Association of Inorganic-Pyrophosphatase Activity with Human Alkaline-Phosphatase Preparations

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1. The inorganic-pyrophosphatase activity of alkaline phosphatases prepared from human liver and small intestine was investigated at different stages of purification. 2. Both liver and intestinal preparations possessed pyrophosphatase activity at all stages of purification, and the two types of activity were not separated by gel filtration or by anion-exchange or cation-exchange chromatography. 3. After starch-gel electrophoresis of the tissue extracts, the zones of pyrophosphatase activity coincided exactly with alkaline-phosphatase zones. 4. Hydrolysis of each type of substrate was inhibited by the presence of the other, and a constant ratio of alkaline-phosphatase activity to pyrophosphatase activity was maintained during inactivation of the enzymes by incubation at 55°. 5. These results are consistent with the view that alkaline phosphatases are also inorganic pyrophosphatases.

It has been assumed for many years that inorganic pyrophosphatase (pyrophosphate phosphohydrolase; EC 3.6.1.1) and alkaline phosphatase (orthophosphoric monoester phosphohydrolase; EC 3.1.3.1) are distinct enzymes, though closely associated in mammalian tissues (Folley & Kay, 1936). Morton (1955) was unable to detect inorganic-pyrophosphatase activity in a purified calf-intestinal alkaline-phosphatase preparation, but little has been reported about the pyrophosphatase activity of alkaline-phosphatase preparations from human sources. Recently, however, the association of an increased urinary excretion of inorganic pyrophosphate with congenital deficiency of alkaline phosphatase in man (Russell, 1965) has revived interest in the possibility that inorganic pyrophosphate is a physiological substrate of alkaline phosphatase, and preliminary results have been reported which suggest that certain human and animal alkaline phosphatases are also pyrophosphatases (Cox & Griffin, 1965). During an investigation involving the purification of alkaline phosphatase from human liver and small intestine, therefore, the inorganic-pyrophosphatase activity of the preparations has been determined at different stages of purification. Some preliminary results of this work have already been reported (Moss, Eaton, Smith & Whitby, 1966*b*).

MATERIALS AND METHODS

Enzyme preparations. Specimens of human liver and small intestine were obtained within 24hr. of death and were stored at −20° until extraction. The tissues were washed with cold tap water and minced, then extracted by a modification of Morton's (1950) butan-1-ol method in which 2l. of water and 1l. of butan-1-ol/kg. of tissue were used for homogenization. The aqueous extracts were fractionated by successive precipitations with acetone at −5° and $(NH_4)_2SO_4$ at room temperature, the active fractions being further purified by gel filtration on columns of Sephadex G-200 (Pharmacia AB, Uppsala, Sweden) followed by ion-exchange chromatography on columns of DEAE-cellulose at three pH values, 5·6, 7·7 and 9·0, and on cellulose phosphate columns at pH 6·0. The ion-exchange columns measured 90cm. long × 2·5cm. diam. and were packed under slight positive pressure increasing to 1lb./in.^{2} during packing. Elution was by an increasing concentration of NaCl, and the buffers used were 0·01M-acetate at pH 5·6 and 6·0 and 0·01M-tris–HCl at pH 7·7 and 9·0. The enzyme activity was eluted from the anion-exchange columns at a chloride concentration of approx. 0·1M. Gel filtration was also carried out on 90cm. × 2·5cm. columns in 0·1M-tris–HCl buffer, pH 7·7, containing NaCl (0·1M). The chromatographic and gel-filtration experiments were performed at 4°. Because of the restricted capacity of the gel-filtration and ion-exchange columns, the enzyme from the precipitation stages of purification was divided into batches for further purification. Table 1 summarizes the enzyme activity and degree of purification obtained at each stage.

