THE MALE FROG AS A TEST ANIMAL FOR THE ASSAY OF CHORIONIC GONADOTROPHIN

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INTRODUCTION

The assay of chorionic gonadotrophins used for clinical purposes is time consuming and expensive. The method generally used for the purpose is based on the "vaginal smear" reaction using immature female rats, as recommended by the Permanent Commission on Biological Standardization of the Health Organization of the League of Nations (42). The present investigation was undertaken to develop an accurate, cheap and rapid method of assay using the Galli-Mainini (61) reaction on male frogs, and to compare results of several assays by the proposed male frog technique with the potency as determined by the rat vaginal smear method.

I. GONADOTROPHINS:

Gonadotrophic hormones may be divided into two main groups, one of which is derived from pituitary and the other from living chorionic tissue. The former includes those obtained directly from the blood and urine of normal men and women at menopause. The second group of gonadotrophins consists of active material of the urine or blood of women during pregnancy and of the blood of pregnant mares (124).

Anterior Pituitary Gonadotrophins: In the early work of Aschner (4) on the effects of hypophysectomy in dogs, atrophy of genital organs was seen. Evans and his coworkers (45) showed enlargement of ovaries and excessive luteinization in normal rats treated with pituitary extracts, and later these investigators indicated the chemical distinction of the gonadotrophic

factor from the growth promoting factor in the extract. Recent advances in our knowledge of the gonadotrophins have been stimulated by the development of techniques for hypophysectomy by various workers using various animals. In 1927 P. E. Smith (117) announced that rats could be conveniently hypophysectomised and that this operation caused atrophy of the gonads. These effects could be reversed by the implantation of rat pituitaries. Much of the recent work is based on this.

Great progress has been made in the fractionation of anterior pituitary extracts. The separation of the hormones has been achieved by fractional precipitation with organic solvents and inorganic salts, and by adjustment of the pH. A detailed account of this work has been given by Li and Evans (88). At least three gonadotrophic hormones have been isolated from crude pituitary extracts. They are the follicle stimulating hormone (FSH), the luteinizing hormone (LH) sometimes referred to as the interstitial-cell-stimulating hormone (ICSH) and luteotrophic hormone (prolactin, lactogenic hormone) (124).

The isolation of FSH in electrophoretically homogenous state from sheep pituitaries has been achieved only recently by Li et al. (88). Its chief function in the female is to induce development of the ovarian follicles up to the point of ovulation, and in the male the development of seminiferous tubles and maintainance of spermatogenesis. If pure it does not cause luteinization directly. In intact animals luteinization may occur eventually due to the liberation of LH from the animals' own pituitary. It was once believed that FSH caused the release of estrogens from the ovaries, but there is evidence that pure

preparations do not do this in hypophysectomised rats and that a small amount of LH must be present for this effect to occur. Pure FSH has thus no effect on the uterus. The gonadotrophins in the urine in female castrates, or at the menopause or in the male urine are probably mainly FSH mixed with a little LH (64).

The luteinizing hormone (LH or ICSH) has been obtained in pure form as a single homogenous protein from pig's pituitaries by Chow et al. (64) and from sheep's pituitaries by Li, et al. (86). The hormones from the two sources, identical in their biological actions, are distinct substances which differ immunologically and in their chemical properties such as molecular weight, isoelectric point, and amino acid composition. It stimulates the interstitial cells in the gonads of either sex of rat. In the male this causes the release of androgens with secondary effects upon the prostate and the seminal vesicle. In the female the effect depends very much on the presence of follicles in the ovary. In young hypophysectomised female rats there are no follicles and, though the effect on the interstitial cells can be detected histologically, there is little or no increase in the weight of the ovary and no release of estrogens. If such rats are first treated with FSH to form follicles, the injection of LH causes ovulation, luteinization, and the release of estrogens. This is called sometimes the synergistic effect (64).

Luteotrophin (lactogenic) Hormone: Corpora lutea formed under the action of FSH and LH do not secrete progesterone until stimulated to do so by a third substance present in the extracts of the anterior pituitary. This substance is called

luteotrophin, but it seems to be identical with lactogenic hormone (A).

Human Chorionic Gonadotrophin (CG): The first known naturally occurring gonadotrophin was that found in the blood and urine of pregnant women, and originally named Prolan by Ascheim and Zondek (3). Its presence was detected by its ability to produce precocious maturity (estrous, follicular development, ovulation and luteinisation) in immature rodents, rats and mice within a period of 96 hours. That it differs from gonadotrophic hormones of anterior pituitary origin was demonstrated by Evans et al. (46) (47). Histochemical studies of Wislocki and Bennett (132) indicated that cytotrophobast is responsible for its secretion. Evidence from tissue culture is in agreement with other evidence in showing that chorionicepithelia, produces the hormone (63) (78). Chorionic gonadotrophin, or CG is now the generally accepted appelation for the hormone.

Claesson et al. (28) claim to have isolated a pure, crystalline, electrophoretically homogenous product. Katzman et al. (82) have described a simple method for the extraction of CG from pregnancy urine, and practical in obtaining a large amount of hormone for further biological and chemical investigations. Their procedure is briefly as follows:-

Urine obtained during the first half of the pregnancy is chilled filtered, and acidified to pH 3.5 with glacial acetic acid. After filtration the clear filtrate is percolated through a column containing Permutit. Adsorption is complete when 10 litres of urine per hour are passed through a column having a

diameter of 4 inches and containing 2 kg. of Permutit. The column is then washed with cold distilled water until the washings are neutral and practically colorless. The hormone is then eluted with 38% ethanol containing 10% ammonium acetate. The hormone in the elute is fractionally precipitated with ethanol. The bulk of the activity precipitates at 70 or 75% ethanol concentration. The hormone thus obtained contains a potency as high as 8500 I.U/mg.

When preparations of CG either from placenta or from pregnancy urine are injected into immature female laboratory animals (rat, mouse, etc.); they produce an effect on the ovaries practically identical with those produced by the pituitary gonadotrophin ICSH (maturation of follicles, formation of corpora lutea). But if tested on the hypophysectomised animal these results do not ensue. The CG injected into male animals, stimulates an increase in size and number of interstitial cells of the testis. It is not the same hormone as obtained from pig or sheep's pituitary, but might possibly be identical with human LH (96).

<u>Equine Chorionic Gonadotrophin (PMS)</u>: This is formed in equine placenta and obtained from pregnant mare's serum. It differs from all the gonadotrophins in the fact that it is not excreted in urine (124). In the normal immature animal, the hormone shows both follicle stimulation and corpus luteum formation. Its main effect on the ovary is like that of FSH, but large doses cause luteinization even in hypophysectomised rats. Its main effect on the testes is like those of LH and human CG (stimulation of interstitial cells with liberation of androgens).

Seminal vesicles are strongly stimulated in the hypophysectomised male and spermatozoa formation may occur (48). A preparation containing 12,000 to 13,500 I.U. per mg. has been made (98).

All the four gonadotrophic hormones (ICSH, FSH, PMS, and CG) are prepared in different degrees of purity. All are glycoproteins, in addition to the amino acid constituents, the substances contain hexose and hexosamine. Destruction of carbohydrate residue by enzymes causes inactivation of the hormones. Similarly, modification of protein groups decreases the gonadotrophic activity. Thus carbohydrate as well as protein parts are essential for the physiological action. Presence of prosthetic groups in these hormones are not indicated. The complete composition of the gonadotrophins has not yet been revealed (87).

II. PHYSIOLOGY OF MALE FROG REPRODUCTIVE SYSTEM:

The anatomy and histology of the reproductive system of male frog Rana pipiens have been thoroughly studied by Rugh (106), but it seems necessary to describe here some of the physiology of the male reproductive system in order to understand the manner of action of the gonadotrophins on the male frog.

The breeding season of the North American frog (Rana pipiens), is usually from the latter part of February to April. The maturing of the spermatozoa and the appearance of sexual instincts of male frog, is correlated with an increased development of the base of the inner digit of the fore leg and an enlargement of certain muscles which are concerned in the clasping reflex - in

which the male frog mounts the back of the female and clasps her tightly about the pectoral region with his fore legs. The clasping reflex has been shown to be a true secondary sexual characteristic and is dependent on the presence of testicular hormones. It is also dependent on the anterior pituitary gonadotrophic hormones (115). The ventral surface of the male's body, especially the pectoral region and ventral surface of forelimbs, are sensitive to the female and cause the clasping reflex. The stimulation spreads from the skin and acts directly on the muscle; the sensory nerves and the central nervous system are not necessary for the reaction. The oviposition movements of the female excite the skin areas of the male and cause the ejaculatory pumps (94).

Nearly all frogs and toads use amplexus without internal fecundation - i.e. the male clasps the female until spawning is over. The discharge of spermatozoa is presumably under the influence of the luteinising hormone (LH), secreted by the anterior pituitary probably in response to nervous stimulation. Just what definite central nervous pathways are involved is not known, but no other type of stimulation, including the excitement caused by handling, is known to release LH (17).

Noble and Aronson (94) believe that a complex of favorable environmental factors stimulate the anterior pituitary in both sexes simultaneously, shortly after emergence from hibernation.

In 1929 Houssay and Lascano Gonzalez (74)showed, that in male toad (Bufo arenarum Hensel) liberation and expulsion of spermatozoa are controlled by the anterior pituitary.
de Robertis et al. (38) in 1946 further showed that injection

of pituitary gonadotrophins causes spermatogenesis at any season of the year.

In controlled experiments these and other investigators, notably Rugh (104), have followed by histological examination, the effects produced on the testes. The spermatozoa normally lie in clusters attached to Sertoli cells. The effect of gonadotrophins is first signaled by swelling of the Sertoli cells, followed by detachment of skeins of spermatozoa into the lumen of the seminiferous tubules. These events occur rapidly, are evident in 10 minutes, and are completed within half an hour. The mature spermatozoa next pass quickly through the vasa deferantia into the ureters, whence they may be voided or stored temporarily in the bladder.

It has been suggested that the peritoneal funnels on the ventral face of the kidneys may carry the injected hormone directly into the blood stream (105). Whether the anterior pituitary hormone acts exclusively through the circulatory system has not been established (106).

Rugh (106) suggests that the mechanism of liberating spermatozoa from Sertoli cells by the anterior pituitary hormone may be due to the stimulation of the muscular contraction of the smooth muscle fibers in the stroma of the testes, forcing mature spermatozoa out of them into the vasa efferentia.

Without discarding the possibility of an increase of the muscular contraction of the testes, de Robertis et al. (38), found that the release of spermatozoa is due to hypertrophy of the Sertoli cells and a progressive vacuolization of the cell with the apical cytoplasm by the action of the anterior pituitary hormone.

III. METHODS OF ASSAY:

Since the discovery (3) of the gonadotrophic hormone in human pregnancy urine, a variety of assay methods have been used by different workers for quantitative determinations. Nearly all investigators present the value determined in terms of units; rat units, mouse units or rabbit units. It is however rarely possible to compare two results of assay with the assurance that the "units" are at all comparable. Studies on the assay of commercial gonadotrophin preparations (20)(33) emphasize the fact that no comparison of activity is possible because of lack of uniformity of the assay methods employed.

In an attempt to lessen confusion in this field, the Permanent Commission on Biological Standardization of the Health Organization of the League of Nations adopted an international unit for gonadotrophin in the urine of pregnant women and that in pregnant mare's serum (42). For both substances, the standard is a mixture of six preparations of gonadotrophin. This is still the standard but U S P is developing its own standard.

For pregnancy urine gonadotrophin the international unit (I.U.) is defined as the "Specific activity of 0.1 mg. (100 gamma) of the standard preparation; for pregnant mare's serum gonadotrophin the I.U. is 0.25 mg. (250 gamma) of the standard preparation."

Two types of tests can be used for the determination of gonadotrophin hormone activity, and thus for the comparison of preparations of unknown potency with standard preparations. According to the League of Nations Memorandum these tests should be based either on (a) the observation of a direct or indirect

gonadotrophic effect as shown by morphological changes in the gonads, or on (b) the observation of secondary changes in the accessory reproductive organs, in animals not deprived of their gonads. When the second type of test is used, the absence of substances directly causing such changes in the accessory reproductive organs should be assured by control tests on animals deprived of their gonads.

