Some Properties of Alkaline Phosphatase of Cow's Milk and Calf Intestinal Mucosa

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The purification of the alkaline phosphatases of cow's milk and calf intestinal mucosa as previously described (Morton, 1950, 1953*a*, 1954*a*) was initiated in order to enable investigation of the properties of these enzymes. The need for such a study has been well recognized (see Moog, 1946, for example), but hitherto all work has been carried out with crude or partially purified enzyme preparations. Moreover, all previous workers have used prolonged autolysis or proteolysis to separate alkaline phosphatases from animal tissues, and the extent of modification of these proteins by such treatments is still uncertain. These undesirable procedures may be avoided, however, by using the butanol procedure (Morton, 1950) to separate alkaline phosphatases from the lipoprotein material with which they are normally associated (Kabat, 1941; Chantrenne, 1947; Hers, Berthet, Berthet & de Duve, 1951; Morton, 1953*b*, 1954*a*, *b*).

Some of the properties of the purified enzymes are described in this paper. Particular attention has been given to the chemical constitution, the activity–pH relationship, metal activation and the effects of alanine and glycine. All these have been the subjects of conflicting statements in the extensive literature concerning alkaline phosphatases.

It has been found that bovine milk and intestinal phosphatases are two different enzymes but of similar substrate specificity. No evidence has been obtained in support of numerous claims for an organic coenzyme of alkaline phosphatase.

MATERIALS AND METHODS

Enzyme substrates

Except where otherwise indicated, these were commercial preparations. They were tested for the presence of contaminant phosphorylated compounds, using paper chromatography as described by Hanes & Isherwood (1949), except in the case of nucleotides, for which the procedure of Markham & Smith (1951) was used. Where necessary, inorganic phosphate was removed from substrates by precipitation with ammoniacal magnesia, and the excess ammonia was then removed over conc. H_2SO_4 *in vacuo*. The concentration of substrate was determined by estimation of organic phosphate.

Buffers

Veronal (sodium diethyl barbiturate). A slight inhibitory effect found in commercial samples was eliminated by the following treatment. The free acid was precipitated from a saturated soln. of veronal by the dropwise addition of conc. HCl and then twice recrystallized from hot distilled water. Buffers were prepared by dissolving the free acid in the appropriate amount of carbonate-free NaOH (2N). The inhibitory action of commercial samples is probably due to traces of heavy metal salts.

Ethanolamine. This was distilled at about 100° under vacuum (approx. 15 mm. mercury). Buffers were prepared by the addition of N-HCl to monoethanolamine (4M).

Enzymes

Unless otherwise indicated, the experiments described in this paper were carried out during 1950 and early 1951, using alkaline phosphatases purified from cow's milk (Morton, 1950, 1953*a*) and calf intestinal mucosa (Morton, 1950, 1954*a*). The enzymes were prepared in 1949 and early 1950, and stored as dry powders over fused $CaCl_2$ in an evacuated desiccator held at about 0°. After twelve months storage, the specific activities (determined as μg. P liberated/min./mg. protein-N) of the preparations were approx. 70% (milk enzyme) and 60% (intestinal enzyme) of the values when immediately isolated.

The alkaline phosphatases from cow mammary gland and from ox kidney were partially purified preparations obtained as previously described (Morton, 1954*a*).

The enzymes were dissolved in dilute (0·05M) veronal–sodium acetate–HCl buffer (Michaelis, 1931), pH 6·8, before use and held at about 0° in stoppered tubes containing a little toluene vapour. They were found to be quite stable for up to 1 month under these conditions, whereas considerable inactivation of the enzyme may occur if held at −15° (Morton, 1954*a*).

Alkaline phosphatase activity. This was generally determined by estimating the rate of hydrolysis of sodium β-glycerophosphate as previously described (Morton, 1953*b*, 1954*a*). As indicated in the appropriate sections, the experimental conditions have been varied as necessary. All activities were determined at 38°. Usually the incubation period was 5 min., although this was extended to 15 min. in some cases as indicated later.

The units of activity and specific activity have been defined previously (Morton, 1953*b*). In order to permit ready comparison of different experiments, the results are reported as rates of hydrolysis *relative* to that of the maximal (or arbitrarily chosen) rate, which is assigned the value of 100. In many cases, this figure approximates to the true initial rate of hydrolysis, expressed as μg. inorganic P liberated/min./ml. stock enzyme solution.

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Phosphate. In determining enzymic activities, the procedure of Fiske & Subbarow (1925) was used. The phosphorus contents of the purified enzymes was estimated using the procedure of Weil-Malherbe & Green (1951), the quantities of materials being reduced proportionately so that the colour was developed in a final volume of 5 ml. instead of 10 ml. The optical density was measured in a Beckman spectrophotometer at 700 mμ. using a 1 cm. cuvette, thus enabling approx. 0·2 μg. phosphorus to be detected.

Nitrogen. This was determined by a micro-Kjeldahl procedure. Initially, the ammonia was determined by distillation and titration. Later, because of the limited amount of purified enzymes available, a modification of the colorimetric procedure described by King (1951) for estimation of serum protein was used. The sample was digested overnight after addition of the sulphuric acid–catalyst reagent (King, 1951). After cooling, 3 ml. of distilled water were added, followed by 3 ml. of the Nessler reagent, added rapidly from a wide-bore pipette. The flask was vigorously agitated at the same time to ensure immediate mixing. The optical density was measured at 480 mμ. using a 1 cm. cuvette. Linear response was obtained over the range 10–60 μg. N, using ammonium sulphate, glycine, glycylglycine, and three-times recrystallized ovalbumin as standards.

pH *Measurement.* Because of the large change of pH with temperature which occurs with most buffers at alkaline pH values, it is important to express pH values for the temperature used in measuring enzymic activity. The pH values of the buffer systems used, at approx. 0·07 M concentration, were estimated both at 20 and 38°. Thereafter, the pH values of the reaction mixtures were measured at approx. 20° and the necessary corrections, based on the determinations with pure buffers, as shown in Table 1, were applied to obtain the pH values at 38°. Unless otherwise indicated, all pH values given refer to 38°.

