# SOME BIOCHEMICAL AND KINETIC PROPERTIES OF SHEEP UTERINE ENDOMETRIAL ALKALINE PHOSPHATASE

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*Abstract*

Sheep uterine endometrial alkaline phosphatase in a purified state displayed optimal activity at pH 10·5. At pH 9·0 activity increased with decreasing substrate concentration.

The enzyme catalysed the hydrolysis of a variety of phosphomonoesters and exhibited pyrophosphatase properties when incubated with ADP and ATP. Metal-complexing agents, such as EDTA, NaCN, *o*-phenanthroline, and L-cysteine inhibited the phosphatase while $Mg^{2+}$ stimulated its activity. Recovery of activity was both rapid and complete when $MgCl_2$ was added to the EDTA-inhibited enzyme suggesting that this alkaline phosphatase is a metalloenzyme with $Mg^{2+}$ as a functional component. No stimulating effect of EDTA was found at any concentration ($10^{-5}$–$10^{-1}$M) used and substrate partially protected the enzyme against its inhibiting properties. $Mg^{2+}$ in low concentrations (1 mM) increased the pyrophosphatase activity of the enzyme when incubated with ADP but decreased it at higher concentrations (2–8 mM). $Mg^{2+}$ was more effective, however, in increasing the orthophosphatase activity of the enzyme and, in this case, did not inhibit activity at higher concentrations. High concentrations (0·1 and 1·0M) of KCl, NaCl, and $NH_4Cl$ inhibited rather than stimulated activity, with the latter salt being the most effective. The enzyme was not stable to heating at 56°C and after 15 min at 65°C all activity was lost. The enzyme preparation was chromatographically and electrophoretically homogeneous and treatment with neuraminidase did not affect these properties. The enzyme exhibited phosphotransferase activity but was unable to catalyse the synthesis of glucose 6-phosphate by direct phosphorylation under the conditions employed.

## I. INTRODUCTION

Previous studies have shown that the activity of alkaline phosphatase (orthophosphoric monoester phosphohydrolase, E.C. 3.1.3.1) in the uterine endometrium of the ewe is influenced by progesterone and increases during the luteal phase of the oestrous cycle and early pregnancy (Hafez and White 1968; Murdoch and White 1968*a*, 1968*b*; Murdoch 1970). Although alkaline phosphatase has been implicated in a number of important cellular processes such as transport of materials across the membranes of cells having a secretory function (Dempsey and Wislocki 1945; Moog 1946; Bradfield 1950), phospholipid synthesis (Malone 1960), RNA synthesis (Gavasto and Pileri 1958), and carbohydrate metabolism (Moog and Wenger 1952; Boshier 1969), its precise role in endometrial metabolic trans-

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formations concerned with the provision of maternal nutriments for the pre-implantation conceptus in particular and in reproductive physiology in general is not known. In view of this, some biochemical and kinetic properties of purified sheep endometrial alkaline phosphatase are presented in this paper.

## II. Materials and Methods

### (a) *Materials*

The following materials were obtained from Sigma Chemical Company, U.S.A.: *p*-nitrophenylphosphate, Tris, 6-phosphogluconate, fructose 1,6-diphosphate, fructose 6-phosphate, glucose 1-phosphate, glucose 6-phosphate, α-glycerophosphate, β-glycerophosphate, β-naphthylphosphate, ribose 5-phosphate, *O*-phospho-L-serine, phosphoenol pyruvate, 3-phosphoglyceric acid, glyceraldehyde 3-phosphate, inorganic pyrophosphate (PPi), AMP, ADP, ATP, L-cysteine, *o*-phenanthroline, and glucose-6-phosphate dehydrogenase (D-glucose-6-phosphate : NADP oxidoreductase, E.C. 1.1.1.49).

Other materials were obtained from the following sources: EDTA and neuraminidase (*N*-acetylneuraminate glycohydrolase, E.C. 3.2.1.18) (purified from *Vibrio cholerae*), British Drug Houses Ltd., England; metal chlorides, Ajax Chemicals Ltd., Australia; sodium cyanide, E. Merck Pty. Ltd., Germany; DEAE-cellulose (medium grade with capacity of 1·0 m-equiv/g), Whatman, England; and Sephadex G-200 beads (particle size, 40–120 μm), Pharmacia, Sweden.

All chemicals were of analytical reagent grade.

### (b) *Preparation of Endometrial Tissue*

Reproductive tracts, obtained from non-pregnant Merino ewes immediately after slaughter at a local abattoir, were placed in polythene bags and packed in ice during transport to the laboratory. The uteri, which arrived at the laboratory within 1 hr following slaughter, were dissected free of fatty and connective tissue and of the attached oviducts and cervix. Each uterine horn was washed through with cold 0·154M NaCl and then placed on a piece of filter paper and opened down the mesometrial side. The exposed endometrium was blotted with filter paper to remove any trace of the flushing fluid or the endometrial secretions. Endometrial tissue from the intercotyledonary areas of each uterine horn was carefully removed by dissection and pooled. No attempt was made to isolate tissue relative to the stage of the oestrous cycle since previous studies indicated no cyclic change in the electrophoretic activity pattern of endometrial alkaline phosphatase (Murdoch 1970).