Enzyme activity determinations. Alkaline-phosphatase activity was determined by measuring the increase in E_{400} after incubation at 37° with disodium *p*-nitrophenyl phosphate (2mM) and $MgCl_2$ (5mM) in 0·05M-Na_2CO_3–$NaHCO_3$ buffer, pH 10. Inorganic-pyrophosphatase activity was determined by incubation at 37° with sodium pyrophosphate (3·3mM) and $MgCl_2$ (1mM) in 0·1M-tris–HCl buffer, pH 8·5, in a final volume of 1ml. There was no visible precipitation of magnesium pyrophosphate at these concentrations. The reaction was stopped by the addition of 3ml. of 2·3M-acetate buffer, pH 4·0, and the liberated

Table 1. *Summary of purification of liver and small-intestinal alkaline phosphatases*

Phosphatase activity is expressed in μmoles of *p*-nitrophenyl phosphate hydrolysed/min.; E_{280} has been taken as a measure of 'protein' concentration. The increase in specific activity for the liver enzyme was 320-fold, and for the intestinal enzyme 130-fold.

	Total enzyme activity	Total 'protein'	Specific activity (activity/protein)	Percentage recovery for each purification stage
Liver:				
Original extract	3940	90900	0·04	—
33–43% (v/v) acetone ppt.	3160	3760	0·84	80
50–70%-satd. $(NH_4)_2SO_4$ ppt.	2470	1720	1·44	80
After gel filtration of part of $(NH_4)_2SO_4$ ppt. on Sephadex G-200	508	143	3·6	93
After chromatography of part of Sephadex-treated enzyme on DEAE-cellulose, pH 7·7	104	7·6	13·7	90
Small intestine:				
Original extract	6830	47600	0·14	—
33–45% (v/v) acetone ppt.	4610	3090	1·49	68
40–70%-satd. $(NH_4)_2SO_4$ ppt.	4540	2040	2·22	98
After gel filtration of part of $(NH_4)_2SO_4$ ppt. on Sephadex G-200	420	46·5	9·0	100
After chromatography of part of Sephadex-treated enzyme on DEAE-cellulose, pH 7·7	107	5·66	18·9	70

inorganic phosphate was measured by the method of Delsal & Manhouri (1958). Similarly incubated reaction mixtures from which enzyme solution was omitted served as controls. For both types of activity results are expressed in μmoles of substrate hydrolysed/min.

Starch-gel electrophoresis. This was carried out on horizontal gels by the method of Smithies (1955) in borate buffer, pH 8·6. The applied voltage was 10 v/cm. for 18 hr. at 4°. Alkaline-phosphatase activity was located on the cut surfaces of the gels after electrophoresis by the method of Estborn (1959). Inorganic-pyrophosphatase activity was located by a modification of the 'direct' method of Allen & Hyncik (1963), in which the substrate solution was sodium pyrophosphate (5 mM), $MgCl_2$ (1 mM) and lead nitrate (3 mM) in 0·1 M-tris–HCl buffer, pH 8·5. The lead phosphate precipitated by incubation of the gels in this solution was converted into lead sulphide by treatment with ammonium sulphide solution. Before certain electrophoretic runs the enzyme solutions were incubated with neuraminidase (EC 3.2.1.18) as described by Moss, Eaton, Smith & Whitby (1966*a*).

RESULTS

Both liver and intestinal preparations possessed inorganic-pyrophosphatase activity at all stages of purification. The ratio of alkaline-phosphatase activity (at pH 10) to pyrophosphatase activity (at pH 8·5) varied during the course of purification from 15:1 in the crude liver extract to 12:1 in the final liver preparation, possibly owing to differential effects on the two activities of ions etc. removed at various stages, but the ratio of activities remained constant in the intestinal preparation at about 3:1. Liver and intestinal preparations each exhibited single coincident peaks of activity towards the two substrates both on gel filtration and on ion-exchange chromatography at all pH values (Figs. 1 and 2). In each chromatographic and gel-filtration experiment the recoveries of alkaline-phosphatase and pyrophosphatase activities were similar, and the ratio of the two activities was constant across the enzyme peaks.

