THE QUANTITATIVE EFFECT OF THE HUMAN CHORIONIC GONADOTROPHIN ON THE MALE TOAD, *BUFO REGULARIS*. A METHOD FOR ITS BIOLOGICAL ASSAY

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SINCE Mainini[1] introduced his test for the rapid diagnosis of pregnancy utilising the male toad *Bufo arenarum* Hanzel, many other members of the anura in various parts of the world have been shown to be equally suitable for the test. These included *Bufo marianus*, *B. calamita*, *B. vulgaris*, *B. regularis*, *B. americanus*, *B. melanosticus*, *B. stomaticus*, *Rana pipiens*, *R. esculenta* and *R. tegerina*. However, in applying the test, a high incidence of false negative results was experienced by many workers, e.g., Mainini,[1] Klopper[2] and Frank and Pollak.[3] The original aim of this work was to find out the probable causes of such results, and thus try to discover the factors that might affect the accuracy of the test.

For this purpose it was first thought advisable to investigate the sensitivity of the response of the male toad which is common in Egypt, *Bufo regularis*, as regards the discharge of spermatozoa in its cloaca, to known graded doses of chorionic gonadotrophin, calculated according to the toad's weight in a proper dose-weight system. When this was done it became at once evident that this response was quantal, so that a satisfactory dose-response curve could be constructed and utilised as the basis of a method for the biological assay of chorionic gonadotrophin.

Robins, Parkes and Bianco[4] had succeeded in using the South African male frog, *Xenopus lævis*, for the assay of this hormone, and even found it many times more sensitive than the standard laboratory animals used for this purpose. Mohanty and Pabrai[5] also reported on the use of the male toad, *Bufo melanosticus* Schneid, for this assay, but, as will be seen later, the technique and criteria of the method here described are different from theirs.

This work was divided into three parts. Part I describes the method and the results obtained by using it to assay preparations of the chorionic gonadotrophic hormone. Part II comprises experiments devised to test the applicability, as well as the reliability, of this method to clinical material, and at the same time elicit its value by comparison with established methods. In Part III is shown the value of the author's modification of the male toad method for the diagnosis of human pregnancy, which is shown in the first part of this work, by comparing it with one of the well-known methods.

PART I

METHOD

In this investigation male toads of the species *Bufo regularis* were employed. Their weights varied between 12 and 31·8 g., with an average of 21·9 g.

Gradually increasing doses of the standard chorionic gonadotrophin were administered, the doses being calculated according to the weights of the toads. For this purpose, the unit weight was considered to be 30 g. Thus, the doses were calculated so that the amount of hormone varied between 5 and 100 I.U./30 g. of body weight. Therefore, for injection, each dose was dissolved in 0·6 per cent. saline solution so that the amount of prepared solution for injection was always 2 ml./30 g. For the test, each toad was examined for the presence of spermatozoa in its cloacal discharge, according to Klopper and Frank's[2] modification of Haines'[6] technique. After weighing it was then isolated throughout the

TABLE I

THE EFFECT OF THE INJECTION OF INCREASING DOSES OF CHORIONIC GONADOTROPHIN ON THE CLOACAL DISCHARGE OF THE MALE TOAD, *Bufo regularis*

Dose (I.U./30 g.)	Average weight of toad g.	Positive response per cent.	Average number of spermatozoa per field	Degree of motility of spermatozoa
5	21	0	—	—
10	18·5	10	—	—
15	17·3	20	3	0
20	25·7	50	2	+
25	22	66·6	10	+
30	19·6	83·3	16	++
35	23	100	5	+++
50	21·7	100	23	++
70	21·3	100	21	++
100	29	100	28	+++

test, without water, in a separate glass jar. The solution of the hormone, prepared as before-mentioned, was then injected into the toad's dorsal lymph sac, following the technique described by Crew,[7] but half of it was injected into one side, and the other half into the other side, in order to eliminate the effect of an abnormally high pressure on one side only.

For each dose of the hormone so tested at least 100 toads, in groups of 10 toads in each test, were used, and their average responses, as regards the criteria to be shortly described, recorded.