### (c) *Enzyme Extraction and Purification*

Alkaline phosphatase was extracted from the endometrial tissue by a modification of the method of Morton (1954). The tissue (105 g) was homogenized in 0·25M sucrose at 4°C. 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 2 hr. The aqueous phase containing the enzyme was removed and retained, and the sediment was again homogenized and re-extracted with n-butanol as before. The aqueous phase from this was pooled with that from the initial extraction and again centrifuged at 1000 *g* for 30 min at 4°C to remove suspended material. The clear solution was brought to 0°C and chilled acetone was slowly added with continuous stirring to 30% (Sussman, Small, and Cotlove 1968). After standing for 1 hr at 0°C, the solution was centrifuged at 1000 *g* for 20 min and the precipitate discarded. The supernatant was treated with more chilled acetone to 50% and, after the precipitate had settled, it was again centrifuged. The resultant precipitate was washed twice with chilled acetone, dissolved in 0·01M Tris–HCl buffer (pH 7·4), and dialysed against the buffer at 4°C to remove traces of butanol and acetone.

The enzyme was further purified following acetone precipitation by chromatography and gel filtration conducted at 4°C. DEAE-cellulose was suspended in water, washed with 1N NaOH to remove coloured material, and then washed free of alkali with water and equilibrated with

0·01M Tris–HCl buffer (pH 7·4). The enzyme was purified on a column (3·5 by 35 cm) of the equilibrated DEAE-cellulose by means of a linear gradient of 0·0 to 0·14M NaCl in the Tris–HCl buffer with 500 ml in each reservoir. The column effluent was collected in fractions (20 ml/fraction) and assayed for enzyme activity and protein (absorbance at 280 nm). Enzyme activity was eluted as a single symmetrical peak and tubes of highest specific activity were pooled, dialysed against distilled water, and concentrated by lyophilization. The enzyme was then purified further by gel filtration on a column (3·5 by 35 cm) of Sephadex G-200 equilibrated with 0·01M Tris–HCl buffer (pH 7·4) containing 0·14M NaCl (Sussman, Small, and Cotlove 1968). Enzyme activity and absorbance at 280 nm were again measured in each fraction (20 ml) and tubes of highest specific activity were pooled, dialysed against distilled water, and concentrated by lyophilization. The enzyme was eluted along with a well-defined peak of protein in advance of a minor protein peak.

Enzyme activity and protein concentration were assessed in the enzyme extract at the end of each purification step. Enzyme activity was assayed at 37°C with *p*-nitrophenylphosphate as substrate (Bessey, Lowry, and Brock 1946; Andersch and Szcypinski 1947) and protein was measured by the method of Lowry *et al.* (1951).

### (*d*) *Starch-gel Electrophoresis*

Aliquots of the purified enzyme were applied to small pieces of Whatman No. 3 paper and subjected to horizontal starch-gel electrophoresis as previously described (Murdoch 1970). Enzymatic activity was located in a longitudinal gel slice by using a solution containing 0·05% (w/v) β-napthylphosphate, 0·005M magnesium chloride, 0·05% (w/v) fast blue RR salt, and 0·06M sodium borate–boric acid buffer (pH 9·7) (Markert and Møller 1959).

### (*e*) *Sucrose-gradient Centrifugation*

Sucrose-gradient centrifugation of purified endometrial alkaline phosphatase was conducted as described by Harkness (1968*a*). A 0·25-ml sample of enzyme (about 0·4 mg protein) was layered on to a linear sucrose gradient (4·6 ml of 5–20% sucrose in 0·002M $MgCl_2$, 0·05M Tris–HCl buffer, pH 7·5) and centrifuged at 39,000 r.p.m. for 16 hr at 1°C in a Beckman L265B ultracentrifuge using a SW50C rotor. After the run, a hole was pierced in the bottom of the centrifuge tube and 10-drop fractions collected. Aliquots of each fraction were then assayed for enzyme activity and protein (absorbance at 212 nm).

### (*f*) *Enzyme Assays*

In most cases, enzyme activity was assayed by the spectrophotometric measurement of hydrolysis of *p*-nitrophenylphosphate. Unless otherwise stated, 0·3 μg of enzyme, appropriately diluted in water, was incubated in 1·0 ml of a mixture containing 50 mM glycine buffer (pH 10·5) and 6·5 mM *p*-nitrophenylphosphate. After incubation at 37°C for 15 min, 10 ml of 0·02N NaOH was added, and the optical density at 405 nm was measured against a reagent blank without enzyme.

The amount of orthophosphate (Pi) liberated from substrates in the presence of enzyme was measured by the method of Fiske and Subba Row (1925). Unless otherwise stated, 2 μmoles of substrate were present in 0·2 ml of an incubation mixture containing 50 mM glycine buffer (pH 10·5), 0·5 mM $MgCl_2.6H_2O$, and 1·0 μg of enzyme protein. After incubation at 37°C for 15 min, 0·8 ml of 10% (w/v) trichloroacetic acid was added and the total 1·0 ml assayed for orthophosphate. This method was also used to obtain Michaelis constants ($K_m$) for a number of compounds hydrolysed by endometrial alkaline phosphatase. At least six levels of substrate (ranging from 1 to 32 μmoles/ml) were used for each compound and Michaelis constants were obtained from linear double reciprocal plots (Lineweaver and Burk 1934).

All solutions were prepared in de-ionized water, twice distilled in glass, and each enzyme assay was done in duplicate.

