THE EFFECT OF THE OESTROUS CYCLE AND OF OESTRADIOL ON THE BREAKDOWN OF SEMINAL GLYCERYLPHOSPHORYLCHOLINE BY SECRETIONS OF THE RAT UTERUS

By J. C. WALLACE,* G. M. STONE,* and I. G. WHITE*

[*Manuscript received June* 29, 1964]

Summary

Glycerylphosphorylcholine (GPC) diesterase activity and protein concentration in the rinsings of the rat uterus were greater during pro-oestrus and oestrus than at metoestrus or dioestrus.

Oestradiol-3,17β significantly stimulated the secretion of this enzyme in ovariectomized rats and maximum levels of activity were observed 24–36 hr after a single injection of the oestrogen. The protein content of the uterine rinsings was also increased by oestradiol, but was greatest 12 hr after injection. The level of GPC diesterase activity was independent of changes in the weight or vascularity of the uterus.

I. INTRODUCTION

Both *in vivo* and *in vitro* techniques have been extensively used to study the effect of oestrogens on the activity of enzymes in the tissues responsive to oestrogen. Much of this work has been aimed at determining the fundamental action of oestrogens [see Talalay and Williams-Ashman (1960) and Villee, Hagerman, and Joel (1960) for reviews] and has often involved a description of the early biochemical events underlying the later gross morphological changes in the uterus and vagina (Mueller 1960). Less attention has been paid to the secretions of the female reproductive tract and there is little information on the hormonal control of their production and composition or on the role they must play in the reproductive processes. However, from the review by Olds and VanDemark (1957) and more recent papers (Bishop 1957; Howard and de Feo 1959; Mastroianni *et al.* 1961; Ringler 1961; Heap 1962; Heap and Lamming 1960, 1963) it is clear that the luminal fluids of the uterus and oviduct are distinct secretions of these organs, varying in composition and quantity with the endocrine state of the animal. The significance of these changes is not yet clear, although it is evident from the report by White and Wallace (1961) that these secretions may have other functions than merely providing a nutrient medium for survival of the gametes until fertilization and the subsequent nidation of the ovum.

This paper deals with the relationship between the ovarian hormone status of the rat and the activity of an enzyme in the uterine secretions responsible for the breakdown of seminal glycerylphosphorylcholine (GPC). This unusual organic compound occurs in high concentrations in mammalian semen (Dawson, Mann, and White 1957) and the occurrence of a GPC-splitting diesterase in the luminal fluid of the female reproductive tract (White and Wallace 1961; Wallace and White,

* Department of Veterinary Physiology, University of Sydney.

unpublished data) would seem to provide a means for making the glycerol component of the molecule available as an energy source for the spermatozoa. Although GPC as such is not utilized by mammalian spermatozoa, glycerol and phosphoglycerol are readily oxidized (White, Blackshaw, and Emmens 1954; Mann and White 1956*a*, 1956*b*; White 1957).

II. Materials and Methods

(*a*) *Animals*

Randomly bred albino rats of the Castle Hill strain were used. Ovariectomy was carried out at 4 weeks of age, the animals primed 2 weeks later with 10 μg oestradiol and used in the experiment 2 weeks after priming. Intact animals with regular 5-day oestrous cycles were used when approximately 12 weeks old. The rats were randomized to experimental groups and housed 10 per cage. Food and water were supplied *ad lib.* Injections of the oestradiol-3,17β were given subcutaneously in the back in 0·1 ml peanut oil. Control animals received 0·1 ml peanut oil 48 hr before being killed. The animals were killed by cervical dislocation and each uterine horn was dissected free of fat and weighed to the nearest 0·1 mg. In experiment 2, dry weights were recorded to the nearest 0·1 mg after 24 hr at 70°C. The vascularity of the uteri was scored immediately after killing and graded on an arbitrary scale of 1–6 (expt. 1) and 1–4 (expt. 2).

