Biochem. J. (1967) **105**, 1307
Printed in Great Britain

Organic Pyrophosphates as Substrates for Human Alkaline Phosphatases

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(*Received* 18 *July* 1967)

1. Purified human liver and small-intestinal alkaline orthophosphatases release inorganic phosphate at appreciable rates from a variety of organic pyrophosphate substrates. 2. The pyrophosphatase action is inhibited by Mg^{2+} ions at concentrations that activate the hydrolysis of orthophosphate substrates by these enzymes. 3. The results of mixed-substrate experiments, denaturation studies with heat or urea and starch-gel electrophoresis suggest that both orthophosphatase and pyrophosphatase activities are, in each preparation, properties of a single enzyme. 4. Intestinal phosphatase shows greater pyrophosphatase activity relative to orthophosphatase than the liver enzyme.

The view that mammalian alkaline phosphatase (orthophosphoric monoester phosphohydrolase; EC 3.1.3.1) does not hydrolyse pyrophosphate substrates has been current for many years (e.g. Folley & Kay, 1936), but it has recently been called in question by a number of observations. Cox & Griffin (1965) found inorganic pyrophosphatase activity to be associated with alkaline phosphatase preparations from a number of human and animal sources, and Moss, Eaton, Smith & Whitby (1967) failed to separate alkaline orthophosphatase and inorganic pyrophosphatase activities during extensive purification of human liver and small-intestinal alkaline phosphatases. Eaton & Moss (1967*a*) found the two types of activity of these preparations to respond similarly to certain inhibitors, and subsequently demonstrated a close correlation between alkaline orthophosphatase and inorganic pyrophosphatase activities in blood sera from patients with elevated serum alkaline phosphatase activities due to liver or bone disease (Eaton & Moss, 1967*b*). Fernley & Walker (1966) also showed that calf intestinal alkaline phosphatase hydrolysed inorganic pyrophosphate and ATP.

The ability of purified human liver and small-intestinal alkaline phosphatases to act on a number of organic di- and tri-phosphates of physiological significance has now been investigated. A preliminary account of part of this work has been presented (Eaton & Moss, 1967*c*).

MATERIALS AND METHODS

Enzyme preparations. Human liver and small-intestinal alkaline phosphatases were purified as described by Moss *et al.* (1967), resulting in an increase in specific activity of 340-fold for the liver enzyme and 135-fold for that from intestine.

Substrates. AMP, ADP, ATP, UMP, UDP and UTP were obtained from Sigma (London) Chemical Co. (London, S.W. 6) as the sodium salts. Thiamine pyrophosphate was obtained from the same supplier. The nucleoside phosphates were each free from detectable amounts of other nucleotides when examined by paper chromatography in ethanol–ammonium acetate (Smith, 1960).

Enzyme activity determinations. The P_i released from the substrates after incubation with the enzyme at 37° for 15min. was measured by the method of Baginski, Foa & Zak (1967). Activities were expressed as μmoles of P_i released/min./ml. of enzyme solution. Comparisons of rates of reaction with *p*-nitrophenyl phosphate as substrate were made as described by Eaton & Moss (1967*a*). In certain experiments Mg^{2+} ions were added to the final concentrations indicated in the Results section. The Na_2CO_3–$NaHCO_3$ buffers defined by Delory & King (1945) were used at a concentration of 25mM in the incubation medium, except when determining pH optima when tris–HCl buffers (final concn. 50mM) were used below pH 9.

Enzyme inactivation. The decline of activity towards the various substrates during incubation of the enzymes at pH 7·7 (tris–HCl buffer) and 54°, and with concentrated urea solutions at 20° (Butterworth & Moss, 1967), was determined.

Starch-gel electrophoresis. This was carried out as described by Moss *et al.* (1967). Zones of alkaline phosphatase activity were located after electrophoresis either with α-naphthyl phosphate as substrate (Estborn, 1959) or with the nucleotide substrates by the method of Allen & Hyncik (1963).

