ACTIVITY OF ENZYMES IN THE ENDOMETRIUM, CARUNCLES, AND UTERINE RINSINGS OF PROGESTOGEN-TREATED AND NATURALLY CYCLING EWES

By R. N. MURDOCH* and I. G. WHITE*

[*Manuscript received August* 17, 1967]

Summary

The activity of several enzymes has been measured in the endometrium, caruncles, and uterine rinsings of ewes at various stages of the oestrous cycle. Ewes were either allowed to cycle naturally or were synchronized by progestogen-impregnated sponges inserted into the vagina. Most (86%) of the progestogen-treated ewes came into oestrus 2 or 3 days after removal of the sponges, and the uterus contained higher levels of amylase and alkaline phosphatase than did naturally cycling ewes. Amylase, succinate dehydrogenase, glutamate–oxaloacetate transaminase, acid and alkaline phosphatase activities were maximal during the luteal phase of the cycle. Endometrial alkaline phosphatase activity followed the growth and retrogression of the corpus luteum more closely than that of the other enzymes studied.

Glycerylphosphorylcholine diesterase activity also increased in the uterine tissues during the luteal phase of the cycle but did not reach a maximum until just prior to oestrus. Lactate dehydrogenase and glucose-6-phosphate dehydrogenase remained unchanged in activity throughout the oestrous cycle.

The levels of amylase, succinate dehydrogenase, glutamate–oxaloacetate transaminase, and protein were greater in the caruncles than in the endometrium, whereas the reverse was true for lactate dehydrogenase and alkaline phosphatase.

The enzyme activity of uterine rinsings was very variable; however, the the activity of glycerylphosphorylcholine diesterase and alkaline phosphatase was found to change significantly during the cycle.

I. INTRODUCTION

In recent years increasing attention has been paid to the influence of the oestrous cycle and ovarian hormones on the activity of enzymes of the female reproductive tract in order to establish their possible role in capacitation, implantation, and foetal development; for reviews see Talalay and Williams-Ashman (1960), Villee, Hagerman, and Joel (1960), Gross (1961), and Hafez (1964).

In one species the luteal phase of the oestrous cycle may stimulate the activity of a particular genital tract enzyme which may decrease or remain unaffected in other species. For example, in the rabbit and rat the carbonic anhydrase activity of the endometrium increases greatly during the luteal phase (Lutwak-Mann and Laser 1954; Lutwak-Mann 1955; Lutwak-Mann and Adams 1957) whereas the uterus of the mouse shows much higher carbonic anhydrase activity in oestrus or after oestrogen treatment (Madjerek and van der Vies 1961). In sheep the enzyme is as

* Department of Veterinary Physiology, University of Sydney, N.S.W. 2006.

active in the non-pregnant endometrium as it is during pregnancy (Lutwak-Mann and Averill 1954; Lutwak-Mann 1955).

Apart from the concurrent work of Hafez and White (unpublished data 1967), no systematic studies seem to have been made of fluctuations in the uterine enzymes of the ewe during the oestrous cycle. The activity of several enzymes in the endometrium, caruncles, and uterine rinsings has therefore been determined when ewes were allowed to cycle naturally or after the removal of progestogen-impregnated sponges inserted into the vagina. The enzymes studied were amylase, succinate dehydrogenase, lactate dehydrogenase, glutamate–oxaloacetate transaminase, acid phosphatase, alkaline phosphatase, glucose-6-phosphate dehydrogenase, and glyceryl-phosphorylcholine diesterase.

II. Materials and Methods

(*a*) *Detection and Synchronization of Oestrus*

Forty-four adult Merino ewes were randomized into two equal groups. Oestrus in group 1 was synchronized by means of sponges impregnated with SC-9880 (17α-acetoxy-19α-fluoro-11β-hydroxy-pregn-4-en-3,20-dione) (Robinson 1965, 1967). Group 2 were untreated. After 16 days the sponges were removed (day 0) and raddled, vasectomized rams were placed with both groups to detect oestrus. Ewes were slaughtered by cutting the throat at 0–2, 3–7, 8–14, and 15–17 days after oestrus.