Table 1. *The influence of temperature on the* pH *values of various buffer systems at approx.* 0·07 M *concentration*

The buffers were prepared according to published directions as follows: glycine–NaOH, Sorensen (1912); veronal–HCl, Michaelis (1930); veronal–Na_2CO_3–HCl, King & Delory (1940); Na_2CO_3–$NaHCO_3$, Delory & King (1945).

pH at approx. 20°	pH at approx. 38°				
	Glycine–NaOH	Ethanolamine–HCl	Veronal–HCl (or NaOH)	Veronal–Na_2CO_3–HCl	Na_2CO_3–$NaHCO_3$
8·5	8·15	8·2	8·4	—	—
9·0	8·65	8·7	8·85	—	—
9·2	8·8	8·85	9·05	—	—
9·4	9·0	9·15	9·2	9·2	9·25
9·6	9·2	9·25	9·4	9·45	9·45
9·8	9·4	9·4	9·6	9·65	9·65
10·0	9·6	9·6	9·8	9·85	9·85
10·2	9·8	9·8	10·0	10·0	10·05
10·4	10·0	10·0	10·2	10·2	10·25
10·6	10·15	10·1	10·3	10·3	—

EXPERIMENTS AND RESULTS

Chemical analyses of the purified phosphatases

The dried enzymes (Morton, 1953*a*, 1954*a*) were dissolved in glass-distilled water. The absorption spectra (see below) and the nitrogen content were determined on suitable portions and the remaining solutions were dialysed in cellophan sacs against large volumes of glass-distilled water at 0·5° for 48 hr. with continuous mechanical stirring. The dry weights were determined by drying 2 ml. of each of the solutions over P_2O_5 *in vacuo* for 48 hr. at 30°. The remaining material was used for the chemical analyses described below.

Nitrogen. The total nitrogen contents were estimated as 16·2% (milk enzyme) and 15·2% (intestinal enzyme).

Phosphorus. No organic phosphate was detected using 0·2 mg. of the milk enzyme and 0·5 mg. of the intestinal enzyme. Since the method previously described would detect 0·2 μg. phosphorus, the phosphorus contents are less than 0·1%.

Carbohydrate. When 0·1 mg. of each enzyme was tested using the Molisch reaction, no carbohydrate could be

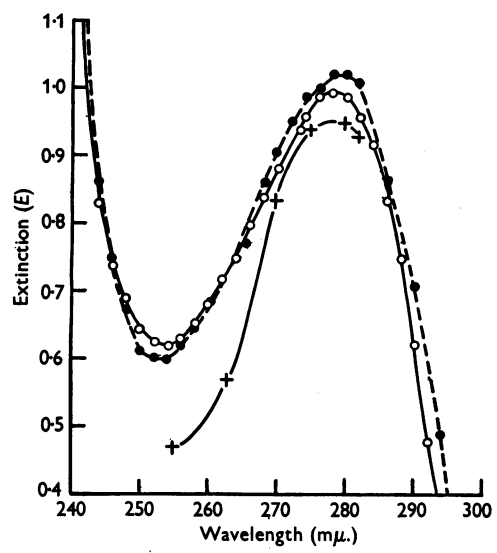

Fig. 1. Absorption spectra of different alkaline phosphatase preparations in distilled water at pH 7·7 and 20°, protein concentration 1 mg./ml. in each case. ○—○, Purified intestinal phosphatase; ●—●, purified milk phosphatase; +—+, partially purified kidney phosphatase.

detected (Morton, 1952). More recently, 0·3 mg. of each enzyme was tested using the anthrone reagent according to the method of Chung & Nickerson (1954). Using as a standard three-times recrystallized ovalbumin (carbohydrate content, 1·8%; Neuberger, 1938), it was estimated that the milk enzyme contained less than 2%, and the intestinal enzyme approx. 2% carbohydrate.

Nucleotides and related compounds. The absorption spectra of the phosphatases, brought to pH 7·7 with 0·01 N-NaOH, were measured using 1 cm. cuvettes with a Beckman DU spectrophotometer. Readings were made against glass-distilled water.

Both protein solutions were colourless and water clear and, as expected, showed no measurable absorption in the visible region of the spectrum. The absorption spectra in the ultraviolet region, given for a protein concentration of 1 mg./ml., are shown in Fig. 1, together with the spectrum of partially purified phosphatase from bovine kidney.

It is apparent from inspection that the preparations are virtually free of nucleotides or related compounds which absorb strongly at or near 260 mμ.

Tyrosine and tryptophan. These were estimated from absorption spectra of the enzymes in 0·1 N-NaOH, using the procedure of Goodwin & Morton (1946) with the modifications of Beavan & Holiday (1952). The values obtained are shown in Table 2.

Table 2. *The tyrosine and tryptophan contents of alkaline phosphatases*

The proteins were dissolved in 0·1 N-NaOH and determinations were made by the spectrophotometric method of Goodwin & Morton (1946).

Protein	Moles/10^5 g. Tyrosine	Moles/10^5 g. Tryptophan	Ratio: tyrosine/tryptophan
Milk phosphatase	27	13	2·1
Intestinal phosphatase	26	11	2·3

The optimum pH *for hydrolysis by phosphatases of different origin*

The optimum pH for hydrolysis varies with the nature of the substrate and with the substrate concentration (Delory & King, 1943; Morton, 1952). However, alkaline phosphatases from different sources may legitimately be compared on the basis of the optimum pH for hydrolysis of a selected substrate at a known concentration.

