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Effect of Perphenazine Treatment of Rats on Serum Ovarian and Adrenal Steroids[1] (37914)

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(Introduced by J. J. Biezenski)

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Perphenazine is a phenothiazine derivative that is an effective sedative in the rat (1). Secretion of prolactin is stimulated by perphenazine as measured by assays of prolactin (2–6) and by the lactogenic response of the mammary glands (7). The response to perphenazine involves depletion of hypothalamic catecholamine stores (8), and decreased concentration (9) and synthesis (10) of prolactin-inhibiting factor. Perphenazine induces prolactin secretion only after inhibition of gonadotropin secretion by estrogen (11) or agents that act similarly (12) and gonadotropins remain inhibited during an ensuing period of pseudopregnancy (13, 14). Some evidence for stimulation of ACTH release has been reported (1), but growth hormone secretion is unaffected by the tranquillizer (8).

The administration of perphenazine provides a useful method for the study of the effects of endogenously released prolactin under conditions of suppressed gonadotropic secretion. Although prolactin is luteotropic in the rat as judged by morphologic criteria (15, 16), the ovarian response has not been characterized in terms of serum concentrations of the steroid hormones.

Materials and Methods. Animals. Rats weighing between 180 and 200 g were obtained from Hormone Assay Laboratories, Chicago. Animal quarters were lighted from 0500 to 1900 hr. Rats exhibiting at least two normal estrous cycles of 4 or 5 days based on evaluation of daily vaginal smears were selected and treatment was begun approximately 12 hr before or 12 hr after the predicted time of ovulation. Perphenazine,[2] 5 mg/kg body wt, was administered subcutaneously once daily for 3 days as a 5 mg/ml solution in 0.03 *N* HCl. On the 4th day, the rats were sacrificed in the same room in which they were housed by decapitation, and blood was collected for analysis. Untreated rats were sacrificed on the 3rd day of diestrus. Serum was separated and stored at −20°.

Technical procedures. Solvents for extraction and chromatography were all Nanograde (Mallinckrodt Chemical Co.) except that diethyl ether of reagent grade was purified by distillation on the day of use. Water was distilled in an all-glass still. Silica gel G (Brinkmann Instruments) was extracted with reagent-grade methanol in a soxhlet apparatus for at least 12 hr before preparing 0.25-mm thickness layers on glass plates. Chromatography was carried out in solvent System A, dichloromethane–diethyl ether (15:7). Steroids were eluted according to the mobility of ^{14}C-labeled standards chromatographed in parallel; the standards were located by the use of a radiochromatogram scanner. Estradiol-17β and corticosterone were eluted with ethyl acetate–water (1:1) and progesterone and 20α-hydroxypregn-4-en-3-one were eluted with 5% methanol in

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dichloromethane. Quantification of radioactivity was carried out by liquid scintillation spectrometry using diotol (17) scintillation fluid.

Analyses. Approximately 5000 cpm each of estradiol-17β-1,2,6,7-^{3}H, progesterone-1,2-^{3}H, corticosterone-1,2-^{3}H, and 20α-hydroxypregn-4-en-3-one-1,2-^{3}H prepared from progesterone-1,2-^{3}H by the method of Wiest (18) were added to 1 ml thawed serum samples. Methanol (2.3 ml) was added to each sample to precipitate serum protein, and after centrifugation at 4°, the supernatants were decanted. The precipitates were resuspended in 70% methanol and centrifuged again. The pooled methanolic supernatant solutions were evaporated to an aqueous residue, 3 ml of water was added, and the steroids were extracted into diethyl ether. After evaporation of the ether, the residue was partitioned between 0.4 *N* aqueous NaOH and benzene–ethanol (25:1). The 0.4 *N* NaOH fraction was neutralized by the addition of 2 *N* NaH_2PO_4 and estrogens were extracted with ethyl acetate. The ethyl acetate solutions were washed once with water, evaporated, and chromatographed on thin-layer plates in System A. After elution, one-fifth of each sample was counted to determine losses during purification, and aliquots of the remaining solution were dried in 12 × 75 mm disposable glass tubes for radioimmunoassay. A 1/50,000 dilution of an anti-estradiol-17β antiserum[3] was employed according to Mishell *et al.* (19).