In the methods that have been proposed for the biological assay of chorionic-gonadotrophin (CG), the test animals have usually been immature or hypophysectomised rats or mice in which circulating gonadotrophins were lacking.

Methods have been based on observation of the presence of corpora lutea (3)(20)(32)(56)(83)(97)(102)(114)(118)(130)(131); increase in weight of ovaries (5)(32)(34)(50)(52)(56)(66)(126)(127), increase in weight of uteri (20)(34)(37)(39)(40)(52)(79)(85)(109); increase in weight of seminal vesicles (10)(32)(34)(35)(84)(109)(126)(128); vaginal smears in rats (5)(21)(27)(32)(34)(56)(66)(109)(125); vaginal smears in vitamin B deficient rats (69)(70); ovulation in the rabbit (59)(72)(107); ovulation in _Xenopus laevis_ (8)(9)(30)(129); increase in weight of the prostate gland in male rats (35)(84)(91); ovarian hypermia in rats (16)(134)(135)(136); release of spermatozoa in the male frog (6)(68).

Estimates of gonadotrophins in blood, urine and tissue extracts are sometimes required in physiological and clinical research. Chorionic gonadotrophin is sometimes present during pregnancy in such overwhelming amounts that blood or urine can be used directly without extraction, but more often it is necessary to make an extract in order

to reduce the bulk and remove toxic substances and other hormones which would interfere with the test. The methods used include precipitation with alcohol or acetone, adsorption on various reagents, salting out with sulphates, and precipitation with tannic acid (18). One simple method which has been found satisfactory for human CG and for the gonadotrophins in urine after menopause, involves adsorption on Kaolin as recommended by Scott (108), followed by precipitation with acetone (36)(91). The adsorption leaves most of the salts behind and the acetone removes oestrogens. A litre of urine can thus be concentrated to about 400 mg. of solid material containing all the gonadotrophin, and quantities corresponding to 200 ml. of urine or more can be injected into each animal.

The first attempt at quantitative assay was made by Zondek and Ascheim (133) in 1927. They employed the vaginal smear method of Allen and Doisy (1) and Allen et. al. (2) based on the discovery of Stockard and Papanicolaon (121) of the relation of vaginal changes to the oestrous cycle.

A separate test was proposed for the fractions designated by them, prolan A and Prolan B. They inject subcutaneously, during 36 hours, six portions of the material into albino rats, four to five weeks old and weighing 30 - 35 g. The end point for prolan A is vaginal oestrous reaction and follicular maturity one hundred hours after the first injection. The follicles should be enlarged and filled with fluid and the uterus hyperaemic and filled with fluid. To test for prolan B, the "luteinizing factor" injections are made into immature mice in a similar manner to the above. The end point is an enlarged hyperaemic

ovary and with projecting lutein bodies, the uterus is thin and unchanged and the vaginal responsive is negative.

Rats or mice can be used for prolan A; but only immature mice, three to four weeks old, weighing 6 - 8 g. should be used for prolan B.

For mixed material (prolan A and B) the vagina should show a positive smear, the ovary exhibit large follicles and corpora and "Blutpunkte", the uterus should be smooth, hyperaemic and some parts filled with secretion.

Zondak describes the unit of activity as, "A mouse or rat unit of prolan is the smallest amount which, divided into six portions and injected during the course of 36 hours, will bring on the vaginal oestrous reaction and follicular maturity 100 hours after the first injection."

Janssen and Loeser (76) estimated the luteinising power of anterior pituitary extracts, using production of corpora lutea as the end point. This was the first biological method to be described for anterior pituitary extracts, which also includes a proposal for a standard preparation. They used female rats of 40 - 50 g., 10 rats in a group. Each rat was injected six times with the preparation to be tested, the injections being made into the peritoneal cavity at regular intervals of eight hours. One hundred hours after the first injection the rats were killed and the ovaries were removed to formol saline. Then they were embedded in paraffin and the sections stained with hematoxylin and eosin. An examination was made for corpora lutea or atretic corpora lutea.

These authors determined a curve relating the dose administered to the percentage of rats in which corpora lutea were found, using a powder prepared by extracting fresh anterior pituitaries. Using this curve as the standard they determined the amount of luteinizing hormone present in the pregnancy urine samples.

Kennedy (83) employs the mouse as a test animal because "they are more sensitive to prolan B." The ovaries of the animal are examined macroscopically for blood-points and corpora lutea, in doubtful cases microscopic examinations is made. He considers the accuracy of the "mouse unit" from a statistical point.

Rowe and co-workers (102) claimed that the use of immature mice is less practical than the use of rats and no more accurate than other methods of assay. Using a commercial product of C.G. (Antuitrin S), they found that four of their rat units (based upon production of corpora lutea in the ovaries of 30 day old female rats, 26 day old at the start of injection) are equivalent to one Katzman and Doisy (80) unit (mouse vaginal smear). In a similar manner they found that the rabbit ovulation unit of Friedman (58). is equivalent of 1 R.U. per kg. of body weight of rabbit.

Several workers (114)(118)(130) have determined the amount of gonadotrophic hormone present, both in urine and blood during pregnancy, and in late pregnancy toxemia using corpora lutea as the end point.

Smith and Smith (118) used 19 - 21 days old rats in which corpora lutea at 96 hours were considered as a positive response. However as these authors point out, the use of serum extracts in amounts of 2 cc., 1.5 cc. give corpora while 0.7, 0.5, and 0.3 cc. give only follicular response. The lowest value 0.2 and 0.1 cc. again give luteinization. The serum thus would contain either 100 or 1000 R.U. per cubic centimeter. The authors state the concentrations at which the serum values show wide variations, but it appears difficult particularly to determine which is the first end point.

Several investigators (5)(20)(32)(34)(128) consider that the corpus luteum method is unsatisfactory for estimating the gonadotrophic activity, and should not be considered.

The corpus luteum end point is not clear cut (20), since, an examination of ovaries under 10 diameters magnification shows stages of transition from hyperaemic follicles through ruptured follicles to more advanced stages of luteinization. Thus if macroscopic examination is relied on, it fails on the score of objectivity, if microscopic sections are made, which is absolutely necessary to be sure of the degree of luteinization, it fails on the score of simplicity. The method is sensitive enough and is applicable to preparations of gonadotrophins from pregnancy urine and anterior pituitary, but individual variation is rather great.

The simplest method of observing the direct effect of gonadotrophic preparations is by determining the increase in the weight of the ovaries.

Hallen-Lawrence and Van Dyke (126) in their method of assay use as an end point the weight of both ovaries free from bursae and fallopian tubes. Nothing is reported concerning assays which are more accurate than crudely quantitative.

The mean weight of both ovaries is given as 16.14 $\pm$ 3.68 g. In a later paper (74) the mean weight is given as 14.74 $\pm$ 3.24 g. The increase in ovarian weight is considered significant if it is greater than 2.75 σ $\pm$ mean weight. This is 16.14 + (2.75 x 3.68) = 26.3 g. for the former and 14.76 + (2.75 x 3.24) = 23.7 g. for the latter mean ovarian weights (the difference between these is not significant). The authors consider vaginal canalization and oestrous a too variable criterian to be used for the end-point for potency determinations.

Deanesly (35) had described the details of methods in which the ovary weight is used to compare preparations from pregnancy urine and also preparations from the anterior pituitary. Groups of 10 - 12 rats per dose are usually employed, at a body-weight of between 40 - 50 g. An injection is given each day, for 5 consecutive days, the animals are killed on the sixth day and the ovarian weight is determined after fixation in Bouin's fluid and immersion in 70% alcohol.

For anterior pituitary extract the relation between dose and mean ovary weight is linear for ovary weights between 30 and 80 mg.; similarly, for pregnancy urine extracts the relation is linear between ovary weights of 20 and 40 mg.

Deanesly found that the standard deviation of weights of ovaries varied with their mean size. For ovaries of 60 mg. mean

weight the standard deviation was 17.3, so that if 12 rats are used for a test the standard error is 5.2. For ovaries of 26 mg. mean weight, the standard deviation was 3.0, and the standard error for 12 rats is 0.85.

The gonadotrophic hormone in pregnant mare's serum differs from those found in most other sources in that ovarian weight increases with dosage over an extremely wide range. It is possible to produce (26) in rats approximately a 15 times increase in mean ovarian weight by increasing the dosage of hormone to not more than 15 times minimum amount necessary to produce a measurable response. It is not surprising that ovarian weight has been most often used in assaying this hormone.

Cole and Saunders (29), Meyer (92), Evans, Gustus, and Simpson (51) Cartland and Nelson (26), have employed assay methods based upon a minimum ovarian response in rats or mice. A unit of this magnitude has the disadvantage of representing approximately a normal maturity change in the ovary. However for quantitative evaluation it possesses the disadvantage of falling in a range where ovarian weight is not utilized to rather large changes in dosage.

Hamburger and Pedersen-Bjergarrd (66) have presented a standardization curve obtained from the results of two different laboratories for two types of gonadotrophic substances, pregnancy urine extract and pregnant mare's serum extract.

They conclude that the ovarian weight response is the best method for standardizing the pregnant mare's serum and that the rat gives the most consistent results, but that the vaginal

smear of immature rats or the determination of corpora lutea in the ovaries of mice are best suited for the assay of gonadotrophins from pregnancy urine.

D'Amour and D'Amour (32) from a comparative study of the assay of gonadotrophins from pregnancy urine, placenta and sheep pituitaries concluded that the immature male rat is more sensitive to the first two, that the corpus luteum formation was a matter of high enough dosage, that the opening of the vagina was a very unreliable criterian, that the positive oestrous vaginal smear was very sensitive for urinary preparations, and that the ovarian weight was the "most objective" criterian although at times a marked ovarian response resulting from glandular preparations gave negative oestrous smears.

Sealey and Sondern (109) studying the action of graded doses of international standard C.G. on infantile female rats found, that the initial ovarian weight decrease from doses of less than 1.5 I.U. is accompained by a sharp rise in uterine weight. An analogous ovarian and uterine response to rat pituitary suspension is reported by Heller et. al. (71) and Cartland and Nelson (26) reported a similar response to pregnant mare's serum extract. Doses above 1.5 I.U. produced a steady rise in the weight of the ovary, the maximum effect being reached at doses near 8.0 I.U. At doses of 1.5 to 8.0 I.U. the curve again approximates a straight line with such a slope that a change in dose of 0.8 I.U. is significant. Thus assays made in ranges of 2 to 8 I.U. equivalents should be accurate within 15 to 20 %.

The ovarian weight method is applicable to preparation of gonadotrophin from pregnancy urine, pregnant mare's serum, and anterior pituitary, the method is completely objective. The sensitivity varies with gonadotrophin concerned, being low for pregnancy urine and it will be noted that the curve rises very slowly, more than 10 I.U. being required to double the ovarian weight (34). Excessively large amount of material therefore must be used. The sensitivity is low at low doses of pregnant mare's serum, but higher with larger doses, and moderate for anterior pituitary gonadotrophin. The method is reasonably simple, although the dissecting out of the ovaries is time consuming.

The test in which injections are made into young rats to observe the opening and cornification of the vagina has been widely applied.

Katzman and Doisy (80) claim that the opening of vagina as the sole endpoint for the method of assay is not reliable because (a) accidental opening is included, and (b) it is difficult to rule out the effect of small traces of theelin and theelol. They adopt both opening of the vagina and oestrous as the criterion of a positive reaction. Mice or rats are used, but the mice are four times more sensitive than the rat. There is no need of sacrificing the animals for examination of the ovaries. Nineteen day old rats used. Six equal doses are spread over three days, the mice are observed on the 22nd. to 24th day of age, the rats by the 27th. day of age. To prove the absence of oestrogenic hormones the material is tested on adult spayed female rats.

