CHANGES IN ACID AND ALKALINE PHOSPHATASE ACTIVITIES IN THE UTERUS AND OVIDUCT OF THE RABBIT DOE DURING EARLY PREGNANCY

By R. N. MURDOCH*

[*Manuscript received November* 5, 1971]

Abstract

Acid and alkaline phosphatase activities in the uterine endometrium of the rabbit doe were studied in relation to the implanting conceptus. The behaviour of these enzymes in the ampulla and isthmus of the oviduct during early pseudopregnancy and pregnancy was also examined.

Acid and alkaline phosphatase activities increased significantly in the implantation site during early pregnancy and on day 9 reached levels almost twice as great as those in the interimplantation endometrium. Activity fell rapidly once implantation was established and only low levels were recorded in the implantation site on day 11 of pregnancy. A slight increase in acid phosphatase activity and decrease in alkaline phosphatase activity occurred in the interimplantation endometrium between days 6 and 11 of pregnancy but the changes in this tissue were not as impressive as those occurring at the implantation site. The enzymes extracted from the interimplantation and implantation sites on day 9 of pregnancy displayed similar biochemical properties suggesting that the phosphatases at both uterine sites are the same protein. Experiments with metal-complexing agents and divalent cations suggest that the endometrial alkaline phosphatase is a Mg^{2+}-containing metalloenzyme.

Small changes in phosphatase activity of dubious physiological importance occurred in the oviduct within several days of injecting human chorionic gonadotrophin (HCG). The presence of the early embryo in the oviduct did not exert any apparent effect on the phosphatases in this tissue. Phosphatase activities and total tissue protein content, however, were significantly greater in the ampulla than in the isthmus of the oviduct and in contrast to the situation found in the uterus, acid phosphatase activity exceeded alkaline phosphatase activity in both sections of the oviduct at all stages studied.

I. INTRODUCTION

Uterine endometrial alkaline phosphatase activity in the mouse and rat rises abruptly following implantation of the conceptus (Christie 1966; Finn and McLaren 1967; Hall 1968; Manning, Steinetz, and Giannina 1969). The activity is localized at the implantation sites and appears to be confined mainly in the decidual cells since the phenomenon may be duplicated by inducing deciduoma formation during pseudopregnancy (Finn and Hinchliffe 1964; Manning, Carter, and Butler 1969; Manning, Calenti, and Carter 1970). A rise in endometrial acid phosphatase also occurs in the rat during early pregnancy (see Manning, Steinetz, and Giannina 1969). The enzyme is probably located within larger decidual cells (Lobel, Tic, and Shelesnyak 1965) and its activity increases in forming deciduomata and decreases during regression (Wood and Barley 1970).

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

Although acid and alkaline phosphatase activities in the endometrium of the rabbit doe have been reported to change during pseudopregnancy and early pregnancy (Hafez and White 1967; Murdoch 1970), knowledge of their behaviour at the implantation site during implantation of the conceptus is limited. The present investigation was undertaken to examine acid and alkaline phosphatase activities in the implantation and interimplantation sites of the rabbit uterus during early pregnancy and to compare the properties of the phosphatases from the two sites.

In the oviduct, fluid produced by the secretory cells may play an important role in the process of fertilization and early embryo development. Since acid and alkaline phosphatases have been associated with cells having a secretory function (see Dempsey and Wislocki 1945; Moog 1946; Bradfield 1950), and since Gupta, Karkun, and Kar (1970) have recently postulated that acid phosphatase in the oviduct of the rabbit may be involved in the process of removal of the denuded cumulus and corona cell debris of ova before they migrate to the uterus, further observations on the behaviour of acid and alkaline phosphatases in the rabbit oviduct during early pseudopregnancy and pregnancy are reported in this paper.

II. Materials and Methods

(*a*) *Experimental Animals*

Virgin albino rabbits, aged 6–8 months, were obtained from the University animal house, Castle Hill. 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 on day 0.

(*b*) *Preparation of Tissue*

After slaughter the ovaries of all animals were examined for evidence of ovulation. The uterus was quickly dissected at the cervix and removed from the animal with the oviducts attached. The excised reproductive tract was dissected free of fatty and connective tissue and the oviducts were then separated from the uterine cornua by section at the uterotubal junction. All subsequent processing was performed at 4°C.

