The Metal Ion Activation of the Alkaline β-Glycerophosphatase of Rabbit Small Intestine

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1. A fraction of intestinal epithelial cells from rabbit small intestine that contained nuclei and microvillus membranes served as a source of alkaline-β-glycerophosphatase activity. 2. The greater part of the enzyme activity could be released from the subcellular particles by disintegration of the latter, followed by centrifugation at 40000*g* and butanol extraction of the resulting sediment. 3. Further purification of the enzyme was achieved by diethylaminoethylcellulose chromatography and by gel filtration. 4. Dialysis of the purified enzyme preparations against EDTA gave an essentially inactive enzyme. High activity could be restored by adding $Zn^{2+}+Mg^{2+}$, $Zn^{2+}+Co^{2+}$, $Mg^{2+}+Co^{2+}$ or Co^{2+} alone to these inactive preparations. Neither Zn^{2+} nor Mg^{2+} added singly to the assay system restored more than a small part of the enzyme activity. 5. The optimum Zn^{2+} concentration was about 0·2–1m-equiv./l., whereas Mg^{2+} and Co^{2+} had optimum concentrations about 30–60m-equiv./l. 6. If added in excess of the optimum concentration, Zn^{2+} strongly inhibited the enzyme under all conditions tested. 7. In the presence of an optimum concentration of Co^{2+} (33m-equiv./l.) in tris buffer at the optimum pH (8·8 at 37°), K_m for the β-glycerophosphatase was 0·3mM.

A review of 30 investigations in which alkaline phosphatase has been assayed shows that in only four investigations published since 1940 has a magnesium salt not been included in the assay solution to provide the activating metal ion for the enzyme (excluded from this count are those assays where omission of magnesium salt constituted a 'control' on an assay in which magnesium salt was included). The investigations in which a magnesium salt was deliberately omitted from a routine assay medium stem from the work of Garen & Levinthal (1960) and are discussed below. Recommended assay procedures (Heppel, 1955; Morton, 1955*a*; Volkin, 1955) each involve the deliberate and obligatory addition of Mg^{2+} to the assay system. Only one of these authors (Morton, 1955*a*) mentions other activating bivalent metal ions but states categorically that added Mg^{2+} give maximum activation. J. W. Porteous & B. Clark (unpublished work) found that only 25–75% of the alkaline-β-glycerophosphatase activity of homogenates of epithelial cells of rabbit small intestine could be recovered from the isolated subcellular fractions of these cells when enzyme assays were performed in the presence of added Mg^{2+}. Satisfactory recovery of the activity among the subcellular fractions was achieved (Porteous & Clark, 1965) when the homogenates and the individual fractions were dialysed against water and assayed in the presence of added Co^{2+}. Accordingly the activation by bivalent metal ions of crude and partially purified preparations of the alkaline β-glycerophosphatase of the epithelial cells of rabbit small intestine was investigated. Preliminary accounts of the results have been published (Clark & Porteous, 1963*a*,*b*).

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EXPERIMENTAL

Isolation and purification of enzyme preparations. Except where otherwise indicated all manipulations were carried out at 2–4°. Fraction III of homogenates of rabbit-intestine epithelial cells (Porteous & Clark, 1965) was dialysed against 5mM-EDTA, pH7·4, for 2hr. and then for 16hr. against glass-distilled water adjusted to pH7·4 with $NaHCO_3$. The non-diffusible material was called fraction IIID and used as a source of crude enzyme.

Enzyme preparations of higher specific activity were prepared from the combined subcellular fractions III and IV (Porteous & Clark, 1965) in the following way. The suspension (approx. 100ml. containing 3–10mg. of protein/ml.) was dialysed for 16hr. against glass-distilled water. The non-diffusible material was called preparation *a*. Preparation *a* was diluted to 200ml. with glass-distilled water,

homogenized in an Ato-Mix Blender (Measuring and Scientific Instruments Ltd.) at full speed for 2min. and filtered through a small plug of glass wool. The filtrate (preparation *b*) was centrifuged ($2{\cdot}4 \times 10^6 g$-min.) to give a sediment containing approx. 80% of the enzyme activity but only 30% of the protein of preparation *a*. The sediment was taken up in glass-distilled water (20–25ml.) to give preparation *c*. Part of this suspension (20ml.) was extracted with butan-1-ol (8ml.). The solvent was added dropwise with efficient stirring and the mixture heated in a water bath (37°) for 20min. After cooling in an ice bath the mixture was centrifuged at 1500*g* for 30min. The upper butan-1-ol layer and the interface layer of solid material were discarded. The lower aqueous phase was dialysed for 3hr. against glass-distilled water. The non-diffusible material (preparation *d*) was centrifuged ($4{\cdot}5 \times 10^6 g$-min.) to give a water-clear supernatant (preparation *e*). The sediment was discarded.

