935), and Stern (1935) considered that although there were drawbacks to the test, it was of some practical value. Weiss noted that urines of high specific gravity were liable to give rise to errors. Stern observed that if nitrites were present in the urine false negative results were common. Renton obtained positive results in pregnancy from the 5th-6th week after conception but considered the best reaction was obtained during the 2nd-3rd month of pregnancy. Other investigators considered the test of no value owing to the high percentage of errors (Ohligmacher, 1934; Bosman, 1935; Ferrari & Francis, 1935; Brandsch, 1935; Gertler, 1936).

Kapeller-Adler (1936a, 1941b) introduced a modification into her original method which overcame the errors due to the presence of nitrites in certain urine samples. Using this improved method, Neuweiler & Grimm (1940) obtained an accuracy of 99% in tests on 272 pregnancy urines, but an error of 15% in 121 samples of non-pregnancy urine. Kraus & Koenigstein (1941) tested the urine from 55 women at various stages of pregnancy from 1½ months onwards; 49 gave positive results. Urine from 28 non-pregnant women with various pathological conditions of the reproductive tract was also tested; 2 samples gave positive tests. Positive tests were frequently observed in tests on urine from children. Kraus & Koenigstein concluded that although the test was not specific for pregnancy it was still of considerable clinical value. Whitacre (1941) on the other hand in a series of 1,054 tests obtained very poor results; only 35% of the tests with pregnancy urine were positive and 11% of the non-pregnancy samples gave incorrect results. More favourable results were obtained by Westberg (1941); 39 negative results were recorded in tests on 615 samples of pregnancy urine and 6 positive results in tests on 257 non-pregnancy samples. Plotz (1941) considered that negative histidine tests were on a more secure basis than negative Aschheim-Zondek tests. Goodfriend & Daniel (1943) considered that the Kapeller-Adler improved
method gave encouraging results and was worth further study. Davey & Daley (1945) had 126 positive tests with samples of urine from 1016 non-pregnant women; of 8 pregnant women only 2 gave positive results.

In addition to the methods for quantitative estimation of histidine devised by Kapeller-Adler, several other quantitative methods have been devised and applied to the diagnosis of pregnancy.

Seidman (1933) developed a simpler technique. Of 102 pregnancy samples tested by this method, 94 gave positive results; of 97 non-pregnancy samples 24 gave incorrect results. Seidman considered that the test was not sufficiently accurate for diagnostic purposes.

Földes (1936a,b) tested over 300 samples of urine using a slightly modified Kapeller-Adler test and a test based more directly on the Knoop reaction; both methods gave results of questionable value.

Krieger (1936) in a comparative study, considered that the Seidman method gave more reliable results than the Kapeller-Adler technique but that neither method was of value in pregnancy diagnosis. Further comparative studies were carried out by Gersh & Lewin (1937) who concluded that Földes's tests were more reliable than the Kapeller-Adler test, and the Kapeller-Adler test was better than the Voge test; but none of them was considered to be sufficiently accurate for routine pregnancy diagnosis.

Other techniques have been described by Niendorf (1939, Racker, 1940), and Chattaway (1947). Langley (1941), using a quantitative modification of the Knoop test, studied the ratio of creatinine to histidine in the urine of 107 pregnant and 61 non-pregnant women. He concluded that although in general the ratio was lower during pregnancy the method could not be recommended as a pregnancy test since about 16% of the urines studied could not be definitely classified.

Page (1943) studied the rates of excretion of histidine in 30 pregnant and 17 non-pregnant women. Preliminary experiments having shown that the Kapeller-Adler test was not reliable for pregnancy diagnosis, Page proposed a new method based on the excretion rate of histidine following an injection of histidine base. By standardizing the diet, Page hopes that it may be possible to obtain useful results by this method.

It would seem reasonable to conclude that the unreliability of the histidine test for pregnancy lies not in the chemical techniques for detecting and estimating histidine but in the fact that histidine is frequently present in the urine of non-pregnant women. Niendorf (1939, 1940) considers that if the analytical method is sufficiently sensitive histidine can be found in all urines. In short the difference between the urine of pregnancy and non-pregnancy, so far
as histidine is concerned, is a quantitative one in which there is no sharply defined border line.

Tasch (1941) has extensively reviewed the literature on the Kapeller-Adler test and its modifications. Using Kapeller-Adler's improved method, Tasch tested urine samples from 621 women. 98% of the pregnancy samples gave correct results but there were 18.6% errors with non-pregnancy samples (33 out of 177).

Specificity.—Armstrong & Walker (1932) and Racker (1941) have concluded that the Knoop reaction as applied to urine is practically specific for histidine. Simola & Mäntylä (1936), however, have shown that the colour produced by the Knoop bromine reaction in pregnancy urine can be extracted with amyl alcohol, whereas the colour produced when histidine is added to a sample of urine and the Knoop reaction carried out cannot be extracted with amyl alcohol. In view of this, Simola and Mäntylä considered that histidine is not the substance in pregnancy urine which gives the positive Knoop reaction. This observation was confirmed by Neuweiler & Grimm (1940), but its significance is not yet clear. Adaka & Kikuti (1940) reported a similar phenomenon using butyl alcohol. Further doubt on the specificity of the test is raised by the parallelism between this test and the iodine reaction (Simola & Närvi, 1936) (see page 132) and the conclusion of Schales & Schales (1944) that the iodine test did not depend on the presence of histidine.

Mechanism of the Increase of Urinary Histidine in Pregnancy.—Kapeller-Adler & Haas (1935) observed that during pregnancy the liver loses its capacity for metabolizing histidine and considered that this might be due to the absence of histidase from the liver. They showed that in the pregnant cat and guinea-pig, which do not excrete histidine during pregnancy (see page 133), histidase is not inhibited during pregnancy. These findings led them to suggest that there might be some direct connection between the gonadotropic hormones and histidase. Further experiments were carried out by Kapeller-Adler & Boxer (1937) when it was demonstrated that the addition of gonadotrophin to a brei of human liver and histidine caused a marked reduction in the histidase activity. The histidine excretion was followed throughout pregnancy in one patient by Kapeller-Adler & Schiller (1935); it was shown that the histidine in the urine was partly exogenous, partly endogenous in origin, the excretion being greater with high protein diets. Page (1946a) has put forward an alternative hypothesis regarding the histidinuria of pregnancy. He postulates that the histidinuria is due to an inhibition of or interference with the renal tubular reabsorptive mechanisms for this particular amino-acid since histidine, given intravenously, disappears from the blood.
stream with equal rapidity in both pregnant and non-pregnant women.

*Urinary Histidine in Pathological Conditions.*—It has already been noted that certain pathological conditions give rise to a high excretion of histidine in the urine. Further information on these conditions will be found in the papers by Brandsch (1935), Tschopp & Tschopp (1938), Niendorf (1940), Westberg (1941), and Kapeller-Adler (1941a,b,c).

It has been demonstrated by Kapeller-Adler (1941b,c) that in toxaemia of pregnancy the histidine in the urine may be replaced by histamine, which is not found in the urine during normal pregnancy. Further discussions on the inter-relationship of histidine, histamine, and histaminase in normal and toxaemic pregnancy will be found in the papers by Kapeller-Adler & Adler (1943) and Kapeller-Adler (1944).

The influence of certain drugs excreted in the urine such as phenols, santonin, quinine, etc., on the test are discussed by Asitaka, Hudii, & Matuyama (1941).

The *Iodine Reaction.*—Simola (1936) observed that if iodine was added to pregnancy urine, the solution boiled and shaken with amyl alcohol, the alcohol layer became red-violet in colour. Over 70% of the samples of pregnancy urine tested by this method gave the colour reaction, whereas the majority of non-pregnant samples gave negative results. This test would appear to be related to the Knoop test for histidine in which bromine instead of iodine is added to the urine. This relationship has been studied by Simola & Närvänänen (1936) who carried out concurrent iodine and bromine tests on 250 samples of pregnancy urine and 100 non-pregnancy samples. They found a distinct parallelism between the colour intensity of the two tests and they considered the reactions were ascribable to the same substance. There was no parallelism between the above tests and the Visscher-Bowman and Friedrich tests (see page 136) which were also carried out on the urine samples. Barnes (1946) also concluded that the iodine reaction was not related to the Friedrich test. A German patent was apparently taken out on this test by Gutschmidt & Gutschmidt (1939) but it is obvious from further experiments by Simola (1943), Schales & Schales (1944), and Barnes (1946) that the order of accuracy is similar to that of the histidine tests and that it is not a reliable test for pregnancy. Schales & Schales concluded from their investigations that the chromogenic substance was not histidine, nor was it identical with any of the known red urinary pigments.

*Domestic Animals.*—Kapeller-Adler & Herrmann (1934) investigated the urines of 2 pregnant mares, 2 pregnant bitches, 2 pregnant cats, 2 pregnant rabbits, 3 pregnant guinea-pigs, and a preg-
naut monkey (*Macaca rhesus*). Only traces of histidine were found. Weiss (1934) also observed that histidine was absent from the urine of the pregnant mare. Jaarsma (1936) studied the estimation of histidine in the urine of animals; he concluded that the Kapeller-Adler and the Lang (1933) techniques were suitable for estimating histidine added to the urine of cows, sows, and bitches. He was, however, unable to find histidine in the urine of pregnant cows, mares, sows, and bitches and he concluded that the test was of no value in the domestic animals.

Foldes (1936a) failed to find histidine in the urine of the following non-pregnant animals: cow, mare, dog, elephant, giraffe, tiger, and antelope.

Kapeller-Adler & Herrmann (1934) correlated the absence of histidine in the urine of pregnant animals with the absence of gonadotrophic hormones in the urine. Further experiments by Kapeller-Adler & Haas (1935) showed that, in contrast to the human, the histidase in the liver of pregnant cats and guinea-pigs was not inhibited during pregnancy—an observation which they considered might explain the absence of histidine in the urine. Kapeller-Adler & Boxer (1937) have shown that gonadotrophins, added to breis of cow and rabbit liver and histidine, inhibit the histidase. That a similar action may occur in vivo is suggested by the experiment of Kapeller-Adler & Herrmann (1934) in which histidinuria was produced in guinea-pigs by giving injections of gonadotrophins over a period of five days.

**GLYCOEURIA TESTS.**

**Woman.**—In 1856 Blot observed that glycosuria occurred in approximately 50% of pregnant women. It has been amply confirmed that glycosuria frequently occurs in the human during pregnancy. Further references on this subject will be found in the papers by Ehrenfest (1924), Lambie (1926), Rowe (1931), and Liston & Chisholm (1941).

The presence of sugar in the urine during pregnancy is not sufficiently regular or characteristic to be of diagnostic value but three methods have been used to exploit the tendency to glycosuria and thus evolve a test for pregnancy—1, The Frank and Nothmann test; 2, The Kaminitzer and Joseph test (Phloridzin or Maturin test); 3, The Roubitschek test (Adrenaline test).

**Frank and Nothmann Test.**—Frank & Nothmann (1920) claimed that when 100 g. of glucose were administered to a fasting patient during the first three months of pregnancy, glycosuria without hyperglycaemia would ensue in the course of two hours. Thirty pregnant women were tested by this method and all gave positive results. Nürnberg (1921) applied the test to 71 women and
obtained glycosuria in all cases more than a month pregnant, and he considered the test very useful in the diagnosis of normal pregnancy. Bauer (1922) tested 120 women, 1-3 months pregnant, and noted glycosuria in every case; tests on 55 women in the later stages of pregnancy were positive in only 66% of the cases and 30 non-pregnant women all gave negative results. Reliable results were also obtained by Wels & Van Nest (1923), Williams (1923), Foyer (1924), and Hirst & Long (1926), who considered that the test was very useful during the first three months of pregnancy. There were, however, other less favourable reports. Seitz & Jess (1922), Hellmuth (1922), Höst (1925), Scheffey (1927), Bokelmann & Rother (1928), and Kleitsman (1929) were in general agreement that the test was unreliable for pregnancy diagnosis, there being a high percentage of errors in both pregnant and non-pregnant cases.

A modification of this test was used by Adlersberg & Porges (1926) in which the urine was tested for acetone in addition to sugar. The patient was placed for one day on a low carbohydrate diet consisting of 200 g. of meat, 2-5 eggs, 50 g. of cheese, 100 g. of butter, with black coffee, tea, soup, and green vegetables. On the following morning the urine was tested for acetone. Then after a breakfast consisting of 60 g. of white bread, and tea with 10 g. of sugar, the patient's urine was tested for sugar. Thirty pregnant women tested by this method all gave positive acetone and sugar tests; of 24 non-pregnant women 2 gave positive acetone tests and 3 positive sugar tests. These incorrect controls were said to be suffering from various pathological conditions.

Kamnitzer and Joseph Test.—Kamnitzer & Joseph (1921a,b) observed that glycosuria could readily be induced in pregnant women by injecting intramuscularly 2 mg. of phloridzin (0.1% solution), a small quantity of local anaesthetic being added to the phloridzin solution to reduce the pain of the injection. Forty-seven pregnant women all gave positive tests up to the end of the third month of pregnancy; of 143 non-pregnant women, a positive test was obtained in only 5. Glycosuria occurred 1-1 hour after the injection. The authors concluded that the method seemed promising and worthy of further investigation. Schilling & Göbel (1922) confirmed these observations but noted that febrile conditions upset the accuracy of the test. Hellmuth (1922), on the contrary, was unable to confirm the accuracy of the test; he obtained 32 positive results in tests on 105 non-pregnant women. Lewin (1929), Küster (1929), Dowig (1923), Williams (1923), Hirst & Long (1926), and Bonaccorsi (1928) all concluded that the test was not reliable and of very limited value. Sacharoff (1923) considered the test was very reliable but the number of cases tested
by him was small. Bronnicoff (1924) carried out over 1,000 tests on 350 persons. Of 122 women less than three months pregnant 90 gave positive tests; positive tests were frequently obtained in non-pregnant women and in males. She concluded that the test was in no way specific for pregnancy and could not be used as a diagnostic method. Crainichianu & Goldenberg (1924) recommended a modification in which 2 mg. of phloridzin were injected. If glycosuria did not follow, a diagnosis of 'non pregnant' could safely be made; if, however, the result was positive the test ought to be repeated again using 1 mg. of phloridzin and if the test was still positive a diagnosis of 'pregnancy' was almost certainly correct, but if the result of the second test was negative then the diagnosis was uncertain.

