- Knott, C. B. & Petersen, W. E. (1945). *J. Dairy Sci.* **28**, 415.
- Kosterlitz, H. W. (1939). *Biochem. J.* **33**, 1087.
- Krebs, H. A. (1950). *Biochim. biophys. Acta*, **4**, 249.
- Krebs, H. A. & Eggleston, L. V. (1940). *Biochem. J.* **34**, 442.
- Krebs, H. A. & Hems, R. (1953). *Biochim. biophys. Acta*, **12**, 172.
- Lehninger, A. L. (1949). *J. biol. Chem.* **178**, 625.
- LePage, G. A. (1949). *Biochem. Prep.* **1**, 5.
- Levene, P. A. & Jacobs, W. A. (1910). *Ber. dtsch. chem. Ges.* **43**, 3150.
- McGeown, M. G. & Malpress, F. H. (1952). *Biochem. J.* **52**, 606.
- Malpress, F. H. (1950). *C.R. du Colloque International sur le mécanisme physiologique de la sécrétion lactée*, p. 115. Strasbourg.
- Malpress, F. H. & Morrison, A. B. (1949). *Biochem. J.* **45**, 455.
- Malpress, F. H. & Morrison, A. B. (1950). *Biochem. J.* **46**, 307.
- Moore, R. O. & Nelson, W. L. (1952). *Arch. Biochem. Biophys.* **36**, 178.
- Müller, A. F. & Leuthardt, F. (1949). *Helv. chim. acta*, **32**, 2289.
- Nelson, N. (1944). *J. biol. Chem.* **153**, 375.
- Ostern, P. (1932). *Biochem. Z.* **254**, 65.
- Partridge, S. M. (1948). *Biochem. J.* **42**, 238.
- Pazur, J. H. (1953). *Science*, **117**, 355.
- Potter, V. R. & Elvehjem, C. A. (1936). *J. biol. Chem.* **114**, 495.
- Reithel, F. J., Horowitz, M. G., Davidson, H. M. & Kittinger, G. W. (1952). *J. biol. Chem.* **194**, 839.
- Roberts, H. R. & McFarren, E. F. (1953). *J. Dairy Sci.* **36**, 620; from *Chem. Abstr.* (1953), **47**, 8112.
- Rutter, W. J. & Hansen, R. G. (1953). *J. biol. Chem.* **202**, 323.
- Somogyi, M. (1945). *J. biol. Chem.* **160**, 61.
- Stadie, W. C. & Riggs, B. C. (1944). *J. biol. Chem.* **154**, 687.
- Terner, C. (1954). *Biochem. J.* **56**, 471.
- Wallenfels, K. & Bernt, E. (1952). *Angew. Chem.* **64**, 28.

The Substrate Specificity and Inhibition of Alkaline Phosphatases of Cow's Milk and Calf Intestinal Mucosa

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It is somewhat surprising, in view of the extensive literature relating to alkaline phosphatases, that the substrate specificity of these enzymes has not been clearly established. Although it has been shown that they hydrolyse a number of different phosphate esters (Folley & Kay, 1936; Roche & Thoai, 1950), this information is derived from studies with crude or partially purified preparations. The widely accepted belief that hydrolysis is limited to orthophosphate monoesters (Roche & Thoai, 1950; Roche, 1950) is not well-founded, especially since Schmidt & Thannhauser (1943) showed that highly purified preparations from calf intestinal mucosa hydrolysed phosphate diesters and pyrophosphates as well as phosphate monoesters.

The substrate specificity has now been determined using purified phosphatases from cow's milk (Morton, 1950, 1953*a*) and calf intestinal mucosa (Morton, 1950, 1954). It has been found that only orthophosphate monoesters and closely related compounds, but not diesters or pyrophosphates, are hydrolysed.

Since these alkaline phosphatases may therefore be regarded as monoesterases of a special type, it

seemed possible that they might be inhibited by compounds which inhibit other esterases. The failure to obtain appreciable inhibition by a range of organic pyrophosphates, phosphites and phosphonates shows the marked difference between these alkaline phosphatases and certain other esterases.