Starch-gel electrophoresis showed that the pyrophosphatase zones coincided exactly with the alkaline-phosphatase zones in extracts of each tissue; the pattern consisted of a major zone of activity, which was rather diffuse in the intestinal extract, and a slow-moving minor zone nearer the origin. After treatment with neuraminidase both the alkaline-phosphatase zones in the liver extract were retarded (Moss *et al.* 1966*a*), and the pyrophosphatase zones coincided exactly with the new positions of the alkaline-phosphatase bands. The mobilities of the alkaline-phosphatase and pyrophosphatase zones of intestinal extract were not affected by neuraminidase treatment.

The pyrophosphatase and alkaline-phosphatase activities of the purified liver and intestinal preparations were measured after incubation at 55° and pH 7·7 (0·01 M-tris–hydrochloric acid buffer) for periods up to 45 min. The ratios of the two types of activity remained constant during incubation for each preparation, though the activities of the liver preparation declined somewhat more rapidly than those of the intestinal preparation (Fig. 3).

Mixed-substrate experiments were carried out

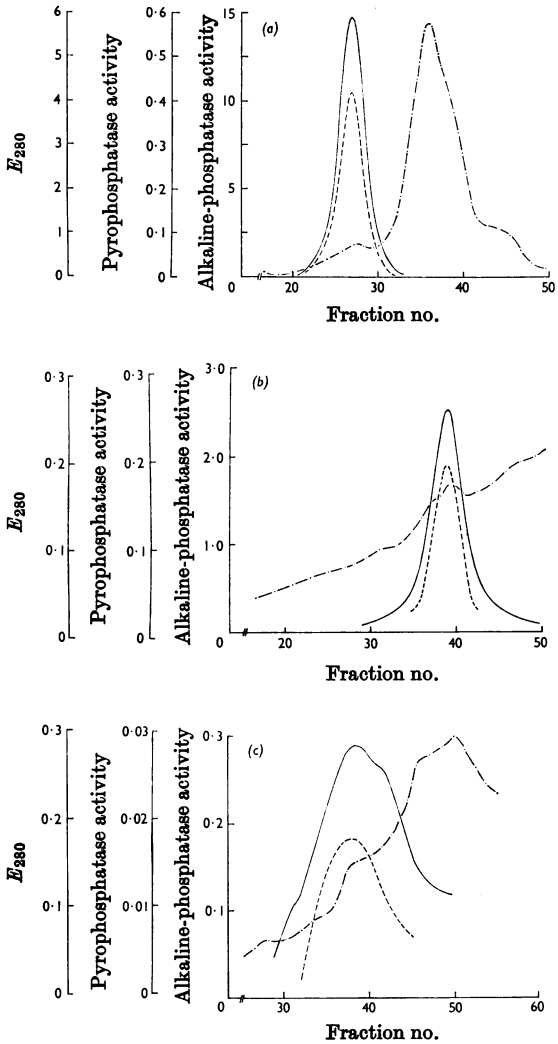

Fig. 1. (*a*) Gel filtration (Sephadex G-200), (*b*) anion-exchange chromatography (DEAE-cellulose, pH 7·7) and (*c*) cation-exchange chromatography (cellulose phosphate, pH 6·0) of liver extract: 15 ml. fractions were collected in each case. ——, Alkaline-phosphatase activity (μmoles of *p*-nitrophenyl phosphate hydrolysed/min./ml.); – – –, inorganic-pyrophosphatase activity (μmole of pyrophosphate hydrolysed/min./ml.); –·–·–, E_{280}.

