Studies on Chorionic Gonadotrophin.

By

E.M. Hobson, B. Sc.

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I should like to thank Professor F.A.E. Crew for the interest he has shown in this work, and also Professor R.J. Kellar and his colleagues for providing much of the clinical material used in the investigation.

Pregnancy Diagnosis Laboratory,
University of Edinburgh.
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INTRODUCTION.

It is commonly assumed that non-clinical methods for the detection of pregnancy are a recent feature of medical science, but this is not so. Some 3,000 years ago the Egyptians attempted to diagnose pregnancy in the human, by adding urine to germinating wheat seedlings; if their growth was accelerated it was concluded that the woman was pregnant. We now know that human urine does contain a growth promoting substance to which plants are sensitive, but it is extremely unlikely that these early pregnancy tests were accurate. Since that time many methods have been tried for the early diagnosis of pregnancy, but none of these were successful until the discovery of a gonadotrophic substance in the urine of pregnant women. Evans (1924) discovered only 30 years ago that extracts of the pituitary had a gonadotrophic action when injected into female rats. Aschheim and Zondek found that the urine of pregnant women would also produce a stimulating effect upon the ovaries of immature female mice, and in 1928 they published an account of the first reliable pregnancy test based upon the presence of gonadotrophin in pregnancy urine. At that time it was thought that this urinary gonadotrophin was produced by the pituitary. It is now known that the gonadotrophin excreted during pregnancy is placental in origin. The term "chorionic hormone" was first used by Hamburger in 1933 for the gonadotrophin of pregnancy. Evans (1935) suggested the name "chorionic gonadotropin" to distinguish it from pituitary gonadotrophin. The Third International Conference on the standardisation of hormones held in Geneva in 1938 adopted the term: gonadotrophin (phin) or gonadotropic (phie) for the
active substances which hitherto had been mentioned by several names.

It is only recently that highly successful methods for the assay of gonadotrophins have been developed. In 1939 an international standard for chorionic gonadotrophin (C.G.) was created and this enabled different workers who had measured their findings in international units (I.U.) to compare their results directly.

Basically, all the successful methods for the qualitative, semi-quantitative, and quantitative estimation of gonadotrophin produced during pregnancy, consist of biological methods utilising laboratory animals, and, these methods rest upon the two fundamental facts, that during pregnancy there is an increase either in the production or excretion of gonadotrophin; and that gonadotrophin produced by the pregnant human female will effect demonstrable changes in lower animals.

Many different methods based upon the presence of C.G. in the urine have been used for pregnancy tests. Few of them are reliable enough for routine purposes. Hogben suggested the use of female Xenopus laevis as a test animal, and this toad is now used for the majority of pregnancy tests in this country. The standardisation of laboratory conditions has ensured a very high standard of reliability and as a result it is not only possible to diagnose pregnancy accurately, but also to assay the amount of gonadotrophin excreted. This has led to the use of the Hogben test as a means of distinguishing between the normal excretion of C.G. during pregnancy, and its excretion during
various pathological conditions. This is not only of 
great value to the clinician but has greatly increased our 
knowledge of the physiology of normal pregnancy, and various 
abnormal conditions in which C.G. is excreted.

In this thesis two main lines of research are 
presented;

(1) The development of methods using the male and female 
South African Clawed Toad (Xenopus laevis) as a test animal 
for the qualitative, semi-quantitative and quantitative 
assay of C.G. in urine and tissues.

(2) The use of such methods to obtain information about the 
excretion of C.G. in normal pregnancy, and from patients with 
hydatidiform mole and chorionepithelioma. The possibility 
of distinguishing between these conditions by such assays is 
discussed. The excretion of C.G. after the surgical or 
spontaneous abortion of hydatidiform moles has been assayed, 
and is shown to be the most reliable indication of retention 
of such tissue. Little is known about the concentration of 
C.G. in molar tissue and such information has always been 
expressed in animal units. The amount of C.G. in 
hydatidiform mole has been assayed in I.U., and compared with 
the amount excreted by the same patients, in an attempt to 
establish a relationship between the level excreted and the 
malignancy of retained tissue. Since C.G. is not produced by 
the normal male its excretion in the urine indicates the 
presence of a teratoma or chorionepithelioma. The presence 
of such tumours and their metastases can be detected by the 
same methods as are used to demonstrate the excretion of C.G. 
in the female. Biological assays done after the removal of 
the primary tumour provide reliable information about the
development of metastases. Several such cases are described here.

Zondek (1926) and Aschheim (1926a) showed that precocious sexual maturity could be induced in immature female mice following the implantation of tissue derived from the anterior lobe of the pituitary (A.L.P.) of mammals. This conclusion was arrived at independently by Smith (1926) and the observations extended and confirmed a few months later (Smith and Engle, 1927).

Aschheim and Zondek (1927, 1928b) investigated the role of the pituitary in pregnant women, and found changes in the reproductive organs of immature female mice after injection with urine collected as early as the 35th day of pregnancy as calculated from the last menstrual period. Reactions caused by the injection of pregnancy urine were considered similar to those of A.L.P. implants. They recorded three grades of response in the ovaries of mice which they classified as follows:

<table>
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<th>Reaction</th>
<th>Description</th>
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<td>I</td>
<td>Follicle stimulation, ovulation and induction of vaginal oestrus.</td>
</tr>
<tr>
<td>II</td>
<td>Ovarian hyperaemia, and haemorrhagic follicles (&quot;Blutpunkte&quot;)</td>
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<tr>
<td>III</td>
<td>Luteinization of the follicles, and formation of corpora lutea atretica.</td>
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On the basis of these separate reactions Zondek postulated that the pituitary produced two gonadotrophic
substances which he called Prolan A and Prolan B, HVR 1
being caused by Prolan A, and HVR 2 and 3 by Prolan B.
Aschheim and Zondek (1928a,b) suggested that the presence or
absence of these pituitary hormones in the urine might be
used for the diagnosis of pregnancy. A positive pregnancy
reaction being HVR 1 - 3 or HVR 2+3. HVR 1 alone was not
sufficient to warrant a positive diagnosis.

We now know that the hormone present in the urine
of pregnant women which caused these reactions was in fact
produced by the placenta. Although the Prolan B of Zondek
originally applied to the luteinizing fraction of the A.L.P.,
the term when used now, includes the luteinizing hormone of
the A.L.P. and of the placenta. In 1928 Aschheim and Zondek
published the results obtained with urines from a series of
known pregnant and non-pregnant women. The biological test
agreed with the clinical diagnosis in 98.5% of cases.

These results stimulated the investigation into the
effects of injected A.L.P. extracts and pregnancy urine into
other laboratory mammals. Evans and Simpson (1930) used
female rats and found them less affected by toxic urines than
mice. Bellerby (1929a,b) produced ovulation, haemorrhagic
follicles and corpora lutea in the rabbit ovary by injecting
extracts of A.L.P. Friedman (1929a,b) found that similar
reactions in the rabbit were produced by multiple
intraperitoneal injections, or a single intravenous injection
of pregnancy urine.

Gonadotrophin in the blood of pregnant women has been
detected by the injection of suitable extracts of blood,
plasma and serum into mammalian test animals. The effect
upon the ovaries of the test animal is identical with that produced by gonadotrophin obtained from other sources, (Aschheim 1926b, Zondek and Aschheim 1927 and Fels 1927). It is claimed that serum can be successfully substituted for urine and is preferable to urine because it gives fewer false negative results (Smith and Smith, 1944). Urine is obviously a more convenient body fluid to collect and use for biological tests than blood.

Following the publication of biological methods using the immature mouse and rabbit as test animals for the early diagnosis of pregnancy, known as the A.Z. and Friedman tests respectively, hundreds of papers appeared, incorporating many modifications of technique, animal husbandry, preparation of the urine prior to injection, etc. The majority of authors are agreed that these tests are reliable methods for the early diagnosis of pregnancy.

Zondek (1930) considered that the injection of mammalian gonadotrophins was without effect on the ovaries of Anurans (Rana esculenta and Rana pipiens). Bellerby (1933b) demonstrated, however, that pituitary gonadotrophins when injected into Rana temporaria induced ovulation but not oviposition. It was necessary to kill the frog to observe the reaction. Shapiro and Zwarenstein (1934b) also found that oviposition did not occur in Rana after injection of pregnancy urine. The observations of Hogben (1930) and Hogben, Charles and Slomes (1931) on the pituitary-gonad relationship in the female South African Clawed Toad (Xenopus laevis), were responsible for the development of a highly successful biological Xenopus test for pregnancy.
These authors noted that ovarian atrophy occurred after removal of the hypophysis. They demonstrated that ovulation and oviposition would occur in the hypophysectomised toad after injections of extracts made from mammalian A.L.P. It was further shown that this reaction could be brought about after injections of pregnancy urine. It was apparent that Xenopus would be a suitable animal for pregnancy diagnosis if it did not ovulate spontaneously when kept under laboratory conditions, ovulated with the extrusion of eggs following the injection of pregnancy urine, and did not ovulate when injected with urine from non-pregnant women.

Bellerby (1933a, b) confirmed that ovulation and oviposition in Xenopus could be brought about by the injection of pituitary extracts. By increasing the temperature at which the animals were kept whilst under test, the time interval between injection and oviposition could be reduced, thus enhancing the value of the reaction for test purposes.

Bellerby (1934a) and Shapiro and Zwarenstein (1934a, b) successfully used Xenopus in a rapid test for pregnancy diagnosis. The usual procedure when making a test was to inject crude urine or aqueous extracts of alcohol or alcohol ether precipitates of the urine; these were injected into the dorsal lymph sac of the toad. The injection of crude urine was far from satisfactory and often had toxic effects upon the toad. Furthermore, unless there was an adequate amount of gonadotrophin in the urine of pregnant women the toad did not respond to the injection by laying eggs. Thus a false negative reaction was given suggesting that the urine was from
a non-pregnant woman. Precipitation methods of
gonadotrophin extraction were normally satisfactory and non
toxic, provided that all traces of alcohol and ether were
removed from the precipitate before injection. These
methods of extraction and concentration of the urine were
superseded by the so called kaolin method of Scott (1940).
Briefly stated, the method takes advantage of the fact that
gonadotrophins (pituitary and chorionic) are adsorbed on
kaolin, and can be readily eluted with N/10 sodium hydroxide.
This is similar to the method proposed by Reiss and Haurowitz
(1929) in which the gonadotrophin was adsorbed on alumina
and eluted with ammonia. Scott's method is identical with
the method of Elden (1933). For some reason Elden's work
has been completely overlooked by all workers in this field,
including myself. I came across it by chance some 2 years
ago.

The reliability of the Xenopus test, as it was then
called, was amply confirmed by numerous reports, Crew (1937,
1939), Landgrebe (1939), Landgrebe and Samson (1944), Scott
(1940), Landgrebe and Hobson (1949). These workers showed
that the toad test was as accurate and reliable as the A.Z.
test, and had many advantages over the mouse test. Crew
(1939) proposed that the Xenopus test should be referred to
as the Hogben test, "in order to bring it into line with the
mouse (Aschheim Zondek), and rabbit (Friedman) tests." This
associated the test with Professor Hogben's name, for it was
in his laboratory in Cape Town that it was first shown that
Xenopus could be used for this purpose.

Male frogs and toads have only recently been used for
the biological assay of C.G. The relationship between the A.L.P. and the production and release of male gametes was demonstrated by Wolf (1929). Wolf observed that transplants of anuran A.L.P. caused spermatogenesis. The same year Houssay and Lascano Gonzalez implanted A.L.P. into the testes of toads and brought about release of sperm into the lumen of the seminiferous tubules. Rugh (1937) confirmed this, and found that injection of pituitary extracts released sperm from the testes of hibernating frogs. This previous work on male anura culminated in the publication, by Galli-Mainini (1947), of a method for diagnosing pregnancy, based upon the expulsion of spermatozoa by male toads when injected with pregnancy urine. It is interesting to note that Robbins, Parker and Bianco (1947) suggested the use of male Xenopus as a new test animal for pregnancy diagnosis and, though they obtained the release of sperm by the injection of chorionic gonadotrophins of pituitary and chorionic origin, they did not use pregnancy urine. Since Galli-Mainini's original paper it has become evident that the appearance of sperm in the urine of male anura, after the injection of chorionic gonadotrophin, is not confined to any genus or species. The universality of this reaction is indicated by the growing list of anurans that have been used.

The use of these biological tests has not been confined to the diagnosis of pregnancy. It has been shown that women with hydatidiform moles or chorionepithelioma excrete abnormally large amounts of chorionic gonadotrophin (C.G.) (Zondek, 1929; Aschheim, 1930). For this reason dilution tests have been used to distinguish a pregnancy from a mole or chorionepithelioma. Zondek (1931) suggested that
a level of 50,000 "mouse units" or more of gonadotrophin per litre of urine, an amount normally sufficient to produce a positive reaction when injected in a dilution of 1 in 100, indicated the presence of either a mole or chorionspithelioma. Despite evidence to the contrary, there is still a general impression that urines from patients with such abnormalities of the chorio-placental system will always give a positive "pregnancy test" in a dilution of 1 in 100, a view held by some clinicians and pathologists. It is now well established that not only may the level of C.G. excreted during a normal pregnancy be more than 50,000 mouse units but also weakly positive and even negative biological reactions may be obtained with urine concentrates from molar and epithelioma cases (Hobson, 1952).

Men with testicular tumours sometimes excrete C.G. in large amounts comparable with the levels found in the urine of pregnant women. This observation was made by Heidrich, Pels and Mathias (1930) who were the first to report the use of the pregnancy test to diagnose tumours in the male. Blood, urine and implants of the primary tumour from the patient were found to give a positive A.Z. test.

Before the international standard in 1939 was established for the gonadotrophic substance for human pregnancy urine, all quantitative estimations were expressed in animal units. The international standard was defined as follows:-- "The specific gonadotropic activity of 0.1 mg (=100 µ) of the standard preparation shall be the international unit for the activities of all gonadotropic preparations of human urine of pregnancy, but only of such."
It is unfortunate that, although the international standard has been available for 16 years, most authors still express amounts of C.G. in animal units. Few investigators have assayed their material against the international standard and expressed their results in international units of chorionic gonadotrophin.

It is extremely difficult to compare the results of quantitative estimations of C.G. in pregnancy and pathological conditions such as hydatidiform mole, and choriocarcinoma, from different laboratories when authors have expressed their findings in these different units. The results obtained with biological methods of assay using such tests as the increase in weight of the prostate and seminal vesicles in the immature rat (Loraine, 1950), the increase in weight of the prostate and seminal vesicles in the hypophysectomised immature rat (Diczfalusy, Hogberg and Westman, 1950), the increase in uterine weight of the immature rat (Delfs, 1941) and the formation of corpora lutea (Diczfalusy, 1953) are valuable. This is particularly true when the authors have expressed their findings in I.U. There is, however, a great need for comparative studies on urine from patients excreting gonadotrophin in which the assays are not only expressed in I.U. but in which the method used has been identical for each pathological condition. The amount of C.G. excreted even in normal pregnancy is so variable, that it is essential that the differences which inevitably occur when different test animals are used, or the same test is performed in different laboratories, should be eliminated.
**EXPERIMENTAL PROCEDURE.**

**Aschheim-Zondek test using the immature female mouse.**

This is essentially the same as the method described by Robson (1947) incorporating the modifications of Landgrebe and Hobson (1949). Four mice are used instead of 5 and are obtained from dealers as 3 weeks old females. The pretreatment of urine (testing with universal indicator, addition of acid, or shaking with ether when toxic) and the addition of 1g. of sulphosalicylic acid to 30 ml of urine, has been discarded in favour of the following method: 4 drops of 20% thymol in alcohol are added to 30 ml of urine as soon as the specimen arrives. This simple modification has reduced the number of urines which prove toxic to the test mice. This is in conformity with the findings of Zondek and Black (1946), who decided that toxicity was not a property of normal urine. The mice tolerate thymol extremely well, and the effect of the injected urine on the ovaries of the test animal seems more clear cut.

**Hogben test using female Xenopus laevis.**

**Husbandry.**

It is important to avoid overcrowding of the test animals. Overcrowding leads to regression of the ovaries and to a diminution of food intake (Alexander and Bellerby, 1938). Although pollution due to overcrowding is a major factor in diminished food intake, Landgrebe (1939) also suggested that some form of interaction between the toads results in fatigue due to the competitive effort before they have eaten much food.
Metal tanks 1ft. x 2ft. x 1ft. are used. They are fitted with a wire mesh cover, to prevent the toads from jumping out. Each tank contains approximately 60 toads, which receive a constant supply of preheated running water; the inflow tap is adjusted so that a complete change of water occurs every 24 hours. Experiments done on groups of *Xenopus* show that those kept in running water increase in weight compared with similar groups kept in still water.

