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JOURNAL OF THE NEW ZEALAND ASSOCIATION OF BACTERIOLOGISTS

CONTENTS

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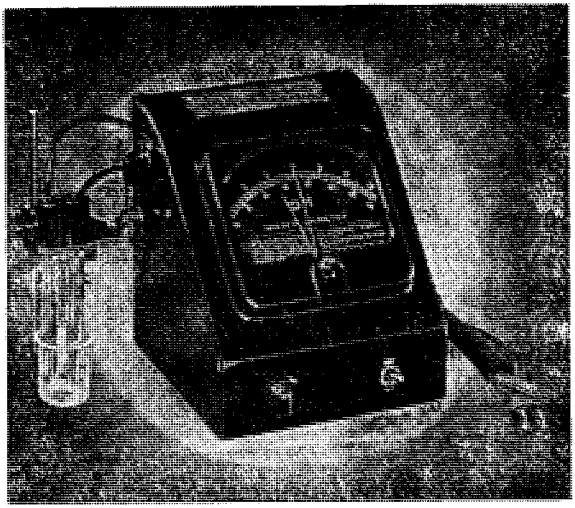

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EDITORIAL

With the Annual Conference taking place next month and the subject of the higher examination likely to provide further discussion, it is time the Association took stock of examinations generally. If one takes the trouble to study the recent examination papers, a striking fact emerges—that in some respects the Intermediate examination is of a higher standard than the Final. Surely this is a ludicrous situation. Now it would appear that a higher examination is to be instituted, but, to what purpose? This question was asked by several members at Dunedin last year, but a satisfactory answer was not supplied. Are all the disadvantages as well as the advantages of a higher examination fully understood by the Association? Now is the time to look to the future and attempt to see what will be the eventual outcome of this system of examinations in relation to the rapid strides which Laboratory Medicine is taking.

As Dr. Pinniger pointed out to the 4th Annual Conference, specialisation is the trend of the day, and it is becoming increasingly difficult, shall we say almost impossible, for any one person to fully grasp all the specialised knowledge required in a large laboratory. Thus two types of laboratory workers are emerging: on the one hand, those who wish to stay in a large laboratory and must, of necessity, specialise in one subject; and on the other hand we have the charge bacteriologists in the small laboratories who must have a good general knowledge.

The obvious course to follow is that of our counterparts in England who sit their final examinations in a specialty such as Bacteriology, Haematology, Biochemistry, Histological Technique, etc. To this we must add a general examination in all these subjects similar to that which is now held, but of slightly higher standard. Any one person would eventually be able to sit and pass all examinations should they so desire. If there must be a higher qualification, let it take the form of a thesis only, on some research the candidate has carried out.

How would you ensure that the distilled water was of the necessary standard of purity for intravenous use?

6. Explain the blue colour in the tube provided.
   In the estimation of non-protein nitrogen by the Folin and Wu method, what is left in the tube after the acid digestion process and what are the fumes given off during digestion?
7. Identify the specimens indicated by the supervisor.

ORAL EXAMINATION

Examiners: Dr. D'Ath, Dr. Lynch, Dr. Mercer, and Dr. Pullar.

Bacteriology:

Enrichment media for Salmonellas; characteristics of Shigella group of organisms; "H" and "O" agglutinins and origin of these symbols; estimation of cells in C.S.F.; media for growth of H. pertussis; characteristics of Cl Tetani, diphtheriae—methods of typing and appearance of colonies on Tellurite media; Diphtheria antitoxin dosage and forms of administering it; meaning of active and passive immunity; virulence test in detail, criteria of good agar; nature of agar; significance of stormy clot reaction; methods of distinguishing human and bovine strains of T.B.; composition of T.B. media; uses of Cragie tubes; production of anaerobiasis; Doremus tube; porosity of filters.

Lab. tests for diagnosis of hydatid disease, the presence and effect on human beings, life cycle of echinococcus granulosus—technique of Casoni test. Trichiniasis—its transmission and manifestation (a) in the primary host, (b) in the intermediate host. Filariasis, prophylaxis in Diphtheria, tetanus, typhoid, cholera and yellow fever; dosage and methods of injection preparation of vaccine; quantitative sugar in urine; uses of merthiolate, detection of bile.

Biochemistry:

Spectrophotometers. Def. of normal solution. Preparation of standard solutions, preparation of sod-lactate $M/_{15}$. Indicators and colour changes; constitution of Topfers reagent and phenolphthalein; protein precipitants. Estimation of serum calcium and phosphorus; blood sugar; T.N.P.N.; serum proteins, and C.S.F. chlorides. White fumes given off in T.N.P.N. digestion, estimation of urea, preparation of hypobromite solution, performance of Friedman test.

Haematology:

Detection of Rh antibodies, photo-electric haemoglobinometer, Newton rings; preparation of Leishman's stain; absolute values; platelet count technique; choice of haematology slides; cell counting apparatus.

Histological stains, microtome knives; frozen sections; section fixatives; staining of frozen sections; Van Gieson's stain.

Percentage total protein of plasma for intravenous infusion; preparation of plasma; sterilisation of Kaolin.

