PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 171, 207–213 (1982)

Ovulation, Ovarian 17α-Hydroxylase Activity, and Serum Concentrations of Luteinizing Hormone, Estradiol, and Progesterone in Immature Rats with Diabetes Mellitus Induced by Streptozotocin (41500)

MARY S. VOMACHKA AND DONALD C. JOHNSON[1]

Departments of Physiology and Obstetrics & Gynecology, The Ralph L. Smith Research Center, The University of Kansas Medical Center, Kansas City, Kansas 66103

Abstract. Immature female rats were injected with streptozotocin (60 mg/kg, iv) 3 to 4 days prior to the injection (iv) of 20 IU of pregnant mare's serum gonadotropin (PMS). Animals were killed at various intervals and the serum levels of estradiol, luteinizing hormone (LH), and progesterone were determined by radioimmunoassays. The ovarian steroid 17α-hydroxylase activity was determined by a tritium exchange assay using pregnenolone as the substrate. Ovulation was determined 72 hr after PMS by flushing of the oviducts. The diabetes mellitus induced by the drug reduced the number of animals ovulating and in some animals the number of ova shed when compared to controls. However, a surge in LH, which reached a peak at 60 hr, was seen in the diabetic animals; a larger peak with the same timing was found in the controls. Changes in ovarian 17α-hydroxylase also indicated that an increase in LH release occurred in the diabetic animals at about 60 hr. Estradiol levels were higher, but progesterone levels lower, in diabetic than control animals. Administration of 1 mg of progesterone to diabetic animals 48 hr after PMS resulted in an increase in the number of animals ovulating and the number of ova shed. The results indicate that hyperglycemia induced by streptozotocin is not inconsistent with production of an LH surge or with ovulation following ovarian stimulation by PMS. However, the lowered production of progesterone, which may be a cause or a result of lowered LH output, appears to be a primary factor in the reduced ovulatory rate.

Female infertility is a well-known consequence of diabetes mellitus regardless of whether the syndrome was induced by pancreatectomy or by pharmacologic agents (1). A consistent finding, and obviously an important factor, in the infertility is a reduction in the percentage of animals ovulating and the number of eggs ovulated per animal (2, 3). In recent studies (4, 5) immature female rats, made diabetic with alloxan, failed to ovulate subsequent to stimulation of the ovary by pregnant mare's serum gonadotropin (PMS), even though follicular maturation and estrogen production were not different from that in control animals. Several lines of evidence indicated that the site of the primary lesion was the hypothalamic–hypophyseal axis. Specifically, the lack of an LH surge, secondary to a reduced pituitary response to hypothalamic LH releasing hormone, was interpreted as the diabetes-induced defect (5). The present study was undertaken to examine the ovarian steroidogenic and ovulatory responses, as well as changes in serum levels of LH, in animals made diabetic with the antibiotic streptozotocin. The latter drug is less toxic than alloxan, but very effective at removing pancreatic beta cell activity (6).

The results demonstrate that treatment with streptozotocin causes a typical syndrome of diabetes mellitus and a reduction in the ovulatory LH surge in immature rats injected with PMS. The number of animals ovulating and the number of ova shed are reduced in these diabetic animals but both indices of ovulatory function can be restored to normal by the administration of progesterone 48 hr after the PMS.

Materials and Methods. Immature (26–30 day) female rats of the Holtzman strain were housed 5–10 animals/cage in

[1] To whom all correspondence should be addressed.

0037-9727/82/100207-07$01.00/0

temperature (25 ± 2°) and light (12 hr light/day) controlled quarters and given free access to food and water. Diabetes mellitus was induced by the intravenous injection (iv) of streptozotocin (Sigma Chemical Co., St. Louis, Mo.). The drug was dissolved immediately before use in 0.01 *M* citrate buffer (pH 4.5) and used at a dose of 60 mg/kg body weight; controls received only the buffer. The animals which received the drug demonstrated polydipsia and polyphagia within 48 hr. Hyperglycemia was verified at the time of sacrifice by determination of the concentration of serum glucose using the glucose oxidase method, with kits obtained from Sigma. Animals having serum glucose concentration levels more than twice that of controls were considered diabetic.

Three to four days after administration of streptozotocin, 20 IU of PMS (Sigma), dissolved in 0.15 *M* NaCl, was injected (iv) while the animals were under light ether anesthesia. The animals were decapitated at various times after injection of the PMS and the blood from the trunk collected in 12 × 75-mm glass tubes. After clotting at room temperature, the blood was centrifuged at 2000*g* for 20 min and the serum stored at −20° until assayed for hormone content.