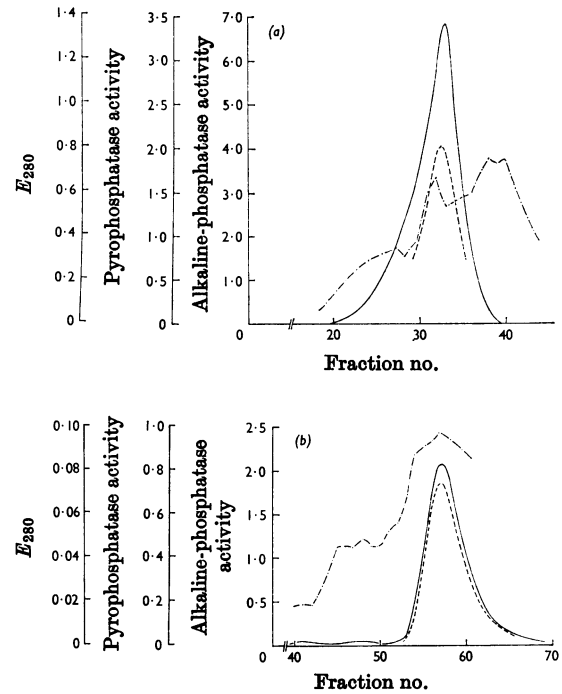

Fig. 2. (*a*) Gel filtration (Sephadex G-200) and (*b*) anion-exchange chromatography (DEAE-cellulose, pH 7·7) of small-intestinal extract: 15 ml. fractions were collected in each case. ——, Alkaline-phosphatase activity (μmoles of *p*-nitrophenyl phosphate hydrolysed/min./ml.); – – –, inorganic-pyrophosphatase activity (μmoles of pyrophosphate hydrolysed/min./ml.); –·–·–, E_{280}.

with the purified enzymes from the two tissues. Inorganic phosphate was measured after incubation with enzyme at 37° and pH 8·5 (0·1 M-tris–hydrochloric acid buffer) with sodium pyrophosphate (2 mM) and with disodium *p*-nitrophenyl phosphate (2 mM) separately, then after incubation with the two substrates simultaneously at the same concentrations. The amount of *p*-nitrophenol formed was also measured, thus allowing individual calculation of the phosphate deriving from alkaline-phosphatase activity and from inorganic-pyrophosphatase activity in the mixed-substrate experiments. The concentration of Mg^{2+} ions was 1 mM throughout. The amount of phosphate released in the mixed-substrate experiments was less than would be expected for independent hydrolysis of the substrates (Table 2), and both types of enzyme activity were inhibited in these experiments; pyrophosphatase activity was more strongly inhibited than alkaline phosphatase, and there was a greater degree of inhibition of liver alkaline phosphatase than of intestinal alkaline phosphatase.

The Michaelis constant, K_m, was determined for the two types of activity at their respective pH values of measurement. The values of K_m obtained were 0·07 mM (pyrophosphatase) and 0·8 mM (alkaline phosphatase) for the liver preparation, and 0·04 mM (pyrophosphatase) and 0·2 mM (alkaline phosphatase) for the intestinal preparation. Thus, for each enzyme, both the relative rates of hydrolysis

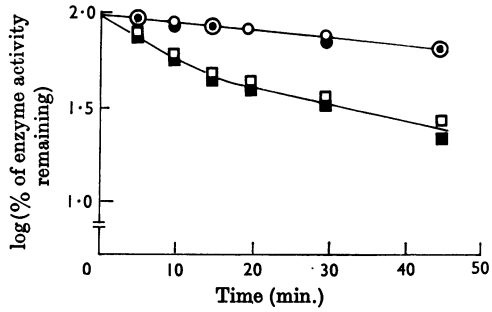

Fig. 3. Effect of incubation at 55° and pH 7·7 (tris–HCl buffer) on alkaline-phosphatase and inorganic-pyrophosphatase activities of purified liver and intestinal enzymes. □, Liver alkaline phosphatase; ■, liver pyrophosphatase; ○, small-intestinal alkaline phosphatase; ●, small-intestinal pyrophosphatase.

