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Inhibition of the Orthophosphatase and Pyrophosphatase Activities of Human Alkaline-Phosphatase Preparations

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1. Inhibition of the pyrophosphatase and orthophosphatase activities of human liver and small-intestinal alkaline-phosphatase preparations by different classes of inhibitors has been studied. 2. Each type of substrate, pyrophosphate or orthophosphate, is a competitive inhibitor of hydrolysis of the other type. 3. L-Phenylalanine is a non-competitive inhibitor of both types of activity of the intestinal preparation, but inhibits neither activity of the liver enzyme. Arsenate is a competitive inhibitor of both activities of both preparations. For a given inhibitor, the values of K_i are independent of the type of substrate used when measurements are made at the same pH. 4. Mg^{2+} ions activate orthophosphatase but inhibit pyrophosphatase, except in very low concentrations. 5. These results are compatible with the presence in each tissue preparation of a single enzyme with one type of active centre, possessing both orthophosphatase and pyrophosphatase activities.

It has been shown recently that preparations of alkaline orthophosphatase (orthophosphoric monoester phosphohydrolase; EC 3.1.3.1) from human tissues also possess inorganic-pyrophosphatase activity, and that the two types of enzymic activity are not separated by fractionation with salts or organic solvents, nor by ion-exchange chromatography, gel filtration or starch-gel electrophoresis (Cox & Griffin, 1965; Moss, Eaton, Smith & Whitby, 1967). These findings pose the question whether the two types of activity are properties of an enzyme with a single type of active centre, or whether two different active centres, presumably located on a single protein because of the failure to separate them by protein fractionation techniques, are involved. An attempt has been made to answer this question by studying the effects of different classes of inhibitors on the orthophosphatase and pyrophosphatase activities of alkaline-phosphatase preparations purified from human liver and small intestine. A preliminary account of part of this work has been presented (Eaton & Moss, 1966).

MATERIALS AND METHODS

Enzyme preparations. Alkaline phosphatase was purified from post-mortem specimens of human liver and small intestine as described by Moss *et al.* (1967). The purification stages included extraction of the tissues with butan-1-ol and water, fractional precipitation with acetone and $(NH_4)_2SO_4$, filtration through Sephadex G-200 gel and anion-exchange chromatography. The increase in specific activity was about 340-fold for the liver enzyme and 135-fold for that from small intestine.

Enzyme activity determinations. Orthophosphatase activity was determined by measuring the increase in E_{400} during 5–10min. incubation of enzyme solution (0·1ml.) with disodium *p*-nitrophenyl phosphate at 37° in the cuvette of a spectrophotometer equipped with a multiple-sample extinction recorder (model 2000; Gilford Instrument Laboratories Inc., Oberlin, Ohio, U.S.A.). In a few experiments, noted in the Results section, disodium α-naphthyl phosphate was used as substrate and the increase in E_{335} was recorded (Moss, 1966). The spectrophotometer records were calibrated by means of standard solutions of *p*-nitrophenol and α-naphthol at the appropriate pH values. Inorganic-pyrophosphatase activity was determined by incubating enzyme solution (0·1ml.) with sodium pyrophosphate at 37° for 15–30min. After termination of the reaction by the addition of 2·3M-acetate buffer, pH4·0, the liberated inorganic phosphate was estimated by the method of Delsal & Manhouri (1958). For both types of enzyme activity results are expressed in μmoles of substrate hydrolysed/min./ml. of enzyme solution. Tris–HCl buffers (0·05M) were used for experiments at pH 8·5 and 8·9, and Na_2CO_3–$NaHCO_3$ buffers (0·05M; Delory & King, 1945) for experiments at pH9·9. Inhibitors and Mg^{2+} ions were added to the final concentrations indicated in the Results section.