The ovaries were removed as quickly as possible after decapitation, cleaned of adhering tissue and weighed to the nearest 0.1 mg on a torsion balance. When 17α-hydroxylase activity was to be measured the ovaries were quickly frozen, and stored, at −20°. In animals killed at 72 hr after PMS the oviducts were flushed with 0.15 *M* NaCl containing hyaluronidase into a depression slide and the number of ova counted with the aid of a dissecting microscope.

Serum LH concentrations were determined by double antibody radioimmunoassays using kits supplied by NIAMDD-Rat pituitary program: details of the procedure have been reported previously (7). Anti-rat LH antiserum No. 3, with an initial dilution of 1:10,000, was used with rat LH-RP-1 as the standard. Hormones were iodinated by a modification of the Butt method (8).

Serum levels of estradiol and progesterone were determined by radioimmunoassay. Tritiated estradiol, (2,4,6,7-^{3}H(N)estradiol-17β; 115 Ci/mmole) and progesterone (1,2,6,7-^{3}(N)progesterone; 97.9 Ci/mmole) were purchased from New England Nuclear Corporation (Boston, Mass.) and used without further purification. Anti-estradiol antiserum was obtained from Dr. D. Exley (University of Liverpool, England) (9) and anti-progesterone antiserum was prepared by Dr. Vernon Stevens (Ohio State University, Columbus). Details of the assay procedure have been published (10).

Ovarian 17α-hydroxylase activity was measured by a tritium exchange assay using pregnenolone as the substrate (11). The ovaries were homogenized in 0.15 *M* KCl and centrifuged at 10,000*g* for 30 min. The supernatant solution was centrifuged at 105,000*g* for 60 min and the pellet (microsomal fraction) was then resuspended in 0.15 *M* Na-K PO_4 buffer (pH 7.4). At least triplicate samples of this suspension were assayed; results were expressed as nanomoles pregnenolone converted per hour per milligram of microsomal protein.

Data were subjected to analysis of variance or χ^2 analyses. The independent Student's *t* test was applied when appropriate. Differences between means with a *P* value less than 0.05 were considered statistically significant.

Results. In two large groups of rats, the 60 mg/kg dose of streptozotocin produced a mean nonfasting serum glucose concentration of 410 ± 27 mg/100 ml (n = 126) compared to 119 ± 3.2 mg/100 ml (n = 116) in the controls. While 24 of the 26 control animals killed at 72 hr after injection of PMS had ovulated an average of 12.6 ± 3.2 ova, only 6 of the 26 diabetic animals had ovulated 11.2 ± 1.2 ova.

The ovarian weight responses to 20 IU of PMS were not significantly different between controls and diabetic animals (Fig. 1). However, the steroidogenic responses did shown differences. In Fig. 2 the changes in serum estradiol levels are shown for groups of 10 rats (experiment A) killed at various times after injection of PMS. The diabetic animals had the same level of es-

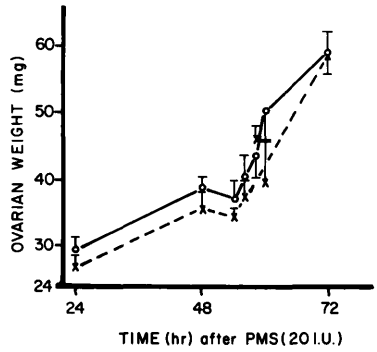

FIG. 1. Ovarian weight increases in control (O) and diabetic (x) immature rats of experiments A and B, injected (iv) at time 0 with 20 IU of PMS. Diabetes was induced by injection (iv) of 60 mg/kg of streptozotocin 3–4 days before the PMS. Vertical lines represent one SEM for groups of 16 rats.

tradiol 24 hr after PMS but by 48 hr it was 32% higher than that in controls ($P < 0.01$). The difference in concentration between control and diabetic animals was not statistically significant at 54 hr, but because of the rapidly falling values which occurred only in controls after 54 hr, differences at 56 and 58 hr were pronounced. The estradiol levels at 60 hr were 95% higher in the diabetic animals but by 72 hr they were the same in the two kinds of animals.