The nonphenolic steroids partitioned into benzene–ethanol (25:1) were chromatographed in System A after evaporation of the solvent. The R_f values of progesterone, 20α-hydroxypregn-4-en-3-one, and corticosterone in this system were 0.58, 0.37, and 0.13, respectively. Aliquots for determination of procedural losses and for assay were taken as in the estradiol-17β assay. Progesterone and corticosterone were assayed by a radioligand method employing serum from women in the last month of pregnancy as a source of steroid-binding globulin. This serum was stripped of endogenous steroids by activated charcoal at 40° before dilution to a final concentration of 0.2% in water for the assay. The assay procedure was essentially that described by Murphy (20). The radioligand in each case was the same as the steroid to be assayed. Florisil was used to separate protein-bound from free steroid. 20α-Hydroxypregn-4-en-3-one was assayed similarly except that serum from male rabbits diluted to a final concentration of 0.5% was used as a source of binding protein. The radioligand used in the assay was corticosterone-1,2-^{3}H. Rabbit serum was also stripped of endogenous steroids before use in the assay.

The assays had the following characteristics. Displacement of estradiol-17β-1,2,6,7-^{3}H from the antibody by 10 pg of estradiol-17β was 28%; displacement by 10 ng of progesterone, 20α-hydroxypregn-4-en-3-one, and corticosterone in the respective assay systems was 43, 38, and 49%. The respective blanks for assays of the above steroids were 0.6 pg, 0.02 ng, 0.22 ng, and 0.00 ng. Coefficients of variation calculated from the duplicate assays of progesterone, 20α-hydroxypregn-4-en-3-one, and corticosterone in rat serum were ±9, ±13, and ±16%, respectively. Because of the low serum concentration of estradiol-17β in these animals, only single determinations could be made on each sample.

Results. The concentrations of progesterone, 20α-hydroxypregn-4-en-3-one, corticosterone, and estradiol-17β in peripheral serum of normal rats on the third day of diestrus are shown in Table I. The corresponding concentrations of these hormones 24 hr after the last of three daily injections of perphenazine is shown in Table II. Retrospectively, it was observed according to the presence of cornified epithelial cells in the vaginal smears that the first four animals in Table II had been given the first injection of perphenazine on 1400 hr of the day before ovulation. The remaining animals received the first injection at 1400 hr after ovulation and leukocytic infiltration was observed in the vaginas on the following day. The level of progestins on the third

[3] Anti-estradiol-17β antiserum was kindly provided by Dr. Ian Thorneycroft, Department of Obstetrics and Gynecology, University of Southern California.

TABLE I. Serum Steroids in Rats on the Third Day of Diestrus.

Rat No.	Progesterone (ng/ml)	20α-Hydroxy-pregn-4-en-3-one (ng/ml)	Corticosterone (ng/ml)	Estradiol-17β (pg/ml)
5	7	66	—	11
8	11	79	55	59
9	16	36	79	2
10	5	46	70	2
20	6	40	136	40
21	7	55	51	23
25	7	48	40	107
27	4	29	38	38
mean	7.9	49.9	67.0	35.2

day of diestrus was less than that observed in another strain previously (21), but the ratio of 20α-hydroxypregn-4-en-3-one to progesterone was the same. Overall, perphenazine administration resulted in more than a 14-fold increase in serum progesterone levels without a significant change in 20α-hydroxypregn-4-en-3-one. However, rats in which treatment was begun after ovulation had a significantly ($P < .05$) greater serum concentration of progesterone than those started on the day before ovulation.

At the time of sacrifice, the untreated rats all contained nucleated epithelial cells in the vaginal smears, thus providing evidence of prior estrogen secretion; rats treated with perphenazine had only leukocytes in vaginal smears on the day of autopsy. Although the serum concentrations of estradiol-17β were variable among the normal rats, the average concentration was about 6 times greater than that assayed in serum of perphenazine-treated rats. The variability is assumed to be due to differences in the rate of follicular maturation in normal rats. Despite the lower average concentration of estradiol-17β in rats treated with perphenazine, detectable concentrations were found in the serum of all animals.

Serum concentrations of corticosterone in perphenazine-treated rats were three-fold greater than in the untreated, normal animals. All rats were handled daily for vaginal examination, and decapitation for collection of serum was performed without

TABLE II. Serum Steroids in Rats Treated for Three Days with Perphenazine.