Test specimens of the cloacal discharge were taken from each toad at intervals of ½ hour after the injection of the hormone. These were immediately examined for (i) the presence or absence of spermatozoa, (ii) their average number per field, and (iii) the degree of their motility. The presence of as few as 2 or 3 spermatozoa in the whole slide was considered as positive, so long as the preliminary examination of the toad's discharge was negative. The number of toads in each group, that gave a positive response as regards the presence of spermatozoa, was recorded, and its percentage calculated. For making spermatozoal counts the enumerating hæmocytometer slide was first employed, but this was later found, for obvious reasons, to be unnecessary, and sometimes impractical. Thus visual enumeration of the spermatozoa in many fields and taking their average, was the method relied upon. For the assessment of the degree of motility of the spermatozoa, it is necessary to examine the slide immediately it is withdrawn from the cloaca. The degree of motility was recorded in four classes: 0, when all the spermatozoa in the prepared

film were non-motile and absolutely inactive; +, when only about one-third of them were active, the rest being inactive and non-motile; ++, when about two-thirds of them were actively motile; and +++, when all the spermatozoa in the film were alive and actively motile.

Either fresh unused toads were employed for the test, or those which had been used for a test 1 to 2 weeks earlier, to ensure the elimination of the effect of the previous dose. Toads could thus be used for the test 6 or 7 times. Sometimes a cross-over test was done using the same dose and the same toads a week later, to exclude individual variation. For the biological assay of preparations of chorionic gonadotrophin of unknown potency by this method, a standard preparation of this substance was run at the same time, employing 2 doses of each.

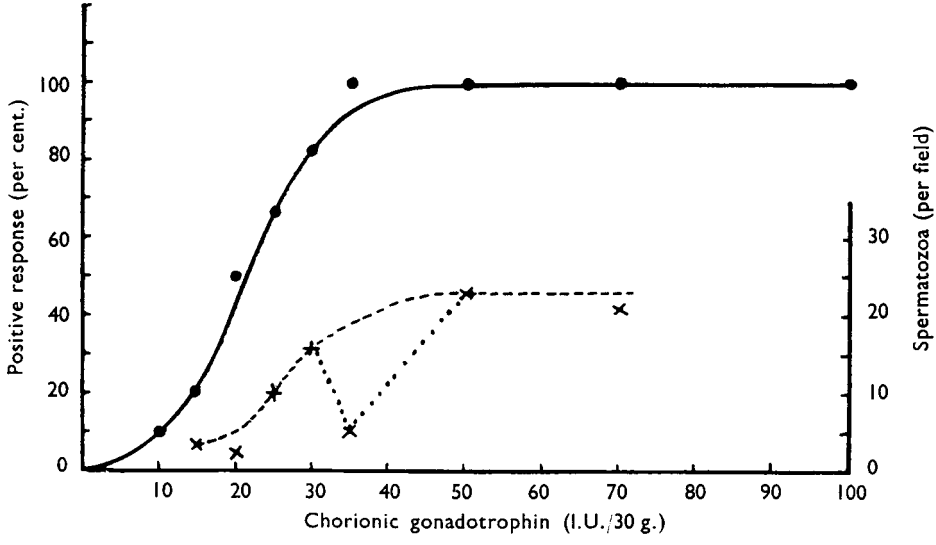

FIG. 1. The quantitative effect of human chorionic gonadotrophin in the male toad, *Bufo regularis*, on the cloacal discharge of spermatozoa. Abscissa = dose of chorionic gonadotrophin in I.U. per 30 g. of body weight. Left ordinate = number of positively reacting toads per cent. Right ordinate = average number of spermatozoa extruded per field of the prepared slide.

RESULTS

The cloacal discharges that were taken just before the tests were always negative for spermatozoa. This proves that during the times of the year in which this work was done, viz., from January to June, spontaneous spermatogenesis never occurred in any of the toads employed in the absence of the female partners. The precaution of a preliminary examination of the animal should always be taken, however, before starting a test.