Rolands and Parkes (103) and Burn (22) have reasoned that the vaginal smear test, because of the secondary effect of the hormone is likely to be extremely variable in different animals. Two variables are involved (a) variation in the amount of oestrin secreted in response to a given quantity of gonado-trophic substance and (b) variation in the effect produced in the secondary organs by the same amount of oestrin. These considerations show that the effect on the vagina is unlikely to be suitable for estimation of the hormone.

Burn (22) has reported the experimental results obtained by Brownlee. He took female rats 21 to 23 days old, weighing 30 to 35 g., the total dose was given in six injections during a course of three days. The vaginal smears were read on the fifth day.

A total dose of 1.2 mg. gave positive vaginal smears in 50% of the animals, while half of this dose, 0.6 gm., gave positive smears in 70% of the animals. The method thus seems unsatisfactory.

With the infant animal the period of cornification lasts only a few hours even when the total dose is spread over three days, and is dependent to a great extent upon the strength of stimulus. When the infantile animal is given a total dose in one injection (70) the period of induced oestrous is still shorter. With this technique of administration much difficulty is experienced in arriving at a satisfactory evaluation of the response because of the persistence of leucocytes even at high dose levels.

The short duration of the cornified period in the immature rat undoubtedly accounts for the inconsistent results obtained by Brownlee (reported by Burn) (22), who made but one observation of the vaginal contents four days after the first of six injections spread over 56 hours.

Heard (70) reports that cornification of vagina is encountered on the fourth or fifth day after the beginning of administration of gonadotrophin. By the recording of smears at least twice daily over the critical period, regular responses have been obtained with infantile animal by Chapman (17), Hamburger and Pedersen-Bjergaard (59) and others.

Chapman (27) has constructed dosage response curves based on vaginal cornification reaction in the rat at different age levels over a range of 10 - 60 days of age, which show a remarkable increase in sensitivity up to puberty. Animals between 32 and 49 days age, exhibit no significant differences in the DR50 (Response dose for 50%), whether used for the first, second or third time. This means that animals used more than once do not change their sensitivity. He further claims that the most accurate age range in which to use rats for A.P.L. assay is 30 to about 50 days. This is contrary to the usual practice of choosing rats 18 - 22 days old. The accuracy of the method is discussed and the statistical analysis shows that the error is between 6 and 12%.

Browne and Venning (21) have studied the excretion of gonadotrophin substances in pregnancy urine. The method of concentration of gonadotrophic substances was the benzoic acid procedure of Katzman and Doisy (81). 21 day old rats were

injected subcutaneously twice daily for 3 days. Vaginal smears were taken on day 4, 5, and 6 and animals with positive smears killed on day 6. A full squamous response (of the vaginal smears) which was usually accompanied by histological evidence of follicle stimulation, was taken as a positive result. The unit is defined as "the smallest amount necessary to produce this result," and recorded in rat units per liter (R.U.L.).

Barlow and Sprague (3) have described a comparison of the responses to the international standard C.G. by four different criteria; the incidence of oestrous smears, degree of uterine hypertrophy, corpora lutea occurrence, and ovarian hypertrophy are of decreasing test value in the order named.

A combined criteria of 80% or more oestrous smears with a 250 to 300% increase in uterine weight appears to be the most reliable index. According to them the 26 - 28 day old infantile rats at the time of initial injections respond more strongly and uniformly than 21 day old rats to unit dosages.

Sealy and Sondern (109) studied the effect of the international standard C. G. substance on immature female rats; the percentage of animals showing vaginal oestrous was significant through the range of 0.5 to 2.0 I.U.

Vaginal smear method is applicable to preparations of gonadotrophins from all three sources. It is reasonably objective, provided one sets as his standard, a full oestrous smear, that is the complete disappearance of leucocytes and their replacement by epithelial cells. The sensitivity is great and compares rather closely to the uterine weight method.

Heard and Winton (69) have investigated the possibility of using adult female rats, rendered anoestrous by the administration of a vitamin B deficient diet, for standardization purposes. Administration of gonadotrophic hormone stimulates the ovaries and brings an oestrous which can be detected by the vaginal smear technique. The animals need not be killed, and can thus be used repeatedly.

With this method it is possible to determine the potency ratio of an unknown with an error range of -24% to + 31% at $P = 0.99$ using a total of 40 animals.

In another study Heard (70) used the vaginal oestrous response of the immature rat, and found a somewhat lower accuracy in the determination of potency ratio. In this study the error range of -35% to +54% was found at $P = 0.99$ when a total of 40 rats were used, twenty on the unknown and twenty on the standard.

In the dietary anoestrous rat, cornification persists of 24 to 48 hours following a single injection of gonadotrophic substance, and hence vaginal smears need be recorded only once each day in order to include all positive reactions.

The increase in uterine size and fluid content as secondary effects on immature rats and mice treated with gonadotrophic substance has been observed by many workers (24)(25)(31)(32)(41)(55)(83)(95)(110)(112)(113)(116).

The same theoretical objections apply to the changes in the weight of the uterus as applied to the vaginal smears; in actual practice, however, the method often yields surprisingly good results (15).

Levin and Tyndale (85) have used the uterine weight response in the immature mouse as a satisfactory assay for the gonadotrophic substances from the castrate urine; the method is less accurate for pregnancy urine and P.M.S. gonadotrophins, as there is more individual variation on minimal dose, although with higher doses the response is more uniform.

Heller, Lauson and Sevringhaus (71) consider the uterine weight method as a satisfactory end point for the assay of rat pituitary gonadotrophic factors.

Hamburger and Pedersen-Bjergaard (66) found in the rat, that the uterine and vaginal responses to C.G., were more reliable as end point than were ovarian changes.

Sealey and Sondern (109) have studied the effect of international standard C.G. substance, based on 96 hour uterine weight response of the infantile rat. According to these workers uterine response is a very sensitive method of estimating the potency of gonadotrophic extracts between 0.5 and 1.0, I.U., since significant responses are observed to changes in doses of 0.1 I.U. Doses of one to two I.U. give a response to changes of 0.25 I.U. Above 2.0 I.U. the weight changes are insignificant.

Delfs (37) has presented an assay method for human serum C.G., based upon the 72 hour response of the immature rat. Female animals of 21 to 23 days have been given injections over 2 days and the uterine weight increase determined at 72 hours. A standard curve for dosage - uterine response has been developed, which is accurate over a 7 - fold dosage. Comparison with international standard C.G. has been made. The method, with

relatively few animals, has been found to be more sensitive and to have a wider useful range than ovarian weight, vaginal smears and corpora lutea method.

Dorfman, Ruban, and Miller (39) have described an assay method of human C.G. both from urinary sources and from the serum, based upon 96 hour uterine weight response.

These workers use the experimental design of Bliss (15); four groups of animals are employed, two groups on the standard and two on the unknown. The total dose of C.G. is given subcutaneously in 6 injections during a course of 3 days, two injections being given daily.

All responses are expressed as the ratio of the uterine weight in mg. to the body weight in g. The log. dose is plotted against uterine response, which gives a straight line relationship.

These investigators found, that the use of a total of 20 animals (10 on the standard and 10 on the unknown) will determine the potency ratio of urinary material with accuracy of -25% to +36% when P = 0.95. Increasing the total number of animals to 40 (20 on the unknown and 20 on the standard) decreased the error range to - 19% to +24%. A somewhat lower range was found in the serum gonadotrophin. For 20 animals a range of - 19% to +25% was found while 40 animals yield a range of -12 to +14% at P = 0.95. The standard employed was the international C.G.

The method although being sensitive and reproducible has the disadvantage of a short useful range in the assay curve, that is only two logarathmic intervals. For this it is frequently necessary to run a preliminary assay to determine the correct

dosage level.

The uterine weight method is applicable to all three types of gonadotrophins, it is completely objective. The sensivitity is great, a six fold increase in weight being obtained on either pregnancy urine or pregnant mare's serum preparations with doses which will double the weight of the seminal vesicles and hardly affect the ovarian weight at all. The individual variation is great, and to overcome this D'Amour (34) suggests the use of sufficiently large number of animals and performance of the assay at sufficiently high dosage level. One disadvantage is the fact that the weight curve reaches a maximum and then declines with larger doses.

An indirect method of estimating gonadotrophic acitivity by means of the effect on seminal vesicles of male rats is described by many workers (10)(32)(35)(84).

Korenechensky et al. (84) have used the seminal vesicle response of 22 to 25 day old male rats for estimating the gonadotrophic factor of pregnancy urine. The animals were injected during a course of 3 days; the total dose being given in 6 injections. The animals were killed at 120 hours after the first injection; the seminal vesicles weighed after fixing in Bouin's fluid for 24 hours.

The male rat unit is defined, and these investigators consider the method satisfactory from a statistical point of view.

Deanesly (35) found that greater effects were produced on the seminal vesical response if the injections were continued for 5 days, but this adds considerably to the length of the experiment.

Sealey and Sondern (109) studied the effect of international standard CG on infantile male rats; the seminal vesicles respond to doses of the standard as low as 1.0 I.U. The response is most significant from doses of 1.5 to 8.0 I.U. Through this range the curve approaches a straight line; a significant change in weight of seminal vesicles occurs with a change of 0.8 I.U. Assays made in range of 2 to 8 I.U. equivalents should there be accurate within 15 to 20%.

Watts and Adair (128) and D'Amour and D'Amour (34) consider the seminal response more reliable. In their colony one I.U. of chorionic gonadotrophin almost exactly doubled the seminal vesicles' weight (34).

The seminal vesicle weight method is applicable to preparations of the gonadotrophins in the urine of pregnancy and that of in P.M.S. It is completely objective, the sensitivity is moderate, and the individual variation is not great.

Following the discovery by Bellerby (7) and Friedman (57) that induction of ovulation in the unmated female rabbit could be brought by pituitary extracts and urine of pregnancy; Hill, Parkes and White (72) proposed a method for the quantitative determination of the gonadotrophic substances. The procedure is to make a single intravenous injection of the preparation in a group of 20 oestrous rabbits, and the ovulation is determined next day by performing sterile laparotomy under ether anesthesia. Animals can be used three times, Standardization curves are constructed for pregnancy urine and anterior pituitary extracts for ovulation in rabbits.

The unit is defined as the ovulation producing activity required to cause ovulation in 50% of a group of not less than ten rabbits.

Friedman recommends (59) that the test should be performed on oestrous post-partum animals. It has been reported by Hammond and Marshall (67) and confirmed by Friedman (58) that oestrous occurs with great regularity in post-partum rabbits and lasts for at least 25 days. The use of post-partum rabbits for gonadotrophic assays largely eliminates individual variation in sensitivity and permits an evaluation of the importance of body weight. For rabbits weighing 2-4 kg., a linear relation between body weight and effective dose is accepted for practical purposes.

With one post-partum rabbit injected at each critical dose level (per kg. body weight), it is possible to define the biological activity of good preparations of human pregnancy urine and P.M.S. 9 times out of 10 with an error less than 30%. The use of a second animal at the critical level provides an accuracy which surpasses that obtainable by the use of 10 isolated rabbits at each dose level.

The rabbit ovulation method is satisfactory, the only disadvantage is of being comparatively expensive.

Recently Loraine (91) has presented a method of assay depending on the increase in the weight of the prostate in immature rats 19-22 day old. The animals are injected subcutaneously once daily for 3 days at approximately 24 hour intervals. The animals are killed at 96 hours, and the prostate weighed, after fixing in Bouin's solution (24 hours), and then dried

thoroughly between pieces of filter paper. The values are expressed as mg. prostate weight per 100 g. rat, which gives more consistent results.

A dose effect curve is constructed from the standard (I.U. of C.G.) doses; plotting mean weight in mg./100 g. against log dose. The mean weights for the pregnancy urine being assayed are also calculated and the corresponding dose read off on this dose effect curve.

The weight of animal appears to be more important than age, and rats weighing 30 - 45 g. are the most suitable; rats weighing less than 30 g. initially tend to lose weight during the test.

On statistical evidence Loraine claims that the method is relatively more accurate than methods depending on seminal vesicles and vaginal smears. The advantage of the method is that it is not affected by oestrogens.