A suitable buffer system must be selected for such determinations. Aebi (1948), using a crude preparation of alkaline phosphatase from rat kidney, observed large differences in the optimum pH values for hydrolysis of sodium β-glycerophosphate (0·016 M) in different buffer systems when activities at 37° were plotted against pH values at 20°. When Aebi's results are re-plotted so as to allow for the differing temperature coefficients of the various buffer systems (see Table 1), the differences in optimum pH values are either eliminated or considerably reduced. However, such plots still show that the optimum pH value in Na_2CO_3–$NaHCO_3$ buffer (0·1 M) is about 0·2 pH unit below that obtained with glycine–NaOH (0·1 M) and veronal–HCl (0·1 M) buffers. A number of preliminary experiments confirmed that activities, as well as optimum pH values, for both milk and intestinal phosphatases are somewhat depressed in Na_2CO_3–$NaHCO_3$ buffer as compared with values obtained with ethanolamine–HCl (0·05 M) buffer. Hence buffers containing carbonate or other inhibitory anions (Zittle & Della Monica, 1950*a*, *b*) were avoided in determining optimum pH values. In a large number of different experiments, very similar results have been obtained with veronal–HCl and ethanolamine–HCl buffers.

Activities were therefore determined in ethanolamine–HCl buffer (0·04 M) with sodium β-glycerophosphate (0·02 M) as substrate. Duplicate tubes were used for measurement of the pH of the reaction mixtures. The activity/pH curves for the purified enzymes from milk and intestinal mucosa are shown in Fig. 2. It is seen that the optimum pH for the milk enzyme (pH 9·65) is higher than that for the intestinal enzyme (pH 9·45). Similar differences in the optimum pH of hydrolysis by these two enzymes are found with most other substrates (Morton, 1955).

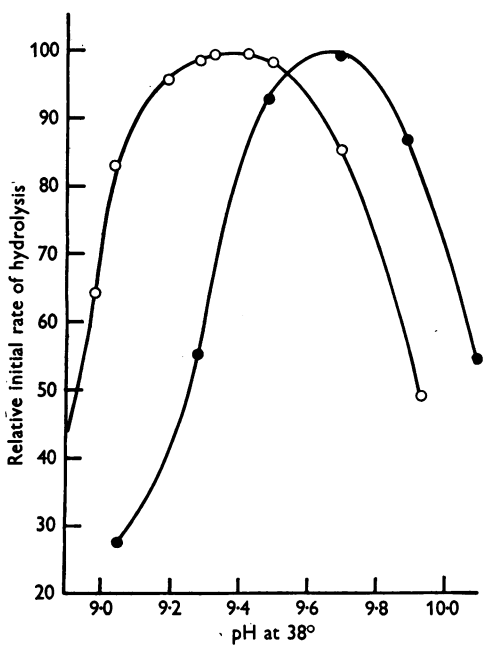

Fig. 2. Comparative activity/pH curves for purified alkaline phosphatases. The reaction mixture (5 ml.) in each case contained sodium β-glycerophosphate (0·02 M), magnesium acetate (0·01 M) and ethanolamine–HCl buffer (0·04 M), with approx. 6 units of enzyme activity. The reaction period was 5 min. at 38°. The results are expressed as relative rates of hydrolysis, the maximal rate being assigned the value of 100. ○—○, Intestinal phosphatase; ●—●, milk phosphatase.

The activity/pH curve for purified milk phosphatase is compared with curves for the partially purified phosphatases from milk, cow mammary gland and bovine kidney in Fig. 3. It is seen that the optimum pH values obtained for these different enzyme preparations are approx. the same (pH 9·65).

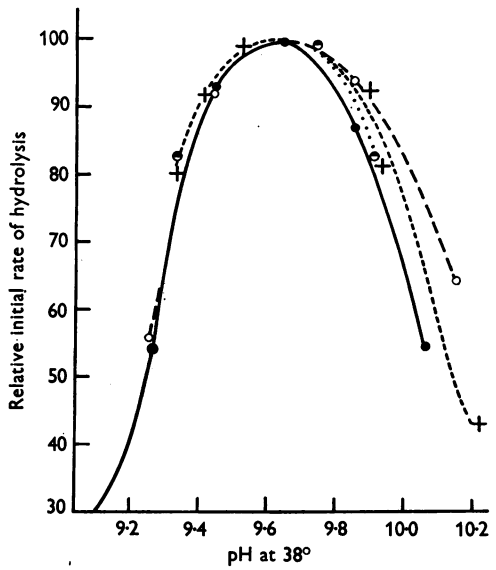

Fig. 3. Comparative activity/pH curves for partially purified alkaline phosphatase preparations, determined from hydrolysis of sodium β-glycerophosphate (0·02 M) in either ethanolamine–HCl (0·04 M) or veronal–HCl (0·04 M) buffers, containing magnesium acetate (0·01 M). The final volume was 5 ml. in each case. Approx. 6 units of enzyme were used in each case, except that 8 units of partially purified milk enzyme were used. The reaction period was 5 min. at 38°. The results are expressed as for Fig. 2. ●—●, Purified milk phosphatase; +···+, partially purified milk phosphatase (Morton, 1953*b*, Table 5, stage 6); ○···○, partially purified kidney phosphatase; ◒ ··· ◒, partially purified mammary gland phosphatase.