(*b*) *Enzyme Assay*

Each uterine horn was rinsed with 1·0 ml of calcium-free Krebs–Ringer phosphate (Umbreit, Burris, and Stauffer 1957) and the rinsings collected in a graduated centrifuge tube. Recovery of the fluid was virtually quantitative. Care was taken to maintain the rinsings at 0–2°C from collection until the incubation period commenced. After removing any cellular debris or blood cells by centrifuging for 20 min at 1500 *g*, an aliquot of the pooled uterine rinsings from each animal was incubated for 3 hr or overnight (15 hr) at 37°C in the presence of 10% v/v ram seminal plasma and 1000 i.u./ml of both penicillin and streptomycin. Seminal plasma was used as a source of GPC because it is the normal physiological vehicle for the uterine diesterase substrate and the cost of the pure compound was prohibitive at the time of these studies. Ram seminal plasma was chosen because it is more readily available than rat seminal plasma and contains high concentrations of GPC but no phosphorylcholine (Dawson, Mann, and White 1957). The solutions were deproteinized by adding 1 volume of 0·15M $ZnSO_4$ and 1 volume of 0·3N $Ba(OH)_2$. The choline liberated from the GPC by the diesterase was used as a measure of enzyme activity. The choline was estimated in the neutral deproteinized filtrate as a complex with potassium triodide by the method of Kushner (1956).

(*c*) *Protein Determination*

The protein concentration in the uterine rinsings was determined by measuring the optical density at 210 mμ (Tombs, Souter, and MacLagen 1959) after preliminary observations had confirmed the applicability of the technique to this biological fluid.

(*d*) *Haemoglobin*

Haemoglobin was estimated by the method outlined in Heilmeyer (1943). Spectral measurements were made with a Hilger Uvispek or a Unicam SP600 spectrophotometer.

(*e*) *Statistics*

Results were transformed to logarithms and the analyses of variance were carried out on the SILLIAC, an automatic digital computer, in the Physics Department, University of Sydney. Non-significant interactions are sometimes grouped in the tables to save space.

TABLE 1

DIESTERASE ACTIVITY (μG CHOLINE AFTER INCUBATION WITH SEMINAL PLASMA) AND PROTEIN CONTENT OF RINSINGS FROM THE RAT UTERUS AND THE WET WEIGHT AND VASCULARITY OF THE UTERUS DURING THE OESTROUS CYCLE

Results are means from 10 animals. The choline content of an equivalent amount of seminal plasma after incubation was 87 μg

Stage of Cycle	Choline (μg)	Protein (μg)	Wet Weight (mg)	Vascularity (1–6)
Dioestrus	134	115	177	1·4
Pro-oestrus	365	201	337	2·8
Oestrus	334	177	288	2·9
Metoestrus	170	103	196	2·0

Analyses of variance of log. transformed results

Source of Variation	Degrees of Freedom	*F* Values			
		Choline	Protein	Wet Weight	Vascularity
Dioestrus *v.* metoestrus	1	0·9	0·2	0	1·0
Pro-oestrus *v.* oestrus	1	0·1	0·1	0·7	0·3
Dioestrus plus metoestrus *v.* pro-oestrus plus oestrus	1	25·9***	12·4***	14·6***	7·7**
Error	36	416·7†	362·4†	334·8†	1·73†

** $0{\cdot}01 > P > 0{\cdot}001$. *** $0{\cdot}001 > P$. † Error mean square.

III. RESULTS

In the first experiment, 10 animals in each of the four stages of the oestrous cycle—dioestrus, pro-oestrus, oestrus, and metoestrus—were selected after the examination of vaginal smears taken on 2 consecutive days. The diesterase activity and the protein concentration of the uterine rinsings and the uterine wet weight and vascularity were recorded. The results and the analyses of variance are summarized in Table 1. There was no difference between dioestrus and metoestrus or between pro-oestrus and oestrus over all parameters, but the difference between the summed results of dioestrus plus metoestrus and pro-oestrus plus oestrus was highly significant.

