7912 C

Factors to be Considered in Immature Female Rat Titration of Pregnancy Urine.*

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The induction of precocious sexual maturity in the female rat or mouse has been used extensively as the foundation for both qualitative and quantitative studies of the gonadotropic activity of blood and urine and of extracts made from these and other substances. Considerable difficulty has been experienced in this and other laboratories in the application of reactions associated with this maturity phenomenon. For the purpose of evaluating several of the criteria suggested for use in estimating the gonad-stimulating potency of urine, an extended series of titrations of pregnancy urines was conducted in immature female rats. Analysis of the data yields information concerning the relative sensitivity of ovarian, vaginal, and uterine reactions; the conditions limiting their reliability and indications for their use, separately or in combination, as endpoints.

Two lots of urine were titrated. One of these (P.U.A.) was a composite of specimens from pregnancies of 2 to 12 weeks' duration. The other (P.U.B.) was a complete 24-hour collection obtained at approximately the seventh week of gestation in a woman with no evidence of malignancy or endocrinopathy. The follicular hormone was extracted with ether. Doses of less than 1 cc. of urine were prepared by so diluting the stock that each lower dose contained one-half as much urine as the preceding one. The test doses were made up to a volume of 5 cc. per animal. Female rats, of a highly inbred strain, weighing 35 to 45 gm. at 24 days of age were given daily subcutaneous injections for 5 days. During the injection period, observations were made for vaginal opening with estrus. The degree and character of uterine and of ovarian response was recorded at necropsy on the sixth day.

It may be seen (Table I) that, although the higher injection levels caused marked ovarian weight increase in all members of the test groups, the percentage of positive vaginal responses was less than that elicited by smaller amounts of urine which induced far less ovarian weight increases. With reduction of urine concentration,

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TABLE I.

Test Urine	Injection Level (cc.)	No. Rats Used	Vaginal Response No. Open	Vaginal Response No. Not Open	Ovarian Response (wt. in mg.) Range	Ovarian Response (wt. in mg.) Group Average
None (Controls)		12	0	12	10.0-17.8	13.32
P.U. A	5.00	12	6	6	34.0-68.0	49.47
"	3.00	12	9	3	26.0-72.2	42.48
"	2.00	12	11	1	28.1-44.0	37.10
"	1.00	12	10	2	24.0-52.0	37.93
"	0.50	12	12	0	16.0-30.0	22.15
"	0.25	12	12	0	11.0-22.7	17.50
"	0.125	12	12	0	20.1-29.2	24.05
"	0.0625	12	11	1	10.0-16.6	14.08
"	0.0312	12	2	10	9.0-17.0	13.20
"	0.0156	12	0	12	9.0-13.3	11.50
P.U. B	5.00	6	3	3	28.3-61.5	39.60
"	1.00	6	2	4	56.0-64.8	60.05
"	0.50	6	6	0	32.8-34.0	40.50
"	0.25	6	6	0	19.5-35.5	26.00
"	0.125	6	6	0	11.1-27.0	21.36
"	0.0625	6	5	1	13.8-23.3	17.63
"	0.0312	6	6	0	15.9-25.5	21.40
"	0.0156	6	6	0	15.0-26.6	20.01
"	0.0078	6	0	6	13.5-18.1	15.55
"	0.0039	6	0	6	14.0-22.0	16.15
"	0.0019	6	0	6	15.7-20.0	17.13
"	0.00038	6	0	6	11.0-13.6	11.78

P.U. A: Pooled specimens from cases of pregnancy of 2 to 12 weeks' duration.
P.U. B: 24-hour specimen from one case of normal pregnancy of approximately 7 weeks' duration.

the increase in group percentage of positive vaginal responses rather closely parallels the increase in follicular elements and the decrease in hypertrophic lutein bodies in the ovaries.

If the ovaries of the P.U.A. series are arranged in order of weights, there are 11 pairs with weights of 50 mg. or more. In one instance only was estrus associated with ovaries of this weight. In 65 rats whose ovaries weighed 20 to 49 mg., positive vaginal reactions were elicited in 64 animals (98%). Below 25 mg. ovarian weight, estrus occurred in all animals until well within the normal weight range. Examination of the ovaries showed that excessive weight and massive luteinization are to be correlated with failure of estrus. When the vaginal reaction occurred in animals with heavy ovaries, there were always visible follicles. It is notable that a significant number of rats gave the estrus response without increased ovarian weight, that is, evidence of functional activity in normal sized ovaries.

The results of the titration of the 24-hour urine P.U.B. differ but little from those obtained with the P.U.A. batch of pooled urines. There is a more abrupt appearance and disappearance of the vaginal

reaction in rats receiving P.U.B. This is probably due to the smaller number of animals in the test groups. The most outstanding difference seems to be related to the potency of the 2 lots. The highest doses of P.U.B. actually caused less weight increase of ovaries as well as fewer estrus responses.

It is to be noted that, at the lowest injection levels of these test urines, neither ovarian nor vaginal changes could be detected although the uteri of several of the rats were extremely hypertrophic with transparent, greatly distended horns which were filled with fluid.

TABLE II.