**Feeding and cleaning.**

Unlike members of other Anuran suborders, *Xenopus* lacks a tongue. Food is thrust into the mouth with both hands and assisted into the oesophagus by depression of the eye balls. Experiment (Landgrebe, 1939), and experience have shown that fresh raw ox liver is the ideal food. This must not be minced but chopped into small cubes, it is dropped into the tanks and enough must be given to provide a small surplus. Toads under test are fed once per week, preferably 2 days after the last injection, or at least 3 days before the next one. This is necessary because *Xenopus* vomits up food when it is injected too soon after feeding. Stock and resting toads are fed 2-3 times per week, and it is advisable, when large numbers of animals are kept to prepare a feeding chart; this prevents feeding at the wrong time and the possibility of some tanks being overlooked.

The day after feeding, all the uneaten liver is removed from the tanks together with the excreta. Cleaning presents little or no problems when toads are kept in running water, as the major part of the bulky faeces and debris is carried away through overflow pipes.
In tanks without a continuous water supply, water must be completely changed the day after feeding and should be renewed once per week. Tanks showing the slightest signs of rusting or paint flaking off are immediately replaced. Twice per day, morning and evening, all tanks are examined for dead or fluid toads; which must be removed.

**Temperature.**

_Xenopus_ is housed in rooms kept at a constant temperature of 25°C. all the year round. The running water supply to the tanks is preheated to 22°C., the most economical temperature consistent with good husbandry. Fluctuations in water temperature should be avoided, as in my experience toads maintained at 22°C. for long periods do not always survive a sudden change of 10°C.-15°C. _Xenopus_, if gradually acclimatized, will survive temperatures from 1°C.-34°C. They eat little or not at all below 12°C.

**Fluid toads.**

Occasionally some of the toads that have been used for Hogben tests are found to have an exaggerated inflation of the dorsal and, less often, ventral lymph sacs. These animals are called "fluid toads." As far as can be ascertained this is most often caused by some substance in the urine which has not been removed by extraction, or by failure to neutralize the concentrate properly. The lymph hearts, of which there are 3 pairs situated in the lumbar region, stop beating and this prevents the injected concentrate getting into the circulation and also allows lymph to accumulate. I have cured many of these animals by withdrawing all the fluid with a syringe, and flushing out
the lymph sac once or twice with distilled water. The toads are then placed in a "hospital" tank and kept under observation. After several months rest those that have recovered are reprimed and returned to the unit.

If the precautions outlined above are taken, the stock can be maintained in a healthy and sensitive condition at all times.

Spontaneous ovulation and oviposition.

Female Xenopus has been used on a large scale for the diagnosis of pregnancy for many years. Only 2 of the numerous workers who have used Xenopus have reported spontaneous oviposition, Dosch (1944) and Thorborg (1950). In both instances toads were kept in glass jars under "ordinary laboratory conditions." Unfortunately these conditions were not described. As Bles (1905) has shown, some Xenopus will lay eggs when the environment is deliberately altered to simulate natural conditions. Spontaneous oviposition has not been observed in Xenopus kept under standardised conditions, and in the past 4 years only 1 false positive test has been reported (Landgrebe and Hobson, 1949). Landgrebe (1953) reported that spontaneous egg laying occurred when male and female Xenopus were kept together in tanks outside. The eggs laid were fertilized and produced normal tadpoles. Four batches of males and females, on 4 separate occasions, were put into tanks in the open between July and September 1953 in Edinburgh. Spontaneous oviposition and fertilization took place twice. Following Thorborg's report (1950) 20 sexually mature females were isolated in glass jars in a room containing croaking males, and were fed and kept under observation for one month.
None of these toads shed ova. The 20 toads were then injected with 70 I.U.C.G.; all oviposited, thus showing that mature eggs were available. In a further communication Landgrebe (1954) reported no significant difference in the dose response curves between females injected with C.G. when croaking males were present, and those injected and isolated from males. When I attempted to produce spontaneous spermatation in male Xenopus by putting males and females in the same container neither spermatation nor oviposition were observed over a period of 7 days. The males were examined twice daily for sperm.

In a further experiment to determine what effect the presence of the male had on oviposition in the female, 5 injected males were paired with 5 uninjected females and 5 uninjected males with 5 injected females. The results are shown below (Table 1).

Table 1

<table>
<thead>
<tr>
<th>No. of Xenopus</th>
<th>Treatment</th>
<th>Spermatation</th>
<th>Oviposition</th>
</tr>
</thead>
<tbody>
<tr>
<td>10 males</td>
<td>None</td>
<td>Nil</td>
<td>Nil</td>
</tr>
<tr>
<td>10 females</td>
<td>None</td>
<td>Nil</td>
<td>Nil</td>
</tr>
<tr>
<td>5 males</td>
<td>C.G.</td>
<td>All positive</td>
<td>Nil</td>
</tr>
<tr>
<td>5 females</td>
<td>None</td>
<td>Nil</td>
<td>Nil</td>
</tr>
<tr>
<td>5 males</td>
<td>None</td>
<td>Nil</td>
<td>All positive</td>
</tr>
<tr>
<td>5 females</td>
<td>C.G.</td>
<td>Nil</td>
<td>All positive</td>
</tr>
</tbody>
</table>

It may be inferred from my own and Landgrebe's observations that spontaneous ovulation and oviposition can be produced under certain special conditions. If, however, a standard method of husbandry is adopted then the toad does not lay eggs unless it is injected with C.G.
As most biological tests for pregnancy depend upon the presence of chorionic gonadotrophin it is essential that they are not affected adversely by other endocrine metabolites. Shapiro and Zwarenstein (1937), Landgrebe (1939) and Burger and Zwarenstein (1955) found that certain steroids would induce ovulation and oviposition in Xenopus. The doses required to effect this are fortunately larger than the amounts excreted in non-pregnant states, and although during pregnancy there is a general increased excretion of these substances their presence is probably advantageous rather than the reverse. Mammalian biological tests have always suffered from the disadvantage of giving some false positive results. These false positive reactions are most commonly obtained with menopausal urines, or conditions promoting the excretion of pituitary gonadotrophin in excessive amounts. This gonadotrophin has in the main a follicle stimulating effect on the mammalian ovary. After the injection of rabbits, rats and mice with sufficiently high concentration of follicle stimulating hormone (F.S.H.) the ovaries are macroscopically similar to those of animals which have been injected with O.G. Female Xenopus unlike male Xenopus is relatively insensitive to F.S.H., and female toads when injected with menopausal urine concentrates do not ovulate. Weisman and Coates (1944) investigated the effect of injected urine concentrates from 89 women at the menopause, and failed to produce egg extrusion in female Xenopus. This lack of response by the female toad to F.S.H. may be explained by the fact that ovulation in Xenopus is caused largely by luteinizing hormone (L.H) which is absent, or present only in
very small amounts, in the urine of menopausal and post
menopausal women. From the complete absence of false
positives it is evident that for all practical purposes
Xenopus may be regarded as reacting specifically to the
chorionic gonadotrophin in the urine of pregnancy.

Collection and preparation of urine.

In accordance with usual laboratory practice first
morning specimens are requested. This usually ensures a
well concentrated specimen. Recently Pedersen-Bjerregaard
and Pedersen-Bjerregaard (1948) and Hamburger (1948) have
questioned the necessity of "first morning urines." The
findings of these authors suggest that C.G. excretion per
unit of time is independent of the corresponding volume of
urine produced. While consider that these observations may
be correct it is important not to overlook the effect of
fluid intake upon urine output. For pregnancy testing as
little as 60 ml of urine is required, an amount that can
easily be produced 30 minutes after drinking a ½ pint of
fluid. During the 2nd and 3rd months of pregnancy, when
peak excretion of C.G. can be expected, (as much as 650 I.U.
C.G/ml/hr. calculated from the data of Pedersen-Bjerregaard
and Pedersen-Bjerregaard, 1948) the amount of urine in which
the gonadotrophin is contained is not important. However,
the advantage of biological tests for pregnancy is that an
accurate diagnosis can be made a few days after the first
missed but expected period. At such times the amount of
gonadotrophin is low.

It is therefore preferable to have a first morning
specimen for routine pregnancy testing. This will obviate
as far as possible false negative results due to a sub
threshold dose of C.G. being injected. Concentration of the urine prior to injection is necessary for two reasons; (1) the toad is less sensitive to C.G. than the mouse. The mean effective dose (M.E.D. 50), required to produce the formation of haemorrhagic follicles in the ovaries of 3 weeks old mice, is 10 I.U.C.G. In female Xenopus the M.E.D. required to cause oviposition in 50 per cent of toads grouped for assay, is 54.5 I.U.C.G. (Landgrebe, 1948) and 29.0 I.U.C.G. (Hobson, 1952a). This difference is due to the fact that Landgrebe used heavier animals. (2) crude urine is very often toxic to the toads and may result either in their death or so upset the function of the lymph hearts that the animal becomes grossly oedematous. Extraction and concentration of C.G. is described in detail and is essentially the same method as that proposed by Elden (1933) and Scott (1940), but it has been modified to suit routine testing.

**Method of extraction and concentration.**

Into a 100 ml cylinder pour 60 ml of urine and dilute to 100 ml with tap water; add 0.5 ml of brom-phenol blue, and acidify to approximately pH 4.0 with 20 per cent HCl, added drop by drop until the blue colour disappears and is replaced by a faint green.

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*Brom-phenol blue solution. 0.1 g. of solid is ground with 4 ml of N/10 sodium hydroxide in a small mortar, and when it is completely dissolved the volume is made up to 100 ml with distilled water.*
The addition of water is only necessary when the turbidity and colour of the urine interferes with the observation of the colour change. In actual practice when large numbers of urines are being extracted daily it is quicker to add water to all specimens. Specimens whose specific gravity is obviously low are not diluted and 100 ml of urine is used. The next step is the addition of 5.0 ml of 20 per cent kaolin upon which the gonadotrophic hormone is absorbed. Maximum absorption occurs about pH 4.0. The whole is well shaken and allowed to stand for half an hour, the supernatant fluid decanted off, and the kaolin suspension transferred to a centrifuge tube and centrifuged for not less than 5 minutes at 1,500 r.p.m. The supernatant fluid is poured off, and the hormone extracted from the kaolin precipitate by adding 5.0 ml N/10 NaOH, and thoroughly stirred for 2 or 3 minutes with a glass rod. Separate rods are used for each specimen, to avoid the transference of any positive concentrate to a negative one. This solution is centrifuged for 20 minutes, and the supernatant fluid poured into another tube; this is made slightly acid to litmus paper with approximately 2 drops of 20 per cent HCl.

The concentrate of 5.0 ml is ready for injection. Failure to neutralize properly will produce "burning" of the animal's dorsal skin due to excessive acidity or alkalinity of the extract. Burning may be mild; small areas of skin appearing blanched and dead. In extreme cases patches of skin are destroyed, revealing the underlying tissue. Toads affected in this way are discarded.
Method and route of injection.

The toad is picked up with the index finger between the hind legs, transferred to the other hand and held firmly by its hind limbs in a dry cloth. The point of the needle, bevel uppermost, is first passed through the thigh muscle so that no fluid escapes from the sac after the needle is withdrawn, tilted upwards, and directed towards the midline of the dorsal surface just under the skin of the back. Care must be taken to ensure that the tip of the syringe needle is actually in the lymph sac before expressing the fluid. The flow of fluid towards the head of the toad indicates that the injection has been properly performed. Some workers inject directly into the peritoneal cavity; this procedure is dangerous, because should the injection be made too near the midline, damage to the large abdominal vein is certain, and the possibility of puncturing and actually making the injection into the viscera is present unless great care is taken. The dilution of the concentrate with peritoneal fluid and its possible escape down the oviducts, via the ostia to the cloaca, should not be overlooked. Furthermore, if the peritoneal route is used in preference to the dorsal lymph sac, twice the dose of chorionic gonadotrophin is required to produce ovulation and oviposition (Landgrebe, 1943).

Selection of test animals.

Before the toads are selected for testing it is essential to distinguish between male and female Xenopus. The mature female in good condition is larger and more pear-shaped than the male, as the body cavity is filled out by
the ovaries. The most characteristic diagnostic features are the cloaca and labia. The female labia are prominent protruding structures, and may or may not be hyperaemic, depending on the sexual condition of the toad. In the male these structures are very small and do not extend beyond the cloaca. A distinguishing feature peculiar to the male is an intense black pigmentation, known as "pads" or "gloves", consisting of closely set hooked pigmented spines covering the ventral surface of the fore limbs from the digits to the axilla. These are present when the animal is in breeding condition. (see Fig. 3).

Failure to obtain reliable results with the Hogben test is often due to workers taking no account of the weight or condition of the toad. Landgrebe (1948) pointed out "that while sensitivity is not proportional to, it is slightly affected by, the weight of the test animal." In this laboratory well-fed toads weighing 60-100 g. are used. Before being used as test animals they must respond by ovulation and oviposition to a priming dose of 70 I.U. of C.G. This procedure not only separates the less sensitive toads from the stock; it also ensures that only animals with mature ovaries are used. Moreover, twice a year, every negative toad in the unit is reprimed; those which do not respond are discarded and replaced by positive toads. These precautions provide a highly sensitive stock.

Test unit.

This unit is made up of numerous sub-units consisting of a "positive" and a "negative" tank for each working day. Positive tanks contain toads which ovulated
when they were last injected; negative tanks being filled with those animals that did not. When test results are checked toads are sorted according to their reaction and placed in the appropriate tank. Positive toads are more sensitive than negative ones, and for this reason negative tests are repeated with positive toads.

Hogben test.

One toad from the negative tank is injected with half the urine concentrate (2.5 ml) and placed in a 2-lb. glass jar, half filled with warm water at 22°C and fitted with a perforated metal screw top. It is important that the test jars are not filled up to the top, as this prevents Xenopus surfacing to obtain air, thus causing death by drowning.

The toad actually rests upon a perforated celluloid platform which allows the eggs as they are extruded, to fall to the bottom of the jar. The platform is a necessary precaution as some toads eat their own eggs, and if this happens a positive test might appear to be negative. If the toad ovulates within 18 hours the result is recorded as positive. Reading the test 18 hours after injection is convenient and has no special significance. Xenopus will deposit eggs within 6-24 hours after an injection of pregnancy urine. The speed of the reaction is related to the amount of C.C. injected and the temperature at which the animal is kept whilst under test. If, however, no ovulation occurs in 18 hours a second toad from the positive tank is injected with the remaining 2.5 ml of the same extract. If both toads are still negative 48 hours after the beginning of the test the result is recorded as
EFFECT OF DOSE ON DURATION OF RESPONSE

(10 Female Xenopus)

Key:
- 100 I.U. C.G.
- 300 I.U. C.G.
- 900 I.U. C.G.

Number of toads ovulating vs Days after injection.
negative. Should the "repeat toad" ovulate and the first toad not, the negative toad is removed and placed in a feeding-up tank for 3 or 4 months, and reprimed before being readmitted to the test unit.

The injection of a second toad only if a negative result is given by the first, enables 25 per cent more tests to be done with the same number of animals without loss of accuracy. It is found that toads which have previously ovulated can be made to deposit eggs if they are injected, 24 hours after their last ovulation, with "negative urine" or distilled water. This is understandable when it is realised that many toads will continue to ovulate and deposit eggs up to 7 days after an injection of C.G. (Fig. 1). It is therefore of the utmost importance that toads should be taken from, and returned to the correct tanks; otherwise many false positive results will be obtained.

Toads have been shown to be more sensitive if they are injected every 10 days instead of at 21 day intervals (Landgrebe, 1943). They are not, however, used continuously, because they lose weight. For routine purposes they are used 3 times in 1 month, and then rested for a month, e.g. animals used on January 3, 13, and 25 will be rested during February and used again on March 1, 11, and 23, resting the following month, and so on. This means that a single Xenopus toad is injected 18 times per year. Some animals have been used in tests over 70 times and are still giving consistent and reliable results.
The interpretation of results.

As it has been shown a negative response is one in which eggs are not shed by either toad within 48 hours after injection. A result is accounted positive when one or both toads lay eggs. The number of eggs a toad lays is related to the amount of C.G. present in the urine. Positive results can be graded (Table 2) and this may occasionally provide additional information when considered in conjunction with the clinical diagnosis, as the results of all "pregnancy tests" should be.

Table 2

<table>
<thead>
<tr>
<th>Grade</th>
<th>Reaction</th>
<th>Number of eggs</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Extremely weak positive</td>
<td>20</td>
</tr>
<tr>
<td>2</td>
<td>Weak</td>
<td>20-50</td>
</tr>
<tr>
<td>3</td>
<td>One plus</td>
<td>50-200</td>
</tr>
<tr>
<td>4</td>
<td>Two pluses</td>
<td>200-500</td>
</tr>
<tr>
<td>5</td>
<td>Three pluses strong</td>
<td>500 or more</td>
</tr>
</tbody>
</table>

For example a weakly positive or negative biological reaction (grades 1 and 2), from what appears clinically to be a normal pregnancy, may be the first sign that all is not as it should be. In a series of 223 cases of confirmed abortion the Hogben test was negative in 75, weakly positive in 29 and positive in 119. Thus in 46 per cent of tests, the negative and weak positive results provided an informed guess about the state and outcome of the pregnancy (Matthew and Hobson, 1953).