The report of Emmens (42) on the international standard C.G. recommends for preparations of pregnancy urine gonadotrophin, the use of the vaginal cornification test; no specific recommendation is made for the preparation of P.M.S. gonadotrophin.

On the whole the vaginal cornification is the most accurate, inexpensive and simple method of assay. It also has the advantage that the animals need not be killed after the test, and may be used for other purposes later. On the other hand, if they are killed, the added test of uterine weight is available as a check on the findings by vaginal cornification. There is one drawback to the test in that its accuracy depends on the absence of oestrogenic material from the preparations tested.

Tests by ovarian weight or corpus luteum formation are independent of the presence of small amounts of oestrogenic material and, in view of their reasonably high accuracy, may be preferred for this reason (122).

Besides the most popular ovarian weight method of assay for the P.M.S. gonadotrophin, other methods for this hormone depend upon vaginal smears, corpus luteum formation, the weight of the uterus or seminal vesicles, and ovulation (42)(43).

Bellerby (8)(9) studied the African clawed toad (Xenopus laevis) and the English frog (Rana temporaria) to determine their suitability as test animals for assay of gonadal stimulating substances. Test of activity of an extract is the extrusion of eggs by the female; ovulation occurring 8 to 24 hours after the injections. The relationship between dose and response has been found to be more constant than that obtained with the mammal as the test animal. Rana is less satisfactory than Xenopus according to Bellerby. Dose response curves have been constructed for anterior pituitary, acid and alkaline extracts.

Female Xenopus has been used as a test animal for pregnancy diagnosis (30)(129), but it has not yet been used for quantitative determination of CG. The method has the advantage of its simplicity, rapidity, and cheapness.

The effect of CG in causing hyperemia of the ovary has been used as a pregnancy diagnosis test by many workers (16)(134)(136). The hyperemic reaction in rat ovary eight hours or less after the administration of CG is pink, after ten hours or more the ovary reaction is a deep red, similar to the color of spleen, kidney or liver.

Zondek and Sulman (136) attempted to standardize the ovarian hyperemic reaction against chorionic gonadotrophin (Winthrop). They found that the sensitivity of the test varies with time of reading. Thus the method fails on the score of objectivity.

Shockart (111) suggested the prepubertal cock (Brown Leghorn, 30 days of age) as a test animal for anterior pituitary extracts. The increase in weight of testicles due the rapid maturation of the semeniferous tubules he uses as the end-point of his test. He does not mention the pregnancy urine extracts.

Hamburger (65) observed that the injection of P.M.S. into young cockerels produced a rapid growth of the testes and comb of the injected animals.

Riddle and Polhemus (99) showed that the testes of the dove is not responsive to the injection of pregnancy urine preparations.

Evans and Simpson (49) showed that the dove testes reacts to the injection of pregnant mare's serum, but that it requires a higher number of rat units of this material than their pituitary extracts to produce the same effect.

Byerly and Burrows (23) studied the response of the one-day-old chick to pregnant mare's serum. They found a straight line relationship when the logarithm of testes weight is plotted against the logarithm of the dose. These workers consider that the response is probably little less sensitive than the ovarian weight response of the 25 day rat. They have defined the chick unit for the hormone.

Besides those discussed here, several workers have described other methods for the biological assay of the gonadotrophins from

anterior pituitary. The existence of so many methods of assay is evidence in itself that none has been particularly satisfactory. The establishment of International Units has eliminated much confusion.

IV. MALE FROG METHOD OF ASSAY:

At the risk of adding still another method to the many proposed the present author undertook the investigation of Galli-Mainini (61) reaction on male frogs as a quantitative method for the determination of the potency of commercial chorionic gonadotrophin preparations.

Galli-Mainini reaction: In 1929 Houssay and Lascano Gonzalez (74) established the relationship between the hypophysis and testes in the toad. They noted that the subcutaneous implantation of the anterior lobe of the hypophysis into the male toad (Bufo arenarum Hensel) stimulated the testes to liberate spermatozoa, which migrate into the bladder and are then excreted in the cloacal fluid. In 1945 de Robertis et al. (38) showed that chorionic gonadotrophin in the human pregnancy urine had the same effect.

In 1947 Galli-Mainini (61) used these facts as a basis for a new pregnancy test. The test depends on the discharge of spermatozoa from the testes and their detection in cloacal fluid, after the injection of human pregnancy urine in a lymph sac of the male toad (Bufo arenarum Hensel). The optimal time recommended for tapping the cloacal contents is three hours. The test has an accuracy of about 99.0%.

Shortly thereafter Miller and Wiltberger (93) and Robbins and Parker (101) showed that the same phenomenon occurred in the North American frog Rana pipiens.

Galli-Mainini (62) has made observations on the effect of urines other than those collected during pregnancy. He reports consistent negative results with urines taken from children, nonpregnant females, women past the menopause, and males. He also observed that the injection of estrogens, progesterone, testosterone, thyroxin, pitressin, insulin, adrenaline and desoxicorticosterone into toads gave negative results. His toads responded to 100 units or more of CG and 500 units or more of serum gonadotrophin. The test thus seems to have a high degree of specificity for chorionic gonadotrophin and LH.

Job (77) studied the Galli-Mainini reaction for normal pregnancy diagnosis and also some pathological cases of pregnancy, using the male frog Rana pipiens. She claims that the test has an accuracy of 95.6%.

The sensitivity of the batrachia to gonadotrophic hormone has been extensively studied and the value of these animals in pregnancy diagnostic tests is now widely recognised (100).

Robbins et al. (100) found the male South African clawed toad (Xenopus laevis) satisfactory. They report that the male African toad is ten times as sensitive as the female rat to CG.

Since frogs (Rana pipiens) are readily available in large numbers and are cheap, Hartmann and Chapman (68) decided to explore the quantitative aspects of the Galli-Mainini reaction as an assay method for the potency of gonadotrophins.

In their investigation they used 10 frogs (average weight 20 gm.), on each of four different doses, a total of 40 frogs. Each frog irrespective of weight received its dose of gonadotrophin (Follutein, Squibb) in international units in 1 ml. of water. In this preliminary work they showed that male frog Rana pipiens is a suitable animal for determining the potency of chorionic gonadotrophin. The frogs may be reused at intervals of seven days; in their work the same frogs were reused five times. The DR 50 of gonadotrophin per frog for a two hour observation period is 9.55 I.U. The average slope "b" of the integrated frequency distribution curve for the two hour observation period is 5.40.

Bedoya and Plaza (6) made use of Rana esculenta for quantitative determination of chorionic gonadotrophin. Animals weighing 30 to 35 gm. and kept at laboratory temperature were used. To study the quantity of gonadotrophin in a test liquid these workers utilized the conventional minimal effective dose method of bioassay. The male frog unit determined is 15 I.U. The male frog is unsuitable for pregnant mare's serum gonadotrophin, as no ejaculation of spermatozoa is produced even when the large amount of 100 I.U. is administered.

EXPERIMENTAL

Animals: The male frogs used in this study were of two different species, obtained from two different sources. The species Rana clamitans came from Oshkosh, Wisconsin, and the species Rana pipiens was from Alburg, Vermont. The male Rana pipiens was identified by the following sexual characteristics which are lacking in the female: stronger forearms, dark pigmentation and enlargement in the inward border of the thumb, "croaking" when grasped between fingers, and sexual reflex of the amplexus. Rana clamitans has a nearly uniform green or brownish color on dorsal surface, marked with small irregular black spots. The most conspicious feature of this species is the very large tympanum, which in the male considerably exceeds the diameter of the eye. In the female the Tympanum is considerably smaller, being about three-fourths the diameter of the eye. Besides the male has stronger forearms, and enlargement in the inward border of the thumb (73).

In Wisconsin the frogs were captured in the fall and kept in tanks with running artesian well water, the temperature of which was around 10°C. (120). The weight of the Rana clamitans used for this study varied on the average from 19 to 30 gm. The weight of Rana pipiens used varied on the average from 28 to 58 gm. After arrival the frogs were placed in a storage tank with running water (19.0° to 21.5°C); no food of any kind was given to the frogs, and because of starvation they gradually

lost weight. On the day before the assay the frogs were weighed and placed in the individual cages in the assay tank. Animals showing the "red leg" disease were discarded.

The assay tank used was so constructed as to accomodate 240 frogs and to automatically maintain a constant temperature of 20° ± 1°C. The tank was divided into eight sections, each containing thirty individual cages in groups of five compartments, each compartment having a hinged lid and open at the bottom. The water level in the individual cages was about 1 cm.

Material: The chorionic gonadotrophin used for these experiments was supplied by AYERST, McKENNA & HARRISON Ltd.; Montreal, Canada. The international standard chorionic gonadotrophin used was obtained from Food and Drugs Laboratory, Department of National Health and Welfare, Ottawa, Canada. For preliminary work Follutein (Lot#15452-2, Squibb) was employed. The chorionic gonadotrophin was preserved in the refrigerator at 8°C. Only sufficient for immediate requirements was taken from the refrigerator at a time, and the weighed material was dissolved in 50% ethyl alcohol, so that approximately 300 I.U. were contained in 1 cc. This stock solution was preserved in the refrigerator. Before injection the dilutions were made with distilled water from this stock gonadotrophin in 50% ethyl alcohol. This diluted gonadotrophin was used for injections within an hour from the time of preparation.

Spontaneous elimination of sperms: Observations were made to determine if spermatozoa were spontaneously eliminated by the frogs used in these experiments. After the arrival of a new shipment of 288 frogs, 30 frogs were placed in the assay

tank at 20°C. and the urine examined for spermatozoa. They were examined twice within a period of five days after their arrival. In this study no occasional spermatozoa (one or two per low power field) were observed in the urine (October 1949 to August 1950). This is contrary to the results obtained by Hartmann and Chapman (68), who observed occasional spermatozoa in urine (February 7th to February 12th 1949). Occasional spermatozoa found after injections of gonadotrophin are considered a negative response in this work.

Injections: Dilutions were made in such a way that each frog received its dose contained in 0.02 cc. per gm. of body weight. The reason for this was to avoid injecting too large or too small a volume of fluid. Injections were administered, starting in the dorsal skin of the leg through the connective tissue into the dorsal lymph sac. This was done to prevent leaks since the frog skin is not elastic. A 1 cc. tuberculin syringe calibrated in 1/100 cc. fitted with a #22 gauge needle was employed.

Urine smears: During assay the frogs were taken out of the assay tank, injected and left in individual cages at laboratory temperature. Two hours after injection the urine of the frog was examined. To obtain the urine the animal is removed from the cage by grasping it gently with the thumb and the second finger just behind the forelegs, ventral side toward the palm of the hand. The hind legs are then grasped with the other hand, the animal held with dorsal surface down on a clean

dry microscopic slide. The urine which is not secreted spontaneously is expressed by gentle pressure with the index finger on the ventral surface just over the cloaca.

A drop of urine thus obtained on the slide is observed under the microscope without a coverslip and with low power magnification. In this work a response is counted as positive when the low power field shows five or more spermatozoa. Each species of frogs was used five times with an interval of about six days.

Procedure: During the preliminary work which was started in January 1950, chorionic gonadotrophin (Follutein) was used to study the dose response curves. Three doses of the drug were used employing 15 animals on each group. The smears were read at 1, 2, 3, 4 hour intervals. From a series of experiments it was found that two hours after the first injection was the most suitable time to read the smears. This is in agreement with the results obtained by Hartmann and Chapman (68).

To establish the approximate potency of the preparation, several groups of five animals were injected with a graded series of doses (Table XIV). The dose which would most likely produce a positive response in fifty per cent of the animals, determined graphically, was selected as the basis for further injections.

This dose hereinafter called the DR50, is the dose which produces positive response in 50 per cent of the animals. From the DR50, doses were calculated, one to produce about twenty per cent response (DR 20), the other about eighty per cent response (DR 80). A previously constructed, tentative, integrated frequency curve for logarithm of dose/probit was used in determining the values of DR 20 and DR 80. This curve was constructed

on a two cycle semi-logarithmic graph paper. It was not expected that the doses would give exactly these responses. If they did not, as frequently happened, the accuracy of the determination was not impaired.