The experiment was repeated one month later using 6 animals per group (experiment B) with quantitatively slightly different results, even though the same lot of PMS was used in both cases. Ovarian weight increases were not different from those found in animals of experiment A and are included with them in the data of Fig. 1. The pattern of changes was the same as in rats of experiment A but higher levels of estradiol were found with the animals of experiment B (Fig. 3). As with experiment A diabetic animals had higher levels of serum estradiol than did controls, with the largest differences occurring between 54 and 60 hr after PMS. While the serum estradiol levels in controls in the two experiments were not different, the diabetic animals of experiment B had four times more estradiol than did diabetics of experiment A when measured at 72 hr. None of the six diabetic animals killed at 72 hr had oviductal ova.

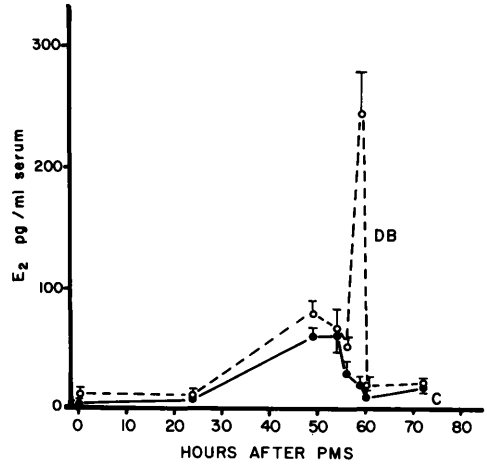

FIG. 2. The serum estradiol (E2) levels, determined by radioimmunoassay, for the control (C) and streptozotocin (DB)-treated rats of experiment A. All animals received 20 IU of PMS at time 0. Vertical lines indicate one SEM for groups of 10 rats.

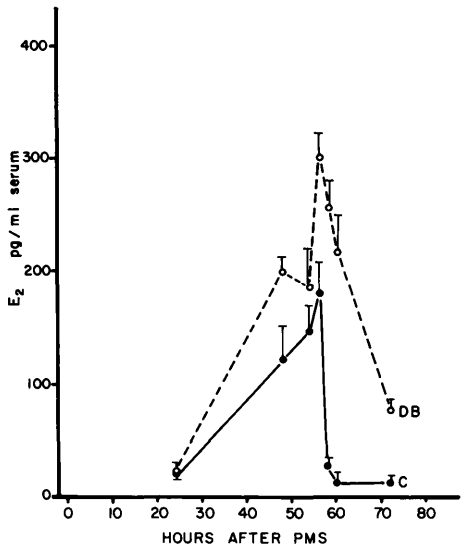

FIG. 3. Estradiol in the sera of females of experiment B. These animals were injected one month later than those of experiment A, with the same dose and lot of PMS. Nonfasting serum glucose levels were not different, nor was the pattern of change in E2 levels, from that of animals in experiment A, but the E2 levels were much higher in the females in this experiment.

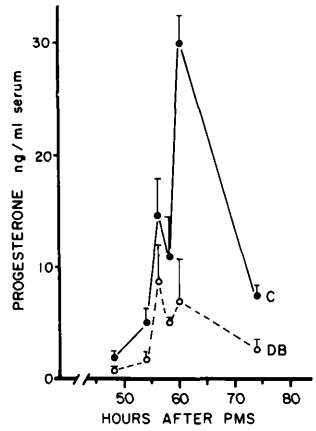

FIG. 4. Serum progesterone concentrations in the animals (10/group) of experiment A. While the pattern of change in the diabetic (DB) and control (C) animals was similar the latter had significantly more of this steroid when measured at 60 and 72 hr after injection of PMS.

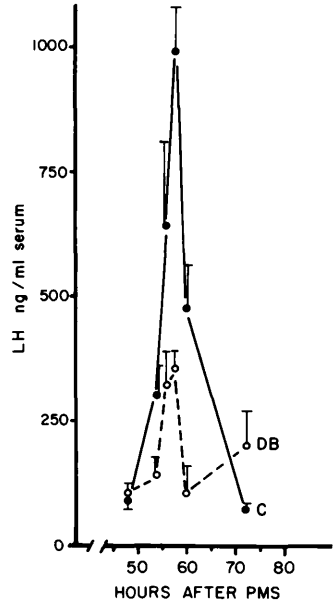

FIG. 5. The serum concentration of LH (as RP-1) in diabetic (DB) and control (C) rats of experiment A (E2 levels shown in Fig. 2).

Changes in serum progesterone concentrations were obtained for only the animals of experiment A (Fig. 4). The trend in the changes in concentration of this steroid in the two kinds of animals was the opposite of that seen for estradiol, i.e., the controls had the larger amount. Even after the decrease in serum progesterone which occurred between 60 and 72 hr in the controls the concentrations were more than double those of diabetic animals.