It is known that there is an enormous difference in the amounts of C.G. excreted by women during pregnancy. This, as would be expected, influences the response of the test
animal, for although the number of eggs shed is not proportional to the amount of gonadotrophin injected, low doses tend to produce weaker reactions than high ones. Furthermore the degree of the reaction is affected by individual variation amongst test animals. This is illustrated in Table 3 which shows the effect of injecting 30 new stock toads grouped by weight, with 70 I.U. of C.C.

Table 3

<table>
<thead>
<tr>
<th>No response</th>
<th>Grades of reaction</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1 2 3 4 5</td>
</tr>
<tr>
<td>2</td>
<td>3 1 9 1:4</td>
</tr>
</tbody>
</table>

Table 3 shows clearly the differential response that can be obtained, and warns against indiscriminate acceptance of graded results when animals are not grouped according to their response. Even careful grouping does not ensure complete uniformity as the response may alter from time to time in the same animal, depending on its previous treatment.

If these things are taken into account when interpreting the results, then a weakly positive test can have some significance for the clinician when it is considered with the case history, and may indicate the necessity for instituting therapeutic measures.

**Accuracy of qualitative tests.**

The most essential feature of any non-clinical method of diagnosing pregnancy is its accuracy and specificity. It is important to the clinician, when making a diagnosis, that the result of a "pregnancy test" is not misleading due to a low degree of accuracy or lack of specificity.
Too often claims are made that a test is 100 per cent correct when it is based on a small series of urines from known cases of pregnancy. The reliability of a method can only be assessed if a large number of urines from cases of early pregnancies, disturbed pregnancies, secondary amenorrhoea of non-pregnancy origin, tumours, cysts and the like, are examined.

Female Xenopus, unlike mice, are insensitive to menopausal concentrates and only lay eggs when injected with C.G. Although little use has been made of the A.Z. test in this investigation the results obtained are useful for purposes of comparison. The following figures are based on tests done between January 1949 and June 1951. During this period some 15,000 A.Z. tests in which a correct result was given in 99.5 per cent of cases were done; and 37,000 Hogben tests with an accuracy of 99.3 per cent were done. There were no false positive Hogben, and false negatives were mainly obtained with specimens sent too early after the missed but expected period, or because the urine was too dilute. False positive results are sometimes obtained with the mouse. Many of these may be due to the spontaneous maturation of the test animals' ovaries (Hobson, 1951).

Semi-quantitative estimation of chorionic gonadotrophin.

(Dilution tests)

Dilution tests are usually requested when a pregnancy is not behaving normally, a hydatidiform mole or choriocarcinoma is suspected. Dilution tests are essentially the same as pregnancy tests, except that the urine or concentrate is diluted with distilled water before
injection. Dilutions in common use are 1 in 10 and 1 in 100. It is common laboratory practice to give the results of semi-quantitative tests as being positive or negative to the particular dilution employed. Providing the sensitivity of the test animal to C.C. is known it is perfectly feasible to express the amount of C.C. present in the urine in International Units per litre. Urines containing approximately 3,000 or more I.U.C.G. per litre will always give a positive Hogben test (Hobson, 1952). It follows therefore that positive responses from specimens diluted 1 in 10 and 1 in 100 will not contain less than 30,000 and 300,000 I.U. per litre respectively. When the level of C.C. falls below 3,000 I.U. per litre of urine, the test is usually negative. Urine giving a positive reaction in a dilution of 1 in 100, is further diluted until no positive response after injection is obtained. Until 1951 dilution tests were done with mice. In view of the need for quicker diagnosis it was decided to develop a dilution test using female Xenopus. To find out whether the mouse could be replaced by the toad without loss of accuracy, a comparison was made between the two tests on a random series of urines.

The following is a brief description of the 2 methods:

(1) Aschheim Zondek. Nine immature 21 day old female mice, subdivided into 3 equal groups, are injected daily for 3 days with 1.0 ml of undiluted urine and with urine in a dilution of 1 in 10 and 1 in 100. The mice are killed on the 5th day and the ovaries examined for haemorrhagic follicles. The presence of 1 or more such
folicles in the ovaries signifies a positive reaction.

(2) Hogben. The method is essentially the same as that described for the qualitative test, except that 66 ml of urine instead of 60 ml are diluted to 100 ml with water. The gonadotrophin is extracted from the kaolin with 5.6 ml \( \text{H}/10 \text{ NaOH} \). Two toads are used and each is injected with 2.5 ml of the neutralized concentrate. To the remaining 0.6 ml 5.4 ml of distilled water is added; 2.5 ml of this (1 in 10 dilution) is injected into 2 other toads. The 1 in 100 dilution is made by adding 0.5 ml of the 1 in 10 solution to 5.5 ml of water; 2 toads are injected as before.

The salient features of these tests are compared in Table (4), and the advantages of using the Hogben dilution test rather than the A.Z. test can be seen. It is more economical if the urines diluted 10 and 100 times, are only injected after a positive result has been obtained with undiluted urine. The test requires 36 hours for completion and is appreciably quicker than the A.Z.

<table>
<thead>
<tr>
<th>ASCHHEIM-ZONDEK</th>
<th>HOGBEN</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Nine mice per test</td>
<td>Six toads</td>
</tr>
<tr>
<td>2. Killed at end of test</td>
<td>Can be used repeatedly</td>
</tr>
<tr>
<td>3. Multiple injections</td>
<td>Single injection</td>
</tr>
<tr>
<td>4. Examination of ovaries</td>
<td>Simple observation of ova</td>
</tr>
<tr>
<td>5. Result in 5 days</td>
<td>Result in 18 hours</td>
</tr>
<tr>
<td>6. Crude urine used</td>
<td>Need to concentrate urine</td>
</tr>
<tr>
<td>7. Occasional false positives</td>
<td>No false positives</td>
</tr>
</tbody>
</table>

One hundred and fifteen urines were examined by both methods and complete agreement was obtained in 109 cases. Clinical confirmation was obtained for the 106 tests which are considered in some detail. (Table 5).
<table>
<thead>
<tr>
<th>Clinical Diagnosis</th>
<th>Tests</th>
<th>Normal</th>
<th>HOGKEN 1/10</th>
<th>1/100</th>
<th>Negative</th>
<th>ASCHHEIM-ZONDEK Normal</th>
<th>1/10</th>
<th>1/100</th>
<th>Negative</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hydatidiform mole</td>
<td>12</td>
<td>12</td>
<td>10</td>
<td>6</td>
<td>-</td>
<td>12</td>
<td>11</td>
<td>5</td>
<td>-</td>
</tr>
<tr>
<td>Follow up after H.M.</td>
<td>24</td>
<td>4</td>
<td>4</td>
<td>-</td>
<td>20</td>
<td>4</td>
<td>1</td>
<td>-</td>
<td>20</td>
</tr>
<tr>
<td>Chorionepithelioma</td>
<td>3</td>
<td>3</td>
<td>1</td>
<td>-</td>
<td>-</td>
<td>3</td>
<td>2</td>
<td>1</td>
<td>-</td>
</tr>
<tr>
<td>Follow up after C.E.</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Normal Pregnancy</td>
<td>18</td>
<td>10</td>
<td>9</td>
<td>1</td>
<td>-</td>
<td>16</td>
<td>9</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>Abnormal Pregnancy</td>
<td>12</td>
<td>9</td>
<td>1</td>
<td>-</td>
<td>3</td>
<td>8</td>
<td>2</td>
<td>-</td>
<td>4</td>
</tr>
<tr>
<td>Testicular Neoplasma</td>
<td>14</td>
<td>2</td>
<td>-</td>
<td>-</td>
<td>12</td>
<td>2</td>
<td>-</td>
<td>-</td>
<td>12</td>
</tr>
<tr>
<td>Not stated</td>
<td>9</td>
<td>4</td>
<td>3</td>
<td>-</td>
<td>5</td>
<td>5</td>
<td>4</td>
<td>1</td>
<td>4</td>
</tr>
</tbody>
</table>

Table 5. Comparison between the results obtained by the Hogben and A.Z. dilution tests.
Under the heading of abnormal pregnancy I have included threatened, missed and incomplete abortion, abnormal foetus, and ectopic pregnancy. Seminomas and teratomas are included under the heading "testicular neoplasms."

Comparing the biological tests with the clinical diagnosis, which was accepted as being correct, the Hogben results agree in 105 cases and the A.Z. in 101. In a case of threatened abortion the Hogben test was negative, the A.Z. giving a weak positive reaction. This was the only incorrect Hogben in the series. Altogether there were 5 incorrect A.Z. tests comprising 1 false positive and 1 false negative in the group for which no clinical diagnosis was made, and 3 false negatives, 2 from cases of normal pregnancy and 1 from patient with an incomplete abortion.

It is evident that the Hogben dilution test can replace the A.Z., and has the advantages of being more accurate and of giving a result in 18-24 hours compared with the 5-7 days required to complete the A.Z. This is an important consideration.

Quantitative estimation of chorionic gonadotrophin.

Chorionic gonadotrophin is assayed by 2 methods using male and female toads. These methods use as criteria, the extrusion of eggs and the release of sperm into the urine.

In the former, extruded eggs can be seen in the water within 24 hours after injection of C.G., and in the latter, microscopic examination of easily obtained specimens of test animals' urine reveals the presence or absence of spermatozoa.
(1) *Xenopus* female.

Data about the excretion of C.G. in normal pregnancy has been obtained by the method described by Landgrebe (1948). Female toads which had previously ovulated to a priming dose of 70 I.U. were divided into 4 groups with 20 animals in each. The toads weighed between 35 and 57 g. To obtain dose response data the animals in each group were injected with graded doses of the International Standard Preparation for C.G. The percentage in each group giving a positive response 24 hours after injection is shown in the table below.

<table>
<thead>
<tr>
<th>Dose in Int. units</th>
<th>Percentage response 24 hours</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Group 1</td>
</tr>
<tr>
<td>20</td>
<td>10</td>
</tr>
<tr>
<td>25</td>
<td>25</td>
</tr>
<tr>
<td>30</td>
<td>30</td>
</tr>
<tr>
<td>40</td>
<td>45</td>
</tr>
<tr>
<td>50</td>
<td>80</td>
</tr>
<tr>
<td>75</td>
<td>100</td>
</tr>
</tbody>
</table>

These toads were found to be more sensitive than those used by Landgrebe to construct his dose response curve. This was mainly due to the fact that his animals were heavier (50-100 g.). The smaller toads gave a 70 per cent response when injected with 40 I.U.C.G.

Twenty four hour specimens of urines were used.

From these a 200 ml aliquot was taken, filtered and the pH adjusted to approximately 4.0 with glacial acetic acid. Precipitation of C.G. was brought about by the addition, with stirring, of 3 vols. of ice cold absolute alcohol and 1 vol. of ether. The mixture was placed in the refrigerator.
and the precipitate allowed to develop overnight. The clear supernatant fluid was poured off and the precipitate collected on a Seitz filter. The precipitate was washed with ice cold acetone of analytical quality, and when no further fluid could be removed by suction it was transferred to a desiccator and dried over phosphorous pentoxide. When dry it was weighed, finely ground in a mortar and if not used immediately, ampouled and stored.

For purposes of assay each group was divided into 2 groups with 10 animals in each. The first group was injected with an amount of the test material which it was calculated would probably cause ovulation in 50 per cent of the toads. The other group was injected with a known dose of the International Standard Preparation (I.S.P.) or my own laboratory standard which I had previously assayed against the I.S.P. The results of the injections were obtained and 10 days later the groups were crossed over, the toads which had previously been injected with the unknown now receiving injections of standard preparation and vice versa. The number of I.U.C.G. excreted per 24 hours was calculated from the combined response of both groups to the unknown and standard preparations by interpolation from a previously constructed dose response curve (D.R.C.). The slope of the D.R.C. was assumed, and the response obtained with the standard was used to fix the position of the dose response line, and to check the response of the group. All the toads used for assay were marked, and the individual response after each injection was recorded. This provided a means of checking the sensitivity of each individual toad as well as that of the group.
Figure 2.
Comparison between semi-quantitative and quantitative methods of estimating C.G.
Quantitative estimations are done when precise information about the excretion of biologically active substances is required. They are, however, time consuming, expensive, and require the keeping of large stocks of animals. When all that is required is a knowledge of the increase or decrease in the excretion of C.G., semi-quantitative tests are adequate.

The following example shows the results obtained by converting "dilutions" into absolute units and comparing them with the results obtained by bio-assay on an aliquot of the same specimen. (Fig. 2).

A closer approximation to the quantitative values can be obtained by narrowing the range of dilutions employed. The curve of C.G. excretion obtained by both methods is similar, and, with the exception of 1 result, quantitative estimations fall within the range of values given by the dilution test. The anomaly is due to a weak positive result being given by the Hogben test with an undiluted urine, suggesting a concentration of approximately 3,000 I.U. per litre. The amount of C.G. in this particular specimen was 2,125 ± 400 I.U. per litre.

There have been many reports that male amura can be used as test animals for pregnancy diagnosis. Preliminary investigations to determine whether male Xenopus was suitable for this purpose failed because the injection of crude urine from pregnant and non-pregnant women resulted in the death of some of the toads.
Several workers have experienced the same difficulty; it appears that there is a species difference amongst anura in their ability to withstand injections of unprepared urine. The impression gained from the literature is that the Bufonidae are more resistant than the Ranidae. Because of this I continued the tests with concentrated extracts of urine using the kaolin method already described. Each male toad was injected with 2.5 ml of the concentrate which was equivalent to 30 ml of urine. Forty two positives and 22 negatives were obtained. As a control measure, 2.5 ml of the same concentrate was tested using the female Xenopus (Hogben test). The results given by the Hogben test were in accordance with the clinical findings. Fifty eight of the tests were positive and 6 were negative.

These results were disappointing and suggested that the males are less sensitive to C.G. than the females. However, when these tests were performed, no information was available about the conditions under which male Xenopus should be kept in order to obtain optimum results. It was therefore decided to investigate the various factors which might modify the response of male Xenopus to injected C.G.

The male Xenopus used in these experiments had been in the laboratory for at least 6 months, during which time they had been fed with raw chopped liver once a week. Before the toads were used, the urine of every toad was examined for spermatozoa by inserting into the cloaca a capillary pipette; "difficult" animals can usually be made to urinate by slackening the grip on the animal and allowing it to struggle slightly. Only toads that had no spermatozoa in their urine were used. Separate pipettes were used for
each examination; slides and cover slips were thoroughly cleaned before use. Examinations were made with a binocular microscope using X10 eyepieces, a half-inch objective and reduced illumination. The injections were made into the dorsal lymph sac, and all the injected material was contained in 1 ml distilled water. The needle first passed through the thigh muscle in order to prevent fluid escaping from the sac after the withdrawal of the needle. The toads were placed in 2 lb glass containers and kept isolated whilst under observation.

**Effect of temperature.**

Landgrebe (1948), investigating the effect of temperature on the speed of response in female *Xenopus* to C.G., found that temperatures higher than 22°C increased the speed of response but did not affect the sensitivity of the toad. In order to determine whether this was also true for the male, 2 similar groups of 10 males were kept for 11 days before use in water maintained at a constant temperature of 16°C and 26°C, respectively. Both groups were then injected with 50 I.U.C.G. and examined for sperm at intervals up to 24 hours after injection. Sixty per cent of the toads kept at the higher temperature had spermated 4 hours after injection, compared with 20 per cent of the controls. After 24 hours the response of the 2 groups differed only very slightly, 30 per cent of the high temperature group responding positively compared with a 60 per cent response for the other group. Because the difference was so small it was decided to keep all male toads in water at a constant temperature of 18°C ± 1°C.
Dose and speed of reaction.

During this investigation it was observed that small amounts of gonadotrophin took longer to produce their effect than large doses, and the number of sperm produced, allowing for individual variation, was related to the dose. Confirmation of this was obtained when 40 males, with similar histories, were split into 4 groups of the same weight, and each group injected with a different dose of C.G. (Table 7).