MATERIALS AND METHODS

Enzymes and buffers

Unless otherwise indicated, the experiments described here were carried out in 1950 and early 1951 using alkaline phosphatases purified from cow's milk (Morton, 1950, 1953*a*) and from calf intestinal mucosa (Morton, 1950, 1954). The method of handling these preparations and of purifying and preparing the buffers used in this work is described in another paper (Morton, 1955*a*).

Substrates

Except where otherwise indicated, commercial preparations were used. They were generally examined for the presence of contaminant phosphorylated compounds (Morton, 1955*a*), and, unless otherwise mentioned, were used without further purification.

Phosphocreatine (PC). The crystalline sodium salt was prepared by the method of Ennor & Stocken (1948).

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Phosphoenolpyruvate (PEP). A preparation of the barium salt, which had been kept in the dry state for some years, was kindly supplied by Dr M. Dixon. It was purified by converting into the sodium salt, precipitating with barium acetate as described by LePage (1949) and subsequently removing inorganic phosphate and pyruvic acid according to Meyerhof & Oesper (1949). All the organic phosphate was iodine-labile when tested as described by Meyerhof & Oesper (1949).

Adenosine triphosphate (ATP). The dibarium salt was prepared from rabbit muscle by the late Mr E. J. Morgan, using the procedure of Lohmann as described by Needham (1942). The preparation also contained some adenosine diphosphate (ADP) and adenosine 5'-monophosphate (AMP).

Adenosine diphosphate. This was prepared by the late Mr E. J. Morgan by the action of rabbit muscle myosin-adenosine triphosphatase on ATP. The preparation also contained some ATP and AMP.

Glucose 1-phosphate (G 1-P). The crystalline potassium salt was kindly supplied by Dr D. M. Needham.

Triose phosphate. A mixture of glyceraldehyde phosphate and dihydroxyacetone phosphate, prepared according to the method of Meyerhof (1938) by the action of aldolase on hexose diphosphate, was kindly supplied by Dr D. M. Needham.

Fructose 1-phosphate. This was prepared synthetically by Dr G. Hers and kindly supplied by him.

2-Phosphoglycerate, 3-phosphoglycerate and diphenyl pyrophosphate. These were gifts from Dr M. Dixon.

Diphosphopyridine nucleotide (DPN). A preparation assaying 33% DPN was prepared by the late Mr E. J. Morgan and kindly supplied by him.

Other compounds

Unless otherwise indicated below, the compounds investigated as potential inhibitors of alkaline phosphatases were commercial preparations.

Trimethyl phosphate and triphenyl phosphate were kindly supplied by Dr M. Dixon. Tetraethyl pyrophosphate, hexaethyl tetraphosphate and diisopropyl fluorophosphate (DFP) were gifts from Dr E. C. Webb. Ammonium fluorophosphate, diethyl phosphonate, tribenzyl borate and the various organic phosphites were gifts from Dr B. C. Saunders, Department of Organic Chemistry, University of Cambridge.

Methods

Hydrolysis of substrates. The reaction was generally commenced by addition of 0.2 ml. of a suitable dilution of enzyme to 4.8 ml. of reaction mixture containing the substrate, magnesium acetate (0.01M) and either ethanolamine-HCl (0.04M) or veronal-HCl (0.04M) buffer. All activities were determined at 38°. Generally, the reaction period was 5 min. but was extended as necessary. Unless otherwise indicated, hydrolysis was measured by estimation of the inorganic phosphate liberated enzymically. Hydrolysis of phenyl phosphate and of PC was usually measured by estimation of liberated phenol and liberated creatine, respectively. All pH values are given for 38°.

Phosphate. The method of Fiske & Subbarow (1925) was generally used. However, where acid-labile materials (PC and all compounds containing pyrophosphate linkages)

were present in the reaction mixture, the method of Weil-Malherbe & Green (1951) was used.

Acid-labile phosphate. This was determined by estimation of inorganic phosphate liberated in 10 min. at 100° in 1N-HCl.

Creatine. The colorimetric method of Eggleton, Elsdon & Gough (1943) was used.

Phenol. The following modification of the colorimetric method of King (1951) was used. The enzymic reaction (total volume, 5 ml.) was stopped by the direct addition of 1.5 ml. of diluted (1/3) phenol reagent. Then 1 ml. of 25% Na₂CO₃ was added, the volume made up to 10 ml. with distilled water and the colour developed at 38° for 20 min.