Effect of chorionic gonadotrophin on the extrusion of spermatozoa. In a preliminary test a solution containing 50 I.U. of chorionic gonadotrophin was administered to a 60-g. toad, and an equal dose into a 15-g. toad. In the former, a negative result, whereas in the latter, a positive one was

obtained. It was found that, as can be seen from Table I, which summarises the results of the tests done, the maximum dose of this hormone that produced no response, as regards spermatozoa excretion, in the toads examined, was 5 I.U./30 g., while the minimum dose producing a 100 per cent. positive response was 35 I.U./30 g. Doses intermediate between these two gave gradually increasing responses directly proportional to the dose administered; so that doses lower than the former were always negative, while those higher than the latter were positive. The medium effective dose (M.E.D.), i.e., the dose which produced a positive response in 50 per cent. of the toads employed, was thus found to be about 20 I.U./30 g.

Figure 1 shows the results of the tests performed, with the doses of the hormone in international units as abscissa, and the percentage of the positive reactions as ordinate. The resulting curve is sigmoid in shape, with the onset of the upper plateau at about the level of the dose of 35 I.U./30 g. When the doses were converted into the corresponding log-doses, a satisfactory linear, gradational dose-response line, with a good slope, was obtained.

The same results were obtained by using either fresh unused toads, or toads that received one or more injections previously, but with intervals of 1 to 2 weeks in between. Furthermore, by using the agreed dose of 2 ml./30 g., no death, either immediate or delayed, occurred in any of the hundreds of toads used in this investigation. This dose-weight relationship was chosen because it was found to be, for many reasons, the most suitable; it represents the average tolerated volume of injected solution, and the commonest average weight encountered.

The potency of an unknown preparation of chorionic gonadotrophin could be arrived at by performing a proper 4-point assay, i.e., administering simultaneously to groups of 10 toads two doses each of a standard chorionic gonadotrophin and of the unknown preparation, one dose being about double the other and both lying between 5 and 35 I.U., recording the results obtained, and calculating the potency of the unknown in relation to the standard. A cross-over test may sometimes be done a week after the first one, although it was found not to change the result materially.

By assaying chorionic gonadotrophin preparations by this method and by the official B.P. method, fairly satisfactory comparable results were obtained.

Sensitivity of the method. From the results shown in Table I, it can be seen that the method detects a difference in the potency of the hormone used, of 5 I.U., which, for practical purposes, is quite satisfactory.

Effect on the number of spermatozoa excreted. It was found that, as a general rule and within the limits specified above, the higher the dose of gonadotrophin given, the larger was the number of spermatozoa excreted. Figure 1 also shows the relationship between the dose in I.U./30 g. and the average number of spermatozoa per field, a relationship which will be seen to be gradational and almost sigmoid.

Effect on the degree of motility of spermatozoa. It was noticed that,

as shown in Table II, as a general rule the degree of motility of the spermatozoa was directly proportional to the dose of chorionic gonadotrophin given. There were, however, exceptions because it so happened that the degree of motility was sometimes higher with smaller doses of the gonadotrophin. It was also noticed that the injection of the smaller doses of the gonadotrophin, e.g., 15 or 20 I.U./30 g., resulted, in most cases, in the expulsion of a mass of sticky mucus in which the dead or almost dead spermatozoa were entangled, and that larger doses, e.g., 40 or 50 I.U./30 g. produced a fluid semen with viable motile spermatozoa, while still larger doses gave very active ones.

TABLE II

THE RESULTS OF THE ASSAY OF 3 SAMPLES OF URINE FROM PREGNANT WOMEN, BY 3 DIFFERENT METHODS. EACH SAMPLE WAS DIVIDED INTO THREE PARTS: (*a*) WITHOUT CHANGE, (*b*) AFTER THE ADDITION OF CHORIONIC GONADOTROPHIN 5000 I.U. PER LITRE, AND (*c*) AFTER ADDING 10,000 I.U. PER LITRE

	Solution used	Amount of chorionic gonadotrophin (I.U./l.)		
		Male toad method	Mouse method	Rabbit method
I	(*a*)	5680	6800	6160
	(*b*)	11,110	11,380	12,120
	(*c*)	14,860	15,320	15,620
II	(*a*)	12,520	11,420	10,940
	(*b*)	16,100	16,810	15,320
	(*c*)	19,620	21,310	19,120
III	(*a*)	13,680	14,180	14,440
	(*b*)	18,920	18,220	18,520
	(*c*)	22,120	21,820	22,920

PART II

METHOD

3 samples of the morning urine were taken from women in their third month of pregnancy, and each sample divided into 3 parts: (*a*) the first was left without change; (*b*) to the second, chorionic gonadotrophin was added in the amount of 5000 I.U./l.; (*c*) to the third was added 10,000 I.U./l.