"THE VALUE OF HYLA AUREA THROUGHOUT THE YEAR IN PREGNANCY TESTING"

S. O. Jarratt

(Pathology Department, Palmerston North Hospital)

The earliest records of the early diagnosis of pregnancy date from attempts to recognise this condition from the comparison of the growth rate of sprouted barley sprinkled with the urine of pregnant women. There was a parallel existing between the auxin and pregnant hormone content, which formed a simple but not accurate test. Excluding the purely chemical tests, there have been many brought to light throughout the centuries, some with an increasing sensitivity and reliability index. Until recently, tests have been confined to the female of the animal species with stimulation of ovarian folliculation by chorionic hormone. These tests were necessarily confined to certain animal species, notably mice, rats and rabbits with suitable functional ovarian rhythm. The earlier test on immature female mice or rats was known as the Ascheim-Zondek test. The next to follow was the Friedman test. Virgin female rabbits were used and this test claimed 80% accuracy at 10 days after the first missed period, increasing to 98% accuracy at the end of the first month. There was no earlier diagnosis with this test. The advantages were: that one animal only was required, and if laparotomy was performed successfully, the same animal could be used again for three or more times after a suitable period of rest to allow for regression of the haemorrhagic follicle. This being three weeks, meant that fairly large stocks of animals had to be maintained if many tests were to be done. The reliability of the test depended on the reactivity of a single animal and hence was liable to variation. The test still occupied three days and was not a rapid test. Two further tests on ovarian stimulation were carried out, one on the Bitterling (Rhodeus amarus) which responded to steroid hormones, the varied character of protrusion of the ovipositor depending on the type of hormone present. As a test for human pregnancy it was not sufficiently specific. The other, by Hogben, Shapiro and others, on the female South African toad (Xenopus laevis), was more reliable than the previous one. Because of various disadvantages, they did not replace the Friedman test in routine work. There followed then the investigation on the male species of Baratrathia with stimulation of spermatogenesis by Galli-Mainini in 1947, using the male toad Bufo arenarus. The main advantage was the rapidity of diagnosis. Following this, attention was focused on the swimming frog, notably by Robbins and Parker, who in 1948 established the reliability of the North American frog (Rana pipiens) in the early diagnosis of pregnancy. The sensitivity of these animals compared more than favourably with any previously tried.

We became interested in the possibilities of the New Zealand frog in July, 1949, but were unable to obtain specimens until

August of that year. The two native species of Liopelma have not yet been investigated, as both are protected.

Hyla Aurea

Common species (Hyla aurea), introduced to New Zealand in 1860, belongs to the Hylidae family. Anatomically it belongs to the tree frog family, but has developed the habits of a grass frog. It is not unusual to find these animals sheltering in small shrubs during the winter. The testes are ventral to the kidneys; spermatozoa pass by the vasa efferentia through the anterior part of the kidney into the Wolffian duct, which functions both as a ureter and as a vas deferens, and opens into the cloaca. Opening ventrally from the cloaca is the bladder, which fills by gravity when the anus is closed. Under the stimulus of certain gonado-trophins, spermatogenesis occurs, the spermatozoa migrating via the kidney and Wolffian duct to the bladder, which is catheterised with ease. The skin is loosely attached to the muscles by bands of connective tissue. This loose arrangement forms a number of communicating lymph sacs. Injection is made dorsally and subcutaneously. Rapid absorption takes place.

Hyla aurea frequent ponds, streams and swamps in large numbers in certain localities during the spring and summer. Water is only required during this time for spawning and the development of their young. In other seasons they may be found miles away from water. They hibernate in mud, under rocks and in small shrubs during the winter. The male frog is smaller than the female and has a dark pigmented nuptial pad on the inner aspect of the "thumb." This pad disappears after the mating season but may be made to reappear until summer by introducing females into a pen of males. This recognition of males by the presence of a pad is not entirely reliable even during the spring, as the pad may not be clearly defined on some. The most reliable method is to inject chorionic hormone and look for spermatogenesis.

The frogs exhibit variations in colour from green to blue and usually have dark brown or gold leaf pigmentation dorsally, while ventrally they are mottled bluish grey to white. They are able to change their colour to suit their surroundings.

Small frogs about two years old appear more sensitive than older males and quicker to respond to stimuli. Their food is varied during the spring, summer and autumn, and they appear to store sufficient food to last them over the winter months. They will only eat food that is moving and will not touch dead insects or substituted food. This has been one of the main worries in keeping large numbers of these animals.

A large proportion appear to be more reactive during the spring, but their level of response remains fairly constant throughout the year. Frogs from localities as far north as Coromandel and as far south as Dunedin have been tested with no detectable difference in reaction.

Sexing Difficulties and Potency Testing

We were misled on several occasions by relying on the nuptial pad of the male to distinguish the sexes. With the idea of further improving the sensitivity of the test we injected chorionic hormone into 200 frogs which had been sexed by the above method, with the following results:—

No. Injected.	++ Reactors.	+ Reactors.	± Reactors.	Negative.
200	160	24	2	14
	80%	12%	1%	7%
		20%		

20% of these were considered unsuitable for the test. 10 International units of chorionic hormone were used in this test.

Variation of Stated Potency of Various Proprietary Hormone Preparations

The potency of proprietary chorionic hormone preparations is not guaranteed over certain periods, due to the large molecule involved and the difficulty of drying this product. Potency is lost by heat or damp. Diluted concentrations deteriorate quickly.

The standardisation of hormone preparations since 1941 has been based on international standards set up by the League of Nations and covers, among others, chorionic and serum gonadotrophin.

The original Rat unit of Gestyl (serum gonadotrophin "Organon") equals 10 present international units.

The original Rat unit of Pregnyl (chorionic gonadotrophin, "Organon") equals the present international unit.

Preparations which were obtainable in 1949 were a mixture of batches, some standardised in Rat units, others in International units. No date of manufacture was available, so that some preparations might have been years old. This probably accounted for the lack of response when a further batch of 100 frogs was potency-tested with 10 Rat units of "Pregnyl." This was evidently an old batch, the potency of which had deteriorated. We found that the majority of frogs reacted to 5 I.U. of "Pregnyl" of fresh preparation, but in order to allow for deterioration, we used twice the dose in subsequent testing.

Hormones, Gonadotrophins

A brief survey of the properties, occurrence and levels found.

Hormones. Chemical substances produced by glandular activity, liberated into the blood stream and transmitted to distant parts of the body, where they are capable of exciting action, the degree of which is determined by the local electrochemical condition of the cells at the time.

E. Laqueur holds that they should meet the following requirements:—

1. They should be capable of extraction by relatively simple means involving no chemical change.

2. When introduced into the body under standard physiological conditions they should elicit morphological or functional changes which appear also in normal life. These changes should follow the administration of minute quantities (mg. or g.) so small as to exclude their being due to volume or to associated impurities.
3. They should only be demonstrable in the body fluids, on their journey, as it were, towards specifically responsive tissues.

