GLYCOGEN, GLYCOGEN-METABOLIZING ENZYMES, AND ACID AND ALKALINE PHOSPHATASES IN THE ENDOMETRIUM OF THE EWE DURING EARLY PREGNANCY

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Summary

The concentration of glycogen and glucose and the activity of several glycogen-metabolizing enzymes have been measured in the endometrium of the ewe during early pregnancy and after the injection of progesterone and 20α-hydroxypregn-4-en-3-one. The activity of acid and alkaline phosphatases following the injection of steroids and the histochemical localization of the latter enzyme in the endometrium during early pregnancy have also been examined.

Glycogen concentration was greatest between days 0 and 14 of pregnancy and decreased significantly on days 22 and 44. The concentration of the polysaccharide on day 31, however, did not differ significantly from that on day 0. Endometrial glucose concentration was maximal on the day of mating and decreased to low levels between days 31 and 44 of pregnancy. Total and active glycogen phosphorylase and glucose-6-phosphatase activities, on the other hand, were maximal 8 days after mating while amylase activity was maximal between days 14 and 22 of pregnancy. The activities of amylase and glucose-6-phosphatase were significantly lower on day 44 than on the day of mating.

The administration of progesterone to ovariectomized ewes significantly increased the activities of total and active glycogen phosphorylase, glucose-6-phosphatase, and acid and alkaline phosphatases but failed to affect the concentration of glycogen or the activity of amylase. 20α-hydroxypregn-4-en-3-one had no statistically significant effect on glycogen concentration or enzyme activity in the endometrial tissue.

Alkaline phosphatase in the intercotyledonary endometrium of the ewe was located in the apical membranes in the cells of the surface and glandular epithelium. The activity of the enzyme increased on day 8 of pregnancy and weak activity was detected in the subepithelial stromal cells during this time.

Acid and alkaline phosphatases occurred in low concentrations in the maternal blood serum during early pregnancy and did not differ significantly between stages studied.

The significance of these observations in relation to histotrophic activity in the endometrium of the ewe during pregnancy has been discussed.

I. INTRODUCTION

The duration of the free life of the conceptus in the uterus of the ewe is extensive when compared with that of many other species. The first contact between the trophoblast and uterine epithelium occurs during the 15th day of gestation

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(Boshier 1969) after which time implantation proceeds slowly until by day 44 there is a "fair degree of adherence between maternal and foetal parts" (Amoroso 1964). Histotrophe, therefore, as opposed to haematotrophe, plays a vital role during early pregnancy in the ewe and for a period of some weeks the conceptus depends predominantly upon the secretory activity of the glandular intercotyledonary uterine endometrium to provide nutrition.

Murdoch (1970) has recently shown that the activity of acid and alkaline phosphatases in the uterine endometrium of the ewe changes considerably during early pregnancy. Since alkaline phosphatase has been associated with the transfer of solutes across the membranes of cells having a secretory function (see Dempsey and Wislocki 1945; Moog 1946; Bradfield 1950), its participation in endometrial metabolic transformations during early pregnancy may be essential for the survival of the pre-implantation embryo. In view of this, the histochemical localization of alkaline phosphatase in the intercotyledonary areas of the uterine endometrium has been examined at various stages of early pregnancy in the ewe. The concentration of glycogen and glucose and the activity of several glycogen-metabolizing enzymes, such as amylase, glucose-6-phosphatase, and glycogen phosphorylase, have also been measured in the endometrial tissue by methods of biochemical assay since changes in these factors may reflect changes in the nutritional demands of the developing conceptus.

Nancarrow and Seamark (1968) showed that the foetal blood of sheep rapidly metabolizes progesterone to produce 20α-hydroxypregn-4-en-3-one (20α-OHP). Murdoch (1970) proposed that this steroid conversion may play a role in regulating the activity of endometrial phosphatases in the ewe between days 22 and 31 of pregnancy. This possibility has been examined in the present paper by comparing the activities of acid and alkaline phosphatase in the endometrium of ovariectomized ewes injected with progesterone and 20α-OHP, separately and in combination. The effect of these steriods on the endometrial concentration of glycogen and activity of several glycogen-metabolizing enzymes has also been studied.

