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*Note.*—The Ediswan machine is commonly used in mental hospitals in India. It has various gadgets controlling the voltage from 70 to 150 volts and time from 0.1 to 1 second. It can be had from Messrs. Cox-Cavendish Electrical Co., Ltd., 45-47, Marylebone High Street, London, W.1. Other similar machines are equally useful for intensified E.C.T. by the method described above, provided the voltage and time range up to 150 volts and 1 second respectively.

## STUDIES ON SEX HORMONES ;

Part I—

### SPERM TEST OF PREGNANCY UTILIZING MALE TOAD, *BUFO MELANOSTICTUS* SCHNEID., AS TEST ANIMAL

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In the early months of pregnancy there is no single sign nor any combination of signs and symptoms to make the diagnosis unequivocal. To overcome this difficulty, a number of tests, biological, biochemical and immunological, have been tried. Of these only three biological tests, viz, Aschheim-Zondek, Friedman and Hogben, have come to stay. The consensus of opinion is that all other tests such as bitterling test (Naidu and D'Souza, 1944), the various intra-dermal tests (Editorial, 1937; Gilli and Howkins, 1937), pregnanediol test (Day and Cains, 1948; McCormack, 1946; Morrow and Benua, 1946), etc., are fallacious and unreliable. The three standard biological tests, viz, A.-Z., Friedman and Hogben, suffer from the disadvantages that they are expensive, time consuming, complicated and can only be performed in well-equipped laboratories under skilled hands. Moreover, in case of Hogben test the unavailability of the test animal, *Xenopus laevis* Daud., outside South Africa, is a serious drawback. The consideration of time factor, above all, is all important, particularly so in the management of emergent cases where these biological tests are of little use as their results can only be known at the earliest after 4 days, 2 days and 1 to 2 days respectively (Crew, 1939; Landgrebe and Hobson, 1949; Weisman *et al.*, 1942).

Two modifications of A.-Z. test have been reported (Hoffman, 1946; Soman, 1944, 1946; Zondek *et al.*, 1945). In these tests, white immature rats are used and the end-point is ovarian hyperaemia instead of appearance of mature follicles or corpus luteum. But the reliability of the tests still awaits confirmation. Even if these tests prove reliable, they are not free from other disadvantages of the standard biological tests.

Recently, Galli Mainini (1947) reported a new biological test of pregnancy. It is based on the reaction of a species of male toad, *Bufo arenarum* Hensel., in shape of ejaculation of sperms when untreated pregnancy urine is injected into it. Other South American workers (Alejandro, 1947; Pinto and Suer Boero, 1948) have corroborated his findings. The test, as reported, seemed to be very accurate, extremely simple, inexpensive, very rapid and highly specific. At that time it seemed to us that the greatest drawback of the test, like that of the Hogben, may be the non-availability of the toad in countries outside South America. In order to find out if any of the locally available species of toads and frogs can be successfully used to replace *B. arenarum* in carrying out this test, it was thought worth while to make a systematic study. This report is based on such trial and embodies the results of our investigations with *Bufo melanostictus* Schneid., the common toad of Orissa (also widely prevalent throughout India, most parts of Pakistan, Ceylon, Burma, Malay, Thailand, Indo-China, South China and Indonesia) and was carried on during the period of 8 months from February to September 1949.

### Method

Untreated samples of urine were subjected to qualitative physical and chemical tests. Healthy male toads, weighing 50 to 70 gm., were put each into a cylindrical glass jar (12 inches high by 8 inches diameter), covered with wire gauze held secure by weight and duly marked with the respective number. By means of a bent glass catheter, samples of urine from the cloaca of each toad were withdrawn and examined under microscope for presence of sperms before the injection of urine. This procedure ensured against any fallacy as each animal served as a control of its own.

By trial and error in the beginning it was found out that it is better to utilize three toads for each specimen of urine in order to guard against occasional refractory and non-reactive animals as well as death of one after injection.

