UTERINE ENDOMETRIAL PHOSPHOMONOESTERASES IN RELATION TO IMPLANTATION IN THE EWE AND RABBIT DOE

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Summary

The activities of acid and alkaline phosphatases in the uterine endometrium of the ewe and rabbit doe were studied during the oestrous cycle and pseudopregnancy and in relation to the implanting conceptus.

Acid and alkaline phosphatase activities greatly increased in the endometrium of the ewe 8 days after oestrus and reached a far greater maximum in pregnant ewes than in non-pregnant ewes. The activity of both enzymes decreased after day 8 of pregnancy but acid phosphatase activity again increased on day 22 and continued to increase until day 31, after which time it declined.

In the endometrium of the rabbit doe, acid phosphatase activity decreased during the first 3 days of pseudopregnancy and then increased until a maximum was reached on day 13. Activity again decreased between days 13 and 19. The activity of acid phosphatase also increased between days 5 and 11 in the endometrium of pregnant does. The pattern of alkaline phosphatase activity, on the other hand, was essentially the reverse to that of acid phosphatase and, in general, was low when acid phosphatase activity was high, and vice versa. There were no significant differences in enzyme activity between gravid and non-gravid uterine horns during early pregnancy in the doe.

Although the activity of alkaline phosphatase exceeded that of acid phosphatase at all reproductive stages studied, the results suggest that, in both the ewe and the doe, endometrial acid phosphatase may become relatively more important than alkaline phosphatase to the conceptus as the embryonic membranes establish closer contact with the maternal tissues.

Starch-gel electrophoretic studies of alkaline phosphatase suggested the possibility of different isoenzymes in the endometrium of the two species with varying sensitivities to circulating hormones.

I. INTRODUCTION

The non-specific orthophosphoric monoester phosphohydrolases, acid and alkaline phosphatases, in the uterine endometrium of the ewe are sensitive to changes in progesterone status and undergo cyclic variation, reaching maximal activity during the luteal phase of the oestrous cycle (Murdoch and White 1968*a*, 1968*b*). Acid and alkaline phosphatases in the endometrium of the rabbit doe, on the other hand, although responsive to changes in progesterone status, appear to depend mainly on oestrogen for the expression of maximal activity (Murdoch and White 1969). Since the duration of the free life of the conceptus in the uterine lumen differs considerably between the ewe and doe, the contrasting hormonal dependence of the enzymes may

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reflect differences in the time at which the phosphatases require stimulation to participate in endometrial metabolic and cellular transformations necessary for the provision of nutriments for the implanting conceptus.

In order to assess the time at which acid and alkaline phosphatases may be of most importance in such transformations, activities in the endometrium of the ewe and doe throughout the oestrous cycle and during early pregnancy have been measured in the present investigation. The electrophoretic activity pattern of endometrial alkaline phosphatase in starch gel has also been examined.

II. Materials and Methods

(*a*) *Experimental Animals*

(i) *Sheep.*—Adult Merino ewes were mated with fertile rams and slaughtered by cutting the throat on the day of mating (day 0) and on days 8, 14, 18, 22, 31, and 44 of pregnancy. Some of the ewes were run with raddled vasectomized rams to establish the date of oestrus and were killed on days 0, 8, and 14 of the oestrous cycle. For a detailed description of events occurring during the above stages of pregnancy in the ewe see Amoroso (1964) and Boyd and Hamilton (1964).

(ii) *Rabbits.*—Virgin albino rabbits, aged 6–8 months, were obtained from the University Animal House, Castle Hill. Following treatment, enzyme activities in the endometrium of gravid cornua were compared with those in the endometrium of non-gravid cornua. This was achieved by ligating one fallopian tube in each experimental rabbit 1 cm anterior to the uterotubal junction thus allowing pregnancy to occur only in the contralateral uterine horn after insemination. Half the number of does in each treatment group had ligatures placed on the right fallopian tube while the remaining half had ligatures placed on the left fallopian tube. All non-pregnant animals were similarly treated to correct for any effect the ligatures may have on the non-gravid uterine horn during pregnancy. Surgery was carried out under pentobarbitone anaesthesia and, after closure of the flank incision, the animals were given 100,000 units of procaine pencillin G and 0·125 g of streptomycin sulphate intramuscularly. The animals were placed in separate cages and allowed 30 days to recover from the surgery before use. Does were made pseudopregnant by intravenous injection of 50 i.u. of human chorionic gonadotrophin (HCG) (Pregnyl) or pregnant by simultaneous insemination with 0·1 ml of semen freshly collected with an artificial vagina (White 1955). The animals were slaughtered by cervical dislocation at appropriate intervals following insemination or injection of HCG.