In experiments conducted to examine the catalysis of phosphorylation and transphosphorylation by the alkaline phosphatase, glucose 6-phosphate formed in the incubation mixture was measured spectrophotometrically with glucose-6-phosphate dehydrogenase (see Horecker and Wood 1957).

## III. Results

### (a) *Purification Details and Sucrose-gradient Centrifugation*

The details of the results of the purification of the enzyme (which achieved 131-fold purity) are given in Table 1. When the purified enzyme was sedimented in a 5–20% sucrose gradient, a single symmetrical peak was obtained with enzyme and protein coinciding.

Table 1

PURIFICATION OF SHEEP ENDOMETRIAL ALKALINE PHOSPHATASE

Specific activities are expressed as μmoles of *p*-nitrophenol released/min/mg of protein

| Fractionation Step | Specific Activity | Yield (%) | Purification |
|---|---|---|---|
| Homogenate | 0·35 | 100 | 1 |
| Butanol supernatant | 1·34 | 71 | 4 |
| 30–50% acetone fraction | 1·78 | 66 | 5 |
| DEAE-cellulose chromatography | 23·75 | 57 | 68 |
| Sephadex G-200 gel filtration | 45·85 | 33 | 131 |

### (b) *pH and Substrate Interaction*

Figure 1 shows the results of an experiment conducted to examine the effects of pH on enzyme activity when incubated in the presence of various substrate concentrations.

Optimal activity was obtained at pH 10·5 for all substrate concentrations. At the lower pH's studied (9·0 and 9·5), activity increased with decreasing substrate concentration but the reverse was true at pH 11·5. At pH 10·5, activity decreased when the substrate concentration reached 13·0 mM.

### (c) *Relative Rates of Hydrolysis of Various Substrates and Michaelis Constants*

The relative rates of hydrolysis of 18 substrates by sheep endometrial alkaline phosphatase are presented in Table 2. All of the phosphate esters tested were hydrolysed by the enzyme and *p*-nitrophenylphosphate was attacked at the most rapid rate. In general, the orthophosphomonoesters were hydrolysed at a greater rate than the nucleoside pyrophosphates, ADP and ATP, and the enzyme displayed very little affinity for inorganic pyrophosphate.

Michaelis constants for nine compounds hydrolysed by the enzyme are also listed in Table 2. The constants varied considerably, depending on the substrate used, and ranged from 0·7 mM for *p*-nitrophenylphosphate to 8·9 mM for glucose 6-phosphate.

### (d) *Effects of Various Compounds*

The effects of several divalent metal ions, sodium cyanide, *o*-phenanthroline, EDTA, and L-cysteine on the activity of endometrial alkaline phosphatase are shown

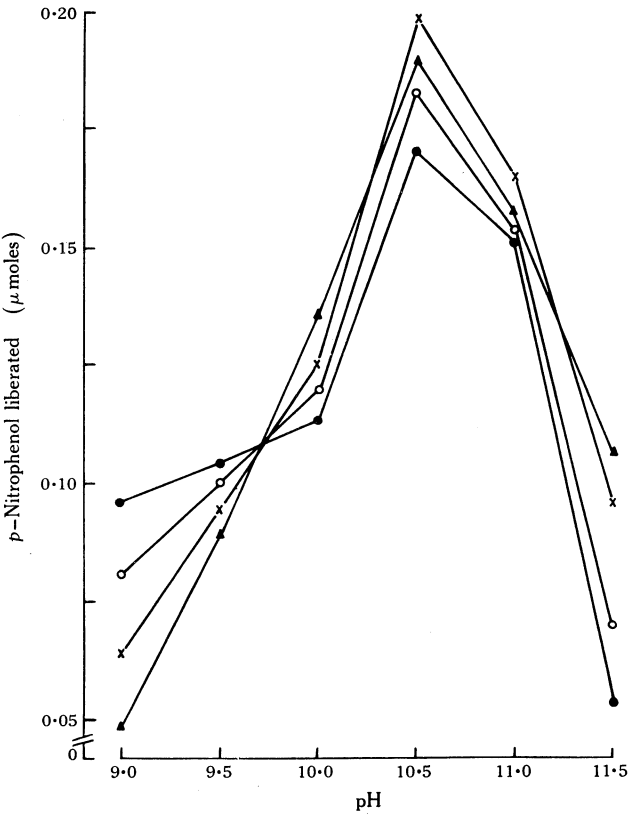


Fig. 1.—Effect of pH and substrate concentration on sheep endometrial alkaline phosphatase activity. Each incubation mixture (1·0 ml) contained 50 mM glycine buffer, 0·5 mM $MgCl_2.6H_2O$, 0·3 μg of enzyme protein, and 1·625 (●), 3·25 (○), 6·5 (×), and 13·0 (▲) mM *p*-nitrophenyl-phosphate. The amount of *p*-nitrophenol liberated was measured as described in the text.