RESULTS

Both liver and intestinal phosphatases released inorganic phosphate from all the substrates at rates that were appreciable compared with the hydrolysis of the reference substrate, *p*-nitrophenyl phosphate,

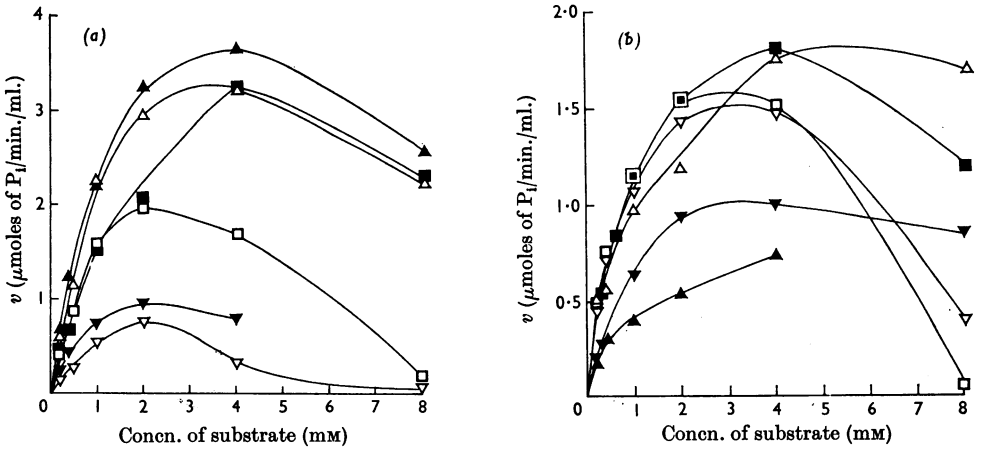

Fig. 1. Relationship between substrate concentration(s) and rate of release of P_i (v) from various substrates for (*a*) liver phosphatase and (*b*) intestinal phosphatase. There was no added Mg^{2+}, and measurements were made at the pH optimum for each substrate at 2 mM concentration. Substrates: △, AMP; □, ADP; ▽, ATP; ▲, UMP; ■, UDP; ▼, UTP.

Table 1. *Relative rates of release of inorganic phosphate from different substrates (concentration 2 mM) by liver and small-intestinal alkaline phosphatases with and without addition of* Mg^{2+} (10 *mM*)

All relative rates are referred to hydrolysis of 2 mM-*p*-nitrophenyl phosphate (in the presence of 10 mM-Mg^{2+}) as 100. K_m values were measured without added Mg^{2+}. Each determination of rate and K_m was made at optimum pH for that substrate at 2 mM concentration. The values given are in each case the means of several estimations. Abbreviations: *p*NPP, *p*-nitrophenyl phosphate; TPP, thiamine pyrophosphate.

Substrate	K_m (mM)	Relative rate: Without Mg^{2+}	Relative rate: With Mg^{2+}
Liver phosphatase			
*p*NPP	—	45	100
AMP	2·0	35	55
ADP	2·1	22	19
ATP	2·0	10	5
UMP	1·1	45	60
UDP	1·5	30	20
UTP	0·8	15	11
TPP	6·3	50	40
Intestinal phosphatase			
*p*NPP	—	77	100
AMP	1·7	95	125
ADP	0·8	150	50
ATP	0·7	115	15
UMP	0·7	45	50
UDP	0·5	130	40
UTP	1·0	75	10
TPP	1·2	110	50

with pH optima in the range 9·1–9·5 at 2 mM substrate concentration. The relative rates of hydrolysis of the various substrates at this concentration and optimum pH are given in Table 1; each of these values represents the mean of several determinations. Michaelis constants, K_m, were calculated from plots of $1/v$ against $1/s$ at pH values corresponding to the optima at 2 mM-substrate concentration (Table 1).

The adenosine pyrophosphates, ADP and ATP, showed marked substrate inhibition of the action of both enzymes at concentrations greater than 2–4 mM (Fig. 1); this effect was less marked with the corresponding uridine derivatives, but was pronounced with thiamine pyrophosphate also. The monophosphates, UMP and AMP, also inhibited the enzymes above 4 mM concentration, but to a smaller extent.

Addition of Mg^{2+} ions to the reaction mixtures changed the apparent pattern of specificity of both enzymes (Table 1). The Mg^{2+} ions activated release of inorganic phosphate from AMP and UMP in a manner comparable with the activation of hydrolysis of the reference substrate. With the pyrophosphate substrates, however, up to about 1 mM-Mg^{2+}, there was some activation, which was slight with the triphosphates with the liver enzyme and with both di- and tri-phosphates with that from intestine, but above this concentration Mg^{2+} ions were inhibitory (Figs. 2 and 3).