(*b*) *Preparation of Endometrium*

After slaughter the ovaries of all animals were examined to check the stage of the oestrous cycle or pregnancy. 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.

(i) *Sheep.*—The uteri from non-pregnant ewes and from ewes on days 8, 14, and 18 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 carefully dissected and, after discarding the embryonic fluids, the embryo and its supporting membranes were carefully separated from the maternal tissues. The endometrium was then rinsed with 0·154M NaCl to remove any contaminating cells or fluid. 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 to remove any traces of flushing fluid and 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. Despite extensive transuterine migration of foetal membranes in ewes at the more advanced stages of pregnancy, the tissue from gravid uterine horns was processed separately from that from non-gravid uterine horns. The endometrial tissue from non-pregnant animals, however, was processed as a pool from both uterine horns.

(ii) *Rabbits.*—The uterine cornua from non-pregnant does and from does on the fifth day of pregnancy were rinsed with 4 ml of 0·154M NaCl to remove any contaminating endometrial secretion and to recover blastocysts. Blastocysts 7, 9, and 11 days old were removed by dissection, taking care to eliminate all trophoblastic tissue. After rinsing with 0·154M NaCl, each horn was placed on a piece of filter paper and opened down the mesometrial side. The exposed endometrium was blotted with filter paper and then carefully scraped off using a scapel blade and fine forceps. Samples were homogenized in 10 parts of distilled water and then filtered through muslin. The tissue from uterine cornua with attached oviducts ligated was processed separately to that from uterine cornua with attached oviducts not ligated.

(c) *Enzyme and Protein Analyses*

Acid and alkaline phosphatase activities were determined in the homogenate after appropriate dilution in distilled water by using *p*-nitrophenyl phosphate 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 of *p*-nitrophenol at 37°C. The protein concentration of the samples was determined by the biuret method (Wales, Scott, and White 1961).

(d) *Electrophoresis of Alkaline Phosphatase*

Extracts of endometrial alkaline phosphatase were made according to the butanol method of Morton (1950). Approximately 20% (v/v) of n-butanol was added to homogenates and extraction was carried out at room temperature for 30 min with agitation. The suspension was allowed to stand for 10 min at 37°C and was then centrifuged at 1000 *g* for 30 min at 4°C. The aqueous layer containing the enzyme was removed after centrifugation and dialysed for 90 min at 4°C against distilled water to remove traces of butanol. The enzyme was then concentrated by dialysis for 60 min at room temperature against polyvinylpyrrolidone powder and was again centrifuged at 1000 *g* for 30 min at 4°C to remove traces of cellular debris.

Aliquots (10 μl) of the clear supernatant were applied to small pieces of Whatman No. 3 paper and subjected to horizontal starch-gel electrophoresis (Smithies 1955) with a discontinuous buffer system as described by Poulik (1957). The bridge buffer (pH 7·95) consisted of 0·3M boric acid, 0·05M sodium hydroxide, and 0·002M magnesium chloride (Chiandussi, Greene, and Sherlock 1962). The gel buffer (pH 8·95) contained 0·076M tris(hydroxymethyl)aminomethane and 0·005M citric acid (Chiandussi, Greene, and Sherlock 1962). Electrophoresis was carried out at room temperature for 17 hr with a voltage drop of 6·3 V/cm.

Following electrophoresis, zones of alkaline phosphatase activity were developed directly on a longitudinal gel slice by using a solution containing 0·05% (w/v) β-naphthyl sodium phosphate, 0·005M magnesium chloride, 0·05% (w/v) fast blue RR salt, and 0·06M (pH 9·7) sodium borate–boric acid buffer (Markert and Møller 1959).

(e) *Statistical Analyses*

The significance of the results has been assessed by analysis 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. The multiple-range test (Duncan 1955) was used to compare enzyme activities at the various reproductive stages studied.