Table 7

<table>
<thead>
<tr>
<th>Dose of C.G.</th>
<th>Percentage Response.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1 Hr.</td>
</tr>
<tr>
<td>25 I.U.</td>
<td>0</td>
</tr>
<tr>
<td>50 I.U.</td>
<td>20</td>
</tr>
<tr>
<td>75 I.U.</td>
<td>20</td>
</tr>
<tr>
<td>100 I.U.</td>
<td>20</td>
</tr>
</tbody>
</table>

The results show that the interval between injection and appearance of sperm is affected, disregarding the effect of temperature, by the amount of hormone injected. This is contrary to the observation of Haines (1948) using Bufo arenarum. He remarks that there is no such reaction as a "weak positive", i.e. no gradation of response, and he tends to assume that there is no difference in the numbers of sperm produced by an individual male when injected with different doses of gonadotrophin, or by individuals of a group to the same dose.

Bieniarz (1950), using Rana esculenta, observed four grades of positive response, varying from + for a few sperm to ++++ for maximum density when the urine was examined under
Ventral Surface of Fore Limb of Male Xenopus showing development of "Gloves"
low-power magnification. Maximum response was obtained after injection from urine produced between the 8th and 12th week of pregnancy, i.e. when concentration of chorionic gonadotrophin is highest. Minimum response was obtained during the second half of pregnancy when the hormone titre in the urine has fallen. There seems to be general agreement between workers using different species that the reaction is complete within 4 hours or remains negative. The impression gained throughout this investigation is that (to doses of less than 50 I.U.C.G.) groups of male Xenopus do not respond fully at 4 hours. Higher doses, however, usually produce the maximum response at 4 hours. (Table 7).

Gloving.

Nuptial exsanguinations, referred to as "pads" or "gloves", and consisting of closely set hooked pigmented spines are to be seen on the ventral surface of the fore-limbs of male Xenopus in breeding condition.

Gloving takes place in three clearly defined stages, pigmentation first occurring in the skin covering the digits and metacarpals, proceeds over the radioulna, and finally covers the humerus as far as the axilla. (Fig. 3). In this investigation these phases are called "hands", "hands forearm", "hands to axilla."

Gloving in anura co-exists with testicular activity and can be produced by injection of gonadotrophins. It was thought that gloved toads might be more sensitive to C.G. than ungloved ones. Eighty toads, weighing 32.7 ± 2.1 g. each, were divided into 2 groups containing 10 "ungloved", "hands",
"hands forearm", and "hands axilla" toads per group. These 2 groups were injected with 2 different doses of C.G. and their urines were examined at intervals for sperm. The results obtained (Table 8) indicate that there is no significant difference between the response of any of the groups with the doses employed.

Table 8

<table>
<thead>
<tr>
<th>Dose C.G. per Toad</th>
<th>Ungloved</th>
<th>Hands</th>
<th>Hands forearm</th>
<th>Hands axilla</th>
</tr>
</thead>
<tbody>
<tr>
<td>25 I.U.</td>
<td>80</td>
<td>100</td>
<td>80</td>
<td>80</td>
</tr>
<tr>
<td>50 I.U.</td>
<td>90</td>
<td>90</td>
<td>90</td>
<td>100</td>
</tr>
</tbody>
</table>

Weight.

It is a well-known phenomenon that the response to injected pharmacological substances may vary according to the weight of the test animal. Landgrebe (1948), using female Xenopus, observed that although sensitivity was not directly proportional to the weight of the toad, it was affected by it.

In order to see whether male toads are so affected, 5 different weight groups were injected with 25 I.U.C.G. (Table 9).

Table 9

<table>
<thead>
<tr>
<th>Number of Toads</th>
<th>Average Weight</th>
<th>Standard Deviation</th>
<th>Percentage Response</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>2 hrs. 4 hrs. 6 hrs. 24 hrs.</td>
</tr>
<tr>
<td>10</td>
<td>17.72</td>
<td>1.7</td>
<td>20 40 60 100</td>
</tr>
<tr>
<td>10</td>
<td>24.95</td>
<td>0.62</td>
<td>30 40 60 90</td>
</tr>
<tr>
<td>10</td>
<td>35.23</td>
<td>2.33</td>
<td>10 40 40 60</td>
</tr>
<tr>
<td>10</td>
<td>44.7</td>
<td>3.08</td>
<td>10 30 30 50</td>
</tr>
<tr>
<td>9</td>
<td>68.2</td>
<td>13.7</td>
<td>0 33 33 44</td>
</tr>
</tbody>
</table>

These figures show that within the usual weight range of 20-50 g. the dose of C.G. required to produce the
response is roughly proportional to the weight of the test animal. While the lighter group gives the greater response, these animals are very small and not easily handled. Unless otherwise stated, all the toads used in this investigation weighed between 20 and 30 g.

Captivity affect.

The male toads used were imported from South Africa during the past 2 or 3 years. A few hundred of them were laboratory-bred males. It was first assumed that different batches of toads of approximately the same weight would be similarly sensitive to C.G. irrespective of the length of time spent in captivity. The following investigation was designed to test this assumption.

The experimental animals consisted of 3 widely differing groups: (a) old stock males kept in the laboratory for approximately 2½ years. They had been injected many times but at the time of this experiment they had not been injected for 6 months; (b) new stock males imported 6 months before this investigation and never previously injected; (c) laboratory-bred males, the first generation offspring of imported parents, which were 2 years old and had never been injected. The 10 toads in each batch were injected with 15 I.U. and examinations for sperm were made at 4 hour and 24 hour intervals. A second dose of 15 I.U. was given 10 days later. The mean 24 hour positive response for both experiments was as follows: old stock 60 per cent, new stock 55 per cent and laboratory-bred stock 30 per cent. This shows that the response of old stock and new stock imported males is similar, but that laboratory-bred Xenopus seem less sensitive.
Effect of dose upon "positiveness", and the motility of spermatozoae.

In order to use the toad repeatedly it is necessary to know how much time must elapse before they again become negative after an effective injection of C.C. Twenty males, which were positive 5 days previously to pregnancy urine concentrates (P.U.C.), and negative upon examination, were divided into 2 groups of 10. One group was used as a control and received 2.0 ml distilled water, the other group was injected with 2.0 ml P.U.C. Twenty-four hours later, 6 toads in the control and 10 in the experimental group had responded. Of the 6 toads in the control group giving positive responses only 2 produced motile sperm, whereas all the sperm in the experimental groups were motile.

A further 24 males were injected with 2.5 ml of pooled P.U.C. and examined daily for sperm. All 24 remained positive for 7 days, and 8 were still positive on the 14th day. On the 22nd day 3 toads were still shedding sperm; the whole group finally becoming negative on the 30th day.

As the amount of C.C. in pregnancy urine varies considerably it was decided to repeat the above experiment, using known amounts of C.C. in order to see if dose influenced both sperm motility and the time taken to become negative. Fifty male Xenopus, 10 per group, were injected with 400, 200, 300, 400 and 500 I.U.C.C. and examined at intervals for sperm. A qualitative estimate was also made of sperm motility. The results are shown in Table (10). Table 10 shows clearly that sperm motility and the time the toads remain positive are both affected by the amount of C.C. injected. Other experiments have shown that toads injected
<table>
<thead>
<tr>
<th>Dose I.U.</th>
<th>Number of toads</th>
<th>Number of toads positive days after injection.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>1</td>
</tr>
<tr>
<td>100</td>
<td>10</td>
<td>10(10)</td>
</tr>
<tr>
<td>200</td>
<td>10</td>
<td>10(10)</td>
</tr>
<tr>
<td>300</td>
<td>10</td>
<td>10(10)</td>
</tr>
<tr>
<td>400</td>
<td>10</td>
<td>10(10)</td>
</tr>
<tr>
<td>500</td>
<td>10</td>
<td>10(10)</td>
</tr>
<tr>
<td>Total</td>
<td>50</td>
<td>50(50)</td>
</tr>
</tbody>
</table>

Table 10 - Effect of Dose Upon "Positiveness" and Motility of Spermatozoa.

(Bracketed figures refer to the number of toads shedding motile sperm.)
Figure 4.
Section through testis of uninjected Xenopus showing mature spermatocytes in unstimulated seminiferous tubules (A). x 100.

Figure 5.
Section through testis after the animal has been injected with C.C. Showing the stimulating effect of C.C. x 100.
Figure 6.
Section of male Xenopus kidney, showing free spermatocytes in vasa efferentia (A).
with 50 I.U.C.G. or less become negative within 5 days and remain so.

As well as remaining positive to higher doses there is a day to day variation in the number and viability of sperm. Many toads stay negative for several days and then suddenly produce motile sperm.

The effect of the size of the dose on duration and percentage response for pregnant mares serum gonadotrophin (P.M.S.) is similar to that of C.G. (Table 11).

<table>
<thead>
<tr>
<th>Dose I.U. P.M.S.</th>
<th>Number of toads</th>
<th>Percentage response days after injection.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>1</td>
</tr>
<tr>
<td>5</td>
<td>30</td>
<td>67</td>
</tr>
<tr>
<td>10</td>
<td>30</td>
<td>100</td>
</tr>
<tr>
<td>20</td>
<td>30</td>
<td>100</td>
</tr>
</tbody>
</table>

In *Xenopus laevis*, sperm is released from the testes and passed through the vasa efferentia to the Malpighian corpuscles of the kidney and into the bladder via the urogenital or Wolffian duct. (Figs. 4, 5 & 6). Unlike *Rana temporaria*, *Xenopus* does not possess seminal vesicles, and any accumulation of sperm must take place in the bladder. It has been suggested that the continued appearance of non-motile sperm in urine samples, after large doses of C.G. is perhaps due to retention of small numbers of sperm in the kidney (Table 10). This is followed by a period of "negativeness" which may last from 2 to 10 days and is almost invariably accompanied by the appearance of large, (i.e., large in proportion to the few non-motile sperm), numbers of
actively swimming sperm. Such a recurrence of motility is suggestive of there being a fresh expulsion of gametes from the testes, possibly in response to some endogenous stimuli, perhaps from the animal's own pituitary.

A much more likely hypothesis is that injection of gonadotrophin mobilises mature sperm and stimulates the production of new spermatocytes by speeding up the maturation of spermatocytes and spermatids. When the interval between the two processes is prolonged, periods of "negativeness" of varying duration prevail. Should the expulsion of mature sperm continue for a length of time sufficiently long to allow a fresh generation of spermatocytes to mature before those first stimulated have been completely expelled, then negative intervals will not occur and the periods of "positiveness" will be extended.

There is some evidence to support this assumption. Two males (used to obtain data for Table 10) were pithed, and the urogenital systems excised and examined histologically. One toad (A) was from the group which had received 200 I.U.C.G and had been negative for 13 days; the second toad (B) was from the group which had received 500 I.U.C.G, and at the time of examination it had remained positive for 30 days. Toad B showed no signs of spermatogenesis, and the tubules of the testes, vasa efferentia, uriniferous tubules, Malpighian corpuscles, Wolffian ducts and the bladder were crowded with free mature sperm. It is suggested that toad B is an example of an animal with overlapping sperm generations, with consequent prolonged spermiation. The microscopical picture presented by toad A is in direct contrast to B, and
is that of an animal featuring all the stages of spermatogenesis, together with a few mature sperm in vasa efferentia and in one or two uriniferous tubules. Although male A remained negative for 13 days, the presence of sperm in the vasa efferentia and kidney tubules strongly suggests that a positive result was to be expected at the next examination.

Whatever the cause, this day to day variation and the time taken to become negative are serious objections to the use of male Xenopus for pregnancy diagnosis. These objections do not arise when small amounts of gonadotrophin are used, and the toad could be used for routine biological assay of C.G., especially when the approximate potency is known.

An observation that may be of some practical value to those wishing to breed Xenopus in the laboratory is suggested by the data shown in Table 10. Forty eight hours after injection the number of toads shedding motile sperm has dropped to less than half. The practice of some workers is to inject males 24 hours or more before mating with injected females. Whilst all the toads in this experiment had motile sperm in their urine 24 hours after injection, the number of sperm per sample was significantly less than it was when they were first examined 6 hours after injection. Female Xenopus injected with 300 I.U. or more C.G. will always ovulate within 8 hours, and often after 6 hours, when they are kept at 24°C. It is suggested therefore that it might be advantageous to inject the male either at the same time, or only a few hours before the female. By so doing the number of ova fertilised might be increased.
Effect of repeated use, and interval between injections and response.

The data given in Table 10 shows that doses of 100 I.U.C.G. or more cause male Xenopus to spermiat© for some considerable time after injection. Small doses (50 I.U. and less) evoke a positive response, but the effect is not prolonged. It is not known whether past injections influence future responses, or if the interval between injections is of importance in qualitative and quantitative work.

Sensitivity of female Xenopus varies with the time interval after the last ovulation. There is a difference between groups injected 15 and 31 days after previous ovulation, the percentage response to 50 I.U. being 40 and 20 respectively. (Landgrebe, 1948).

Male Xenopus has been shown to behave similarly. A large batch of negative new stock males which had never previously been injected were given 30 I.U. Twenty four hours later the positive toads were isolated and divided into groups. Five days later one group was given 20 I.U.; 10 days after the first injection, another group was given 20 I.U. and this was continued for further groups at 5 day intervals. Similar groups, used as controls, were given distilled water and remained negative throughout. The 24 hour response is recorded in Table 12.
### Table 12

<table>
<thead>
<tr>
<th>Interval after last Injection</th>
<th>Number of toads</th>
<th>Per cent Response</th>
<th>Int. Unit equivalents</th>
</tr>
</thead>
<tbody>
<tr>
<td>5 days</td>
<td>20</td>
<td>65</td>
<td>18.5</td>
</tr>
<tr>
<td>10 days</td>
<td>18</td>
<td>78</td>
<td>21.0</td>
</tr>
<tr>
<td>15 days</td>
<td>10</td>
<td>40</td>
<td>12.5</td>
</tr>
<tr>
<td>20 days</td>
<td>10</td>
<td>50</td>
<td>15.0</td>
</tr>
<tr>
<td>25 days</td>
<td>20</td>
<td>15</td>
<td>5.0</td>
</tr>
<tr>
<td>30 days</td>
<td>10</td>
<td>20</td>
<td>6.0</td>
</tr>
</tbody>
</table>

It will be seen from the table, that there is a significant decline in the sensitivity of the toad from the 15th day onwards. If the percentage response is converted to I.U.G.G. from the dose response data (Table 14), the difference between groups is accentuated. The group having the smallest percentage of positives was that injected with 20 units on day 25, and which gave a response equivalent to 5 I.U.G.G. This falling off of sensitivity in the male does not seem to have been recognised previously, and may well account for some false negative "pregnancy" tests if the interval between positive injections has not been considered. That this is a true decline in the sensitivity of testicular tissue to chorionic gonadotrophin and is not due to lack of mature sperm, is supported by the data in Table 13. This information was obtained in the following way with males from the same imported stock as those used to compile Table 14. Three consecutive injections at 10 day intervals were made in 4 groups of 10 males, each group representing a different dose level. Examinations for sperm
were made at 4 hours and 24 hours, the total positive at 24 hours being recorded.

Table 13

<table>
<thead>
<tr>
<th>Group</th>
<th>Dose Int. units</th>
<th>1st injection</th>
<th>2nd injection</th>
<th>3rd injection</th>
<th>Mean percentage response</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>30</td>
<td>70</td>
<td>70</td>
<td>100</td>
<td>80</td>
</tr>
<tr>
<td>2</td>
<td>15</td>
<td>50</td>
<td>50</td>
<td>40</td>
<td>43</td>
</tr>
<tr>
<td>3</td>
<td>10</td>
<td>40</td>
<td>40</td>
<td>30</td>
<td>33</td>
</tr>
<tr>
<td>4</td>
<td>5</td>
<td>20</td>
<td>20</td>
<td>10</td>
<td>165</td>
</tr>
</tbody>
</table>

Except for a greater response to the last injection in the 30 I.U. group the results are remarkably consistent, and lend support to the view that there is a reduction in sensitivity of the gonadal mechanism (Table 13) and that the optimum time between injections is 10 days (Table 12). Toads injected at this interval show no "exhaustion" (lack of sperm?). There are two reasons why injections should be made every ten days in preference to any other interval: firstly, males injected too frequently do not all give a "true" response when related to the 10 day figures, and secondly, groups given C.G. on and after day 15 show a diminution in the number responding positively.

Dose Response curve.