II. Materials and Methods

(a) *Experimental Animals*

Adult Merino ewes were mated with fertile rams and slaughtered by cutting the throat and dislocating the cervical vertebrae either on the day of mating (day 0) or on days 8, 14, 22, 31, and 44 of pregnancy. For a detailed description of events occurring during these stages of pregnancy see Amoroso (1964) and Boyd and Hamilton (1964).

Some of the ewes were ovariectomized and, after 30 days, randomized into four equal groups and treated according to the following schedule. All groups were injected with 30 μg of oestradiol benzoate on day 1 and killed on day 6.

Group 1: controls; no further treatment received.

Group 2: 5 mg of progesterone in the morning and evening on days 3, 4, and 5.

Group 3: 5 mg of 20α-OHP (Sigma Chemical Company, St. Louis, Missouri) in the morning and evening on days 3, 4, and 5.

Group 4: 5 mg of progesterone and 5 mg of 20α-OHP in the morning and evening on days 3, 4, and 5.

The steroids were administered by intramuscular injection in 1·0 ml of peanut oil.

(*b*) *Preparation of Uterus, Endometrial Tissue, and Maternal Blood Serum*

Just prior to slaughter, a sample of blood was collected from the jugular vein of the pregnant ewes. It was allowed to clot in a centrifuge tube and, after separating the clot from the wall of the tube by means of a glass rod, was centrifuged at 6°C for 30 min at 1000 *g* to obtain the serum. Acid and alkaline phosphatase activities in the serum were assayed immediately following centrifugation.

After slaughter the uteri were removed, placed in crushed ice, and quickly taken to the laboratory where the uterine horns were immediately dissected free of fatty and connective tissue and of the attached oviducts and cervix. All subsequent processing of the tissue was performed at 4°C. The uteri from ovariectomized ewes and from ewes on days 0, 8, and 14 of pregnancy were washed through with 10 ml of 0·154M NaCl to remove any contaminating endometrial secretion and to recover blastocysts. The uteri from ewes at more advanced stages of pregnancy were dissected and, after removing the embryonic fluids, the embryo and its supporting membranes were carefully separated from the maternal tissues. The endometrium was then gently rinsed with 0·154M NaCl to remove any contaminating cells or fluid. Sections of each uterine horn from pregnant ewes were then taken for histochemical studies of alkaline phosphatase and were placed in cold 95% ethanol to fix the tissue. The remainder of each uterine horn was placed on a piece of filter paper and opened down the mesometrial side. The exposed endometrium was blotted with filter paper and the endometrial tissue from the intercotyledonary areas was carefully dissected using fine scissors and forceps. Samples of tissue were homogenized in 10 parts of distilled water with a Potter–Elvehjem homogenizer and then filtered through muslin.

(*c*) *Analytical Methods*

The glycogen concentration of the endometrial tissue was determined with the anthrone reagent of Seiffer *et al.* (1950) after digesting the tissue in 30% (w/v) KOH and precipitating the polysaccharide with ethanol.

The glucose concentration of the homogenate was measured by the glucose oxidase method described by Huggett and Nixon (1956) after deproteinizing the sample with 5% (w/v) $ZnSO_4.7H_2O$ and 0·3N $Ba(OH)_2$.

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 hydrolyses 0·2 mg of amylose in 15 min at pH 7·0 and 37°C.

Total and active glycogen phosphorylase activities were measured by methods described by Leonard (1957) and are reported as micrograms of Pi liberated from glucose 1-phosphate per 1 mg of tissue protein per hour.

Glucose-6-phosphatase activity was determined by the method outlined in Bergmeyer (1963) and is reported as micrograms of Pi liberated from glucose 6-phosphate per 1 mg of tissue protein per hour.