(*b*) *Preparation of Tissues and Uterine Rinsings*

After slaughter the ovaries were examined to check the stage of the oestrous cycle. The uteri were then quickly removed, placed in crushed ice, and all subsequent processing of the tissue was performed at 4°C. The uterine horns were dissected free of fatty and connective tissue and of the attached oviducts and cervix. Calcium-free Krebs–Ringer solution (4 ml) buffered to pH 7·0 with Tris (122 mM NaCl, 5 mM KCl, 1 mM $MgSO_4$, 25 mM Tris, 23 mM HCl) was introduced into each horn with a syringe. The uterine rinsings were passed along the length of each horn by gently squeezing, and were collected into graduated centrifuge tubes at 4°C (taking care to avoid contaminating the samples with blood) and centrifuged at 400 *g* for 10 min at 4°C to remove contaminating cells or debris (Wallace and White 1965). The uterine horn was then placed on a piece of filter paper and opened down the mesometrial side. The exposed endometrium and caruncles were blotted with filter paper to remove any trace of the flushing fluid or the endometrial secretions. Caruncular and endometrial tissue from the intercotyledonary areas were carefully dissected using fine scissors and forceps. Samples of tissue were homogenzied in five parts of calcium-free Krebs–Ringer–Tris with a Potter–Elvehjem homogenizer and then filtered through cheesecloth.

(*c*) *Enzyme Analyses*

Enzymes were assayed either directly or after appropriate dilution in calcium-free Krebs–Ringer–Tris by assay procedures which, except for succinate dehydrogenase, are set out in Bergmeyer (1963).

Amylase was estimated by the change in iodine colour of an amylose solution (Street and Close 1956) using one-tenth of the volume prescribed in Bergmeyer (1963) for the micromethod. Under these conditions one Street–Close unit would be contained in 10 ml of sample when 0·1 ml hydrolysed 0·2 mg of amylose in 15 min at pH 7·0 and 37°C.

The reduction of neotetrazolium chloride to formazan with sodium succinate as substrate was used to determine succinate dehydrogenase (SDH) activity (Sobel and Eckstein 1959). The SDH activity of the homogenate was expressed as micrograms of formazan formed during 20 min at 37°C.

Lactate dehydrogenase (LDH) activity was measured by the decrease of optical density at 340 mμ due to the oxidation of NADH in the presence of pyruvate. A unit is the amount of LDH which changes the optical density of NADH at 340 mμ by 0·001 in 1 min in a 3-ml assay mixture at 24–27°C (Wróblewski and La Due 1955).

Glutamate–oxaloacetate transaminase (GOT) activity was measured by determining the rate of formation of oxaloacetate colorimetrically as the 2,4-dinitrophenylhydrazone (Reitman and Frankel 1956). The optical density at 546 mμ was converted to GOT units from the table in Bergmeyer (1963). A GOT unit is the amount of transaminase in 1 ml homogenate or uterine rinsings, which decreases the optical density of NADH at 340 mμ by 0·001 in 1 min in a 3-ml assay mixture at 25°C (La Due, Wróblewski, and Karmen 1954).

Acid and alkaline phosphatase activities were determined by using *p*-nitrophenylphosphate as substrate (Bessey, Lowry, and Brock 1946; Andersch and Szcypinski 1947). One phosphatase unit is defined as being the amount of enzyme contained in 1000 ml sample, which liberates 1 m-mole (139 mg) *p*-nitrophenol at 37°C.

Glucose-6-phosphate dehydrogenase (GDH) activity was determined by measuring the rate of formation of NADPH spectrophotometrically (Warburg, Christian, and Griese 1935). One unit of activity is the amount of enzyme in a 1-ml sample which at 25°C in a 3-ml assay mixture changes the optical density of NADPH at 340 mμ by 0·001 in 1 min (La Due, Wróblewski, and Karmen 1954).

Glycerylphosphorylcholine (GPC) diesterase activity was determined by incubating aliquots (1 ml) of homogenate or uterine rinsings for 15 hr at 37°C in the presence of antibiotics (1 mg/ml of both penicillin and streptomycin) with or without ram seminal plasma as a source of GPC (Wallace and White 1965). The samples were deproteinized by adding 1 volume of $ZnSO_4.7H_2O$ solution (5 g/100 ml) 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 and was estimated in the neutral filtrate as a complex with potassium triiodide by the method of Kushner (1956).