RESULTS

Each type of substrate (orthophosphate or pyrophosphate) is a competitive inhibitor of the hydrolysis of the other type. Fig. 1(*a*) shows a plot

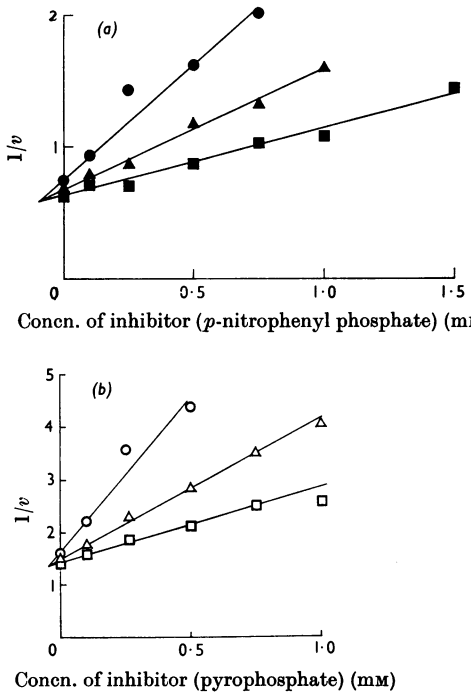


Fig. 1. (*a*) Plot of $1/v$ against i for inhibition of inorganic-pyrophosphatase activity of intestinal alkaline phosphatase by disodium *p*-nitrophenyl phosphate at pH 8·5. Substrate (pyrophosphate) concns. (mM): ●, 1·0; ▲, 2·0; ■, 3·0. (*b*) Plot of $1/v$ against i for inhibition of orthophosphatase activity of intestinal alkaline phosphatase by inorganic pyrophosphate at pH 8·9. Substrate (*p*-nitrophenyl phosphate) concns. (mM): ○, 0·1; △, 0·25; □, 0·5. Initial velocity, v, is expressed as μmoles/min./ml.

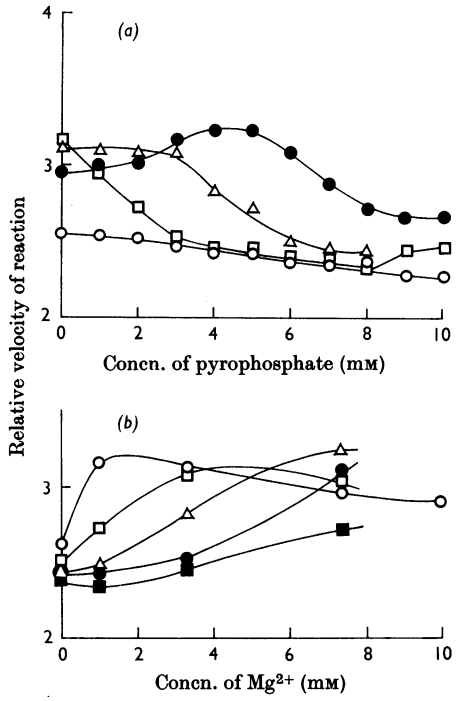


Fig. 2. (*a*) Relationship between orthophosphatase activity (substrate, 3·3 mM-α-naphthyl phosphate) and inorganic pyrophosphate concn. at pH 9·9. Mg^{2+} concns. (mM): ○, 0; □, 1·0; △, 3·3; ●, 6·7. Intestinal enzyme was used. (*b*) Replotted as v against Mg^{2+} concn. Pyrophosphate concns. (mM): ○, 0; □, 2; △, 4; ●, 6; ■, 8.

of $1/v$ against inhibitor concentration, i (Dixon, 1953), for the inhibition of inorganic-pyrophosphatase activity of intestinal alkaline phosphatase by *p*-nitrophenyl phosphate at pH 8·5, and Fig. 1(*b*) illustrates the inhibition by pyrophosphate of orthophosphatase activity at pH 8·9. These pH values, which are remote from the pH optima for the breakdown of the inhibitors, disodium *p*-nitrophenyl phosphate and sodium pyrophosphate respectively, were chosen so as to minimize changes in the concentrations of the inhibitors during the experiments. The incubation periods, particularly for the study of the inhibition of pyrophosphatase by *p*-nitrophenyl phosphate, were also kept as short as possible. These experiments were carried out without addition of Mg^{2+} ions.