The total amount of chorionic gonadotrophin in each of these parts in the 3 samples of urine tested was assayed by 3 different methods: (i) the male toad method, as previously described in Part I of this paper; (ii) the mouse method of Aschheim and Zondek[8]; (iii) the rabbit method of Friedman.[9]

RESULTS

The results of the assay of the 3 samples of urine by the 3 methods are summarised in Table II. It will be seen from this table that the amount of chorionic gonadotrophin in each part of every sample of urine was, within reasonably narrow limits, more or less equal in the 3 methods, and the results obtained by them were nearly parallel. These experiments indicate, first, that the male toad method of biological assay of chorionic gonadotrophin is as well applicable to clinical material as the mouse and the rabbit methods, and, secondly, that it can be as safely and dependably employed for this purpose.

PART III

METHOD

Morning samples of untreated urine from 8 women who missed their menstrual periods by 10 to 30 days were simultaneously tested for pregnancy both by the rabbit method of Friedman[9] and by the modified male toad method as described in Part I of this paper. The main point in this modification, it should be recalled, is the injection of the urine according to a definite dose-weight system (2 ml./30 g. of toad's weight). All cases were then followed up and the diagnoses of the conditions present confirmed.

RESULTS

The male toad and the rabbit methods were employed simultaneously on each of the 8 samples of urine. In 6 both methods gave negative reactions, and in 2 the male toad method was positive for pregnancy while the rabbit method was negative. Observation and follow-up of these women confirmed the absence of pregnancy in the former 6 and its presence in the latter 2 cases.

DISCUSSION

All workers who have so far reported on the male toad test for the diagnosis of pregnancy in the human, used a constant volume of the female's urine for injection into each of a group of male toads, without taking into consideration the toads' individual weights. Considering the fact that adult toads of the same species varied in weight between 10 and 60 g., or even more, the method did not seem to the author to be scientifically sound, because the same amount of chorionic gonadotrophin which the injected urine contained could thus be distributed into a toad's body which is 6 times the weight of another, and thus could have a much less powerful effect in one than in the other.

This is where the present work started. Thus when gradually increased doses of human chorionic gonadotrophin were then injected into groups of male toads, in a proper dose-weight system according to the method described, it was at once found that the number of reacting toads, as regards spermatozoal extrusion, was directly proportional to the amount of the hormone injected, and thus a gradation in the quantitative response of the male toads to the hormone was clearly shown, and a satisfactory dose-response curve arrived at. A method for the biological assay of human chorionic gonadotrophin was therefore devised and scrutinised for its applicability in practice. It was also noticed that different doses of this substance had a different effect on the number of spermatozoa extruded per field, as well as their degree of motility. Thus, the method is now built up on three criteria, the presence or absence of spermatozoa in the toads' cloacal discharge, their number per field and their degree of motility.

While this work was approaching completion, Mohanty and Pabrai[5] reported their results on the use of *Bufo melanosticum* Schneid for this

purpose. However, as is seen from this work, the technique followed here is different, as regards the arrangement and the conduct of the experiment, dilution of the hormone, etc., as well as the criteria taken for the evaluation of the results.

The dosage system followed here (i.e., 2 ml./30 g. of toad's weight in all cases) was chosen because it was found to be the most satisfactory, since it was the best that could be tolerated without adverse effects on the toads; no toads died either during the test or for many weeks after.

Most workers on the male toad for the diagnosis of pregnancy considered a result positive only when the slide prepared from the cloacal discharge "swarmed with spermatozoa swimming in all directions." But with the standardised technique here employed, the presence of even as few as 2 or 3 non-motile extruded spermatozoa in the whole field was, and should be, considered as positive, provided always that the preliminary examination of the toad's cloacal discharge had been negative. This is so, because the presence of even such a small number of spermatozoa still showed that chorionic gonadotrophin was present, though in a very low concentration. That is why a careful search for spermatozoa should always be made in the film before making a decision.