Inhibition of phosphatase activity. Activities were determined as follows. The diluted enzyme, in veronal-sodium acetate-HCl buffer (0.02M, pH 7.8) was treated with the inhibitor, and held at room temperature (about 18°) for 20 min. The tube was then brought to 38° and the reaction commenced by the addition of the buffered substrate-magnesium acetate mixture. Volumes were adjusted so that the concentration of the inhibitor during the activity test was half that during prior treatment of the enzyme. Final concentrations of the reactants, in a final volume of 1.0 ml., were as follows: phenyl phosphate, 0.02M; magnesium acetate, 0.01M; ethanolamine-HCl buffer (pH 9.95), 0.04M. The reaction period was 5 min. Activity was estimated by determination of liberated phenol, except where interfering substances, such as cysteine, were present. In these cases, liberated inorganic phosphate was measured.

RESULTS

Substrate specificities of purified phosphatases

In order to determine the types of substrates which are hydrolysed by the purified milk and intestinal phosphatases, representative phosphorylated compounds were examined at selected substrate concentrations. As far as possible, each substrate was examined over a range of pH values chosen to include the optimum pH for the substrate concentration used. The results are shown in Table 1. In some cases a number of substrate concentrations were used, and the apparent K_m (Michaelis's constant) value determined from a Lineweaver & Burk (1934) plot as modified by Dixon (1953a). These are shown in Table 2.

All orthophosphate monoesters examined, as shown in Table 1, were found to be rapidly hydrolysed by both purified phosphatases. Especial interest attaches to the results obtained with compounds other than true orthophosphates, and these are briefly considered in greater detail.

Phosphoenolpyruvate. The rapid hydrolysis of this compound by both enzymes confirms previous findings by Meyerhof & Green (1950), using partially purified phosphatase from calf intestinal mucosa.

Phosphoamides. Phosphocreatine was chosen as representative of the known biological compounds of this class. Because of the considerable lability of PC under acid conditions, the experiment was

carried out as follows. The enzymic reaction was commenced by the addition of enzyme (0.1 ml.) and stopped by addition of 0.2 ml. of 20% trichloroacetic acid (TCA) to the reaction mixture (2 ml.). After 15 sec., the mixture was brought to pH 8.0 by addition of the appropriate amount of 0.2N-NaOH, and the liberated creatine or inorganic phosphate determined as indicated under Methods. Both phosphatases rapidly hydrolysed PC, and

with both enzymes the rate of hydrolysis was markedly dependent on substrate concentration, increasing up to the maximum (0.05M) used. The activity/pH curve for hydrolysis of PC (0.015M) is compared with those for hydrolysis of β -glycerophosphate (0.02M) and phenyl phosphate (0.025M) in Fig. 1. It is seen that the pH has somewhat less influence on the rate of hydrolysis of PC by intestinal phosphatase than on the rate of hydrolysis

Table 1. *Compounds examined as substrates for purified milk and intestinal phosphatases*

Each compound, at the concentration indicated, was examined as described under Methods, either at an arbitrarily chosen pH value or over a range of pH values from about 8.2 to 10.2.