One group of 57 frogs was divided into three sub groups and injected with standard and another group of 57 was used for the test sample. Thus each assay required 114 frogs; standard and the test sample were injected simultaneously. This design of (3 + 3) dose assay type was used in all the assays carried out in this study.

DISCUSSION

1. Mathematical treatment of results: Much commendable work has been done in the application of statistical techniques to the design and evaluation of biological assays based on a quantal or "all-or-none" response. The earliest approach to the general problem of finding the dosage-response curve in such cases was made by Trevan (123), Gaddum (60) and Bliss (11); and further contributions to the theory have been made by Irwin (75) and Finney (53). The methods which have been used differ in detail, but all the methods agree in transforming the proportional responses into normal equivalent deviations or probits, and in finding the regression line relating them to the logarithm of the dose. It was shown that by use of these transformations the dosage response curve became approximately linear over most of its range.

A quantal response is taken to mean one in which the result of the test on any one animal can be expressed only as positive or negative. The results for analysis are therefore the number of positive reactions expressed as a percentage of the total number of reactions for any one dose of drug. Trevan (123), Gaddum (60), Bliss (14), and others have pointed out that successful treatment of such data must not only express potency by some particular dose such as DR50, but also, because of uncontrollable animal variations, must give some idea of the precision of this figure. The following three values are particularly useful: (1) The DR50, which depends on the over-all susceptibility of

the animals used, (2) the slope b, which depends upon the uniformity of the response, and (3) the error associated with the potency which depends upon the standard error and the slope b. A particularly comprehensive treatment concerning these requirements has been given by Bliss (11)(12)(13).

In the present work the liberation of spermatozoa in the male frog by different doses of gonadotrophin is an all-or-none type response, and the experimental data can be evaluated by the methods described by Bliss (11)(12), Irwin (75) and Litchfield (90). The former two methods are algebraic, while the latter one is graphical. These calculations were made on the results of the various assays listed in the Tables I to XIII.

In the following example the data of Table II is calculated to illustrate the method of Bliss (11)(12), in which three doses of International Standard were used versus U. M. Standard. The percentage of frogs reacting positive to any particular dose of gonadotrophin is plotted on the ordinates in probits on a two cycle semi-logarithmic graph paper. The probits corresponding to any percentage response are given by Bliss (11) in his Table I, page 138, and are called empirical probits. The doses in International Units are read directly on the logarithmic scale of the paper. The straight line which has the best fit by observation for the points so plotted, then is constructed. From this constructed line the value of expected probits (Y) are obtained, by reading them off from each dose level. From the empirical and expected probit at each dose level a corrected probit (y) is computed, which is used in the actual calculation of regression line. The weighing coefficient z is obtained

from Bliss (11) Table III, page 151, corresponding to the expected probit at each dose level. The dose in column 2 is multiplied by 10 in order to avoid negative logarithm of the dose. The weight (W) in column 9 is obtained by multiplying columns 1 and 8. Weighted log. dose (WX) in column 9 and weighted probit response (Wy) in column 11 are obtained by multiplying W in the columns 4 and 7 respectively. In column 12 and 13 are the weighted values of Xy and X^2 respectively. The slope of a dose-response line, relating Y to X is termed "b". M is the logarithm of the ratio of the potency of the U.M. Standard (U) to that of the International Standard (S). SM is the error of M. "bs" is the combined slope obtained by averaging b for U. and S. Ss(W) is the S(W) for the International Standard and Su(W) is the S(W) the U.M. Standard. S in all cases represents summation. The calculations were made on a "Friden" automatic calculator.

The data for International Standard from Table II were calculated as shown on the following page.

Data for assay No. II from Table II.
International Standard

1	2	3	4	5	6	7	8
n No. Frogs	Dose x 10 in I.U.	% Resp.	X Log 10 Dose	Empirical Probit	Y Expected Probit	y Corrected Probit	z Weight Coeff.
20	2.2	35.0	.3424	4.615	4.5	4.618	.581
17	3.1	70.59	.4914	5.541	5.4	5.537	.601
20	4.3	85.0	.6335	6.036	6.28	5.996	.3428

	9	10	11	12	13
	W zn	WX	Wy	WXy	WX^2
	11.62	3.9787	53.6612	18.3736	1.3619
	10.2170	5.0206	56.5715	27.7990	2.4674
	6.8560	4.3433	41.1086	26.0424	2.7513
S =	28.6930	13.3426	151.3413	72.2150	6.5804

$\bar{X} = \frac{S(WX)}{S(W)} = 0.4650$

$\bar{Y} = \frac{S(Wy)}{S(W)} = 5.2745$

$A = S(WX^2) - \bar{X}S(WX) = 0.3761$

$S(WXy) - \bar{X}S(Wy) = 1.8413$

$b = \frac{S(WXY) - \bar{X}S(Wy)}{A} = 4.8957$

$\log 10 \text{ DR50 } X = \frac{5 + b\bar{X} - \bar{Y}}{b} = 0.4089$

$S(W) = 28.6930$

$S(WX) = 13.3426$

$S(Wy) = 151.3413$

$\bar{X}S(WX) = 6.2043$

$\bar{X}S(Wy) = 70.3737$

$S(WX^2) = 6.5804$

Anti log 0.4089 = 2.564 I.U.
Correcting for multiplying by 10 in column 2 DR50 = .2564 I.U.

$\sigma b = \sqrt{\frac{1}{A}} = 1.630$

$bc = \frac{bS + bU}{2} = 5.1759$

$V(bc) = \frac{1}{As + AU} = 1.3175$

$\sigma bc = \sqrt{V(bc)} = 1.148$

M (log Potency ratio) $= \bar{X}s - \bar{X}u - \frac{1}{bc}(\bar{Y}s - \bar{Y}U) = -(0.0102)$

$= \bar{1}.9898$

Antilog $\bar{1}.9898 = 0.9768$ or 97.68 I.U.

$SM = \frac{1}{bc}\sqrt{\frac{1}{Ss(W)} + \frac{1}{Su(W)} + \frac{(\bar{Y}s-\bar{Y}u)^2\,V(bc)}{b^2c}} = 0.0507$

Log Sm at $p = 0.95 = (0.0507) \times (1.96) = 0.0993$

Anti log $0.0993 = 1.257$, or 125.7 I.U.

Antilog of recip. $(\bar{1}.9007) = .7960$, or 79.6 I.U.

Limits of Error = 79.6 to 125.7 I.U.

Fiducial limits of Potency = (79.6) (0.9768) = 77.75 I.U.

(125.7) (0.9768) = 122.78 I.U.

The U. M. Standard would be reported as 97.68 I.U. of the International Standard and 95 chances out of 100 the true potency is not less than 77.7 I.U. or more than 122.8 I.U. of this value or the fiducial limits of potency is 77.7 I.U. to 122.8 I.U.

Irwin's Method:- This method of calculation is the most precise of all the methods used. It differs from Bliss in the system of weighting used. Bliss (11) advocates obtaining an approximate line graphically and then using the weights calculated from it to obtain a further approximation. Irwin (75) suggests using the line, calculated from the observed weights as a first approximation, recalculating the weights from this line and fitting again, repeating the process until two successive approximations agree sufficiently accurately.

In this study the provisional line was fitted graphically, weights and corrected probits were computed from this line instead of using weights and probits corresponding to the actual observations in the calculation of a provisional line. Irwin's method involves a series of approximations, but with a good provisional line it is often unnecessary to repeat the process more than once, thus eliminating some tedious calculations.

In this study the results obtained were sufficiently accurate that the provisional line could be fitted graphically and the Chi square test for 2 degrees of freedom at P = 0.05 at the end of first approximation was significant. However, the results were carried to the second approximation for the sake of accuracy.

The data from table XII of U. M. Standard is calculated here to illustrate the method. The description is taken from Emmens (44). The plotting of the results is done in the same way described previously for the method of Bliss. In column 9, nw is the weight. In column 14 is the weighted value for the square of the corrected probit. For Chi square test the table IV in Fisher and Yates' (54) tables is referred for n equal to 2 degrees

of freedom. If Chi square is smaller than the value in the column for P equal to 0.05, the data must be consistent with the straight line that has been fitted, and a second approximation is not required. In the example given below the Chi square is significant at the end of 1st approximation, but the calculations are carried to the second approximation to illustrate the procedure. After having secured the "b" value for first approximation, the expected probits Y_1, Y_2, Y_3 for the second approximation are obtained by substituting the values of the corresponding log doses X_1, X_2 and X_3 in the equation as shown below. Using these new expected probits, new corrected probits and new weights are computed for the second approximation using the tables given by Bliss (11) as described before. The rest of the calculations are the same as described for the 1st approximation. M, the logarithm of the potency ratio of the test sample to the U. M. Standard is determined in the same way as for the method of Bliss. The SM, the standard error of M is determined in the same manner as described before. The calculations are shown on the following page.

Data for assay No. XII from Table XII
U. M. Standard, first approximation

1	2	3	4	5	6	7	8
n No. of frogs	Dose x 10 in I.U.	% Resp.	X Log_{10} Dose	Empirical Probit	Y Expected Probit	Yc Corrected Probit	w Weight Coeff.
20	7	30	0.8451	4.476	4.19	4.5072	.4998
17	11	58.82	1.0414	5.223	5.35	5.2195	.6085
20	17	90	1.2304	6.282	6.48	6.2494	.2756

	9	10	11	12	13	14
	nw Weight	nwX	nwYc	nwXYc	nwX^2	$nwYc^2$
	9.996	8.4476	45.0539	38.0750	7.1391	203.0667
	10.3445	10.7727	53.9409	56.2281	11.2186	281.8173
	5.5120	6.7819	34.4467	42.3828	8.3446	215.2711
S =	25.8525	26.0022	133.4415	136.6859	26.7023	700.1551

$\bar{X} = \frac{S(nwX)}{S(nw)} = 1.0057$

$\bar{Y}c = \frac{S(nwYc)}{S(nw)} = 5.1616$

$Snwx^2 = SnwX^2 - \bar{X}SnwX = 0.5519$

$Snwyc^2 = SnwYc^2 - \bar{Y}cSnwYc = 11.3799$

$Snwxyc = SnwXYc - \bar{Y}cSnwX = 2.4730$

$b = \frac{Snwxyc}{Snwx^2} = 4.4808$

$\bar{X}SnwX = 26.1504$

$\bar{Y}cSnwx = 134.2129$

$\bar{Y}cSnwYc = 688.7716$

$\chi^2 = Snwyc^2 - b(SnwxYc) = 0.2989$

$Y_1 = \bar{Y}c + b\ (X_1 - \bar{X}) = 4.442$

$Y_2 = \bar{Y}c + b\ (X_2 - \bar{X}) = 5.3216$

$Y_3 = \bar{Y}c + b\ (X_3 - \bar{X}) = 6.1684$

Data for assay No. XII
U. M. Standard, second approximation

1	2	3	4	5	6
n	X	Empirical Probit	Y	Yc	w
20	0.8451	4.476	4.442	4.4765	.5676
17	1.0414	5.223	5.3216	5.2209	.6127
29	1.2304	6.282	6.1684	6.2735	.3811

7	8	9	10	11	12
nw	nwX	nwYc	nwXYc	nwX^2	$nwYc^2$
11.3520	9.5936	50.8172	42.9457	8.1076	227.4827
10.4159	10.8471	54.3804	56.6316	11.2960	283.9145
7.6220	9.3781	47.8166	58.8335	11.5389	301.5518
29.3899	29.8188	153.0142	158.4108	30.9425	812.9490

$\bar{X} = \frac{S(nwX)}{S(nw)} = 1.0145$

$\bar{Y}c = \frac{S(nwYc)}{S(nw)} = 5.2063$

$Snwx^2 = SnwX^2 - \bar{X}SnwX = .6914$

$Snwyc^2 = SnwYc^2 - \bar{Y}cSnwYc = 16.3112$

$SnwxYc = SnwXYc - \bar{Y}cSnwX = 3.1652$

$b = \frac{Snwxyc}{Snwx^2} = 4.5779$

$\bar{X}SnwX = 30.2511$

$\bar{Y}cSnwX = 155.2456$

$\bar{Y}cSnwYc = 796.6378$

$\chi^2 = Snwyc^2 - bS\,(nwXyc) = 1.8213$

$\sigma b = \sqrt{\frac{1}{Snwx^2}} = 1.150$

$$bc = \frac{bUM + bTS}{2} = 5.1967$$

$$V(bc) = \frac{1}{Snwx^2UM} + \frac{1}{Snwx^2TS} = 0.8333$$

$$\sigma bc = \sqrt{V(bc)} = .9128$$

$$M = \bar{X}(UM) - \bar{X}(TS) - \frac{1}{bc}(\bar{Y}UM - \bar{Y}TS) = 0.0253$$

Antilog 0.0253 = 1.060 or 106.0 I.U.