Rat	Treatment started	Progesterone (ng/ml)	20α-Hydroxy-pregn-4-en-3-one (ng/ml)	Corticosterone (ng/ml)	Estradiol-17β (pg/ml)
11	Before ovulation	96	38	115	—
15	Before ovulation	87	27	316	14
17	Before ovulation	69	30	331	9
26	Before ovulation	87	44	141	5
mean		84.8	34.7	225.7	9.3
6	After ovulation	66	18	165	1
23	After ovulation	176	54	314	—
24	After ovulation	133	28	60	1
28	After ovulation	152	37	287	4
30	After ovulation	148	47	123	5
32	After ovulation	150	61	348	10
mean		137.5	40.8	216.2	4.2

exciting the animals. In order to compare the effect of the treatment paradigm with normal hormone levels, normal rats were not injected with the 0.03 *N* HCl vehicle. However, since the last injections were made 24 hr before sacrifice, the effect of the injections on adrenal function is considered to be minimal. Nevertheless, the component of the treatment paradigm responsible for elevated corticosterone levels is not established by the experiment.

Discussion. The effect of perphenazine administration on prolactin secretion by the rat pituitary has been well-documented (2–7). Estrous cycles are suspended by the administration of perphenazine (13, 14) or by prolactin (22–24), indicating that the normal preovulatory release of LH is inhibited. Since similar effects can be achieved by the administration of as little as 1.5 mg of progesterone per day (25), inhibition of ovulation may be brought about by an increase in progesterone secretion. Despite the failure of prolactin added to the incubation medium to stimulate steroidogenesis *in vitro* (26), evidence that *in vivo* treatment of rats with prolactin results in an increased capacity for *in vitro* steroidogenesis has been reported (27), and by morphologic criteria, i.e., vaginal mucification of estrogen-treated hypophysectomized rats (16) and deciduomata development in hypophysectomized rats (15), prolactin free of gonadotropic activity has been shown to stimulate progestin secretion.

In the present study, perphenazine administration, which may induce serum prolactin levels as high as 400 ng/ml, (3), resulted in 17.5 times the concentration of progesterone present in normal rats when treatment was begun after ovulation. According to the studies of Armstrong *et al.* (27), prolactin administration alone increased *in vitro* synthesis of progesterone only at the expense of its reduction product, 20α-hydroxypregn-4-en-3-one, although they also found that serum progesterone levels were elevated to a greater extent than 20α-hydroxypregn-4-en-3-one levels were decreased. The extremely high serum progesterone values obtained after perhenazine treatment in the present study were observed with little change in the concentration of 20α-hydroxypregn-4-en-3-one. High levels of serum progesterone would be expected to inhibit preovulatory release of LH as well as LH-dependent follicular maturation (28, 29). In fact, the serum estradiol-17β concentration in perphenazine-treated rats was on the average only one-sixth of that in normal rats. From this it must be concluded that the serum concentrations of progesterone observed were obtained under conditions of very low serum LH concentration. The mechanism by which prolactin promotes steroidogenesis is not well-understood, but availability of precursor cholesterol appears to be a factor (27). Even so, the rate-limiting step is thought to be conversion of cholesterol to pregnenolone (30), a process which requires LH or HCG. The results, therefore, raise an important question about the rate-limiting steps in steroidogenesis in other than acute *in vitro* conditions.

The greater serum concentrations of progesterone observed in rats that were started on perphenazine treatment after ovulation as compared with those started before ovulation (all rats treated for 3 days, injections made between 2 and 4 P.M.) indicate that the response increases with time of exposure of the newly formed corpora lutea to prolactin as found previously (31). In both groups, prolactin secreted in response to perphenazine presumably hastened luteolysis of corpora lutea from the previous cycle (32). Rat No. 6 had a vaginal smear indicative of late estrus or early metestrus at the time treatment was begun; treatment may have been begun too late for an adequate stimulatory effect by prolactin.

Elevation of corticosterone in perphenazine-treated rats may be the results of increased ACTH secretion (1), although obviously the stimulation of secretion of ACTH is not as great as the increase in prolactin secretion. Alternatively, the production of adrenocortical steroids may be increased via a decrease in adrenal 5α-reductases as a result of elevated serum prolactin (33). Since adrenal steroidogenesis was increased only sufficiently to increase

serum corticosterone three-fold it is apparent that most of the increase in serum progesterone was of ovarian origin.

Summary. A comparison of the concentration of steroids in peripheral serum was made between normal rats in the third day of diestrus and rats injected subcutaneously for 3 days with 5 mg/kg body wt/day perphenazine. Progesterone levels were most markedly altered. When perphenazine treatment was begun on the afternoon following ovulation, the average progesterone level was increased 17-fold over that of untreated control rats; when treatment was begun on the afternoon preceding ovulation, the concentration of progesterone was increased only ten-fold. 20α-Hydroxypregn-4-en-3-one was only slightly decreased by the treatment. Irrespective of the day treatment was initiated, corticosterone was increased (three-fold) and estradiol-17β was decreased (six-fold) by perphenazine administration. The results are discussed in relation to the known effects of perphenazine administration on prolactin secretion.