	Compound tested	Concentration (M)	pH of reaction	
			Intestinal phosphatase	Milk phosphatase
	Hydrolysed			
Orthophosphate monoesters and amides	Phenyl phosphate	2.5×10^{-3}	9.8*	10.05*
	<i>p</i> -Nitrophenyl phosphate	1.0×10^{-3}	9.6	9.6
	Phenolphthalein phosphate	1.0×10^{-3}	9.6	9.6
	α -Glycerol phosphate	2.0×10^{-2}	9.3*	—
	β -Glycerol phosphate	2.0×10^{-2}	9.45*	9.65*
	2-Phosphoglycerate	Approx. 1×10^{-4}	9.0	—
	3-Phosphoglycerate	Approx. 1×10^{-4}	9.0	—
	Triose phosphate	Approx. 4×10^{-4}	9.0	—
	Glucose 1-phosphate	1.7×10^{-3}	9.3*	9.5
	Glucose 6-phosphate	1.3×10^{-3}	9.2*	9.5
	Fructose 1-phosphate	Approx. 2×10^{-4}	9.45	—
	Fructose 6-phosphate	Approx. 5×10^{-4}	9.45	—
	Fructose 1,6-diphosphate	1.2×10^{-3}	9.15	9.5
	Adenosine 3'-phosphate	5.2×10^{-4}	9.45*	9.5
	Adenosine 5'-phosphate	4.5×10^{-4}	9.45*	9.5
	PC	1.5×10^{-2}	9.55*	9.55*
	PEP	1.0×10^{-2}	9.65	9.5
	Not hydrolysed			
Orthophosphate diesters	YNA	†	—	—
	Diphenyl phosphate	Approx. 1×10^{-4}	—	—
Orthophosphate triesters	Trimethyl phosphate	Approx. 1×10^{-2}	—	—
	Triphenyl phosphate	Approx. 1×10^{-2}	—	—
Polyphosphates	Sodium pyrophosphate	5.0×10^{-4}	—	—
	Sodium hexametaphosphate	Approx. 1×10^{-5}	—	—
	Diphenyl pyrophosphate	Approx. 1×10^{-4}	—	—
	ATP	Approx. 6×10^{-4}	—	—
	ADP	Approx. 5×10^{-4}	—	—
	DPN	Approx. 6×10^{-4}	—	—

* Optimum pH values for hydrolysis at the substrate concentration shown.

† 120 μ g. organic phosphate per 2 ml. final volume.

Table 2. *K_m values for some substrates of alkaline phosphatases*

K_m values were estimated graphically according to Lineweaver & Burk (1934) as modified by Dixon (1953a).

Substrate	pH	K _m value (M)	
		Intestinal phosphatase	Milk phosphatase
Phenyl phosphate	10.0	8.6×10^{-4}	6.1×10^{-4}
β -Glycerol phosphate	9.0	1.7×10^{-2}	—
Adenosine 5'-phosphate	9.45	1.1×10^{-2}	—
Adenosine 3'-phosphate	9.45	2.0×10^{-4}	—
Phosphocreatine	9.55	3.5×10^{-2}	3.5×10^{-2}

of other typical orthophosphate monoesters when examined at approximately the same substrate concentration.

The K_m values at pH 9.6, obtained with milk and intestinal phosphatases, are considerably greater than those obtained for other substrates examined (Table 2), indicating a rather low affinity of these phosphatases for PC.

Fig. 1. Activity/pH curves for hydrolysis of phosphocreatine (PC), β -glycerophosphate and phenyl phosphate by purified intestinal alkaline phosphatase. The reaction mixtures contained magnesium acetate (0.01 M), ethanolamine-HCl buffer (0.04 M) and substrates at the concentrations shown. The reaction period was 5 min. at 38°. Hydrolysis was estimated as described under Methods. Results are expressed as μ g. P liberated in 5 min. by the quantity of enzyme indicated. ●—●, PC (0.015 M), with approx. 12 μ g. enzyme; ○—○, β -glycerophosphate (0.02 M), with approx. 2.1 μ g. enzyme; +—+, phenyl phosphate (0.025 M), with approx. 1 μ g. enzyme.

It is noteworthy that PC was hydrolysed by a partially purified acid phosphatase from human prostate gland (Morton, 1952). At pH 5.5, the initial rate of hydrolysis with 0.005 M PC was 102 μ g. and with 0.05 M PC, 20 μ g. P/min./0.1 ml. enzyme. High PC concentration thus causes marked inhibition of hydrolysis of PC by this enzyme, in marked contrast to the results obtained with the alkaline phosphatases.

Orthophosphate diesters. A commercial preparation of partially degraded yeast nucleic acid (YNA) was used as a source of naturally occurring phosphate diesters. The preparation was found to contain some free purines, adenylic acids *a* and *b*, and polynucleotides.

With a large amount of enzyme (approx. 20 μ g. purified intestinal phosphatase) at pH 9.3, approx.

30% of the total organic phosphate (120 μ g.) was liberated in 90 min., and 40% after 18 hr. The experiment was repeated by adding crystalline ribonuclease (approx. 2 μ g.) before the addition of phosphatase. The initial rate of hydrolysis was not greatly increased (45% of the total phosphate liberated in 90 min.), but 80% of the total phosphate was liberated after 18 hr.