Inhibition of orthophosphatase activity at pH 9·9 by pyrophosphate appears to be due to removal of Mg^{2+} ions, which activate alkaline orthophosphatase; without added Mg^{2+} there is little inhibition of

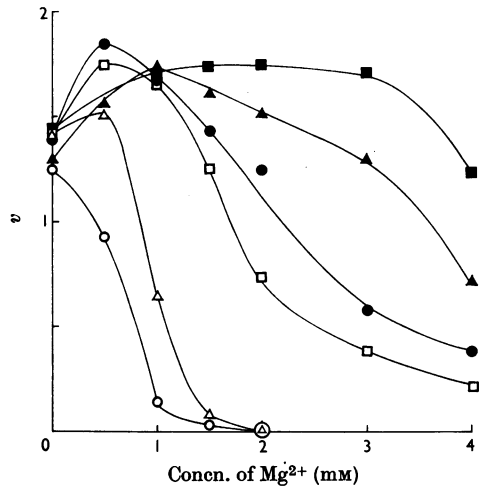
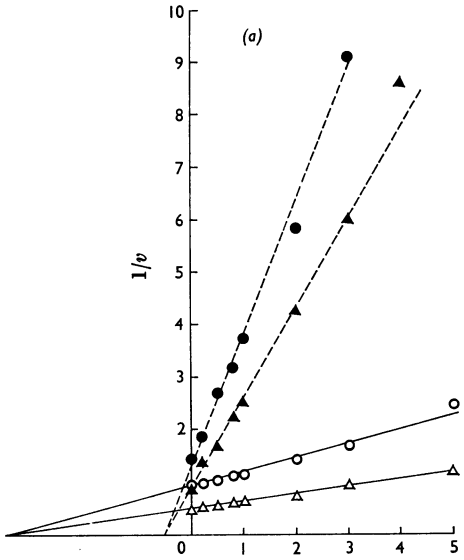


Fig. 3. Relationship between Mg^{2+} concn. and rate of hydrolysis of inorganic pyrophosphate by intestinal phosphatase at pH 8·5. Substrate concns. (mM): ○, 0·5; △, 1·0; □, 1·5; ●, 2·0; ▲, 3·0; ■, 4·0. Initial velocity, v, is expressed as μmoles/min./ml.

orthophosphatase activity by pyrophosphate, and presumably little binding of pyrophosphate by the enzyme (Fig. 2). The increase in orthophosphatase activity over the range 0–4mM-pyrophosphate at 6·7mM-Mg^{2+} ion concentration (Fig. 2*a*) may be due to the decrease in Mg^{2+} concentration by combination with pyrophosphate, effecting a release of inhibition by excess of Mg^{2+}, since at zero pyrophosphate concentration there is some indication of inhibition of the enzyme by an excess of Mg^{2+} ions (Fig. 2*b*). In contrast with the well-known activating effect of Mg^{2+} on alkaline orthophosphatase (Roche, 1950), this cation is strongly inhibitory towards pyrophosphatase except in very low concentrations. The inhibition by an excess of Mg^{2+} is offset by increasing pyrophosphate concentration. Fig. 3 illustrates this relationship for the intestinal enzyme preparation; similar results were obtained with liver enzyme that had previously been dialysed against EDTA to remove endogenous metal ions, EDTA being removed subsequently by gel filtration. There was no visible precipitation of magnesium pyrophosphate in any of these experiments.



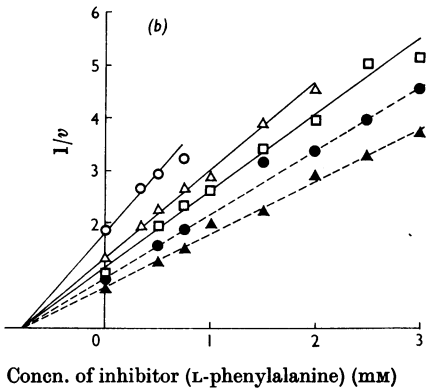


Fig. 4. (*a*) Plots of $1/v$ against i for inhibition of intestinal orthophosphatase and pyrophosphatase by L-phenylalanine at pH9·9 and pH8·5 respectively. ○ and △, Orthophosphatase; ● and ▲, pyrophosphatase. Substrate concns. (mM): *p*-nitrophenyl phosphate: ○, 0·25; △, 2·0; pyrophosphate: ●, 0·5; ▲, 5·0. (*b*) Inhibition of both intestinal enzyme activities by L-phenylalanine at pH8·9. Substrate concns. (mM): *p*-nitrophenyl phosphate: ○, 0·02; △, 0·1; □, 0·5; pyrophosphate: ●, 0·5; ▲, 3·0. Initial velocity, v, is expressed as μmoles/min./ml.