Before using male toads for quantitative estimations of C.G. it was necessary to establish the relation between dose and response. When male and female Xenopus were injected with graded doses of C.G. and the percentage response plotted arithmetically against the dose, the relation between the two variables assumed the form of a sigmoid curve. Such
<table>
<thead>
<tr>
<th>Dose Int. Units</th>
<th>Number of toads</th>
<th>Mean percentage response</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>4 hrs.</td>
</tr>
<tr>
<td>5</td>
<td>40</td>
<td>2.5</td>
</tr>
<tr>
<td>10</td>
<td>40</td>
<td>20</td>
</tr>
<tr>
<td>15</td>
<td>90</td>
<td>18</td>
</tr>
<tr>
<td>20</td>
<td>10</td>
<td>40</td>
</tr>
<tr>
<td>25</td>
<td>60</td>
<td>41</td>
</tr>
<tr>
<td>30</td>
<td>40</td>
<td>50</td>
</tr>
<tr>
<td>35</td>
<td>10</td>
<td>70</td>
</tr>
<tr>
<td>50</td>
<td>40</td>
<td>47.5</td>
</tr>
<tr>
<td>75</td>
<td>10</td>
<td>100</td>
</tr>
<tr>
<td>100</td>
<td>20</td>
<td>85</td>
</tr>
<tr>
<td>200</td>
<td>10</td>
<td>90</td>
</tr>
<tr>
<td>300</td>
<td>10</td>
<td>80</td>
</tr>
<tr>
<td>400</td>
<td>10</td>
<td>100</td>
</tr>
<tr>
<td>500</td>
<td>10</td>
<td>100</td>
</tr>
</tbody>
</table>

\[
b = \text{slope} \quad \quad \quad m = \log \text{ED} \quad \quad \quad M = \text{ED} \quad \quad \quad 95 \text{ per cent fiducial limits of } M.
\]

\[
\text{Int. Units} \quad \text{Int. Units}
\]

\[
4 \text{hrs. } 1.85 \pm 0.24 \quad 1.5730 \pm 0.21 \quad 37.4 \quad 30.9-45.3
\]

\[
24 \text{hrs. } 3.03 \pm 0.15 \quad 1.1546 \pm 0.0284 \quad 14.3 \quad 12.5-16.2
\]

Table 14.- Four and 24 Hr. Dose Response Data obtained with *Xenopus* Males.
<table>
<thead>
<tr>
<th>Dose Int. Units</th>
<th>Number of toads</th>
<th>Mean percentage response 24 hrs.</th>
</tr>
</thead>
<tbody>
<tr>
<td>15</td>
<td>20</td>
<td>25</td>
</tr>
<tr>
<td>20</td>
<td>20</td>
<td>30</td>
</tr>
<tr>
<td>25</td>
<td>40</td>
<td>40</td>
</tr>
<tr>
<td>30</td>
<td>10</td>
<td>50</td>
</tr>
<tr>
<td>35</td>
<td>10</td>
<td>60</td>
</tr>
<tr>
<td>40</td>
<td>30</td>
<td>60</td>
</tr>
<tr>
<td>45</td>
<td>20</td>
<td>70</td>
</tr>
<tr>
<td>50</td>
<td>30</td>
<td>86</td>
</tr>
<tr>
<td>70</td>
<td>20</td>
<td>95</td>
</tr>
<tr>
<td>100</td>
<td>20</td>
<td>100</td>
</tr>
</tbody>
</table>

\[ b = \text{slope} \quad m = \log \text{ED} \quad M = \text{ED} \]

| 24hrs. | 3.50±.494 | 1.4628±.0285 | 29.0 | 25.5-33.0 |

95 per cent fiducial limits of M.

Int. Units.

|  
|  
|  

Table 15 - Twenty-four Hr. Dose Response Data obtained with Xenopus Females.
data, when dose was transformed into log dose, was converted to a straight line over the useful working range of doses. The dose response data of Table 14 were obtained with males weighing 20-34 g., the percentage positive at 4 hours and 24 hours being recorded.

Some 300 individual males, from widely differing stocks, were used. There was considerable variation in the experimental history of these toads; a few had never been injected previously; the majority had at least one previous injection, and had been used for obtaining other data pertinent to this investigation. Table 4 shows that the response at 24 hours, to differing dose levels, is more constant than at 4 hours; furthermore, using a 4 hour dose response curve, instead of a 24 hour curve for quantitative estimations of C.G., results in an assay neither so accurate nor so precise. Moreover, the estimated number of I.U.C.G. needed to give a 50 per cent response, at 4 hours, is more than 2½ times that required at 24 hours. \( \chi^2 \) calculated from the 4 hour observations gives some (though not significant) evidence of heterogeneity of response of the animals. Table 15 records the 24 hour response to C.G. of Xenopus females; the animals used to obtain these results weighed 50-77 g. and had been injected many times previously.

Comparison between male and female Xenopus 24 hour responses, shows that the male is more sensitive to C.G. (Tables 14 & 15). The linear dose response relation of the male and female is not significantly different. From the
data contained in the above tables it is calculated that male toads should always give a positive result with concentrated pregnancy urine containing approximately 1,500 or more I.U. C.C. per litre, compared with 3000 or more units per litre for the female.

Specificity.

It would appear from the available data that the male toads and frogs used so far, only react positively to gonadotrophins of the pituitary, pregnant mares serum and chorionic gonadotrophin. There are two exceptions, Rana pipiens and Xenopus laevis, which react to some adrenergic drugs (Robbins and Parker, 1949, 1952). Table 16 shows response of male Xenopus to certain hormones of widely differing origin.

<table>
<thead>
<tr>
<th>Substance injected</th>
<th>Dose</th>
<th>Number of Toads</th>
<th>Toads responding positively</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ethinyl oestradiol</td>
<td>0.01 mg</td>
<td>15</td>
<td>10</td>
</tr>
<tr>
<td>Methyl testosterone</td>
<td>5.0 mg</td>
<td>17</td>
<td>3</td>
</tr>
<tr>
<td>Isopropyl nor-adrenaline sulphate</td>
<td>0.0001 mg</td>
<td>20</td>
<td>10</td>
</tr>
<tr>
<td>Adrenaline (1:1000)</td>
<td>0.001 mg</td>
<td>30</td>
<td>13</td>
</tr>
<tr>
<td>D.O.C.A.</td>
<td>1.0 mg</td>
<td>15</td>
<td>5</td>
</tr>
</tbody>
</table>

Unlike Xenopus females, male Xenopus readily respond to injections of pituitary (gland and urinary extracts), serum and chorionic gonadotrophins by releasing sperm into their urine within 24 hours. Comparative data were obtained with groups of males using the following preparations:

(1) International Standard Pregnant Mares Serum Gonadotrophin (P.M.S.).
(11) International Preparation Ox Anterior Lobe Pituitary (as a suspension in water) (A.L.P.).


(1IV) A dry extract of gonadotrophin from menopausal urine kindly supplied by Organon Limited, and designated MEN. 15/16.

### Table 17

<table>
<thead>
<tr>
<th>Dose I.U. P.M.S.</th>
<th>Number of Animals</th>
<th>Number positive</th>
<th>Percentage response</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>80</td>
<td>22</td>
<td>27</td>
</tr>
<tr>
<td>4</td>
<td>130</td>
<td>83</td>
<td>58</td>
</tr>
<tr>
<td>6</td>
<td>40</td>
<td>36</td>
<td>90</td>
</tr>
<tr>
<td>8</td>
<td>40</td>
<td>38</td>
<td>95</td>
</tr>
</tbody>
</table>

### Table 18

<table>
<thead>
<tr>
<th>Dose I.U. C.G.</th>
<th>Number of Animals</th>
<th>Number positive</th>
<th>Percentage response</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>60</td>
<td>6</td>
<td>10</td>
</tr>
<tr>
<td>10</td>
<td>60</td>
<td>14</td>
<td>23</td>
</tr>
<tr>
<td>15</td>
<td>110</td>
<td>54</td>
<td>49</td>
</tr>
<tr>
<td>20</td>
<td>30</td>
<td>21</td>
<td>70</td>
</tr>
<tr>
<td>25</td>
<td>80</td>
<td>70</td>
<td>87</td>
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<td>30</td>
<td>60</td>
<td>51</td>
<td>85</td>
</tr>
<tr>
<td>35</td>
<td>30</td>
<td>30</td>
<td>100</td>
</tr>
</tbody>
</table>

### Table 19

<table>
<thead>
<tr>
<th>Dose in mg. MEN. 15/16</th>
<th>Number of Animals</th>
<th>Number positive</th>
<th>Percentage response</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>40</td>
<td>5</td>
<td>12.5</td>
</tr>
<tr>
<td>5</td>
<td>50</td>
<td>13</td>
<td>26</td>
</tr>
<tr>
<td>7</td>
<td>60</td>
<td>33</td>
<td>55</td>
</tr>
<tr>
<td>10</td>
<td>30</td>
<td>27</td>
<td>90</td>
</tr>
</tbody>
</table>
Figure 7.
Percentage of animals showing a positive response, as measured by spermiation, 24 hours after injection of P.M.S., C.C., M.E.N. 15/16 and A.L.P. The dose required to produce a 50 per cent response is given in parenthesis.
The above tables (17,18,19 & 20) give the data from which the log dose lines in Figure (7) are drawn. In Figure (7) the dose required to produce a 50 per cent response is given in brackets.

Procedure for routine assay of C.C.

Male Xenopus in good condition, weighing between 30 and 40 g., were used. As the sensitivity of the animal is approximately inversely proportional to its weight, the animals should be either matched in groups or the dose calculated according to weight. (Table 9). The animals were examined for the presence or absence of sperm before test; any positives were rejected. This rarely occurred, but was found sometimes if the animals on a previous occasion had been given a large dose of any substance producing a positive response.

Animals were injected with the material under test (all doses made up in 4.0 ml distilled water), into the dorsal lymph sac and placed in separate jars. Four hours later, a few drops of urine were collected with a capillary pipette by slightly irritating the cloacal folds with the tip of the pipette, and the urine was placed upon a slide and.
Figure 8.
Unstained toad spermatozoa. x 700.
examined microscopically. A clean pipette was used for each animal to avoid contamination. The appearance of sperm constitutes a positive reaction (Fig. 8) (a small air bubble in the field makes focusing easier). Animals under test which were negative when examined at 4 hours were returned to their jars and re-examined 24 hours after injection. Some animals take 24 hours to respond to a suitable dose, but the 4 hour reading is useful as it sometimes saves time. A response greater than 50 per cent at this stage indicates that too large a dose has been given. A reduced dose can then be immediately administered to another group of test animals. A further advantage of the 4 hour reading is that animals are examined twice before being recorded as negative.

One great advantage of Xenopus is that it can be used repeatedly for this purpose. A 10 day interval between successive tests is most convenient, and allows adequate feeding of the toad between tests. A positive reaction always becomes negative in 10 days, provided suitable doses are used and very large ones avoided (Table 10). The use of the animal at 10 day intervals makes it possible to obtain reasonable accuracy with economy of test animals by making use of cross-over tests. For those occasions when a result is required in 24 hours, the slope of the dose response curve is assumed. Each toad from a group of 10 or 20 is injected with the same dose of the standard preparation, estimated to give about a 50 per cent response, to fix the position of the line. Each animal from a further group of 10 or 20 receives the same dose of the unknown. The result can be used to determine the potency, provided the number of animals responding is between 20 and 30 per cent. Such a rapid
assay using 20-30 animals matched by weight in each group, gives an error of about 20 per cent, which is adequate for most purposes. Further accuracy can be obtained by crossing over the groups 10 days later, and the use of similar doses reduces the percentage error to about 10 per cent.

**Tissue Extracts.**

Mole tissue was obtained from 7 patients. This was placed in acetone of analytic quality soon after removal. After complete homogenisation in fresh ice cold acetone, the tissue residue was filtered off and placed in a desiccator until dry. This material was ground up in a mortar with freshly made ice cold distilled water and was stirred for 10 minutes at 2,000 r.p.m. The residue was centrifuged off and extracted as before, the supernatant fluids from both extractions were combined, and the pH of the solution adjusted to 4.0 with glacial acetic acid. The gonadotrophin was precipitated from the supernatant fluid by the addition, with stirring, of 5 volumes of ice cold acetone and 1 volume of ice cold ether, and the precipitate allowed to develop overnight in a cold room at 5°C. The precipitate so formed was collected in a Seitz filter, washed with acetone and dried. The dry powder was ground and kept in a desiccating cabinet until required. For comparison 2 full term placentae were extracted and prepared in exactly the same way. Before assaying, the precipitates obtained were dissolved in distilled water and the dose to be injected contained in 1 ml.

Extracts were freshly made up before each assay and the potency expressed in I.U.C.G. per gram of acetone dried tissue.
The excretion of C.6. in 632 cases of normal pregnancy as estimated by the dilution test.
RESULTS.

Excretion of C.G. during pregnancy.

Semi-quantitative estimations.

Figure (9) shows the results obtained by single semi-quantitative estimations from 632 pregnancies from the 3rd to the 40th week. It will be seen that urines from some pregnancies contain enough C.G. to produce a biological response in a dilution of 1 in 100, and sometimes in a dilution of 1 in 150. Urines positive in such dilutions may appear as early as the 5th, or as late as the 14th, week after the last menstrual period. The majority occur in the 2nd and 3rd months when there is a temporary peak excretion of C.G. (see Fig. 10). It will further be seen that with the exception of the 1st month of pregnancy, positive results in a dilution of 1 in 10 are obtained right up to term, indicating that post peak values of 30,000 I.U. C.G. per litre are not exceptional.

Quantitative estimations.

Biological assays, using female Xenopus, were done on 48 twenty four hour urines collected from 6 pregnant patients. In addition assays were done on 4 first morning specimens from one patient (P9). These estimations were done between the 3rd and 17th week of pregnancy and the amount of C.G. excreted per 24 hours or per litre (P9) plotted on a logarithmic scale against weeks of pregnancy (Fig. 10). The curves of C.G. excretion for all patients have certain characteristics in common; there is a constant and steady rise in the amount of C.G. excreted until a brief peak is
Figure 10.
Quantitative estimation of C.g. excretion in 7 pregnant women, between the 3rd and 17th week of pregnancy.
reached. This occurs as early as the 45th day of pregnancy (case P4) or as late as the 70th day (cases 5 and 6).

Following the peak, a dramatic fall in C.G. concentration is observed, and between 6,000 - 16,000 I.U. per 24 hours are excreted. In the case of patient P9 only 2,390 I.U. of C.G. in 24 hours were excreted, and the following day abortion occurred. Patient P9 has a particular interest; she was suspected of having a hydatidiform mole and the biological tests showed that the excretion of C.G. was increasing rapidly. The first estimation, about the 12th week of pregnancy, was within the range of values that might be expected at the peak. A second estimation a week later showed that the concentration of C.G. had increased almost threefold from 320,000 to 880,000 I.U. per litre. A third estimation showed that the C.G. level was still rising and the urine assayed 1,225,700 I.U./litre. In view of the extreme toxaemia experienced by the patient, and the abnormally high C.G. values, the uterus was evacuated. A normal twin pregnancy was terminated.

**Excretion of C.G. by women with hydatidiform moles.**

**Semi-quantitative estimations.**

The data presented here are obtained from 105 patients with hydatidiform mole. A further 100 mole cases are not included because not enough of the clinical information was obtainable. The C.G. in I.U. per litre was estimated semi-quantitatively in 841 urines and the values obtained are compared with those collected during normal pregnancy between the 3rd and 40th week (Fig. 11). When the excretion
Figure 11.

O.G. excretion in 37 pregnancies and 35 cases of hydatidiform mole which were positive in dilutions of 1 in 100 or more.
Figure 12.
The excretion of C.G. before and after complete removal of hydatidiform mole.

Figure 13.
The excretion of C.G. in 3 cases where removal of hydatidiform mole was incomplete.
(C. Curettage, H. Hysterectomy).
of C.G. from 67 patients before the removal of the mole, obtained between the 5th and 35th week of gestation, is compared, it is found that most of them fall within the range of values obtained from normal pregnancies. The distribution of these cases of pregnancy and mole which are positive in a dilution of 1 in 100 or more is shown in Figure 11.

In only 3 pregnancies was a result positive in a dilution of 1 in 150. With two exceptions urines collected after the 12th week of pregnancy were positive only in a dilution of 1 in 10. The exceptions were urines from 2 multiple pregnancies giving a positive response in dilutions of 1 in 100 in the 17th and 20th weeks. Figures 12 and 13 illustrate, in the immediate post partum period, the use of dilution tests in estimating C.G. levels in urine. The excretion pattern as exemplified by those patients in Figure 12 is typical of the uncomplicated case. The urines from 43 patients were negative within 1 month after the removal of the mole. The earliest date at which a test was found to be negative was 2 days after removal of the mole. It was impossible to estimate how soon these patients stopped excreting C.G. as urines were sent in at varying times after the evacuation of the mole.

In those patients where some chorionic tissue is retained, there is a continued excretion of gonadotrophin until the tissue is removed or regresses spontaneously. Figure 13 depicts urinary C.G. levels after incomplete removal of the mole. These patients excreted C.G. for a considerable time, and in case 83, 2 temporary negative phases occurred. This emphasises the need for doing more than 1 follow up test because a single negative result may be misleading.
Figure 15.
Quantitative estimation of C.G. in a patient with retained chronic tissue.
Figure 15.
Semi-quantitative estimations of C. C., excreted a woman with hydatidiform mole in situ, compared with that excreted in 2 normal pregnancies.
When the C.G. titre falls below 5,000 I.U. per litre, and it is necessary to know the concentration in the urine, bio-assays are done. Figure 14 shows the results obtained in this way; Hogben tests were also done on each specimen. With the exception of urine collected on day 71, which contained 2,850 I.U.C.G. per litre, all specimens were negative.