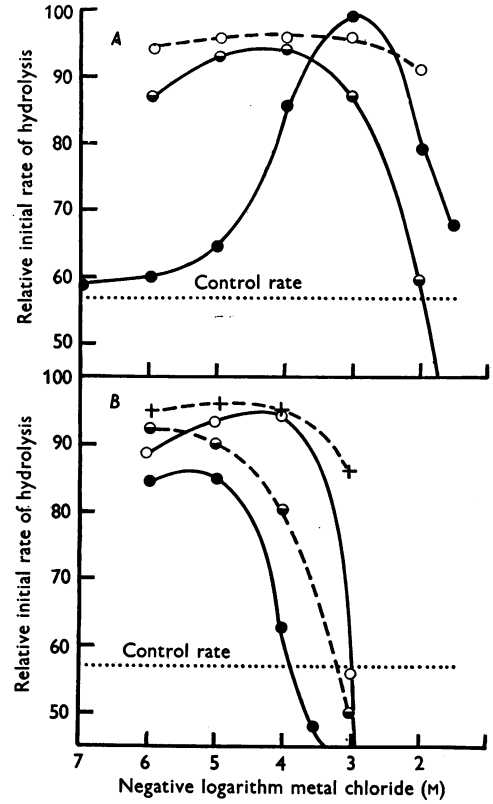

Fig. 4. Influence of added metal chlorides on the hydrolysis of sodium glycerophosphate by partially purified milk phosphatase (Morton, 1953*b*, Table 5, stage 6). The reaction mixture (5 ml.) contained the substrate (0·02 M), veronal–HCl buffer (0·02 M, pH 9·65), and about 5 units of enzyme. The reaction period was 15 min. at 38°. Metal chlorides were added to give the concentrations indicated. When included with other metal salts, the concentration of magnesium chloride was 10^{-3} M. Results are expressed as relative rates of hydrolysis, the rate with magnesium chloride (10^{-3} M) being assigned the value of 100. *A*: ○, calcium and magnesium; ●, magnesium; ◒, calcium. *B*: ○, zinc; ●, cobalt; ◒, cobalt and magnesium; +, zinc and magesium.

The activation of alkaline phosphatases by metal cations

A partially purified preparation of the milk enzyme, obtained as previously described (Morton, 1953*b*, Table 5, stage 6) was used for the initial studies of the influence of added metal chlorides on the hydrolysis of sodium β-glycerophosphate (0·01 M) in veronal–HCl (0·02 M) buffer. The reaction was commenced by the addition of about 6 units of enzyme (in 0·2 ml.) to 4·8 ml. of the buffered substrate. The reaction period was 15 min. The results are shown in Fig. 4.

It is seen that partially purified milk phosphatase resembles other impure alkaline phosphatases in the lack of specificity towards the metal activator. The maximal activity is obtained with magnesium chloride (10^{-3} M). When the latter salt is included in the test system, the inhibitory effects of high concentrations of calcium and zinc salts are largely abolished.

Further studies were carried out with purified enzymes which had been stored in the dried state for about 3 years. About 2 mg. enzyme was dissolved in 0·5 ml. glass-distilled water and dialysed in a small cellophan sac at about 2° for 14 hr. with continuous mechanical stirring. The stock enzyme solution was diluted in distilled water just prior to use so that 0·1 ml. contained approx. 0·02 μg. intestinal enzyme or 0·08 μg. milk enzyme. The reaction was commenced by the addition of this amount of enzyme (about 0·1 unit) to 1·9 ml. of buffered substrate. The final reaction mixture (2 ml.) contained phenyl phosphate (0·0025 M) and ethanolamine–HCl buffer (0·05 M) either at pH 9·65 (intestinal enzyme) or pH 9·9 (milk enzyme). Dilutions of metal salts were used to give a range of concentrations increasing from 10^{-10} M. Phenol liberated enzymically in 15 min. was estimated by the addition of 0·5 ml. of phenol reagent (King, 1951) followed by 0·5 ml. of 15% Na_2CO_3. Colours were developed at 38° for 20 min. and read at 750 mμ., using 1 cm. cuvettes in a Beckman spectrophotometer.

At the very low concentration of protein used in these studies, some difficulty was experienced at first in obtaining consistent results, owing to physical denaturation of the enzyme. This denaturation was largely avoided by mixing the reactants with a rotary motion and avoiding shaking the test tube. In view of the nature of the experiments, the addition of inert protein or other protective agents was not considered desirable.

A very low concentration of enzyme was used in these experiments so that the dissociation of any metal or other group remaining bound to the enzyme would be favoured. Moreover, it was desired to ascertain whether a large change in enzyme concentration would influence the concentration of magnesium salt (ascertained as 10^{-2} M in earlier studies) required for optimum activation. It should be noted that longer dialysis than that described was avoided since, especially at low protein concentrations, prolonged dialysis of the purified enzymes leads to irreversible loss of activity, probably due to protein denaturation.

The results are shown in Figs. 5 and 6, for metal concentrations greater than 10^{-3} M, results obtained at lower concentrations being very similar to those at 10^{-3} M. The possibility of the substrate or the buffer containing metal contaminants at this order of concentration cannot be excluded.

It is seen that, with both enzymes, maximum activity is obtained with magnesium chloride at approx. 10^{-2} M, while there is only slight decline of activity at higher concentration of metal. The behaviour of the purified milk enzyme therefore differs from that of the crude enzyme, which shows maximum activity at 10^{-3} M magnesium chloride and decreased activity with higher concentrations of this salt (Fig. 4). Other differences in the behaviour of the crude and purified milk enzymes are also evident. The relative activation by magnesium chloride is very much greater in the case of the purified enzyme. Although the effects of added calcium and zinc salts are qualitatively the same, maximum activity occurs at rather different metal concentrations (cf. Figs. 4 and 5). The relative activation by zinc or calcium chlorides is greater in the case of the crude enzyme.