DEAE-cellulose column chromatography. Preparation *e* was applied to a DEAE-cellulose column (1·5cm. × 10cm.) that had been equilibrated with 0·05M-tris buffer, pH7·1. The column was then washed with 0·05M-tris buffer, pH7·1 (200ml.), before applying a linear concentration gradient of NaCl (0–0·7M) in 0·05M-tris buffer, pH7·1. The enzyme was eluted as a water-clear solution between 0·2M- and 0·4M-NaCl. The contents of the tubes containing enzyme activity were combined to give preparation *A*, which contained 31% of the activity of preparation *e* and showed a 50-fold increase in specific activity (enzyme units/mg. of protein) over preparation *a*. No further activity could be eluted from the DEAE-cellulose columns with NaCl concentrations up to 1·0M. The remainder of the activity could be eluted as a clear solution with 1·0M-NaCl containing 0·4% of sodium deoxycholate or sodium taurocholate. Removal of the deoxycholate or taurocholate by dialysis left a non-diffusible turbid enzyme preparation.

Gel filtration. For this 20g. of Sephadex G-100 (Pharmacia, Uppsala, Sweden) was suspended in 0·05M-tris buffer, pH7·1, and allowed to stand for 24hr. A column (2·2cm. diam.) was then filled with the suspension to a height of 55cm. and 0·05M-tris buffer, pH7·1, allowed to pass through the column for 16hr. Previously concentrated fraction *A* (1·5ml. containing 112 units of enzyme activity) was then applied to the column. Elution was carried out with 0·05M-tris buffer, pH7·1, at a flow rate of 6–12ml./hr. Fractions were collected every 20min. and assayed for enzyme activity. The contents of the tubes containing activity were combined to give preparation *S* (55 enzyme units). The water space external to the Sephadex beads (V_o) was determined by measuring the volume of buffer necessary to elute a diluted sample of indian ink applied to the top of the column.

Enzyme activity measurements. The following method was used for tissue preparations [fractions I–X described by Porteous & Clark (1965) and dialysed preparations of these fractions] and for relatively crude enzyme preparations such as *a*, *b* and *c* described in the present paper (Table 5). The enzyme was preincubated at 37° for 20min. in tris buffer (43 or 66mM) containing (unless otherwise specified) $CoSO_4$ (17mM). Sodium β-glycerophosphate was then added to give a concentration of 17mM and to bring the volume to 3ml. The final pH value was 8·8 at 37°. The reaction was terminated after 20min. by the addition of 50% trichloroacetic acid (0·5ml.). The assay tubes were cooled in ice and the contents filtered after 10min. A sample of the filtrate (2ml.) was then analysed for inorganic orthophosphate by the method of King (1932). Control assay tubes from which substrate or enzyme had been omitted were incubated for each assay.

The same method was used for the assay of partially purified preparations (preparations *d*, *e*, *A–D* and *S*; Table 5) except that the tris buffer concentration was raised to 170mM and that deproteinization and filtration were found to be unnecessary and were omitted; enzyme activity was terminated by the addition of 2N-H_2SO_4 (1ml.). Ammonium molybdate (0·5ml. of a 5% solution) and 0·1% aminonaphtholsulphonic acid reagent (0·5ml.) were then added in succession. The tubes were left at room temperature for 20min. before measuring the extinction (Unicam SP.300 colorimeter; Unicam Instruments, Cambridge) with a red filter (Ilford no. 204).

Preliminary experiments were carried out to ensure that the liberation of orthophosphate was linear with respect to time of incubation and protein concentration, and that the assay was carried out at the optimum substrate concentration and pH value. The experimentally determined Michaelis constant (preparation *A*; Table 5) for β-glycerophosphate was approx. 0·3mM at the optimum Co^{2+} ion concentration (34m-equiv./l.) and at pH8·8 (37°). It was necessary to preincubate the preparation for 20min. at 37° in the presence of tris buffer and metal salt, but in the absence of β-glycerophosphate, to obtain maximum enzyme activity.

Variations in the composition of the enzyme assay system are described in the Results section and principally involve replacement of the $CoSO_4$ by other specified salts.

Enzyme units. One unit of enzyme activity was defined as that amount of enzyme which released 1μmole of inorganic orthophosphate when incubated for 20min. at 37° and at pH8·8 with sodium β-glycerophosphate (17mM) and $CoSO_4$ (17mM) in tris buffer (43–170mM).

Buffer solutions. Tris buffer consisted of tris base (C. F. Boehringer und Soehne G.m.b.H., Mannheim, Germany) adjusted to the required pH with HCl and diluted to the molarity indicated.

Determination of protein. Protein was determined by the method of Lowry, Roseborough, Farr & Randall, as modified by Miller (1959). Elution of protein from DEAE-cellulose columns was followed by measuring the extinction value of each fraction at 280mμ.

Determination of pH *values.* The pH measurements were made with a direct-reading pH-meter (model 23A; Electronic Instruments Ltd., Richmond, Surrey).

Reagents. DEAE-cellulose was prepared by the method of Peterson & Sober (1961), with 100–200 mesh Solka Floc (Brown Co., Boston, Mass., U.S.A.) as the starting material. The prepared DEAE-cellulose had pK_a 9·6 and a base content of approx. 0·7m-equiv./g. EDTA, aminonaphtholsulphonic acid and sodium β-glycerophosphate were laboratory reagents of high purity. Other chemicals used were of AnalaR grade. Glass-distilled water was used throughout.