These results, obtained early in 1950, may now be explained in terms of the structure of YNA, which has since been elucidated (see Davidson, 1953). The intestinal phosphatase rapidly removed the monoester groups of the mononucleotides, larger polynucleotides and YNA. In the preparation of YNA used, this accounted for some 40% of the total organic phosphate. The failure of the purified phosphatase to liberate any further inorganic phosphate, even after prolonged incubation, indicates the absence of diesterase activity.

It is clear from the results obtained in the presence of added ribonuclease, a specific phosphate diesterase, that diesterase action, together with phosphatase, would liberate up to 80% of the total organic phosphate of the YNA. The failure to hydrolyse all the organic phosphate links is due to the ribonuclease-resistant 'core' of YNA.

The absence of diesterase activity from the intestinal phosphatase preparation has been further confirmed by Dr R. H. Markham (personal communication), using a phosphate diester of guanosine and uridine as substrate. Likewise diphenyl phosphate was not hydrolysed by intestinal phosphatase.

Pyrophosphates. Inorganic (0.0005 M) and diphenyl (0.0001 M) pyrophosphates were each examined, over the pH range 7.5–10.2. There was no enzymic hydrolysis by either milk or intestinal phosphatase after 90 min. or after 18 hr. incubation, using 10 μ g. of purified enzyme in 3 ml. reaction mixture.

In testing the action of the purified phosphatases on ATP and ADP, approx. 10 μ g. of protein was added to the reaction mixture (3 ml.) over the pH range 8.4–10.2. Inorganic phosphate was estimated after 15 min. With both enzymes there was maximum liberation of inorganic phosphate from the ATP preparation at pH 8.8. The course of the reaction was therefore followed at pH 8.8. As shown by the results in Fig. 2, there was an initial rapid reaction during the first 15 min., after which little further inorganic phosphate appeared. Approx. 5% of the total organic phosphate was liberated after 90 min. However, after 17 hr. at pH 8.8, approx. 19% of the total organic phosphate had appeared as inorganic phosphate. Similar results were obtained with both enzymes.

The results indicate that ATP is not hydrolysed by either phosphatase. The initial rapid liberation

of inorganic phosphate is attributed to the hydrolysis of AMP, shown by chromatography to be present. Moreover, by comparison of the acid-labile phosphorus (197 $\mu\text{g.}$) with total organic phosphorus (320 $\mu\text{g.}$) it was estimated that the preparation contained between 5 and 10% AMP. The liberation of 19% of the organic phosphate, following prolonged incubation (17 hr.) appears to be due to the non-enzymic splitting of ATP in alkaline solution, giving inorganic pyrophosphate and AMP, the latter being hydrolysed by intestinal

phosphatase. Since essentially the same amount of inorganic phosphate was liberated with twice the amount of enzyme (Fig. 2), it cannot be attributed to enzymic hydrolysis of ATP.

Somewhat similar results were obtained with the ADP preparation, as shown in Fig. 2. In this case, however, the inorganic phosphate liberated enzymically after 90 min. amounted to 15% of the total organic phosphate. Since similar results were obtained with two enzymes, and with intestinal phosphatase at two concentrations (approx. 10 and 20 $\mu\text{g.}$ protein per tube), the enzymic reaction had effectively reached completion.

AMP was shown to be present in the ADP preparation, and comparison of the acid-labile phosphorus (81 $\mu\text{g.}$) with the total organic phosphorus (185 $\mu\text{g.}$) indicated that between 10 and 20% of the organic phosphate was probably present as AMP. The results therefore show that ADP, like ATP, is not hydrolysed by either milk or intestinal phosphatase. The inorganic phosphate liberated is attributed to hydrolysis of contaminant AMP which, as shown in Fig. 2, is rapidly and completely hydrolysed by both phosphatases.

When a preparation of DPN, containing 214 $\mu\text{g.}$ organic phosphorus per reaction, was examined with both phosphatases under the same experimental conditions as those used with ATP, the results were essentially similar to those obtained with ATP. The inorganic phosphorus (38 $\mu\text{g.}$) liberated after 90 min. incubation with 10 $\mu\text{g.}$ intestinal phosphatase was undoubtedly due to the presence of contaminant AMP. Further evidence that DPN is not hydrolysed by either milk or intestinal phosphatase is presented in another paper (Morton, 1955b).