The Gonadotrophic Hormones

The anterior lobe of the pituitary gland stimulates the ovary and testis by means of the following specific gonadotrophic hormones, which are believed to be secreted by the basophilic cells of this gland:

1. Gametokinetic Gonadotrophin (Follicle-Stimulating Hormone, FSH: Prolan A; Thylakentrin). This, in the female, stimulates growth and maturation of ovarian follicles and oestrogen production, and in the male stimulates spermatogenesis.

2. Interstitial Cell-Stimulating Hormone (ICSH) (Luteinizing Hormone, LH; Prolan B; Metakentrin). This, in the female, stimulates certain interstitial cells of the ovary to undergo luteinization and, in the male, stimulates the interstitial cells of Leydig to produce the male sex hormone.

The chorionic cells of the placenta in the human also produce a gonadotrophic hormone, which is termed "chorionic gonadotrophin." Superficially, it behaves much like the luteinizing hormone of the pituitary and is therefore frequently referred to as "anterior pituitary-like hormone" or "APL." It differs from the pituitary lutenizing hormone in that it will not produce lutenization in the hypophysectomized animal.

Several hormones are proved to exist in the placenta. They are likely to have been produced in that organ itself and to be partly responsible for the changes that occur during pregnancy. The placental content varies from species to species.

The rich content of oestrogen (q.v.) and progesterone (q.v.) may account for the fact that human pregnancies do not necessarily abort after castration.

The androgen content is low. The content of gonadotrophins (q.v.) may be very large indeed. The established methods of pregnancy diagnosis are based on the hyper-excretion of this chorionic gonadotrophin by the human kidney. Except the giraffes, no other mammals excrete gonadotrophin in the urine.

A fourth type of gonadotrophic hormone is produced by the placenta of the pregnant mare (equine gonadotrophin) and is present in the blood of that animal during pregnancy. It differs from human pregnancy gonadotrophin (chorionic gonadotrophin) in that it has both follicle-stimulating and luteinizing properties, and also in that it is not excreted in the urine of the pregnant mare.

The gonadotrophic hormones are complex glycoproteins, which have not yet been isolated in pure form. They are soluble in water and may be precipitated by alcohol or tannic acid. They lose their potency on standing in solution and are readily destroyed by heat. It would appear from the above literature that where excessive concentrations of Gametokinetic Gonadotrophin occur, false positive reactions may be expected. In the normal non-pregnant state, however, they are present in such small amounts that the urine or blood must be concentrated or extracted before detection.

Gonadotrophic Levels (other than Chorionic Gondatrophin)

In children up to the onset of puberty, amounts are not detectable except in certain cases of precocious development.

In normal adult males and females, amounts are present in detectable amounts after concentration of urine, but methods involve tests other than the frog test.

In certain conditions, mentioned below, gonadotrophic levels may become greatly increased and lead to a positive frog test.

Chorionic Gonadotrophin in Normal Pregnancy

It is believed that in pregnancy the chorionic gonadotrophin manufactured by the placenta suppresses the pituitary gonadotrophin. Chorionic gonadotrophin enters the maternal bloodstream as soon as the chorionic villi have established contact with the maternal circulation. This usually occurs during the last week of the cycle.

As early as ten days after conception, an increase in gonadotrophin may be demonstrated in the blood and urine, and within ten days after the missed period there is a sufficient concentration in the urine to give a positive "pregnancy testing." By the sixth week of gestation values as high as 10,000 mouse ovarian units per 100 cc. of blood serum (and urine) are commonly present and often a transient peak, up to 30,000 units, may be encountered between the sixth and twelfth weeks of gestation.

After the twentieth week the hormone level falls, and during the last half of the period of gestation the values remain quite constant, at from 300 to 500 mouse ovarian units per 100 cc. After delivery of the placenta, gonadotrophins fall rapidly, and within a week they have usually reached non-pregnant levels, although occasionally titers sufficient to give a positive "pregnancy testing" may persist for several weeks, particularly if fragments of placenta have been retained.

Pregnancy Tests.

The demonstration of increased amounts of gonadotrophin in the blood or urine constitutes the basis for the reliable tests for pregnancy.

Since pregnancy testing indicates merely the presence of large amounts of gonadotrophin, it is apparent that it is not specifically diagnostic of pregnancy. Fortunately, there are but few other

conditions in which gonadotrophic hormone is produced in amounts sufficient to give a positive reaction. Rarely menopausal women will produce a sufficient amount of hormone to give a weakly positive reaction. If the test is repeated, using a smaller quantity of blood or urine, such false positives can usually be distinguished from pregnancy reactions. Chorionic tumours of all kinds, including hydatidiform chorionepithelioma and chorionic tumours of the testes, frequently give positive results. It is frequently stated that positive tests are sometimes obtained in pituitary tumours and hyper thyroidism, but this is rare.

A false negative test for pregnancy may be obtained if the test is performed too early, within ten days after the first missed period, or if intrauterine foetal death has occurred. However, in the latter instance the pregnancy test may remain positive for several days to several weeks after death of the foetus. False negatives may also occur if the specific gravity of the urine is below 1.020.

Gonadotrophic values indicate chorionic activity and not foetal life, but quantitative values fall just shortly after or just before foetal death.

Increase in Gonadotrophic Values Other Than Normal Pregnancy

1. Pituitary tumours—rarely met, but must be borne in mind.
2. Functional hyperactivity of the pituitary, as in—
   (a) Menstrual disorders accompanied by increased gonadotrophins.
   (b) Menopause and the male climateric.
3. Toxaemia of pregnancy.
4. Hydatidiform Mole,—This being a tumour of chorionic tissue usually produces an even greater amount of gonadotrophic hormone than is observed in pregnancy, up to several million mouse units per 24 hours in the urine and up to 100,000 units per 100 cc. in the serum. Fleshy or degenerative moles may show smaller amounts of hormone, while vesicular types show high titres.
5. Chorionepithelioma.—Very high values have been obtained also in chorionepithelioma of the placental tissue, up to 100,000 R.U. or several million mouse units per litre of urine. This is a malignant degeneration of the chorion, inasmuch as this condition may follow the expulsion of a mole, miscarriage, or normal pregnancy. Increased quantities of gonadotrophin have been found in eclampsia and in diabetic women during the last half of pregnancy.