(This test is frequently referred to as the 'Maturin' test as ampoules of phloridzin solution plus local anaesthetic were sold under the trade name 'Maturin'.)

Roubitschek Test.—Another modification of the glucose tolerance test was introduced by Roubitschek (1922) who claimed that during pregnancy glycosuria could be induced by giving to the patient 10 g. of glucose followed 20 minutes later by an injection of 0.5 ml. of adrenaline (1 in 1,000), the urine being tested three-quarters of an hour after the injection of adrenaline. Twenty pregnant women tested by this method all gave positive tests. Künstner (1922) carried out 150 tests on 125 pregnant women; up to the 33rd week of pregnancy the test was correct in 97% of cases. Hellmuth (1922), Römmert (1923), Williams (1923), and Hirst & Long (1926) considered the method quite unreliable.

Comparative trials of the three glycosuria tests for pregnancy have been made by Hellmuth (1922), Williams (1923), and Hirst & Long (1926). All were in agreement that none of the three methods gave a sure diagnosis, but the least inaccurate was the Frank and Nothmann test.

Domestic Animals.—Glycosuria tests have been applied to the diagnosis of pregnancy in the cow but in the light of more recent investigations of the urine of cows it is obvious that such tests are not applicable to ruminants. One of the earliest reports of glycosuria in the cow during pregnancy is that of Blot (1856). Boddie (1933) has, however, demonstrated that reducing sugar is present in more than 50% of samples of urine from normal cows.

It must also be noted that Fehling's and Benedict's reagents are quite unreliable for testing for sugar in the urine of the cow owing to the presence of glucuronides in the urine.

The Kämmlitzer and Joseph test was applied to the cow by various investigators; the majority concluded that the test was quite unreliable and valueless (Söntgen, 1922; Eberhardt, 1923; Bräuer,
Miller (1923) is said to have considered the phloridzin test of some value in the sheep. Vollmer (1923) and Kiss (1926) concluded that the phloridzin test was of no value for diagnosis of pregnancy in the mare.

Zuravelj (1934) tested 263 pigs by the phloridzin test and obtained an average accuracy of 85 to 90%. He considered the test required further improvements.

Breuer (1923) considered that the phloridzin test was reliable in the bitch in early pregnancy, the accuracy declining as pregnancy advanced.

Küstner (1923) observed that the rabbit frequently showed glycosuria during pregnancy.

Visscher-Bowman Test.

Visscher and Bowman described a chemical test for pregnancy. The theory on which the test was based was not discussed, although it was inferred that the reaction was due to the reducing properties of hormones present in pregnancy urine. The test was carried out as follows: To 1 ml. of urine were added 1 drop of 0.5% hydrogen peroxide, 5 drops of a 1% aqueous solution of phenylhydrazine hydrochloride, 5 drops of a 5% aqueous solution of methyl cyanide, and 5 drops of concentrated hydrochloric acid; the mixture was placed in a boiling water bath for 25 minutes. If a russet color developed and a flocculent precipitate appeared the test was regarded as positive; a negative test was indicated by the development of a straw color and by the absence of a precipitate or the presence of a powdery one. A titration method based on the above reaction was also described which was said to be rather more sensitive. The authors tested 317 urines by this method and obtained an accuracy of 93%.

On account of its simplicity the test aroused considerable interest and was investigated by numerous workers. It is not necessary to review in detail the results obtained by the individual investigators. Several workers obtained fairly reliable results with the test but the majority were of the opinion that it was unreliable. An accuracy of approximately 90 to 93%, thus confirming the figure given by Visscher & Bowman, was obtained by the following authors: Menken (1934, 1935), Dolf (1935), Dodds (1936), Friedrich (1936), Wiesener (1936), Frech (1937), Mashuko (1938). In general these authors considered the test was useful but required further research. Others considered the test quite useless, in some cases errors being in the neighborhood of 50% (Frankl & Engel, 1936; Josef, 1936; Simola & Närvänen, 1936; Eissner, 1936;
There have been several modifications of the Visscher-Bowman test. Friedrich (1936) claimed that the only reagent necessary for the test was hydrochloric acid, a positive result being indicated by a red or brown colour with no precipitate. An accuracy of about 93% was obtained in tests on over 300 samples of urine. Simola & Närvinen (1936) found the Friedrich modification quite unreliable. Ink (1938) using a slight modification of the Friedrich method obtained an accuracy of 97 to 99% in a series of 402 tests. Rodecurt (1938) on the contrary concluded that the Friedrich method was worthless, an accuracy of only 50% being attained. Leunbach & Koepppe (1939) considered the Friedrich method positive only when the precipitate obtained was insoluble in a 50% solution of urea, and obtained 358 correct results in tests on 350 pregnant women. Angelis (1940) used Leunbach & Koepppe's method with an accuracy of 90%.

Hughes & Eskin (1937) modified the Visscher-Bowman technique by adding, at the end of the reaction, 2 drops of a 1 in 1000 solution of methylene blue. If the solution remained blue or muddy the test was negative; when positive the colour became green and then faded. This modification was claimed to be very useful in interpreting borderline cases.

Pátkay (1939) examined the precipitate obtained in the test with a Kuhn-Voithe agglutinoscope. He considered that the type of precipitate was much more important than the shade of colour. Urine samples from 238 women were tested with an accuracy of 95%.

There is considerable evidence that this test gives no information of the presence or absence of hormones in the urine (Ofládal, 1937; Messinger, Presberg, & Fellows, 1938; Ink, 1938; Krieger, 1939). Several investigators have noted a correlation between the specific gravity of the urine and the result of the test, and it has been claimed by some investigators that by adjusting the specific gravity of the sample the accuracy of the test was improved (Friedrich, 1936; Wagner, 1937; Dunn & Northway, 1938; Pátkay, 1939; Angelis, 1940). The presence of sugar in the urine affects the test and is believed by some workers to be largely responsible for the reaction (Loehle, 1936; Ofládal, 1937; Krieger, 1939). Febrile or inflammatory conditions and the presence of protein in the urine also influence the test (Friedrich, 1936; Bodó, 1937; Mashuko, 1938; Messinger, Presberg, & Fellows, 1938; Dunn & Northway, 1938; Ink, 1938). Urinary
pigments may be involved in the reaction (Sheehan, 1937; Krieger, 1939). (See page 48 for further chemical tests for gonadotrophic hormones.)

**Domestic Animals.**—Josef (1936) tested the urine of 6 non-pregnant cows and 9 pregnant cows (10 to 41 weeks pregnant) by the Visscher-Bowman test but found it impossible to distinguish between the urine of pregnant and non-pregnant animals.

Busch (1936) applied the test to mares and cows. Of 12 non-pregnant cows 2 gave positive tests; of 10 pregnant cows only 5 gave positive results. Seven of 18 pregnant mares (2 to 8 months pregnant) gave positive results; of 12 non-pregnant mares 10 gave negative results and 2 doubtful results; 3 of 8 geldings gave positive tests. Busch concluded that the Visscher-Bowman test was valueless for the diagnosis of pregnancy in cows and mares.

**Other Tests Using Urine.**

**Dienst Test.**—See Dienst Test on blood (page 140).

**Sodium Thiosulphate Test.**—Bolliger (1929) observed that commencing from the 10th to the 21st day of pregnancy in the bitch there was a distinct depression of the thiosulphate excretion after the injection of 1 g. of thiosulphate intravenously. This depression lasted up to delivery, the maximum depression occurring from 3 to 1 weeks before parturition. By this means, in experimental animals, Bolliger was able to diagnose pregnancy. The method would scarcely be applicable as a routine test.

**Nagamatsu Reaction.**—Nagamatsu (1938) claimed to have developed a reagent which gave characteristic colour reactions with gonadotrophic, oestrogenic and progestogenic hormones. This reagent was incorporated in test-papers by which rapid tests could be made on samples of urine. Nagamatsu reported that tests using urine samples from 500 pregnant women gave an accuracy of 98%, while tests on samples from 120 non-pregnant women were 100% correct. The original paper is in Japanese, but it appears from an abstract (in German) in another Japanese journal that Nagamatsu did not publish any details about the chemical nature of his reagent.

Unfortunately, this remarkable reagent did not prove reliable when tested by Manaka (1939); of 56 pregnancy urines tested only 31 gave positive results. Manaka concluded that the colour changes were dependent on the pH of the test samples.

**Tétry Red Blood Cell Test.**—Tétry (1943) described an empirical reaction which is claimed to be of value in the diagnosis of pregnancy. The urine under test is added to sheep red blood cells and the mixture incubated for 1 hour at 37° C. If the urine is from a pregnant woman the red colour produced after haemolysis
will persist; if from a non-pregnant woman the red colour changes to a dirty yellow.

**Manoilov Test.**

*Woman.*—The Manoilov test for pregnancy appears to have been developed from the Manoilov reaction which was a test by which it was claimed the sex of an animal or human could be diagnosed from the blood (Manoilov, 1924; Rastegaeff, 1928).

Manoilov (1930a,b), described the following test for pregnancy diagnosis. To 0.3 ml of fresh serum is added 1 ml of a 2% solution of diuretin, the mixture is shaken, one drop of 0.2% alcoholic solution of Nile blue is added and the mixture allowed to stand for a few minutes. If the serum is from a non-pregnant woman the colour will remain blue or pink-blue, but if from a pregnant woman the colour of the solution will change to yellow or rose-yellow. Manoilov considered that the test was based on the degree of alkalinity of the serum. He carried out tests on 2,288 different sera with an accuracy of 94% (Manoilov, 1930b).

The conclusions of subsequent investigators fall roughly into two classes, (a) that the test was not very reliable during the first three months of pregnancy but that in later months the results were more accurate and the test was of some clinical value, and (b) that the test was quite unreliable and of no value at any stage of pregnancy.

Francesco (1930) tested serum samples from 131 pregnant and 54 non-pregnant women. In the first third of pregnancy an accuracy of 24% was obtained; in the last third the accuracy was 96%. Over 10% of the non-pregnant women gave positive tests. Schamarina (1930) is credited with having obtained an accuracy of 94% in tests on 500 sera. In 1931 Manoilov claimed that he had tested 2,696 sera in all, with an average accuracy of 94%. He now considered that the test depended partly on the alkalinity of the serum and partly on an enzymic action on the Nile blue. Browkin (1931), Kabisch (1931), Moschkow (1931), Gymnich (1931), Goldschmidt-Fürstner (1931), Luh (1931), and Zelić (1931) were in general agreement that the test was not accurate in early pregnancy but became more accurate as pregnancy advanced. Other investigators have concluded that the test is quite unreliable (Fretwurst & Otto, 1930; Fusco, 1930; Rodecurt & Jernakoff, 1930; White & Severance, 1931; Szathmáry, 1932; Gernez, 1934; Leon, 1935).

Manoilov (1932) considered that the high percentage of errors obtained by investigators with sera from cases of early pregnancy was due to lack of experience in reading the delicate colour changes. He described a modification of the test which consisted
of the addition of 0.5 ml. of a 1% aqueous solution of diethylbarbituric acid to the reagents already specified. Manoiloy tested 482 samples with this modification with an accuracy of 95.5%. The reaction could also be carried out with a similar accuracy on serum which had been allowed to dry on a watch-glass.

Fretwurst & Otto (1930) and Leon & Dambrosi (1931) were unable to confirm Manoiloy's claim that the test was based on the alkalinity of the serum.

Domestic Animals.—Manoiloy (1930b) tested sera from 12 pregnant cows and 12 non-pregnant cows and reported that the results were good. Other workers, however, have failed to confirm this statement. Fiore (1933) obtained 61 positive, 65 negative, and 24 doubtful results in tests on 150 non-pregnant cows. Of 20 pregnant cows tested 9 gave positive results, 6 gave negative, and the remaining results were doubtful. Positive results were also obtained with sera from bulls and calves. Kalusch (1933) tested 221 sera from cows and concluded that the test was of no value. Lucas (1934) tested 3 pregnant cows, all giving negative results, and 15 non-pregnant cows, of which 10 gave positive results. Busch (1936) tested 126 samples of bovine sera; the results proved to be very inaccurate and he decided that the test was of no value. Hovorka (1937) obtained rather better results although they were not sufficiently accurate for practical purposes. Fifty-six tests were carried out on 40 pregnant cows and 42 were positive; 24 tests were made on non-pregnant cows and calves of which 2 were incorrect.

Fiore (1933) tested 6 non-pregnant ewes; 1 gave a positive result. Four non-pregnant and 5 pregnant goats were tested; 3 of the former and 3 of the latter gave negative results. Fiore concluded the test was of no value in sheep or goats. Lucas (1934) tested 11 goats and 4 ewes and came to a similar conclusion.

After testing 292 samples of mare sera, Kalusch (1933) concluded that the Manoiloy test was of no value for the diagnosis of pregnancy in the mare.

Dienst Biuret and Ninhydrin Tests.

Woman.—Dienst (1922) claimed that by performing the biuret reaction with sodium hydroxide and copper sulphate solutions on a mixture of 10 ml. of water and 2 drops of serum, a lilac colour would develop if the serum was from a pregnant woman; if from a non-pregnant woman then the colour would be blue. An alternative method of carrying out the test was to add ninhydrin instead of the biuret reagents to the water-serum mixture, and boil the mixture; if pregnancy serum had been used the precipitate of serum-proteins had a definite blue colour, but with non-
pregnancy serum the precipitate was snow-white. Dienst claimed
that the tests depended on the presence of antithrombin which was
said to show a marked increase in pregnancy. Dienst gave no data
to indicate the accuracy of the method.