In view of the report by Dawson (1956) of GPC diesterase activity in rat erythrocytes, care was taken to exclude this possible source of the enzyme from confusing the interpretation of the results. The cell-free rinsings taken for protein estimation

TABLE 2

EFFECT OF THE TIME OF INCUBATION AND THE DOSE AND TIME OF INJECTION OF OESTRADIOL-3,17β ON THE DIESTERASE ACTIVITY (μg CHOLINE AFTER INCUBATION WITH SEMINAL PLASMA) OF UTERINE RINSINGS FROM OVARIECTOMIZED RATS

Values are means for four rats per group, the rinsings from two being pooled for each estimation. The choline content of an equivalent amount of seminal plasma was 313 μg after 3 hr incubation; 293 μg after 15 hr incubation, and 302 μg before incubation

Incubation Time (hr)	Dose of Oestradiol (μg)	Time after Injection (hr): 12	24	36	48
3	Control (oil)				459
	0·1	304	472	404	357
	10·0	350	623	623	514
15	Control (oil)				308
	0·1	359	395	320	305
	10·0	679	1259	1805	364

Analysis of variance of log. transformed results

Source of Variation	Degrees of Freedom	Mean Square	F
Dose of oestradiol (D)	1	11289·1	128·7***
Time after injection			
Linear (T_L)	1	37·8	0·4
Quadratic (T_Q)	1	6520·6	74·3***
Cubic (T_C)	1	2·8	0
Dose × time			
$D \times T_L$	1	4·5	0·1
$D \times T_Q$	1	2526·1	28·8
$D \times T_C$	1	588·6	6·7*
Error (a)	8	88·1†	
Incubation time (I)	1	712·5	11·0**
$I \times$ dose	1	1471·5	22·9***
$I \times$ dose × time			
$I \times D \times T_Q$	1	810·0	12·5**
Remainder	4	223·7	3·5
Error (b)	8	64·7†	

* $0·05 > P > 0·01$. ** $0·01 > P > 0·001$. *** $0·001 > P$.
† Error mean square.

were pooled within the same stage of the oestrous cycle, and concentrated. The bands of the visible spectrum characteristic of haemoglobin were examined, but it was evident that although the absorption at 410 mμ and at 540 mμ relative to

that at 210 mμ was higher for the rat uterine rinsings than for a purified protein (bovine serum albumin), there was little difference between the four stages. Nor was there any indication of absorption maxima at 410, 540, and 580 mμ which were characteristic of haemoglobin.

As the increase in diesterase activity paralleled the increasing oestrogen dominance of the oestrous cycle, in the next experiment the change in the enzyme activity was measured at varying times after a single subcutaneous injection of oestradiol to ovariectomized rats. The design was a 4×2 factorial with four times of injection (12, 24, 36, and 48 hr) and two doses of oestradiol-3,17β (0·1 and 10 μg). There were four animals per group, the tissues from two being pooled to allow a comparison of two incubation times (3 and 15 hr) in a split-plot design (Cochran and Cox 1957).