(6) **Tumors of the Testicle.** Certain malignant tumours of the testicle produce markedly increased amounts of gonadotrophic hormone, often enough to give a positive "pregnancy test." Values up to 300 mouse units per 100 cc. of serum (or up to 10,000 units per 24 hours' urine) are not rare. In the majority of instances these tumours are believed to be chorionepitheliomas, but because of existing difficulties in histologic classification there is some

question as to whether other embryonal tumours may not produce gonadotrophic hormones. As a general principle, the largest amounts of hormone are produced by the more embryonal types of tumour. Metastases are often accompanied by marked increase in hormone concentration, while a decrease often follows removal or irradiation of the testicular tumour.

It must be emphasised that not all malignant tumours of the testis are associated with increased gonadotrophin values. Moreover, early in the course of development of those tumours that do produce gonadotrophin, the values may not be sufficiently high to give a positive reaction with the ordinary qualitative "pregnancy tests," and quantitative assay of the urine or blood may yield helpful information.

Technique of the Test

Two male frogs of known potency are inoculated with 2 ccs. of urine or serum subcutaneously, into the dorsal region of the animal. Fresh urine is preferred, or particularly an overnight specimen, or that from a patient where fluids have been restricted for 24 hours to concentrate the specimen. High protein diets are to be avoided. One early false negative in our series was due to a urine with a specific gravity of 1004, which on concentration produced a positive results. A seven-day later specimen with a specific gravity of 1020 also gave a positive result. A 10 cc. B.D. springe and 25-gauge ½in. needle is used. The animal is held in the left hand and the needle inserted under the outer skin with the needle pointing towards the flank. The skin is lifted and the contents inserted slowly. Too rapid an injection using refrigerated urine, or not allowing refrigerated animals to reach room temperature, causes discomfort and even death. Urines which are badly contaminated, toxic, or contain antiseptics, may also account for deaths. No alteration of the pH was made on urines, but all deposits of urates were mixed before injecting. We did not find that increasing the dose from 2 to 4 ccs. gave any better response, but did find that larger doses produced torpidity and delayed response. Latterly, after carrying out a series on known positives, 2 ccs. of serum was used with a consequent fall in the death rate. It was noticeable, however, that on very hot days animals seemed to succumb more easily to injection. The animals were placed in wide-mouthed needle jars (half-size Agee jars are suitable) covered with a piece of gauze, secured by a rubber band. Half an inch of water is added to promote absorption, and later, urination. The jars are placed in a large covered receptacle. Strong light seems to retard the speed of the reaction.

The animals are catheterised by means of a small glass pipette tapered to about 1 mm. in diameter, the end being annealed. No suction is applied. The pipette is gently moved downwards, or slight pressure is applied until the animal voids urine. Catheterisation is carried out at 1, 2 and 3-hour intervals.

Slides are examined under high power, and as the operator

becomes efficient, under low power.

The reaction appears to be all or nothing. The appearance of a few sperms indicates a positive result in the absence of negative cloacal smears initially. Reactions were classified as +++ and ++ if the smear swarmed with sperms and + if 5 - 10 were present per H.P.F. This, of course, should be influenced to some extent by the quantity of urine passed by the frog during the test. Not all the sperms are motile, and in weak reactions dead sperms may predominate.

Not more than one minute is required for each examination. We prefer to isolate males for three days before testing and not to use again for a further three days.

It is advisable always to catheterise each animal before testing, although we have not as yet seen spontaneous spermatogenesis in any frog after separation from females for three days. However, if females are accidentally included in a pen this may occur.

Microscopical Appearance of Sperms

Low power: Positive reactions show few, many, motile or non-motile banana-shaped sperms with a long delicate whip-like tail.

High power: The spermatozoa are curved, banana-like, motile or non-motile, with a long whip-like undulating flagella attached to the thicker portion. The banana shape distinguishes it from other flagellates sometimes seen.

Preliminary Testing

In order to ascertain the reactivity of the species, serum gonadotrophin "Gestyl" and chorionic gonadotrophin "Pregnyl" were first tried using 10 I.U. of each. As our stock of "Pregnyl" appeared more reliable, subsequent tests were made using this preparation. Large doses of each, ranging from 500 I.U., 100 I.U., 50 I.U. and 10 I.U., were tried, using each type. Positive responses were found in each case.

Further tests were made to discover the response to other than chorionic gonadotrophin or A.P.L. hormone. Oesterone, Ethinyl, oestradiol, progesterone, testosterone and stiboestrol were tried with negative results.

Tests were carried out on a series of known pregnancies next. In a series of 23 known positives of 5--9 months' duration, 23 positive results were obtained (detailed below).

\+ Reactions were encountered in 16 speciments at 1 hour and 18 at 2 hours.

++ Reactions were encountered in 18 specimens at 1 hour and 28 at 2 hours.

6 frogs were + after 1 hours and 4 were + at 2 hours.

1 frog was ++ after 1 hour and 2 were + at 2 hours.

1 frog was negative after 1 hour.

In 10 patients of 8 months' duration,

12 frogs + 1 hour, 10 frogs + 2 hours, 10 frogs ++ 2 hours.

6 frogs ++ 1 hour.
2 frogs negative 1 hour.
In 3 patients of 7 months' duration,
6 frogs ++ 1 hour, 6 frogs ++ at 2 hours.
In 3 patients of 6 months' duration,
2 frogs + 1 hour, 6 frogs ++ at 2 hours.
In 3 patients of 5 months' duration,
1 frog + 1 hour, 2 frogs + 2 hours.
4 frogs ++ 1 hour, 4 frogs ++ 2 hours.
1 frog negative 1 hour.