(i) *Uterus.*—The uterine cornua from does on day 6 of pregnancy were carefully opened down the mesometrial side to expose the preimplantation blastocysts. The uterine tissue surrounding the area of each blastocyst was separated from the remaining tissue and the endometrium from each site was recovered for study as described below. Blastocysts at more advanced stages of pregnancy were removed by dissection, taking care to eliminate all trophoblastic tissue. The implantation sites were separated from the interimplantation areas of the uterus and after rinsing with 0·154M NaCl each tissue section 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 scalpel blade and forceps. Samples were homogenized in 10 parts of distilled water and then centrifuged at 1000 *g* for 10 min. The supernatant was retained for phosphatase and protein analyses.

(ii) *Oviducts.*—All oviducts were rinsed with 0·154M NaCl heated to 37°C immediately following their removal from rabbits and the flushings were examined microscopically for evidence of ova fertilization. The ampulla and isthmus of each oviduct were then separated by section and dried between pieces of filter paper. The tissues were minced thoroughly with fine scissors and forceps, weighed, and homogenized in 10 parts of distilled water. Samples were centrifuged at 1000 *g* for 10 min and the supernatants retained for enzyme and protein analyses.

(c) *Enzyme Extractions*

In experiments where some biochemical properties of the acid and alkaline phosphatases in the implantation and interimplantation sites of the uterus on day 9 of pregnancy were examined, enzyme extractions, using pools of tissue from five rabbits, were continued as described in the following methods.

(i) *Acid Phosphatase.*—The supernatants of homogenates of tissue from each uterine site were treated with 0·1 vol. 2% (w/v) protamine sulphate and allowed to stand at 4°C for 10 min (see Kuo and Blumenthal 1961). The precipitate which formed was removed by centrifugation and the supernatant, which contained the acid phosphatase activity, was treated slowly with 0·5 vol. of absolute ethanol (−20°C) and allowed to stand at 4°C for 2 hr. The precipitate was then removed by centrifugation and more ethanol was added to the supernatant to bring the alcohol concentration to 66% (v/v). The mixture was allowed to stand for 2 hr without further mixing and, after removing the clear supernatant by siphoning and centrifuging, the precipitate was washed twice by dispersing and centrifuging in 75% (v/v) ethanol, dissolved in distilled water, and dialysed against 10 vol. of 0·02M acetate buffer (pH 5·6). Aliquots of this preparation were used in subsequent analyses for acid phosphatase.

(ii) *Alkaline Phosphatase.*—Alkaline phosphatase was extracted from endometrial homogenates by a modification of the method of Morton (1954). The homogenate was adjusted to pH 6·5 with acetic acid and extraction was then carried out in the presence of n-butanol (1·5 ml/g tissue) for 1 hr at room temperature with agitation. The resulting suspension was incubated at 37°C for 1 hr, kept overnight at 4°C, and centrifuged at 1000 *g* for 1 hr. The aqueous phase containing the enzyme was removed, dialysed against distilled water for 24 hr at 4°C, and then centrifuged again to remove any remaining particulate material. Aliquots of this preparation were used in subsequent analyses for alkaline phosphatase.

(d) *Enzyme and Protein Analyses*

Enzyme activities were assayed by the spectrophotometric measurement of hydrolysis of *p*-nitrophenyl phosphate (Bessey, Lowry, and Brock 1946; Andersch and Szcypinski 1947). Unless otherwise stated, the following conditions of assay were employed.

(i) *Acid Phosphatase.*—An aliquot (0·1 ml) of the enzyme preparation, appropriately diluted in water, was incubated in 1·0 ml of a mixture containing 25 mM citrate–citric acid buffer (pH 4·8) and 6·5 mM *p*-nitrophenyl phosphate. After incubation at 37°C for 30 min, 4 ml of 0·1N NaOH was added, and the optical density at 405 nm was measured against a reagent blank without enzyme.

(ii) *Alkaline Phosphatase.*—An aliquot (0·1 ml) of the enzyme preparation, appropriately diluted in water, was incubated in 1·0 ml of a mixture containing 50 mM glycine buffer (pH 10·5), 0·5 mM $MgCl_2.6H_2O$, and 6·5 mM substrate. After incubation at 37°C for 30 min, 10 ml of 0·02N NaOH was added, and the optical density at 405 nm was measured against a reagent blank without enzyme.