TABLE 2

RELATIVE REACTION RATES AND MICHAELIS CONSTANTS FOR VARIOUS SUBSTRATES WITH SHEEP ENDOMETRIAL ALKALINE PHOSPHATASE

Enzyme activity was assayed by measuring the amount of orthophosphate liberated from the substrate under incubation conditions described in the text. Rates are listed as relative to the hydrolysis of *p*-nitrophenylphosphate

| Substrate | Relative Rate | $10^3K_m$ | Substrate | Relative Rate | $10^3K_m$ |
|---|---|---|---|---|---|
| *p*-Nitrophenylphosphate | 100 | 0·7 | α-Glycerophosphate | 50 | 5·4 |
| 6-Phosphogluconate | 87 | 4·6 | AMP | 48 | — |
| Fructose 1,6-diphosphate | 85 | 3·4 | Glucose 6-phosphate | 47 | 8·9 |
| β-Glycerophosphate | 84 | 5·7 | Fructose 6-phosphate | 39 | 5·8 |
| β-Naphthylphosphate | 78 | — | Glyceraldehyde 3-phosphate | 41 | — |
| Glucose 1-phosphate | 69 | 5·5 | 3-Phosphoglyceric acid | 33 | — |
| Ribose 5-phosphate | 63 | 5·0 | ADP | 33 | — |
| *O*-Phospho-L-serine | 62 | — | ATP | 21 | — |
| Phosphoenol pyruvate | 51 | — | Inorganic pyrophosphate | 5 | — |

in Table 3. Enzyme activity was greatly enhanced when $Mg^{2+}$ ions were included in the reaction mixture but was slightly depressed in the presence of $Zn^{2+}$, $Co^{2+}$, and $Ni^{2+}$ ions. Cadmium ions were extremely effective in depressing activity. Sodium cyanide, *o*-phenanthroline, and EDTA also depressed enzyme activity while L-cysteine almost completely inhibited the enzymic reaction.

TABLE 3

EFFECT OF DIVALENT METAL IONS AND OTHER COMPOUNDS ON THE ACTIVITY OF SHEEP ENDOMETRIAL ALKALINE PHOSPHATASE

Enzyme activity was assayed by measuring the amount of *p*-nitrophenol liberated from *p*-nitrophenylphosphate as described in the text. Rates are listed as relative to the hydrolysis of substrate in the absence of any added compound

| Compound ($10^{-3}$M) | Relative Rate | Compound ($10^{-3}$M) | Relative Rate |
|---|---|---|---|
| None | 100 | NaCN | 49 |
| $MgCl_2$ | 167 | *o*-Phenanthroline | 42 |
| $ZnCl_2$ | 86 | $CdCl_2$ | 24 |
| $CoCl_2$ | 83 | EDTA | 13 |
| $NiCl_2$ | 65 | L-Cysteine | 3 |

### (*e*) *Effect of Substrate Concentration on EDTA Inhibition*

Figure 2 shows that enzyme activity with 1 μmole of *p*-nitrophenylphosphate was completely inhibited by $10^{-3}$M EDTA within 1 min at room temperature. Increasing substrate concentrations (3 and 9 μmoles), however, partially protected the enzyme against this inhibition.

### (*f*) *Effect of Divalent Cations on EDTA Inhibition*

Incubation of alkaline phosphatase with $10^{-5}$M EDTA in the absence of substrate for 15 min at room temperature almost completely inhibited enzyme activity (Table 4). The addition of various divalent cations at $10^{-4}$M concentration increased the activity of the enzyme to various extents but only $Mg^{2+}$ ions were effective in restoring it 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.

### (*g*) *Effect of EDTA Concentration*

Human placental alkaline phosphatase displays a progressive gain in activity with increasing concentration of EDTA (Conyers *et al.* 1967). The present experiment was conducted to examine the possibility of a similar phenomenon occurring with sheep endometrial alkaline phosphatase.

When incubated in the presence of 6·5 mM *p*-nitrophenylphosphate at pH 10·5, EDTA at a concentration of $10^{-5}$M was ineffective in altering enzyme activity. However, no stimulating effect of the compound was found at any concentration used and, between $10^{-4}$ and $10^{-1}$M, EDTA greatly inhibited activity.

### (*h*) *Effect of $Mg^{2+}$ Concentration*

Nayudu and Miles (1969) have shown that the pyrophosphatase activity of mouse duodenal alkaline phosphatase is activated by $Mg^{2+}$ ions but is strongly