The protein concentration of the samples was determined by the biuret method (Wales, Scott, and White 1961).

(*d*) *Statistical Analysis*

The experiment was of factorial design and the significance of the results has been assessed by analysis of variance. All main effects and their first- and second-order interactions were isolated and tested for significance using the within-group error mean square to calculate variance ratios.

III. Results

(*a*) *Synchronization of Oestrus*

Figure 1 shows the onset of behavioural oestrus in ewes following the withdrawal of sponges (group 1) and of normally cycling ewes (group 2). Of the 22 ewes in group 1, 20 came into oestrus between the second and fourth day after removing the sponges. The two remaining ewes came into oestrus on days 19 and 20. The onset of behavioural oestrus in group 2 was distributed over a period of 16 days.

(*b*) *Description of Ovaries at Various Stages of the Oestrous Cycle*

The ovaries of all ewes were examined after slaughter from 0–17 days after coming into heat and had the following characteristics:

0–2 days: recently ruptured follicles or blood-spotted eruptions with corpora lutea forming;

3–7 days: corpora lutea small and red, no follicles;

TABLE 1

ENZYME ACTIVITY OF HOMOGENATES OF ENDOMETRIUM AND CARUNCLES DURING OESTROUS CYCLE

Values represent the means of five ewes and are calculated as units per milligram protein (see text for definition of units)

Days of Cycle	Tissue	Amylase $\times 10^4$	SDH	LDH	GOT	Acid Phosphatase $\times 10^4$	Alkaline Phosphatase $\times 10^4$	GDH	GPC Diesterase	Protein (mg/g wet wt.)
					Synchronized (Group 1)					
0–2	Endometrium	12·8	0·85	573	28·4	19·8	146	29·8	42·1	85·6
	Caruncles	12·8	1·27	459	28·9	18·6	49	26·9	48·1	93·2
3–7	Endometrium	9·0	0·95	534	27·1	17·1	248	22·9	40·7	94·6
	Caruncles	14·0	1·50	485	31·3	18·5	93	29·2	50·9	101·3
8–14	Endometrium	15·3	0·77	623	22·5	28·6	491	30·9	78·6	80·4
	Caruncles	19·9	1·22	589	25·2	28·2	171	30·9	59·2	82·1
15–17	Endometrium	11·1	0·58	645	22·0	25·3	227	31·4	97·7	84·1
	Caruncles	17·6	0·81	498	21·6	21·5	92	31·3	73·3	87·6
					Natural (Group 2)					
0–2	Endometrium	7·6	0·81	682	22·6	16·2	101	30·0	29·8	79·9
	Caruncles	9·1	1·07	547	24·2	17·2	53	28·8	43·1	89·0
3–7	Endometrium	9·2	0·88	724	24·1	25·2	220	25·1	66·0	80·9
	Caruncles	13·4	1·39	565	26·5	22·3	103	30·2	36·0	90·4
8–14	Endometrium	11·8	0·76	626	21·2	26·2	281	28·8	63·6	85·9
	Caruncles	13·4	0·96	523	25·8	21·7	106	24·8	30·0	94·0
15–17	Endometrium	11·4	0·55	513	21·4	23·2	186	27·1	103·1	80·2
	Caruncles	15·9	0·69	437	24·6	23·7	82	25·9	99·0	86·9