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.

Michaelis constants (K_m) were obtained from linear double reciprocal plots (Lineweaver and Burk 1934) using at least six levels of substrate, ranging from 0·5 to 16 μmoles/ml.

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

All analytical determinations were performed in duplicate.

(e) *Statistical Analyses*

Where necessary, 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.

III. Results

(a) *Phosphatases in the Interimplantation and Implantation Sites*

Figure 1 shows the activity of acid and alkaline phosphatase per milligram of tissue protein in the interimplantation and implantation sites of the rabbit uterus between days 6 and 11 of pregnancy.

Acid phosphatase activity in the interimplantation endometrium significantly increased ($P < 0{\cdot}01$) with increasing gestational age until a maximum was reached on day 10. Evidence of a slight decrease in activity was apparent on day 11. The rate of increase in the activity of acid phosphatase in the implantation site, however, was significantly greater ($P < 0{\cdot}01$) than in the interimplantation area between days 7 and 9 of pregnancy and on day 9 activity in the former tissue was almost double that in the latter tissue. Following day 9 of pregnancy activity in the implantation site fell rapidly until on day 11 it was less than that in the interimplantation area.

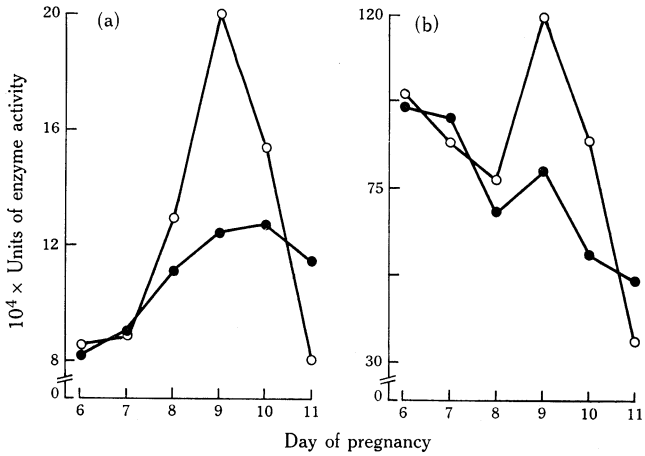

Fig. 1.—Activity of acid phosphatase (*a*) and alkaline phosphatase (*b*) per milligram of tissue protein in the uterine endometrial implantation site and interimplantation area of pregnant rabbits. ○ Implantation site. ● Interimplantation area. Values represent the means of four rabbits.

The pattern of alkaline phosphatase activity in the uterus of the doe during early pregnancy was different in many respects from that of acid phosphatase. Alkaline phosphatase activity in the interimplantation endometrium decreased significantly ($P < 0{\cdot}01$) between days 6 and 11 of pregnancy and tended to fluctuate during this period more noticeably than acid phosphatase activity. Activity in the implantation site also decreased similarly between days 6 and 8 but on day 9 increased to levels greater ($P < 0{\cdot}01$) than those recorded in the interimplantation area. Following day 9 of pregnancy alkaline phosphatase activity, like that of acid phosphatase, fell rapidly until only relatively low levels were recorded on day 11. The activity of alkaline phosphatase was greater than that of acid phosphatase in both uterine sites and at all stages of pregnancy studied.

(b) Properties of the Uterine Enzymes

(i) *Effect of pH.*—Figure 2 shows the effect of pH on the activity of acid and alkaline phosphatases extracted from the interimplantation and implantation sites of the rabbit uterus on day 9 of pregnancy. Incubation of acid phosphatase in citrate–citric acid buffers of pH ranging from 4·2 to 6·2 isolated optimum activity for the enzyme from both sources at pH 5·4. Differences in the pH of the reaction mixture, however, did not produce profound changes in the activity of this enzyme. The activity of acid phosphatase extracted from the implantation site was greater than that from the interimplantation endometrium at all pH levels studied.