Ganssle (1924) tested sera from 20 pregnant and from 20 non­
pregnant women with both the biuret and ninhydrin tests, but
neither method gave reliable results. Timofeew (1925) using the
ninhydrin test examined 95 sera; he concluded that the test was
of some aid in diagnosis, a negative result ruling out pregnancy
in 90% of cases. Fink (1926) carried out 170 tests and found both
techniques quite valueless. A similar conclusion was reached by
Vogel (1926a). Dienst (1926) re-affirmed the value of his test,
recommending especially the ninhydrin method. Subsequent
investigators, however, were all unable to confirm Dienst’s claims
(Derankova, 1927, 1930; Witte, 1929; Motta, 1929; Geruez
1934).

Dienst (1927, 1928a,b,c), claimed that the ninhydrin reaction
could also be carried out using urine, when in positive tests an
intense dark lilac colour developed. Narimatsu (1929) obtained
positive results in only 69% of tests using 106 pregnancy urines;
of 50 non-pregnancy samples, 17 gave positive results. He con­
cluded the test was of no value. Results even less accurate were
obtained by Gragert & Zander (1930) in a series of 115 tests.
Derankova (1930) reported that 50% of non-pregnant females gave
positive ninhydrin urine tests.

Vogel Test.—Vogel (1926b) claimed that more accurate results
could be obtained with the Dienst ninhydrin test if the method of
reading the test was altered; he claimed that with non-pregnancy
sera the precipitate consisted of large grey-white floccules which
precipitated from a bluish fluid whereas with pregnancy sera the
protein remained in suspension. Tests on 281 samples of sera
from pregnant women were 97.8% positive and tests on 212 non­
pregnancy samples were 97.7% correct. Oettingen (1926), Cordua
(1928), Gragert (1928), and Motta (1929) were unable to confirm
the specificity of the test.

Observations on the Dienst Tests.—As has already been stated,
Dienst considered that the tests depended on the presence of anti­
thrombin in the serum; further details of the supposed underlying
theory of the test will be found in Dienst’s papers already men­
tioned (see also Dienst, 1929). It is obvious, as has been pointed
out by Fink (1926), Narimatsu (1929), and Gragert & Zander
(1930) that the biochemical methods used are in no way specific for
antithrombin, and positive tests will be obtained with practically
any soluble protein and many amino-acids.
**Domestic Animals.**—Kriisa (1934) tested 21 cows by the Dienst method and concluded that the test was quite non-specific.

**Costa Test.**

Woman.—Costa (1923) described a reaction with blood which was said to occur only in pregnancy and in certain diseased conditions. It is said to consist of a sedimentation of haematin in presence of a solution of novocaine. To 1.5 ml. of a 2% solution of novocaine is added 3 drops of a 5% solution of sodium citrate and 3 drops of the blood to be tested; the mixture is shaken and centrifuged, and 1 drop of pure formalin is added. The reaction is considered positive if in 15 minutes a grey or greyish-yellow precipitate forms. It has not been possible to obtain the journal containing the original paper describing this test and the underlying theory has not been ascertained. This test was not satisfactory for the diagnosis of pregnancy in women, the accuracy being only 80%.

Domestic Animals.—Mariani (1930) applied the Costa reaction to the diagnosis of pregnancy in the cow and mare. In 27 of 34 animals it was possible to detect correctly the presence or absence of pregnancy at the first test; 2 results were incorrect and 5 doubtful. Mariani observed that the test became positive from the 26th to the 68th day of pregnancy. In cows it remained positive 36-57 days after parturition. Eleven samples from stallions and geldings, and 14 from non-pregnant cows and heifers, were all negative.

Kriisa (1934) tested 99 cows by this reaction; 65.3% of the positive tests and 71.8% of the negative tests were reliable.

The Costa test was modified by Voloskov (1935), but it has not been possible to ascertain details of this. Using the Voloskov modification, Kerov (1939) tested 104 pregnant and 30 non-pregnant mares: the average error was 31.4%, the errors being all false positives.

**Mertz Phosphotungstic Acid Test.**

Mertz (1928) postulated that there was a difference in the acid-combining properties of pregnancy and non-pregnancy sera and he evolved a test for pregnancy based on this assumption. To 1 ml. of serum is added 1 ml. of a 0.28% solution of phosphotungstic acid, the mixture is allowed to stand at room temperature for half-an-hour, and then 2-3 drops of bromophenol blue are added. If the serum is from a non-pregnant woman, the solution will remain a clear blue colour: if it is from a pregnant woman then the solution will become opaque and dark-blue to green. One hundred and fifty tests were carried out by this
method; 9 gave incorrect results and these were from cases of carcinoma.

Rodecurt (1928) failed to obtain reliable results; 106 tests gave an overall accuracy of 70%. Schmidt (1929) on the other hand obtained an accuracy of 92%. He considered that by using this test in combination with the Lütte & Mertz Alcohol-Extract Reaction (see page 117) and the erythrocyte sedimentation test (see page 170) an accuracy of 98% could be achieved in the diagnosis of pregnancy from the 6th week onwards to about the 6th month, after which results became less reliable. Fusco (1930) and Lewinski (1930) considered the test unreliable. Bebtschuk (1931) tested 216 pregnant women; 15 gave incorrect results and of these 13 were in the first two months of pregnancy. Of 303 non-pregnant women tested, 37 gave incorrect results. An accuracy of about 90% was obtained by Meo (1934) in a series of 500 tests. Less favourable results were reported by Kilduff & Steinig (1932) who tested 259 sera; although 90% of non-pregnant women gave negative tests only 7% of pregnant women gave positive results.

The Mertz phosphotungstic acid test is referred to by some workers as a modification of the Abderhalden test. While it may have developed from the Lütte & Mertz Alcohol-Extract Reaction, the phosphotungstic acid test would seem to be unconnected with the protective enzyme theory.

VARIOUS ASPECTS OF BLOOD BIOCHEMISTRY DURING PREGNANCY.

It is of interest to consider other modifications in the biochemistry of the blood which occur during pregnancy to find whether any are likely to provide information of value in pregnancy diagnosis.

Ammonia.—Stanojevic (1931) noted a slight increase of ammonia in the blood of women during pregnancy, but as this increase did not occur until the end of pregnancy it would be of no diagnostic value.

Parnas & Heller (1924) observed that the blood ammonia in the rabbit was increased during pregnancy.

Cholesterol.—It has been known for some time that during pregnancy there is an increase in blood cholesterol. This increase is, however, not specific for pregnancy as it occurs in various pathological conditions (Chauffard, Laroche, & Grigaut, 1911, 1920; Kürten, 1924; Salomon & Potter, 1926; Guilhem, Bugnard, & Colombies, 1935; Gautschi, 1945). (For the effect of pregnancy urine on the blood cholesterol of test animals see page 95.)

An increase in blood cholesterol during pregnancy has also been observed in the mare, but the variations observed are so great that...
the method is of no value as a pregnancy test (Brocq-Rousseau, Roussel, & Gallot, 1938a; Mühlböck, 1937a,b).

**Choline.**—There may be a slight fall in blood choline during pregnancy but it would appear to have no diagnostic value (Eufinger & Gottlieb, 1933; Eagle, 1941).

**Lactic Acid.**—An increase in the lactic acid content of the blood has been reported during pregnancy but it does not appear to have any diagnostic value (Schultze, 1926; Loeser, 1926). There is also a slight increase in the urinary lactic acid (Holtermann, 1932).

Observations on other changes in the biochemistry of the blood during pregnancy will be found in the reviews by Rowe (1931) and Gellé & Driessens (1940).

**ALCOHOL-MILK TEST.**

**Cow.**—The alcohol-coagulation test has been used in the dairy industry for many years as a rapid test for acidity and as a guide as to the suitability of the milk for various commercial purposes. The principle of the test depends on the coagulation of the milk in alcohol which occurs under certain conditions of acidity or composition (see Davies, 1939).

Linkies (1930) claimed that the reaction could be used as a means of diagnosing pregnancy in the cow. A small quantity of milk was drawn from the udder into a glass, an equal volume of absolute alcohol added, and the mixture was shaken and allowed to stand for 1/2 to 3 hours. If the milk was from a non-pregnant cow no change would be observed in the mixture; if from a pregnant cow, coagulation would occur. It was claimed that diagnosis could be made as early as the 21st day of pregnancy by this method. Eighteen cows were tested, 12 of which were pregnant, and all gave correct results. Linkies, however, added a warning to the effect that reliable results were only obtained in cows that were absolutely healthy; mastitis or any general pathological condition was said to invalidate the test. It is obvious that such a stipulation could be used to explain any errors which might occur.

A similar test was described by Palmeri (1932).

Rutz (1932) carried out 400 tests by the Linkies method on 57 different cows; 91% of the tests were positive (i.e., coagulation was observed) although only 45% of the cows were pregnant. Positive results were obtained in many cows in which there were no clinical signs of udder disease or any other pathological condition. Rutz concluded that the test was quite useless. A similar conclusion was reached by Palmer (1933) who tested 77 pregnant and 143 non-pregnant cows; 57% of the non-pregnant cows gave positive results and 70% of the pregnant cows gave negative results.
OTHER BIOCHEMICAL TESTS

KOSJAKOV TEST.

Woman.—It has been difficult to obtain a complete picture of the history and development of this test since many of the earlier papers are in Russian journals which are not available in this country, but a brief outline of the development of the test has been obtained from a few papers by the workers concerned which were published in German journals, and from references obtained from the few Russian journals which are available.

The Kosjakov test is said to be based on a difference in the sulphur content of the hair during pregnancy. Kosjakov (1928, 1929) maintained that there was a sex difference in the amount of sulphur present in human hair, it being considerably higher in the male.

The simplest test used by Kosjakov to determine the quantity of sulphur present was based on the time required for an alkaline solution of the hair to decolorize a solution of methylene blue. Whether such a test can indicate, even approximately, the sulphur content of the hair is very much open to question. Data on the specificity of the test have not been seen although they may have been provided in the original papers. Further information on the sulphur content of the hair of the two sexes will be found in the papers by Kresiment (1930), Piepenborn (1931), and Radeff (1932).

While investigating this sex difference in the composition of the hair, Kosjakov (1930) noted that in 9 of 15 tests the hair of pregnant women had a high sulphur content, as does the hair of males. This observation was utilized as a test for pregnancy by Kosjakov (1934) and Afanasjevskii (1934) who reported that the hair of 96 to 97% of pregnant women tested in early pregnancy had a higher sulphur content than the hair of non-pregnant women.

The test is very simple and easy to carry out: 0.1 g. of hair is added to 1 ml. of 10% potassium hydroxide and boiled for about a minute; 1 ml. of distilled water is added and the mixture boiled again; a further 15 ml. of distilled water is then added and the mixture shaken. To 1 ml. of this mixture is added one drop of a 1% alcoholic solution of methylene blue and a few drops of 4% hydrochloric acid. If the hair is from a pregnant woman there will be a rapid decoloration in a few seconds; if from a non-pregnant woman the time required will be several minutes. Several variations of this technique have been described.

The reliability of the test for pregnancy diagnosis in the human has not been confirmed by subsequent work. Tortora (1937) tested hair samples from 30 pregnant women and 24 gave correct results, but of 50 samples from non-pregnant women only 7 gave correct results. Sanna (1937) also concluded that the test was unreliable.
Domestic Animals.—While studying the sex difference in the sulphur content of hair, Kosjakov (1930, 1931) observed that, in the human, hair from the male had a higher sulphur content than hair from the female, whereas the opposite was true in the case of the lower animals where the hair from the female contained a greater percentage of sulphur than hair from the male, except in the case of apes where the content of sulphur was similar in the two sexes. This observation was partly confirmed by Radeff (1932), who observed a high content of sulphur in the hair of 26 of 37 non-pregnant mares; of 112 stallions tested, 70% had a low sulphur content in the hair. Of 98 pregnant mares tested 48 gave a low sulphur content similar to the male. From these figures it would appear that in animals the sulphur content of the hair is generally higher in the female, but that during pregnancy the sulphur content may fall towards the level characteristic for the male sex.

The application of the Kosjakov test to the diagnosis of pregnancy in animals is confused since most of the investigators have assumed that the sex difference in the sulphur content of the hair in animals was similar to that in the human. Whether this assumption was based on experiments or arose from ignorance of Kosjakov’s experiments on animals it has not been possible to ascertain, since not all the original papers have been available.

Afanasjevskii (1934) and Nikolaevskii (1938), working on the assumption that in animals the sulphur content of the hair increased during pregnancy as in the human, claimed an accuracy of about 90% in diagnosing pregnancy in domestic animals. Runge (1939) using Nikolaevskii’s technique was unable to confirm the accuracy of the test; 27 of 30 pregnant cows gave correct results, but of 26 non-pregnant cows only 2 gave correct results. Runge concluded that the test was non-specific.

Timofeev, Gubarević, & Ciradze (1935) also assumed that the sex differences in the sulphur content of the hair in animals were similar to the differences in the human. They carried out a series of experiments on cows, mares, goats, sows, and bitches, using the technique described by Kosjakov in conjunction with a modification of their own. 141 tests were made on cows, 27 on goats, 273 on mares, 58 on sows, and 66 on bitches. The accuracy of tests carried out on pregnant animals varied from 49 to 94%; in non-pregnant animals the accuracy was extremely low, varying from 6 to 36%. The authors’ modification of the test was in no way superior to the original test; although it increased the accuracy of the tests on pregnant animals it decreased the accuracy of the results of tests on non-pregnant animals. Owing to the ambiguous method of presenting the results it is possible to interpret these experiments
in a more favourable way than above, as has been done in the abstract of this work in *Anim. Breed. Abstr.*, 1938, 4: 154, in which case the accuracy of the results in non-pregnant animals would be 64 to 94%. I believe the former interpretation is the correct one. The conclusions of the authors are non-committal; with further work on the method they hope a high percentage of correct answers will be reached.