Hobson (1952) has pointed out the difficulties of collecting data, over a period, about the excretion of C.G. with the mole in situ. In the present investigation, where the ante-partum excretion of C.G. in 67 patients was estimated, it was not possible to obtain more than 2 or 3 specimens of urine from each patient before the mole aborted or was removed. The following case is of particular interest because semi-quantitative estimations were made on 10 specimens of urine collected between the 17th and 22nd weeks after the L.M.P. and before the removal of the mole. These results are compared with those obtained from 2 normal pregnancies of the same duration. (Figure 15).

It has been made quite clear that estimations on a single specimen of urine are usually of little use in predicting the presence of a mole unless the test is positive in high dilutions (1 in 200 or more). In the case illustrated (Figure 15) a confident diagnosis of hydatidiform mole was made as a result of the first specimen being positive in a dilution of 1 in 300. However, had the first test been positive with undiluted concentrate only, as was the second specimen, it might have been thought that this was a normal pregnancy. A third specimen collected 3 days later contained not less than 450,000 I.U.C.G. per litre, and with the
Figure 16.
Ante and post partum quantitative estimations of C.G. excreted by 7 women with hydatidiform moles, and 2 women with normal pregnancies.
Figure 17.
The rise of C₃G₂ excretion during normal pregnancy compared with that excreted in the presence of a hydatidiform mole.
exception of the urine obtained at 16½ weeks of pregnancy the amounts of C.G. excreted remained at a pathological level until the removal of the mole. After the expulsion of the mole daily estimations were done, and the first negative test was obtained 6 days post partum; subsequent tests have remained negative.

**Quantitative estimations.**

Quantitative estimations were done on 88 urines from 7 patients with a history of hydatidiform mole, and 10 urines from 2 pregnancies. Approximate values were first determined from the results given by dilution tests, and the final values obtained with groups of male Xenopus. Specimens from each case were collected at intervals of not more than 3 days and the cases were followed until the amount of C.G. fell below 300 I.U. per litre of urine. The results of these assays are shown in Figure 16.

Removal of the mole or placenta results in a sudden fall in the urinary level of C.G., all cases having a similar post partum excretion pattern. Negative Hogben tests (3,000 I.U./litre) were obtained with the urines of all cases by the 10th day post partum, and, with the exception of case 1 remained so. Case 1 is of particular interest because a second mole, the presence of which was detected by the dilution test, was obtained 24 days after the removal of the first. Excretion of C.G. during the growth of this mole is shown in detail (Figure 17) where it is compared with a case of normal pregnancy (P3). The C.G. titre in case 1
<table>
<thead>
<tr>
<th>Case</th>
<th>Tissue</th>
<th>Gestation</th>
<th>I.U.C.G. per gram</th>
<th>I.U.C.G. per litre of urine.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Ante partum</td>
</tr>
<tr>
<td>205</td>
<td>Moles</td>
<td>24 weeks</td>
<td>32,100</td>
<td>600,090(12)</td>
</tr>
<tr>
<td>29</td>
<td>&quot;</td>
<td>15 &quot;</td>
<td>20,034</td>
<td>34,000(1)</td>
</tr>
<tr>
<td>40</td>
<td>&quot;</td>
<td>24 &quot;</td>
<td>14,300</td>
<td>87,500(1)</td>
</tr>
<tr>
<td>3</td>
<td>&quot;</td>
<td>11 &quot;</td>
<td>3,750</td>
<td>30,000(3)</td>
</tr>
<tr>
<td>41</td>
<td>&quot;</td>
<td>23 &quot;</td>
<td>2,710</td>
<td>40,000(1)</td>
</tr>
<tr>
<td>2</td>
<td>&quot;</td>
<td>17 &quot;</td>
<td>2,452</td>
<td>2,550,000(1)</td>
</tr>
<tr>
<td>6</td>
<td>&quot;</td>
<td>18 &quot;</td>
<td>1,550</td>
<td>9,050(4)</td>
</tr>
<tr>
<td>F2</td>
<td>Placenta</td>
<td>Full term</td>
<td>136</td>
<td>2,350(1)</td>
</tr>
<tr>
<td>F1</td>
<td>&quot;</td>
<td>&quot;</td>
<td>106</td>
<td>10,000(1)</td>
</tr>
</tbody>
</table>

Table 21: C.G. Concentration per gram of tissue in 2 normal placentae and 7 hydatidiform moles. (Figures in parenthesis refer to day on which estimations were made).
rose from 2,125 - 65,000 IU/litre in 13 days before its removal, and from 4,170 - 72,000 IU/litre in 48 days during pregnancy, (P3).

Tissue extracts.

Table 21 shows the concentration of C.G. in moles and placentae. Results of assays are expressed in I.U. per gram of dry tissue as only part of the mole was available.

There is from 14 to 300 times more C.G. per gram of tissue in the moles than in the placentae. However, the 2 placentae were "full term" and are not strictly comparable with the 7 moles obtained between the 11th and 24th weeks of gestation. The concentration of C.G. per gram wet weight of placental tissue remains fairly constant from the 20th week until term. Concentrations during the second month of pregnancy are approximately 100 times greater than those found at full term. (Dicafaluay, 1953). Although ante and post partum levels of C.G. were estimated, there is little correlation between the amount of C.G. per gram and per litre. This was expected, because if there is a direct relationship between the amount of C.G. in the tissue and that found in the urine, then it will only be discovered when moles are assayed in their entirety and the amount of C.G. contained in them compared with that circulating in the blood and the amount excreted during a period of not less than 24 hours prior to the evacuation of the mole.

Urinary excretion of C.G. by women with chorionepithelioma.

The generic term "chorionepithelioma" is applied rather loosely to two fairly distinct conditions. In this
<table>
<thead>
<tr>
<th>Patient</th>
<th>Previous Pregnancy</th>
<th>Urines Tested Pre</th>
<th>Post</th>
<th>I, U. C. G. per Litre</th>
<th>Test</th>
<th>Survived after Hysterectomy</th>
</tr>
</thead>
<tbody>
<tr>
<td>CC 1</td>
<td>Normal Delivery</td>
<td>2 0</td>
<td></td>
<td>&gt;30,000 &lt;300,000</td>
<td>No hysterection</td>
<td>Not known</td>
</tr>
<tr>
<td>CC 2</td>
<td></td>
<td>2 8</td>
<td></td>
<td>&gt;300,000 &lt;600,000</td>
<td>&gt;30,000 &lt;300,000</td>
<td>12 weeks</td>
</tr>
<tr>
<td>CC 3</td>
<td></td>
<td>1 0</td>
<td></td>
<td>3,173,000</td>
<td></td>
<td>1 day</td>
</tr>
<tr>
<td>CC 4</td>
<td>Miscarriage</td>
<td>0 7</td>
<td></td>
<td>No test</td>
<td>1,987</td>
<td>No information</td>
</tr>
<tr>
<td>CC 5</td>
<td></td>
<td>0 2</td>
<td></td>
<td>No test</td>
<td>&gt;30,000 &lt;45,000</td>
<td>8 weeks</td>
</tr>
<tr>
<td>CC 6</td>
<td>Ectopic</td>
<td>0 18</td>
<td></td>
<td>No test</td>
<td>1,330,000</td>
<td>All positive</td>
</tr>
<tr>
<td>CC 7</td>
<td>Hydatidiform mole</td>
<td>0 5</td>
<td></td>
<td>No test</td>
<td>&gt;30,000 &lt;300,000</td>
<td>5 weeks</td>
</tr>
<tr>
<td>CC 8</td>
<td></td>
<td>1 7</td>
<td></td>
<td>2,150</td>
<td>&gt;30,000 &lt;300,000</td>
<td>27 weeks</td>
</tr>
<tr>
<td>CC 9</td>
<td></td>
<td>0 5</td>
<td></td>
<td>No test</td>
<td>1,150,000</td>
<td>3 weeks</td>
</tr>
<tr>
<td>CC 10</td>
<td></td>
<td>5 1</td>
<td></td>
<td>&gt;3,000 &lt;30,000</td>
<td>&gt;30,000 &lt;300,000</td>
<td>7 weeks</td>
</tr>
</tbody>
</table>

Table 22 Levels of C.G. excreted by 10 patients with Choriocarcinoma.
investigation the distinction has been made between choriodenoma destruens (="malignant or invasive mole") and
choriocarcinoma. All cases diagnosed by the clinician as
"chorionepithelioma" have been treated as a separate group.
The urine from the 26 women who made up the three groups
was tested for C.G. Most of the 148 specimens, 40 collected
before treatment and 108 after treatment, were assayed semi
quantitatively. The results of the biological tests, and
certain other information, for all patients are given in the
following tables. (Tables 22, 23, and 24).

Group (1) choriocarcinoma.

The 10 women in this group were between 21 and 49
years of age. All except 1 patient (CC1) were hysterectomised
Metastases were present in all patients. In 2 patients
cystic ovaries were noted, the ovaries of 5 patients appeared
normal, and no information was available about ovarian
hypertrophy in the remainder. Eight of the patients are now
dead, no information is to be had about the 9th patient
(CC1). The condition of patient CC2 is deteriorating,
although she is still alive (May 1955). Secondary shadows
are reappearing in the chest and she is losing weight.

It is unfortunate that such a small number of
estimations for C.G. were done in this group before
hysterectomy, as it would have been useful to compare the
amounts excreted before and after hysterectomy. Apart from
patient CC1 who died 7 months after normal delivery, patient
CC2 who is living and whose Hogben test is at present
negative, patient CC3 who died the day following hysterectomy
and patient CC4 about whom no information is available, the
post hysterectomy values of C.G. excretion are extremely
Figure 18.
The quantitative estimation of C.C. excreted by a woman with choriocarcinoma. ■■■ areas indicate X-ray therapy.
high. If these results are compared with the post partum excretion of C.G. from those patients who had hydatidiform moles (Figure 16) it will be seen that the patients with choriocarcinoma excreted much larger amounts. The amount of C.G. per litre of urine in each case remained high until the death of the patient, except for a temporary fall after hysterectomy (CC9) or as the possible result of radiotherapy (patients CC4 and CC8).

Some information was obtained about the excretion of C.G. during X Ray therapy. Twenty five urines, 18 from patient CC6, and 7 from patient CC8, were obtained during treatment and assayed. Figure 18 shows the excretion of C.G. in I.U. per litre. Twenty four hour specimens were not obtainable before, during and after treatment.

The first impression is that the X Ray treatment was successful in arresting the growth of the metastases with a consequent drop in the amount of C.G. excreted. However, within 3 weeks after the start of the treatment the C.G. level in the urine was above the pre-treatment value. The highest value obtained was on the 141st day after hysterectomy and this was followed by a sharp fall in the amount of C.G. excreted. The excretion of C.G. continued to rise during the next and last course of treatment until the pre-treatment level was once more passed. This patient died 7 months after hysterectomy. The second patient CC8, developed a choriocarcinoma of the uterus one year after the abortion of a hydatidiform mole. Follow up assays done after the expulsion of the mole and until hysterectomy a year later, showed a fairly constant urinary level of C.G. between 1,600 and 3,200 I.U./litre.
<table>
<thead>
<tr>
<th>Patient</th>
<th>Previous Pregnancy</th>
<th>Urines Tested</th>
<th>I. U. C. G. per Litre</th>
<th>Test Negative</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Pre</td>
<td>Post</td>
<td>Maximum pre Hysterectomy value</td>
</tr>
<tr>
<td>G A 1</td>
<td>Missed Abortion</td>
<td>0</td>
<td>3</td>
<td>No test</td>
</tr>
<tr>
<td>G A 2</td>
<td>Hydatidiform</td>
<td>0</td>
<td>16</td>
<td>No test</td>
</tr>
<tr>
<td>G A 3</td>
<td></td>
<td>4</td>
<td>0</td>
<td>&gt; 27,000</td>
</tr>
<tr>
<td>G A 4</td>
<td></td>
<td>1</td>
<td>1</td>
<td>&gt; 3,000</td>
</tr>
<tr>
<td>G A 5</td>
<td></td>
<td>4</td>
<td>5</td>
<td>&gt; 30,000</td>
</tr>
<tr>
<td>G A 6</td>
<td></td>
<td>11</td>
<td>5</td>
<td>&gt; 1,200,000</td>
</tr>
</tbody>
</table>

Table 23: Levels of C.G. excreted by 6 patients with Chorio-adenoma-destructans.
The patient was free from metastases at this time. Three
months after hysterectomy the patient was excreting between
3,000 and 6,000 I.U. C.G./litre. This was followed by a
sudden rise 4 weeks later to between 15,000 and 30,000 I.U./
litre. The X Ray showed that multiple secondary deposits
were present in the lung. Deep therapy was given during
the next three months and the Hogben test was negative 27
weeks after hysterectomy. At the same time the chest
secondaries were reported to have cleared. A test one month
later was positive with undiluted urine, though negative in a
dilution of 1 in 10. For the next month the amount of C.G.
excreted fluctuated between 5,330 and 10,000 I.U./litre, and
rose in the next 4 weeks to 30,000 I.U./litre. An X Ray of
the chest showed that the secondaries had reappeared.

Following a second course of treatment the amount of C.G.
excreted dropped to between 3,000 and 6,000 I.U./litre. The
patient died 12 months after hysterectomy and secondary
deposits were found in the brain, lungs, kidneys and spleen.
In both patients the level of C.G. excreted in the urine was
temporarily depressed as a result of the X Ray therapy.

Group (2) Chorio-adenoma destruens.

The 6 women in this group were between 22 and 45
years of age. All were hysterectomised, and are free from
metastases. Cystic ovaries were noted in 1 patient, not
present in 2 patients; no information is available about the
ovarian condition of the other 3 patients. Five patients in
this group are known to be living, no information is to be had
for the 6th (2A3).
<table>
<thead>
<tr>
<th>Patient</th>
<th>Previous Pregnancy</th>
<th>Urines Tested</th>
<th>I. U. C. G. per Litre</th>
<th>Test Negative</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Pre Operative</td>
<td>Maximum pre Hysterectomy value</td>
<td>Maximum post Hysterectomy value</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Post Operative</td>
<td>No test</td>
<td>&gt;30,000 &lt;300,000</td>
</tr>
<tr>
<td>C E 1</td>
<td>Normal Delivery</td>
<td>0</td>
<td>1</td>
<td>No test</td>
</tr>
<tr>
<td>C E 2</td>
<td>&quot;</td>
<td>1</td>
<td>3</td>
<td>&gt;3,000 &lt;30,000</td>
</tr>
<tr>
<td>C E 3</td>
<td>Miscarriage</td>
<td>1</td>
<td>1</td>
<td>Hogben Negative</td>
</tr>
<tr>
<td>C E 4</td>
<td>&quot;</td>
<td>0</td>
<td>6</td>
<td>No test</td>
</tr>
<tr>
<td>C E 5</td>
<td>&quot;</td>
<td>0</td>
<td>2</td>
<td>No test</td>
</tr>
<tr>
<td>C E 6</td>
<td>&quot;</td>
<td>1</td>
<td>2</td>
<td>Hogben Positive (No dils)</td>
</tr>
<tr>
<td>C E 7</td>
<td>&quot;</td>
<td>1</td>
<td>2</td>
<td>&gt;3,000 &lt;30,000</td>
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<tr>
<td>C E 8</td>
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<td>0</td>
<td>2</td>
<td>No test</td>
</tr>
<tr>
<td>C E 9</td>
<td>Hydatidiform Mole</td>
<td>1</td>
<td>4</td>
<td>&gt;3,000 &lt;30,000</td>
</tr>
<tr>
<td>C E 10</td>
<td>&quot;</td>
<td>0</td>
<td>4</td>
<td>1,500,000 &lt;1,800,000</td>
</tr>
</tbody>
</table>

Table 24. Levels of C.G. excreted by 10 patients with "Chorionepithelioma."
In this group post operative convalescence was not complicated by secondary growths or by the excretion of excessive amounts of C.G. The pattern of C.G. excretion is similar to that of patients who have had hydatidiform moles completely evacuated; all biological tests, with one exception, becoming negative 2 - 31 days after hysterecctomy. The apparent exception is case 641, the first negative test being obtained 7 weeks after hysterecctomy. This is probably due to the fact that this patient was followed up only at monthly intervals. These women, like those patients with hydatidiform moles, and unlike those with a choriocarcinoma, recovered and no deaths have so far occurred.