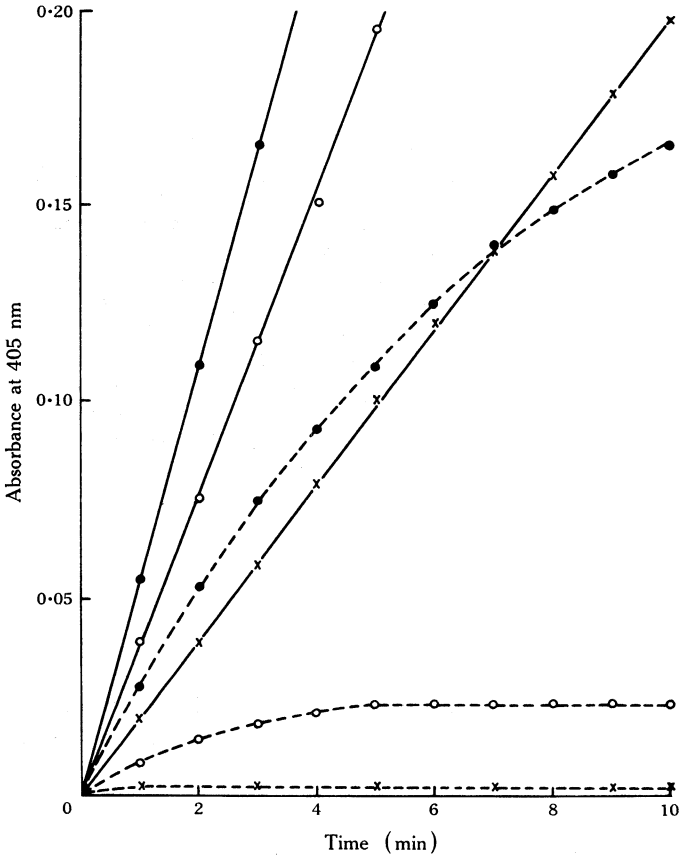


Fig. 2.—Effect of substrate concentration on the inhibition of sheep endometrial alkaline phosphatase by EDTA. Each 3·0 ml of incubation mixture contained 50 mM glycine buffer (pH 10·5), 0·3 μg of enzyme protein, and 9 (●), 3 (○), and 1 (×) μmoles of *p*-nitrophenylphosphate. Tubes at each substrate concentration were prepared to contain 0·0 mM EDTA(———), and 1·0 mM EDTA (– – – –) and the reactions were followed in a spectrophotometer at 405 nm.

TABLE 4

EFFECT OF DIVALENT METAL IONS ON THE INHIBITION OF SHEEP ENDOMETRIAL ALKALINE PHOSPHATASE BY EDTA

| Conditions of Experiment | Addition | Relative Rate |
|---|---|---|
| Each incubation mixture (1·0 ml), containing 50 mM glycine buffer (pH 10·5), $10^{-5}$M EDTA, and 0·3 μg of enzyme protein, was held for 15 min at room temperature. *p*-Nitrophenylphosphate (6·5 mM final concentration) and various metal chlorides ($10^{-4}$M final concentration) were then added and the amount of *p*-nitrophenol liberated was measured as described in the text after incubating the mixtures for a further 15 min at room temperature. Rates are listed as relative to the hydrolysis of substrate in the absence of any added compound | None | 100 |
| | EDTA | 3 |
| | EDTA+$Cd^{2+}$ | 6 |
| | EDTA+$Ni^{2+}$ | 15 |
| | EDTA+$Co^{2+}$ | 41 |
| | EDTA+$Zn^{2+}$ | 60 |
| | EDTA+$Mg^{2+}$ | 105 |
| | $Mg^{2+}$ | 186 |

inhibited as the $Mg^{2+}$/PPi ratio increases. Similarly, the pyrophosphatase activity of sheep endometrial alkaline phosphatase, incubated with ADP, was increased in

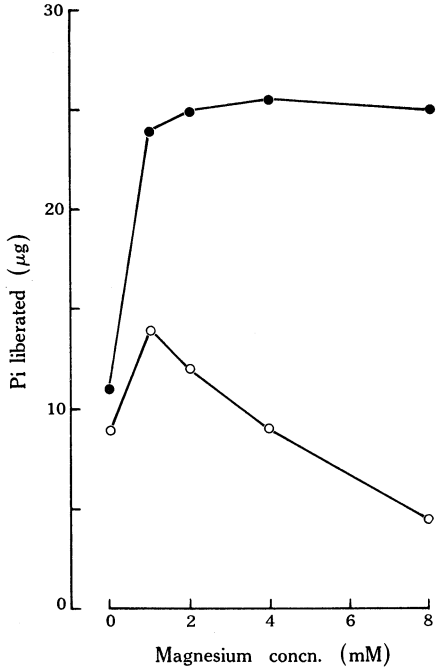


Fig. 3.—Effect of $Mg^{2+}$ concentration on the pyrophosphatase and orthophosphatase activity of sheep endometrial alkaline phosphatase. Each 1·0 ml of incubation mixture contained 50 mM glycine buffer (pH 10·5), 1·0 μg of enzyme protein, and $MgCl_2.6H_2O$ in concentrations indicated. Two μmoles of *p*-nitrophenyl-phosphate (●) and ADP (○) were added separately to the mixture and, after incubating at 37°C for 15 min, all reactions were stopped with 10% (w/v) trichloroacetic acid. The amount of orthophosphate liberated was measured as described in the text.

the presence of 1 mM $MgCl_2$ and decreased with increasing concentration of the cation (Fig. 3). Orthophosphatase activity of the enzyme, however, was stimulated to a

TABLE 5

EFFECT OF SALT CONCENTRATION ON THE ACTIVITY OF SHEEP ENDOMETRIAL ALKALINE PHOSPHATASE

Enzyme activity was assayed by measuring the amount of *p*-nitrophenol liberated from *p*-nitrophenylphosphate as described in the text. Rates are tabulated as relative to the hydrolysis of substrate with no salt added

| Salt Concentration (M) | Relative Rate | | |
|---|---|---|---|
| | KCl | NaCl | $NH_4Cl$ |
| 0·0 | 100 | 100 | 100 |
| 0·001 | 102 | 103 | 109 |
| 0·01 | 105 | 110 | 101 |
| 0·1 | 104 | 72 | 27 |
| 1·0 | 61 | 37 | 2 |

much greater extent by 1 mM $MgCl_2$ in the presence of *p*-nitrophenylphosphate and increasing concentrations of $Mg^{2+}$, in this case, failed to alter activity.