Summary of Analysis of Variance

Source of Variation	Degrees of Freedom	Variance Ratios								
		Amylase	SDH	LDH	GOT	Acid Phosphatase	Alkaline Phosphatase	GDH	GPC Diesterase	Protein
Natural *v.* synchronized oestrus (*A*)	1	10·1**	0·7	0·8	3·6	0·03	6·9*	1·1	0·2	2·3
Caruncles *v.* endometrium (*B*)	1	15·5**	7·8**	11·5**	4·4*	1·0	62·2**	0·02	2·9	13·9**
Days of oestrous cycle (*C*)	(3)									
(i) 0–2 *v.* 3–7	1	0·7	1·1	0·1	0·6	2·6	9·5**	0·9	0·8	3·6
(ii) 0–2 *v.* 8–14	1	14·7**	0·2	0·4	2·1	20·9**	46·6**	0·0	4·0*	0·3
(iii) 0–2 *v.* 15–17	1	8·4**	4·3*	1·1	5·4*	9·2*	5·5*	0·0	37·7**	0·9
Interactions:										
$A \times B$	1	0·2	0·2	1·1	0·4	0·0	3·2	0·2	0·3	0·9
$A \times C$	3	1·7	0·02	3·5*	1·8	3·2*	2·7	1·3	1·9	6·0**
$B \times C$	3	1·2	0·3	0·4	0·4	0·2	4·2*	1·5	1·5	0·3
$A \times B \times C$	3	0·4	0·02	0·2	0·2	0·7	0·5	0·2	1·2	0·1
Within-group error	64	$0{\cdot}15 \times 10^{-6}$	3·09	17,200	24·8	$0{\cdot}33 \times 10^{-6}$	$65{\cdot}8 \times 10^{-6}$	43·7	731	63·4

* $P < 0{\cdot}05$. ** $P < 0{\cdot}01$.

9–14 days: no recent ovulations, corpora lutea pink and greater in diameter, no follicles;

15–17 days: prominent follicles and only small yellow degenerate corpora lutea.

(c) *Endometrium and Caruncles*

Table 1 shows the activity of amylase, SDH, LDH, GOT, acid phosphatase, alkaline phosphatase, GDH, and GPC diesterase per milligram tissue protein, together with the protein concentration per gram of wet tissue, in the uterine endometrium and caruncles of the ewe at 0–2, 3–7, 8–14, and 15–17 days after the onset of oestrus when the ewes were allowed to cycle naturally or had oestrus synchronized. The summary of the analysis of variance is also presented in Table 1.

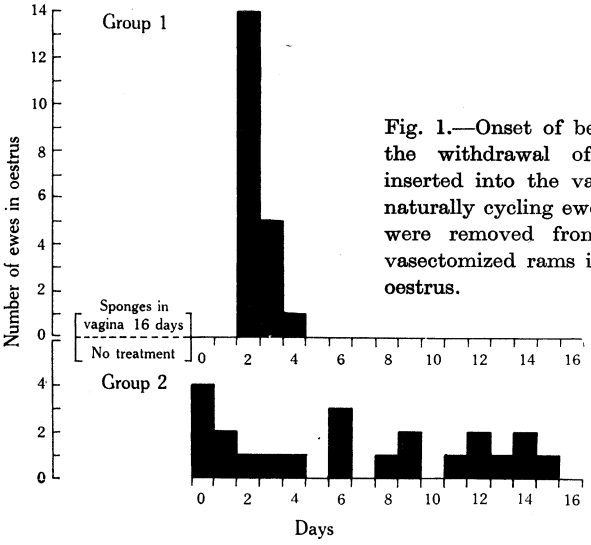

Fig. 1.—Onset of behavioural oestrus in ewes following the withdrawal of progestogen-impregnated sponges inserted into the vagina for 16 days (group 1) and in naturally cycling ewes (group 2). On day 0, the sponges were removed from the treated ewes and raddled, vasectomized rams introduced to both groups to detect oestrus.

The most striking changes in the uterine tissues were the increases in amylase, and acid and alkaline phosphatase activities during the luteal phase (days 3–14), reaching a maximum in mid-cycle. GPC diesterase also increased in activity until the follicular stage (days 15–17) of the cycle. SDH and GOT activity decreased during the follicular phase after reaching a maximum soon after oestrus. LDH and GDH activity remained unchanged throughout the cycle.

The amylase and alkaline phosphatase activities of both the endometrium and caruncles were greater at most stages of the cycle in synchronized ewes (group 1) than in ewes cycling naturally (group 2). The protein concentration of both the endometrium and caruncles was also higher in synchronized ewes than in naturally cycling ewes during the early stages of the cycle. The LDH activity was less in the early stages of the oestrous cycle in synchronized ewes than in naturally cycling ewes, while the reverse was true after mid-cycle.