During this time early pregnancies were sought and the following were recorded: One 4 days overdue, and one 6 days overdue. One series involving 30 tests were carried out daily on a non-pregnant female in the reproductive age group, completing the menstrual cycle with negative results. Ten urines from non-pregnant females at mid-cycle were also negative. One series occupying one month from a lactating female: negative. One series on a heavily jaundiced patient. Ten urines from menopausal states. Three series from adolescent males and females. Three series from the climateric age group of males. Four specimens from uterine fibroids. Three from ovarian cysts and others from carcinoma of the cervix, prostate, etc., all gave negative results. We have not recorded a positive test in any condition not liberating chorionic hormone. Of the 120 known negatives no positive result was recorded.

In a series of 18 tests run in parallel with Friedmans:

Friedmans:	Frog:
9 positive	8 positive
9 negative	10 negative

There was not 100% agreement in this small series, as in one case of this series the frog test was negative and the Friedman was considered positive. There may not have been any doubt about the Friedman had not the frog test proved negative. Repeat on (1), an untreated, and (2), a concentrated specimen, proved negative. The specific gravity of the first urine was 1022 and the second specimen 1020. The large haemorrhagic follicle appearing on the ovary of the first rabbit, in view of the negative frog test in this case, was considered to be of a "regressive" type. Smaller follicles such as described sometimes appear on the ovaries of older rabbits which have been used several times. It is hard to account for its appearance on this rabbit, as it was a maiden doe used for the first time, and had been strictly isolated. No positive Friedmans were injected at the same time, so that this must be considered a "false Friedman." This is the first large "regressive" follicle seen in a series of several hundred Friedmans. There is evidence of a slight superiority of the frog test in this small series.

It is hoped to carry out a larger series to determine the sensitivity of the test and to run these in parallel with Friedman tests. There is evidence that the frogs used may vary in their response

to small stimuli. In view of this, duplicate tests are always made.

Order of Sensitivity

In order to test the sensitivity of the animal, 50 frogs in one batch were tested with fresh "Pregnyl" at 1 I.U., 2 I.U., 3 I.U., 4 I.U., 5 I.U., 10 I.U. These were caught during the mating season and appeared very active.

Dose	1 I.U.	2 I.U.	3 I.U.	4 I.U.	5 I.U.	10 I.U.
% Reactors	Nil	Nil	34%	96%	100%	100%

It would appear that the reactivity of this species is one of the most sensitive per test so far recorded. Especially where potency selected frogs are used, the test may be relied upon to detect at least 1.5 I.U. of chorionic gonadotrophin per cc. and should compare favourably with the Friedman test, which has been stated to react to a minimum of 1 I.U. per cc. using a 15 cs. test dose.

Alternatively, Hyla aurea may be said to react to 3 I.U. and the rabbit to 15 I.U.

Hyla aurea also compares more than favourably with other frogs and toads investigated elsewhere.

The test has the advantage of being easy to perform and gives results in 1--2 hours compared with 3 days in the Friedman test. The animals are easy to obtain during the breeding season and are cheap to maintain. Our stock has been maintained on fresh water, sods and occasional woodlice, for two months. We are not satisfied with this and hope to arrive at a better diet. We have noticed that fresh animals which react strongly at first become lethargic under laboratory conditions and give less positive reactions when kept for a long time, and also that young males seen to be better than older males.

Of the 352 frogs injected only 40 deaths were encountered. Some had been used as many as 6--8 times. Shortage of animals necessitated this. We hope to secure larger stocks of these animals and we are experimenting with an outside pen.

In all 352 frogs were injected from 12.10.49 to 6.12.49, involving 176 separate tests, of which 56 positive tests and 120 negative tests were encountered. Our total stock of animals was 89, of which 11 came from Coromandel and 78 from around Palmerston North. They all possessed the same degree of reactivity initially.

The accuracy shown on the 176 trials up to 6.12.49 was 100%. The above constituted the preliminary report to 6.12.49.

Large-scale Clinical Trials to 6.8.50 Covering Spring to Winter Months

During this time we carried out 528 tests involving 1056 frog injections. The over-all percentage of deaths due to injection was approximately 7%. This lower death-rate has been largely due to refusal to test badly-infected urines and to the use of serum instead of urine. The following tests occupied spring, summer, autumn and winter months. Potency-tested frogs were used throughout.

Date.	Negs.	Pos.	Inconclusive, not able to be repeated.	Frogs. died.	Numbers injected.
Spring, 12.10.49) Summer, 16.12.49)	120	56	1	14	354
Autumn to 16.5.50	153	91	2	25	492
Winter to 1.8.50 ...	44	58	3	29	210
	317	205	6	68	1056

False Negatives in Above Series

To date 2 false negatives were obtained:

1. Due to lowered specific gravity of urine in an early pregnancy within 10 days.
2. As yet an unexplained negative—urine (1022 sp. gravity) and serum both remained negative up to 14 days over the expected period.

Doubtful False Positive

One only has been recorded and as yet unexplained. Patient with amenorrhoea for two months, followed by excessive bleeding. Pregnancy test weakly positive in one animal only. Test recorded as doubtful positive. Repeating with fresh urine the result was negative. An ovarian cyst was diagnosed and removed.

Persistent Corpus Luteum

The persistence of the corpus luteum apart from pregnancy results in the formation of cysts. The continuous production of corpus luteum hormone causes amenorrhoea and other symptoms which may suggest extra-uterine pregnancy. Finally metrorrhagia may arise from a normal or over-developed secretory endometrium. Presumably this takes place only during the regression of the corpus luteum cyst. In some cases of corpus luteum cyst an excess of oestrogenic hormone seems to be elaborated. An increased production of gonadotrophic hormone (with a positive pregnancy test) may perhaps be looked upon as the direct cause of the persistence of the corpus luteum and has often been demonstrated. In this connection attention may be drawn to the fact that large corpus luteum cysts occur in cases of hydatidiform mole, and chorionepithelioma. Curiously, corpus luteum cysts seem capable of producing gonadotrophic hormone themselves (Zondek).