RESULTS

Metal ion activation of crude alkaline β-glycerophosphatase. The effect of several bivalent metal ions on the alkaline-phosphatase activity of fraction IIID [fraction III of a homogenate of rabbit-

intestine epithelial cells (Porteous & Clark, 1965) dialysed against EDTA and water; see the Experimental section] is shown in Table 1.

Each metal salt was added to the enzyme assay system to give a final concentration of 17mM-salt (a cation concentration of 34m-equiv./l.). At this concentration, Co^{2+} had the greatest effect on the activity of the enzyme; Ca^{2+}, Ba^{2+} and Fe^{2+} were each more effective than Mg^{2+}. The effects of different concentrations of added Co^{2+}, Ca^{2+}, Fe^{2+} or Mg^{2+} on enzyme activity are shown in Fig. 1.

At all concentrations of added cations (from 6·6 to 66m-equiv./l.) the enzyme activity was greatest in the presence of added Co^{2+}. Lower activities were obtained with Ca^{2+} and Fe^{2+}, and added Mg^{2+}

Table 1. *Metal ion activation of alkaline β-glycerophosphatase in fraction III D*

The preparation of fraction III D and the enzyme assay procedure are described in the Experimental section. The assay system consisted of fraction III D (0·1 ml. containing 0·6mg. of protein), tris buffer, pH 9·3 (43mM), sodium β-glycerophosphate (17mM) and one of the metal salts indicated (17mM) in a final volume of 3ml. The relative activity of 100 corresponded to the liberation of 0·8μmole of inorganic orthophosphate in the assay system after incubation at 37° for 20min. The specific activity of the enzyme in the presence of Co^{2+} (34m-equiv./l.) was 1·3units/mg. of protein. —, Not measured.

Metal salt added	Relative enzyme activity	
	Expt. 1	Expt. 2
None	15	8
Magnesium acetate	38	31
$CaCl_2$	73	75
$BaCl_2$	46	46
$Ni(NO_3)_2$	12	—
$CoSO_4$	100	100
$MgCl_2$	35	—
Zinc acetate	31	—
$CuSO_4$	19	—
$FeSO_4$	73	72

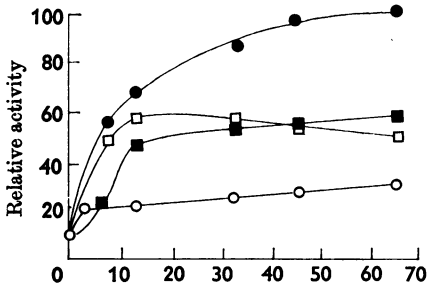

Fig. 1. Metal ion activation of the alkaline β-glycerophosphatase of fraction III D (Table 5). The assay system was the same as that given in Table 1 except that the concentrations of $CoSO_4$ (●), $CaCl_2$ (■), $FeSO_4$ (□) and magnesium acetate (○) were varied as indicated. The relative activity of 100 for this enzyme preparation is defined in Table 1.

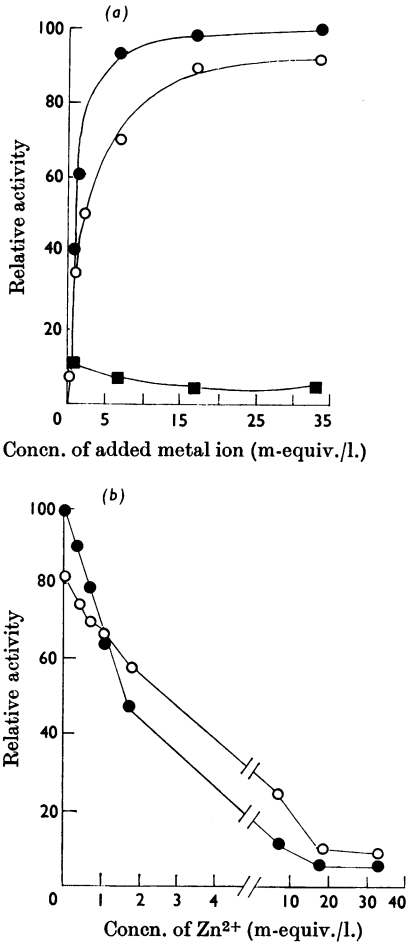

Fig. 2. Metal ion activation of the alkaline β-glycerophosphatase of preparation *A* (Table 5). Alkaline-β-glycerophosphatase activity was determined as described in the Experimental section except that 17mM-$CoSO_4$ was replaced by the salts indicated. The relative activity of 100 corresponded to the release of 0·93μmole of inorganic orthophosphate in the assay system. In the presence of added Co^{2+} (34m-equiv./l.) the specific activity of the enzyme was 57units/mg. of protein (cf. Table 1). Metal ion concentrations were varied as follows. (*a*): ●, Increasing $MgSO_4$ concentration; ○, increasing $CoSO_4$ concentration; ■, increasing $ZnSO_4$ concentration. (*b*): ●, $ZnSO_4$ concentration varied in the presence of a constant concentration of $MgSO_4$ (17mM); ○, $ZnSO_4$ concentration varied in the presence of a constant concentration of $CoSO_4$ (17mM).

gave the lowest activities at all concentrations tested.