### (*i*) *Effect of Salt Concentration*

Heppel, Harkness, and Hilmoe (1962) have shown that the phosphatase from *Escherichia coli* is stimulated by high salt concentration. At concentrations of 0·001 and 0·01M, KCl, NaCl, and $NH_4Cl$ had no effect on sheep endometrial alkaline phosphatase activity (Table 5). At higher concentrations (0·1 and 1·0M), however, the salts inhibited rather than stimulated phosphatase activity with $NH_4Cl$ being the most effective.

### (*j*) *Effect of Heat*

Purified human placental alkaline phosphatase is resistant to heating at 56°C for 15–30 min (Moss and King 1962; Neale, Clubb, and Posen 1964; Neale *et al.* 1965; Harkness 1968*b*). Purified sheep endometrial alkaline phosphatase, however, does not possess this property since on heating the enzyme at pH 6·0, 7·0, and 9·0 for 15 min at 56°C, it was found that 87, 81, and 76% of its activity was lost respectively. All enzyme activity was lost after heating at 65°C for 15 min.

### (*k*) *Effect of Neuraminidase*

A number of reports have shown that neuraminidase treatment decreases the electrophoretic mobilities of certain alkaline phosphatases (Robinson and Pierce 1964; Butterworth and Moss 1966; Moss *et al.* 1966; Ghosh and Fishman 1969) and affects their chromatographic properties on DEAE-cellulose (Saraswathi and Bachhawat 1968) indicating an association with *N*-acetylneuraminic acid or other neuraminic acid derivatives (see Gottschalk 1960).

Sheep endometrial alkaline phosphatase was treated with neuraminidase as described by Ghosh and Fishman (1969) and subjected to electrophoresis on starch gel. Treated and control digests were also subjected to chromatography on DEAE-cellulose and gradient elution was carried out as described by Saraswathi and Bachhawat (1966, 1968). Only one zone of enzyme activity was resolved following electrophoresis on starch gel and it migrated about 2 cm from the origin towards the anode over a 17-hr period with a potential drop of 6·3 V/cm. Neuraminidase treatment did not affect the activity of the enzyme or its electrophoretic migration. Moreover, treatment of the enzyme with neuraminidase failed to influence its elution pattern during chromatography on DEAE-cellulose.

### (*l*) *Phosphorylation and Transphosphorylation Reactions*

Incubation of enzyme with relatively high concentrations of phosphate and glucose at 37°C (see Harkness 1968*b*) failed to produce any glucose 6-phosphate in detectable amounts (Table 6). However, when β-glycerophosphate was added to the incubation mixture, the hexose phosphate accumulated slowly and increased with time. Synthesis of glucose 6-phosphate was more rapid when phosphate was excluded from the incubation mixture and again increased with increasing time of incubation. No measurable amounts of glucose 6-phosphate were detected in mixtures incubated without enzyme.

Inorganic phosphate is known to inhibit many non-specific alkaline phosphatases (Ahmed and King 1960; Harkness 1968*b*). Although the endometrial enzyme retained

72% of its activity when incubated in the presence of 25 mM Pi for 15 min at 37°C, no glucose 6-phosphate was formed by direct phosphorylation of glucose when the phosphatase was incubated with the sugar in the presence of 25, 50, 100, or 200 mM Pi.

TABLE 6

SYNTHESIS OF GLUCOSE 6-PHOSPHATE BY DIRECT PHOSPHORYLATION AND BY TRANSPHOSPHORYLATION WITH SHEEP ENDOMETRIAL ALKALINE PHOSPHATASE

| Conditions of Experiment | Time (min) | Glucose 6-Phosphate Formed (μmoles) | | |
|---|---|---|---|---|
| | | Pi | β-Glycerophosphate +Pi | β-Glycerophosphate |
| Each incubation mixture (1·0 ml) contained 50 mM glycine buffer (pH 10·5), 0·5 mM $MgCl_2.6H_2O$, 1000 μmoles of glucose, 170 μg of enzyme protein, and, where indicated in the table, 250 μmoles of $Na_2HPO_4$ (Pi) and 125 μmoles of β-glycerophosphate. All tubes were incubated at 37°C for the times indicated. The tubes were then placed in a water-bath at 90°C for 3 min and stored at −20°C until assayed for glucose 6-phosphate | 0 | 0·00 | 0·00 | 0·00 |
| | 15 | 0·00 | 0·07 | 0·43 |
| | 30 | 0·00 | 0·18 | 1·08 |
| | 60 | 0·00 | 0·35 | 2·09 |
| | 120 | 0·00 | 0·86 | 2·34 |

## IV. DISCUSSION

Although the results of the present investigation clearly place sheep endometrial alkaline phosphatase in the category of the non-specific alkaline phosphomonoesterases, it must also be regarded as a pyrophosphatase in view of its ability to catalyse the hydrolysis of ADP, ATP, and, to a lesser extent, PPi. Purified preparations of alkaline phosphatase from a number of sources have been shown to hydrolyse PPi (Butterworth 1968; Fernley and Bisaz 1968; Harkness 1968*b*; Sussman and Laga 1968). Nayudu and Miles (1969) showed that the pyrophosphatase activity of mouse duodenal alkaline phosphatase was activated by low concentrations of $Mg^{2+}$ but was inhibited as the $Mg^{2+}$/PPi concentration ratio increased. The pyrophosphatase activity of sheep endometrial alkaline phosphatase was similarly affected by $Mg^{2+}$ when incubated with ADP as substrate and supports the view that the true substrate for many pyrophosphatase enzymes is the complex ion $MgP_2O_7^{2-}$ (Block-Frankenthal 1954; Pynes and Younathan 1967; Butterworth 1968; Nayudu and Miles 1969).