Excluding the "Inconclusive, not able to be repeated tests," the over-all accuracy in positive results appears to be 98.5% and in negative results approximately 99%.

Where both animals have died in tests, repeat specimens have been requested.

This series also includes the following early tests:

Positive Tests.	Days Over Estimated Cycle.
2	2
2	3-4
3	5-6

12	7-8
15	9-10
21	11-14
55	

Quantitative Tests

At present only 6 comparisons have been made. The results have been encouraging in (1) the separation of moles and chorionepitheliomas from the pregnant state, (2) the recognition of ectopic pregnancies, (3) the detection of threatened, complete and incomplete abortion. When sufficient cases are investigated it is intended to record these results at a later date.

Parallel Friedman Tests. 2nd series to 1.7.50		Friedman Test.	Frog Test.
	Positives. 10	10	10
	Negatives. 16	16	16
	26	26	26

Ectopic Pregnancies

6 cases of proved ectopic pregnancy: 4 gave positive results and 2 were negative.

Comparison of methods of keeping animals and their reactive response.

(1) The reactive response of "penned" frogs on a two-hour test.
(2) The reactive response of refrigerated frogs on a three-hour test.

The tests were made to discover advantages in either method. Unfortunately comparisons cannot be made, as these tests were not carried out under identical conditions. It can be inferred, however, that advantages or disadvantages may be found in either method.

Two groups of 50 frogs were selected and potency tested with 10 I.U. of Pregnyl. Only ++ or over reactors were kept for the test. The succeeding tests occupied a period of three months, and this length of time may account for the loss of reactivity in some.

No. 1: % Positive Reaction in Penned Frogs on Two-hourly Examinations.

Number of sperms present.	Reaction.	Percentage.
Large numbers per H.P.F.	+++	32%) useful
10 numbers per H.P.F.	++	42%) for test-
5--10 numbers per H.P.F.	+	16%) ing, 90%
5 or less per H.P.F.	+	3%
Negative reactors	—	2%

As a precaution all negative reactors were checked at three hours with no change in the result.

No. 2: % Positive Reaction in Refrigerated Frogs on Three-hourly Examinations.

Number of sperms present.	Reaction.	Percentage.
Large numbers per H.P.F.	+++	22%) 80% use-
10 numbers per H.P.F.	++	50%) ful for
5--10 numbers per H.P.F.	+	8%) testing.
5 or less per H.P.F.	+	14%
	—	
Negative reactors	—	6%

Unfortunately the refrigerated frogs were not kept under ideal conditions. They were kept in large glass jars placed on their sides and in biscuit tins. This latter method was discontinued when it was discovered that frogs kept in contact with tin showed an abnormally high death rate. Because of lack of outside space, we were attempting to keep about 200 frogs this way. The refrigerator temperature varied from 4 to 6 degrees C. It became increasingly clear, as the end of the three months approached, that frogs could not be satisfactorily kept in this way at this temperature (blood bank frig.) when they were frequently taken out or disturbed many times during the day, with constant fluctuation of temperature.

In our case failure of this method was due possibly to:

1. The use of frogs collected during the spring, which did not allow time for the animals to accumulate sufficient food for their enforced hibernation.
2. They had been kept under poor conditions before refrigeration.
3. Repeated thawing and refrigeration for test done.
4. Overcrowding.
5 Too frequent use of animals.
6. Too frequent disturbance.

These six facts may account for the higher percentage of non-reactors in the 2nd series. The three hours' test must possess an advantage over the two-hour one, especially with reference to weak reactions. We have carried this a stage further. When weak reactions are found at two hours, we place the test animals in the refrigerator till next morning and examine before the animals have time to void any urine. We have frequently found this has increased the strength of the reaction from ± to a + or ++ reaction.

In our opinion frogs could be kept successfully in the refrigerator provided

(1) They were kept in all-glass containers plus sufficient water or wet moss to keep them moist.
(2) That frogs kept were collected in autumn when they should have collected their hibernating food storage.
(3) That they are kept at a temperature of 6 to 8 degrees C. and not disturbed too frequently.
(4) That only ++ or +++ reactors are stored and these should be checked at monthly intervals to discover loss of reactivity.

(5) That they should have ample space.
(6) That they should be used two or three times only and then discarded.

Reactive Response Levels

A series of 20 potency-tested frogs from an outside pen were tested during the summer to discover their minimum response. This was followed up, on the same frogs, during the winter, and their response charted. The same batch of Pregnyl was used in each case.

	10-I.U.	4 I.U.	3 I.U.	2 I.U.
Summer Testing: Numbers Positive	20	14	6	Nil
Autumn Testing: Numbers Positive	20	16	4	Nil
Winter Testing: Numbers Positive	19	15	4	Nil

In the last test, carried out in July, 1950, one frog remained negative.

It may be assumed that even carefully-tested frogs lose potency on prolonged storage. It is evident that:

1. The test must be carried out in duplicate.
2. Only potency-tested frogs should be used (especially in winter months).
3. Three-monthly checks should be carried out on stored frogs during the middle winter and early spring period.

Comparison with Fijian Toad

We were fortunate in securing two male Fijian toads from the Auckland Hospital, but were not successful in maintaining these animals, and both died within one month. They were potency tested at the same time as the winter test of the frogs and were negative to 4, 10, 20 and 40 I.U. and positive at 60 I.U.

The minimum response of these two toads was between 40 and 60 I.U. We are hoping to secure more specimens in order to compare their sensitivity. It may lie somewhere between 10-20 times less sensitive than the frog.