Each toad was taken in turn and 10 mil. of untreated urine from the corresponding sample was injected into its dorsal lymph sac by means of a hypodermic syringe and a small hæmostat applied for half a minute or so over the point of needle puncture to prevent leakage. After ½ hour, by catheterization, cloacal samples from each toad were taken and examined microscopically for the presence of sperms. A sample was considered positive if any one or more of the three toads used showed sperms in the cloacal samples. If negative, fresh cloacal samples were examined every half hour till 4 hours. If still negative, the urine was treated otherwise for extraction of hormone according to the method of Barbosa de Castro (1947) with the modification that rectified spirit was used in place of absolute alcohol (hereinafter

referred to as 'hormone extraction method' or 'extraction method'); 100 mil. of urine from each specimen were subjected to this treatment and the extract injected into two additional toads. Specimens of urine treated and untreated from puerperal cases were injected into toads using the same technique.

Urines from healthy regularly menstruating women in various phases of menstrual cycle, from healthy males and samples of frog saline\* were also similarly subjected to the test in order to find out if any of these gave positive reaction.

Samples of urine of known and suspected cases of pregnancy and of puerperal cases were obtained from the obstetrics wards and antenatal clinics of the college hospital and from private sources. Non-pregnant urines were collected from the nursing staff while male urines were secured from members of the staff and others.

Lastly, in order to find out the particular hormone or hormones responsible for this biological reaction and the degree and nature of its specificity, trial was made with standardized preparations of various hormones in varying doses, viz, chorionic gonadotropin of pregnancy urine, oestrone, oestriol, progesterone, stilboesterol and desoxycorticosterone.

Further, with a view to knowing whether the used toads can be re-utilized after rest and with proper feeding for a few days under artificial conditions, the following method was tried :

*Housing and feeding the toads.*—Toads already subjected to test were placed in a galvanized iron tank containing 4 inches to 6 inches deep water and provided with taps for emptying and refilling water daily. The cover of the tank was fitted with wire netting of 1 inch mesh. Inside the tank there were two ladders leading to two platforms fixed to its sides. A narrow piece of wood bridged the two platforms. An electric bulb was provided inside the tank. The windows of the room containing the tank were kept open at night and the electric light of the tank was kept burning when other lights were put out. The whole idea was to attract insects to the tank so that the toads could help themselves to them. This device has proved a success. The toads are getting on fairly well for the last two months and are being utilized every 10 to 12 days with satisfactory results.

In the beginning mortality of the toads was 9.26 per cent with untreated urine. With the idea that the bacterial content of urine might be one of the causes of mortality, we started adding 5 drops of 20 per cent thymol in alcohol to each 100 mil. of urine. The toads tolerated the thymol satisfactorily without any change in their reactivity. Reaction of the toads as

regards ejaculation of sperms to injections of thymol solutions in frog saline or thymol solution added to male and non-pregnant urine was studied and was always found to be negative.

#### Observations

Positive cases were characterized by the presence of sperms in cloacal samplings when examined microscopically. The sperms are elongated curved bodies with a flagellum or tail springing from the middle piece and their movement is quite brisk and characteristic of the positive cases. 87.98 per cent showed presence of sperms in  $\frac{1}{2}$  to  $1\frac{1}{2}$  hours after injection, the earliest being 25 minutes and the latest 4 hours.

In all, 407 specimens of urine were subjected to this test. Of this, 63 samples from 43 suspected cases of early pregnancy and 262 specimens from 108 known cases of pregnancy were examined. In addition, 26 specimens collected from 6 cases on different days of early puerperium and 36 specimens collected during various phases of menstrual cycle from 12 healthy regularly menstruating women and 20 samples of male urine were also tested.

*Suspected early cases.*—Of the 63 samples from 43 suspected cases of early pregnancy, subsequent history proved 4 to be cases of delayed menstruation, 3 others turned out to be cases of amenorrhoea due to causes other than pregnancy and 1 was a case of incomplete abortion. Sixteen samples from these 7 cases, both by treated and untreated methods, gave persistently negative results and one sample from the case of abortion gave negative result with untreated urine. Of the remaining 46 specimens of urine from 35 cases of early pregnancy, confirmed by subsequent history, 44 (from 33 cases) showed positive results with untreated urine and two samples collected 5 days and 8 days after missed period from 2 cases gave positive reaction by hormone extraction method (*vide* table I); these two cases might be too early with very little chorionic tissue to give satisfactory concentration of hormone in urine to be positive by untreated method.