III. Results

(a) *Phosphatases in the Endometrium of the Ewe*

Figures 1(*a*) and 1(*b*) show the activity of acid and of alkaline phosphatase per milligram of tissue protein in the uterine endometrium of ewes at 0, 8, and 14 days after the onset of oestrus and in the endometrium of gravid and non-gravid uterine horns of ewes at 0, 8, 14, 18, 22, 31, and 44 days of pregnancy.

The activity of endometrial acid and alkaline phosphatases closely followed the growth and retrogression of the ovarian corpus luteum throughout the oestrous cycle and reached a maximum 8 days after the onset of oestrus. Activity also increased

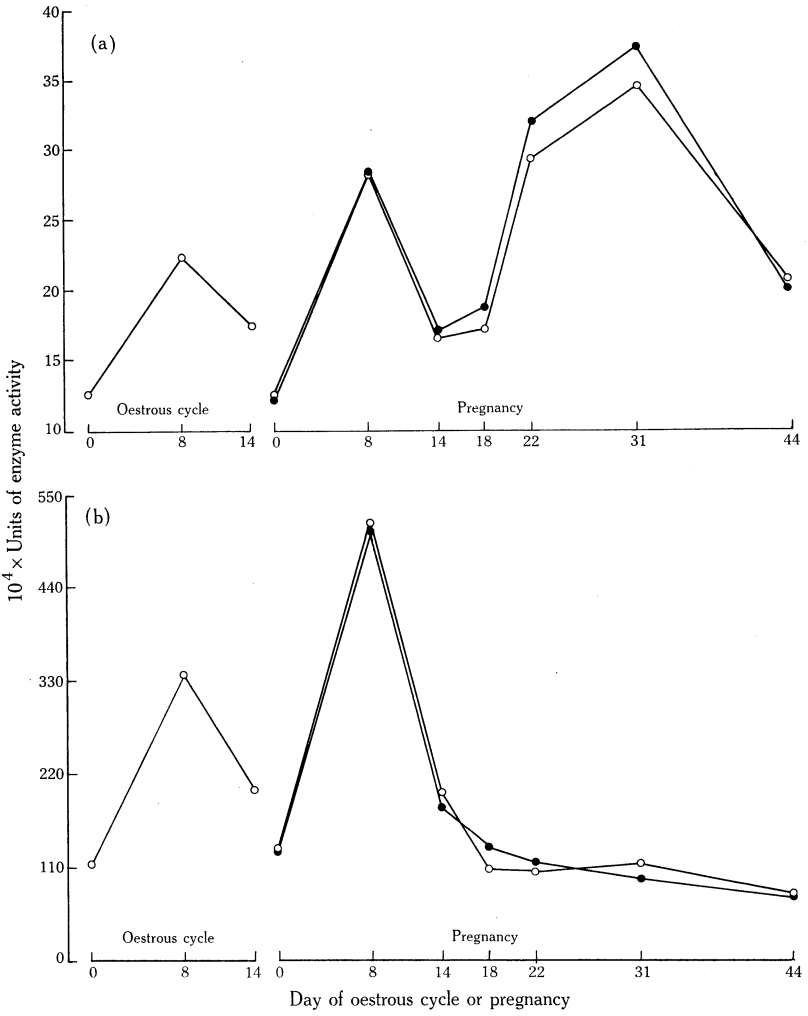

Fig. 1.—Activity of acid phosphatase (*a*) and alkaline phosphatase (*b*) per milligram of tissue protein in the uterine endometrium of the ewe at various stages of the oestrous cycle and early pregnancy. ○ Non-gravid uterine horn. ● Gravid uterine horn. Values represent the means of four ewes. The multiple-range test gave the following ranking for acid phosphatase activity on the following days of the oestrous cycle or of pregnancy:

Oestrous cycle: 8 > 0 = 14 (5% level of significance).
Pregnancy: 8 = 22 = 31 > 0 = 14 = 18 = 44 (5% level).