The progress of phosphate release from each of the substrates was linear over the period used for the assay of activity; beyond this the rate of reaction declined. There was no progressive increase in

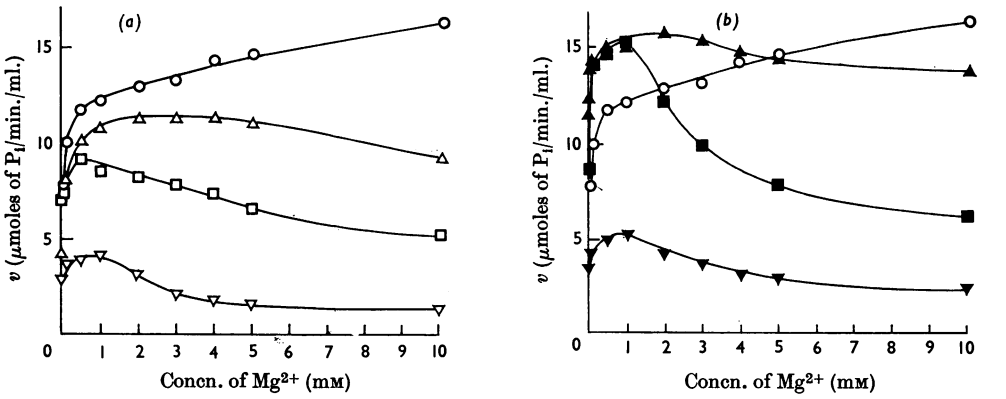

Fig. 2. Effect of Mg^{2+} ions on rate of release of P_i from (*a*) adenine nucleotides and (*b*) uridine nucleotides (concn. of each 2mM) by liver phosphatase. Rates for AMP are observed values × 2, and those for ADP, ATP, UMP, UDP and UTP are observed values × 5. Substrates: △, AMP; □, ADP; ▽, ATP; ▲, UMP; ■, UDP; ▼, UTP; ○, *p*-nitrophenyl phosphate (hydrolysis measured by release of *p*-nitrophenol).

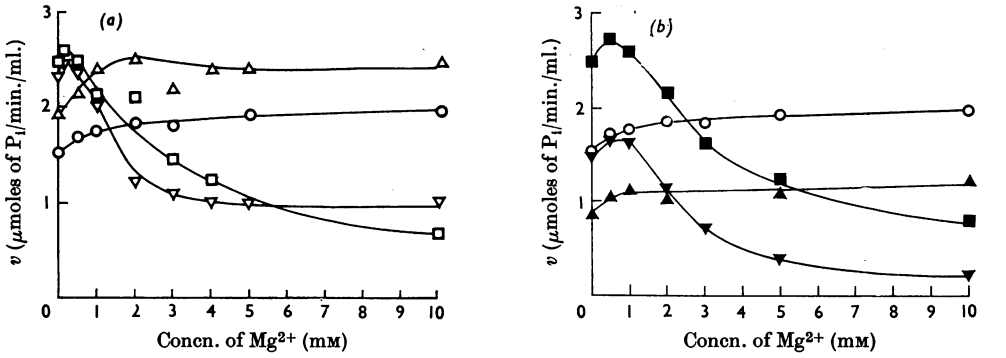

Fig. 3. Effect of Mg^{2+} ions on rate of release of P_i from (*a*) adenine nucleotides and (*b*) uridine nucleotides (concn. of each 2mM) by intestinal phosphatase. Substrates: △, AMP; □, ADP; ▽, ATP; ▲, UMP; ■, UDP; ▼, UTP; ○, *p*-nitrophenyl phosphate (hydrolysis measured by release of *p*-nitrophenol).

velocity as might have been expected if the course of the reaction gave rise to intermediates that were attacked more rapidly, e.g. the production of ADP and AMP during breakdown of ATP by liver phosphatase. Attempts to demonstrate intermediates in the hydrolysis of di- and tri-phosphates by paper chromatography showed the appearance of AMP during the hydrolysis of ADP and ATP, and of UMP during hydrolysis of UDP and UTP. The corresponding diphosphates could not be identified with certainty during the hydrolysis of ATP and UTP, owing to the rather poor resolution of the di- and tri-phosphates on the chromatograms.