L-Phenylalanine inhibits intestinal alkaline orthophosphatase, but not alkaline phosphatases from other tissues (Fishman, Green & Inglis, 1962). Plots of $1/v$ against i show that inhibition of the intestinal enzyme is of the non-competitive type, and that the pyrophosphatase activity of this preparation is also inhibited non-competitively (Fig. 4). When determined at the same pH, the values of K_i are similar whichever type of substrate, *p*-nitrophenyl phosphate or sodium pyrophosphate, is used (Fig. 4*b*). L-Phenylalanine was without effect on either activity of the liver phosphatase preparation.

Sodium arsenate inhibits competitively both activities of the enzymes from each tissue (Figs. 5 and 6). When inhibition by arsenate was studied at the same pH value for both pyrophosphate and orthophosphate hydrolysis, similar values of K_i were obtained whichever substrate was used (Figs. 5*b* and 6*b*).

Values of K_i under various conditions, calculated from the plots of $1/v$ against i by the method of least squares, are collected in Table 1.

DISCUSSION

The similarity of behaviour of the two types of phosphatase activity of a particular tissue extract towards the inhibitors arsenate and L-phenylalanine in these experiments supports the view that, for each preparation, the two activities are properties of a single enzyme. If a single inhibitor-binding site is involved, for a fully competitive inhibitor this would also be the active centre of the enzyme, and the value of K_i for a particular inhibitor should be independent of whether orthophosphate or pyrophosphate is used as substrate in its determination, provided that differences in pH of measurement with the two substrates can be eliminated. This involves lowering the pH of measurement of orthophosphatase activity to values at which pyrophosphatase activity, which has a much lower

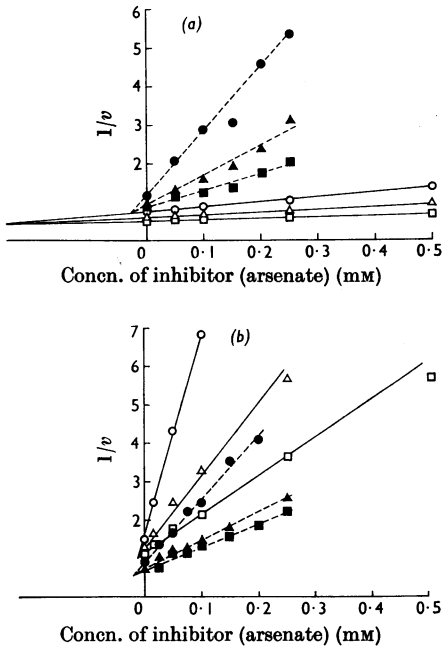


Fig. 5. (*a*) Plots of $1/v$ against i for inhibition of intestinal orthophosphatase and pyrophosphatase by arsenate at pH 9·9 and pH 8·5 respectively. ○, △ and □, Orthophosphatase; ●, ▲ and ■, pyrophosphatase. Substrate concns. (mM): *p*-nitrophenyl phosphate: ○, 0·5; △, 1·0; □, 2·0; pyrophosphate: ●, 0·5; ▲, 1·0; ■, 3·0. (*b*) Inhibition of both intestinal enzyme activities by arsenate at pH 8·9. Substrate concns. (mM): *p*-nitrophenyl phosphate: ○, 0·1; △, 0·25; □, 0·5; pyrophosphate: ●, 0·5; ▲, 1·0; ■, 3·0. Initial velocity, v, is expressed as μmoles/min./ml.