Changes in serum levels of LH for animals of experiments A and B are shown in Figs. 5 and 6, respectively. At 48 hr after PMS serum LH was the same in diabetic and control animals; in both the level was about four times that of immature controls not given PMS. The concentration rose quickly to a peak at 58 hr in the control and diabetic animals of experiment A but the quantitative differences were quite profound (Fig. 5). The level of LH in diabetic animals killed 72 hr after PMS was nearly three times that of controls ($P < 0.01$). The pattern of changes in serum LH in animals of experiment B were similar to those of experiment A but the peak level in B, which was only about half that found in animals of experiment A, was achieved 2 hr later. LH levels in diabetic animals of experiment B were not significantly different from those found in animals of experiment A.

Previous studies had demonstrated that steroid 17α-hydroxylase activity in the ovary decreased dramatically following an LH surge (11). Assay of the enzyme in immature diabetic females treated with PMS

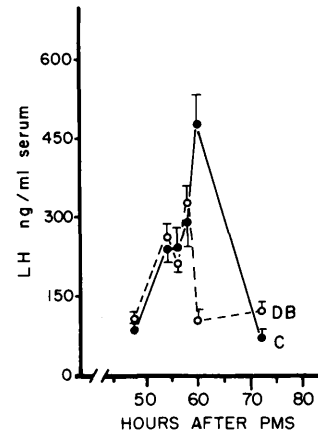

FIG. 6. Serum LH levels in females of experiment B, which had much higher levels of serum E2 (see Fig. 3).

revealed a pattern similar to that found in normal females. That is, after an initial decline in ovarian 17α-hydroxylase activity, an increase, which reached a peak at 60 hr was seen. The enzyme activity at 72 hr was significantly reduced, even though the ovaries of the two animals of the group which had ovulated were not included in the homogenate used for the assay.

In a third experiment, performed 3 months later and with a different lot of hormone, 7 of 8 control and 6 of 11 diabetic females had oviductal ova when examined at 72 hr after the injection of 20 IU of PMS (Table I). The number of ova shed was reduced by 66% ($P < 0.03$) in the animals treated with streptozotocin. Both kinds of females had received (sc) 0.1 ml of sesame seed oil 24 hr before autopsy. Injection of 1 mg of progesterone, dissolved in 0.1 ml of oil, 48 h after administration of PMS increased slightly (nonsignificantly) the number of ova shed by normal females. In the diabetic animals, however, ovulation was increased by the progesterone to the extent that 14 of the 16 ovulated. Furthermore, the number of ova released was increased by 135% ($P < 0.05$) compared to diabetic animals not receiving progesterone. With progesterone treatment the number of ova was not significantly different from the number released by normal females.

Discussion. The results of the present study confirm and extend the findings of Kirchick *et al.* (4, 5). These authors found that ovarian weight and estrogen production were similar in diabetic and normal immature rats when measured about 50 hr after a single injection of PMS. Further, they noted that serum progesterone levels were below normal in diabetic animals when measured either at 1200 or 2100 hr on the day of presumed proestrus. However, in contrast to their results with alloxan treatment, the present study has shown that animals made diabetic with streptozotocin produced an LH surge on the second day after injection of PMS and that some of the animals ovulated within 72 hr. The lower levels of serum LH in the diabetic animals may have accounted for the lowered percentage of animals ovulating and the reduced number of ova shed in some diabetic animals (Table I). In adult female rats with normal estrous cycles only 11 to 14% of the ovulatory LH surge on proestrus is required for ovulation (12). If this is also true for immature rats stimulated by PMS we would have expected a higher ovulatory rate because in both experiments with diabetic animals the peak LH levels were greater than 30% of those in control animals.