∴ Test Sample is 106.0 I.U. of U. M. Standard

∴ The test sample is (106 x (1.01) = 107.06 I.U. of Int. Std.

$$SM = \frac{1}{bc}\sqrt{\frac{1}{SUnw} + \frac{1}{STnw} + \frac{(\bar{Y}UM-\bar{Y}TS)^2 V(bc)}{bc^2}} = 0.0528$$

Log Sm at p = 0.95 = (0.0528) x (1.96) = 0.1034

Antilog 0.1034 = 1.269, or 126.9 I.U.

Antilog recip. ($\bar{1}$.8966) = .788, or 78.8 I.U.

Limits of Error = 78.8 to 126.9 I.U.

Fiducial limits of Potency = (78.8) x (1.0706) = 84.3 I.U.

= (126.9) x (1.0706) = 135.8 I.U.

The test sample would be reported as 107.06 I.U. of International Standard and 95 chances out of 100 the true potency is not less than 84.3 I.U. or more than 135.8 I.U. of this value.

Litchfield's Method:- Recently a rapid graphical method has been reported by Litchfield (90) for evaluating dose-effect experiments. The data from Table II for the International Standard is illustrated as an example.

The logarithmic - probability paper designed by Hazen, Whipple and Fuller (No. 3128, Codex Book Co., Inc. Norwood, Mass.) is used for plotting the data. The dose is plotted directly

against percent effect. Then with a transparent ruler the line of best fit is drawn. From the line on the graph the dose for 16, 50 and 84 percent effects (ED16, ED50, and ED84) are read. The slope function S is then calculated using the equation

$$S = \frac{ED84/ED50 + ED50/ED16}{2}$$

N' is the total number of animals tested at those doses whose expected effects were between 16 and 84 percent. fED50 is a factor for ED50. P.R. is the potency ratio. The slope "b" of the regression line is determined by taking the reciprocal of the logarithm of the slope function S. The calculations are shown on the following page.

Data for assay No. II from Table II
International Standard

$ED16 = 0.158$

$ED50 = 0.25$

$ED84 = 0.396$

$N' = 37$

$S = \frac{ED84}{ED50} + \frac{ED50}{ED16} = 1.5831$

$b = \frac{1}{\log S} = \frac{1}{\log .15831} = 5.012$

$fED50 = S^{2.77/\sqrt{N'}} = S^{Exponent}$

$= S^{0.455}$

$fED50 = 1.230$ (From nomograph #2)

$PR = \frac{ED50_1 \text{ of Int. Std}}{ED50_2 \text{ of U.M. Std}} = \frac{0.25}{0.26} = 0.9615$ or 96.15 I. U.

$fPR = 1.33$ (Using $fED50_1$ and $fED50_2$ read fP.R. from the center scale of Nomograph #4)

The Fiducial limits of Potency ratio are given by

P.R. x fP.R. = upper
P.R./fP.R. = lower
} limit for 19/20 Probability

(.9615) x (1.33) = 127.8 I.U.

(.9615)/1.33 = 72.3 I.U.

2. Comparison of results with rat method of assay: For this study four different samples of chorionic gonadotrophin were kindly supplied by Ayerst, McKenna & Harrison Ltd., Montreal, Canada with the potency for each sample as determined in their laboratory by the immature female rat vaginal smear method as described on page 18. These samples were assayed using the male frog method developed in this work (Tables I, II, XI, VIII, X, IX, XII), and the potency compared with that of the results obtained on the rat assays (Table XIII).

The U. M. Standard (A. Mck. & H. Lot #54372) was assayed three times by the frog method in comparison with the International Standard and its average potency was found to be 101.0 international units per milligram. The U. M. Standard was employed as a reference standard in the remainder of the work reported here. Because of the accuracy of the calculations the results obtained by Irwin's method (75) were used in comparing potencies.

The potency of sample #54372 determined on frogs showed a difference of 41.61% from that of the potency determined on rats, while the sample #58540 showed a difference of 45.43%. The sample #859822 showed a difference of 42.97%. The results on these samples obtained by using frogs showed a very close agreement with those obtained by the rat method. Sample #11414 showed a difference in potency by 432.4%. Only one assay was done on frogs on this Lot #11414 because of lack of sufficient sample. If more assays had been done the deviation of 32.4% probably could be reduced. This lot had been greatly diluted with lactose and if the mixture were not uniform the difference between frog and rat methods could be as great as observed.

Except for Lot #11414 there is a very good agreement between the proposed frog method and commonly accepted rat vaginal smear method (Table XIII). It will be noted that the proposed frog method always gave a slightly higher result than the vaginal smear method. This difference however is not significant; it will require many more assays to determine if this positive difference is significant.

The fiducial limits of the potency and the 'b' value on rat assays were not determined, hence these data were not included in Table XIII.

No comparison was made with assays on rats for the two commercial samples of Follutein (Lot #15452-2 and Lot #9G10072). The average potency for three assays determined on frogs for sample #15452-2 was 118.2 international units per milligram, and the average potency for sample #9G10072 from two assays was 119.1 international units per milligram. Generally commercial samples of chorionic gonadotrophin are 10-15% stronger than the labelled potency; this is in close agreement with the results obtained on the frog assays for the above two samples of Follutein.

3. Weight of frogs: It was observed during this work that the average weight of the frogs decreases with the number of times used. The frogs Rana clamitans showed an average loss in weight of 24.3% after being used six times (Table I and VII), while the frogs Rana pipiens showed an average loss in weight of 41.8% after being used five times (Table VIII and XII). The frogs were kept without food under constant conditions of environment and temperature. It can not be said definitely that the loss

of weight is due to the injection of chorionic gonadotrophin. It was observed by Hartmann and Chapman (68) that frogs kept under similar conditions to those injected with chorionic gonadotrophin do not lose weight to the same extent as lost by the injected frogs over the same period of time.

In the present study healthy Rana pipiens with an average weight at the beginning of the work of 37 gm. were used. It was observed that the DR 50 did not vary significantly with the loss of weight of the animal (Table VIII and XI). Several assays were done using Rana clamitans with an average weight of 29 gm. at the beginning (Tables I to VII). Here also it was noticed that there was no significant change in DR 50 with loss of weight (Table IV and VII). This is contrary to the results obtained by Hartmann and Chapman (68) that the DR 50 generally increases as the animal becomes smaller.

4. Number of times used and interval between use: In the present study, Rana clamitans was used six times at an interval of 6-7 days (Tables I to VII). It can be seen from these tables that there is no significant change in the DR 50. The b value when the animals are used at an interval of six to seven days may vary or may remain constant. Rana pipiens were used five times for assaying samples of chorionic gonadotrophin. Those used for assay No. IX (Table IX) were used again after an interval of three days, assay No. X (Table X), while the rest of the assays were performed at an interval of six days. The results of assay No. X does not show much variation in b value from assay No. IX. The potency of test sample, A.Mck. & H.Lot#58540, determined in assay No. X is 958 I. U. per mg., while the potency for

the same sample determined when rats were used was 921 I. U. per mg. This shows that Rana pipiens used for the second time after a three day interval gives comparable results to the rat vaginal smear assay, but this should be confirmed by more assays using the same time interval. Although frogs after having been used five times lose considerable weight, they still give consistent results for the potency of the test sample (Table VI an VII, XI).

5. Seasonal Variation: The frogs used in this study were kept approximately at 20°C, and isolated from the females. Under these conditions it might be expected that the pituitary would not be excited to produce gonadotrophin which would be additive to the action of injected gonadotrophin. However, as the frogs approach the breeding season (March to April), one might expect that the anterior pituitary gland would increase its activity. This is apparently the case as can be determined from the values of DR 50 obtained in the preliminary work on the species Rana pipiens using Follutein.

From January 1, 1950 to February 26, 1950, it was observed that the DR 50 varied from 0.95 I. U. to 1.05 I. U. per gm. frog weight. Suddenly at the end of February the value of DR 50 dropped to 0.57 I. U. From the beginning of March to the middle of April (which corresponds to the breeding season) the value of DR 50 varied from 0.40 I.U. to 0.57 I.U. An increase in the value of DR 50 to 0.85 I.U. was observed in the beginning of May. From the latter half of May through the rest of the summer up to 21st of September the DR 50 varied from 0.93 I.U. to 1.1 I.U. Thus during winter and summer the DR 50 is approximately one I.U.

(0.93 - 1.1), there is a sudden drop in DR 50 at the beginning of the breeding season to half this value, (i.e. 0.4 to 0.57 I.U.), rising again to a high value after the end of the breeding season.

Sampson (B) studied the seasonal variation in the sensitivity of the frog, Rana pipiens, to chorionic gonadotrophin. According to him this species is quite sensitive to chorionic gonadotrophin during the autumn, winter and spring season. In summer, the animal is relatively insensitive and responds to five times as much gonadotrophin. This is contrary to the observations in this study, where it is shown that the value of DR 50 during spring (the breeding season) drops to approximately half of its normal range observed during winter and summer. It was observed by Hartmann and Chapman (68) that the uniformity of the animals during the breeding period remains approximately constant, because the slope, "b", of the characteristic curves are not significantly different.

6. DR 50 and b value: The uniformity of a group of frogs in their response to chorionic gonadotrophin is expressed by the slope of the integrated curve or the "b" value. The value for b is a measure of the individual variation in the test animal, the greater the value of b the less the individual variation. DR 50 is the best value to represent the sensitivity of a group of animals.

The average b value with standard deviation for assays done on Rana clamitans was 5.464 ± 1.35 (Tables I to VII), and the average b value for all the assays done on Rana pipiens (Tables VIII to XII) was 4.72 ± 0.654. Thus Rana clamitans

shows a statistically significant greater uniformity of response as shown by the higher average slope than _Rana pipiens_. The individual b values for both standard and test sample along with their individual standard deviation (σb) are given in Tables I to XII. The σb for individual slopes varies from 2.4 to 1.16 for _Rana clamitans_, while it varies from 1.2 to 1.14 for Rana pipiens. This change in the slope of the curve (σb) for the same species of frog from time to time indicates that the uniformity of response to chorionic gonadotrophin varies significantly. Therefore a "Standard" or "Characteristic" curve (b value) cannot be employed for this assay. The three dose method for both standard and sample is to be preferred. By this procedure b for standard and sample is calculated for each assay.

During summer the DR 50 for U.M. Standard varied from 0.225 I.U. to 0.256 I.U. on _Rana clamitans_, while on _Rana pipiens_ for the same standard it varied from 0.93 I.U. to 1.08 I.U. There is therefore a small, statistically not significant shift in the sensitivity of both species to chorionic gonadotrophin during summer. However the difference in sensitivity between both species is quite marked (statistically significant). The average DR 50 for _Rana clamitans_ was 0.242 I.U. and 1.06 I.U. for _Rana pipiens_. Therefore in employing this method of assay a constant DR 50 cannot be assumed for each species of frog.

Rana clamitans were used in this study from the middle of June to the end of July, the seasonal variation in _Rana clamitans_ remains to be studied.