Metal ion activation of preparation A. Both Co^{2+} and Mg^{2+} activated this preparation but Zn^{2+}, added at concentrations up to 33m-equiv./l., caused little activation of the enzyme (Fig. 2*a*). The enzyme activity in the presence of added Mg^{2+} rose to a maximum at a concentration of 33m-equiv./l. The same activity was obtained with a Mg^{2+} concentration of 66m-equiv./l. This maximum activity was assigned an arbitrary value of 100 and the activity of preparation *A* in the presence of various other metal ions expressed relative to this value (Figs. 2, 3 and 4, and Tables 2 and 3). Alkaline-β-glycerophosphatase activity in the presence of added Co^{2+} reached a maximum value of 93, again at a concentration of 33m-equiv./l. Enzyme activity in the presence of optimum Co^{2+} or Mg^{2+} (33m-equiv./l.) was markedly decreased by further additions of Zn^{2+} (Fig. 2*b*).

Dialysis of portions of preparation *A* against various concentrations of EDTA at pH 7·4 gave preparations with enzyme activities shown in Fig. 3. After dialysis against 0·5mM-EDTA, the addition of Co^{2+} to the previously determined optimum concentration gave an activity of 70, whereas Mg^{2+} in place of Co^{2+} gave an activity of only 5. Dialysis of preparation *A* against glass-distilled water for up to 3 days caused only a 10% loss of activity when assayed with Mg^{2+} and no loss of activity when assayed with Co^{2+}.

Preparation *B* was obtained by dialysing a larger portion of preparation *A* against 0·5mM-EDTA, pH 7·4, for 4–16hr. Preparation *B* was then assayed in the presence of added Co^{2+} and Mg^{2+} (Fig. 4): Co^{2+} (33m-equiv./l.) gave an activity of 92; Mg^{2+} alone (0·7–250m-equiv./l.) did not restore the activity of preparation *B*, but the further addition of Zn^{2+} (2m-equiv./l.) restored the activity to 75% of its original value (Expt. 1 in Table 2). The alternative further addition of Cu^{2+}, Ca^{2+}, Mn^{2+} or Fe^{2+} (all at 2m-equiv./l.) had little or no effect on the activity of preparation *B* in the presence of Mg^{2+} (66m-equiv./l.). The change in activity with change in Zn^{2+} concentration in the presence of a fixed concentration of Mg^{2+} (66m-equiv./l.) is shown in Table 2 (Expt. 2); almost complete (99%) reactivation was obtained by the addition of Zn^{2+} at 1m-equiv./l. Higher concentrations of Zn^{2+} reversed this activation. Since a small amount of EDTA was present in preparation *B* (approx. 0·17 mM in the final assay) the apparent optimum Zn^{2+} concentration was probably higher than it would have been in the complete absence of EDTA. When added alone Zn^{2+} caused only a small activation of preparation *B* (Expt. 2 in Table 2).

Portions of preparation *B* were dialysed at 4° for 16hr. against glass-distilled water to remove traces of EDTA and then against either 5mM-zinc sulphate or 5mM-cobalt sulphate at 4° for 8hr. Any metal ions not tightly bound to the protein were removed by subsequent dialysis against glass-distilled water at 4° for 16hr. The portion of preparation *B* treated

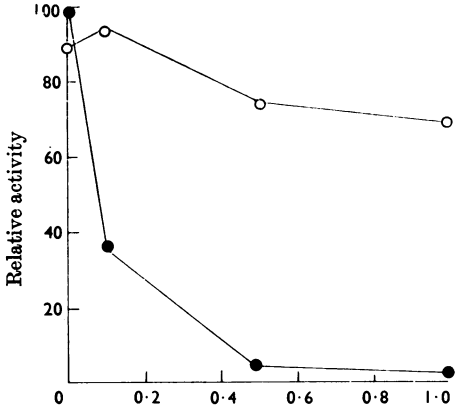

Fig. 3. Effect of dialysis (at 4° for 16hr. against various concentrations of EDTA, pH 7·4) on the alkaline-β-glycerophosphatase activity of preparation *A* (Table 5). The activity of dialysed preparations was assayed: ●, in the presence of added $MgSO_4$ (17mM); ○, in the presence of added $CoSO_4$ (17mM). The assay system was that described in the text except for the variations in the added metal ions. The relative enzyme activity of 100 is defined in Fig. 2.