**Group (3) choriocarcinoma.**

The 10 patients in this group were between 20 and 43 years of age. All were hysterectomised, and patients 652, 3, 5, 7 and 9 had metastases. Cystic ovaries were present in 4 patients, absent in 2; no information was to be had about the remainder. Nothing is known about the survival of these patients as they were only followed for a short time post operatively. Like the two previous groups, more information is available about the excretion of C.G. in the post operative period. The post hysterecctomy values for the excretion of C.G. were often greater than 30,000 and less than 300,000 I.U. per litre. Such titres are considerably higher than those obtained with the urines from patients in the chorio-adenoma group. The maximum post hysterecctomy value in this group, was obtained with a urine collected 26 days before the evacuation of a hydatidiform mole, and approaches that obtained with urines from patient 3 in the choriocarcinoma group.
<table>
<thead>
<tr>
<th>Type of Tumour</th>
<th>I. U. C. G. per litre.</th>
<th>Negative</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>&gt; 3,000</td>
<td>&gt; 300,000</td>
</tr>
<tr>
<td></td>
<td>&lt; 30,000</td>
<td>&lt; 300,000</td>
</tr>
<tr>
<td>Choriocarcinoma</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>Mixed</td>
<td></td>
<td>1</td>
</tr>
<tr>
<td>Seminoma</td>
<td>5</td>
<td>1</td>
</tr>
<tr>
<td>Teratoma</td>
<td>9</td>
<td>3</td>
</tr>
<tr>
<td>Total</td>
<td>15</td>
<td>5</td>
</tr>
</tbody>
</table>

Table 25. Semi-quantitative estimations of C.G. excreted by 102 males with testicular neoplasms.
The excretion of C.C. by males with testicular neoplasms.

The urines from 210 males, suspected of having testicular tumours, were examined for the presence of chorionic gonadotrophin. The dilution test using female Xenopus was done, and unless otherwise mentioned these estimations were all pre-operative. In the series, 102 males had miscellaneous testicular conditions of a non neoplastic type; the biological test for all these urines was negative. The results obtained with the remaining 108 cases are shown in Table (25).

It will be seen that in only 25 cases was a positive biological result obtained. This is not surprising when nearly 50 per cent of the tumours were seminomas. The fact that any positive results should have been obtained with urines from the group classified on histological grounds as seminoma, casts doubt upon the accuracy of the pathological diagnosis in my opinion. (See page 110 of discussion for an elaboration of this statement.) In this group the two males who excreted between 300,000 and 4,500,000 I.U.C.C./litre are known to have died from chest and abdominal metastases.

In the teratoma group C.C. was excreted in amounts varying from 3,000 - 9,000 IU/litre. A certain amount of clinical and biological data were obtained about 4 of the patients excreting large quantities of C.C. Post operative specimens of urine from 3 of these males contained between 30,000 and 300,000 I.U.C.C./litre, and at the time of estimation 2 of them had secondaries in the lungs and para aortic glands; the 3rd had secondary deposits in the lungs.
and cerebellum. More details are available about the 4th patient and a brief case history is given below.

21.5.52. Right testis removed, pathological report - teratoma, no metastasis.

14.8.52. Urine contained more than 3,000, less than 30,000 I.U.C.G./litre.

4.12.52. Urine contained more than 3,000, less than 30,000 I.U.C.G./litre.

20.5.53. Urine contained more than 900,000, less than 1,000,000 I.U.C.G./litre.

25.5.53. Patient dead. Lung secondaries.

The diagnosis for all 4 patients was malignant teratoma.

The "mixed" tumour group contained 6 males with tumours made up of seminoma plus teratoma and 1 male with seminoma plus choriocarcinoma. With the exception of 1 male with a seminoma plus teratoma, all the biological tests were negative. It should be mentioned that in the case of the male with seminoma plus choriocarcinoma the only estimation was one done 17 days post operatively.

Of the 4 males (A15, G15, C15, and F18) for whom a diagnosis of choriocarcinoma was made, 3 are dead. No information is available about the 4th (F18) and the only estimation done 17 days post operatively was negative.

The first estimation for patient (A15) was done immediately after operation and was negative. No chest secondaries were reported one month after removal of the left testis, the site of the choriocarcinoma. A second estimation made 11 weeks post operatively showed that between 3,000 and 30,000 I.U.C.G./litre of urine was being excreted.
No further tests were done and the patient died 3 months later with extensive chest secondaries. Only a single estimation was done for patient (015) and this patient excreted more than 300,000 and less than 450,000 I.U.C.G./litre. When this estimation was done he had metastases in lungs and abdomen.

The 4th patient (09) in this group was provisionally diagnosed as "semionoma with pulmonary metastases." A 24 hour specimen of urine was sent in for estimation of pituitary gonadotrophin. However, as "true" seminomas seldom present pulmonary metastases it was decided to treat this as a possible choriocarcinoma and the urine was assayed for C.G.

1.4.54. 27,640 I.U.C.G./per 24 hours. Following this report orchidectomy was carried out.

15.4.54. The pathological report was "chorioncarcinoma."

15.4.54. Urine assayed 7,400 I.U.C.G./litre — chest secondaries worse.

19.4.54. 12 days post operative urine assayed 1,45,000 I.U.C.G./litre.

20.4.54. — 26.4.54. 6 doses of radiotherapy were given in an effort to reduce chest metastases.

27.4.54. Urine assayed between 300,000 and 450,000 I.U.C.G./litre.

General condition much worse and patient discharged.

3.5.54. Patient died.

Of the 25 patients who excreted C.G., 10 are known to be dead. Attempts to follow up the remaining 15 patients have been unsuccessful.
DISCUSSION.

When the A.Z. and Hogben tests for pregnancy diagnosis are done under the conditions described here, they are correct in 99 out of every 100 tests. Other authors using female Xenopus have independently confirmed the high degree of accuracy that can be obtained. Weismen and Coates (1944) using female Xenopus over a period of 5 years correctly diagnosed 99 per cent of very early pregnancies. Schwalbacher (1951) analyzed the results of 6,443 tests and estimated that the Hogben test was 99.48 per cent correct.

Spontaneous egg laying will sometimes occur if female Xenopus are stimulated by the male, or kept under natural conditions. False positive reactions due to such causes can be completely avoided by the separation of male stock from female stock and by standardising methods of husbandry. Injections of concentrates from menopausal urines do not normally provoke egg laying in the female. Gonadotrophins having a follicle stimulating effect on the mammalian ovary have to be injected in large quantities before Xenopus lay eggs. For example P.M.S. which is mostly, if not entirely follicle stimulating will produce spermatiation when 3-4 I.U. per male toad are injected. This dose has to be increased to about 900 I.U. per toad to cause oviposition in 50 percent of females. The same is true of preparations made from menopausal urines, when these are injected into the female toad in doses 15-20 times greater than that required to produce a MRD 50 in the male toad, or to double the weight of the immature mouse uterus compared with the control, they have no effect. However, female Xenopus can be made to lay
eggs after injections with exceedingly large amounts of steroids derived from the ovary and adrenal cortex. This is claimed to be a direct effect upon the ovary and is not mediated via the toads' own pituitary. Shapiro and Zwarenstein (1937) and Burgers and Zwarenstein (1955) observed ovulation by excised and isolated ovaries when they were placed in saline containing steroid material. Although these and similar steroids are produced and excreted in large amounts during pregnancy the quantity contained in 30 ml of pregnancy urine would not be sufficient to cause ovulation, and for all practical purposes the toad can be regarded as reacting specifically to C.G. It may be argued, however, that although the presence of steroids or their metabolites in pregnancy urine are of no consequence when a qualitative result is required, they may considerably affect the results of quantitative assays. I have eliminated this possibility by injecting extracts of urine from which C.G. has been precipitated by alcohol and ether, and from which the steroid hormones are consequently removed.

Apart from the lack of false positives, the superiority of the Hogben test, over methods which employ mammals are used, is due to the speed with which a positive result can be obtained. Depending upon the concentration of C.G. in specimens tested a result may be obtained within 6 hours of an injection or in most cases within 12 hours. The toad can also be used repeatedly and the animal is not killed in order to observe the response. The only advantage that can be claimed for the A.Z. test is that unconcentrated urine can be used. This is not without its disadvantages as some mice die during the test due to the toxicity of the specimen. Unlike the female toad, immature mice
occasionally give false positive reactions after injections with urines rich in pituitary gonadotrophin (s). This may be due to the presence of some L.H. in the injected urine, or because the pituitary of the mouse has been stimulated to produce its gonadotrophin. Haemorrhagic follicles are occasionally found in the ovaries of uninjected 3 week old mice, and false positive reactions can be due to the spontaneous maturation of the immature mouse ovary.

In most mammalian assay methods for C.G., the test animal is killed in order to observe or measure the biological reaction. These methods may be qualitative; the amount of C.G. present may be judged by the formation of the corpora lutea in the ovary of the immature rat (Smith and Smith, 1934) or mouse (Hamburger and Federsen-Bjergaard, 1937) or hyperaemia of the immature rat ovary (Albert and Berkson, 1951). When a quantitative end point is used, organs such as ovaries, uteri, seminal vesicles or prostate glands have to be dissected out and weighed. These methods prohibit the use of a cross over technique and therefore large numbers of animals have to be employed. Mammalian bioassays like mammalian pregnancy tests have the disadvantage that 4-5 days must elapse before a result is obtained. Unless pilot assays are first done, the injection of preparations of unknown potency may cause minimum or maximum responses in the test animals. If this happens either the assay must be repeated or the investigator must be content with a rough approximation of potency that could have been obtained with less elaborate methods. When the results of assays are required for clinical purposes, e.g. the patient is suspected of having a hydatidiform mole or chorionepithelioma, then the time taken to do any assay is of importance.
A further disadvantage of mammals as test animals is that it is not possible to test the assumption that the same group of animals would give the same response when repeatedly injected with a standard dose. Because test animals are killed it is not possible to select groups according to their sensitivity to injected C.G. before being used for assay purposes. Some authors recognise that there is a seasonal variation in response to C.G. amongst their test animals, (Hamburger, 1950, Lorraine, 1950, and Diencalusy, 1953). Hamburger (1950) has pointed out that "statements such as the minimal limits of error to be expected from an assay" have no absolute validity; even within the same strain of test animals the "limits of error" may change in the course of months or years." Such variation is an additional reason for employing the I.S.P. and expressing the results of assays in I.U. and not in "rat or mouse units."

The results obtained with male and female Xenopus show that this amphibian can be employed with advantage for the assay of C.G. in urine or tissue. The outstanding features of Xenopus as test animals are that they can be used, if required, every 10 days. Maximum or near maximum responses can be avoided by examining the urine of male Xenopus for sperm 4 hours after injection. A group response of 50 per cent or more at this time means that too large a dose has been injected. This is corrected by injecting lower doses into new test groups, and the time elapsing before the result of the assay is known is only prolonged for a further 4 hours. The same is true of the female; examination of the groups at intervals during the 24-hour period after
injection, will indicate at once whether too low or too high doses have been used. Not only can the sensitivity of the same group be checked at intervals with a standard preparation, but by marking each toad in the group the response of an individual can also be recorded. Furthermore, because the toad is not killed at the end of an assay, it is possible to test the response of the same group to the I.S.P. of C.G. and the unknown preparation. This is done by injecting groups with the I.S.P. and 10 days later injecting the same groups with the unknown.

Chorionic gonadotrophin has been shown to be excreted in increasing amounts by the pregnant woman until a peak is usually reached during the 3rd month of pregnancy. This peak excretion is followed by an exceedingly rapid fall in excreted C.G. and for the rest of pregnancy the level of excretion is fairly constant. Lorainè's (1950) mean of 95 estimations made after the peak was 7,400 I.U. per 24 hours, with a normal range of approximately 4,000 - 11,000 I.U. per 24 hours. Smith, Albert and Randall (1951), obtained values between 2,000 - 15,000 I.U. per 24 hour specimen, from the 90th day onward. In this investigation the results of the semi-quantitative and quantitative estimations show that peak values may be encountered as early as the 45th day of pregnancy (case P4) or as late as the 70th day (cases P5 and P6). C.G. excretion per 24 hours at the peak period range from 42,000 - 330,000 I.U. per 24 hours. Occasionally 450,000 I.U. per litre was recorded by the semi-quantitative method (Figure 11). The values obtained at the peak by different authors who quantitatively assayed C.G. in urine or serum,
<table>
<thead>
<tr>
<th>Authors</th>
<th>Number of women investigated</th>
<th>Range of C.G. excretion at the peak.</th>
<th>Mean Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hobson (1955)</td>
<td>7</td>
<td>42,000–330,000</td>
<td>151,000</td>
</tr>
<tr>
<td>Loraine (1950)</td>
<td>5</td>
<td>25,000–35,000</td>
<td>30,000</td>
</tr>
<tr>
<td>Jones, Delfs &amp; Stran (1944)</td>
<td>24</td>
<td>24,000–600,000</td>
<td>150,000</td>
</tr>
<tr>
<td>Smith, Albert, &amp; Randall (1951)</td>
<td>6</td>
<td>50,000–450,000</td>
<td>250,000</td>
</tr>
</tbody>
</table>

Note: Twin Pregnancy (P9) not included.

Table 26. Ranges of values for C.G. in urine and serum obtained during the peak.
and expressed their results in I.U. is shown in Table 26.

G.G. may be excreted in vary large amounts in multiple pregnancies, as well as in hydatidiform mole and choriocarcinoma. The G.G. values for patient (P9) who had a twin pregnancy were unusually high although the placentae showed no hydatid change. Although the amounts of G.G. excreted are very rarely as high as this in normal pregnancy, this case emphasises the extreme importance of considering the biological result together the clinical findings.

The results obtained show that a single dilution test as a means of distinguishing between a normal pregnancy and a mole is of little value. It has been suggested that positive reactions from highly diluted urines obtained after the peak excretion of gonadotrophin in normal pregnancy might be more reliable. (Hobson, 1954). Since this work was done urines from 2 multiple pregnancies have given positive reactions in dilutions of 1 in 100, and one in a dilution of 1 in 400 (Case P9, Figure 10); these tests were done in the 14th, 17th and 20th weeks of pregnancy. Hamburger (1944) has reported a single case of normal pregnancy in which 900,000 I.U. per litre was excreted. Values of the order of 450,000 I.U.C.G. per 24 hours are only occasionally reported. (Smith, Albert and Randall, 1951). Urines from 3 cases described here were positive in dilutions of 1 in 150 but negative in 1 in 200 i.e. they contained between 450,000 and 600,000 I.U.C.G. per litre. It would seem therefore that a urine, particularly if collected after the 14th week of pregnancy, which gives a positive reaction in a dilution of 1 in 200, indicates the
presence of a mole. It should, however, be borne in mind that urine from some multiple pregnancies may excrete C.G. in quantities sufficiently large to produce positive reactions when injected in dilutions of 1 in 400. Tests positive in a dilution of 1 in 10 are of no value because the urines from a small number of pregnancies are positive in such dilutions right up to term. Weakly positive and even negative biological reactions can be obtained with urine concentrates from mole cases. In the present series the urine from 3 patients with moles in situ gave repeated negative biological reactions. This may have been due to the fact that only small amounts of actively secreting tissue were present, or possibly because the C.G. was in some way inactivated. A negative result does not exclude the presence of mole tissue, just as repeatedly negative results are occasionally encountered with perfectly normal pregnancies (Hobson, 1954). Caution must be exercised when interpreting the results of dilution tests. They provide information to be considered together with the clinical data.

The impression that a urine which gives a positive result in dilutions of 1 in 100 or even 1 in 150 indicates the presence of a mole has led to the termination of several pregnancies. Dilution tests are most useful in detecting the presence of some retained tissue after the evacuation of the major part of the mole. I have found that it is not unusual for urines from post molar cases to give positive when responses even there is no chorionic tissue in curettings. Because of this some clinicians assume that the test has given a "false positive" result. Complete evacuation of the tissue producing the gonadotrophin must result in a drop in the circulation and excretion of C.G., and prolonged excretion
of C.G. can only be due to retained chorionic tissue.
Negative biological tests are obtained within 7 to 96 hours after delivery of the placenta (Crew, 1936). The excretion of C.G. after its injection into non-pregnant women, normal males and males with hypogonadism ceases between $3\frac{1}{2}$ to 7 days after injection (Lloyd, Hughes, Eva and Lobotsky, 1949; Leach, Tokuyama and Maddock, 1954). Really accurate information on the excretion of C.G. can only be obtained when tests are done, on a large series of patients, at intervals of not less than 3 days following the removal of the mole. This can only be achieved by the closest cooperation between the clinician and the laboratory. One molar case (Figure 12) is of particular interest because bleeding first occurred during the 9th week of pregnancy, a time at which the allantois and trophoblast fuse to form the chorion. Ewart (1917) and Hartig (1950) have pointed out that this is the most common time at which pathological foetuses abort. The chorions of these defective foetuses show hydatid swelling and constitute the early phase of hydatidiform mole. In view of these observations it might be advisable to follow up carefully cases of pregnancy in which bleeding occurs at this time. Whilst the majority of such pregnancies will abort, or recover and go to term, those pregnancies which eventually become hydatidiform moles, might be diagnosed earlier if dilution tests were used.