The decrease in ovarian 17α-hydroxylase activity seen in diabetic rats between 60 and 72 hr after PMS (Fig. 7) is another indication of an increase in LH release. This decrease in enzyme activity occurs in normal females between 48 and 60 hr after PMS, immediately following the LH surge. The enzyme activity continues to increase in hypophysectomized animals until at least

TABLE I. THE EFFECT OF PROGESTERONE UPON OVULATION IN NORMAL AND DIABETIC IMMATURE RATS INJECTED WITH PMS

Treatment	Group	No. of rats	Body weight	Serum glucose	Ova per animal	Ovulation rate (%)
Control	1	8	77.5 ± 2.1^a	86 ± 8^a	14.8 ± 4.4^a	87.5
Diabetic	2	11	62.9 ± 3.0^b	361 ± 21^b	4.9 ± 2.4^b	54.5
Control + progest.	3	14	80.4 ± 3.2^a	93 ± 2^a	21.5 ± 2.9^a	100
Diabetic + progest.	4	16	57.4 ± 2.3^b	349 ± 2^b	11.5 ± 3.4^a	87.5

Note. Results are means ± SEM. Diabetic animals were injected (iv) with streptozotocin (60 mg/kg) 3 days prior to injection (iv) of 20 IU of PMS. Progesterone (progest.) was injected (1 mg, sc) 48 hr after the PMS. Ova were flushed from the oviducts 72 hr after injection of PMS. Groups with the same superscript are not significantly different from each other, $P < 0.05$.

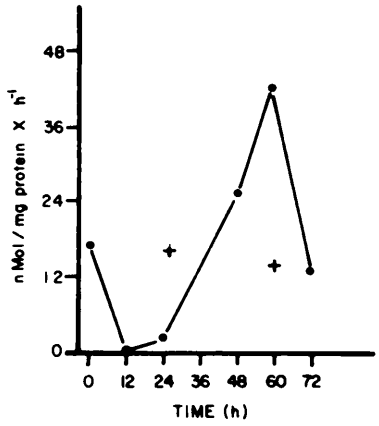

FIG. 7. Steroid 17α-hydroxylase activity in the microsomal fraction of ovaries from immature female rats made diabetic with streptozotocin; 17α[^{3}H]pregnenolone was the substrate. The pattern of change is similar to that for normal females (11) except that the decline associated with the LH surge is less pronounced and is delayed until 72 hr. Controls injected with saline (+) had no significant change in enzyme level. SEM for triplicate samples does not exceed the area covered by the symbols.

60 hr, and usually to 72 hr after injection of PMS before it gradually declines ((11) and unpublished data). In the present study, the ovarian hydroxylase activity of diabetic animals of experiment B at 48 hr post-PMS was 36.0 ± 0.4 nmole/mg protein/hr, while control ovaries had 32.0 ± 0.4. By 60 hr enzyme activity had decreased to 4.7 in controls but it had increased to 41.9 in the diabetic females. This delay in the ovarian response to LH may reflect the lower levels of this gonadotropin in the diabetic, and it may account for the high serum estrogen concentrations seen at 60 hr. However, it may also reflect an altered ovarian response to the gonadotropin because there is no difference in the timing of the LH surge between diabetic and normal females (Figs. 4, 5).

Kirchick *et al.* (5) concluded that the lack of an LH surge in alloxan diabetic rats was due to a pituitary insensitivity to hypothalamic gonadotropin-releasing hormone (GnRH). Furthermore, they demonstrated that the insensitivity was not secondary to a lack of estrogen stimulation of the pituitary. The present results also indicate that the ovaries of the diabetic respond to PMS by increasing production of estradiol. Possibly the estradiol levels were too high (Figs. 2 and 3) and rather than being stimulatory for LH release they may have become inhibitory, as shown by Wyss and Pincus (13).

The stimulatory effect of progesterone on ovulation in the diabetic rat suggests that the lack of this steroid may be a primary factor in the reduction of the LH surge. Grayburn and Brown-Grant (14) reported that a single injection of 0.5 mg of progesterone 50 hr after PMS increased the proportion of animals ovulating and the number of ova shed. The problem is understanding whether the low progesterone levels in the diabetic females are a consequence or a cause for the low LH levels. If an ovarian response was altered by the diabetic state and progesterone production was curtailed we would expect an inhibiting effect on ovulation at the ovarian level (15). On the other hand a reduction in LH release may account for the low progesterone levels which would result in the lack of an increasingly effective positive feedback loop. In either situation the administration of LH or progesterone would be expected to bring about normal ovulation rates; this is the case as shown by Kirchick *et al.* (4) and the results in Table I.

A role for the adrenal in loss of the ovulatory response in diabetics must be considered. Diabetes has long been recognized as a stress and to produce elevated serum levels of corticosteroids (16). Furthermore, in immature female rats, similar to those used in the present experiments, adrenal secretion of corticosterone was increased in response to PMS administration (17). The level was not high enough to inhibit ovulation, however, and after the LH surge in these animals adrenal function decreased dramatically: an ovarian product was responsible for the latter effect (17). If the progesterone from the heavily luteinized ovaries was responsible for lowering of the serum corticosterone levels, a point which has not been clearly established (see discussion in (17)), then the

stimulatory effect of progesterone on ovulation in the diabetic may involve yet another mechanism in addition to its effect on the release of LH and its direct effects upon the ovary. We have not measured serum corticosterone levels in the immature diabetics used in the present experiments.