Comparison of Figs. 5 and 6 shows important differences in the behaviour of the purified milk and intestinal enzymes. In general, the relative activation by magnesium and manganese is very much greater in the case of the milk enzyme. While both enzymes are inhibited by beryllium and zinc salts at concentrations greater than 10^{-6} M, the milk enzyme is more strongly inhibited by zinc, and the intestinal enzyme by beryllium. In the case of the milk enzyme, added magnesium chloride (10^{-3} M) largely abolishes the inhibitory action of both beryllium and zinc chlorides at

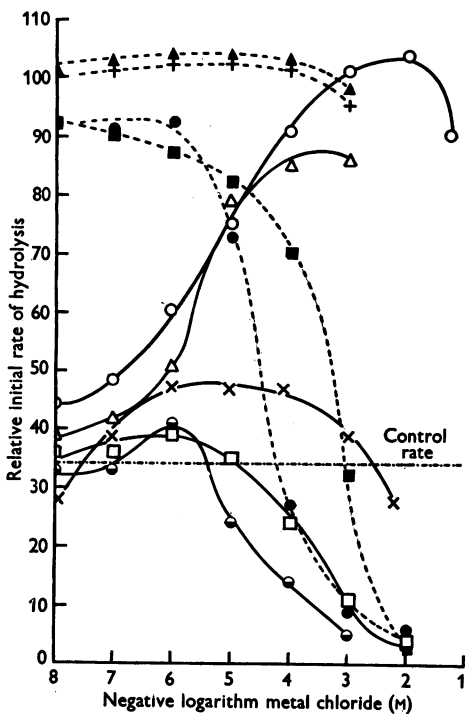

Fig. 5. Influence of added metal chlorides on the hydrolysis of disodium phenyl phosphate by purified milk phosphatase. The reaction mixture (2 ml.) contained the substrate (0·0025 M), ethanolamine–HCl buffer (0·05 M, pH 9·9) and approx. 0·1 unit of enzyme. The reaction period was 15 min. at 38°. All other details as for Fig. 4. ○, Magnesium; ●, zinc and magnesium; ◒, zinc; □, beryllium; ■, beryllium and magnesium; ×, calcium; +, calcium and magnesium; △, manganese; ▲, manganese and magnesium.

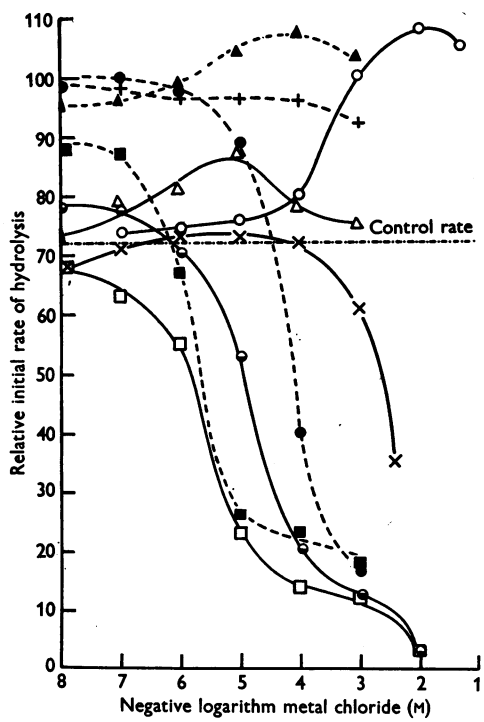

Fig. 6. Influence of added metal chlorides on the hydrolysis of disodium phenyl phosphate by purified intestinal phosphatase. Approx. 0·1 unit of enzyme was used in the 2 ml. reaction mixture as described for Fig. 5. All other details as for Fig. 5.

10^{-5}M and at lower concentrations. While the same concentration of magnesium chloride overcomes the inhibitory influence of zinc chloride at 10^{-5}M and lower concentrations on the intestinal enzyme, it fails to reverse the effect of beryllium chloride except at concentrations less than 10^{-6}M.

The effect of alanine and glycine on alkaline phosphatases

Since it has been reported that partially purified intestinal phosphatase from dog intestinal mucosa is markedly activated by alanine (Thoai, Roche & Roger, 1947), the influence of alanine and glycine on the purified enzyme of calf intestinal mucosa has been studied.

Initially, tests were carried out under conditions closely resembling those used by Thoai *et al.* (1947). The enzyme was incubated for 1 hr. at 38° in Na_2CO_3–$NaHCO_3$ buffer (0·05M) with varying concentrations of alanine or glycine and with varying concentrations of magnesium acetate. At high concentrations of amino acids there was some shift of pH of the system during incubation, but this was always adjusted so that the pH during the activity test was 9·2, as used by Thoai *et al.* (1947).

The results are shown in Table 3. It is seen that, both in the presence and absence of added magnesium acetate, activity increased with increasing concentration of alanine, up to 5×10^{-2}M. Glycine caused only slight activation.

It seemed possible that these results were dependent on the particular experimental conditions used, especially

Table 3. *The influence of pre-incubation for* 1 *hr. with alanine and glycine on the activity of intestinal alkaline phosphatase*

Approx. 10 units of enzyme was pre-incubated for 1 hr. at 38° in 1 ml. of Na_2CO_3–$NaHCO_3$ buffer (0·05M) containing magnesium acetate and varying concentrations of alanine or glycine as indicated. The pH during pre-incubation was 8·7 to 9·05. The reaction was commenced by the addition of 4 ml. of sodium β-glycerophosphate in a similar reaction mixture to that used for pre-incubation of the enzyme but at pH 9·2. The final substrate concentration in 5 ml. reaction mixture was 0·02M at pH 9·2. The reaction period was 5 min. at 38°. The results are expressed as relative rates of hydrolysis, the rate with alanine (10^{-2}M) and magnesium acetate (10^{-2}M) being designated as 100 units.