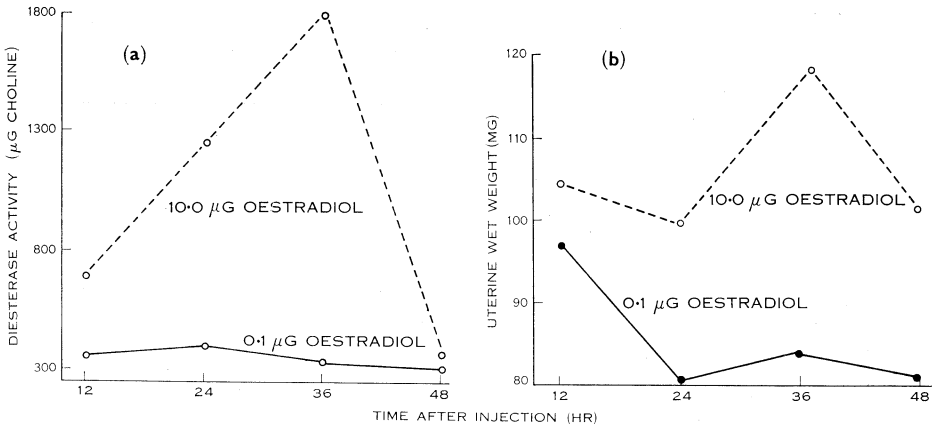

Fig. 1.—Effect of the dose and the time of injection of oestradiol-3,17β on the diesterase activity (μg choline) of the rinsings of the uterus (*a*) and on the uterine wet weight (*b*) of ovariectomized rats. There were four rats per group and the tissues from two were pooled for the estimation of enzyme activity. The values for the control animals are: (*a*) 308 μg; (*b*) 67·5 mg; these animals received 0·1 ml peanut oil 48 hr before being killed.

Increasing the dose of oestradiol significantly increased the enzyme activity of the uterine rinsings and this activity reached a maximum 24–36 hr after the injection (Table 2; Fig. 1). The longer incubation time provided a more sensitive assay since these effects were more pronounced after 15 hr of incubation, as seen in the significant effect of incubation time and the interactions with incubation time in the analysis of variance (Table 2). These changes in enzyme activity did not parallel the changes in the protein content of the uterine rinsings, which at both doses showed a significant decrease from 12 to 48 hr after injection (Table 3). The observed increases in wet weight at the higher dose were not significant in the analysis of variance (Table 3), although at all dose–time combinations the wet weight was higher than the control values. There was no significant change in the vascularity of the uterus with time after injection of oestradiol. Animals which received oestradiol treatment had a greater development of uterine vascularity than the controls but surprisingly the lower dose had a significantly greater effect. Table 4 shows that the enzyme activity (calculated from the 15-hr incubation) per unit wet and dry weight of the uterus increased with the dose of oestrogen and also reached a peak 24–36 hr after injection.

IV. Discussion

Although few studies have been made of the luminal fluid of the female reproductive tract (Olds and VanDemark 1957; Howard and de Feo 1959; Polge 1960; Heap and Lamming 1963) and even fewer reports of studies made of enzymic activity in this secretion, it is evident that its composition is much influenced by the ovarian hormones. The results presented in this paper show that in the rat the secretion of a luminal fluid enzyme breaking down seminal GPC is stimulated by both endogenous

Table 3

Effect of the dose and the time of injection of oestradiol-3,17β on the protein content of rat uterine rinsings, and on the uterine wet and dry weight and vascularity

There were four rats per group and the rinsings and tissues from two were pooled for each estimation. The values are means for the totals of each pair of rats

Dose of Oestradiol (μg)	Time after Injection (hr)	Protein (μg)	Wet Weight (mg)	Dry Weight (mg)	Vascularity (2–8)
0·1	12	131	194·8	16·05	5·0
	24	56	161·0	16·75	7·0
	36	54	167·6	17·53	4·0
	48	45	162·2	20·85	8·0
10·0	12	317	208·8	13·40	2·5
	24	110	199·2	18·33	5·0
	36	98	235·6	15·98	6·0
	48	90	203·4	18·88	5·0
Control (oil)	48	60	67·5	14·32	2·5

Analyses of variance of log. transformed results

Source of Variation	Degrees of Freedom	*F* Values			
		Protein	Wet Weight	Dry Weight	Vascularity
Dose of oestradiol	1	5·6*	3·0	5·2	7·8*
Time after injection					
Linear	1	5·9*	0·4	0	1·6
Remainder	5	0·4	0·5	1·2	2·7
Error	8	83·8†	53·1†	42·2†	1·81†

* $0{\cdot}05 > P > 0{\cdot}01$. † Error mean square.

and injected oestrogen. This finding may explain the function of this important component of the semen of many species (Wallace and White, unpublished data) as the occurrence of maximal diesterase activity, i.e. at pro-oestrus and oestrus, coincides with the period when seminal GPC would be expected in the female tract.