Kerov (1939) carried out tests on 170 pregnant and 97 non-pregnant mares using both the original Kosjakov technique and the modification described by Timofeev *et al.* (1935). Neither method proved of the slightest value, the average error being 41-45%.

Kosjakov, Tatarko, & Ivanova (1939) questioned the validity of much of the experimental work dealing with the application of the Kosjakov test to the domestic animals since, as already indicated, practically all of it was based on the assumption that pregnancy would produce an increase of sulphur in the hair. They stressed again the earlier experimental work of Kosjakov, which had shown that in animals the sulphur content of the hair was higher in females, and asserted that if the Kosjakov test was to be applied to the diagnosis of pregnancy in animals, it must be demonstrated that pregnancy produced a decrease in the sulphur content of hair to a level approaching that found in the male. These authors studied the application of the test to cows, mares, and sows. Twenty three cows and 17 mares were tested over a period of 12 to 14 months; in addition to these protracted experiments, tests were carried out on 28 pregnant and 9 non-pregnant cows and 76 pregnant and 46 non-pregnant mares. No correlation was noted between the sulphur content of the hair and whether or not these animals were pregnant, despite the use of various methods of carrying out the test and of obtaining the samples. Kosjakov *et al.* (1939) concluded that it was not possible to diagnose pregnancy by this method in cows and mares. The tests on swine, however, gave more promising results. The sulphur content of the bristles of 48 pregnant sows, 23 non-pregnant sows, and 7 boars was investigated. The sulphur content of the bristles from the boars was the lowest, the average time required for the decoloration of the methylene blue being 45 seconds; the bristles from non-pregnant sows had the highest sulphur content, the average time required for decoloration of the solution being 7 seconds, and the sulphur content of the bristles from the pregnant sows was intermediate, giving an average decoloration time of 24 seconds. These results confirmed the earlier findings of Kosjakov regarding the sex differences in the sulphur content of the hair of animals. The difference in sulphur content of the bristles in the pregnant and
non-pregnant sows proved to be well defined, there being only one error in each category. Supplementary tests on the bristles of swine using an iodometric method revealed that the sulphur content of the bristles of non-pregnant sows averaged 2.015% as compared with 1.887% in pregnant sows' bristles. The authors consider that further investigations are required before the method can be used in practice.

Giannotti (1942) employed the Kosjakov test and various modifications for the diagnosis of pregnancy in the domestic animals but obtained no useful results. He concluded that the rate of the decoloration was more dependent on the temperature at which the test was carried out than on the state of pregnancy.
CHAPTER 8. TESTS BASED ON VARIOUS PHYSIOLOGICAL PHENOMENA

Basal Body Temperature.

Woman.—The cyclical variations in the basal temperature which occur during the sex cycle in women have been employed as a means of detecting the time of ovulation and for providing information on ovarian dysfunction in cases of sterility. The literature dealing with this field has been briefly reviewed by Tompkins (1944).

More recently a study of the basal temperature has been recommended as an aid to the diagnosis of early pregnancy. One to 2 days before the onset of menstruation the basal temperature begins to fall, reaching a low level 1 to 2 days after the onset of menstruation. This low level continues to the mid-interval. About the time of ovulation there is a further slight drop in temperature followed by an abrupt rise which continues for 24-36 hours, reaching a plateau where it remains until 1-2 days before the onset of the next menstrual period. In cases where regular basal temperature records have been kept, the persistence of the post-ovulatory elevated temperature during the first week of the missed period is strong evidence in favour of pregnancy (Vollmann, 1940; Vollmann & Vollmann, 1942; Tompkins, 1944; Davis, 1946).

Cow.—Studies on the relationship between the sex cycle and the basal body temperature in domestic animals appear to have been made only on the cow. Vollmann & Vollman (1942) carried out extensive observations on 6 heifers and 30 cows. In 54-60% of the oestrous cycles studied a fall in temperature of 0.2°-0.5° C. occurred 0-3 days before the onset of oestrous symptoms, and the temperature rose again to its initial level 2-10 days after the beginning of the external signs of oestrus. During the 2nd to the 5th week of pregnancy the basal temperature appeared to be slightly higher than in the beginning of metoestrus; in the 6th to the 19th week it fell to the level noted at the beginning of metoestrus. From the 20th week onwards the basal temperature rose slowly until the 25th week when the rise was more rapid, the maximum being reached during the last five weeks of pregnancy. In the cow, therefore, the maximum basal temperature occurs at the end of gestation, whereas in the human it occurs at the beginning of gestation. The basal temperature in the cow during pregnancy was also investigated by Blaxter & Price (1945) who concluded that an increase was only apparent during the last 6 weeks of pregnancy.

It would appear that in the cow the temperature changes associated with the sex cycle and fertilization are less characteristic and
less regular than in the human and it is unlikely that information regarding pregnancy can be obtained from basal temperature records in the cow.

It should be noted that the readings of body temperature if they are to be used for demonstrating cyclical variations associated with the sex cycle must be taken under certain standard conditions. In the human the term "basal temperature" usually indicates the body temperature as recorded, either rectally or orally, immediately on awakening. In animals the time of taking temperatures must be fitted into a regular timetable with feeding, milking, and other activities, otherwise any cyclical changes will be completely masked by changes due to other causes.

Cycloscope Test.

**Woman.**—Samuels (1937a,b,c, 1938a,b) described a test for the early diagnosis of pregnancy, based on the reduction time of oxyhaemoglobin. It was postulated that the reduction time was dependent on the hormone level in the blood. To measure the reduction time Samuels devised an instrument which he called the "cycloscope." Essentially this was a spectroscope mounted on a special stand. The reading was obtained by transilluminating the interdigital web between the thumb and finger; the circulation in a small area of the web was momentarily obstructed, and the reduction of the oxyhaemoglobin followed by studying the changes in the absorption bands in the spectrum. Samuels claimed that the reduction times showed a cyclical variation throughout the sex cycle and from a study of these variations it was possible to determine the time of ovulation. If conception occurred characteristic changes in the reduction time ensued, the reduction time increasing and cyclical variations ceasing, and from these changes it was possible to diagnose pregnancy at a very early date.

Although Samuels gave his instrument wide publicity (essentially the same description and claims appeared in at least five different journals) there appear to be few subsequent references to the method. Imbach (1938) and Wenner (1939) both completely failed to confirm Samuels' claim about the value of the method for the diagnosis of ovulation and pregnancy or as an indication of the hormone level in the blood.

Other factors influencing the reduction time of blood in the capillaries of the skin have been studied by Ray, Ray, & Johnson (1946).

**Domestic Animals.**—No reference to this or any similar test being employed for the diagnosis of pregnancy in domestic animals has been found.
PHYSIOLOGICAL TESTS

Uterine Response to Posterior-Pituitary Extract.

Woman.—Hoehne (1925) observed that as early as the second month of pregnancy the human uterus responded to the intravenous injection of posterior-pituitary extract by marked contractions which could be readily detected by bimanual examination. Zorn (1926) confirmed this observation and concluded that it served as a useful aid in the diagnosis of pregnancy. Eleven cases of pregnancy, of 5 to 8 weeks' duration, were accurately diagnosed by this method. Thirty-two cases tested in the second half of pregnancy all gave positive tests by the method. Zorn concluded that the test was of considerable value in practice and free from danger. Löwin (1927) further studied the method in a series of 50 to 60 cases with very successful results. Küster (1930), Molfino & Boero (1931), Rodent (1931), and Reeb (1932) recommended the method as a very useful test in the clinical diagnosis of pregnancy and provided the method was used only in early pregnancies, it was considered harmless. White & Pratt (1936) used the test in a series of 119 cases, and considered the method very helpful in the differential diagnosis of pregnancy but not a conclusive test for pregnancy. They found no evidence of any risk or danger in the method.

Studies on the effect of posterior-pituitary extract on isolated strips of human uterine muscle by Robson (1933) indicate that both the oxytocic and the pressor principles in the posterior-pituitary extract may be responsible for the uterine contraction observed after the intravenous injection of extracts. Muscle strips from the earliest stages of pregnancy showed an absence of reactivity to both oxytocin and vaso-pressin; as pregnancy advanced a greater reactivity to vaso-pressin was observed and, later on, there was a gradual increase in response to oxytocin, reaching a maximum at parturition.

Further information on the effect of posterior-pituitary extract on the human uterus in situ will be found in the papers by Murphy (1941) and Henry & Browne (1943).

Cow.—Petrych (1936) employed the uterine contractions following the intravenous injection of posterior-pituitary extract for the diagnosis of pregnancy in cows. Thirty i.u. of posterior-pituitary extract were injected into the jugular vein of the cow to be tested; immediately afterwards the hand was inserted into the vagina and the cervix palpated. In pregnant cows within 1½-2½ minutes of injecting the extract a marked constriction and stiffening of the cervix occurred; this contraction lasted for about half a minute and passed off in 3-4 minutes. In the non-pregnant cow no contraction was observed. Fifty-seven cows were tested by
this method. Of 18 which reacted negatively to the test, 4 were pregnant but at the time of testing they were only 3 to 17 days pregnant. All the cows in which contraction of the uterus was noted were pregnant, the earliest positive result being in a cow served 16 days previously.

Further references to the use of the method in veterinary practice have not been found.

Information on the response to posterior-pituitary extract of isolated strips of uteri of various laboratory animals will be found in the review by Robson (1940).

**Placental Sign.**

*Rat.*—The "placental sign" was first described as a test for pregnancy in the rat by Long & Evans (1920).* About the 14th day of pregnancy, vaginal examination with a speculum revealed a bright red coloration of the ventral wall of the vagina near the cervix. This sign appeared abruptly and persisted to the 16th day, the reddish colour changing to brown. Histological smears made from this region showed considerable numbers of red blood cells. Further details were given by Long & Evans (1922). They believed that, in all probability, the blood was of placental origin, reaching the vagina *via* the cervix, and they pointed out that this was the earliest infallible sign of pregnancy in the intact living rat. It is present only from the 14th to the 16th or 17th days of pregnancy. Red blood cells are not found in the vaginal smear of the rat at any other stage of pregnancy or at any phase of the oestrous cycle. (For further references see review by Venable, 1939).

*Woman.*—Hartman (1928a,b) as a result of observations on macaque monkeys suggested that the possibility of the "placental sign" occurring to a limited extent in the human was worth investigating. No such investigation appears to have been carried out until Kulitzky (1931) published the results of 75 examinations of the vaginal secretion in 30 women in early pregnancy. In 72% of the smears from pregnant women red blood cells were present. In 16% of the smears from non-pregnant women made during the intermenstruum, red blood cells were present. Kulitzky concluded that the "placental sign" was not sufficiently reliable to be of diagnostic value in the human. Bartelmez (1937) considers that "placental sign" occurs in varying degrees in women and in certain cases is mistaken for menstruation.

*It has recently been pointed out by Courrier (1945) that the occurrence of this phenomenon in certain rodents during pregnancy was observed by Lataste in 1887.*
Cow.—Asdell & Madsen (1933) found no evidence that the "placental sign" was present in the cow during pregnancy. Ajello (1940) examined vaginal smears from 105 pregnant cows; only in 5 cases were red blood cells present. He concluded that the test was of no value for pregnancy diagnosis in the cow.

Monkey.—The "placental sign" has been described in the macaque. It appeared about the 18th to the 20th day after the assumed date of conception and lasted about three weeks. Evidence was obtained to show that the red blood cells reached the lumen of the uterus from the uterine glands and leaked through the cervix (Hartman, 1928a,b, 1929; Wislocki & Hartman, 1929).

Prostigmine Test.

Woman.—It will be obvious that this test would be applicable, so far as animals are concerned, only to certain of the primates in which menstruation occurs. However, for the sake of completeness, brief reference will be made to it.

Soskin, Wachtel, & Hechter (1940) introduced this test, which is based on the claim that, in cases of delayed menstruation which are not due to pregnancy, menstruation will commence 1/2 to 78 hours after the intramuscular injection of prostigmine methylsulphate. The theory underlying the test is that the hyperaemia of the endometrium which occurs before menstruation may be largely under the control of the parasympathetic nervous system and that most cases of delayed menstruation not due to pregnancy are due to decreased vascular responsiveness of the uterus. By injecting prostigmine, the cholinesterase is inhibited and the naturally occurring acetylcholine in the uterine endometrium is thus potentiated and uterine hyperaemia ensues. One to three injections of prostigmine may be required. This treatment was said to have no effect on pregnant women. The authors stressed that this test could only be used in selected cases where the possibility of the amenorrhoea being due to prolonged endocrine dysfunction or local organic changes had been excluded.

This test has been widely investigated. It is generally agreed that the method cannot replace the biological methods of diagnosis owing to the difficulty of eliminating cases of endocrine dysfunction and pelvic abnormalities. The test has been criticized on the grounds that sufficient evidence has not been provided to show that prostigmine is harmless in cases of early pregnancy. There have, however, been several reports confirming the value of the test as a means of diagnosing early pregnancy. The opinion seems now to be growing that prostigmine is more valuable as a therapeutic agent for the induction of menstruation after a diagnosis of non-pregnancy has been made (Weisman, 1940;
VAGINAL SMEAR AND VAGINAL BIOPSY.

Woman.—As a result of the widespread interest aroused by the detailed descriptions of the cyclical changes occurring in the histology of the vaginal walls and the vaginal smears of guinea-pigs (Stockard & Papanicolaou, 1917) and rats (Long & Evans, 1922), numerous studies have been made on the histology of the vagina and vaginal smear of the human, to investigate whether reliable information could be obtained on the time of ovulation, on the diagnosis of pregnancy, and on the detection of hormonal abnormalities. In this review, only the aspect of pregnancy diagnosis will be considered.