The case of abortion referred to above is an interesting one. The patient was admitted to the hospital on 16th August, 1949, with complaint of intermittent bleeding accompanied by pain of 10 days' duration preceded by history of 3 to 4 months' amenorrhoea. Examination of urine of this case by hormone extraction method could not be done as the quantity was small and before collection of further sample, the abortion became complete. The products of conception along with the decidua came out *en bloc* as a cast of the uterine cavity. Detailed study of this cast is being carried on by workers of other departments of this college. Private discussion with these workers has revealed that the foetus seems to be of much earlier age than the period of amenorrhoea.

\*0.6 per cent sodium chloride solution in distilled water without any adjustment of pH.

*Clinically undoubted cases of pregnancy.*—The clinical diagnosis of the 'known' cases was unequivocal. All of them were in-patients of the hospital and their period of gestation varied

The consideration of tables I and II reveals that, in a series of 308 samples from 143 cases of early and late pregnancy, the result was cent per cent positive.

TABLE I

Showing results obtained with urines of early cases of pregnancy confirmed by subsequent history

Period of gestation (after missed period)	Number of cases	Results with						Net result (total)	
		Untreated urine			Treated urine*				
		Number of samples tested	Number of samples positive	Per- centage of samples positive	Number of samples tested	Number of samples positive	Per- centage of samples positive	Number of samples positive	Per- centage of samples positive
5 days ..	1	1	nil	..	1	1	100.0	1	100.0
8 " ..	1	1	nil	..	1	1	100.0	1	100.0
8 " to 2 weeks ..	4	5	5	100.00	..	..	..	5	100.0
3 to 8 weeks ..	17	25	25	100.00	..	..	..	25	100.0
9 to 14 " ..	12	14	14	100.00	..	..	..	14	100.0
TOTAL ..	35	46	44	95.65	2	2	100.0	46	100.0

\* 'Treated urine' means urine subjected to hormone extraction method, and only specimens giving negative results with untreated urine were subjected to this treatment.

from 20th to 40th week. Of the 262 samples collected from this group, 234 gave positive results with untreated urine; the remaining 28 became positive by hormone extraction method. These 28 specimens were collected a few hours to 5 weeks before the onset of labour from 12 cases (see table II).

*Puerperal cases.*—Examination of samples of urine collected during early puerperium, as presented in table III, shows that most of them became negative after 4th day; only one sample gave positive result up to the 8th day of puerperium by treated method. In this exceptional case, some live chorionic tissue

TABLE II

Showing results obtained with urines from undoubted cases of pregnancy in later half of the term

Period of gestation in weeks *	Number of cases	Results with						Net result (total)	
		Untreated urine			Treated urine				
		Number of samples tested	Number of samples positive	Percentage of samples positive	Number of samples tested	Number of samples positive	Percentage of samples positive	Number of samples positive	Percentage of samples positive
20-28	11	30	30	100.00	..	..	..	30	100.0
29-36	32	110	108	98.18	2	2	100.0	110	100.0
37	13	32	30	93.75	2	2	100.0	32	100.0
38	15	29	25	86.21	4	4	100.0	29	100.0
39	18	33	24	72.73	9	9	100.0	33	100.0
40	19	28	17	60.71	11	11	100.0	28	100.0
TOTAL	108	262	234	89.31	28	28	100.0	262	100.0

\* Calculated from the first day of the last menstruation.

TABLE III

Showing results obtained with urine collected on different days of puerperium from 6 cases

Day of puerperium	Number of samples	Results with			Total number of samples positive	Remarks
		Untreated urine	Treated urine			
		Number of samples positive	Number of samples tested	Number of samples positive		
2nd	6	3	2	1	4	Some of the samples, negative with untreated urine, could not be examined by hormone extraction method as the quantity was small.
3rd	3	1	2	nil	1	
4th	5	2	3	1	3	
5th	6	1	3	nil	1	
6th	3	1	1	nil	1	
7th	1	nil	1	1	1	
8th	1	nil	1	1	1	
9th	1	nil	1	nil	nil	

might have been retained in the wall of the uterus.

*Urine from non-pregnant women.*—Samples of urine collected from healthy regularly menstruating women and those from males gave negative results both by treated and untreated methods and by the former method in doses 4 times that of pregnancy cases (equivalent to 200 mil. of untreated urine).