	Results with alanine		Results with glycine	
Mg^{2+} (M) ...	10^{-4}	10^{-2}	10^{-4}	10^{-2}
Amino acid concentration (M)				
0	39·2	70·0	39·2	70·0
10^{-4}	43·3	72·5	46·5	—
10^{-3}	48·1	82·0	46·2	70·5
5×10^{-3}	65·8	95·3	—	—
10^{-2}	79·5	100	55·6	79·6
5×10^{-2}	100	108	47·8	76·1
10^{-1}	—	—	48·0	41·6

Table 4. *The effect of alanine, with short and long pre-incubation, on the activity of intestinal alkaline phosphatase*

Approx. 10 units of enzyme were pre-incubated at pH 9·0 and 38° in 1 ml. of buffer containing magnesium acetate (10^{-2}M), with or without alanine as indicated. In treatment *A*, pre-incubation was 2 min. and, in treatment *B*, 1 hr. The reaction was commenced by addition of 4 ml. of substrate in a similar buffer to that used for pre-incubation of the enzyme, but adjusted so that the final reaction pH was as indicated. In the final reaction mixture (5 ml.) the concentration of sodium β-glycerophosphate was 0·02M, magnesium acetate 10^{-2}M, alanine (where added) 10^{-2}M, and buffer as shown. The reaction period was 5 min. at 38°. Results are expressed as in Table 3.

		Treatment *A*		Treatment *B*	
Buffer	pH of reaction	With alanine	Without alanine	With alanine	Without alanine
Na_2CO_3–$NaHCO_3$ (0·05M)	8·9	47·8	46·1	6·66	39·4
Na_2CO_3–$NaHCO_3$ (0·05M)	9·5	134	99	114	104
Veronal–HCl (0·04M)	9·5	146	152	124	114

Table 5. *Partial reversal by amino acids of metal inhibition of purified intestinal alkaline phosphatase*

The enzymes were the same preparations as used for metal-activation studies (see text: 'The activation of alkaline phosphatases by metal cations'). 0·1 ml., containing 0·08 unit of activity, was added to 1·9 ml. of buffered substrate so that the final concentrations of reactants were: ethanolamine–HCl buffer, 0·05M; phenyl phosphate, 0·0025M; metal chlorides, where added, 10^{-3}M; alanine, 10^{-3}M; histidine, 5×10^{-4}M. The final pH was 9·65 (intestinal enzyme) or 9·8 (milk enzyme). The reaction period was 15 min. at 38°. Liberated phenol was estimated as described in the text. The results are expressed as relative rates of hydrolysis, the rate with magnesium chloride (10^{-2}M) in the absence of inhibitor or amino acid being designated as 100 units.

		No inhibitor		Zn^{2+}		Be^{2+}	
	Amino acid	No Mg^{2+}	With Mg^{2+}	No Mg^{2+}	With Mg^{2+}	No Mg^{2+}	With Mg^{2+}
Results with intestinal phosphatase	None	69	100	12	20	8	13
	Alanine	75	102	40	63	8	13
	Histidine	70	110	48	50	16	11
Results with milk phosphatase	None	32	100	5	10	10	25
	Alanine	47	107	15	40	30	65
	Histidine	62	114	22	44	40	55

since the use of Na_2CO_3–$NaHCO_3$ buffer is undesirable in that it depresses both the activity and the optimum pH for activity (p. 575). Comparison was therefore made of the effects of alanine, with and without pre-incubation with the enzyme, in Na_2CO_3–$NaHCO_3$ and veronal–HCl buffers. As shown by the results in Table 4, addition of alanine, either with or without prior incubation with the enzyme, gave no greater activity than that obtained in veronal–HCl buffer with addition of magnesium salt (10^{-2}M). Table 4, however, confirms that alanine exerts some protective action during pre-incubation of the enzyme, especially in the presence of carbonate ions.

Alanine caused no increase of activity beyond that obtained with magnesium acetate (10^{-2}M) alone when purified milk phosphatase was tested with sodium β-glycerophosphate at pH 9·65 using veronal–HCl buffer (0·07M).

It seemed likely that the inhibitory action of beryllium and zinc salts on both milk and intestinal phosphatases could be partly abolished by the addition of amino acids (or other metal-chelating agents). When alanine (10^{-3}M) or histidine (5×10^{-4}M) were included in the test system, together with magnesium chloride, the marked inhibitory effects of high concentrations (10^{-3}M) of beryllium and zinc chlorides were somewhat reduced, as shown in Table 5.

DISCUSSION

Electrophoretic and other evidence which suggests that the purified alkaline phosphatase preparations used in these studies are free of detectable contaminant proteins has been considered in earlier papers (Morton, 1953*a*, 1954*a*). Further evidence for the homogeneity of the enzyme from calf intestinal mucosa has recently been provided by the work of Schramm & Armbruster (1954). Using a procedure which combined the autolysis method of Albers & Albers (1935) with the butanol treatment of Morton (1950), followed by further fractionation with salts and organic solvents and electrophoretic separation, these workers isolated a very active fraction from calf intestinal mucosa. This material, of specific activity 46000 units/mg. N (57000 units/mg. N following activation with alanine), was found by Schramm & Armbruster to behave as a single component both on electrophoresis and on sedimentation. It is clear, therefore, that the material used in the present studies, which has a specific activity of 83500 units/mg. N (Morton, 1950, 1954*a*), must be regarded as essentially a single component. The markedly lower activity of the preparation of Schramm & Armbruster is possibly due to the undesirable exposure of the enzyme to autolysis during the purification procedure (see later).

Chemical constitution of the enzymes

The absorption spectra (Fig. 1) and the chemical analyses indicate that both enzymes are typical unconjugated proteins, substantially free of nucleotides or related compounds. Assuming molecular weights of about 60000 (see later), the phosphorus contents (less than 0·1%) show that there would be less than two gram atoms of phosphorus per mole of enzyme. These analyses therefore exclude both choline pyrophosphate (Kutscher & Sieg, 1950) and a diphosphonucleoside of uracil (Lora-Tamayo & Municio, 1951; Lora-Tamayo & Alvarez, 1954), considered to be the 'coenzymes' of alkaline phosphatases from muscle and kidney respectively. Unlike the absorption spectrum presented by Lora-Tamayo & Alvarez (1954), that of the highly active preparation of kidney alkaline phosphatase obtained by the butanol procedure (Morton, 1954*a*) shows no evidence of an absorption peak at or near 260 mμ. (see Fig. 1).