Preliminary investigations on the histology of the vaginal smear in the human during pregnancy were carried out by Papanicolaou (1925), who concluded that soon after the onset of pregnancy there was a distinct tendency for the vaginal cells to assume characteristic forms. Further investigations showed, however, that the variations observed in the vaginal smear were so great as to render the early diagnosis of pregnancy by that method impracticable. Papanicolaou & Traut (1943) conclude that beginning from the 35th or 40th day (counting from the first day of the last menstrual period) the vaginal smear gradually takes a more characteristic form, but there are many modifications of the typical pattern caused by the prevalence of various bacterial or trichomonad infections, and certain hormonal factors such as high blood oestrogen. All these factors make the diagnosis of pregnancy from the study of the vaginal smear difficult and somewhat uncertain. In a more recent review, Papanicolaou (1946) has again stressed that great care and experience are required for the interpretation of vaginal smears in the human, and that so far as pregnancy diagnosis is concerned, progress has been slow and at present the vaginal smear is only a guide in this question.

Further information will be found in the papers by Papanicolaou (1933), Schilling (1935), Sjövall (1938), Rubenstein (1940), Mack (1943a,b), Schuman (1944), Rakoff, Feo, & Goldstein (1944), Hall (1945), and Chapin (1946).

The histological changes occurring in the walls of the vagina during pregnancy have also been studied. As in the case of the vaginal smear, histological changes do take place, but owing to the wide variations observed and the difficulties of interpretation it seems unlikely that a reliable test for early pregnancy will be
evolved from this work (Smith & Brunner, 1934, 1937; Davis & Pearl, 1938; Rakoff, Feo, & Goldstein, 1944).

The presence of glycogen in the vaginal smear during the menstrual cycle has been studied and used as an index of oestrogenic function, but little appears to be known about the glycogen content of the vaginal smear during pregnancy (Lehmann, 1921; Miura, 1928; Cruickshank & Sharman, 1934a,b; Mack, 1943a,b; Rakoff, Feo, & Goldstein, 1944).

**Mare (Kurosawa Test).**—The histological study of the vaginal smear has proved of considerable value in the diagnosis of pregnancy in the mare. In 1931, Kurosawa described his technique for diagnosing pregnancy in mares by macroscopic and microscopic examination of the vagina. Only the microscopic part of the test will be considered in this section, since the macroscopic test which is similar to the method of diagnosis employed by Benesch has already been reviewed in the section on clinical diagnosis (page 18). Smears of the cervical secretion are stained with either haematoxylin and eosin or Giemsa stain. In smears from pregnant mares, ciliated epithelial cells of various shapes and darkly staining mucous globules are present; these features are said to be characteristic of pregnancy. Kurosawa claimed that this test could be applied as early as the 7th day after service; from the 6th to the 10th day after service an accuracy of 86% was obtained and from the 11th to the 15th day an accuracy of 90%, after which the accuracy was in the neighbourhood of 100%.

Kurosawa's test has been extensively used with considerable success by Russian veterinarians and horse-breeders. Nikitin (1932) and Barulin (1933-34) in preliminary reports confirmed the reliability of the test. Rastjapin & Rjazanceva (1933, 1936a,b) examined over 250 mares and obtained an accuracy of about 100% from the third week after service. Barulin & Burčenko (1935a,b), Barulin, Burčenko, & Popov (1936), and Barulin (1936, 1937) carried out over 8,000 Kurosawa tests. The results were checked in over 2,500 mares; in studs with high-grade stock or where conditions of husbandry were good the incidence of error was 0 to 12%, but under less favourable conditions of husbandry the error rose to 16%. These authors concluded that although this test was not so reliable as the hormone test for pregnancy in the mare, it was of considerable value because of its simplicity. The method was also considered satisfactory by Suetin (1936). Rjazanceva (1939) examined 8,000 mares by the Kurosawa method with an accuracy of 90-100%, reliable results being obtained 10 or 20 days after service. Kerov (1940) tested 475 mares with an error of 14%; he recommended the combined use of both vaginal and rectal
methods of examination from the 20th to 30th day after service. The Kurosawa test has been used in this country by Miller & Day (1939) and Day & Miller (1940). 765 tests were carried out on 545 mares; there were 59 incorrect positive tests and 20 incorrect negative tests. From the 20th to the 40th day of pregnancy an accuracy of 78% was obtained, the accuracy improved as pregnancy advanced, and at the 90th to the 100th day an accuracy of 97% was obtained.

Suetin (1936) believed that the presence of the ciliated epithelial cells in the smear was the most conclusive evidence of pregnancy since mucous globules were occasionally found in smears from barren mares. Riazanceva (1939) and Kawahara (1941), on the contrary, believed that the mucous globules were the most reliable sign of pregnancy since ciliated epithelial cells might occur in smears from mares suffering from cervicitis and endometritis.

The various sources of error, especially pathological conditions, are discussed at length by Rastjapin & Riazanceva (1936a,b). Barulin, Burčenko, & Popov (1936), and Barulin (1937).

Minakov (1936) simplified the test by dispensing with the staining of the smear. By examining the smear with a darkened field (i.e., with the iris diaphragm of the microscope almost closed), the mucous globules could be readily seen. Minakov claimed that this considerably speeded up the test and did not reduce the accuracy of the results.

Lescouyries & Wiktor (1947) have reported their findings with the Kurosawa test in 150 examinations of about 30 mares. Seldom did they observe signs characteristic of pregnancy before the 21st day. After this period, ciliated cells became apparent in the smears, but mucous globules were not seen till after the 40th day. They considered this method of diagnosis very reliable after the 40th day.

Cow.-Hart (1923) studied the histology of the vaginal smears in pregnant and non-pregnant cows, but was unable to detect any change denoting the establishment of pregnancy. From the tables given by Frei & Metzger (1926) it does not appear that they observed changes in the vaginal smear of the cow, characteristic of pregnancy. Hammond (1927) concluded that during pregnancy there were no distinctive changes in the cytology of the vaginal smear. Similar conclusions were reached by Schatalow (1933). Jankowski (1932) studied the vaginal smears of cows from the first day of pregnancy to the 4th month. He detected ciliated epithelial cells and mucous globules in the smears from pregnant cows from about the 20th day. The method was not sufficiently reliable to be used alone, but Jankowski considered it of value when used in conjunction with the clinical methods of diagnosis. Mirskaja, Kozlova, & Smirnov (1935) also claimed
some success in the diagnosis of bovine pregnancy from a study of the vaginal smear. Using the Kurosawa technique (see Mare section, page 155) they were unable to detect cells of ciliated epithelium in cervical smears, but smears from pregnant cows were characterized by aggregates of mucus which either contained or were covered with leucocytes. In a series of 71 pregnant (29 to 190 days) and 22 non-pregnant cows tested by this method an accuracy of 57 to 92% was obtained, the lowest accuracy occurring with cows 121 to 150 days pregnant. In another series of cows studied at slaughter, 13 to 14% errors were encountered with animals 3 to 6 months pregnant and 4% with animals 7 to 8 months pregnant. Róza (1935) also found that studies of smears from the cervix were helpful in the diagnosis of early pregnancy. Often as early as the 20th day the appearance of nucleated epithelial cells and disappearance of leucocytes would be noted. This was considered a valuable indication of pregnancy. Ajello (1940) examined vaginal smears from 60 non-pregnant and 105 pregnant cows and concluded that pregnancy could be diagnosed by the absence of leucocytes and the rarity of epithelial cells. Only in 8 out of 105 cases might a smear, from a pregnant animal, have been mistaken for a smear from a non-pregnant cow.

It is obvious that the findings of the above-mentioned investigators are not entirely in agreement and it may be concluded that in the cow the vaginal smear is not as reliable an index of pregnancy as it is in the mare, although to those experienced in its interpretation it may serve as a guide in diagnosis.

Vaginal biopsy studies do not appear to have been carried out in the cow, but from the histological studies of the vagina (Hammond, 1927; Cole, 1930; Richter, 1939) it seems unlikely that any of the changes which occur during pregnancy would be sufficiently characteristic to serve as a means of diagnosing pregnancy.

Glycogen is absent from the vagina of the cow (Cruickshank & Sharman, 1934a).

Ewe.—From a study of vaginal smears in ewes, Richter & Rittau (1933) concluded that the non-appearance of cornified cells in the smears after service was a good indication of pregnancy. Cole & Miller (1935) found that the vaginal smears from pregnant ewes were similar to smears from ewes in the late dioestrous period.

Glycogen is absent from the vagina of the ewe (Cruickshank & Sharman, 1934a).

Sow.—Scholz (1934) examined 43 smears from non-pregnant sows during various stages of the oestrous cycle and 30 from pregnant sows. Changes sufficiently characteristic to be of value for pregnancy diagnosis were not observed.
Glycogen is absent from the vagina of the sow (Cruickshank & Sharman, 1934a).

Bitch.—Evans & Cole (1931) have demonstrated that histological changes occur in both the vaginal smear and the vaginal walls during pregnancy in the bitch, but it is doubtful whether these are sufficiently characteristic to be of value in pregnancy diagnosis.

Guinea-pig.—Kelly (1928, 1929) has demonstrated that pregnancy can be diagnosed in the guinea-pig after the 3rd week from a study of biopsy preparations of the vaginal mucosa.

Monkey.—The vaginal smear and vaginal mucosa in macaques have been studied by Hartman (1928b) and Davis & Hartman (1935). It would appear that the changes in the histology of the smear or biopsy preparations would not be sufficiently characteristic to allow a reliable diagnosis of early pregnancy.

Glycogen has been demonstrated in the vagina of the macaque (Robertson, Maddux, & Allen, 1930; Van Dyke & Ch’en, 1936).

**Högler Vasomotor Test.**

Högler (1932) described a vasomotor phenomenon which he considered of value in the diagnosis and differential diagnosis of pregnancy. A mixture of a dilute solution of boric acid and adrenaline is introduced into the patient’s urinary bladder. A ureteral cystoscope is then introduced into the bladder and the mucous membrane is streaked with the end of the cystoscope. In pregnant women a white ischaemic streak will appear on the mucous membrane. 250 pregnant women were examined by this method and 82% gave positive results during the second month of pregnancy. The test became much less reliable as pregnancy advanced. Of 100 non-pregnant women tested, only five gave a positive test.

Högler also attempted to develop a vasomotor test on the mucous membrane of the nose, but results were only 72% correct during the second month of pregnancy.

Studies on the physiology of the capillaries during pregnancy were carried out by Melbärd (1936), who observed vascular changes in many pregnant women after the second month.

**Intradermal Saline Test.**

Various types of skin-sensitivity tests for pregnancy are considered in the section dealing with "immunological tests" (see page 162). The intradermal saline test, however, would appear to be fundamentally different from allergic tests based on the reaction to foreign proteins and will be considered in this section of the review.

In 1923, McClure & Aldrich described experiments in which
0.2 ml. of normal saline was injected intradermally and the time required for the bleb to disappear was studied in health and in pathological conditions, with special reference to the cases of oedema. Guggenheimer & Hirsch (1926) found the test of value in the diagnosis of heart and kidney diseases when latent oedema could be detected by an increase in the rate of disappearance of the saline bleb. This was considered due to an increased affinity of the tissues for water. Lash (1926) employed the test as a diagnostic aid in the toxaemias of pregnancy. Further references to the diagnostic value of the test in various diseases are given by McClure & Aldrich (1927).

This test was employed for the diagnosis of pregnancy by Obladen (1927, 1928), who claimed that in pregnant women all sign of the saline bleb had disappeared within 50 minutes of the injection, whereas in non-pregnant women the bleb remained for more than 50 minutes. Of 84 women tested in the first three months of pregnancy, 78 were correctly diagnosed by the test; 60 non-pregnant women were tested and in every case the bleb lasted for more than 50 minutes. Obladen considered that the test was worthy of further investigation.

Corinaldesi & Greco (1933) confirmed that the saline bleb disappeared somewhat more quickly in the first three months of pregnancy than before pregnancy and that the duration was further reduced as pregnancy advanced.

Owing to the various pathological conditions which may influence the test it seems unlikely that the reaction will be of value in the diagnosis of pregnancy.

No reference to the use of this test in the domestic animals has been found.

Pupillary Reaction Tests.

Bercovitz Test.—Bercovitz (1930) described the following reaction which he claimed was useful in the diagnosis of pregnancy. A few drops of blood serum obtained from the patient were instilled into her conjunctival sac and the reaction of the pupil noted. (It was, of course, necessary to standardize the conditions of lighting.) If the patient was pregnant there was either a marked dilatation or contraction or a marked activity of the pupil, after instilling the serum; if the woman was non-pregnant none of these changes occurred. Seventy-two non-pregnant women at all stages of the sex cycle gave negative tests; of 68 non-pregnant women with amenorrhoea two gave positive tests. Fifty-four of 68 pregnant women gave positive tests. Results almost as accurate were obtained when adrenaline was instilled into the eye instead of serum.
The theoretical basis of the test is somewhat obscure, but it was postulated that some adrenaline-like substance, to which the pupil becomes highly sensitive, circulates in the blood during pregnancy.

Meneghini, Tullio, & Camillo (1931) repeated Bercovitz's experiments with both serum and adrenaline, and concluded that positive results were about 90% accurate, but negative results were less reliable. Similar conclusions were reached by Gordon & Emmer (1931) who tested 90 cases; all positive tests were correct, but several negative tests proved incorrect. King (1933) tested 215 patients and obtained 4 false positive and 22 false negative results. White & Severance (1931), however, considered the reaction of no diagnostic value.

Bercovitz (1933) reported that it was unnecessary to use serum, but that the patient's whole blood diluted with sodium citrate would serve just as well. Bercovitz concluded that the evidence was all in favour of positive reactions being reliable indications of pregnancy.

Further reports on the test were made by Spitzer (1934) and Skapska (1936) who concluded that the test was quite unreliable, by Pouliot (1935) who concluded that positive reactions were wonderfully accurate, and by Triantaphilopoulos (1938) who considered the reaction was not sufficiently accurate for practical purposes.

No reference to the use of the Bercovitz reaction for the diagnosis of pregnancy in domestic animals has been found.