*Reactivity of toads and intensity of reaction.*—Up to a certain limit, there seems to be some positive correlation between the concentration of gonadotropic hormone in urine and the intensity of positive reaction as reflected by the number of sperms in the cloacal samples. In

positive samples, concentration of sperms as seen under the microscope varied widely from 'very abundant' to 'very scanty' as shown in table IV.

Other factors, besides the concentration of gonadotropin in urine which may possibly influence the intensity of reaction, are concentration of urine, age, health and nutrition of the toads, the season in relation to their breeding habit, and individual variations in reactivity. But concentration of urine does not seem to influence much the intensity of reaction; very dilute samples from early cases of pregnancy gave positive reaction graded as 'abundant' or 'moderate'. Health and nutrition of the toads

TABLE IV

Showing reactivity of toads and intensity of reaction

Type of urine (1)	Number of toads used (2)	Percentage of toads showing						Remarks (9)
		Very abundant (3)	Abundant (4)	Moderate (5)	Scanty (6)	Very scanty (7)	Negative (8)	
<b>A. Untreated urine of :</b>								
1. Early pregnancy	138	8.69	46.71	24.64	10.86	5.79	3.31	Figures under column (8) include refractory, dead and moribund toads.
2. Late pregnancy	836	5.14	13.28	38.28	24.16	7.42	11.72	
3. Puerperium ..	78	..	..	2.56	15.36	10.24	61.84	
<b>B. Treated urine of :</b>								
1. Early pregnancy	6	..	33.33	66.67	..	..	..	
2. Late pregnancy	56	..	46.43	46.43	5.36	..	1.78	
3. Puerperium ..	28	..	..	17.86	14.28	..	67.86	

Very abundant = More than 100 sperms per field under high power.  
 Abundant = 20-100 sperms per field under high power.  
 Moderate = 5-20 sperms per field under high power.  
 Scanty = 1-5 sperms in most of the fields under high power.  
 Very scanty = 5-10 sperms in 20-30 fields under high power.  
 Negative = No sperms seen.

no doubt affects their reactivity. Results with a few toads, starving for about a week, were very poor whereas healthy toads with the very samples of urine gave satisfactory response. Season does not seem to influence the reactivity of the toads but the authors hesitate to be emphatic on this point till further experience is gathered in mid-winter. Individual variation in reactivity is no doubt a factor but the percentage of such refractory toads is very low (about 3 per cent).

Variation in colour among male *B. melanostictus* is quite frequent, which may be due to differences either in nutritional or ecological factors. Observation of the authors is that earthy dark coloured male toads, with yellowish red colour over the skin covering the floor of the mouth and subjacent vocal sacs, are very reactive. Experience enables them to pick up toads, 99 per cent of which turn out to be reactive.

**Mortality among toads.**—In the beginning, 9.26 per cent of toads put up for the test with untreated urine became moribund or died within 10 to 20 minutes after injection before giving positive reaction. With the addition of thymol (*vide supra*), the mortality came down to 3.29 per cent. The residual mortality might be due to pH of urine, individual susceptibility or elimination of metabolites, drugs or bacterial toxins in urine. It is apparent by comparative study with frog saline that the volume of urine injected is not responsible for the death. Individual susceptibility is no doubt a factor as it has been noticed, at times, one toad dying out of three injected with an equal quantity of urine from the same specimen.

#### Discussion

It is significant that most of the early cases of pregnancy, after the second week after the missed period, gave more intense reaction (*i.e.* 'very abundant', 'abundant' or 'moderate') whereas the reaction in majority of the cases in later half of pregnancy was less intense (*i.e.* 'moderate', 'scanty' or 'very scanty'). Some cases towards the end of the term gave 'false negative' results by untreated method while 4 days after delivery almost all cases became negative even by hormone extraction method. This signifies that the excretion and concentration of chorionic gonadotropin in urine is more in early months of pregnancy, particularly between 2 to 16 weeks after the missed period, than in the later months and it almost disappears from urine about 4 days after delivery. This is in conformity with the findings of Evans *et al.* (1937) and Browne and Venning (1936) who made quantitative study of urinary excretion of gonadotropin in pregnancy.

No 'false positive' result was recorded in the present series with urine for non-pregnant women and urine for males.