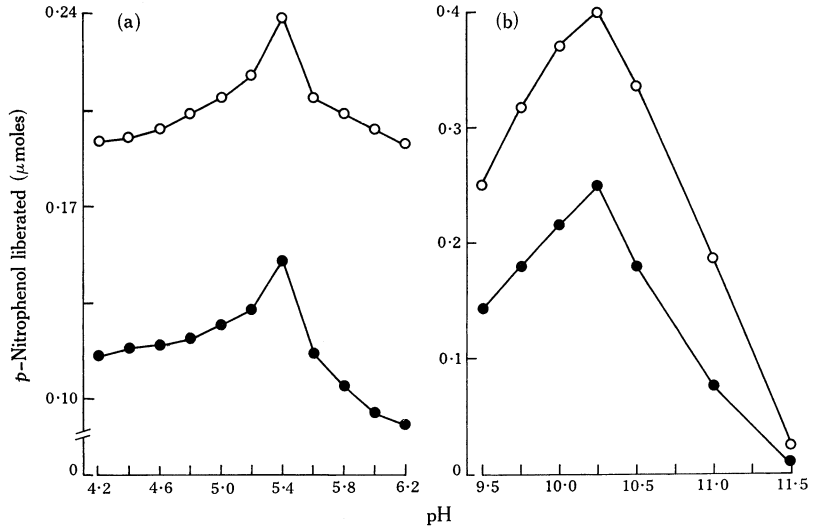

Fig. 2.—Effect of pH on the activity of acid phosphatase (*a*) and alkaline phosphatase (*b*) following extraction from the implantation and interimplantation sites of the rabbit uterus on day 9 of pregnancy. ○ Implantation site. ● Interimplantation site.

Alkaline phosphatase from both uterine sources displayed optimal activity in a glycine buffer mixture at pH 10·25. Small changes in the pH of the reaction mixture in this case had very marked effects on the activity of the enzyme and at pH 11·5 almost no activity could be detected. The activity of alkaline phosphatase extracted from the implantation site was greater than that from the interimplantation site over the range of pH studied.

(ii) K_m *Values.*—The enzyme extracts from both uterine sources were adjusted to yield similar activities at their respective optimal pH and K_m values were estimated for each enzyme. A mean K_m of 0·41 mM was found for acid phosphatase with *p*-nitrophenyl phosphate as substrate and differences in the uterine source of the enzyme had no effect on the estimate. Similarly, no differences between uterine sites could be detected with respect to the determination of K_m for alkaline phosphatase and a mean value for this enzyme was found to be 0·59 mM *p*-nitrophenyl phosphate.

(iii) *Effects of Various Compounds.*—The effects of several divalent metal ions, L-phenylalanine, sodium cyanide, *o*-phenanthroline, EDTA, and L-cysteine on the activity of acid and alkaline phosphatases from the interimplantation and implantation sites of the rabbit uterus on day 9 of pregnancy are shown in Table 1. None of these compounds had a profound effect on acid phosphatase from either uterine source.

TABLE 1

EFFECT OF DIVALENT METAL IONS AND OTHER COMPOUNDS ON THE ACTIVITY OF ACID AND ALKALINE PHOSPHATASES EXTRACTED FROM THE IMPLANTATION SITE (I.S.) AND INTERIMPLANTATION SITE (I.I.S.) OF THE RABBIT UTERUS ON DAY 9 OF PREGNANCY

Values are listed as rates relative to the hydrolysis of substrate in the absence of any added compound

Compound (10^{-3} M)	Acid Phosphatase		Alkaline Phosphatase	
	I.I.S.	I.S.	I.I.S.	I.S.
None	100	100	100	100
$MgCl_2$	109	106	166	164
$ZnCl_2$	102	102	120	112
$CaCl_2$	—	—	105	99
$NiCl_2$	—	—	77	77
$CoCl_2$	—	—	74	66
$BeCl_2$	100	102	38	38
$CdCl_2$	97	97	23	20
L-Phenylalanine	—	—	93	98
NaCN	102	104	69	69
o-Phenanthroline	—	—	39	40
EDTA	102	104	11	11
L-Cysteine	102	105	1	1

Alkaline phosphatase activity, however, was greatly enhanced when Mg^{2+} ions were included in the reaction mixture and also slightly increased in the presence of Zn^{2+} ions. Activity was unaffected in the presence of Ca^{2+} ions and L-phenylalanine and depressed in the presence of Ni^{2+}, Co^{2+}, Be^{2+}, and Cd^{2+} ions and NaCN, *o*-phenanthroline, and EDTA. L-Cysteine almost completely inhibited the enzymic reaction. Alkaline phosphatase extracted from the implantation site responded to the compounds in a similar manner to that extracted from the interimplantation area.