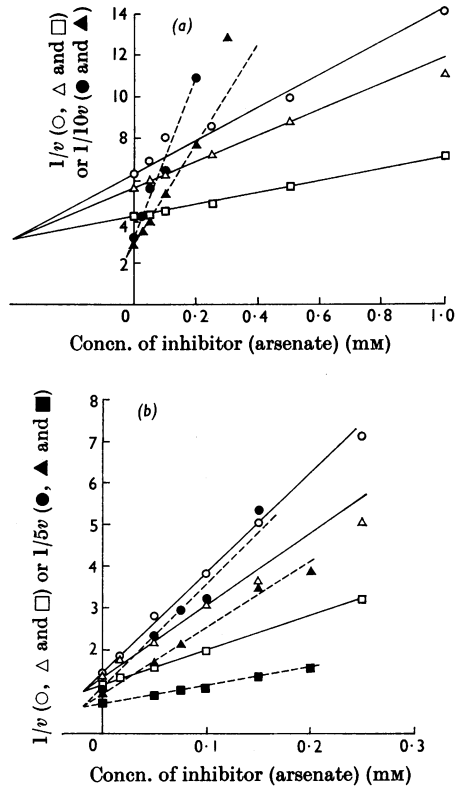


Fig. 6. (*a*) Plots of $1/v$ against i for inhibition of liver orthophosphatase and pyrophosphatase by arsenate at pH 9·9 and 8·5 respectively. ○, △ and □, Orthophosphatase; ● and ▲, pyrophosphatase. Substrate concns. (mM): *p*-nitrophenyl phosphate: ○, 0·5; △, 1·0; □, 2·0; pyrophosphate: ●, 1·0; ▲, 3·0. (*b*) Inhibition of both liver enzyme activities by arsenate at pH 8·9. Substrate concns. (mM): *p*-nitrophenyl phosphate: ○, 0·1; △, 0·25; □, 0·5; pyrophosphate: ●, 0·5; ▲, 1·0; ■, 3·0. Initial velocity, v, is expressed as μmoles/min./ml.

optimum pH (in the region of pH 8·0–8·5) and is measured by a less sensitive assay procedure, can be determined. When nearly saturating concentrations of substrate (e.g. greater than about 1 mM) are used, the pH–activity curves at different substrate concentrations for alkaline orthophosphatase intersect in the region of pH 9 (Motzok, 1959; Moss, Campbell, Anagnostou-Kakaras & King, 1961), so that converging plots of $1/v$ against i at different substrate concentrations cannot be obtained. At lower substrate concentrations, e.g. below 1 mM, the pH optima of alkaline orthophosphatases are shifted to a more acid range and the pH–activity curves at different substrate concentrations no longer intersect in the pH 9 region. In the present work, converging plots of $1/v$ against i for inhibition of orthophosphatase were obtained at pH 8·9 by the use of *p*-nitrophenyl phosphate concentrations of 0·5 mM and below, and pyrophosphatase proved to be sufficiently active at pH 8·9 to allow its inhibition also to be studied at this pH. Under these conditions, similar K_i values were obtained for a given inhibitor for inhibition of both pyrophosphatase and orthophosphatase (Table 1).

A complex relationship between Mg^{2+} and substrate concentrations has been noted previously for the inorganic pyrophosphatase of erythrocytes (Bloch-Frankenthal, 1954), but the present results differ from those reported for erythrocyte pyrophosphatase in that addition of Mg^{2+} does not appear to be essential for hydrolysis of pyrophosphatase by liver and intestinal alkaline-phosphatase preparations, even when the enzyme has been pretreated by dialysis against EDTA. Inorganic pyrophosphate has been used as a specific inhibitor of alkaline orthophosphatase, e.g. in methods for assaying enzyme activity (Huggins & Talalay,

Table 1. *Values of K_i for inhibition of alkaline-phosphatase and pyrophosphatase activities of purified human small-intestinal and liver preparations by different classes of inhibitors*