The activities of several endometrial and myometrial enzymes are similarly related to the oestrous cycle (see, for example, Jenner 1948; Harris and Cohen 1951; Hadek 1955; Bo 1959; Albers, Bedford, and Chang 1961); however, the secretion of some uterine enzymes (e.g. carbonic anhydrase) is depressed by oestrogens (Leonard

and Knobil 1950; Harris and Cohen 1951; Lutwak-Mann 1955; Herbener and Atkinson 1959; Miyake and Pincus 1959; Ogawa and Pincus 1962). In the present studies the increase in the GPC diesterase activity of the uterine rinsings following

TABLE 4

EFFECT OF THE DOSE AND THE TIME OF INJECTION OF OESTRADIOL-3,17β ON THE DIESTERASE ACTIVITY (CALCULATED AS μg CHOLINE AFTER 15 HR INCUBATION) PER MILLIGRAM OF WET AND DRY WEIGHT OF UTERUS AND PER MICROGRAM OF PROTEIN IN THE UTERINE RINSINGS

There were four rats per group and the rinsings from two were pooled for each estimation

Dose of Oestradiol (μg)	Time after Injection (hr)	μg Choline per mg Uterus Wet Weight	μg Choline per mg Uterus Dry Weight	μg Choline per μg Protein
0·1	12	1·85	10·2	3·70
	24	2·47	22·4	3·02
	36	1·92	8·8	6·40
	48	1·89	8·9	9·25
10·0	12	3·22	19·2	2·38
	24	10·90	28·1	10·32
	36	7·71	39·8	21·15
	48	1·76	9·5	5·13
Control (oil)	48	4·56	21·5	5·10

Analyses of variance of log. transformed results

Source of Variation	Degrees of Freedom	*F* Values		
		μg Choline per mg Uterus Wet Weight	μg Choline per mg Uterus Dry Weight	μg Choline per μg Protein
Dose of oestradiol (D)	1	26·0***	91·0***	1·8
Time after injection				
Linear (T_L)	1	2·6	8·2*	5·9*
Quadratic (T_Q)	1	21·0***	50·9***	4·7
Cubic (T_C)	1	0·3	17·6**	1·0
Dose × time				
$D \times T_L$	1	1·8	2·7	0·1
$D \times T_Q$	1	12·8**	30·1***	8·2*
$D \times T_C$	1	0·3	9·7*	0
Error	8	186·6†	43·6†	64·4†

* $0{\cdot}05 > P > 0{\cdot}01$. ** $0{\cdot}01 > P > 0{\cdot}001$. *** $0{\cdot}001 > P$.
† Error mean square.

oestrogen injection was independent of the wet and dry weight and vascularity of the uterus and the protein content of the rinsings. Similarly at pro-oestrus and oestrus the increase in enzyme activity was relatively greater than the changes observed in the other parameters.

The changes in total diesterase activity and activity per unit weight (Table 2; Fig. 1) are similar to those in uterine metabolism in the rat after the injection of oestradiol as demonstrated by Mueller (1960). This author showed that ribonucleic acid accumulation is at a peak 20–24 hr after injection and immediately precedes a phase of rapid protein synthesis. The decline in diesterase activity by 48 hr is reminiscent of the studies of Mueller (1960) on the incorporation of [^{14}C]glycine into the uterus and not unlike the results of Scott and Lisi (1960) with glucose 6-phosphate dehydrogenase and phosphogluconate dehydrogenase in the rat uterus.

The greater protein concentration of the uterine rinsings at pro-oestrus and oestrus compared with dioestrus and metoestrus contrasts with the changes in protein concentration reported by Heap and Lamming (1963) for the luminal fluid of the rat. However, Ringler (1961) found an increase in luminal fluid protein after oestrogen and in the present experiments the luminal fluid protein was elevated 12 hr after the injection of oestradiol.

The haemoglobin estimations on the uterine rinsings from the first experiment exclude erythrocytes as a source of the diesterase enzyme. Confirmation of this was obtained in the second experiment, in which there was no relationship between trends in enzyme activity and uterine vascularity after oestradiol administration.

V. Acknowledgments

The authors are indebted to Professor C. W. Emmens for his interest and advice, and to the Lalor Foundation and the Rural Credits Development Fund of the Commonwealth Bank of Australia for financial assistance. Two of us (J.C.W. and G.M.S.) were supported by a Commonwealth Post-Graduate Award.