Mintscheff Reaction.—Mintscheff (1932-33, 1934, 1937) described a curious pupillary reaction in several of the domestic animals which is said to be characteristic of pregnancy.

The mare is kept in the dark for 4-5 minutes to allow the pupils to dilate fully. The eyes are then subjected to direct illumination. If the mare is three or more months pregnant the pupils will not react immediately to the light, but will remain widely dilated for about one minute; the more advanced the state of pregnancy the more resistant is the pupil to light. As a result of examining 200 pregnant mares, Mintscheff considers that a marked mydriasis is characteristic of pregnancy. Further experiments by Mintscheff showed that when one eye of a non-pregnant mare is illuminated the pupils of both eyes will contract, but the pupil of the non-illuminated eye will remain slightly larger than the pupil of the illuminated eye: when the same test is applied to a pregnant mare the pupil of the illuminated eye will remain dilated for some time, but the pupil of the other eye will contract and be distinctly smaller. In brief, to obtain contraction of the pupil of one eye of a pregnant mare, the other eye must be illuminated.
Mintscheff (1937) found that the pupils of the cow were generally slow in reacting to light and he was unable to find any distinct difference in response between pregnant and non-pregnant cows.

Mintscheff (1932-33, 1934, 1937) noted that the behaviour of the pupil in the bitch and cat during pregnancy was rather similar to that in the mare.

Aglialoro (1934, 1935) was unable to find any significant difference in the pupil size or behaviour in pregnant and non-pregnant women.

Piraino & Santomauro (1935) observed that in a third of the cases of extra-uterine pregnancy examined by them there was a mydriasis of the pupil on the same side as the extra-uterine pregnancy.

Pupillary Reactions in Test Animals.—Bercovitz (1930) observed that serum from a pregnant woman when instilled into the eye of a non-pregnant woman, rabbit, or cat would give similar pupillary reactions to those observed when the serum was instilled into the patient's own eye (see Bercovitz test, page 159).

Takakusu (1931) described a test for pregnancy based on the dilatation of the pupils which occurred when freshly extirpated frog eyes (Rana esculenta) were placed in pregnancy urine. The mydriasis became maximal after 15 minutes. In a series of 200 tests results equal in accuracy to those obtained with the Aschheim-Zondek reaction were claimed. King & King (1932), using the extirpated eyes of Rana pipiens, were quite unable to confirm the good results of Takakusu and they concluded that the method was not reliable for pregnancy diagnosis.

Ferrigno (1933) also found the Takakusu test unsatisfactory.

The observations of Konsuloff (1934b) and Davis, Konikov, & Walker (1934) on the effect on the pupil of injections of human pregnancy urine into frogs and rabbits respectively have already been noted (see pages 110 and 37).

Blood Picture.

Szepeshelyi (1934) concluded that it was not possible to diagnose pregnancy from a study of the blood picture in cows or bitches.

Further references to the literature dealing with the blood picture during pregnancy in the human and domestic animals are given by Morris (1944).

It does not appear that the blood picture can give information of value on the early diagnosis of pregnancy.
CHAPTER 9. TESTS BASED ON IMMUNOLOGICAL PHENOMENA

The possibility of developing a test for pregnancy based on an allergic reaction in the mother to foetal, placental, and other proteins has received considerable attention.

It has been found convenient to divide such allergic tests carried out on the mother into three types: skin tests, eye tests, and "haemoclastic" tests.

SKIN TESTS (Woman).

The skin tests have generally been carried out by injecting the antigen intradermally, but in certain cases subcutaneous injections have been used.

Fromme (1911) observed that when 0.2 ml. of sterilized serum (bovine) was injected subcutaneously, pregnant women generally showed a greater reaction at the site of injection—oedema, swelling, and erythema—than did non-pregnant women. This reaction occurred in about 78% of women over 33 weeks pregnant; about 26% of non-pregnant women showed a reaction at the site of injection. Further experiments were carried out by Esch (1912) using foetal serum and foetal extracts injected intradermally. He was unable to find any evidence of hypersensitivity in pregnant women.

The earliest attempts to use such skin tests as a means of diagnosing pregnancy were made by Falls & Bartlett (1914) and Engelhorn & Wintz (1914). Falls & Bartlett used various types of placental extracts both intradermally and subcutaneously; the results obtained were considered of no value owing to the high percentage of non-specific reactions. Engelhorn & Wintz, on the other hand, claimed that extract of placenta injected intradermally gave specific reactions from the 7th week of pregnancy. The test was read 36 hours after the injection; in positive cases there was swelling, redness, and light brownish staining round the site of injection. Seventy women, 2-10 months pregnant, all gave positive tests; 53 non-pregnant women all gave negative tests. Esch (1914), Eben (1914), and Bar & Ecalle (1919) were unable to confirm the findings of Engelhorn & Wintz.

Interest in skin tests abated after the unsatisfactory results obtained by the Engelhorn & Wintz method, but the method was again investigated when the success of the hormonal tests became apparent. The theoretical basis of the method was considerably revised and the tests were said to be based on the allergic reaction to injections of hormones. Gonadotrophic hormone preparations
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were generally used, although it is not always clear whether the preparations were of pituitary or placental origin.

The first of these tests was described by Porges & Pollaczek (1930) in 1929. “Prolan” was injected intradermally and the site examined 2-4 hours later. The interpretation of the test differed from previous tests in that positive reactions occurred in non-pregnant women (i.e., swelling, inflammation, and erythema), whereas in the pregnant woman negative results were to be expected since gonadotrophins were already present in large quantities in the body and therefore the skin hypersensitivity was no longer present. The results were not satisfactory; tests on 90 cases in early pregnancy resulted in an error of 28%, and there was an error of 4.6% in a series of 175 non-pregnant women. Deutsch (1929) tested 45 pregnant women and 65 non-pregnant women by this method, but the results were hopelessly inaccurate. Strauss (1930) used both chorionic and pituitary gonadotrophins in skin-sensitivity tests in a series of 524 cases. He concluded that the reaction was neither definite nor reliable, a very large number of doubtful reactions being obtained. Dowell (1933) claimed satisfactory results with anterior-pituitary extract, but gave no data to support his claim.

Since 1936 the skin tests for pregnancy fall into three categories: The chorionic-gonadotrophin test (“Antuitrin-S”) of Gilfillen & Gregg; the placental-extract test of Gruskin; and the colostrum test of Falls, Freda, & Cohen.

*Chorionic-Gonadotrophin Test of Gilfillen and Gregg.*—The use of gonadotrophins for skin-sensitivity reactions in the diagnosis of pregnancy was “rediscovered” by Gilfillen & Gregg (1936), who injected 0.12 ml. of “Antuitrin-S” (a commercial preparation of chorionic gonadotrophin prepared from human pregnancy urine) intradermally and read the test in ¼-1 hour. In pregnant women there was no reaction or only a small bleb; in non-pregnant women the site of injection was surrounded by a zone of erythema 2-3 cm. in diameter. Reliable results were obtained in a series of 30 to 40 patients.

Numerous workers have investigated the claims of Gilfillen and Gregg but the majority have failed to obtain accurate results, although there was a tendency for pregnant women to show no reaction to the injection of gonadotrophin. It must be concluded that this method is of no value in the early diagnosis of pregnancy. Further details will be found in the papers by the following workers:—

Saglik & Scipiades (1937), Weisman & Yerbury (1937), Schneider & Cohen (1937), Gill & Howkins (1937), Graffagnino & Haam (1938), Bronstein (1938), Gersh (1938), Frank & Wahringer
Pregnancy Diagnosis

(1938), Hoffmann & Fouch (1938), Morehead (1938), Sharman (1938), Lass, Enderle, & Kurzrok (1938), Rezende (1939), Parsons (1939), Hadley (1940), Mandy & Mandy (1941).

Placental-Extract Test of Gruskin.—Gruskin (1936) described a skin-sensitivity test for pregnancy using a placental extract as antigen. It was claimed that this test was independent of the presence of gonadotrophins in the extract since the extract remained active after boiling. The method of reading the test also differed from previous methods. In pregnant women, within 10 minutes of injecting the extract intradermally, a slight area of inflammation was noted and pseudopodia had formed on the circumference of the original bleb. In non-pregnant women pseudopodia did not occur. It was essential to control the reaction with an injection of saline; if pseudopodia developed on the saline bleb the patient was considered unsuitable for the test. Gruskin claimed excellent results in tests on 200 patients. Schwartz (1936) obtained an accuracy of 90 to 96%; he considered it a valuable test but requiring considerable skill to interpret. Subsequent investigators on the whole have failed to substantiate the accuracy of the test (Pangalos, 1938; Lass, Enderle, & Kurzrok, 1938; Gersh, 1938; Graffagnino & Haam, 1938). Pevaroff & Biskind (1939), however, obtained more satisfactory results; they considered the Gruskin test was the most reliable of the skin tests although it did not compare favourably with the Aschheim-Zondek or Friedman tests. Naidu & D'Souza (1944) also claimed reliable results in a series of 126 cases.

Colostrum Test of Falls, Freda, and Cohen.—Falls, Freda, & Cohen (1941) described a test for pregnancy based on the allergic reaction to an intradermal injection of a preparation of human colostrum. Of 265 pregnant women tested by this method, 98% gave no reaction; of 358 non-pregnant patients 96% gave a characteristic wheal and areola formation at the site of injection. Readings of the test were taken 10, 30, and 60 minutes after the injection. It was observed that children before puberty gave negative tests, i.e., as in pregnancy. Subsequent investigators have not substantiated the accuracy of the test and it would appear to be of little practical value. For further details see the papers by Weisman (1941), Weisman & Snyder (1941), Goldman, Kessler, & Wilder (1942), Pulver & Posner (1942), Allen & Donaldson (1943), Davey & Daley (1945) and Ottolenghi (1945).

Other Hormonal Antigen Tests.—Sharman (1938) used solutions of Pituitrin (posterior-pituitary extract) and adrenaline as antigens for intradermal injection but did not obtain satisfactory results.
Frank & Wahrsinger (1938) injected intradermally an extract of corpus luteum, but results were not satisfactory.

Hoffmann & Fouch (1938) injected Antuitrin-S and oestrogen simultaneously, with totally unreliable results.

**SKIN TESTS (DOMESTIC ANIMALS).**

*Placental Antigens.*—Jong (1914) investigated the possibility of diagnosing pregnancy in the cow by the skin reaction to intradermal injections of bovine maternal- or foetal-placenta extracts. The method proved quite unreliable, being only 50% accurate.

Schrannm (1921) studied the effects on the skin of placental extracts administered by scarification and intradermal injection. Pregnant and non-pregnant cows, goats, mares, and dogs were tested. While in general a higher percentage of positive results were obtained in pregnant animals there were numerous doubtful results and it must be concluded that neither method of administering the antigen gave results sufficiently reliable for practical purposes.

Nagl (1931) used various placenta extracts and emulsions as antigens for intradermal tests in cows and mares, but the results were not reliable.

More promising results were obtained by Dzanošija (1935) who prepared from the placenta an antigen which was specific for the pregnant organism and in which much of the non-specific antigenic activity had been destroyed by treatment with immune serum. 0.5 ml. of the antigen was injected subcutaneously under the tail. In pregnant cows a reddening and swelling of the skin, 3 cm. in area, was produced; the reaction appeared in about 6 hours and lasted 18 to 29 hours. Thirty-four pregnant cows were tested, of which 3 were under 1 month pregnant and 14 from 1-3 months pregnant. From these cows 4 incorrect results were recorded, 3 of which were from cows ½-1 months pregnant. 83 non-pregnant cows were tested and all gave negative results except two which gave a delayed reaction after 24 hours. Dzanošija believed the test would have considerable value.

*Choronic Gonadotrophin Antigen.*—Saglik & Scipiades (1937) tested pregnant and non-pregnant monkeys, rabbits, rats, and guinea-pigs with the "Antuitrin-S" intradermal reaction. The results were all negative in both pregnant and non-pregnant animals.

*Colostrum Antigens.*—Asdell, Elliot, & Cupps (1942) used the intradermal colostrum test on 43 pregnant and non-pregnant cows and concluded that the results were not sufficiently reliable for practical purposes.

Frost (1943) commenced an investigation on the use of the
colostrum test in cows; his work, however, was interrupted by the war and only preliminary results were obtained. The colostrum antigen was injected intradermally in the neck and in the caudal fold. Contrary to the findings of Falls et al. (1941) in the human, positive results were obtained predominantly in pregnant cows. Owing to the limited number of animals tested it was not possible to give any conclusions on the value of the test.

Cairy (1945) carried out a series of 322 tests on 35 cows and 17 tests on 2 freemartins. Various types of colostrum antigen were used and the injection was made into the mucosa 6-12 mm. inside the vulvar orifice. Unless an easily recognized reddening or swelling developed, the cow was considered pregnant. There were marked differences in the responses of some animals to certain of the antigen preparations. Of the initial tests carried out 37 were correct and 18 incorrect. It is concluded that the reaction is not sufficiently accurate to be used as a test for pregnancy.

OTHER DIRECT ALLERGIC REACTIONS.

Eye Tests.—Mandy & Mandy (1941) studied the reaction of the conjunctiva after 2 drops of "Antuitrin-S" had been instilled. After several hundred tests had been carried out the method was abandoned since responses were very poor.

Jong (1914) studied the conjunctival reaction in the cow to extracts of bovine maternal and foetal placenta, but the reactions were of no value in pregnancy diagnosis.

(See also other eye tests described in section on Physiological Methods, page 159.)

"Haemoadlastic" Test.—Lengi (1928) described a haemoadlastic test for pregnancy, carried out by making leucocyte counts before and after the intravenous injection of extracts of ovary or mammary gland. In non-pregnant women the number of leucocytes was unaltered or only slightly reduced after the injection; in pregnant women a marked leucopenia ensued. Although the number of cases tested was small the results were all correct and the author believed the test might prove of value.