Mixed-substrate experiments were carried out in which the rate of phosphate release from pairs of substrates present together was compared with that from single substrates; Mg^{2+} ions were omitted. For each pair of substrates the amount of phosphate released was decreased below that expected for independent hydrolysis when both substrates were present (Table 2).

During inactivation of both enzymes by heat (Fig. 4) and by urea (Fig. 5) the activities towards all the substrates declined in parallel with the possible exception of activity of the intestinal enzyme towards UMP during denaturation by urea. However, the greater resistance to denaturation by urea of intestinal phosphatase compared with liver phosphatase (Butterworth & Moss, 1967) was shown whatever substrate was used.

The pattern of enzyme zones after starch-gel electrophoresis was independent of the nature of the

Table 2. *Hydrolysis by purified phosphatase preparations of mixtures of pairs of substrates at* 37° *and* pH 9·3 *(carbonate–bicarbonate buffer)*

The concentration of each substrate was 1 mM, and results are expressed as μmoles of P_i liberated/min./ml. of enzyme soln. Abbreviation: *p*NPP, *p*-nitrophenyl phosphate.

	Liver		Intestine	
	Observed	Expected for independent hydrolysis	Observed	Expected for independent hydrolysis
AMP	2·79		1·72	
ADP	2·06		2·28	
AMP+ADP	3·54	4·85	1·89	4·00
AMP	1·80		0·413	
ATP	0·53		0·462	
AMP+ATP	1·75	2·33	0·472	0·875
*p*NPP	5·29		1·04	
UMP	3·34		0·66	
*p*NPP+UMP	5·56	8·63	1·00	1·70
*p*NPP	5·89		1·10	
UDP	2·03		2·12	
*p*NPP+UDP	5·26	7·92	1·49	3·22
*p*NPP	5·13		1·21	
UTP	1·17		1·33	
*p*NPP+UTP	3·61	6·30	1·28	2·54

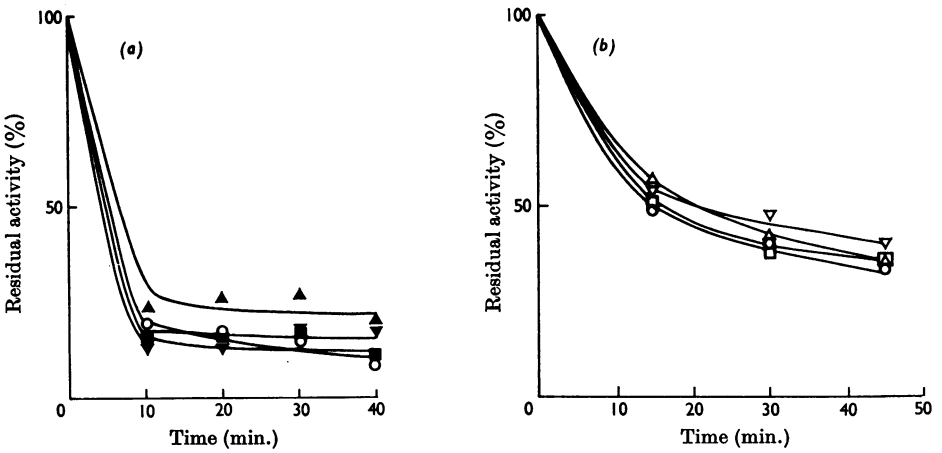

Fig. 4. Inactivation of (*a*) liver and (*b*) intestinal alkaline phosphatases during incubation at 54°. Substrates: △, AMP; □, ADP; ▽, ATP; ▲, UMP; ■, UDP; ▼, UTP; ○, *p*-nitrophenyl phosphate (hydrolysis measured by release of *p*-nitrophenol).

substrate, whether nucleotide or α-naphthyl orthophosphate, used to demonstrate it (Fig. 6). Both enzymes were without phosphate-releasing action on diphenyl phosphate and NAD.