7. _Accuracy of the methods of calculation:_ In this study the methods of Bliss (11), Irwin (75), and Litchfield (90) are used for calculating the results of assays (Tables I to XII).

The former two methods are algebraic and very precise. The latter method is graphical and rapid (10 - 15 minutes), its accuracy is commensurate with the nature of the dose - per cent effect data. Referring to results of calculations by the above three different methods (Tables I to XII), it appears that the results calculated by Litchfield's method are good and very close to those calculated by the other two supposedly more accurate methods. By Litchfield's (90) method the following values can be calculated in a short time. DR 50, b value and their fiducial limits. The experimental data are used throughout this method in their original form without transforming to logarithms and probits. Means are provided for plotting and using zero and hundred per cent effects. The calculations are simplified by use of nomographs. The method provides for the rapid test of parallelism of both curves and easy computation of the relative potency with its fiducial limits.

The methods of Bliss (11) and Irwin (75) while more accurate, are so time consuming and laborious that the busy experimenter is deterred from using them. From the view point of many non-mathematically minded biologists, such procedures are not pleasant to contemplate because the calculations are difficult, tedious, and often quite incomprehensable. To do away with all these difficulties Litchfield's method serves the purpose admirably.

8. Advantages and accuracy of the frog method: The proposed frog method developed in this study has the advantage of being rapid, cheap and accurate, as compared to the generally employed rat vaginal smear method. The rat method usually takes 96 hours for an assay, and requires considerable experience for reading

the vaginal smears; it is time consuming and laborious. It takes about 7 hours to complete an assay using the male frogs, which includes the time for weighing the animals and reading the smears. The end point is quite simple and requires very little experience to determine the presence of spermatozoa.

In the rat method 19-21 days old immature female rats are used, and these cannot be used again for another assay. This makes the assay expensive. The male frogs can be used five times which reduces the cost of the assay. The frogs are easier to get and are very cheap compared to rats. Fiducial limits of potency are consistent with good bioassay procedure, and approximately the same as rat vaginal smear method. With much less consumption of time and money, one can reduce the fiducial limits of the potency by employing a greater number of frogs on each dose.

Another advantage of the frog method of assay is that it does not respond to oestrogens which may be present as contaminants in samples of chorionic gonadotrophin. The frog method can also be used to determine the amount of chorionic gonadotrophin quantitatively in a mixture containing oestrogens. The rat vaginal smear method will not differentiate chorionic gonadotrophin and oestrogens.

The disadvantage of the frog method is that it requires large quantities of gonadotrophin compared to the rat method. One I.U. is necessary to obtain a positive vaginal smear in the rat and one I.U./gm. frog (<u>Rana pipiens</u>) will give a positive response; for an average frog of 30 gm. 30 times as much gonadotrophin is required than for the rat. <u>Rana clamitans</u> requires during the summer 1/4 the quantity of hormone as that required by <u>Rana pipiens</u>. Therefore the use of the <u>Rana clamitans</u> in place

of *Rana pipiens* will reduce the quantity of chorionic gonado-
trophin required without impairment of the accuracy of the test.

SUMMARY

1. A new method of assay for chorionic gonadotrophin using Galli-Mainini reaction on male frog is described.

2. A group of 57 frogs divided into three sub groups is injected with standard and another group of 57 is used for the test sample. Thus each assay requires 114 frogs; standard and test sample are injected simultaneously. This design of (3 + 3) dose assay type is used in all the assays.

3. Dose of chorionic gonadotrophin injected into dorsal lymph sac, is contained in 0.02 cc. of water per gm. frog weight. The end point is indicated by the presence of spermatozoa, two hours after injection.

4. Results are computed mathematically and graphically, and DR 50, slope (b) of the integrated frequency curves, its standard deviation, σ b, for individual assays are calculated.

5. Results are calculated by using methods of Bliss, Irwin, and Litchfield; close agreement is seen in results obtained by these methods.

6. Two different species of frogs - Rana clamitans and Rana pipiens can be conveniently used for the purpose of determining potency. The same results are obtained with either species of frog.

7. The suitability of this method of assay is demonstrated by estimation of the potencies of unknown test sample of chorionic gonadotrophin as compared with a reference standard. The fiducial limits of potency are consistent with good bioassay procedure and approximate those determined by rat

vaginal smear method.

8. Comparison is made between the potencies of the test samples as determined by the proposed frog method and rat vaginal smear method.

9. An assay can be conveniently completed in one day by this method.

10. In the interest of economy it is demonstrated, that both species of frogs can be used five times at an interval of six days. Although frogs after having been used five times lose considerable weight, they still give consistent results for the potency of the test sample.

11. The average b value with its standard deviation, for assays done on Rana clamitans is 5.464 ± 1.35, and the average b value for all assays done on Rana pipiens is 4.72 ± 0.654.

12. In both species of frog the loss of weight, and number of times used during the summer, have no significant change in the value of DR 50. However the difference in sensitivity between both species is quite marked (statistically significant).

13. Seasonal variation in Rana pipiens to chorionic gonadotrophin is studied. During winter and summer the DR 50 is approximately one International Unit (0.93 to 1.1 I.U.); there is a sudden drop in DR 50 at the beginning of the breeding season to half this value (i.e. 0.4 to 0.57 I.U.), rising again to a high value after the end of the breeding season.

14. The proposed method has the advantage of being rapid, accurate and cheap.

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Table I

International Standard vs. U. M. Standard (A.Mck. & H.Lot#54372)
Chorionic Gonadotrophin

Assay I

Frog Species - Rana clamitans

Date - June 20, 1950

Times used - First

International Standard			U. M. Standard		
Ave. Wt. in Gm. of frogs	Dose in I.U. per gm. frog	No. positive out of total injected	Ave. Wt. in Gm. of frogs	Dose in I.U. per gm. frog	No. positive out of total injected
30.1	0.32	10/20	26.3	0.32	11/20
27.3	0.45	15/17	28.0	0.45	14/17
25.2	0.60	20/20	31.1	0.60	20/20

Results calculated by following methods

	Bliss	Irwin (2nd Approx.)	Litchfield
bS		8.8894	8.13
bUM		7.3551	8.071
σbS		2.424	-
σbUM		2.01	-
bc		8.0483	-
σbc		1.630	-
DR50 S		-	0.316
DR50 UM		-	0.326
Potency of U.M. Std. in I.U./mg.		99.38	96.93
Fiducial limits of Potency in I.U./mg.		83.63 to 118.06	77.5 to 121.1

Table II

International Standard vs. U. M. Standard
Chorionic Gonadotrophin

Assay II — Frog Species - Rana clamitans

Date - June 22, 1950 — Times used - First

International Standard			U. M. Standard		
Ave. Wt. in Gm. of frogs	Dose in I.U. per gm. frog	No. positive out of total injected	Ave. Wt. in Gm. of frogs	Dose in I.U. per gm. frog	No. positive out of total injected
30.2	0.22	7/20	26.3	0.22	6/20
27.3	0.31	12/17	28.0	0.31	12/17
23.2	0.43	17/20	31.3	0.43	17/20

Results calculated by following methods

	Bliss	Irwin (2nd Approx.)	Litchfield
bS	4.8957	4.9924	5.012
bUM	4.4510	5.5050	5.005
σbS	1.630	1.5414	-
σbUM	1.613	1.5423	-
bc	5.1759	5.2485	-
σbc	1.148	1.0902	-
DR50 S	0.2564	-	0.25
DR50 UM	0.2658	-	0.26
Potency of U.M. Std. in I.U./mg.	97.68	97.6	96.15
Fiducial limits of Potency in I.U./mg.	77.75 to 122.7	78.3 to 121.5	72.3 to 127.0

Table III

U. M. Standard vs. Test Sample (Follutein Lot #15452-2)
Chorionic Gonadotrophin

Assay III

Date - June 28, 1950

Frog Species - Rana Clamitans

Times used - Second

U. M. Standard			Test Sample		
Ave. Wt. in Gm. of frogs	Dose in I.U. per gm. frog	No. positive out of total injected	Ave. Wt. in Gm. of frogs	Dose in I.U. per gm. frog	No. positive out of total injected
26.6	0.17	4/20	24.4	0.17	5/20
27.5	0.26	10/17	26.7	0.28	12/17
26.0	0.38	19/20	28.7	0.38	18/20

Results calculated by following methods

		Bliss	Irwin (2nd Approx.)	Litchfield
	bUM	6.8684	6.8929	6.7613
	bTS	5.5801	5.7260	6.747
	σ bUM	1.474	1.5444	-
	σ bTS	1.367	1.3905	-
	ρ bc	6.1752	6.2486	-
	σ bc	1.0001	1.0334	-
	DR50 UM	0.2307	-	0.234
	DR50 TS	0.2192	-	0.226
A	Potency of T.S. in terms of U.M. Std. in I.U./mg.	103.5	104.25	103.54
B	Potency in terms of Int. Std. in I.U./mg. (1.01 x Value of A)	104.5	105.3	104.57
C	Fiducial limits of Potency in I.U./mg.	85.4 to 127.6	86.13 to 128.8	82.3 to 129.4

Table IV

U. M. Standard vs. Test Sample (Follutein, Lot #9010072)
Chorionic Gonadotrophin

<u>Assay IV</u> — Frog Species - Rana Clamitans

Date - July 3, 1950 — Times used - Third

U. M. Standard			Test Sample		
Ave. Wt. in Gm. of frogs	Dose in I.U. per gm. frog	No. positive out of total injected	Ave. Wt. in Gm. of frogs	Dose in I.U. per gm. frog	No. positive out of total injected
24.6	0.17	4/20	20.7	0.17	8/20
23.9	0.26	10/17	23.8	0.26	13/17
22.5	0.38	17/20	26.6	0.38	18/20

Results Calculated by following methods

	Bliss	Irwin (2nd Approx.)	Litchfield
bUM	5.0775	5.3971	4.11
bTS	4.2634	4.5169	4.15
σ bUM	1.40	1.3381	-
σ bTS	1.509	1.3497	-
bc	4.8528	4.9608	-
σ bc	1.006	0.9503	-
DR50 UM	0.2411	-	0.255
DR50 TS	0.1888	-	0.204
A Potency of T.S. in terms of U.M. Std. in I.U./mg.	124.3	124.2	125.0
B Potency in terms of Int. Std. in I.U./mg. (1.01 x Value of A)	125.5	125.4	126.25
C Fiducial limits of Potency in I.U./mg.	97.1 to 161.6	97.9 to 160.3	87.4 to 178.0

Table V

U. M. Standard vs. Test Sample (Follutein, Lot #9010072)
Chorionic Gonadotrophin

<u>Assay V</u> — Frog Species - Rana Clamitans

Date - July 7, 1950 — Times used - Fourth

U. M. Standard			Test Sample		
Ave. Wt. in Gm. of frogs	Dose in I.U. per gm. frog	No. positive out of total injected	Ave. Wt. in Gm. of frogs	Dose in I.U. per gm. frog	No. positive out of total injected
26.8	0.17	3/20	25.3	0.17	4/20
23.9	0.26	10/17	21.5	0.26	11/17
24.0	0.38	14/20	23.2	0.38	16/20

Results calculated by following methods

		Bliss	Irwin (2nd Approx.)	Litchfield
	bUM	4.2175	4.4511	4.327
	bTS	4.7778	4.8994	4.430
	σbUM	1.395	1.2787	-
	σbTS	1.380	1.2964	-
	bc	4.4997	4.6722	-
	σbc	0.9828	0.9104	-
	DR50 UM	0.2685	-	0.27
	DR50 TS	0.2404	-	0.238
A	Potency of T.S. in terms of U.M. Std. in I.U./mg.	111.9	111.7	113.4
B	Potency in terms of Int. Std. in I.U./ mg. (1.01 x Value of A)	113.0	112.8	114.5
C	Fiducial limits of Potency in I.U./mg.	86.2 to 148.0	87.8 to 144.7	83.4 to 154.2