Alkaline phosphatases are generally regarded as metalloenzymes (Mathies 1958; Engstrom 1961; Trubowitz *et al.* 1961; Plocke, Levinthal, and Vallee 1962; Harkness 1968*b*) and are inhibited by metal-complexing agents (Wallach and Ko 1964; Agus, Cox, and Griffin 1966; Conyers *et al.* 1967; Harkness 1968*b*). The inhibition of sheep endometrial alkaline phosphatase activity by EDTA, *o*-phenanthroline, NaCN, and L-cysteine and its stimulation by $MgCl_2$ suggests that this

phosphatase is also a metalloenzyme with $Mg^{2+}$ as a functional component. Although Agus, Cox, and Griffin (1966) concluded that EDTA probably removes the metal from metalloenzymes, the rapid recovery of the phosphatase in the present study upon addition of $Mg^{2+}$ to the EDTA-inhibited enzyme suggests that a metal chelate complex was formed on the enzyme and that the metal was not actually dissociated from the enzyme. A similar explanation has been proposed for the instantaneous recovery of *E. coli* phosphatase from KCN inhibition (Plocke, Levinthal, and Vallee 1962) and human placental phosphatase from EDTA inhibition following the addition of $Zn^{2+}$ (Harkness 1968*b*).

The protective effect of substrate against EDTA inhibition of sheep endometrial alkaline phosphatase is similar to that described for the human placental phosphatase (Harkness 1968*b*) and indicates that substrate and EDTA compete for the active metal-containing sites of these enzymes. The failure of the sheep endometrial phosphatase to progressively gain in activity with increasing concentration of EDTA, however, is the reverse response to that displayed by the human placental enzyme (Conyers *et al.* 1967) and various non-phosphatase enzymes (Milstein 1961; O'Sullivan and Morrison 1963; Bright 1965).

Sheep endometrial alkaline phosphatase does not possess the stable heat-resistant protein structure of the human placental enzyme (Moss and King 1962; Neale, Clubb, and Posen 1964; Neale *et al.* 1965; Harkness 1968*b*) and also differs from the alkaline phosphatase in *E. coli* in being inhibited, rather than stimulated, by high salt concentration (Heppel, Harkness, and Hilmoe 1962). Moreover, *N*-acetylneuraminic acid does not appear to constitute a part of the enzyme as has been reported for a number of other alkaline phosphatases (Robinson and Pierce 1964; Butterworth and Moss 1966; Moss *et al.* 1966; Saraswathi and Bachhawat 1968; Ghosh and Fishman 1969). The possibility that *N*-acetylneuraminic acid or other sialic acids may have been lost from the enzyme during extraction, however, cannot be overlooked since in a previous study using a relatively crude preparation of endometrial alkaline phosphatase (Murdoch 1970), two zones of activity were resolved following electrophoresis on starch gel. This is somewhat at variance with the results of the present investigation where the purified phosphatase was found to be electrophoretically and chromatographically homogeneous. Further studies are necessary to assess whether the heterogenicity of the enzyme in the crude state arises from non-specific binding or from an association with sialic acid.

Sheep endometrial alkaline phosphatase exhibits phosphotransferase activity but does not appear to be capable of catalysing the synthesis of glucose 6-phosphate by direct phosphorylation. The apparent inability of the enzyme to catalyse phosphorylation under the conditions employed in the present experiments is not a result of the inhibition of the enzyme by Pi since at a concentration of Pi of 25 mM 72% of the enzyme activity is retained but no glucose 6-phosphate is formed. Although alkaline phosphatases from other sources have been shown to catalyse the synthesis of phosphate esters by direct phosphorylation, synthesis by transphosphorylation is more rapid (Morton 1958; Harkness 1968*b*). Harkness (1968*b*) proposed that the more rapid synthesis of phosphate esters with phosphoryl-donor compounds is evidence that phosphoryl transfer takes place directly and not through the intermediate formation of Pi.

The precise role of the endometrial alkaline phosphatase in reproductive processes in the ewe remains uncertain, but its low specificity is consistent with the view (Murdoch 1970) that the enzyme may play an important role in histotrophic activity during early pregnancy in the ewe whereby essential nutriments are provided for the growth and development of the pre-implantation conceptus. The interacting effects of substrate concentration and pH on the activity of the enzyme also indicate that it may be functional in endometrial cells where low substrate concentration and pH levels below the usually measured optimum prevail (see Ross, Ely, and Archer 1951; Harkness 1968*b*).