(iv) *Effect of Cations on EDTA Inhibition of Alkaline Phosphatase.*—Incubation of alkaline phosphatase from the interimplantation and implantation sites of the rabbit uterus with 10^{-5}M EDTA in the absence of substrate for 15 min at room temperature caused a marked inhibition of enzyme activity (Table 2). The addition of Ni^{2+} and Co^{2+} ions at 10^{-4}M concentration failed to influence enzyme activity while Zn^{2+} ions alleviated the inhibitory effect of EDTA to some extent. However, only Mg^{2+} ions were effective in restoring alkaline phosphatase activity to the original level. When no EDTA was present, the addition of Mg^{2+} ions greatly increased enzyme activity above that incubated without added cations. Enzyme extracted

from the implantation site responded to the cations and EDTA in a similar manner to that extracted from the interimplantation area.

TABLE 2

EFFECT OF DIVALENT METAL IONS ON THE INHIBITION BY EDTA OF ALKALINE PHOSPHATASE EXTRACTED FROM THE IMPLANTATION SITE (I.S.) AND INTERIMPLANTATION SITE (I.I.S.) OF THE RABBIT UTERUS ON DAY 9 OF PREGNANCY

Conditions of experiment	Addition	Relative rates	
		I.I.S.	I.S.
Each incubation mixture (1·0 ml), containing 50 mM glycine buffer (pH 10·25), 10^{-5}M EDTA, and enzyme, was held for 15 min at room temperature. Substrate (6·5 mM final concentration) and various metal chlorides (10^{-4}M final concentration) were then added and enzyme activity was assessed as described in the text after incubating the mixtures for a further 30 min at room temperature. Rates are listed as relative to the hydrolysis of substrate in the absence of any added compound	None	100	100
	EDTA	51	54
	EDTA + Ni^{2+}	52	53
	EDTA + Co^{2+}	54	54
	EDTA + Zn^{2+}	78	77
	EDTA + Mg^{2+}	104	105
	Mg^{2+}	175	177

(c) *Phosphatases and Protein in the Oviduct during Early Pseudopregnancy and Pregnancy*

Small but statistically significant changes in acid and alkaline phosphatase activities were detected in the rabbit oviduct between days 0 and 4 of pseudopregnancy and pregnancy (Table 3). Acid phosphatase activity decreased slightly

TABLE 3

ACTIVITY OF ACID AND ALKALINE PHOSPHATASE AND CONCENTRATION OF PROTEIN IN THE AMPULLA AND ISTHMUS OF THE RABBIT FALLOPIAN TUBE DURING EARLY PSEUDOPREGNANCY AND PREGNANCY

Values represent the means of four rabbits and are expressed as follows: acid and alkaline phosphatase, $10^4 \times$ units/mg protein; protein, mg/g of tissue (wet weight)

Day of pseudopregnancy or pregnancy	Acid phosphatase		Alkaline phosphatase		Protein	
	Ampulla	Isthmus	Ampulla	Isthmus	Ampulla	Isthmus
			Pseudopregnancy			
0	8·48	7·91	2·62	1·90	99	75
1	9·05	7·52	3·95	2·96	97	75
2	8·76	7·47	2·54	1·64	95	81
3	7·39	6·02	2·88	2·27	105	77
4	7·54	6·77	3·03	2·45	100	75
			Pregnancy			
0	8·55	8·54	2·72	1·78	95	73
1	9·03	7·52	3·82	3·74	93	73
2	8·57	7·52	2·71	1·76	99	84
3	7·87	6·35	2·89	2·39	99	75
4	7·21	6·61	3·32	2·50	99	83

($P < 0·01$) in both the ampulla and isthmus during this period while alkaline phosphatase activity increased 1 day after injection of HCG or insemination, then

fell and increased again on day 5 ($P < 0{\cdot}05$). There were no significant changes in the protein concentration of the oviduct during this period and the presence of the early embryo had no apparent effect on any of the parameters measured. Phosphatase activities (acid, $P < 0{\cdot}01$; alkaline, $P < 0{\cdot}05$) and total tissue protein content ($P < 0{\cdot}01$) were significantly greater in the ampulla than in the isthmus and, unlike the situation found in the uterus, acid phosphatase activity exceeded alkaline phosphatase activity in both sections of the oviduct at all stages studied.