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 of sample, which liberates 1 mmole (139 mg) *p*-nitrophenol at 37°C.

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

Inorganic phosphate was measured by the method of Fiske and Subba Row (1925).

(*d*) *Histochemical Method*

Fresh tissues for histochemical studies were placed in fixative at 4°C. Paraffin sections were cut at 7 μm in a microtome and alkaline phosphatase was demonstrated by the azo-coupling technique described by Culling (1963). Control solutions lacking the substrate were prepared with each section. Since it has been reported that fixing and paraffin-embedding of tissues before sectioning destroys variable amounts of the enzymes present (Pearse 1960), all the technical manipulations were carefully standardized to ensure that the proportional loss of enzyme activity was the same in all pieces of tissue studied.

(e) *Statistical Analyses*

Where necessary the primary data were converted to logarithms and the significance of the results assessed by analyses of variance. All main effects and their first-order interactions were isolated and tested for significance using the within-group error mean square to calculate variance ratios. In the first experiment, the within-group error mean square has been used to calculate the standard errors of the difference between means and this has been used in *t*-tests.

III. Results

(a) *Biochemical Changes in the Endometrium during Early Pregnancy*

Table 1 shows the concentration of glycogen and glucose and the activities of amylase, total and active glycogen phosphorylase, and glucose-6-phosphatase in the intercotyledonary areas of the uterine endometrium of ewes at 0, 8, 14, 22, 31, and 44 days of pregnancy.

TABLE 1

CONCENTRATION OF GLYCOGEN AND GLUCOSE AND ACTIVITY OF SOME GLYCOGEN-METABOLIZING ENZYMES IN THE ENDOMETRIUM OF THE EWE DURING EARLY PREGNANCY

Values represent the means of four ewes and are expressed as follows: glycogen and glucose, mg/100 g tissue; amylase, $10^4 \times$ units/mg protein; active and total glycogen phosphorylase and glucose-6-phosphatase, μg Pi liberated/hr/mg protein

Day of Pregnancy	Glycogen	Glucose	Amylase	Glycogen Phosphorylase		Glucose-6-Phosphatase
				Active	Total	
0	48·9	11·01	13·5	5·6	25·2	33·4
8	53·8	8·73	12·5	17·1**	34·3*	68·7**
14	46·5	8·58	20·0*	6·4	23·4	41·8
22	30·4**	7·57	19·1*	5·8	18·9	31·0
31	42·8	4·46**	14·1	6·2	25·5	27·7
44	30·0**	4·56**	7·7*	6·6	29·0	22·6**
Standard errors†	0·056	0·126	0·081	0·168	0·066	0·052

* Significantly different from day 0 of pregnancy, $P < 0{\cdot}05$.

** Highly significantly different from day 0 of pregnancy, $P < 0{\cdot}01$.

† Derived from the logarithmically transformed data.

Glycogen concentration was greatest between days 0 and 14 of pregnancy and decreased significantly on days 22 and 44. The concentration of the polysaccharide on day 31, however, did not differ significantly from that on day 0. Endometrial glucose concentration was maximal on the day of mating and decreased to low levels between days 31 and 44. Total and active glycogen phosphorylase and glucose-6-phosphatase activities, on the other hand, were maximal 8 days after mating while amylase activity was maximal between days 14 and 22 of pregnancy. Although active glycogen phosphorylase showed little change in activity between days 14 and 44, total glycogen phosphorylase activity displayed a tendency to increase after having reached a minimum on day 22. The activities of amylase and glucose-6-phosphatase, however, were significantly lower on day 44 than on the day of mating.