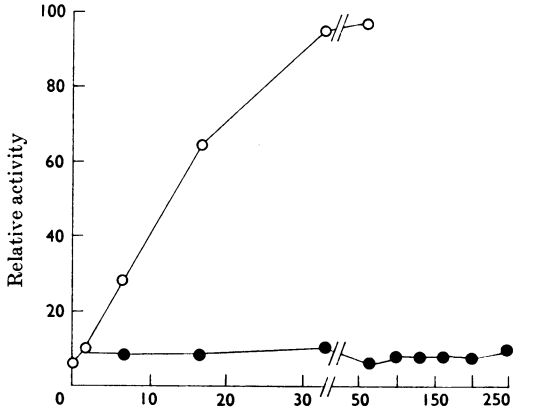

Fig. 4. Metal ion activation of the alkaline β-glycerophosphatase of preparation *B* (Table 5). The relative enzyme activity of 100 is defined in Fig. 2. The assay system was that described in the Experimental section except for the variations in metal ion concentration indicated in the Figure. ●, $MgSO_4$ added to the assay system; ○, $CoSO_4$ added to the assay system.

Table 2. *Metal ion activation of the alkaline β-glycerophosphatase of preparation* B (*Table* 5)

The assay system for Expts. 1 and 2 was that given in the Experimental section except that $CoSO_4$ was replaced by $MgSO_4$, $ZnSO_4$, $CuSO_4$, $CaCl_2$, $MnSO_4$ or $FeSO_4$ as indicated. The relative enzyme activity of 100 is defined in Fig. 2.

Expt. no.	Addition(s) (m-equiv./l.)	Relative activity
1	Mg^{2+} (66)	1
	Mg^{2+} (66)+Zn^{2+} (2)	75
	Mg^{2+} (66)+Cu^{2+} (2)	2
	Mg^{2+} (66)+Ca^{2+} (2)	1
	Mg^{2+} (66)+Mn^{2+}(2)	1
	Mg^{2+} (66)+Fe^{2+} (2)	1
2	Mg^{2+} (66)	5
	Mg^{2+} (66)+Zn^{2+} (0·2)	40
	Mg^{2+} (66)+Zn^{2+} (0·4)	96
	Mg^{2+} (66)+Zn^{2+} (1·0)	99
	Mg^{2+} (66)+Zn^{2+} (2·0)	81
	Mg^{2+} (66)+Zn^{2+} (4·0)	46
	Mg^{2+} (66)+Zn^{2+} (8·0)	18
	None	4
	Zn^{2+} (1)	16
	Zn^{2+} (2)	13
	Zn^{2+} (4)	28
	Zn^{2+} (10)	25

Table 3. *Metal ion activation of the alkaline β-glycerophosphatase of preparations* A, B, C *and* D (*Table* 5)

The assay system was that given in the Experimental section except that the added metal ions were those shown in the Table. The relative enzyme activity of 100 is defined in Fig. 2.

Metal ion(s) added (m-equiv./l.) Preparation...	Relative activity *A*	*B*	*C*	*D*
None	23	3	13	22
Mg^{2+} (33)	100	14	138	177
Mg^{2+} (33)+Zn^{2+} (1)	68	92	80	105
Co^{2+} (33)	77	130	108	148

with zinc sulphate was called preparation *C*, and that treated with cobalt sulphate preparation *D*.

With preparations *A*, *C* and *D* (but not with preparation *B*) Mg^{2+} added alone gave high activity (Table 3). The concomitant addition of Zn^{2+} decreased the activity of preparations *A*, *C* and *D*, but increased the activity of preparation *B*; both preparations *C* and *D* had higher activities in the presence of added Mg^{2+} or Co^{2+} than did the original preparation *A*. Preparation *D* with added Mg^{2+} gave the highest activity of all (177). When added alone Co^{2+} gave a high activity with each of the four preparations.

Sephadex G-100 filtration. A portion of preparation *A* was further purified by passage through

Table 4. *Metal ion activation of the alkaline β-glycerophosphatase of preparation* S (*Table* 5) *before and after dialysis against EDTA*

The assay system was that described in the Experimental section except for variations in the kind and quantity of added metal ion as indicated in the Table. Each appropriate assay vessel received 0·1 ml. of enzyme preparation containing approx. 1 μg. of protein. The relative activity of 100 corresponded to the liberation of 0·2 μmole of inorganic orthophosphate in the assay system. The specific activity of the enzyme in the presence of Co^{2+} (33·3 m-equiv./l.) was approx. 120 units/mg. of protein (cf. Table 1 and Fig. 2).

Preparation	Addition(s) (m-equiv./l.)	Relative activity
S (undialysed)	Mg^{2+} (66)	100
S (EDTA-treated)	None	5
	Mg^{2+} (66)	10
	Mg^{2+} (66)+Zn^{2+} (0·07)	65
	Mg^{2+} (66)+Zn^{2+} (0·17)	92
	Mg^{2+} (66)+Zn^{2+} (0·33)	89
	Mg^{2+} (66)+Zn^{2+} (1·7)	85
	Mg^{2+} (66)+Zn^{2+} (3·3)	78
	Mg^{2+} (66)+Zn^{2+} (6·6)	58
	Mg^{2+} (66)+Zn^{2+} (16·6)	37
	Mg^{2+} (66)+Zn^{2+} (33·3)	12
	Zn^{2+} (0·17)	17
	Zn^{2+} (0·7)	16
	Zn^{2+} (3·3)	13
	Zn^{2+} (16·6)	7
	Zn^{2+} (33·3)	9
	Mg^{2+} (66)+Co^{2+} (0·7)	44
	Mg^{2+} (66)+Co^{2+} (1·7)	54
	Mg^{2+} (66)+Co^{2+} (3·3)	56
	Mg^{2+} (66)+Co^{2+} (6·6)	77
	Mg^{2+} (66)+Co^{2+} (16·6)	82
	Mg^{2+} (66)+Co^{2+} (33·3)	84
	Co^{2+} (1·7)	21
	Co^{2+} (6·6)	30
	Co^{2+} (33·3)	59