Urine Tested	Injection Level (cc.)	End Points: Vaginal Opening and Estrus — Positive (% of Test Group)	End Points: Vaginal Opening and Estrus — R.U./Liter Test Urine	End Points: 100% Ovarian Weight Increase (20-35 mg. ovaries) — % of Test Group	End Points: 100% Ovarian Weight Increase (20-35 mg. ovaries) — R.U./Liter Test Urine
P.U. A	0.50	100		66⅔	2,000
"	0.25	100		16⅔	
"	0.125	100		100	8,000
"	0.0625	92	16,000	0	
"	0.0312	16		0	
P.U. B	0.125	100		83⅓	8,000
"	0.0625	83⅓		33⅓	
"	0.0312	100		66⅔	
"	0.0156	100	64,000	66⅔	64,000
"	0.0078	0		0	

The data assembled in Table II permit consideration of the vaginal reaction and of the ovarian weight response as end-points for the estimation of the gonadotropic potency of P.U.A. and of P.U.B. in rat units per liter (R.U.L.). Katzman and Doisy,[1] in defining their vaginal opening-estrus criterion of activity, include neither the number of rats used per test group nor the percentage response required. The concentration of active substance in P.U.A., therefore, cannot be stated with certainty in Katzman-Doisy units, since with the use of 12 rats per test group, failure to induce vaginal response is more gradual than in the case of the 6 animal titration groups used in the P.U.B. series. If the Hamburger[2] requirement that the unit dose elicit estrus in at least one-half of the injected animals be applied, the potency of P.U.A. may be estimated at 16,000 R.U.L. and that of P.U.B. at 64,000 R.U.L.

The data concerning the ovarian weight end-point (Table II) il-

[1] Katzman, P. A., and Doisy, E. A., *J. Biol. Chem.*, 1932, **98**, 751.

[2] Hamburger, C., *Acta Path. et Microbiol. Scand.*, Supplement XVII, 1933.

lustrates the difficulty of using this response alone for determining the unit concentration of gonad-stimulating substance in pregnancy urine. In the titration of P.U.A., the injection of 0.25 cc. induced 100% ovary weight increase in but 16 2/3% of the rats, while twice as much of the urine effected this degree of ovarian growth in 2/3 of the test group. Such results might justify the acceptance of the latter dose as the minimal effective dose, had the titrations not been continued at higher dilutions. The 100% ovary weight increase again appeared in all of the rats receiving 0.0625 cc. of P.U.A. but was not encountered at 4 lower injection levels. Similar results were obtained in the P.U.B. titration series. Comparison of the ovarian morphology of the rats injected at the 2 levels of positive ovarian weight response in each series indicates that 0.5 cc. of P.U.A. and 0.125 cc. of P.U.B. are the lowest levels at which discrete corpora lutea are seen. The highest dilutions of these urines (0.0625 cc. P.U.A. and 0.0156 cc. P.U.B.) to produce 100% ovary weight increases are also correlated with the final detection of follicles of macroscopic size. Thus the luteinizing activity of P.U.A. would appear to be 2000 R.U.L. and that of P.U.B. 8000 R.U.L., while their respective follicle-stimulating capacities would be 8000 R.U.L. and 64,000 R.U.L.

In the titration of P.U.A., the vaginal response serves as a somewhat more sensitive indicator of functioning follicles in the ovaries than does the increased weight with development of follicles of macroscopic size. In the P.U.B. series, these 2 end-points are in perfect agreement.

Summarization of the findings of these titration studies of the gonadotropic effects of graded doses of 2 batches of pregnancy urine leads to several conclusions. (1) Failure to induce vaginal opening with estrus may be due to the injection of too little active material or to inhibition of follicular development by rapid and excessive luteinization. The cause of negative vaginal reactions, therefore, should be investigated by ovary observation. (2) Vaginal opening with estrus is an indication of follicular activity. No information as to the relative follicle-stimulating or luteinizng potency of urines can be gained by means of the vaginal reaction alone. (3) The 100% ovarian weight increase fails at the levels just below the minimum dose required to produce discrete corpora lutea but reappears at a lower urine concentration, which also represents the minimum dose for visible follicles. Failure to induce 100% ovary weight increase may indicate urine concentration inadequate for luteinization or injection of amounts insufficient to produce enough follicu-

lation to effect the ovary weight. (4) The follicle-stimulation dose, as determined by ovary weight and development of follicles to macroscopic proportions, approximates the unit for follicle stimulation as determined by vaginal opening with estrus. Hence, the latter end-point may be used alone for testing dilutions known to be below the level of the minimum luteinizing dose. Such a combination of these 2 end-points may be used for the estimation of the relative concentration of the luteinizing and follicle-stimulating potentialities of test urines. (5) The uterine reactions to given doses of pregnancy urine are extremely variable so cannot be incorporated into the criteria of the unit dose. However, uterine hypertrophy in animals giving neither vaginal nor macroscopic ovarian responses should be considered as indication for microscopic investigation of the ovaries.

7913 P

Theelin and Progestin Injections on Uterus and Mammary Glands of Ovariectomized and Hypophysectomized Rabbits.

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The work of Gardner and Turner[1] suggests that the immediate stimulus for mammary growth is due to the ovarian hormones, theelin and progestin. But Corner[2] and others have obtained growth with anterior pituitary preparations in rabbits which have not been exposed to the influence of corpora lutea. The possibility arises that the ovarian hormones act through the pituitary. To test this point we ovariectomized 40 rabbits and kept them for 2 months to exhaust their supply of ovarian hormones. Then by the use of Firor's[3] technique we attempted to remove their pituitaries, We were completely successful in 10 cases; of these successes, 2 occurred while we were perfecting our techniques, 4 died during the 2 months after the operation before we commenced injections. This left us with 4 successful operations which were used for injection work. We sectioned the contents of the sella turcica in each case after the

1 Gardner, W. U., and Turner, C. W., *Mo. Ag. Exp. Sta. Res. Bull.* 196, 1933.
2 Corner, G. W., *Am. J. Physiol.*, 1930, **95**, 43.
3 Firor, W. M., *Am. J. Physiol.*, 1933, **104**, 204.