(b) Biochemical Changes in the Endometrium following Injection of Steroids

Table 2 shows the concentration of glycogen and activity of amylase, total and active glycogen phosphorylase, glucose-6-phosphatase, and acid and alkaline phosphatases in the intercotyledonary areas of the uterine endometrium of ovariectomized

TABLE 2

EFFECT OF INJECTIONS OF PROGESTERONE AND 20α-OHP ON THE GLYCOGEN CONCENTRATION AND ENZYME ACTIVITY IN THE ENDOMETRIUM OF OVARIECTOMIZED EWES

Values represent the means of three ewes and are expressed as follows: glycogen, mg/100 g tissue; amylase, acid phosphatase, and alkaline phosphatase, $10^4 \times$ units/mg protein; glycogen phosphorylase (active and total) and glucose-6-phosphatase, μg Pi liberated/hr/mg protein

Treatment	Glycogen	Amylase	Glycogen Phosphorylase		Glucose-6-Phosphatase	Acid Phosphatase	Alkaline Phosphatase
			Active	Total			
None	50·7	12·3	2·60	18·4	18·6	11·2	101
Progesterone	55·2	15·0	12·91	29·9	38·7	22·2	360
20α-OHP	51·1	8·5	2·66	18·2	21·0	10·7	100
Progesterone +20α-OHP	45·7	9·5	10·13	29·4	43·5	20·8	399

Summary of the analyses of variance

Source of Variation	D.F.	Variance Ratios						
		Glycogen	Amylase	Glycogen Phosphorylase		Glucose-6-Phosphatase	Acid Phosphatase	Alkaline Phosphatase
				Active	Total			
Effect of progesterone	1	0·5	0·4	101·7**	108·5**	37·5**	89·0**	132·8**
Effect of 20α-OHP	1	3·0	2·5	0·5	0·0	1·3	0·6	0·2
Interaction	1	3·9	0·0	0·8	0·0	0·0	0·0	0·2
Within-group error†	8	*0·0058*	*0·030*	*0·011*	*0·0012*	*0·0081*	*0·0029*	*0·0076*

** $P < 0·01$.

† Within-group error mean squares derived from the logarithmically transformed data are given in italics.

ewes after injecting progesterone and 20α-OHP separately and in combination. The summary of the analyses of variance is also presented in Table 2.

Progesterone significantly increased the activity of total and active glycogen phosphorylase, glucose-6-phosphatase, and acid and alkaline phosphatases but failed to affect the concentration of glycogen or the activity of amylase. 20α-OHP had no statistically significant effect on glycogen concentration or enzyme activity in the endometrial tissues and did not alter their response to progesterone.

(*c*) *Acid and Alkaline Phosphatase Activity in Blood Serum*

Acid and alkaline phosphatases occurred in very low concentrations in the maternal blood serum of sheep and did not differ significantly between days 0, 8, 14, 22, 31, and 44 of pregnancy. The blood serum of 24 sheep was examined and alkaline phosphatase ($1 \cdot 76 \pm 0 \cdot 16$ units/1000 ml) was always present in greater concentration than acid phosphatase ($0 \cdot 22 \pm 0 \cdot 06$ units/1000 ml).

(*d*) *Histochemical Studies of Alkaline Phosphatase*

Alkaline phosphatase activity was localized predominantly in the apical regions of the uterine epithelial cells during early pregnancy in the ewe and reached a maximum on day 8. Some activity also occurred in the subepithelial stromal cells on day 8 of pregnancy, but none was demonstrated in the intercotyledonary endometrial capillaries or myometrial smooth muscle at any stage of pregnancy studied. Enzyme activity was greater in the superficial areas of the glandular epithelium near the lumen of the uterus than in the glandular areas further from the lumen and nearer the myometrium. There appeared to be little difference in the activity of the enzyme between days 0, 14, 22, 31, and 44 of pregnancy.

IV. Discussion

The relatively high concentration of glycogen and glucose in the uterine endometrium of the ewe at a period when the conceptus is solely dependent upon histotrophic activity for its nutrition and their gradual diminution with increasing anastomosis of the embryo with the maternal system suggests that the polysaccharide, through its conversion to glucose, acts as a source of nutrition for the pre-implantation conceptus. The occurrence in the endometrium of glycogen-degrading enzymes, such as glycogen phosphorylase (see Stetten and Stetten 1960), amylase, and glucose-6-phosphatase, further supports this suggestion.