Sephadex G-100. The V_o for this Sephadex column was approx. 55 ml. The enzyme activity first appeared in the eluate when 56 ml. of buffer had passed down the column. A further 20 ml. of buffer was required to elute the remaining activity as a narrow symmetrical peak. The enzyme thus appeared to be completely excluded from Sephadex G-100, suggesting a molecular weight greater than 100000. The β-glycerophosphatase activity of the Sephadex G-100 eluate (preparation *S*) was taken as 100, with Mg^{2+} added to a concentration of 66 m-equiv./l. This preparation was dialysed against 5 mM-EDTA, pH 7·4, for 4 hr. and then against glass-distilled water for 16 hr.

The dialysed preparation *S* was assayed for activity in the presence of various concentrations of added Mg^{2+}, Co^{2+} and Zn^{2+}, as shown in Table 4. The addition of Mg^{2+} alone (66 m-equiv./l.) to the

EDTA-treated preparation *S* gave an activity of 10, but the further addition of Zn^{2+} ions (0·17m-equiv./l.) gave 92% of the original activity. Higher Zn^{2+} concentrations inhibited the enzyme. Added Zn^{2+} alone did not markedly activate the enzyme. When added alone to the EDTA-treated preparation *S* Co^{2+} (33m-equiv./l.) gave an activity of 59. Increased concentrations of Co^{2+} in the presence of a fixed concentration (66m-equiv./l.) of Mg^{2+} gave greater increases in activity than did the addition of Co^{2+} alone. The activity was restored to 84 by the concomitant addition of Co^{2+} (33m-equiv./l.) and Mg^{2+} (66m-equiv./l.).

DISCUSSION

Our results indicate that addition of cobalt salt to crude cell homogenates and to isolated subcellular fractions that had been dialysed against EDTA or against water (Fig. 1 and Table 1; see also Porteous & Clark, 1965), or to various partially purified enzyme preparations (Figs. 2*a*, 3 and 4, and Tables 3 and 4), invariably gave high activity though not necessarily the highest activity obtainable. Partially purified enzyme preparation *A* that had not been dialysed against EDTA (Table 5) gave higher activity with added Mg^{2+} than it did with added Co^{2+} (Table 3 and Fig. 2*a*); preparation *B* (Table 5), on the other hand, was not activated by added Mg^{2+} but was activated by added Co^{2+} (Figs. 3 and 4) or by concomitant addition of Mg^{2+} and Zn^{2+} (Tables 2 and 3), provided that Zn^{2+} was added in low concentration.

We interpret these results to mean that partially purified preparation *A* (Table 5) contained the alkaline β-glycerophosphatase in the form of a metallo-enzyme or metal–enzyme complex (Vallee, 1955) that required the addition of a bivalent metal ion (Mg^{2+} or Co^{2+}) before high activity could be elicited. Treatment of preparation *A* with EDTA (Table 5) then removed any endogenous metal ion affecting the activity of the enzyme and gave essentially inactive preparations (Fig. 4 and Tables 2 and 3) to which full activity could be restored only by adding any pair of the metal ions Co^{2+}, Mg^{2+} and Zn^{2+} (provided that Zn^{2+}, if added, was present in low concentration) or by adding Co^{2+} alone (Figs. 3 and 4, and Tables 2 and 3). This interpretation is supported by the results of experiments in which attempts were made to remove residual EDTA from dialysed enzyme preparations and to equilibrate the presumably metal-free enzyme with Zn^{2+} or Co^{2+} (preparations *C* and *D* in Table 3) and by the results obtained (Table 4) before and after dialysis of preparation *S* (Table 5) against EDTA and water. The results obtained with crude enzyme preparations (Table 1 and Fig. 1) appear to be consistent with this interpretation of the requirement for bivalent metal ions for full activity of the enzyme under the conditions of assay employed.

Table 5. *Summary of the origin of various alkaline-β-glycerophosphatase preparations from fractions III and IV of a homogenate of intestinal epithelial cells (Porteous & Clark, 1965)*

Full details of the preparations are given in the Experimental and Results sections.