VI. References

Albers, H. J., Bedford, J. M., and Chang, M. C. (1961).—*Amer. J. Physiol.* **201**: 554.
Bishop, D. W. (1957).—*Int. J. Fertil.* **2**: 11.
Bo, W. J. (1959).—*J. Histochem. Cytochem.* **7**: 403.
Cochran, W. G., and Cox, G. M. (1957).—"Experimental Designs." 2nd Ed. (Burgess Publ. Co.: Minnesota.)
Dawson, R. M. C. (1956).—*Biochem. J.* **62**: 689.
Dawson, R. M. C., Mann, T., and White, I. G. (1957).—*Biochem. J.* **65**: 627.
Hadek, R. (1955).—*Anat. Rec.* **121**: 187.
Harris, R. S., and Cohen, S. L. (1951).—*Endocrinology* **48**: 264.
Heap, R. B. (1962).—*J. Endocrinol.* **25**: 367.
Heap, R. B., and Lamming, G. E. (1960).—*J. Endocrinol.* **20**: xxiii.
Heap, R. B., and Lamming, G. E. (1963).—*J. Endocrinol.* **25**: 57.
Heilmeyer, L. (1943).—"Spectrophotometry in Medicine." (Adam Hilger Ltd.: London.)
Herbener, G., and Atkinson, W. B. (1959).—*Proc. Soc. Exp. Biol., N.Y.* **100**: 736.
Howard, E., and de Feo, V. J. (1959).—*Amer. J. Physiol.* **196**: 65.
Jenner, R. (1948).—*Biochim. Biophys. Acta* **2**: 439.
Kushner, D. J. (1956).—*Biochim. Biophys. Acta* **20**: 554.
Leonard, S. L., and Knobil, E. (1950).—*Endocrinology* **47**: 331.
Lutwak-Mann, C. (1955).—*J. Endocrinol.* **13**: 25.
Mann, T., and White, I. G. (1956*a*).—*Nature* **178**: 42.
Mann, T., and White, I. G. (1956*b*).—*Biochem. J.* **65**: 634.
Mastroianni, L., Beer, F., Shah, U., and Clewe, T. H. (1961).—*Endocrinology* **68**: 92.
Miyake, T., and Pincus, G. (1959).—*Endocrinology* **65**: 64.

MUELLER, G. C. (1960).—In "Biological Activities of Steroids in Relation to Cancer". (Eds. G. Pincus and E. Vollner.) (Academic Press, Inc.: New York.)
OGAWA, Y., and PINCUS, G. (1962).—*Endocrinology* **70**: 359.
OLDS, D., and VANDEMARK, N. L. (1957).—*Amer. J. Vet. Res.* **18**: 87.
POLGE, C. (1960).—*J. Reprod. Fertil.* **1**: 113,
RINGLER, I. (1961).—*Endocrinology* **68**: 281.
SCOTT, D. B. M., and LISI, A. G. (1960).—*Biochem. J.* **77**: 52.
TALALAY, P., and WILLIAMS-ASHMAN, H. G. (1960).—*Rec. Progr. Hormone Res.* **16**: 1.
TOMBS, D., SOUTER, F., and MACLAGEN, N. F. (1959).—*Biochem. J.* **73**: 167.
UMBREIT, W. W., BURRIS, R. H., and STAUFFER, J. F. (1957).—"Manometric Techniques and Tissue Metabolism." 3rd Ed. (Burgess Publ. Co.: Minneapolis.)
VILLEE, C. A., HAGERMAN, D. O., and JOEL, P. B. (1960).—*Rec. Progr. Hormone Res.* **16**: 49.
WHITE, I. G. (1957).—*Amer. J. Physiol.* **189**: 307.
WHITE, I. G., BLACKSHAW, A., and EMMENS, C. W. (1954).—*Aust. Vet. J.* **20**: 85.
WHITE, I. G., and WALLACE, J. C. (1961).—*Nature* **189**: 843.