1. Smith, R. L., Maickel, R. P., and Brodie, B. B., J. Pharmacol. Exp. Ther. **139**, 185 (1963).

2. Danon, A., Dikstein, S., and Sulman, F. G., Proc. Soc. Exp. Biol. Med. **114**, 366 (1963).

3. Ben-David, M., Danon, A., Benveniste, R., Weller, C. P., and Sulman, F. G., J. Endocrinol. **50**, 599 (1971).

4. Ben-David, M., Danon, A., and Sulman, F. C., Neuroendocrinology **6**, 336 (1970).

5. Blackwell, R., Vale, W., Rivier, C., and Guillemin, R., Proc. Soc. Exp. Biol. Med. **142**, 68 (1973).

6. Arimura, A., Dunn, J. D., and Schally, A. V., Endocrinology **90**, 378 (1972).

7. Ben-David, M., Endocrinology **83**, 1217 (1968).

8. MacLoed, R. M., Fontham, E. H., and Lehmeyer, J. E., Neuroendocrinology **6**, 283 (1970).

9. Mittler, J. C., and Meites, J., Proc. Soc. Exp. Biol. Med. **124**, 1433 (1964).

10. Sulman, M., Ben-David, M., Danon, A., Dikstein, S., Givant, Y., Khazen, K., Mishkinsky-Shani, J., Nir, I., and Weller, C. P., *in* "Hypothalamic Control of Lactation," (F. G. Sulman, ed.), p. 4. Springer–Verlag, New York (1970).

11. Danon, A., Weller, C. P., and Sulman, F. C., J. Endocrinol. **48**, 365 (1970).

12. Ben-David, M., Danon, A., Sulman, F. G., J. Endocrinol. **51**, 719 (1971).

13. Velardo, J. T., Fert. Steril. **9**, 60 (1958).

14. Ben-David, M., J. Endocrinol. **41**, 377 (1968).

15. Evans, H. M., Simpson, M. E., Lyons, W. R., and Turpeinen, K., Endocrinology **28**, 933 (1941).

16. Astwood, E. B., Endocrinology **28**, 309 (1941).

17. Herberg, R. J., Anal. Chem. **32**, 42 (1960).

18. Wiest, W. G., *in* "Methods in Enzymology," (S. P. Colowick and N. O. Kaplan, eds.), p. 638. Academic Press, New York (1969).

19. Mishell, D. R., Jr., Nakamura, R. M., Crosignani, P. G., Stine, S., Kharma, K., Nagata, Y., and Thorneycroft, I. H., Amer. J. Obstet. Gynecol. **111**, 60 (1971).

20. Murphy, B. E. P., J. Clin. Endocrinol. **27**, 973 (1967).

21. Chatterton, R. T., Jr., MacDonald, G. J., and Greep, R. O., Endocrinology **83**, 1 (1968).

22. Lahr, E. L., and Riddle, O., Proc. Soc. Exp. Biol. Med. **34**, 880 (1936).

23. MacDonald, G. J., Yoshinaga, K., and Greep, R. O., Proc. Soc. Exp. Biol. Med. **136**, 687 (1971).

24. Lyons, W. R., Simpson, M. E., and Evans, H. M., Proc. Soc. Exp. Biol. Med. **48**, 634 (1941).

25. Phillips, W. A., Amer, J. Physiol. **119**, 623 (1937).

26. Huang, W. Y., and Pearlman, W. H., J. Biol. Chem. **237**, 1060 (1962).

27. Armstrong, D. T., Miller, L. S., and Knudsen, K. A., Endocrinology **85**, 393 (1969).

28. Dempsey, E. W., Amer. J. Physiol. **120**, 126 (1937).

29. Astwood, E. B., and Fevold, H. L., Amer. J. Physiol. **127**, 192 (1939).

30. McKerns, K. W., *in* "The Gonads," (K. W. McKerns, ed.), p. 137. Appleton–Century–Crofts, New York (1969).

31. Chatterton, R. T., Jr., Miller, T. T., and Harris, J., Endocrine Society Abstracts, p. A-203 (1973).

32. Wuttke, W., and Meites, J. Proc. Soc. Exp. Biol. Med. **137**, 988 (1971).

33. Witorsch, R. J., and Kitay, J. I., Endocrinology **91**, 764 (1972).

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