Of the various hormones tried to evoke this reaction in toads only chorionic gonadotropin of

pregnancy urine has been found to be successful. Equine gonadotropin and anterior pituitary gonadotropin have not yet been tried due to unavailability of the products locally. Although pregnancy urine gonadotropin is predominantly LH (luteinizing hormone), yet chemically and pharmacologically it differs from the LH of anterior pituitary. It also differs from equine gonadotropin which is mainly FSH (follicle stimulating hormone). It is not possible to pronounce any opinion without further study whether anterior pituitary gonadotropin and equine gonadotropin will give the reaction in toads as well. But whatever may be the final outcome of further study and elucidation, it is purely of academic interest and will not affect in the least the practical value and utility of this test because in human urine in pregnancy the question of equine gonadotropin does not arise and anterior pituitary gonadotropin is seldom present in any quantity in urine to evoke this reaction by itself.

Study with the standard chorionic gonadotropin of urine in pregnancy (unpublished work) convinces the authors that the minimum quantity to evoke this reaction is much less than what it is in the case of Hogben test which requires a minimum of 70 I.U. (Landgrebe and Hobson, 1949). Further, they have observed that the intensity of reaction runs parallel with the increase in dose up to a certain limit. This lends support to the authors' conclusion (*vide supra*) that 'within certain range positive correlation exists between the excretion and concentration of chorionic gonadotropin in urine and intensity of reaction'.

As mentioned before, one sample from a case of incomplete abortion gave negative result by untreated method which implies that the concentration of hormone in urine was low. The history of the case with the coming out of the products of conception along with decidua in the form of a cast lends support to the assumption that the moorings of the chorionic tissue in the wall of the uterus were severed a few days before the urine was collected, thus preventing further elaboration of the hormone and its entry into maternal circulation. In the meantime, the pre-existing placental gonadotropin in maternal body fluid might have come down to a very low level due to rapid elimination. Moreover, as discussed before, some specimens of urine towards the end of the term gave negative results by untreated but positive by treated method, which evidently was due to low concentration of hormone in urine. Towards the end of the term, placenta becomes senile with concomitant impairment of its function. This might be the cause of low concentration of hormone towards the end of the term. Viewed together, these two points lead to the conclusion that actively functioning chorionic tissue in biological continuity with the maternal tissue is necessary for satisfactory positive results.

Although there is no 'false positive' result in the present series of cases, its possibility cannot be entirely ruled out. Conditions other than pregnancy in which this hormone is present in urine in enormous quantities, *viz.*, chorionic epithelioma, hydatid mole and testicular teratoma, are likely to give strong positive results.

Besides, there are other conditions as menopause, castration in both sexes, genital carcinoma in women, certain benign tumours of ovary and pituitary, organic disease of the hypothalamus, tumour of adrenal cortex, etc., in which urinary gonadotropin content, either LH or FSH, rises appreciably. Whether the urine of such cases, which at times gives positive results with other biological tests of pregnancy, will show positive reaction with this test is yet to be seen.

During the progress of this work, the authors came across two more papers (Brody, 1949; Wiltberger *et al.*, 1948) reporting the suitability of *Rana pipiens* for the test. Other species of Ranidae and Bufonidae families are likely to be found suitable for this test. This will simplify the matter as to the availability of the test animal which can be had everywhere.

#### Summary and conclusions

1. Suitability of male toad, *Bufo melanostictus*, the common toad of India, in the biological test of pregnancy, based on the principle of Galli Mainini reaction, is reported.

2. In the present series of 308 samples from 143 cases of pregnancy, no 'false negative' result was obtained. With 92 samples of non-pregnant and male urine and frog saline, no 'false positive' result has been recorded.

3. Within a certain limit, a positive correlation exists between the concentration of the hormone in urine and the intensity of reaction.

4. In the first weeks of pregnancy and towards the end of the term, in some cases, the reaction has been found to be negative with untreated urine but positive by hormone extraction method, implying that concentration of hormone in urine is low during this period. The reaction becomes negative early in puerperium.

5. The distinct advantages of the test are its simplicity, inexpensiveness, rapidity and very high degree of accuracy. The result can be known within  $\frac{1}{2}$  to 4 hours. The end-point is sharp and clear-cut. The test animal is available almost everywhere.

6. The same toads can be re-utilized every 10 to 12 days with proper feeding.

7. It is suggested that this biological test of pregnancy with various species of toads and frogs be termed as 'the Sperm Test of Pregnancy'.

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