The same test gave the following ranking for alkaline phosphatase activity:

Oestrous cycle: 8 > 0 = 14 (5% level).
Pregnancy: 8 > 0 = 14 = 18; 14 > 22 = 31 = 44 (5% level).

significantly 8 days after mating ewes to fertile rams but the levels reached in the endometrium of these ewes were much greater than those reached in the endometrium

of non-pregnant cyclic ewes. The activity of both enzymes rapidly decreased after day 8 of the oestrous cycle and pregnancy and on day 14 was similar to that recorded

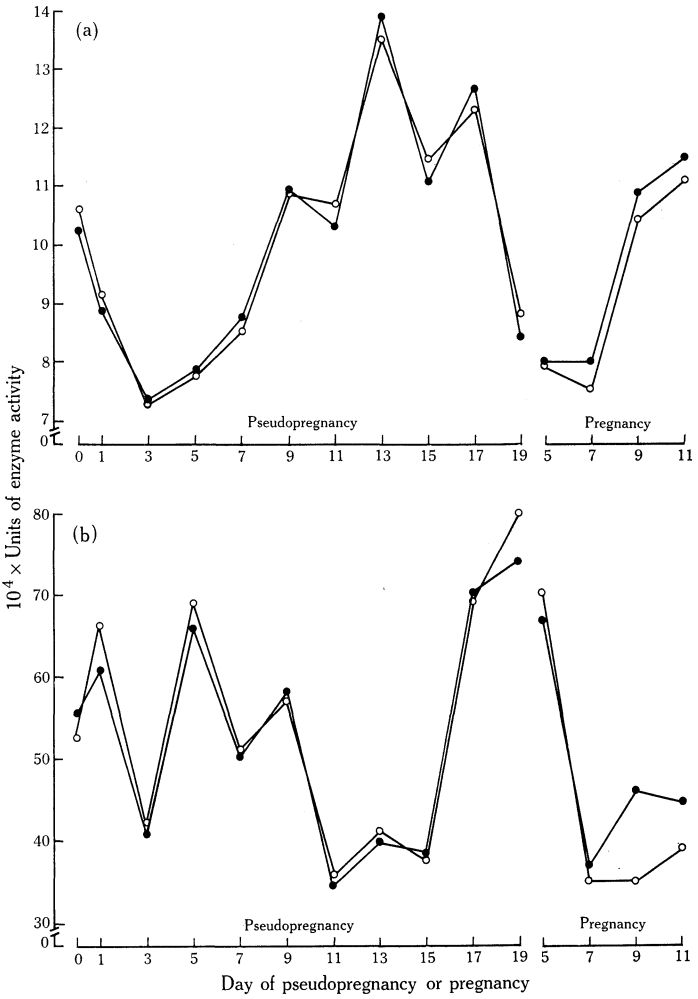

Fig. 2.—Activity of acid phosphatase (*a*) and alkaline phosphatase (*b*) per milligram of tissue protein in the uterine endometrium of the rabbit doe at various stages of pseudopregnancy and early pregnancy. ○ Attached oviduct ligated. ● Attached oviduct not ligated. Values represent the means of four rabbits. The multiple-range test gave the following ranking for acid phosphatase activity on the following days of pseudopregnancy or early pregnancy (P):

13 > 0 = 9 = 9P = 11 = 11P = 15 > 3 = 5 = 5P = 7 = 7P = 19 (5% level).
13 = 17 > 0 = 1 (5% level).

The same test gave the following ranking for alkaline phosphatase activity:

19 = 5 = 5P = 17 > 3 = 7P = 9P = 11 = 11P = 13 = 15 (5% level).
19 = 0 = 1 = 9 (5% level).
19 > 7 = 7P (5% level).

at oestrus. Alkaline phosphatase activity continued to decrease slightly between days 14 and 44 of pregnancy. Acid phosphatase activity, however, again increased

on day 22 of pregnancy and reached a maximum on day 31. Activity again decreased on day 44.

The activity of alkaline phosphatase exceeded that of acid phosphatase at all stages studied. Furthermore, there were no significant differences in enzyme activity at any stage of pregnancy between the endometrial tissue from gravid and non-gravid uterine horns.

(b) Phosphatases in the Endometrium of the Doe

Figures 2(*a*) and 2(*b*) show the activity of acid and of alkaline phosphatase per milligram of tissue protein in the endometrium of rabbit uterine cornua with attached oviducts, either ligated or not ligated, during pseudopregnancy and early implantation stages of pregnancy.