The effect of certain esterase inhibitors on milk and intestinal alkaline phosphatases. The experiments were carried out as described under Methods, using approx. 0.55 $\mu\text{g.}$ of intestinal phosphatase and 2.5 $\mu\text{g.}$ of milk phosphatase per tube. The results are shown in Table 3. None of the organophosphorus compounds examined inhibited appreciably except at relatively high concentrations, i.e. at greater than 10^{-3}M concentration. However, cysteine and iodine caused 72 and 84% inhibition respectively at 10^{-3}M concentration.

DISCUSSION

The substrate specificity of alkaline phosphatases

The results reported in this paper were obtained with enzyme preparations which appear to be substantially homogeneous (Morton, 1952, 1953a, 1954). It is clear from Table 1 that both purified enzymes hydrolysed all the true orthophosphate monoesters examined. Related compounds containing a primary orthophosphate group, such as

Fig. 2. The action of purified alkaline phosphatases on ATP, ADP, AMP and PC. The reaction mixture (3 ml.) contained ethanolamine-HCl buffer (0.04M), magnesium acetate (0.01M) and the amount of organic phosphorus shown.

ATP (320 $\mu\text{g. P}$) at pH 8.8: $\circ-\circ$, with milk phosphatase (approx. 10 $\mu\text{g.}$); $\bullet-\bullet$, with intestinal phosphatase (approx. 10 $\mu\text{g.}$); $\times-\times$, with intestinal phosphatase (approx. 20 $\mu\text{g.}$).

ADP (185 $\mu\text{g. P}$) at pH 8.8: $\square-\square$, with milk phosphatase (approx. 10 $\mu\text{g.}$); $\blacksquare-\blacksquare$, with intestinal phosphatase (approx. 10 $\mu\text{g.}$); $\blacksquare-\blacksquare$, with intestinal phosphatase (approx. 20 $\mu\text{g.}$).

AMP (195 $\mu\text{g. P}$) at pH 9.3: $\triangle-\triangle$, with milk phosphatase (approx. 10 $\mu\text{g.}$); $++$, with intestinal phosphatase (approx. 5 $\mu\text{g.}$).

PC (328 $\mu\text{g. P}$) at pH 9.5: $\ominus-\ominus$, with intestinal phosphatase (approx. 20 $\mu\text{g.}$).

Table 3. *The effect of certain organophosphorus and other compounds on the hydrolysis of phenyl phosphate by purified alkaline phosphatases*

Most of the organophosphorus compounds were made up as concentrated solutions in ethanol. Dilutions were prepared using veronal-sodium acetate-HCl buffer (0.02M) at pH 7.8. Then 0.1 ml. of the compound at the appropriate dilution was added to 0.4 ml. of the diluted enzyme in the same buffer at pH 7.8, the reactants mixed and held for 20 min. at room temp. (approx. 18°). The activity test was carried out by addition of 0.5 ml. solution containing phenyl phosphate, magnesium acetate, and ethanolamine-HCl buffer (pH 9.95) and sufficient 0.1N NaOH so that the final pH of the reaction was 9.95. Final concentrations for the activity test were: disodium phenyl phosphate, 0.02M; magnesium acetate, 0.01M; ethanolamine-HCl buffer (0.04M). Hydrolysis was estimated by determination of phenol or of inorganic phosphate (where cysteine or iodine was present) liberated enzymically in 5 min. at 38°. Each tube contained sufficient enzyme to liberate approx. 2 μ moles phenol (or inorganic phosphate)/5 min. in the absence of inhibitor.