Table VI

U. M. Standard vs. Test Sample (Follutein, Lot #15452-2)
Chorionic Gonadotrophin

Assay VI — Frog Species - Rana Clamitans

Date - July 14, 1950 — Times used - Fifth

U. M. Standard			Test Sample		
Ave. Wt. in Gm. of frogs	Dose in I.U. per gm. frog	No. positive out of total injected	Ave. Wt. in Gm. of frogs	Dose in I.U. per gm. frog	No. positive out of total injected
21.6	0.17	6/20	22.3	0.17	8/20
19.1	0.26	10/17	22.0	0.26	12/17
22.5	0.38	17/20	22.5	0.38	18/20

Results calculated by following methods

	Bliss	Irwin (2nd Approx.)	Litchfield
bUM	4.4545	4.4360	4.430
bTS	4.4009	4.3778	4.410
σbUM	1.283	1.280	-
σbTS	1.330	1.332	-
bc	4.4288	4.4075	-
σbc	0.9237	0.9241	-
DR50 UM	0.2257	-	0.226
DR50 TS	0.1945	-	0.1950
A Potency of T.S. in terms of U.M. Std. in I.U./mg.	116.6	115.8	115.9
B Potency in terms of Int. Std. in I.U./mg. (1.01 x Value of A)	117.7	116.95	117.06
C Fiducial limits of Potency in I.U./mg.	90.0 to 153.7	89.3 to 152.9	84.6 to 158.7

Table VII

U. M. Standard vs. Test Sample (Follutein, Lot #15452-2)
Chorionic Gonadotrophin

<u>Assay VII</u>

Frog Species - Rana Clamitans

Date - July 21, 1950

Times used - Sixth

U. M. Standard			Test Sample		
Ave. Wt. in Gm. of frogs	Dose in I.U. per gm. frog	No. positive out of total injected	Ave. Wt. in Gm. of frogs	Dose in I.U. per gm. frog	No. positive out of total injected
22.2	0.14	3/20	18.0	0.14	4/20
18.6	0.21	8/17	21.6	0.21	10/17
22.4	0.34	15/20	21.3	0.34	16/20

Results calculated by following methods

	Bliss	Irwin (2nd Approx.)	Litchfield
bUM	4.3696	4.7276	4.54
bTS	4.2079	4.3274	5.08
σbUM	1.192	1.172	-
σbTS	1.20	1.163	-
bc	4.2894	4.5125	-
σbc	0.8465	1.140	-
DR50 UM	0.2319	-	0.235
DR50 TS	0.1865	-	0.207
A Potency of T.S. in terms of U.M. Std. in I.U./mg.	112.8	113.5	113.5
B Potency in terms of Int. Std. in I.U./mg. (1.01 x Value of A)	113.9	114.6	114.6
C Fiducial limits of Potency in I.U./mg.	86.4 to 149.8	88.8 to 154.3	85.3 to 150.1

Table VIII

U. M. Standard vs. Test Sample (A.Mck. & R. Lot #58540)
Chorionic Gonadotrophin

Assay VIII — Frog Species - Rana pipiens

Date - September 1, 1950 — Times used - First

U. M. Standard			Test Sample		
Ave. Wt. in Gm. of frogs	Dose in I.U. per gm. frog	No. positive out of total injected	Ave. Wt. in Gm. of frogs	Dose in I.U. per gm. frog	No. positive out of total injected
36.9	0.7	3/20	34.9	0.7	3/20
34.3	1.1	10/17	35.2	1.1	11/17
36.4	1.7	16/20	35.4	1.7	17/20

Results calculated by following methods

		Bliss	Irwin (2nd Approx.)	Litchfield
	bUM	4.8533	4.8557	4.10
	bTS	5.3915	5.4099	4.63
	σ bUM	1.206	1.209	-
	σ bTS	1.214	1.258	-
	bc	5.1206	5.1219	-
	σ bc	0.8537	0.8714	-
	DR50 UM	1.083	-	1.1
	DR50 TS	1.028	-	1.04
A	Potency of T.S. in terms of U.M. Std. in I.U./mg.	105.5	105.4	105.7
B	Potency in terms of Int. Std. in I.U./mg. (1.01 x Value of A)	106.5	106.45	106.7
C	Fiducial limits of Potency in I.U./mg.	84.5 to 134.0	84.3 to 137.27	76.5 to 145.8

Note: Original sample was diluted 9 times for the purpose of this assay, therefore 106.45 x 9 = 958.05 I.U. per mg. of Test Sample.

Table IX

U. M. Standard vs. Test Sample (A.Mck. & H. Lot#11414)
Chorionic Gonadotrophin

Assay IX

Frog Species - Rana pipiens

Date - September 2, 1950

Times used - First

U. M. Standard			Test Sample		
Ave. Wt. in Gm. of frogs	Dose in I.U. per gm. frog	No. positive out of total injected	Ave. Wt. in Gm. of frogs	Dose in I.U. per gm. frog	No. positive out of total injected
36.9	0.7	3/20	30.8	0.7	6/20
34.3	1.1	10/17	32.8	1.1	12/17
36.4	1.7	16/20	32.6	1.7	18/20

Results calculated by following methods

		Bliss	Irwin (2nd Approx.)	Litchfield
	bUM	4.8533	4.8557	4.10
	bTS	4.7600	4.3503	4.36
	σ bUM	1.206	1.209	-
	σ bTS	1.261	1.247	-
	bc	4.8087	4.6110	-
	σ bc	0.8714	0.8680	-
	DR50 UM	1.083	-	1.1
	DR50 TS	0.8851	-	0.89
A	Potency of T.S. in terms of U.M. Std. in I.U./mg.	122.5	123.64	123.6
B	Potency in terms of Int. Std. in I.U./mg. (1.01 x Value of A)	123.5	124.8	124.8
C	Fiducial limits of Potency in I.U./mg.	95.8 to 158.8	95.34 to 160.1	87.04 to 175.5

Note: The dilution of Test Sample was 1/5 as much as that for the Standard because the Test Sample had 1/5 the potency of the Standard, therefore $\frac{124.8}{5} = 24.9$ I.U. per mg. of Test Sample

Table X

International Standard vs. Test Sample (A.Mck. & H.Lot#58540)
Chorionic Gonadotrophin

Assay X

Frog Species - Rana pipiens

Date - September 4, 1950

Times used - Second

International Standard			Test Sample		
Ave. Wt. in Gm. of frogs	Dose in I.U. per gm. frog	No. positive out of total injected	Ave. Wt. in Gm. of frogs	Dose in I.U. per gm. frog	No. positive out of total injected
31.6	0.7	2/20	37.3	0.7	4/20
30.1	1.1	9/17	38.5	1.1	10/17
33.3	1.7	15/20	36.1	1.7	15/20

Results calculated by following methods

	Bliss	Irwin (2nd Approx.)	Litchfield
bS	4.9091	4.9265	4.20
bTS	3.5549	3.9129	4.12
σbS	1.298	1.220	-
σbTS	1.276	1.163	-
bc	4.2202	4.3764	-
σbc	0.9097	0.8250	-
DR50 S	1.179	-	1.20
DR50 TS	1.080	-	1.1
Potency of T.S. in I.U./mg.	109.4	109.5	109.0
Fiducial limits of Potency in I.U./mg.	81.7 to 146.3	84.07 to 144.5	78.9 to 150.4

Note: Original sample was diluted 9 times for the purpose of this assay, therefore 109.5 x 9 = 985.5 I.U. per mg. of Test Sample.

Table XI

International Standard vs. U. M. Standard
Chorionic Gonadotrophin

Assay XI

Frog Species - Rana pipiens

Date - September 16, 1950

Times used - Fourth

International Standard			U. M. Standard		
Ave. Wt. in Gm. of frogs	Dose in I.U. per gm. frog	No. positive out of total injected	Ave. Wt. in Gm. of frogs	Dose in I.U. per gm. frog	No. positive out of total injected
32.5	0.7	4/20	33.0	0.7	5/20
27.6	1.1	9/17	34.7	1.1	10/17
28.6	1.7	16/20	33.9	1.7	16/20

Results calculated by following methods

	Bliss	Irwin (2nd Approx.)	Litchfield
bS	4.7751	4.3402	4.325
bUM	3.9368	3.9387	4.382
σbS	1.165	1.160	-
σbUM	1.170	1.146	-
bc	4.3580	4.1297	-
σbc	0.8231	0.8292	-
DR50 S	1.086	-	1.08
DR50 UM	1.014	-	1.02
Potency of U.M. Std. in I.U./mg.	106.5	106.1	105.9
Fiducial limits of Potency in I.U./mg.	81.65 to 138.87	79.85 to 140.9	80.2 to 139.7

Table XII

U. M. Standard vs. Test Sample (A.Mck. & H.Lot B59822)
Chorionic Gonadotrophin

Assay XII — Frog Species - Rana pipiens

Date - September 21, 1950 — Times used - Fifth

U. M. Standard			Test Samples		
Ave. wt. in Gm. of frogs	Dose in I.U. per gm. frog	No. positive out of total injected	Ave. wt. in Gm. of frogs	Dose in I.U. per gm. frog	No. positive out of total injected
31.2	0.7	6/20	29.2	0.7	5/20
30.9	1.1	10/17	29.0	1.1	12/17
32.9	1.7	18/20	32.2	1.7	19/20

Results calculated by following methods

	Bliss	Irwin (2nd Approx.)	Litchfield
bUM	4.5004	4.5779	4.30
bTS	6.0428	6.0379	4.58
σ bUM	1.346	1.150	-
σ bTS	1.412	1.1403	-
bc	5.2350	5.1967	-
σ bc	0.9740	0.9128	-
ED50 UM	0.9328	-	0.90
ED50 TS	0.9016	-	0.84
Potency of T.S. in terms of U.M. Std. in I.U./mg.	105.9	106.0	107.1
Potency in terms of Int. Std. in I.U./mg. (1.01 x Value of A)	106.95	107.06	108.1
Fiducial limits of Potency in I.U./mg.	83.15 to 134.81	84.3 to 135.8	77.6 to 147.7

Note: Original sample was diluted 5.5 times for the purpose of this assay, therefore 107.06 x 5.5 = 588.5 I.U. per mg. of Test Sample.

Table XIII

Comparison of the potency of chorionic gonadotrophin as determined by using male frogs and female rats.

	Potency in International Units per milligram			
	No. of times assayed	Male frog method	Rat method	Percent deviation of frog potency from rat potency
A.Mck. & H. Lot.#54372	1	99.4	100.0	
	2	97.6	98.8	
	3	106.1	-	
Average		101.03	99.4	+1.61
A.Mck. & H. Lot#58540	1	958.0	1000.0	
	2	985.5	875.0	
	3	-	889.0	
Average		971.5	921.0	+5.43
A.Mck. & H. Lot#11414	1	24.9	19.4	
	2	-	18.1	
Average		24.9	18.8	+32.4
A.Mck. & H. Lot#B59822	1	588.5	572.0	+2.97

Note: For the data on frog assays refer to tables I, II, XI, VIII, X, IX, VII. The results of the rat assays were supplied by Ayerst, McKenna & Harrison Ltd., Montreal, Canada.

Table XIV

ORIENTING TESTS

Sample: A.Mck. & H.Lot#54372 Date: June 14, 1950

Frog Species: Rana clamitans

Dose in I.U. per gm. frog	No. positive out of total injected	DR 50
0.4	2/5	
0.6	4/5	
0.8	5/5	0.45 I.U.
1.1	5/5	
1.2	5/5	

Sample: A.Mck. & H.Lot#54372 Date: August 27, 1950

Frog Species: Rana pipiens

Dose in I.U. per gm. frog	No. positive out of total injected	DR 50
0.6	1/5	
0.8	1/5	
1.0	2/5	1.11
1.2	2/5	
1.4	4/5	
1.6	4/5	

Sample: A.Mck. & H.Lot#11414 Date: August 27, 1950

Frog Species: Rana pipiens

Dose in I.U. per gm. frog	No. positive out of total injected	DR 50
0.6	1/5	
0.8	1/5	
1.0	2/5	1.09
1.2	5/5	
1.4	3/5	
1.6	4/5	