TABLE 2

ENZYME ACTIVITY OF RINSINGS OF THE UTERUS DURING OESTRUS

Values represent the means of five ewes and are calculated as units per milligram protein (see text for definition) of units

Days of Cycle	Amylase $\times 10^4$	LDH	GOT	Acid Phosphatase $\times 10^4$	Alkaline Phosphatase	GDH	GPC Diesterase	Protein (mg/ml rinsings)
				Synchronized (Group 1)				
0–2	35	556	32·0	26·7	0·16	11·0	155	0·14
3–7	216	433	60·3	13·6	0·83	11·3	663	0·18
8–14	110	431	28·6	29·4	1·06	10·4	140	0·23
15–17	132	437	19·3	30·4	0·36	8·9	102	0·28
				Natural (Group 2)				
0–2	134	669	36·1	7·6	0·07	16·2	56	0·21
3–7	116	632	62·9	131·9	0·59	10·2	221	0·23
8–14	58	450	24·8	22·5	0·46	14·1	22	0·49
15–17	67	201	7·2	43·3	0·58	4·3	128	0·31

Summary of Analysis of Variance

Source of Variation	Degrees of Freedom	Variance Ratios							
		Amylase	LDH	GOT	Acid Phosphatase	Alkaline Phosphatase	GDH	GPC Diesterase	Protein
Natural *v.* synchronized oestrus (*A*)	1	0·4	1·5	0·0	0·8	1·9	0·1	4·0	3·3
Days of oestrous cycle (*B*)	(3)								
(i) 0–2 *v.* 3–7	1	1·6	0·4	2·3	1·7	11·3**	0·8	9·1**	0·2
(ii) 0–2 *v.* 8–14	1	0·0	2·1	0·2	0·0	13·3**	0·2	0·1	5·5*
(iii) 0–2 *v.* 15–17	1	0·1	6·1*	1·3	0·2	4·1	4·6*	0·0	2·4
Interaction:									
$A \times B$	3	0·9	0·8	0·1	1·1	1·8	0·8	1·6	0·9
Within-group error	32	$2{\cdot}13 \times 10^{-4}$	69,400	1,652	$8{\cdot}94 \times 10^{-4}$	0·159	56·9	62,227	0·031

* $P < 0{\cdot}05$. ** $P < 0{\cdot}01$.

Amylase, SDH, GOT, and protein occurred in greater concentrations in the caruncles than in the endometrium whereas LDH and alkaline phosphatase activity was less in the caruncles than in the endometrium, particularly during the luteal phase in the case of the alkaline phosphatase. There was no significant difference in the activities of acid phosphatase, GDH, or GPC diesterase between the endometrium and caruncles at any stage of the cycle.

(*d*) *Uterine Rinsings*

The enzyme activity of the uterine rinsings was extremely variable but several significant differences emerged (Table 2).

LDH and GDH activity decreased during the follicular phase of the oestrous cycle while alkaline phosphatase activity and protein content increased during the luteal phase, reaching a maximum about mid-cycle. GPC diesterase, although showing maximum activity at 15–17 days after oestrus in the endometrium and caruncles, reached peak activity at days 3–7 in the uterine rinsings. There was no significant variation in amylase, GOT, and acid phosphatase activity during the oestrous cycle. Synchronization of oestrus also had no significant effect on the activities of the enzymes measured in the rinsings. Values for SDH are not recorded in Table 2 since we were unable to detect any activity in the rinsings from the uterus.

IV. Discussion

The evidence suggests that progesterone from the corpus luteum stimulates the production of amylase and acid and alkaline phosphatase in the uterine endometrium and caruncles of the ewe. Of these enzymes alkaline phosphatase appears the most sensitive to progesterone, particularly in the endometrium where a much higher peak was reached than in caruncular tissue. Alkaline phosphatase activity has also been found to parallel the growth and retrogression of the corpus luteum in the endometrium of the cow, reaching maximum activity during the luteal phase of the cycle (Moss, Wrenn, and Sykes 1954; Skjerven 1956). In women and sows, however, maximum activity has been found during the follicular phase (Atkinson and Engle 1947; Hall 1950; Goode, Warnick, and Wallace 1965) suggesting that, in these species, uterine alkaline phosphatase is stimulated by oestrogen. Endometrial acid phosphatase, on the other hand, appears to be influenced by progesterone in women and sows (Goldberg and Jones 1956; McKay *et al.* 1956; Goode, Warnick, and Wallace 1965). Alkaline phosphatase has been linked with several physiological processes such as the formation of fibrous proteins, DNA turnover in the nucleus, and secretion (see Danielli 1953). Bradfield (1950) and Borghese (1957) have suggested that the enzyme plays a part in the transfer of material across the placenta, and the induction of the decidual reaction also appears to be associated with alkaline phosphatase (Finn and Hinchliffe 1964).