Errors in Performing Routine Tests

1. Too rapid an injection causing spasm in animals.
2. Holding animal too tightly, so forcing injected urine out.
3. Not allowing one hour for refrigerated frogs to reach room temperature.
4. Not allowing one hour for refrigerated urine to reach room temperature.
5. Not using potency-tested animals, or, using potency-tested animals stored too long without a periodic check.
6. Using animals in poor condition.
7. Using urine which is toxic, or of a specific gravity lower than 1.020.
8. Using animals too frequently for repeated testing.
9. Not catheterising animals before each test. We have found that refrigerated animals may exhibit sperms up to 10 days after a positive test when kept in the refrigerator and up to 5 days in outside pens during cold weather.

10. Failure to rinse out pipettes thoroughly after a positive test before catheterising the next test.
11. Insufficient chorionic hormone produced at the time of test to give a positive result.

Advantages Over Friedman Test

Although a large enough parallel series has not yet been carried out, it seems likely that the Frog Test will be as reliable and perhaps even more so than the Friedman Test. It has many advantages, namely:—

1. Time-saving. (Actual time taken to carry out Frog Test, 1min.; Friedman Test, ½ hour.)
2. Earlier reporting of results. In 50% of cases positives were obtained in ½ to 1 hour (2 hours compared with 3 days).
3. No messy operative procedure.
4. May be carried out in laboratory—no special operating table or room necessary.
5. Easier to handle, house and feed.
6. Reduced feeding and maintenance cost of animals.
7. Less discomfort to the animal.
8. Detects early pregnancies as accurately as the Friedman Test, and possibly more so.

Advantages to the Surgeon and Obstetrician of a 2-Hour Test

1. Early recognition of moles and chorionepitheliomas.
2. Early report on pregnancy tests and foetal death.
3. The pre-operative separation of ectopic pregnancies from other confusing abdominal symptoms has been one of the main advantages.
4. The early recognition of complete or incomplete miscarriage.

Housing

There are several methods which may suggest themselves, and one may be influenced by the opportunities or facilities available. Of the several methods, such as storage in the animal house, the basement of the refrigerator, we have found the outside pen simulating natural conditions superior to others. The essential seems to be the provision of a darkened space where the animals may hibernate during the winter months, as it is during this period that the greatest care should be taken.

Refrigeration, in slowing down the animal's metabolism, reduced the necessity of providing food during this enforced hibernation. It possesses several disadvantages.

A cool basement in which the humidity can be maintained may prove a better solution than refrigeration. We found that where moisture is provided along with shelter from the sun, the outside pen animals remained in better condition. We lost a number of frogs under animal house and refrigeration conditions. Under laboratory conditions animals become noticeably lethargic, lost condition, and their reactive levels dropped more noticeably. It would seem that an even cool temperature, provision of moisture, avoidance of strong sunlight (by provision of pipes, etc.,

in the house) are the ideal conditions and possibly the nearest approach to natural conditions is the best.

Feeding

We were not able to find any practical food which would replace the natural diet of these animals. Their daily requirements are practically nil if outside pens are used, where they may catch their own wing-born diet. To encourage this wing-born supply, we devised several fly-traps. The simplest and most successful consisted of a shallow dish filled with equal parts of sugar solution and stale beer, covered with a glass kidney-shaped bowl raised about two inches from the surrounding ground surface. Flies were attracted by the solution and trapped in the glass bowl. The frogs soon discovered their waiting meal. We found, however, that some frogs were crowded out and losing condition, so their diet was supplemented by weekly feeding with worms and woodlice. Two average-size worms seem to be sufficient for one frog per week. It is advisable to feed frogs during the week-end only, as animals tested immediately after feeding, particularly with toxic urines, will regurgitate their food.

Summary

A brief summary of the methods used in the biological test for pregnancy in addition to the use of the introduced common frog (Hyla aurea) of New Zealand as a test animal is given. The types of hormone and the levels found in normal conditions and in others related and unrelated to pregnancy are discussed. The procedure of the test, the specificity of the test animal (Hyla aurea) to chorionic gonadotrophin, the sensitivity per cent. to small doses of "Pregnyl" ("Organon"), and comparisons with a small series of Friedman tests are recorded.

Sexing difficulties, potency testing, reactive levels, and loss of potency, potency comparisons of "penned" and refrigerated frogs, housing and feeding difficulties are dealt with. Errors in technique, the advantages over the Friedman test, and the advantages of the rapidity and accuracy of the test to the clinician are evaluated.

The value of the Hyla aurea as a test animal throughout the year, its near constancy of reactive level in selected animals, has been demonstrated. The specificity of the animal as yet, toward chorionic gonadotrophin, and the minimal reactive level in selected animals to 3 I.U. and to the general level of 4 I.U. is recorded. Quantitative estimations using this species are to be published at a later date.

Conclusions

1. Studies on the males species of Hyla aurea indicate marked sensitivity to chorionic gonadotrophins.
2. Chorionic gonadotrophin stimulates spermatogenesis in the male species. Spermatozoa may be demonstrated by catheterisation of the cloaca.
3. To date, this reaction has been obtained with human chorionic

gonadotrophin and that, liberated by the placenta of the mare; and not as yet, with pituitary gonadotrophins, as these were not available.

4. The reaction of the animal is influenced by the individual "reactive response," the dose, temperature and illumination.
5. A positive response is indicated by the appearance of spermatozoa in the urine and constitutes a positive test.
6. Results as yet are specific for chorionic gonadotrophin. In the absence of chorionic gonadotrophin, as yet, a negative response is obtained.
7. The reaction indicates activity only of the chorion. In general, but not always, gonadotrophic levels fall at parturition, during abortion, death of the foetus, expulsion or extirpation of mole or chorion epithelioma.
8. In 528 tests carried out, of which 205 were positives at all stages of pregnancy, the corrected accuracy was 98.5%. In 317 negatives the corrected accuracy was 99%.

The preliminary report up to 6.12.49 and the first large-scale clinical trials covering spring, summer, autumn and winter to 1.8.50 on this species is outlined.

The above was the subject matter of a paper presented at the Annual Conference of the N.Z. Association of Hospital Basteriologists (Inc.) at Dunedin in August, 1950.