DISCUSSION

The most probable interpretation of these observations is that human liver and intestinal alkaline orthophosphatases are also capable of hydrolysing organic pyrophosphates. This conclusion is in agreement with the observations made by Fernley & Walker (1966) with calf intestinal alkaline phosphatase, and extends the findings of Cox & Griffin (1965) and Moss *et al.* (1967) that human alkaline phosphatases possess inorganic pyrophosphatase activity. As is the case with inorganic pyrophosphate, the intestinal enzyme

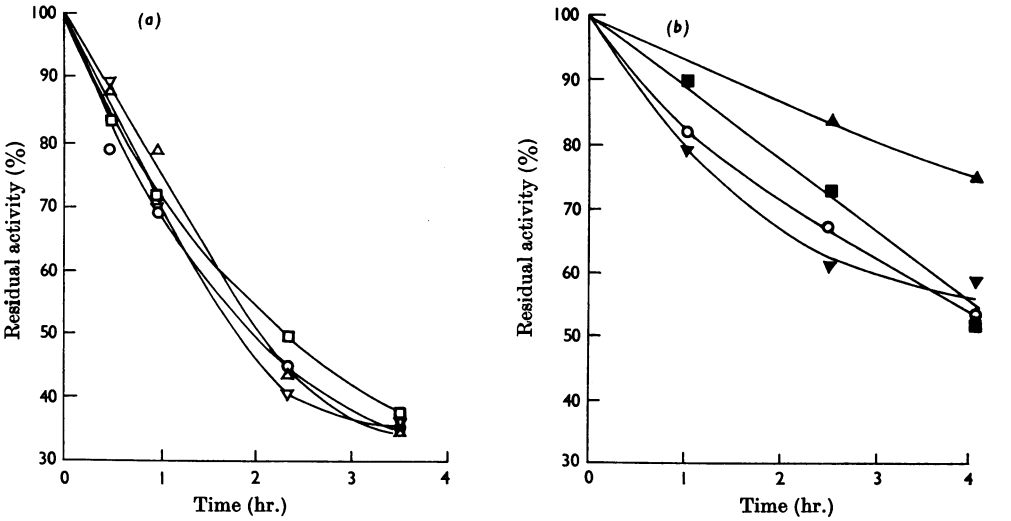

Fig. 5. Inactivation of (*a*) liver alkaline phosphatase with 3M-urea and (*b*) intestinal alkaline phosphatase with 6M-urea during incubation at 20° and pH 7·7. Substrates: △, AMP; □, ADP; ▽, ATP, ▲; UMP; ■, UDP; ▼, UTP; ○, *p*-nitrophenyl phosphate (hydrolysis measured by release of *p*-nitrophenol).

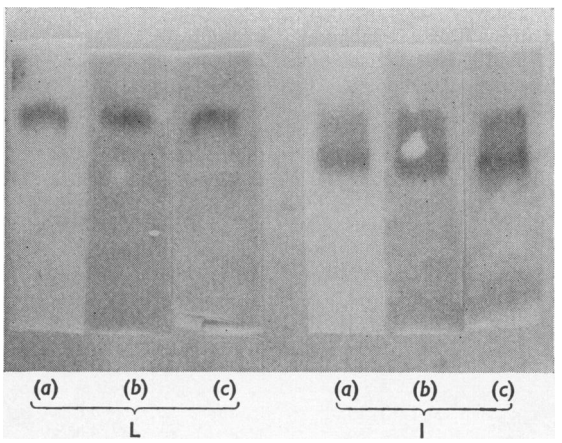

Fig. 6. Alkaline phosphatase zones in liver (L) and small-intestinal (I) preparations after starch-gel electrophoresis at pH 8·6. Substrates: (*a*) α-naphthyl orthophosphate; (*b*) ADP; (*c*) UTP. Anodes are at the top, sample insertion slots at the bottom.

exhibits greater activity towards the organic pyrophosphate substrates relative to its orthophosphatase activity than does liver phosphatase. Sandler & Bourne (1962) noted that ATP, ADP and orthophosphate substrates were hydrolysed by a single enzyme zone after starch-gel electrophoresis of calf intestinal alkaline phosphatase. These authors found, however, that after electrophoresis of rat kidney homogenates the orthophosphatase zone did not show this multiple specificity. Kidney alkaline phosphatase resembles liver phosphatase rather than intestinal phosphatase in many respects, e.g. in denaturation by urea (Butterworth & Moss, 1967), and the failure to detect pyrophosphatase activity in the kidney orthophosphatase zone may therefore have been due to a relatively lower pyrophosphatase activity by this enzyme compared with intestinal phosphatase, as is the case with liver alkaline phosphatase.