During the period in which the corpus luteum is functionally active, SDH and GOT activity was maximal suggesting that the production of these enzymes in the ewe is also stimulated by progesterone. The activity, however, did not follow the development and retrogression of the corpus luteum as closely as did acid and alkaline phosphatase.

Although the role of amylase in the female genital tract is not known with any certainty, in the rabbit it may be concerned with capacitation (Kirton and Hafs 1965; Dukelow, Chernoff, and Williams 1966). Amylase has been found in the fallopian tubes of women (McGeachin *et al.* 1958) and its activity in cervical mucus may depend on progesterone in this species (A. T. Gregoire, personal communication).

GPC diesterase activity in the uterine tissues increased during the luteal phase but did not reach a maximum until just prior to oestrus. In the uterine rinsings, however, maximum activity was reached during the first half of the oestrous cycle (3–7 days). This suggests that release of the enzyme into the uterine lumen does not occur until several days after optimal activity is reached in the tissues. Wallace and White (1965) found that the activity of GPC diesterase in the uterine rinsings of the ewe was greatest at or just after oestrus (0–2 days) and have suggested that the enzyme may make glycerol and phosphoglycerol components of seminal GPC available to spermatozoa as an additional energy source in the female genital tract. Because of the variability, uterine rinsings are of limited value for quantitative enzyme studies and luminal fluid collected by means of a cannula might prove more satisfactory. However, the present study demonstrates that all the enzymes found in the uterine tissue are also present in the lumen and that the activity of alkaline phosphatase and GPC diesterase in the lumen fluctuates during the oestrous cycle.

The effective synchronization of behavioural oestrus in ewes 2 or 3 days after removing sponges impregnated with the progestogen SC 9880 from the vagina confirms the work of Robinson (1965, 1967). The greater activity of uterine amylase and alkaline phosphatase in synchronized ewes than in naturally cycling ewes suggests that SC 9880 may specifically stimulate the synthesis of these enzymes as well as uterine protein generally. The apparent depression of LDH synthesis was transitory and after mid-cycle the levels in the uterus of synchronized ewes was above that of the control group. It is tempting to speculate that these alterations in the enzyme pattern of the endometrium and caruncles by the progestogen may in some way be related to the lowered fertility during the first oestrus following removal of the sponges (see Robinson 1967). The appearance of the ovaries at the various stages of the oestrous cycle agree with the observation of Restall (1964) and provides evidence that both groups of ewes ovulated normally and that oestrus was effectively detected by the ram.

Implantation is a lengthy procedure in the ewe and the precise time of its occurrence is difficult to define. Although some degree of attachment has been reported as early as the tenth day after mating (Green and Winters 1945) erosion of the maternal tissues occurs only when allanto–chorionic fusion is complete at about the twenty-second day (Davies 1952) resulting in closer attachment. Progesterone, secreted by the corpus luteum or the placenta or both, is required not only for implantation but is also important in the maintenance of pregnancy (see Marshall and Moir 1952). Since some of the uterine enzymes of the ewe are apparently dependent on progesterone for maximal activity, they may participate in implantation and foetal development. The significance of difference in activity between the endometrium (the glandular intercotyledonary area) and caruncles (non-glandular areas to which the foetal cotyledons become attached) is unknown.

V. Acknowledgments

The authors are indebted to Professor C. W. Emmens for his interest and advice. The work has been aided by grants from the Australian Wool Board. One of us (R.N.M.) was supported by an F. H. Loxton Post-Graduate Studentship. Progestogen-impregnated sponges were generously donated by G. D. Searle and Company.

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