ALLERGIC REACTIONS USING TEST ANIMALS.

Skin Tests.—Rodríguez (1944) injected 0.2 ml. of serum into an immature female guinea-pig and a pregnant guinea-pig. He claimed that if the serum was from a pregnant woman the immature guinea-pig would show a red wheal 1-2 cm. in diameter at the site of injection within 6 hours; the pregnant guinea-pig should show no reaction. Serum from non-pregnant women would give no reaction in either immature or pregnant guinea-pigs. Rodriguez tested sera from 50 supposedly pregnant women (from the 8th day
IMMUNOLOGICAL TESTS

...to the second month after the missed period); all the samples gave positive results in immature guinea-pigs. 48 of these tests were later checked and all were correct. Tests with sera from non-pregnant women were all negative.

Rodriguez believes that the test is antihormonal in character.

"Haemolastic" Test (Nito).—Nito (1936) described a rapid test for pregnancy based on the leucopenia which occurred after the intravenous injection of 5-10 ml. of pregnancy urine into a rabbit. The reaction was evident 2-4 minutes after the injection of pregnancy urine. Non-pregnancy urine was inactive. Nito tested 100 urine samples by this method with an accuracy of 90%.

Nito claimed that this test did not depend on the presence of sex hormones, but was due to the presence in the urine of a substance which caused a fall in blood pressure. This substance was said to be produced by the lymphatic system as a result of stimulation by proteins which gained access to the maternal bloodstream from the placenta. Further particulars of this substance are given by Nito (1935).

Subsequent investigators all agree that the test is not reliable (Emmrich, 1937; Donno, 1937; Hauser, 1937; Martzy & Pap, 1938; Panajotou, 1938; Katranski, 1939; Wattenwyl, 1939).

Hauser (1937) investigated the possibility of pregnancy diagnosis in the cow by means of the Nito reaction. Rabbits and guinea-pigs were used as test animals. Urine samples from 41 pregnant cows gave 39 correct results and samples from 20 non-pregnant cows gave 13 correct results, when tested on rabbits. A few tests on guinea-pigs gave less accurate results. Hauser concluded that the test was of no value for pregnancy diagnosis in the cow. Similar conclusions were reached by Stieglecker (1938) who tested 20 cows and Katranski (1939) who tested 120 cows.

SEROLOGICAL REACTIONS.

There have been several attempts to develop tests for pregnancy based on serological reactions.

Fieux & Mauriac (1910a, b) postulated that the blood of women during the early stages of pregnancy contained an antibody to the young chorionic villi. Using an extract of villi from a 2-months-old embryo they developed a complement-fixation test with which it was said pregnancy could be diagnosed from the 2nd to the 4th month. After the 4th month the antibody was absent from the blood and diagnosis was no longer possible.

The accuracy of the complement-fixation tests for pregnancy was not confirmed. Further references to the method will be found in the reviews by Bar (1912), Murray (1913), Bar & Écalle (1919), and Vozza (1929).
Further experiments were carried out by Gentili (1922) and Valcarenghi (1931) on serological tests for pregnancy diagnosis but the results were not promising.

In a recent publication, Cohen & Freda (1940) have described experiments in which they attempted to detect placental proteins in the blood and urine of pregnant women with an antiserum which reacted strongly with placental autolysate. Positive tests were obtained in a number of cases with blood and urine from pregnant women, whereas samples from healthy non-pregnant women and from males gave negative results. Positive tests were obtained with blood and urine samples from women with certain pathological conditions. The authors conclude that the method requires further research to improve the specificity before it can be of any diagnostic value.

*Cobra-Venom Test.*—Heynemann (1910) observed that cobra venom did not haemolyse equine red blood cells which had been washed free from serum, but if serum from a pregnant woman were added the haemolytic properties of the cobra venom would be activated. He did not consider the reaction sufficiently specific for diagnostic purposes. Bar & Ecalle (1919) investigated 46 sera and found that pregnancy sera from the third month of pregnancy were generally more active in producing haemolysis than non-pregnancy sera. These authors considered that the method could have no great value for the diagnosis of pregnancy.

*Bacillus Proteus OX 19 Agglutination Test.*—An interesting observation was made by Gratch (1943) who, while studying the incidence of rickettsial diseases in certain districts in America with the Weil-Felix reaction$, noted that Bacillus proteus OX 19 was agglutinated by the sera of pregnant women. 288 serum samples from non-pregnant women all gave negative tests; of 512 samples which gave positive tests, 7 samples proved to be from non-pregnant women (all of whom were affected with malignant tumours). Of 512 sera from healthy men, 390 gave no agglutination. Gratch stressed that further work was necessary before the method could be regarded as having value as a pregnancy test.

This phenomenon has been further investigated by Nelson & Cruickshank (1945) in this country. They agreed with Gratch that agglutinins to B. proteus OX 19 were found with greater frequency in the sera of pregnant women than in normal sera, but the agglutinins were not constantly present nor in high titre as reported by Gratch. Nelson & Cruickshank consider that the differences may be due to a difference in the strain of B. proteus. They do not

think that the test using their strain of *B. proteus* would have any application in the early diagnosis of pregnancy.

Hoare (1945) and White (1945) consider that this reaction is not sufficiently regular to prove of value in the diagnosis of pregnancy.

**Leuco-diagnosis.**—Achard, Bénard, & Cagneux (1910) studied the behaviour *in vitro* of leucocytes after the addition of certain gland extracts. They observed that the leucocytes from the blood of pregnant women were greatly activated by the addition of placental extract; this phenomenon was said to appear at the end of the second month of pregnancy, and was suggested as a method of pregnancy diagnosis.

The tryptic power of the leucocytes is increased during pregnancy (Celentano, 1935) but it does not appear that this reaction is sufficiently characteristic to be used as a test for pregnancy.

**Animals.**—Serological reactions during pregnancy in the rabbit were studied by Shinoda (1924). These would not appear to be of value in pregnancy diagnosis.
CHAPTER 10. TESTS BASED ON PHYSICAL INVESTIGATIONS OF BODY FLUIDS AND TISSUES

Tests Using Blood.

Red Cell Sedimentation Rate.—Fähraeus (1918a,b) studied the sedimentation rate of the red blood cells in samples of blood from 15 men, and 37 non-pregnant and 51 pregnant women. He found, in general, that the cells in the blood from males had a lower rate of sedimentation than the cells in the blood of non-pregnant women, and the cells of non-pregnant women were slower than cells of pregnant women. Linzenmeier (1920) confirmed that there was an increase in the sedimentation rate of the red blood cells during pregnancy. Günzale (1922, 1923) considered the test was of no value for the early diagnosis of pregnancy although it was frequently useful in differential diagnosis in the second half of pregnancy. It is now generally agreed that the red blood cell sedimentation rate is not sufficiently specific to be used as a test for pregnancy, since the sedimentation velocity is affected by various disease conditions.

For further information and references on the red blood cell sedimentation rate in pregnancy the following papers may be consulted: Linzenmeier (1923), Kürtén (1924), Honda & Yanagi (1928). Fähraeus (1929), Witte (1929), Kaplan (1930), Mathieu Trotman, Haskins, Osgood, & Albert (1931), Vollmar (1932), Vogt (1941), Nichols (1942).

The increase in the red blood cell sedimentation velocity is not sufficiently characteristic or specific to be of value in the diagnosis of pregnancy in the cow (Franz, 1921; Falcoianu, 1922).

The red blood cell sedimentation test is not a reliable means of diagnosing pregnancy in the mare (Kaaij, 1926).

Surface Tension.—In a few experiments on the blood of women in advanced pregnancy, Kisch & Remertz (1914) found the surface tension similar to that in non-pregnancy. Eufinger (1928) found a slight reduction in the surface tension of the blood during the later stages of gestation.

Mucha (1922) found that in general there was a decrease in the surface tension of the blood of cows during pregnancy but because of the individual variations the method did not present a reliable means of diagnosing pregnancy.

Galke (1928-24) measured the surface tension of the blood serum of 29 pregnant mares and concluded that pregnancy produced little or no alteration.

Osmotic Pressure.—The colloid osmotic pressure of human blood serum during pregnancy has been investigated by Orrù (1933)
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and Rottschaefer & Bethell (1936). A slight lowering of the pressure may occur.

Viscosity.—The viscosity of the blood falls slightly during pregnancy in women but the change is not sufficiently characteristic or specific to be of diagnostic value (Esiaschwili, 1933; Evans, 1942).

Novotný (1935) studied the blood viscosity in bitches and rabbits during pregnancy; a lowering of the viscosity generally occurred.

Colloid Lability.—Honda & Yanagi (1928) described a test based on the colloid lability of the blood serum during pregnancy. To a series of tubes containing graduated dilutions of saline and alcohol are added equal volumes of the serum to be tested. The tubes are incubated at 60° C and examined after 1, 2, 3, and 24 hours to ascertain whether a "floating fleecy matter" is present on the surface. The authors claimed that early diagnosis of pregnancy was possible with this test.

"Sero-electric" Test.—Kumagai (1928) has described a "sero-electric" test for pregnancy in women. An alcoholic extract of placenta is diluted 25 times with normal saline, producing a milky solution with large numbers of microscopic emulsoid particles. This solution is allowed to stand one hour at room temperature and the blood serum to be tested is then added in the proportion of 1: 20 and the mixture left for 3 hours. A drop of this mixture is then placed on a slide, an electric current is passed through the liquid and the movement of the particles observed under the microscope. When pregnancy serum is added to the placental extract the movement towards the cathode is considerably decreased as compared with the movement observed after the addition of non-pregnancy serum. Kumagai claimed that this test could be used with certainty after the 1st month of pregnancy. 105 tests were carried out, and all were correct.

Kumagai (1928) claimed that his test was suitable for pregnancy diagnosis in rabbits, positive results being obtained 72 hours after conception.

Refractive Index.—Demmel (1932) studied the refractive index of the blood serum of cows during the oestrous cycle and pregnancy. The values tended to be higher in pregnancy. The changes do not appear to be sufficiently characteristic to be of diagnostic value.

Crystallographic Tests.—Bergauer, Boucek, & Podrouzek (1935) observed that variations in the configuration of crystals, formed when drops of human blood serum and saline were allowed to dry, were produced by hormones present in the serum. Bergauer, Stich, & Zlabek (1935) described the types of crystal configuration noted when pregnancy serum and saline was allowed to dry.

Investigations on the crystal configuration of copper chloride
Following the addition of blood, and on the application of the technique for diagnostic purposes were carried out by Pfeiffer (1935). Trumpp & Rasche (1936) employing the Preiifer technique studied the effect of pregnancy urine on the crystallization of copper chloride. Sixty-six tests were carried out with 5 errors. The authors considered that the method might prove of considerable value in the diagnosis of pregnancy.

Schönsweeter (1943) studied the effect of the blood of horses on the crystallization of copper chloride; numerous disease conditions were investigated but no mention is made of the effect of pregnancy.

Mitogenetic Radiation Test.—Zalkind & Beshlebnov (1941) have claimed that the mitogenetic radiation of dried haemolysed blood from pregnant mares is much greater than the radiation from the blood of non-pregnant mares as measured by the effect on agar cultures of yeast. Blood from pregnant mares is said to affect the cultures after 5-15 seconds exposure compared with 3-5 minutes required with blood from non-pregnant animals. It is recommended that this phenomenon be utilized as a method of pregnancy diagnosis.

(Certain plant and animal tissues under certain conditions, are said to emit the so-called "mitogenetic rays," which are able to stimulate growth in tissue cells placed in a position favourable for their absorption. The literature on this subject has been extensively reviewed by Bateman (1935). Bateman concludes that "the unsatisfactory state of mitogenetic literature makes it advisable to regard all that has been written in support of the existence of mitogenetic radiation with the greatest scepticism".

A similar conclusion is reached by Topley & Wilson (1946) who write "while not denying the existence of this effect, we feel bound to adopt a strictly sceptical attitude until quantitative results that will stand the usual tests of statistical significance are forthcoming.

"Guttadiaphot" Test.—The "Guttadiaphot" test is carried out by allowing a drop of blood to fall on a specially prepared piece of red absorbent paper and allowing the paper to dry slowly in air. The blood stain on the paper is examined by transmitted light at intervals up to 24 hours and the appearance, colour, and structure of the stain and the sharpness of its perimeter are noted. Further details of this test will be found in the papers by Meyer, Bierast, & Schilling (1928, 1929), Schilling & Bruch (1928), Gesenius (1928), and Schilling (1928, 1929). From this study of the blood stain, useful information regarding the presence or absence of certain diseases was said to be obtained.

Baucks (1930) observed that the blood from pregnant women tended to give characteristic stains and he considered the method
useful in the diagnosis of pregnancy. Kurzel (1931) employed the test in 124 cases and obtained an accuracy of 97.5%; the majority of the samples were from women in the last two months of pregnancy. Kurzel considered that negative results were more reliable than positive results since certain pathological conditions gave stains simulating those from pregnant women. Ponzi (1932) tested over 200 patients and considered the method useful but requiring further research.

No reference to the use of this technique in domestic animals has been found.

**Tests Using Urine or Milk.**

*Surface Tension of Urine.*—The surface tension of human urine has been studied by several workers and no changes have been observed which are characteristic or specific for pregnancy (Schemensky, 1920a,b,c; Bechhold & Reiner, 1920; Hahn, 1926; Selous & Perryman, 1935).

Candido & Piccoli (1947), using a modification of the Schemensky method, investigated the surface tension of the urine of non-pregnant and pregnant women, and of women with pregnancy toxaemia. The stalagmometric quotient was lowest for non-pregnancy urine, highest for urine from patients with pregnancy toxaemia, and of intermediate value for urine from women 8 to 9 months pregnant.

Fiege (1922) investigated the changes which took place in the surface tension of the urine of cows and mares during pregnancy. Lactation and certain pathological conditions were also observed to have a considerable influence on the surface tension. Fiege thought that the method might be of some value in the diagnosis of pregnancy in mares which were not lactating.