There is no direct evidence that the enzyme preparations are free of non-phosphorylated lipid material. However, in view of the successive use of organic solvents in the preparative procedures, and the high nitrogen contents it seems unlikely that either preparation contains any large amount of lipid.

Schmidt & Thannhauser (1943) found that their partially purified preparations of alkaline phosphatase from calf intestinal mucosa regularly contained about 22% of polysaccharide (expressed as glucose). Other workers (Abul-Fadl & King, 1949*a*; Roche & Bouchilloux, 1950) have described partially purified preparations of intestinal phosphatase as free of carbohydrate, and Schramm & Armbruster (1954) found less than 5% of carbohydrate in their highly active preparation. The present work shows that the enzyme from milk, as well as that from intestinal mucosa, has a very low carbohydrate content.

Schramm & Armbruster (1954) have reported that the molecular weight of intestinal phosphatase is 60000. This figure is probably a reliable estimate, even though the activity of the preparation is well below that used in the present studies. In the absence of evidence to the contrary, it is assumed that the milk enzyme has a molecular weight of the same order, that is, 60000. The estimates of Mathies & Goodman (1953) of molecular weights of 5×10^5 and greater for kidney and intestinal phosphatases were based on studies with impure preparations and cannot be accepted as true of the purified enzymes.

Activation of alkaline phosphatases by metals

It is now clearly established that many alkaline phosphatases, even when partially purified, are activated by a number of metal cations (see reviews by Folley & Kay, 1936*a*; Roche, 1945; Roche & Thoai, 1950; and Lehninger, 1950). The present investigations have extended previous studies.

As shown in Fig. 4, crude milk phosphatase is activated by a number of metals, for each of which there is an 'optimum' concentration for maximal

enzymic activity. Comparison of these results with those obtained with the purified milk enzyme (Fig. 5) shows that the presence of impurities can markedly influence both the qualitative and quantitative effect of added metal salts.

Comparison of Figs. 5 and 6 shows that the activity of the intestinal enzyme is relatively much greater in the absence of added metal salts than is the activity of the milk enzyme. Both preparations were highly purified, dialysed against glass-distilled water, and generally treated in an identical manner. The difference may be due to stronger binding of an activating metal by the intestinal enzyme and this may be related to the much higher activity of the intestinal enzyme (83 500 units/mg. N) as compared with that from milk (15 300 units/mg. N). However, in spite of this difference in the 'control' levels, both enzymes show maximal activity with the same concentration (about 10^{-2} M) of added magnesium salt. This concentration of magnesium salt appears to be partly independent of the enzyme concentration, since earlier studies (Morton, 1952), using twenty times the concentration of purified enzyme as that used for Fig. 5, had given a similar result. The molar concentration of metal salts giving maximal effects (Figs. 5 and 6) are of the order of 10^7 to 10^8 the molar concentration of enzyme, and there is little influence of added metals at the same concentration as the enzyme, i.e. about 2×10^{-10} M.

The results suggest that, as in the case of pyrophosphatases (Bauer, 1937) and peptidases (Smith, Davis, Adams & Spackman, 1954), metal is necessary for the formation of active phosphatase–substrate complexes.

The inhibition of crude and partially purified alkaline phosphatase by beryllium salts has been extensively investigated (DuBois, Cochran & Mazur, 1949; Grier, Hood & Hoagland, 1949; Klemperer, Miller & Hill, 1949; Schubert & Lindenbaum, 1954), particularly since this inhibition may be related to the pathological condition resulting from contact with beryllium salts (Lindenbaum, White & Schubert, 1954). It is noteworthy, however, that zinc salts, which are relatively non-toxic when applied externally, also strongly inhibit purified milk and intestinal phosphatases (Figs. 5 and 6) as well as partially-purified enzymes (Sanadi, 1952). It seems unlikely that beryllium inhibition is merely due to interaction of precipitated beryllium hydroxide and the enzyme (Veerkamp & Smits, 1953). In the experiments described in Figs. 5 and 6, the copious precipitates of magnesium and manganese hydroxides which occurred at the highest concentrations of these metals did not cause much inhibition. The results obtained by Veerkamp & Smits (1953) may be due to the use of crude enzyme preparations, which are very readily adsorbed on to a variety of materials (Morton, 1953*b*, 1954*a*).

Amino acid activation and the so-called 'coenzyme' of alkaline phosphatase

There have been numerous claims for identification of organic coenzymes of alkaline phosphatases. Such a component was claimed to explain reversible loss of activity induced by dialysis at low pH as observed by Albers (1936) using partially purified preparations of kidney phosphatase. Using impure preparations from a variety of sources, different workers have claimed to identify the 'coenzyme' as organic material, possibly nitrogenous (Abul-Fadl & King, 1949*b*), alanine (Thoai *et al.* 1947), histidine (Akamatsu & Kobayashi, 1952) or a histidyl peptide (Akamatsu & Aso, 1953), a peptide (Ek, Euler & Hahn, 1949), choline pyrophosphate (Kutscher & Sieg, 1950), a diphosphonucleoside (Lora-Tamayo & Municio, 1951) and uridylic acid (Lora-Tamayo & Alvarez, 1954).

As pointed out earlier, the chemical analyses show that neither choline pyrophosphate nor any nucleotide are present in either purified phosphatase. The claims for amino acids or peptides as coenzymes require separate consideration.

Thoai *et al.* (1947) found that a partially purified preparation of alkaline phosphatase from dog intestinal mucosa lost most of its activity on prolonged dialysis against distilled water. After pre-incubation of the dialysed enzyme with magnesium chloride and alanine, the activity was restored to a level appreciably higher than before dialysis. Roche & Bouchilloux (1950, 1953) therefore regularly employ pre-incubation with magnesium chloride and alanine in determining phosphatase activity.