It seems less probable that the several types of phosphatase activity are the result of the presence of a number of enzymes, each of restricted specificity, since the enzymes were prepared by an extensive purification procedure that involved fractionation according to both net molecular charge and size (Moss *et al.* 1967). Such variations in the relative rates of loss of the several types of activity during denaturation by heat or urea as are seen (e.g. with UMP and intestinal phosphatase; Fig. 5*b*) are considered to fall within the observational error of this type of experiment, which involves the estimation of decreasing enzyme activities. An alteration in the specificity of the enzyme during denaturation cannot, however, be ruled out on the evidence of these data. The progress of inactivation by heat does not follow first-order kinetics, particularly for the liver enzyme (Fig. 4*a*). This may represent an initial, rapid, transition to a more stable form of the enzyme, since activity towards all the substrates seems equally affected.

The alkaline phosphatase preparations were free from Ni^{2+}-inhibited AMP-hydrolysing activity (Campbell, 1962) and the observations of optima in the range pH 9·1–9·4 for AMP and UMP further confirm that the preparations were not contaminated by 5′-nucleotidase (EC 3.1.3.5). Similarly, the ATP phosphohydrolases (EC 3.6.1.4) that have been described in various animal tissues (e.g. Perry, 1952; Caffrey, Tremblay, Gabrio & Huennekens, 1956) are characteristically activated by Mg^{2+} ions and have pH optima close to pH 7·5, and so are unlikely to be responsible for the adenosine triphosphatase activity of the present preparations, and the nucleoside diphosphatase (EC 3.6.1.6) of liver or kidney (Gibson, Ayengar & Sanadi, 1955) also requires Mg^{2+} ions and has only slight activity towards ADP. The specific phosphatase that most resembles the present enzymes in properties is the alkaline adenosine triphosphatase (EC 3.6.1.8), discovered in bull seminal plasma by Heppel & Hilmoe (1953), which has a pH optimum in the range 8·4–9·0 and probably also hydrolyses ADP, but which is unaffected by Mg^{2+}.

Sandler & Bourne (1962) suggested that the broader specificity of calf intestinal alkaline phosphatase compared with rat kidney phosphatase after starch-gel electrophoresis might be the result of denaturation of the former enzyme during purification, resulting in a loss of specificity. However, Moss *et al.* (1967) did not observe a change in the relative activities of human intestinal phosphatase towards orthophosphate and inorganic pyrophosphate substrates during purification, and it is concluded, as mentioned above, that the explanation of the observations of Sandler & Bourne (1962) lies in the relative rates of hydrolysis of different classes of substrates by intestinal and non-intestinal phosphatases.

The inhibitory effect of Mg^{2+} ions on organic pyrophosphate hydrolysis resembles the effect of this ion on the inorganic pyrophosphatase activity of these enzymes (Eaton & Moss, 1967*a*) and on the breakdown of ATP by calf intestinal phosphatase (Fernley & Walker, 1966), an effect that is presumably due, at least partly, to formation of complexes between Mg^{2+} and triphosphates. This inhibition may have been responsible for previous reports (e.g. Morton, 1955) that mammalian alkaline phosphatases were without action on ATP and ADP, since relatively high concentrations of Mg^{2+} ions were used in this earlier work.

There are few significant variations in the K_m values for the several substrates, apart from a tendency to slightly higher values on the whole for liver phosphatase. The change of the non-phosphate part of the nucleotide substrates from adenosine to uridine is similarly without a pronounced effect on K_m or, in general, on relative rates of hydrolysis, except that UMP is a poorer substrate for intestinal phosphate than AMP. The rapid hydrolysis of AMP relative to *p*-nitrophenyl phosphate seen with the intestinal enzyme is in accordance with earlier reports (Landau & Schlamowitz, 1961), and the different patterns of activity towards the various substrates of the phosphatases from liver and small intestine is further evidence of the separate identities of these two enzymes.

We thank the Medical Research Council for generous financial support.

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