IV. Discussion

The results demonstrate significant rises in acid and alkaline phosphatase activities in the implantation site of the rabbit uterus during early pregnancy which are coincident with the time of implantation of the conceptus in this species. The rise in phosphatase activity at the implantation site does not appear to depend solely on circulating hormones produced during pregnancy since the enzymes in the interimplantation endometrium failed to display similar patterns of activity. However, as suggested by Manning, Steinetz, and Giannina (1969) for the enzymes in the rat, a progesterone-conditioned endometrium may be necessary for the occurrence of this phenomenon. The slight rise in acid phosphatase activity and fall in alkaline phosphatase activity in the interimplantation endometrium of the rabbit uterus during early pregnancy are consistent with the results of a previous report (Murdoch 1970) and indicate that these enzymes are possibly regulated by factors different from those regulating the phosphatases in the implantation site. Although the processes responsible for the stimulation of acid and alkaline phosphatases in the implantation site appear to last for only a relatively short period, elucidation of their nature and mechanism of action could yield important information about the implantation process. The rapid fall in phosphatase activity in the implantation site between days 9 and 11 of pregnancy is difficult to understand and does not appear to be associated with any regressive changes in the decidual cells, since growth of induced deciduomata and the normal maternal placenta continue until about day 16 in the rabbit (Huggett and Hammond 1964). Such enzyme changes in the implantation site are probably an inherent part of the decidual reaction, as similar changes may be duplicated in the mouse and rat by inducing deciduoma formation during pseudopregnancy (see Manning, Steinetz, and Giannina 1969).

Endometrial acid phosphatase activity has also been observed to rise during early pregnancy in the ewe and may be important to processes leading to the modification of the maternal epithelium during implantation (Hafez and White 1968; Boshier 1969; Murdoch 1970). A similar role may possibly be assigned to the increased acid phosphatase activity in the implantation site of the rabbit. The increased alkaline phosphatase activity may play a role in the transfer of solutes (see Dempsey and Wislocki 1945; Moog 1946; Bradfield 1950) and increase the availability of nutriments for the implanting embryo.

Manning *et al.* (1969) claim that the decidual alkaline phosphatase in the rat is either released or activated from a latent precursor protein and that *de novo* nucleic acid or protein synthesis is not necessary for the formation of the active enzyme molecule. It is not known whether this phenomenon also applies in the rabbit but since

the enzymes extracted from the interimplantation and implantation sites on day 9 of pregnancy in this species display similar biochemical properties it is possible that the phosphatases at both of these uterine areas are the same protein molecule. The inhibition of alkaline phosphatase activity by EDTA, *o*-phenanthroline, NaCN, and L-cysteine and its stimulation by $MgCl_2$ suggests that this phosphatase is a metalloenzyme with Mg^{2+} as a functional component. Similar properties for the alkaline phosphatase from sheep endometrium have also recently been reported (Murdoch 1971). The absence of any effects of divalent cations and metal-chelating agents on acid phosphatase activity supports the general belief that these enzymes do not depend upon divalent metal ions for functional activity.

In the oviduct, acid and alkaline phosphatase activities do not appear to be influenced by the presence of the early embryo. The slight changes which were found to occur within several days of injecting HCG are of dubious physiological importance and are not consistent with the findings of Gupta, Karkun, and Kar (1970), who reported rather substantial increases in acid phosphatase activity in the ampulla, ampullary–isthmus junction, and isthmus within 24–70 hr post coitus. Since these investigators compared all their observations with enzyme activities measured at 14 hr post coitus rather than with those measured at oestrus, as was the case in the present study, the discrepancy between the two sets of results may be explained if a fall in acid phosphatase activity occurs in the oviduct between 0 and 14 hr after mating or injection of HCG in response to rapidly increasing levels of 20α-hydroxypregn-4-en-3-one (Hilliard, Hayward, and Sawyer 1964). The present results also fail to support the proposal by Gupta, Karkun, and Kar (1970) that acid phosphatase in the oviduct may be involved in the denudation of ova and removal of cumulus and corona cell debris. The activity of the enzyme decreases rather than increases during the first 3 or 4 days of pregnancy and ova are transported through the ampulla to the isthmus of the tube, where phosphatase activities are comparatively lower, within only a few minutes of their entry into the oviduct (Harper 1965). A period of 3–4 days is then required for transport to the uterus (Pincus 1930; Chang and Harper 1966). The low concentration of alkaline phosphatase and its small changes in activity in the oviduct during early pseudopregnancy and pregnancy further suggest that this enzyme may not be involved in oviducal secretory processes to any great extent.