Table 2. *Hydrolysis by purified phosphatase preparations of* p-*nitrophenyl phosphate, sodium pyrophosphate and a mixture of the two at* 37° *and* pH 8·5 (*tris–hydrochloric acid buffer*)

The concentration of Mg^{2+} ions was 1 mM throughout. Enzyme activities are expressed in μmole of inorganic phosphate (P_i) or *p*-nitrophenol (*p*NP) liberated/min.

	Liver enzyme	Intestinal enzyme
Sodium pyrophosphate (2 mM):		
(μmole of P_i/min.)	0·0299	0·0155
p-Nitrophenyl phosphate (2 mM):		
(μmole of P_i/min.)	0·0148	0·0034
(μmole of *p*NP/min.)	0·0166	0·0032
Sodium pyrophosphate (2 mM) + *p*-nitrophenyl phosphate (2 mM):		
(μmole of P_i/min.)	0·0101	0·0055
(μmole of *p*NP/min.)	0·0082	0·0024
Calculated inhibition (%) of each type of activity in mixed-substrate experiments:		
Pyrophosphatase	94	80
Alkaline phosphatase	52	25

and the K_m values differ for the two types of substrate under the conditions of measurement, and there are differences in these quantities between the enzymes from the two tissues.

DISCUSSION

The statement that non-specific alkaline phosphatase is free from inorganic-pyrophosphatase activity has been repeated in the literature since 1928, although it was early recognized that the two types of activity were closely associated in animal tissues (see review by Folley & Kay, 1936). The main ground for ascribing the two activities to different enzymes appears to have been the difference in pH optima for the hydrolysis of the two substrates. This argument loses its force, however, with the demonstration that the pH optimum of alkaline phosphatase varies when different orthophosphate esters are used as substrates (Delory & King, 1943; Walker & King, 1950), and also that the pH optimum depends on substrate concentration (Ross, Ely & Archer, 1951). In the present studies, pH optima of 9·4 and 8·0 for the hydrolysis of *p*-nitrophenyl phosphate (2 mM) and sodium pyrophosphate (3·3 mM) respectively were found.

It has also been stated that alkaline-phosphatase activity is less stable than inorganic-pyrophosphatase activity in extracts of pig liver, and that keeping the extracts for 24 hr. at 37° and pH 9 selectively destroys alkaline-phosphatase activity (Roche, 1950), but the thermal-inactivation experiments reported above did not reveal any such difference in stability. It is also difficult to explain Morton's (1955) observation that highly purified alkaline phosphatase from calf intestine was free from inorganic-pyrophosphatase activity, but recently Cox & Griffin (1965) have reported that purified calf-intestinal alkaline phosphatase does act as a pyrophosphatase. It is considered that the conditions used by Morton (1955) to test for pyrophosphatase action (i.e. 0·5 mM-substrate and 10 mM-Mg^{2+} ion concentrations) were significantly less favourable than those used either in the present work or in the experiments of Cox & Griffin (1965) because, when Morton's (1955) conditions were used to investigate the preparations described in the present paper, very little pyrophosphatase activity could be detected.

The failure to separate the two types of enzymic activity, by methods depending both on molecular size and on net charge at various pH values, together with the results of mixed-substrate experiments, thermal-inactivation studies and modification of electrophoretic mobility by treatment with neuraminidase, strongly supports the view that human liver and intestinal phosphatases are also inorganic pyrophosphatases. If this property is shared by bone phosphatase also, it may help to explain the physiological role of alkaline phosphatase in the calcification of bone, since inorganic pyrophosphate is an inhibitor of bone mineralization in tissue culture (Fleish *et al.* 1964) and is found in excess in the urine of patients deficient in alkaline phosphatase (Russell, 1965): alkaline phosphatase may therefore function by removing the inhibitor from the sites of mineralization. Human alkaline phosphatase may also possess pyrophosphatase activity towards organic pyro-

phosphates (e.g. nucleotide di- and tri-phosphates), and these possibilities are being investigated.

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