Magnus (1920) studied the surface tension of urine samples from 20 pregnant mares and 26 non-pregnant mares. He concluded that the method was not reliable as a means of pregnancy diagnosis owing to the many factors affecting the surface tension.

*Crystalllographic Test on Milk.*—Sagorsky (1942) studied the effect of milk on the crystallization of copper chloride. Crystallization patterns said to be characteristic were obtained with milk from cows which were four months pregnant.

**Cervical and Vaginal Secretions.**

In the section dealing with the clinical examination for pregnancy, it has been noted that certain changes in the appearance and in the physical properties of the cervical and vaginal secretions occur during pregnancy. Chemical and physical studies have been made on these secretions and these will now be reviewed.
Chemical Properties.—Woodman & Hammond (1925) produced evidence to show that the cervical secretions of the cow may contain a muco-protein belonging to the class of mucin bodies containing mucolitin sulphuric acid. The effects of numerous chemical agents on the mucous secretion were studied but no differences in the behaviour of samples from non-pregnant and pregnant cows were reported.

Experiments were carried out by Blair and co-workers (1940-1943) on the chemical properties of bovine cervical secretion. Determinations of the nitrogen and dry-matter content of secretions, various iodine titration tests, studies on the buffering power and the effect of various chemical agents on the secretions were carried out, but none of these tests revealed chemical differences between samples from non-pregnant and pregnant animals of sufficient regularity to serve as a basis for a test for pregnancy. Further work by Boyland (1946) has also failed to reveal an easy chemical test to differentiate the secretion of pregnant and non-pregnant cows.

Various aspects of the chemistry of the cervical mucus in women have been studied by Zweifel (1908), Raab (1928), Nürnberger (1932), Hochloff (1932), Pommerenke (1948), and Pommerenke & Viergiver (1948b), but no characteristic difference between samples from non-pregnant and pregnant women has been recorded.

Rheological Properties.—That changes occur in the physical properties of the cervical secretions during the oestrous cycle and pregnancy in the cow has long been recognized by veterinarians and physiologists. At oestrus, the secretion is translucent, "glassy," fairly fluid and is present in large quantities. It has the property of forming long strings or threads (spinnbarkeit); these strings of mucus may frequently be seen hanging from the vulva about the time of oestrus. In mid-cycle the quantity of secretion is greatly reduced; it is more viscous, whitish in colour and slightly adhesive to the touch. During pregnancy the secretion becomes very viscid, opaque, and is generally said to be peculiarly adhesive when palpated with the finger tip.

In the clinical examination for pregnancy, when these changes are considered, it will be obvious that the estimation as distinct from measurement of such properties as "thickness" and "stickiness," is highly subjective. (It may be compared to the method of ascertaining whether the body temperature of the cow is above normal by placing the palm of the hand for a few seconds on the animal's back—a practice now rendered obsolete by the introduction of a simple but accurate objective method, the use of the clinical thermometer.) An extensive investigation of the rheological properties of these secretions by accurate objective methods.
has recently been carried out by Blair and co-workers (1940-1943) with a view to ascertaining whether by such methods it was possible to detect rheological changes which might be of value in the early diagnosis of pregnancy in the cow. These studies have not as yet led to the evolution of a test of sufficient accuracy in the early stages of gestation to be of practical value, nevertheless findings of considerable importance have been recorded.

Details of the changes of viscosity and flow-elasticity which occur in the secretion during the oestrous cycle have been described by Blair, Folley, Coppen, & Malpress (1941) and Blair, Folley, Malpress, & Coppen (1941). Viscosity values reached a minimum about the time of oestrus while flow-elasticity values were then maximal. (Cervical secretions are not true fluids and cannot be said to have a single viscosity since the viscosity is not independent of variations in stress and strain conditions, but by using a Blair emptying-tube viscometer, a viscosity value under arbitrarily standardized conditions can be obtained.) The marked increase in the flow-elasticity of the secretion has proved of great value in the detection of oestrus. To enable the flow-elasticity to be easily, rapidly and accurately measured the above-mentioned workers devised a simple instrument known as the "oestroscope." In the cow the symptoms of oestrus are frequently not obvious, although the ovarian cycle is normal, and valuable time may be wasted before the cow is served or inseminated. Hitherto the only reliable means of detecting oestrus has been to put the cow to the bull—a practice which may not always be practicable or even possible in small herds where no bull is kept. The practical application of the oestroscope in such conditions is obvious. It may be noted here that the vaginal-smear method of detecting oestrus which is of great value in the small laboratory animals is not applicable to the cow (Hart, 1923; Hammond, 1927; Cole, 1930).

Certain aspects of the subsequent investigations into the rheological properties of the secretions during pregnancy have already been published (Blair, Cowie, & Coppen, 1942; Blair, Cowie, & Folley, 1942; Blair, 1944). These investigations, based mainly on studies of the flow-curves of secretions tested in a Blair emptying-tube viscometer, revealed that plasticity predominated in pregnancy and elasticity in non-pregnancy. For details of the technique and of the interpretation of the flow-curves, the original papers should be consulted. Tests were carried out on 399 samples of secretion from 182 cows and heifers. 200 samples from non-pregnant animals were tested; 184 gave correct results, 7 were incorrect, and 18 inconclusive. In 182 samples from pregnant cows, 132 correct results were obtained, 20 were incorrect, and 30 inconclusive. In the pregnancy samples all the incorrect
results with one exception occurred with samples from animals in the first half of pregnancy (2 weeks to 4½ months); from the middle of the third month the accuracy was over 80%. Although this test would appear to be one of the most accurate laboratory tests yet devised for the diagnosis of pregnancy in the cow it is not sufficiently accurate in the early stages of pregnancy to be of practical value since by the third month accurate diagnosis is possible by clinical methods. Every effort has been made to improve the method, a photographic technique of recording the flow-curves was developed and other rheological properties were studied in conjunction with the above test, but a significant improvement in accuracy has not been obtained.

In addition to the rheological studies already published many other aspects of the complex rheological properties of the secretions were examined. These included investigations on the elastic recovery, work-hardening, elastic hysteresis, structural viscosity, yield-value, thixotropy, stickiness, and spinnavbar properties of the samples. Torque, extension, tearing, and sectility tests were also carried out and studies were made of the surface tension (static and dynamic), refractive index, and vapour pressure. The results of these investigations, however, were less encouraging from the point of view of obtaining a test for pregnancy than the studies on the elastic and plastic properties mentioned in the previous paragraph.

(Readers who are unfamiliar with the rheological terms should consult the books on rheology by Blair (1938, 1944) and the paper by Clift (1945).)

Various observations on the physical properties of human cervical secretion have been made and attempts to measure these properties reported, mainly in connection with ovulation and sperm penetrability; these papers have been reviewed by Clift (1945), Pomerinke & Viergiver (1946b), and Abarbanel (1946). The effect of pregnancy on the physical properties of secretions does not appear to have been investigated until, as a result of the interesting findings in connection with bovine secretions, preliminary rheological studies were carried out on human secretion by Blair, Moir, & Meares (see Blair, 1944). These studies have been considerably extended by Clift (1945), who has shown that in the woman the flow-elasticity and spinnavbarkeit of the cervical secretion are at a maximum about the time of ovulation, as in the cow, and that by the use of an instrument "the mestroscope" (a miniature oestroscope which gives an accurate objective measurement of the flow-elasticity) the approximate time of ovulation can be detected. In addition Clift has demonstrated that changes occur during pregnancy in the cervical secretions. These are
recognizable from about the 6th to the 7th week of pregnancy and are essentially similar to the changes observed in the cow—the mucus becomes opaque, more cohesive and the elastic properties disappear. It is considered that with further investigation a simple accessory test for pregnancy may be developed.

pH.—It is clear from a study of the literature that the pH values recorded for the cervical and vaginal secretions of the cow depend considerably on whether indicators or electrometric methods have been used in their measurement (Smith & Asdell, 1941; Brown, 1944). In addition, in the case of the glass electrode method, it appears that different results are obtained if the measurement of the pH of the secretion is carried out in vitro or in vivo, since the values obtained by Smith & Asdell (1941) and Cowie (1943), who obtained the secretions manually from the vagina and measured the pH in vitro with the glass electrode, indicated that normally the vagina of the cow was alkaline whereas Dougherty (1941) and Brown (1944), using a special glass electrode which allowed the pH of the secretion to be measured in situ, obtained results which showed that the vagina tended to be acid. (A possible explanation of this difference may be afforded by the observation of Barton & Wiesner (1945) that the exterior surface of the cervical mucus (in the woman) is acid while its interior is alkaline.)

So far as pregnancy is concerned, in experiments where the pH of the secretions before and during gestation has been measured by the same technique, there does not appear to be any change sufficiently characteristic or specific on which to base a diagnostic method for pregnancy (Falaschini, 1934; McNutt, Schwarte, & Eveleth, 1939; Dougherty, 1941; Cowie, 1943).

Küpper (1939) observed that in 75% of non-pregnant mares the vaginal secretions were slightly alkaline. As soon as 3 days after service the reaction became slightly acid, a change which Küpper believes may be of value in pregnancy diagnosis. Studies of the vaginal pH in non-pregnant mares have also been made by Berthelon & Juteau (1945).

The changes in the pH of the human cervical and vaginal secretions during pregnancy are not sufficiently characteristic to be of diagnostic value; see Oberst & Plass (1936), Trussell & MacDougal (1940), Rakoff, Feo, & Goldstein (1944), Chappaz, (1946).

Effect of Hormones.—The cyclical nature of the physical changes observed in the cervical secretions during the sex cycle is suggestive of a hormonal control mechanism. Experimental evidence is now accumulating in support of this view.

Cesa (1936) showed that when combined oestrogenic and pro-
gestogenic extracts were injected into ovariectomized or immature guinea-pigs, large quantities of mucus were secreted by the cervix. Palmer (1941, 1944) and Palmer & Marcille (1941) reported that the clear threads of mucus noted in the region of the cervix of women about the time of ovulation, could be produced by the injection of oestrogens and would disappear when progesterone was injected. Pommerenke & Vieglier (1946a) observed that after ovariectomy in the woman the amount of cervical secretion was decreased, but when oestrogens were injected quantities of translucent mucus were produced. Moricard, Moricard, & Vassy (1942) have shown that the refractive index of the cervical mucus appears to depend on the oestrogen-progesterone equilibrium in the blood.

It has been noted by Hammond (Jr.) & Day (1944) that the cervical and vaginal secretions of cows implanted with tablets of synthetic oestrogen differ from those of natural oestrus. Measurement with the oestroscope of the flow-elasticity of samples of mucus from oestrogen-implanted cows has confirmed that although the flow-elasticity increases soon after implantation and may attain the high values associated with oestrus, this high value is not maintained but falls to a level only slightly greater than the average value observed during mid-cycle (Cowie, 1943). Preliminary experiments with an ovariectomized heifer have shown that the flow-elasticity is increased after the injection of oestrogens but that the amount of secretion produced is very small in contrast to the quantities generally present during normal oestrus. It may well be, as postulated by Woodman & Hammond (1925) and Hammond (1927), that the hormone of the corpus luteum is largely responsible for the secretion of mucus by the cervical glands. With the onset of oestrus and the predominance of oestrogenic hormones, the secretion "liquefies" and the properties associated with the mucus at the time of oestrus develop. Owing to the cost of progesterone it has not yet been possible to test this theory experimentally in ovariectomized cows and heifers.

If with further study and experiment the various rheological phenomena known to occur in the cervical secretion can be shown to reflect specific alterations in the balance of hormones in the blood, a most valuable method for the study of the physiology and pathology of the reproductive system in the woman and the domestic animals will be made available.
SUMMARY AND CONCLUSIONS

SUMMARY AND CONCLUSIONS.

MARE.

In the mare, the experienced clinician may diagnose pregnancy in the second month.

The following tests are reliable in the mare:

1. **The Kurosawa Cervical Smear Test** which gives reliable results about a month after service, when used in conjunction with the Benech clinical method.

2. **The Gonadotrophin Test** which depends on the detection of gonadotrophic hormones present in the serum of pregnant mares from the 50th to 100th day of pregnancy (approximately).

3. **The Oestrogen Test** which depends on the detection of oestrogenic hormones which are present in large quantities in the urine of pregnant mares from the 60th day onwards. From the 60th to the 120th day, biological methods are generally required to detect the oestrogen but afterwards it can be equally well detected by the more simple Cuboni chemical test.

COW.

The diagnosis of pregnancy is generally possible in the cow from the third month of gestation by clinical examination.

As yet no laboratory test, hormonal or otherwise, has been found of any value in the early diagnosis of bovine pregnancy.

EWE AND GOAT.

Pregnancy may be diagnosed by clinical methods during the last two months of gestation.

As in the cow, no laboratory test is available for the ewe and the goat.

SOW.

Clinical methods are of little or no value.

**The Oestrogen Test** may be helpful if carried out between the 21st and 30th days after service, as there is evidence that in the pregnant sow oestrogens are present in the urine during this period.
Dog and Cat.

In some animals diagnosis by clinical methods may be possible from the third week of pregnancy.

Radiography will not give certain diagnosis until the 7th or 8th week of gestation.

There is some slight evidence that oestrogens in small quantities may be present in the urine of the bitch from the third week of pregnancy, but in general hormonal tests are of no value for the diagnosis of pregnancy in the dog or cat.
1. Where authors are quoted at second-hand, the source of citation or abstract is indicated at the end of the reference. A.B.A. = Animal Breeding Abstracts.

2. Russian names and titles have been transliterated by the Serbo-Croat system, as used by the Commonwealth Bureau of Animal Breeding and Genetics. As a result, certain names may appear in a form unfamiliar to some readers. This system, however, has the advantage over the more usual phonetic systems in that it allows accurate re-transliteration into Russian—an essential requisite if titles are to be included—and avoids the confusion arising from the several spellings of a name which inevitably occur with phonetic systems which vary from country to country.


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