This work has shown that every patient with a mole should have monthly tests for 6 months after evacuation of the mole. The continued excretion of C.G. in the absence of a new pregnancy means either that there is a secondary growth or retained chorionic tissue. One in every 40 molar pregnancies is said to become a chorionepithelioma compared with 1 in
160,000 pregnancies (Hertig, 1950). The regular use of dilution tests after the removal of a mole is advocated because in the majority of cases these tests will detect the presence of metastases before the condition is clinically evident.

Chorionepithelioma was described by Sanger (1889) as a sarcoma of the uterus; he believed that it originated from decidual cells, and used the term "deciduoma malignum" to describe this type of tumour. The correct origin of deciduoma malignum was recognised by Marchand (1895) as being derived from chorionic tissue, and he renamed it chorionepithelioma. Ewing (1910) suggested that a distinction should be made between the two types of tumour included under the heading of chorionepithelioma, and proposed the terms chorio-adenoma-destruens and choriocarcinoma.

Chorio-adenoma or invasive mole is seldom if ever fatal and metastases are comparatively rare. When metastases of chorio-adenoma do occur they are usually found in the pelvic region, and occasionally in the lung. Choriocarcinoma is a highly malignant tumour which produces widespread and fatal metastases.

The possible development of a chorionepithelioma, a term which should be restricted to the fatal tumour originally described by Marchand, from any previous or of pregnancy is well recognised, although different authors disagree in their estimates of the incidence of the malignant change. Hertig (1950), from data collected on 200 cases of hydatidiform mole, estimated that the true chorionepithelioma arises from the various types of pregnancy in the following ratios:
1:40 hydatidiform moles, 1:15,333 ectopic pregnancies, 1:15,326 abortions and 1:160,000 normal pregnancies and concluded that the more abnormal the pregnancy the greater the likelihood of a chorionepithelioma developing.

Biological tests for C.G. can be a useful diagnostic aid in cases of hydatidiform mole and chorionepithelioma. These tests do not distinguish before hysterectomy between chorio-adenoma and choriocarcinoma. Positive post operative tests are often the first indication that metastases are present, and by this time the disease is well advanced. Unless the distinction is made between chorio-adenoma and choriocarcinoma there will be added to the literature further claims that patients with choriocarcinoma have been cured. In this investigation such a distinction has been made and where the diagnosis was not clearly defined by the clinician a separate group of patients have been included under the heading "chorionepithelioma."

In the choriocarcinoma group 8 deaths have so far occurred, compared with none in the chorio-adenoma group. No information is available regarding the survival of the patients with "chorionepithelioma." The ranges of values of C.G. excretion in all 3 groups before hysterectomy overlap, and are similar to amounts excreted by patients with hydatidiform moles. In fact, with the exception of cases C03, G06 and G010 the amounts excreted are within the range of values obtained from the urine of normal pregnancies at the peak. The excretion of C.G. after hysterectomy presents a different pattern. The amount excreted by the patients in the chorio-adenoma group is indistinguishable from that excreted by patients who had a hydatidiform mole completely
removed; with the exception of patient CA1, the urine from all the women in this group contained no measurable amount of C.G. within 4 weeks of operation. The excretion of C.G. by patients in the choriocarcinoma group can be compared with that from patients in whom the mole was incompletely removed (Figure 1). In the patients with choriocarcinoma the C.G. was being produced by secondary deposits of chorionic tissue. In the other group remnants of the original mole tissue were responsible for the continued excretion of C.G.

Because the histological diagnosis was uncertain in the "chorionepithelioma" group no real comparison can be made with the other groups. The fact that within this group patients GE2, 3, 5, 7 and 9 had secondaries, and GE2, 5 and 17 were excreting between 30,000 - 300,000 I.U. C.G. per litre 2, 4 and 17 weeks after hysterectomy respectively, suggests that they had choriocarcinoma. The response of choriocarcinoma to radiation is not great and any regression of metastases is of a temporary nature. This is shown by the fact that the amounts of C.G., in the urine of patients undergoing such treatment, decreased, indicating either that the growth of the carcinoma had been temporarily arrested or that the secretion of C.G. had been suppressed. Kullander (1948) treated 2 women who had choriocarcinoma and secondary deposits in the chest, with large daily doses of stilboestrol, up to 1,000 mgs per day being given orally. He found that while stilboestrol did not prevent the growth of vaginal metastases, some regression of the metastases in the lungs was obtained. In 1 patient there was a temporary decrease in the amount of C.G. in the urine. It has also been reported that oestrogens and androgens suppress the production of C.G. but that
nevertheless patients so treated died of chorionic metastases. (Peel, Dawson and Mather, 1955, and Hunter and Bockarty, 1955). One must conclude that in the present state of therapeutic knowledge the chances of a patient with choriocarcinoma recovering are extremely small.

The chief value of biological assays for the amount of C.G. excreted by such patients lies in the fact that they enable the clinician to distinguish between chorio-adenoma and choriocarcinoma after the removal of the primary tumour by hysterectomy, and it also provides a measure of the effectiveness of the treatment.

Little attention has been paid to the concentration of C.G. in placentae, hydatidiform moles and chorionepithelioma. The diagnosis of malignancy in these tissues is usually based upon histological evidence. Zondek (1942) suggested that it would be necessary to show the presence of at least 800 mouse units of C.G. per g. in fresh tissue obtained by curettage, in order to make a diagnosis of malignant degeneration. He believed that the concentration of C.G. in extra genital tumours, was about 100 mouse units C.G. per g. of fresh tissue.

Allison (1955) could not distinguish macroscopically or microscopically between moles from 2 patients, and they appeared to be identical. It is of interest that when extracts were made from the 2 moles, and an assay made to determine the concentration of C.G. in the tissue, 1 mole contained 400 times more than the other. Allison estimated the amount of C.G. in several intact moles and found that they
contained from 235-4,324 I.U.C.G. per g. (wet weight) of tissue. When the vesicular fluid was removed from these moles and the concentration of C.G. in the remaining tissue was estimated, it was found that most of the C.G. was in the vesicular fluid.

Allison also estimated the concentration of C.G. in both the primary tumour and metastases from a patient who had choriocarcinoma. The primary tumour contained 1,333 I.U. C.G. per g., compared with 133 and 440 I.U.C.G. per g. in the secondary tumours in kidneys and lungs.

Information about the concentration of C.G. in the placenta is sparse, though Zondek (1931), Augustin (1941) and Diczfalusy (1953) are agreed that the concentration per g. of tissue is highest at the beginning of pregnancy. I have found that there is no correlation between the concentration of C.G. in mole and placental tissue, and the amounts excreted in the urine before and after removal of the tissue (Table 21). Because the complete mole was not available the amount of C.G. in the tissue was expressed in I.U. per g., 24 hour specimens of urine could not be obtained and the excretion of C.G. was estimated per litre. The amount of C.G. excreted depends upon the contact of the mole with the maternal circulation and also upon the level of renal clearance of C.G. in each individual patient. Some patients with hydatidiform mole have extreme toxaemia and, as Loraine (1950a) has shown, the renal clearance of C.G. in patients with pre-eclamptic toxaemia is significantly lower than in normal pregnant patients. Allison (1955) reports an instance of a patient with choriocarcinoma in which the urine contained between 16,000 and 32,000 I.U.C.G. per litre and the
serum was negative. Tumour tissue removed from the patient contained no demonstrable amount of C.G. It would be unwise to assume that this finding is typical until further work has been done on a large number of patients.

According to Tenny and Parker (1939) the amount of C.G. excreted by women with hydatidiform mole or chorioepithelioma corresponds roughly with the amount of active trophoblast cells in the tissue. The authors do not provide quantitative information and were content to make the following statement, "a mole with cystic villi and with slight trophoblastic proliferation gives a low titre, while one with more trophoblastic tissue gives a higher one."

They believe that the activity and amount of trophoblastic tissue is not necessarily related to its malignant potentialities, and found that some patients with benign moles excreted large amounts of C.G. (no values given) and that one patient with a malignant mole excreted a small amount of C.G.

I thought that some correlation might be found between the histological appearance of the tissue and the amount of gonadotrophin excreted before the removal of the mole. I obtained histological sections of molar tissue from 4 patients, and curettings from a 5th patient. These sections were sent to Dr. A.F. Anderson of the Department of Obstetrics and Gynaecology, Edinburgh, who was requested to place them in order of activity. Dr. Anderson had no other information than that they were sections of mole tissue. The order in which he placed them and the results of bio-assays done on the urine before the mole was removed, are shown in the following Table 27
Table 27

<table>
<thead>
<tr>
<th>Reference Number</th>
<th>I.U.C.G. per litre</th>
<th>Pathological report.</th>
</tr>
</thead>
<tbody>
<tr>
<td>A 9909</td>
<td>3,000,000</td>
<td>Active regular cells, very little anyway.</td>
</tr>
<tr>
<td>A 8902</td>
<td>300,000</td>
<td>More activity and more irregularity or anaplasia.</td>
</tr>
<tr>
<td>B 7184</td>
<td>30,000</td>
<td>Ordinary hydatid villi but one area of greater activity and anaplasia on decidual plate.</td>
</tr>
<tr>
<td>A 8652</td>
<td>3,000</td>
<td>Very anaplastic and makes one wonder if she is being carefully followed up.</td>
</tr>
</tbody>
</table>

The report on the curettings from the 5th patient, which were taken 75 days after the removal of the mole was "hydropic chorionic villi, and one or two clusters of quite irregular trophoblast cells." The amount of C.G. excreted by this patient was 2,825 I.U. per litre (Figure 14).

It seems reasonable to postulate that since the C.G. excreted in the urine is produced by the tumour or its metastases, there will probably be some similarity between the amount in the urine and blood, and in the tumour itself. While the pattern of excretion of C.G., even in an individual patient is so variable, while only small amounts of the primary tumour are usually available for histological examination, and while the difficulties of obtaining 24-hour specimens of urine is so great, there is little hope of finding any correlation between the histological structure of the tumour and its malignancy as estimated by the amount of C.G. excreted. Such information as we do possess is largely contradictory and it is essential that further work should be done on a large series of patients.
The literature concerning testicular tumours and the excretion of gonadotrophin is an extensive though confused one. C.G. is not excreted by the normal male, therefore when C.G. is excreted by a male, its presence in the urine is an indication that it is being produced by chorionic tissue. Almost all the earlier attempts to correlate the type of testicular tumour with the amounts of gonadotrophin excreted failed. It was thought that the pituitary was the source of gonadotrophin during pregnancy, and that, the findings of an excess of gonadotrophin in the urine when chorionic tissue was present in the body, meant, by inference, that there was a hyperactivity of the pituitary, similar to that which was thought to occur during pregnancy.

It was not then known that the injection of gonadotrophin of pituitary or chorionic origin might produce the same macroscopical picture in the ovaries of non hypophysectomised rats and mice. It is now known that the injection of urines, in which there/large amounts of pituitary gonadotrophin, into mammalian test animals produces an effect upon the ovaries similar to that of pregnancy urine. In this way, false positive A.Z. reactions were given by some post menopausal urines. In the same way, when C.G. was injected into immature rats or mice in amounts sufficient to produce uterine enlargement, without the formation of haemorrhagic follicles or corpora lutea, then the effect seemed to be due to pituitary gonadotrophin. It is now known that there may be an excess of pituitary gonadotrophin in the urine when the patient has a seminoma, and an excess of C.G. when chorionic tissue is present. It was not
possible to distinguish between these two types of tumours, except on histological grounds, until the two types of gonadotrophin were recognised.

Evans and Simpson (1929) first pointed out that the stimulating effect of hypophyseal tissue implants upon the immature female rat ovary differed from the effect produced by the injection of pregnancy urine concentrates. Montpellier and Harlant (1933) reported that the urine from a patient with seminoma, when injected into immature rats or mice caused ripening of the follicles only, whereas the urine from males with chorionepithelioma of the testis provoked the appearance of haemorrhagic follicles and corpora lutea. Fluhmann and Hoffmann (1934) concluded that the gonadotrophin in the urine of males with a teratoma of the testis, had the same characteristics as the gonadotrophin in the urine of pregnant women. No such distinction was usually made between the effects of C.G. and pituitary gonadotrophin upon the ovaries of the test animal, and most authors investigating the excretion of gonadotrophin in males with testicular tumours expressed their results in terms of "Prolan." The majority did not distinguish between the effect of "Prolan" A and B.

Hamburger (1938) showed that the urine of males with seminoma of the testis contained pituitary gonadotrophins, in amounts usually higher than those excreted by normal males. He showed that the urine of males with chorionepithelioma contained C.G., and Hamburger emphasised that the "chorionic hormone" excreted in certain cases of testicular tumours had the same biological effects as the gonadotrophin found in
pregnancy urine, and that the hormone was present in the
tumour tissue itself. Furthermore the pituitaries of such
males did not contain any gonadotrophin and were therefore
similar in this respect to the pituitary of the pregnant
woman. Males with seminoma and differentiated teratomatous
tumours of the testis, excreted excess pituitary
gonadotrophin, and were found to have gonadotrophin in their
pituitaries but no gonadotrophin was present in the tumour.
The amount of C.G. excreted by males with chorionepithelioma
often reached the same high values as are found in women with
hydatidiform mole and chorionepithelioma. Similarly the
amount of pituitary gonadotrophin excreted by males with
other testicular tumours reached the levels excreted by
castrates and post menopausal women.

The results of my own investigation (Table 25)
confirm Hamburger's observations that males can excrete C.G.
in amounts comparable with those found in the urine of
women with hydatidiform mole and choriocarcinoma. Like
Hamburger I found that males with seminoma of the testis may
excrete from 50 mouse units, about the maximum for the normal
male, to 340 mouse units of pituitary gonadotrophin per litre
of urine. The mean values obtained by the same technique,
for female castrates and post menopausal women was 460 and
377 mouse units per 24 hours respectively.

Hamburger (1938) found that two of his males, for
whom the diagnosis on histological grounds was seminoma,
excreted C.G. and not pituitary gonadotrophin. Both these
males developed metastases, and continued to excrete C.G.
after the removal of the tumour. In my own series of 59
males with seminoma of the testis, 8 excreted C.G. and 2
are known to have died from metastases. It is now generally thought that males with seminoma excrete pituitary gonadotrophin. This gonadotrophin is not produced by the tumour tissue, and the excretion of excess pituitary gonadotrophin is undoubtedly a secondary effect due to the decreased production of androgenic steroids. Testicular tumours are usually large and pathologists doing routine work take only a few representative sections from such tissue. Chorionic tissue may not be demonstrated in the primary growth because it may be only a small part of it, because the entire tumour is not examined microscopically, or because it is impossible to differentiate chorionic tissue from the multiple and varied tissues of a testicular tumour. For these reasons, and the fact that female Xenopus laevis do not regularly respond to injected pituitary gonadotrophin, it is probable that these so-called seminomas described here were in fact wrongly diagnosed and were composed mostly of seminomatous tissue and some chorionic tissue. They were fact "mixed tumours."

SUMMARY.

It has been shown that the normal pregnant female excretes C.G. and that chorionic tissue in both males and females may become pathological and malignant producing large amounts of the hormone. The chorionic tissue in the female is derived from foetal trophoblast and the presence of C.G. in the urine is the earliest sign we have that the female is pregnant. The amount of C.G. excreted during a normal single pregnancy increases until about the 3rd month. It
then falls rapidly to lower levels. At the peak as much as 450,000 I.U.C.G. per litre may be excreted, or in the case of a multiple pregnancy, as much as 1,200,000 I.U.C.G. per litre. Normal chorionic tissue may become abnormal at any stage of pregnancy. If hydatid degeneration of the placenta takes place the amount of C.G. excreted may rise to about 5,000,000 I.U.C.G. More rarely the chorionic tissue becomes malignant and metastasises. Patients with choriocarcinoma may excrete even more C.G. than those with hydatidiform mole. Chorionic tissue is not normally present in the male and its origin is uncertain. It is thought that these tumours arise from primitive cells which have essentially the same capacity as the developing ovum and that the malignant trophoblast elements of these tumours are derived from ectoderm in the same way as the comparable tissue in ordinary pregnancy. The presence of chorionic tissue in the male is usually only recognised when it has become malignant. It may form part of a teratoma, or it may appear in the testis or more rarely elsewhere. Such males with choriocarcinoma excrete extremely large amounts of C.G.

The use of accurate bioassay methods for the estimation of C.G. has not only contributed useful information about the physiology of normal pregnancy, but has also become a useful diagnostic aid in the detection of pathological chorionic tissue.
REFERENCES.


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