In the human, uterine glycogen is maximal during the luteal phase of the menstrual cycle (Zondek and Stein 1940), which coincides with a high production of progesterone, while in the rat, rabbit, mouse, and sheep its synthesis is dependent upon oestrogen (Hall 1965; Bitman *et al.* 1967; Gregoire, Ramsey, and Adams 1967; Cecil and Bitman 1968). The failure of progesterone to change the concentration of glycogen in the endometrium of the ovariectomized ewe is consistent with earlier results for the rat and rabbit (Bo and Atkinson 1952; Bo 1961). Thus, the decreased concentration of glycogen on days 22 and 44 of pregnancy in the ewe may reflect insufficient oestrogen-induced glycogenesis rather than progesterone-induced glycogenolysis.

Total glycogen synthetase activity in the rat uterus is increased following either progesterone or oestrogen treatment (Bo and Ashburn 1968) but, as mentioned previously, an increase in uterine glycogen only occurs after the injection of oestrogen

in this species. It is possible that a glycogen synthetase with a similar hormonal sensitivity to that in the rat may also exist in the endometrium of the ewe since the glycogen content of this tissue remained high when the glycogen-degrading enzymes, glycogen phosphorylase (both total and active) and glucose-6-phosphatase, were at maximal activity. Hence, an increase in glycogen breakdown due to the increased glycogen phosphorylase activity on day 8 of pregnancy, or after the injection of progesterone, may be masked by a simultaneous increase in glycogen synthesis through the action of the glycogen synthetase enzyme, thus resulting in no net increase or decrease in uterine glycogen. Since the activities of many endometrial enzymes in the ewe reach a maximum for a relatively brief period of time 8 days after mating (Murdoch and White 1968; Murdoch 1970; present study), histotrophic activity during this time of pregnancy in this species may be of greater importance to the survival of the conceptus than at any other stage prior to implantation.

The hormonal regulation of amylase activity in the endometrium of the ewe is problematic. Amylase activity did not alter after progesterone was administered to ovariectomized ewes but increased significantly between days 14 and 22 of pregnancy. This peak of activity occurs later than that of glycogen phosphorylase, glucose-6-phosphatase, acid phosphatase, and alkaline phosphatase (day 8) but earlier than the second peak of acid phosphatase activity between days 22 and 31 of pregnancy (Hafez and White 1968; Murdoch 1970). Further studies of the regulation of endometrial amylase and its physiological significance during early pregnancy in the ewe are clearly warranted.

The administration of 20α-OHP to ovariectomized ewes in the present investigation failed to alter significantly the enzyme activity or glycogen content of the endometrium, indicating that this steroid, through its production from progesterone in foetal blood (Nancarrow and Seamark 1968), is not responsible for the regulation of acid phosphatase activity between days 22 and 31 of pregnancy in the ewe as was proposed by Murdoch (1970). Changes in the vascularity of the uterus also do not appear to account for any of the change in activity of acid and alkaline phosphatases in the endometrium during early pregnancy since their concentration in the maternal blood serum was low at all stages of pregnancy studied.

The histochemical demonstration of increased endometrial alkaline phosphatase activity on day 8 of pregnancy confirms the results of earlier experiments (Murdoch 1970) and shows that the response occurs predominantly within the secretory elements of the endometrial tissue (see Boshier 1969). This observation, together with the finding that activity is greatest in glandular areas closer to the uterine lumen, supports the general belief that alkaline phosphatase plays a role in the transfer of solutes across the membrane of cells having a secretory function and in endometrial metabolic processes which provide the necessary nutriments for the survival and development of the pre-implantation embryo. It is difficult, on the other hand, to assign a role for the alkaline phosphatase activity in the subepithelial stromal cells on day 8 of pregnancy.

The regulation and role of acid and alkaline phosphatases in histotrophic processes in the endometrium of the ewe during early pregnancy remain the subjects of further investigation.

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