The gradation in the quantitative response of the male toads was found to be satisfactory only between 2 dose levels, 5 and 35 I.U./30 g., which is rather a narrow margin; but it was so regular that a difference in the injected hormone as small as 5 I.U./30 g. was easily detectable, a definite difference in the percentage of the response obtained being observed.

Conflicting opinions have been given as regards the minimum effective dose of chorionic gonadotrophin on male toads. While Mainini[1] obtained positive results only in two-thirds of his toads with 500 I.U., Klopper and Frank[2] obtained 100 per cent. of positive results with 20, 100 and 2000 I.U., whereas Mohanty and Pabrai[5] showed that in their toads the minimum effective dose was 15 I.U. The present author found this in his toads to be between 5 and 10 I.U./30 g. This variation is due both to species and individual variation in the toads used, but it may also be due to the different criteria taken by these authors in considering a result positive. It was also found that both the minimum dose that produced a 100 per cent. response and the maximum dose that produced no response were smaller in this case than those obtained by Mohanty and Pabrai.

For all these reasons, it is suggested that the male Egyptian toad, *Bufo regularis*, when employed according to the method described, is a more sensitive and discriminating test animal both for the assay of the chorionic gonadotrophin in pure solution or in clinical material, and for the diagnosis of pregnancy; and its reliability for this purpose is proved in Part II of this paper.

This method has certain advantages over the other methods in common use. It is much less time-consuming, as the results can be reported on the same day as the test is begun, instead of after many days in the older methods. A number of specimens can thus be assayed on the same day. It is far less expensive, as the toads are cheaper and easier

to obtain locally than the other animals employed in the older methods. The test animals, too, require less care and expense to keep and maintain; and the same animal can be repeatedly and satisfactorily used for the test 3 or 4 times, or even more, at weekly intervals so as to ensure the complete elimination of the effect of the previous dose. It is simple, and, as other workers have previously shown, seems to be specific; the end-point is definite and easy to read; and the method is shown to be accurate and reliable enough for routine work, and is applicable to pure solutions of the hormone as well as to clinical material.

The results of Part III of this paper show that the male toad test for the diagnosis of pregnancy, when employed according to the modifications here specified, will probably be more sensitive and accurate than both the original unmodified method and the Friedman's rabbit method.[9] Therefore, in performing this test, it is recommended that the volume of urine injected should be calculated according to a definite dose-weight system.

It must further be noted that since this work showed that the minimum dose of chorionic gonadotrophin that produced a 100 per cent. positive response in our toads was 35 I.U./30 g., therefore, in the diagnosis of pregnancy by this method, the volume of urine injected (2 ml.) must contain an amount of gonadotrophin higher than this level, because if it is lower, the result might be negative, a likely occurrence during the first few days after the first missed menstrual period. In such cases, if the test were repeated a week or two later, when the concentration of gonadotrophin in the urine had increased, it would most probably be positive.

The neglect of this point might have been the major cause for the high incidence of false negative results that some workers obtained, amounting sometimes to 20 per cent. with one author (Pollak[3]), who consequently emphasised the limited usefulness of male amphibia for pregnancy tests as compared with rabbits. By employing this standardised technique, confidence is again placed in the male toad test, and it is not only possible to diagnose the presence or absence of pregnancy or, more correctly, of demonstrable amounts of chorionic gonadotrophin, but also to estimate its amount in the urine.

SUMMARY

1. Egyptian male toads, *Bufo regularis*, were tested for the effect of gradually increasing doses of chorionic gonadotrophin. A satisfactory and regular quantal dose-reponse curve was established. This was used as a method for the biological assay of this hormone, with accurate and repeatable results. The advantages of the method are its simplicity, speed, cheapness, convenience and, apparently, reliability.

2. A modification in the technique of the male toad test for the diagnosis of pregnancy is recommended, based on the calculation of the volume of urine injected according to the toad's weight. This is likely to eliminate the causes of the false negative results that might occur, and renders the test very reliable.

3. On comparing the results of the assay of the chorionic gonadotrophin content of 3 samples of urine taken from normal pregnant women, by the male toad, the mouse and the rabbit methods simultaneously, parallel results were obtained.

4. A comparison was made of the thus modified male toad test for pregnancy and the rabbit test of Friedman, which showed the former to be probably more reliable than the latter.

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