## V. Acknowledgments

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## VI. References

Agus, S. G., Cox, R. P., and Griffin, M. J. (1966).—*Biochim. biophys. Acta* **118**, 363.
Ahmed, Z., and King, E. J. (1960).—*Biochim. biophys. Acta* **45**, 581.
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.
Bloch-Frankenthal, L. (1954).—*Biochem. J.* **57**, 87.
Boshier, D. P. (1969).—*J. Reprod. Fert.* **19**, 51.
Bradfield, J. R. G. (1950).—*Biol. Rev.* **25**, 113.
Bright, H. J. (1965).—*J. biol. Chem.* **240**, 1198.
Butterworth, P. J. (1968).—*Biochem. J.* **110**, 671.
Butterworth, P. J., and Moss, D. W. (1966).—*Nature, Lond.* **209**, 805.
Conyers, R. A. J., Birkett, D. J., Neale, F. C., Posen, S., and Brudenell-Woods, J. (1967).—*Biochim. biophys. Acta* **139**, 363.
Dempsey, E. W., and Wislocki, G. B. (1945).—*Am. J. Anat.* **76**, 277.
Engstrom, L. (1961).—*Biochim. biophys. Acta* **52**, 36.
Fernley, H. N., and Bisaz, S. (1968).—*Biochem. J.* **107**, 279.
Fiske, C. H., and Subba Row, Y. (1925).—*J. Biol. Chem.* **66**, 375.
Gavasto, F., and Pileri, A. (1958).—*Cancer* **11**, 222.
Ghosh, N. K., and Fishman, W. H. (1969).—*Can. J. Biochem.* **47**, 147.
Gottschalk, A. (1960).—In "The Enzymes". 2nd Edn. (Eds. P. D. Boyer, H. Hardy, and K. Myrbäck.) Vol. 4. p. 461. (Academic Press: New York.)
Hafez, E. S. E., and White, I. G. (1968).—*J. Reprod. Fert.* **16**, 59.
Harkness, D. R. (1968*a*).—*Archs Biochem. Biophys.* **126**, 503.
Harkness, D. R. (1968*b*).—*Archs Biochem. Biophys.* **126**, 513.
Heppel, L. A., Harkness, D. R., and Hilmoe, R. J. (1962).—*J. biol. Chem.* **237**, 831.
Horecker, B. L., and Wood, W. A. (1957).—In "Methods in Enzymology". (Eds. S. P. Colowick and N. O. Kaplan.) Vol. 3. p. 105. (Academic Press: New York.)
Lineweaver, H., and Burk, D. (1934).—*J. Am. chem. Soc.* **56**, 658.
Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951).—*J. biol. Chem.* **193**, 265.
Malone, T. E. (1960).—*Am. J. Anat.* **106**, 41.
Markert, C. L., and Møller, F. (1959).—*Proc. natn. Acad. Sci. U.S.A.* **45**, 753.
Mathies, J. C. (1958).—*J. biol. Chem.* **233**, 1121.
Milstein, C. (1961).—*Biochem. J.* **79**, 584.
Moog, F. (1946).—*Biol. Rev.* **21**, 41.
Moog, F., and Wenger, E. L. (1952).—*Am. J. Anat.* **90**, 339.

MORTON, R. K. (1954).—*Biochem. J.* **57**, 594.
MORTON, R. K. (1958).—*Biochem. J.* **70**, 139.
MOSS, D. W., EATON, R. H., SMITH, J. H., and WHITBY, L. G. (1966).—*Biochem. J.* **98**, 32c.
MOSS, D. W., and KING, E. J. (1962).—*Biochem. J.* **84**, 192.
MURDOCH, R. N. (1970).—*Aust. J. biol. Sci.* **23**, 1089.
MURDOCH, R. N., and WHITE, I. G. (1968*a*).—*Aust. J. biol. Sci.* **21**, 123.
MURDOCH, R. N., and WHITE, I. G. (1968*b*).—*J. Endocr.* **42**, 187.
NAYUDU, P. R. V., and MILES, P. L. (1969).—*Biochem. J.* **115**, 29.
NEALE, F. C., CLUBB, J. S., HOTCHKIS, D., and POSEN, S. (1965).—*J. clin. Path.* **18**, 359.
NEALE, F. C., CLUBB, J. S., and POSEN, S. (1964).—*Proc. Aust. Ass. clin. Biochem.* **1**, 71.
O'SULLIVAN, W. J., and MORRISON, J. F. (1963).—*Biochim. biophys. Acta* **77**, 142.
PLOCKE, D. J., LEVINTHAL, C., and VALLEE, B. L. (1962).—*Biochemistry, N.Y.* **1**, 373.
PYNES, G. D., and YOUNATHAN, E. S. (1967).—*J. biol. Chem.* **242**, 2119.
ROBINSON, J. C., and PIERCE, J. E. (1964).—*Nature, Lond.* **204**, 472.
ROSS, M. H., ELY, J. O., and ARCHER, J. G. (1951).—*J. biol. Chem.* **192**, 561.
SARASWATHI, S., and BACHHAWAT, B. K. (1966).—*J. Neurochem.* **13**, 237.
SARASWATHI, S., and BACHHAWAT, B. K. (1968).—*Biochem. J.* **107**, 185.
SUSSMAN, H. H., and LAGA, E. (1968).—*Biochim. biophys. Acta* **151**, 281.
SUSSMAN, H. H., SMALL, P. A., and COTLOVE, E. (1968).—*J. biol. Chem.* **243**, 160.
TRUBOWITZ, S., FELDMAN, D., MORGENSTERN, S. W., and HUNT, V. M. (1961).—*Biochem. J.* **80**, 369.
WALLACH, D. P., and KO, H. (1964).—*Can. J. Biochem.* **42**, 1445.