Starting material	Treatment	Preparation
Fraction III	Dialysed against EDTA, then against water	III D
Fractions III+IV	Dialysed against water	*a*
a	Disintegrated and filtered	*b*
b	Centrifuged; retained sediment	*c*
c	Butanol extracted; aqueous phase dialysed against water; non-diffusible material retained	*d*
d	Centrifuged; supernatant retained	*e*
e	Eluted from DEAE-cellulose	*A*
A	Dialysed against EDTA	*B*
B	Dialysed against water, then against $ZnSO_4$ and once more against water	*C*
B	Dialysed against water, then against $CoSO_4$ and once more against water	*D*
A	Gel filtration through Sephadex G-100	*S*
S	Dialysed against EDTA then against water	Dialysed *S*

Since other alkaline phosphatases have been shown to be zinc metallo-enzymes (Mathies, 1958; Trubowitz, Feldman, Morganstern & Hunt, 1961; Engström, 1961*a*,*b*; Plocke, Levinthal & Vallee, 1962) it is possible that the rabbit-intestinal alkaline phosphatase also contains zinc as an integral part of its structure; direct evidence for the presence of enzyme-bound zinc is lacking. Most of our results are consistent with this possibility, but it is clear (Tables 3 and 4) that added Zn^{2+} alone confers only low activity on a presumably metal-free enzyme preparation and is, further, a powerful inhibitor of the Co^{2+}–Mg^{2+}-, Co^{2+}-, Zn^{2+}–Mg^{2+}- and Zn^{2+}–Co^{2+}-activated enzyme (Fig. 2 and Tables 2, 3 and 4). It is also clear that the presumably metal-free enzyme cannot be activated by added Mg^{2+} alone (Tables 2, 3 and 4, and Figs. 3 and 4) under the conditions of assay used. That high enzyme activity could be obtained by adding Co^{2+} alone to presumably metal-free enzyme preparations [Figs. 1, 3 and 4, and Tables 1 and 3 (preparations *B* and *D*)] implies that this metal ion can fulfil the functions otherwise fulfilled jointly by a pair of bivalent metal ions (e.g. Mg^{2+} and Zn^{2+}) in the assay system employed. The

concentration of Co^{2+} required to elicit maximum phosphatase activity from the presumably metal-free enzyme was about 50m-equiv./l. in the absence of other added bivalent metal ions (Fig. 4) or in the presence of added Mg^{2+} (Table 4), whereas Zn^{2+}, at least in the presence of Mg^{2+}, was required at a concentration of 0·1–1·0m-equiv./l. (Tables 2 and 4). In the presence of endogenous metal ions (Fig. 2*a*) the optimum concentration of added Mg^{2+} (or Co^{2+}) was about 25m-equiv./l.

Porteous & Clark (1965) prepared a cell homogenate in a sucrose–EDTA medium, isolated subcellular fractions from the homogenate and dialysed each preparation against water. When alkaline-β-glycerophosphatase activity was assayed in the presence of added Mg^{2+} large discrepancies were observed (unpublished work) between the activity of the homogenate and the sum of the activities of the isolated subcellular fractions; the discrepancy was eliminated when assays were carried out in the presence of added Co^{2+} rather than added Mg^{2+}. Endogenous activating metal ions might have been lost in the form of metal ion–EDTA chelates during dialysis of the various preparations, but the discrepancy alluded to cannot be accounted for by a proportional decrease in the activity of each preparation assayed in the presence of added Mg^{2+}. The large amount of adventitious protein in the homogenate was differentially distributed among the subcellular fractions (Porteous & Clark, 1965). It is necessary to assume that endogenous activating metal ions were also differentially distributed during isolation of the subcellular fractions (or differentially lost during dialysis) in such a way that one or more of the fractions containing alkaline phosphatase retained none or a disproportionately small amount of the endogenous activating metal ions retained in the homogenate; it must also be assumed that activating metal ions retained in the dialysed homogenate and subcellular fractions were freely available to the enzyme under assay conditions. In the light of the preceding discussion incomplete recovery of activity among the isolated subcellular fractions would then be obtained when assays were performed in the presence of added Mg^{2+} alone, whereas assays performed in the presence of added Co^{2+} alone would give complete recovery of activity in the homogenate and in the isolated subcellular fractions.

Formation of a non-diffusible chelate with a high stability constant for the activating metal ion(s) should also inactivate the enzyme. It follows that β-glycerophosphatase might be activated or inhibited by adding, or by forming *in situ*, suitable ligands (including other proteins) to chelate with inhibitory or activating metal ions. A change in enzyme activity might then also depend on the period of incubation.

Other results on the activation of alkaline phosphatase. The present results are similar in many respects to those obtained by Cloetens (1941*a*,*b*) with a purified kidney β-glycerophosphatase that was inactivated by dialysis against cyanide and reactivated by added magnesium–zinc, magnesium–cobalt or magnesium–mercuric salt mixtures (and to a considerably smaller extent by added cobalt salt alone). The detailed results were interpreted to mean that the enzyme possessed two metal ion-binding sites, each of which had to be occupied by appropriate metal ions to achieve full enzyme activity. Thoai, Roche & Roger (1947) claimed that an inactive and purified dog-intestinal alkaline phosphatase was activated (after preincubation with alanine) by adding only one of the following: Mg^{2+}, Zn^{2+}, Ca^{2+}, Fe^{2+} and Mn^{2+}. Thoai *et al.* (1947) and Roche & Thoai (1950) suggested that the single true metal ion activator of Cloetens' enzyme was made unavailable to the enzyme because a non-diffusible protein–cyanide–metal ion complex was formed; they further suggested that a second concomitant metal ion was required solely to displace the true metal ion activator of the enzyme from this complex. It might be argued that a protein–EDTA–metal ion complex was similarly formed; this interpretation of the mode of activation of a β-glycerophosphatase by more than one metal ion seems to be incompatible with the present results (see particularly those shown in Tables 2, 3 and 4).