Acknowledgments are due to Dr. T. H. Pullar Pathologist, Palmerston North Hospital, and to the Board of the Palmerston North Hospital, where much of the work was done.

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D'Arcy, E. N. S., and Grönwall, F.A.: "A Simple, Rapid Test for Human Pregnancy. The Use of the Common New Zealand Male Frog (Hyla aurea)." New Zealand Medical Journal, December, 1949, Vol. X4VIII., No. 268.

COUNCIL MEETING

A meeting, attended by full Council, was held in Wellington on Saturday 14th April, 1951, at 10 a.m.

Following a discussion on the Intermediate Examination it was arranged that the several Bacteriologist examiners bring down a report on the examination for the benefit of candidates who will be sitting this examination in future. It was further suggested that the Director-General of Health be asked to agree to the passes in the Senior examination be graded 1, 2 and 3.

Mr. I. W. Saunders was nominated for the position of Bacteriologist examiner, October, 1951.

The present members of the Salaries Advisory Committee, Messrs. Buxton, Whillans and McKinley, were re-nominated to that position. Mr. H. T. G. Olive and Mr. H. Ward were also re-nominated as deputies.

The following new members were elected:

Senior: Mr. J. Perry-Johnson and Mr. C. Felmingham.

Junior: Misses J. Munro, S. Cook, A. Wiseman, M. Lindberg, R. Graham and A. Reid; Messrs. L. Paul, R. T. Kennedy, G. Cameron, V. Darley.

The following resignations were received with regret:—Mrs. Roberts (nee MacKenzie) and Miss J. Bailey.

Conference dates of August 16th and 17th, at New Plymouth, were approved and arrangements put in train for a successful meeting. The method of Preferential voting put forward by a Committee was approved.

It was approved that the *Journal* be printed in future by Messrs. Percy Salmon, Wills and Grainger, of Auckland.

Messrs. Whillans and McKinley were appointed as a deputation to meet the Pathologists at their Conference in Palmerston North late in June. An agenda was then arranged for this meeting.

The Council then spent much of the rest of the time on proposals for a higher examination and proposals were put forward that the Association should consist of Junior members, Senior members and Fellows, the latter to be elevated by (a) Examination or (b) thesis. The fee for Examination to be £5/5/-.

Finally, submission to the Salaries Advisory Committee were discussed, these to be put forward by the three representatives at a meeting early in July.

The meeting closed at 10 p.m. after an exhausting day.

INTERMEDIATE EXAMINATION FOR HOSPITAL LABORATORY TRAINEES

Medical School, Dunedin, 25th and 26th May, 1951.

Examiners: Drs. M. Watt and R. Rodda and Mr. G. W. McKinley.

Theoretical Paper—3 hours.

1. Discuss the growth of bacteria on culture and the factors influencing this. What practical applications of this knowledge are made use of in the laboratory?
2. Draw a diagram of an autoclave. Describe the method of operating this autoclave, so that efficient sterilisation will result.
3. Describe one method for estimating the blood sugar in a specimen of oxalated blood.
4. How would you determine the cells, protein and chloride in a specimen of spinal fluid?
5. Describe in detail one recognised routine method of determining the quantity of haemoglobin present in the blood. What are the underlying principles of the method? Discuss likely errors and outline how these may be minimised and periodically checked.
6. Write *brief* notes on
   (a) Detection of bile in the urine.
   (b) Mean cell haemoglobin concentration.
   (c) Seitz filter.
   (d) Pasteurisation.

Practical Paper—3 hours.

1. Smear K is from a cerebrospinal fluid. This organism has been grown on culture A. Culture A_1 is from a test for sensitivity to penicillin, aureomycin, chloromycetin and streptomycin. Culture A_2 is a fermentation reaction with glucose, A_3 with maltose, and A_4 with saccharose.

   Report on this organism and state what further investigations, if any, you would wish to carry out.

   (The organism was N. meningitidis.)
2. Report on the organisms in culture B_1 and B_2. What further investigation of these organisms might be done.

   (The organisms, growing on blood agar and broth, were Streptococcus viridans and a haemolytic streptococcus.)
3. Examine the urine C for albumin, sugar, and deposit.

   (Albumin negative, sugar positive and deposit showed a number of red cells.)
4. (a) What is the reagent D, and how is it used?

   (Nessler's solution.)

   (b) Test specimen E for the presence of occult blood.

   (Specimen of faeces, strongly positive for occult blood.)
5. Perform a leucocyte count on the blood F.

   (Count was about 10,500 per c. mm.)

   Stain the film F with Leishman and do a differential count.

   (Eosinophilia.)

   What abnormality do you recognise in the stained film G?

   (Film from a case of chronic myeloid leucaemia.)
6. Write brief notes on slides W, X, Y, and Z.

   (Vincent's organisms, pneumococcus capsule stained, E. typhi, flagellar stained, and N. gonorrhoeae, intracellular.)

ORALS.

Each candidate was examined for approximately thirty minutes by each of the three examiners separately, i.e., each candidate had approximately ninety minutes of orals, in three separate sessions of thirty minutes. The examiners questioned candidates further concerning their theoretical and practical papers.

Questions asked in the orals included the following:—

Sterilisation; the microscope; graduated and volumetric glassware; weights and measures; vernier scales; balances; colorimeters; cleaning of glassware; culture media; nomenclature of bacteria; isolation of intestinal pathogens; growth conditions of the gonococcus and of Br. abortus; specific gravity of urine; acetone in urine; hypobromite method for urea; streptomycin and penicillin sensitivity tests; haematology, including errors in the estimation of haemoglobin; errors in red counts; origin and development of cells, reticulocytes, platelets; haematocrit techniques and M.C.V., M.C.H. and M.C.H.C. values; blood grouping; gastric analysis; T.N.P.N.; protein precipitation; the sedimentation rate; antidotes covering accidental poisoning in the laboratory; the packing and posting of laboratory specimens.

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