Source of enzyme	Substrate	Inhibitor	pH	K_i (mM)	Type of inhibition
Small intestine	Sodium pyrophosphate	*p*-Nitrophenyl phosphate	8·5	0·09	Competitive
	p-Nitrophenyl phosphate	Sodium pyrophosphate	8·9	0·05	Competitive
	Sodium pyrophosphate	L-Phenylalanine	8·5	0·5	Non-competitive
	Sodium pyrophosphate	L-Phenylalanine	8·9	0·8	Non-competitive
	p-Nitrophenyl phosphate	L-Phenylalanine	8·9	0·8	Non-competitive
	p-Nitrophenyl phosphate	L-Phenylalanine	9·9	3·5	Non-competitive
	Sodium pyrophosphate	Arsenate	8·5	0·025	Competitive
	Sodium pyrophosphate	Arsenate	8·9	0·02	Competitive
	p-Nitrophenyl phosphate	Arsenate	8·9	0·02	Competitive
	p-Nitrophenyl phosphate	Arsenate	9·9	0·25	Competitive
Liver	Sodium pyrophosphate	Arsenate	8·5	0·02	Competitive
	Sodium pyrophosphate	Arsenate	8·9	0·02	Competitive
	p-Nitrophenyl phosphate	Arsenate	8·9	0·02	Competitive
	p-Nitrophenyl phosphate	Arsenate	9·9	0·4	Competitive

1945), but, as Fig. 2 shows, there is little inhibition of alkaline phosphatase at pH 9·9 in the absence of added Mg^{2+} ions. Since it has been suggested (Bloch-Frankenthal, 1954) that pyrophosphate is bound to pyrophosphatase in the form of a magnesium complex, an attempt was made to demonstrate inhibition of alkaline orthophosphatase at pH 9·9 by maintaining an equimolar ratio of Mg^{2+} ions and sodium pyrophosphate while increasing their concentrations, but enzyme activity was independent of Mg^{2+} and pyrophosphate concentration. At pH 8·9, pyrophosphate inhibition of alkaline orthophosphate appears not to depend on the addition of Mg^{2+} ions (Fig. 1*b*); pyrophosphate can presumably be bound by the enzyme at this pH, which is closer to the optimum for pyrophosphatase activity.

The differential effect of Mg^{2+} on pyrophosphatase and orthophosphatase activities may perhaps be interpreted as indicating the existence of separate active centres for the two activities, but in spite of these differences the most reasonable interpretation of the present results appears to be that, in each of the tissue preparations, inorganic-pyrophosphatase and orthophosphatase activities are properties of a single enzyme having only one type of active centre. The different response of intestinal phosphatase compared with liver phosphatase to inhibition by L-phenylalanine, together with the different ratios of pyrophosphatase activity to orthophosphatase activity for the two enzymes (Moss *et al.* 1967), is further evidence of the separate identities of liver and intestinal alkaline phosphatases.

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REFERENCES

Bloch-Frankenthal, L. (1954). *Biochem. J.* **57**, 87.
Cox, R. P. & Griffin, M. J. (1965). *Lancet*, ii, 1018.
Delory, G. E. & King, E. J. (1945). *Biochem. J.* **39**, 245.
Delsal, J. L. & Manhouri, H. (1958). *Bull. Soc. Chim. biol., Paris*, **40**, 1623.
Dixon, M. (1953). *Biochem. J.* **55**, 170.
Eaton, R. H. & Moss, D. W. (1966). *Biochem. J.* **100**, 45P.
Fishman, W. H., Green, S. & Inglis, N. I. (1962). *Biochim. biophys. Acta*, **62**, 363.
Huggins, C. & Talalay, P. (1945). *J. biol. Chem.* **159**, 399.
Moss, D. W. (1966). *Enzymologia*, **31**, 193.
Moss, D. W., Campbell, D. M., Anagnostou-Kakaras, E. & King, E. J. (1961). *Biochem. J.* **81**, 441.
Moss, D. W., Eaton, R. H., Smith, J. K. & Whitby, L. G. (1967). *Biochem. J.* **102**, 53.
Motzok, I. (1959). *Biochem. J.* **72**, 169.
Roche, J. (1950). In *The Enzymes*, 1st ed., vol. 1, part 1, p. 485. Ed. by Sumner, J. B. & Myrbäck, K. New York: Academic Press Inc.