V. Acknowledgments

The author is indebted to Professor C. W. Emmens and Associate Professor R. G. Wales for interest and criticism. The work was aided by a grant from the Ford Foundation.

VI. References

Andersch, M. A., and Szcypinski, A. J. (1947).—*Am. J. clin. Path.* **17**, 571.
Bessey, O. A., Lowry, O. H., and Brock, M. J. (1946).—*J. biol. Chem.* **164**, 321.
Boshier, D. P. (1969).—*J. Reprod. Fert.* **19**, 51.
Bradfield, J. R. G. (1950).—*Biol. Rev.* **25**, 113.
Chang, M. C., and Harper, M. J. K. (1966).—*Endocrinology* **78**, 860.
Christie, G. A. (1966).—*J. Reprod. Fert.* **12**, 279.
Dempsey, E. W., and Wislocki, G. B. (1945).—*Am. J. Anat.* **76**, 277.
Finn, C. A., and Hinchliffe, J. R. (1964).—*J. Reprod. Fert.* **8**, 331.

FINN, C. A., and MCLAREN, A. (1967).—*J. Reprod. Fert.* **13**, 259.
GUPTA, D. N., KARKUN, J., and KAR, A. B. (1970).—*Am. J. Obstet. Gynec.* **106**, 833.
HAFEZ, E. S. E., and WHITE, I. G. (1967).—*Anat. Rec.* **159**, 273.
HAFEZ, E. S. E., and WHITE, I. G. (1968).—*J. Reprod. Fert.* **16**, 59.
HALL, K. (1968).—*J. Endocr.* **41**, 53.
HARPER, M. J. K. (1965).—*Endocrinology* **77**, 114.
HILLIARD, J., HAYWARD, J. N., and SAWYER, C. H. (1964).—*Endocrinology* **75**, 957.
HUGGETT, A. ST. G., and HAMMOND, J. (1964).—In "Marshall's Physiology of Reproduction". 3rd Ed. (Ed. A. S. Parkes.) Vol. 2. p. 312. (Longmans, Green & Co.: London.)
KUO, M-H., and BLUMENTHAL, H. J. (1961).—*Biochim. biophys. Acta* **52**, 13.
LINEWEAVER, H., and BURK, D. (1934).—*J. Am. chem. Soc.* **56**, 658.
LOBEL, B. L., TIC, L., and SHELESNYAK, M. C. (1965).—*Acta endocr., Copenh.* **50**, 517.
MANNING, J. P., CALENTI, L., and CARTER, S. G. (1970).—*Proc. Soc. exp. Biol. Med.* **133**, 1031.
MANNING, J. P., CARTER, S. G., and BUTLER, M. C. (1969).—*Acta endocr., Copenh.* **61**, 164.
MANNING, J. P., CARTER, S. G., CALENTI, L., and ANDERSON, J. I. (1969).—*Enzymologia* **36**, 249.
MANNING, J. P., STEINETZ, B. G., and GIANNINA, T. (1969).—*Ann. N.Y. Acad. Sci.* **166**, 482.
MOOG, F. (1946).—*Biol. Rev.* **21**, 41.
MORTON, R. K. (1954).—*Biochem. J.* **57**, 594.
MURDOCH, R. N. (1970).—*Aust. J. biol. Sci.* **23**, 1089.
MURDOCH, R. N. (1971).—*Aust. J. biol. Sci.* **24**, 331.
PINCUS, G. (1930).—*Proc. R. Soc.* B **107**, 132.
WALES, R. G., SCOTT, T. W., and WHITE, I. G. (1961).—*Aust. J. exp. Biol. med. Sci.* **39**, 455.
WHITE, I. G. (1955).—*Aust. J. exp. Biol. med. Sci.* **33**, 367.
WOOD, J. C., and BARLEY, V. L. (1970).—*J. Reprod. Fert.* **23**, 469.