Acid phosphatase activity significantly decreased during the first 3 days of pseudopregnancy and then increased until a maximum was reached on day 13. Except for a slight rise on day 17, acid phosphatase activity decreased between days 13 and 19 of pseudopregnancy. The activity pattern of the enzyme in the endometrium of the pregnant uterus was similar to that in the endometrium of the pseudopregnant uterus between days 5 and 11 following the administration of HCG. Oviductal ligation did not significantly influence acid phosphatase activity in the endometrium of attached uterine cornua and, during pregnancy, no differences in enzyme activity were observed between gravid and non-gravid uterine horns.

The pattern of alkaline phosphatase activity in the uterine endometrium of the rabbit doe was essentially the reverse to that of acid phosphatase. In general, alkaline phosphatase activity was low when acid phosphatase activity was high and vice versa. Alkaline phosphatase activity fluctuated considerably between days 1 and 9 of pseudopregnancy and was minimal on day 11. Although the activity of the enzyme varied little between days 11 and 15, it again increased on day 17 and reached a maximum on day 19. During pregnancy, alkaline phosphatase activity decreased between days 5 and 7 in both gravid and non-gravid uterine horns, but failed to vary significantly between days 7 and 11. Oviductal ligation did not significantly influence endometrial alkaline phosphatase activity.

The activity of alkaline phosphatase exceeded that of acid phosphatase at all stages studied and there were no significant differences in enzyme activity between endometrial tissue from right and left uterine horns.

(c) Distribution of Alkaline Phosphatase Activity in Starch Gel

The activity distribution of sheep and rabbit endometrial alkaline phosphatase after electrophoresis in starch gel is shown in Figure 3.

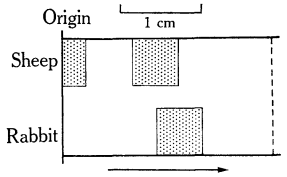

Fig. 3.—Distribution of endometrial alkaline phosphatase activity following electrophoresis in starch gel. Arrow indicates direction of migration.

With sheep endometrium, two zones of alkaline phosphatase activity were resolved by electrophoretic separation in starch gel. One zone remained near the origin and appeared to stain with slightly greater intensity than the broader zone of

activity which migrated toward the anode. With rabbit endometrium, on the other hand, only one broad zone of enzyme activity was resolved by electrophoretic separation and had a slightly greater mobility than the mobile zone in the endometrium of the ewe. These patterns of activity were characteristic of all reproductive stages studied.

IV. Discussion

The increase in the activity of acid and alkaline phosphatases in the uterine endometrium of the ewe during the luteal phase of the oestrous cycle confirms previous results (Murdoch and White 1968*a*) and supports the claim that the enzymes are sensitive to changes in progesterone levels in this species (Murdoch and White 1968*b*). Progesterone concentrations in peripheral blood plasma, ovarian vein blood, and corpora lutea of the ewe are greatest between days 8 and 14 of the oestrous cycle and decline rapidly on day 15 or about 48 hr before the onset of oestrus (Moore *et al.* 1969; Smith and Robinson 1969; Stabenfeldt, Holt, and Ewing 1969). The fall in phosphatase activity on day 14 of the oestrous cycle presumably reflects regression processes occurring in the corpus luteum which begin on day 12 or 13 (Deane *et al.* 1966).

Information about luteal function during early pregnancy in the ewe is limited. However, the greater phosphatase activity in the endometrium on day 8 of pregnancy than on day 8 of the oestrous cycle suggests that the conceptus exerts a very early effect on luteal function to induce the corpus luteum to produce greater amounts of progesterone in order to prepare the uterus for implantation. Moor and Rowson (1964) have indicated that the embryo must be in the uterus by day 12 if the corpus luteum of the cycle is to be converted into one of pregnancy. The increased acid phosphatase activity in the endometrium between days 22 and 31 of pregnancy indicates that the corpus luteum may again increase its production of progesterone during this time. It is not clear, however, why alkaline phosphatase failed to display a similar response. Nancarrow and Seamark (1968) have shown that the foetal blood of sheep rapidly metabolizes progesterone to produce 20α-hydroxypregn-4-en-3-one. Since the foetal heart beat begins on day 21 of pregnancy in the ewe (Green and Winters 1945), it is possible that this conversion may play a role in regulating the activity of endometrial phosphatases between days 22 and 31 of pregnancy. Other workers have also reported an increase in endometrial acid phosphatase activity during early pregnancy in the ewe and gilt (Goode, Warnick, and Wallace 1966; Hafez and White 1968; Boshier 1969). Although the activity of alkaline phosphatase exceeded that of acid phosphatase at all stages studied in the present investigation, it appears that acid phosphatase may become relatively more important than alkaline phosphatase in the endometrium of the ewe between days 22 and 31 of pregnancy or as the embryonic membranes establish a firmer contact with the maternal tissues.