Most claims that alkaline phosphatases were activated by only one (added) metal ion (usually Mg^{2+}) were made before it was realized that the enzyme from several sources contained zinc as an integral part of its structure (Mathies, 1958; Trubowitz *et al.* 1961; Engström, 1961*a*,*b*; Plocke *et al.* 1962; Plocke & Vallee, 1962; but see also Roche, Bouchilloux & Roger, 1948), and that this indigenous metal was required to activate the enzyme. Any requirement for a second activating metal ion may then depend on the conditions of assay employed. Thus Hove, Elvehjem & Hart (1940), working with crude enzymes, showed a dependence of metal ion activation on the kind of phosphate ester substrate employed; Morton (1955*b*) discussed the effect of extraneous amino acids on metal ion activation of alkaline phosphatases; Garen & Levinthal (1960) showed that purified *Escherichia coli* alkaline nitrophenyl phosphatase was fully active in the presence of Zn^{2+} and M-tris, pH 8·0, but was fully active in 0·01M-tris, pH 8·0, only if Mg^{2+} was also present (see also Plocke *et al.* 1962); Plocke & Vallee (1962) demonstrated that metal ion activation or inhibition of *E. coli* alkaline nitrophenyl phosphatase was partly dependent on the stability constant of the metal ion complex of the buffer components employed in the assay system.

The metal ions shown to activate various alkaline

phosphatases may fulfil one or more of the following roles: (*a*) bonding of the inactive enzyme to an organic activator (Hofstee, 1955) or displacement of an inhibiting metal ion from an organic activator; (*b*) stabilization of the enzyme (Gomori, 1952; Garen & Levinthal, 1960; Levinthal, Signer & Fetherolf, 1962); (*c*) occupation of a specific site in the structure of the enzyme (Mathies, 1958; Trubowitz *et al.* 1961; Engström, 1961*a*,*b*; Plocke *et al.* 1962; Plocke & Vallee, 1962; but see also Roche, Bouchilloux & Roger, 1948); (*d*) displacement of an inhibiting metal ion from the enzyme; (*e*) formation of a chelate or salt with the phosphate ester to give the true substrate (Mathies, 1958); (*f*) formation of a chelate with organic inhibitors (including some substrates), so indirectly activating the enzyme (Gomori, 1952), or displacement of a true activating metal ion from a concomitant inhibitor (Thoai *et al.* 1947; Roche & Thoai, 1950).

Situations (*a*), (*b*), (*c*) and (*d*) appear to be independent of the substrate. Situation (*a*) as originally proposed involved a low-molecular-weight activator that was specific for alkaline phosphatase (see also Abul-Fadl & King, 1949; Alvarez & Lora-Tomayo, 1958). Morton (1955*b*) came to the conclusion that there was no substantial evidence for the existence of such specific organic activators of alkaline phosphatase. Situation (*a*) could possibly be rewritten as: (I) promotion by a metal ion of the reversible dissociation of the active enzyme into inactive or partially active molecules (Reithel, 1963). Such metal ion-dependent reversible associations could conceivably account for several observations that the enzyme consists of several fractions (Gomori, 1952; Mathies, 1958; Engström, 1961*a*; Moss, Campbell, Anagnostou-Kakaras & King, 1961; Grossberg, Harris & Schlamowitz, 1961; Moss & King, 1962). Situations (*b*), (*c*) and (*d*) might be subsumed as: (II) promotion by a metal ion of a conformation of the protein that is necessary for enzyme activity. Situations (*e*) and (*f*) could be summarized as: (III) formation of chelates of metal ions with various molecules so as to permit or promote catalytic hydrolysis of phosphate esters, one or more of the chelates being involved in the mechanism of ester hydrolysis. Situations (I), (II) and (III) are not, of course, mutually exclusive.

The simplest interpretation of the results obtained in the present investigation would be that the rabbit-intestinal β-glycerophosphatase contains a bivalent metal (possibly zinc) as an integral part of its structure, and that a second bivalent metal ion (Mg^{2+} or Co^{2+}) is required to activate the enzyme under the conditions of assay employed; further, if the indigenous metal is removed from the enzyme then full activity may be restored by adding Co^{2+} alone (or by adding appropriate concentrations of any pair of the metal ions Zn^{2+}, Mg^{2+} and Co^{2+}) to the assay system used in the present work. If this interpretation is valid it seems reasonable to suppose that Zn^{2+} or Co^{2+} can fulfil the roles described in (I) and (II) and that Mg^{2+} or Co^{2+} can fulfil the role described in (III).

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