The results in Table 4 show that alanine causes no activation of freshly prepared intestinal alkaline phosphatase when activity is determined in veronal–HCl buffer at near-optimum pH conditions in the presence of magnesium salt. However, alanine undoubtedly exerts some protective action under certain conditions of treatment of the enzyme and is especially effective in reducing the inhibitory effect of carbonate anions (Tables 3 and 4). The slight activation by alanine observed by Schramm & Armbruster (1954) may be due to the use of sodium bicarbonate in the buffer used in determination of activity. In the absence of magnesium salt, activation by alanine may be quite considerable (Tables 3 and 4) especially with enzyme stored for long periods (Table 5; cf. Gomori, 1952). However magnesium salts alone cause much greater activation (Table 5) and the possibility that activating metals are introduced with the amino acids has not been excluded. This seems the more likely, since the milk enzyme, which responds to low concentrations of metal more than does the intestinal enzyme (Figs. 5 and 6), also shows greater activation by added amino acids (Table 5).

The partial reversal of metal inhibition of phosphatases (Table 5) by added alanine and histidine (Table 5) suggests that, in certain circumstances, activation by amino acids may be due to removal of metal inhibitors. As distinct from Thoai *et al.* (1947), Abul-Fadl & King (1949*b*) and Akamatsu & Kobayashi (1952) observed greater activation of dialysed crude and partially purified alkaline phosphatase preparations by histidine rather than alanine. Table 5 shows histidine to be more effective than alanine with the inhibited enzymes.

The variety of different compounds claimed to be the 'coenzymes' of alkaline phosphatases strongly suggests that the re-activation of dialysed preparations is not specific, and thus no true organic coenzyme exists. The chemical analyses, maximal activation by magnesium salt only (Table 4), the 'protective' action of certain amino acids (Tables 3 and 4), and the activation of inhibited enzyme (Table 5) all support this view. From the established metal dependency of alkaline phosphatases (Figs. 5 and 6) and the ability to form stable complexes with inhibitory metals and certain organic acids (Schubert & Lindenbaum, 1954), it is evident that the products liberated by autolysis or proteolysis of tissues would greatly modify the behaviour of these enzymes. It is not unexpected, therefore, that different results have been reported by various workers, according to the experimental procedures and the purity of the enzyme preparations used. The results and conclusions given in this paper are based on enzymes purified without use of proteolysis.

Alkaline phosphatases of different origin

In this investigation, results were obtained with purified bovine enzymes isolated from cow's milk and calf intestinal mucosa. These enzymes resemble each other showing a similar substrate specificity (Morton, 1952, 1955), activation by metal cations (Figs. 5 and 6) and phosphotransferase activity (Morton, 1953*c*). However, the intestinal enzyme is about 5 times as active as the milk enzyme, the respective specific activities being 83 500 units/mg. N (Morton, 1954*a*) and 15 300 units/mg. N (Morton, 1953*a*). Moreover, the enzymes show differences in optimum pH values for hydrolysis of different substrates (Fig. 2), in the type of response to added metal salts (Figs. 5 and 6) and in the K_m values with the one substrate at selected pH values (Morton, 1952). Clearly the enzymes are quite different. Moog (1946) has discussed previous evidence for the existence of different alkaline phosphatases and has rejected these claims, rightly pointing out that 'the uncertainty as to the components of phosphatase extracts necessitates that claims of differences must, to be acceptable, be based on thoroughly purified preparations'. This requirement has now been met for the first time.

SUMMARY

1. The properties of purified alkaline phosphatases from cow's milk and calf intestinal mucosa have been studied. Chemical analyses show that both enzymes are colourless unconjugated proteins. Both are substantially free of organic phosphorus, and of nucleotides or related compounds. They may contain small amounts of carbohydrate. The tyrosine and tryptophan contents are very similar. The two enzymes differ in their activity/pH relationships.

2. The influence of magnesium, manganese, calcium, zinc and beryllium on the activities of the two enzymes has been investigated, using both crude and purified enzyme preparations. Maximum activity of the purified enzymes is obtained with magnesium chloride (10^{-2} M). Beryllium and zinc salts both cause considerable inhibitions.

3. Pre-incubation of the purified enzymes with alanine and magnesium causes no greater activity than that obtained using magnesium acetate alone without any pre-incubation. Alanine protects the enzyme under certain treatments. The effects observed with alanine and other amino acids is discussed in relation to the experimental procedures used by various workers.

4. No evidence has been obtained to support numerous claims for the existence of dissociable organic 'coenzymes' of alkaline phosphatases.

5. Evidence is presented to show that the milk and intestinal enzymes are distinctly different, although having similar specificities.

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Minor Constituents of Quebracho Tannin Extract

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Previous papers in this series (White, 1949; Kirby, Knowles & White, 1951; White, Kirby & Knowles, 1952) have shown that each so-called 'vegetable tannin' is a complex mixture of many substances—far more so than has been generally realized. The two most important tannin extracts, 'Mimosa' or 'Wattle' extract from the bark of *Acacia mollissima* and 'Quebracho' extract from the heartwood of *Schinopsis Lorentzii* Engl., were selected for detailed study of this complexity. Two-dimensional paper chromatography enabled characterization of the numerous substances of unknown nature present in each extract and provided, for the first time, a method of assessing the efficiency of fractionation procedures applied to them. Using this approach each extract was split into fractions by solvent extraction or by countercurrent distribution between water and organic solvents (Kirby, Knowles & White, 1952, 1953). The components present in any fraction and the degree of component overlapping from one fraction to the next were determined by two-way paper chromatography, the presence of the components being detected by their fluorescence in ultraviolet light or by reaction with appropriate spray reagents. In the specific case of Quebracho tannin extract the material could be