In the rabbit, as in the ewe, implantation of the conceptus depends upon progesterone secretion from the corpora lutea which, during pregnancy increases steadily in the first week and becomes maximal during the third week (Mikhail, Noall, and Allen 1961; Hilliard, Spies, and Sawyer 1968). The activities of a number of enzymes in the endometrium of the rabbit are increased by progesterone rather than by oestrogen (Lutwak-Mann and Laser 1954; Albers, Bedford, and Chang 1961;

Hafez and White 1967; Murdoch and White 1969) but acid and alkaline phosphatases are increased by both oestrogen and progesterone, with the former hormone having a more profound effect (Giering and Zarrow 1958; Murdoch and White 1969). The decrease in alkaline phosphatase activity during pseudopregnancy and early pregnancy is understandable in view of this hormonal regulation but the reason for the increase in acid phosphatase activity is problematic. Hafez and White (1967) also reported a decrease in alkaline phosphatase activity in the endometrium of the doe on the tenth day of pregnancy. The similar levels of phosphatase activity in the endometrium of gravid and non-gravid uterine cornua of the unilaterally pregnant rabbits indicate a function of circulating hormones and suggest that the conceptus, at this time of pregnancy, fails to exert any marked local effect on the rate of synthesis of these enzymes. However, a local effect of the conceptus on alkaline phosphatase activity at implantation sites has been clearly demonstrated in the rat (Manning, Meli, and Steinetz 1966). The present results imply that, as in the ewe, acid phosphatase may become relatively more important than alkaline phosphatase to the process of implantation as the embryonic membranes establish closer contact with the maternal tissues.

The differences between the ewe and rabbit doe with respect to the differentiation of endometrial alkaline phosphatase activity following electrophoresis in starch gel may, if the resolved zones of activity do in fact represent various molecular forms of the enzyme explain, in part, why the enzyme differs in its hormonal dependence between the two species. Thus, the observations suggest the possibility of different isoenzymes in the endometrium of the two species with varying sensitivities to the hormonal stimulus. The failure to detect changes in the starch-gel activity pattern during the various reproductive stages examined further indicates that the increased alkaline phosphatase activity at day 8 of the cycle and of pregnancy in the ewe is due to an increase in the synthesis of existing enzymes rather than the induction of a new enzyme.

The precise physiological relationship of endometrial acid and alkaline phosphatases to implantation remains to be determined. Alkaline phosphatases are known to catalyse the hydrolysis of a variety of phosphate esters (see Stadtman 1961) and have been associated with carbohydrate metabolism (Boshier 1969) and with the transfer of solutes across the membranes of cells having a secretory function (see Dempsey and Wislocki 1945; Moog 1946; Bradfield 1950). The high alkaline phosphatase activity in the endometrium of the ewe and rabbit doe during very early pregnancy may, therefore, be implicated in metabolic transformations concerned with the provision of maternal nutriments for the pre-implantation conceptus. On the other hand, the rise in endometrial acid phosphatase activity during the later stages of pregnancy may, as Boshier (1969) suggested, be important to processes leading to the modification of the maternal epithelium during implantation of the conceptus. Numerous studies have associated acid phosphatase activity with lysosomal activity and have implicated the enzyme in secretory and autolytic or regressive processes (De Duve 1959; Lobel, Rosenbaum, and Deane 1961; Gahan 1967). Acid phosphatase may also be involved in the hydrolysis of phosphate esters during the pre-implantation stages of pregnancy in the ewe to increase the availability of energy substrates for the developing blastocyst.

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