In conclusion, the present study has shown that hyperglycemia induced by streptozotocin is not inconsistent with an LH surge or with ovulation. The difference in responses to those found with hyperglycemia induced by alloxan (4, 5) may be due to differences in the degree of pancreatic beta cell destruction induced by streptozotocin and consequently to the severity of the diabetes induced. Understanding the causes for ovulation reduction in uncontrolled diabetic animals will obviously require investigations into ovarian responses as well as those involving the hypothalamic–hypophyseal axis.

Thanks are due to Pierre Kremers of the University of Liege for supplying the substrate used in the 17α-hydroxylase assay to Drs. D. Exley and V. Stevens for anti-steroid antibodies and to the Rat Pituitary Program of the NIAMDD, National Institutes of Health, for supplying radioimmunoassay kits. The expert technical assistance of Mrs. Murriel Wagoner is gratefully acknowledged.

1. Chiere RA, Pivetta OH, Foglia VG. Altered ovulation patterns in experimental diabetes. Fertil Steril 20:661–666, 1969.
2. Davis EM, Fugo NW, Lawrence KB. Effect of alloxan diabetes on reproduction in the rat. Proc Soc Exper Biol Med 66:638–641, 1947.
3. Lawrence DM, Contopoulos AN. Reproductive performance in the alloxan diabetic female rat. Acta Endocrinol 33:175–184, 1960.
4. Kirchick HJ, Keyes PL, Frye BE. Etiology of anovulation in the immature diabetic rat treated with pregnant mare's serum gonadotropin: Absence of the preovulatory LH surge. Endocrinology 102:1867–1873, 1978.
5. Kirchick HJ, Keyes PL, Frye BE. An explanation for anovulation in immature alloxan-diabetic rats treated with pregnant mare's serum gonadotropin: Reduced pituitary response to gonadotropin-releasing hormone. Endocrinology 105:1343–1349, 1979.
6. Hoftiezer V, Carpenter A.-M. Comparison of streptozotocin and alloxan induced diabetes in the rat, including volumetric quantitation of the pancreatic islets. Diabetologia 9:178–184, 1973.
7. Mallampati RS, Johnson DC. Serum and pituitary prolactin, LH and FSH in androgenized female and normal male rats treated with various doses of estradiol benzoate. Neuroendocrinology 11:46–56, 1973.
8. Johnson DC. Maintenance of functional corpora lutea in androgenized female rats treated with PMSG. J Reprod Fertil 56:263–269, 1979.
9. Exley D, Johnson AW, Dean PDG. Antisera highly specific for 17β-estradiol. Steroids 18:605–620, 1971.
10. Grotjan HE Jr, Johnson DC. Temporal variations in reproductive hormones in the immature male rat. Proc Soc Exp Biol Med 152:381–384, 1976.
11. Johnson DC. Temporal changes in ovarian steroid 17α-hydroxylase in immature rats treated with pregnant mare's serum gonadotropin. Proc Soc Exper Biol Med 159:484–487, 1978.
12. Grieg F, Weisz J. Preovulatory levels of luteinizing hormone, the critical period and ovulation in rats. J Endocrinol 57:235–245, 1973.
13. Wyss HT, Pincus G. Effect of pregnant mare's serum gonadotropin, estradiol and progesterone on superovulation in the immature rat. Endocrinology 75:586–591, 1964.
14. Grayburn JA, Brown-Grant K. The role of oestrogen in the induction of ovulation in the gonadotrophin-treated immature rat. J Endocrinol 42:409–416, 1968.
15. Takahashi M, Ford JJ, Yoshinaga K, Greep RO. Induction of ovulation in hypophysectomized rats by progesterone. Endocrinology 95:1322–1326, 1974.
16. Devecerski MC, Frawley TF. Adrenal steroid production in rats with alloxan diabetes. Endocrinology 73:368–391, 1963.
17. Ramaley JA, Olson J. Adrenal function in rats given PMS before puberty: Response to ether stress. Neuroendocrinology 14:1–13, 1974.

Received June 17, 1982. P.S.E.B.M. 1982, Vol. 171.