	Compound	- Log* concn. (M)	Percentage inhibition	
			Intestinal phosphatase	Milk phosphatase
Phosphates	Disodium phosphate	2	25	—
	Trimethyl phosphate	2	0	0
	Triphenyl phosphate	2	11	15
Polyphosphates	Sodium pyrophosphate	2	36	—
	Tetraethyl pyrophosphate	2	0	—
		3	0	0
	Hexaethyl tetraphosphate	2	36	—
		4	2	0
Phosphites	Sodium hypophosphite	2	0	—
	Sodium phosphite	2	2	—
	Diisopropyl phosphite	2	51	—
		3	10	4
		4	3	—
		5	0	—
	Triisopropyl phosphite	2	17	—
		3	4	—
		4	0	—
	Tri- <i>n</i> -butyl phosphite	3	1	—
	Triphenyl phosphite	3	27	32
		4	0	—
Phosphonates	Diethyl phosphonate	2	2	0
	Ammonium fluorophosphonate	2	28	28
	Diisopropyl fluorophosphonate	3	20	26
Other compounds	Sodium borate	2	13	—
	Tribenzyl borate	2	38	24
	Sodium arsenate	2	74	—
		3	0	—
	Sodium arsenite	3	13	—
	<i>p</i> -Nitrophenyl sulphate	3	0	—
	Cysteine	2	84	—
		3	72	—
		4	0†	—
	Iodine (in potassium iodide)	2	100	—
		3	84	—
		4	0‡	—

* Concentration during treatment of enzyme, before activity test.

† 15% activation.

‡ 20% activation.

PEP and PC, were also hydrolysed. However, Table 1 also shows that, under the experimental conditions used, both enzymes failed to hydrolyse any orthophosphate diesters or triesters or inorganic or organic polyphosphates. Hence both enzymes have well-defined substrate specificities and are true orthophosphate monoesterases.

There is no reason for doubting that both phosphatases will hydrolyse all true orthophosphate monoesters. However, the rates of hydrolysis (Fig. 1) and the K_m values (Table 2) vary with different substrates, as observed earlier by Delory & King (1943). As shown by comparison of the values obtained with adenosine 3'- and 5'-phosphates (Table 2), the K_m values (and the rates of hydrolysis) may differ considerably even for closely related compounds.

The classification of alkaline phosphatases as monoesterases by Folley & Kay (1936) was based on observations of more rapid hydrolysis of mono- than of di-esters or polyphosphates by crude or partially purified preparations from various tissues. Where the substrate specificity of highly active preparations was adequately examined, however, it was found that YNA, ATP and inorganic pyrophosphate were hydrolysed as well as phosphate monoesters (Schmidt & Thannhauser, 1943). Indeed, such is the pyrophosphatase activity of certain preparations of intestinal alkaline phosphatase that these have been used to obtain evidence for a pyrophosphate link in coenzyme A (Novelli, 1953). However, the results shown in Table 1 indicate that the purified intestinal phosphatase, as well as the milk phosphatase, is devoid of pyrophosphatase activity. Likewise, the extensive hydrolysis of nucleic acids by intestinal phosphatase preparations (Schmidt & Thannhauser, 1943; Zittle, 1946) can now be attributed to contaminant enzymes in the phosphatase preparations. Diesterase activity may similarly accompany the alkaline phosphatase of milk, even after considerable purification, as shown by Zittle & Della Monica (1952).

Since this work was completed and reported elsewhere (Morton, 1952), Schramm & Armbruster (1954) have also shown that a highly active alkaline phosphatase preparation obtained from calf intestinal mucosa hydrolyses diphenyl phosphate and YNA at a rate which is negligible compared with that of the hydrolysis of monophenyl phosphate, glycerophosphate and adenylic acid.

Folley & Kay (1936) classified the enzymes hydrolysing phosphoamides, as distinct from the orthophosphate monoesterases. Gomori (1948) and Holter & Li (1950, 1951) have both claimed to have demonstrated specific tissue phosphoamidases, using phospho-*p*-chloroanilide as substrate. However, as shown in Table 1 and Fig. 2, the naturally

occurring phosphoamide PC is rapidly hydrolysed by both the purified milk and intestinal enzymes. Winnick (1947) had earlier suggested that PC is hydrolysed by alkaline phosphatases since the activity of different tissue extracts with PC was found to be proportional to their activity with phenyl phosphate. Since a highly purified acid phosphatase preparation from prostate gland also rapidly hydrolysed PC at pH 5.5 (Morton, 1952), it seems not unlikely that PC is attacked at acid pH by the enzyme of wide substrate specificity rather than by a specific phosphoamidase. Furthermore, PC, like phenyl phosphate, acts as a donor for the transfer reactions catalysed by both milk and intestinal alkaline phosphatases and by prostate-gland acid phosphatase (Morton, 1952, 1953*b*). None of the substrate-specific phosphatases investigated catalysed such a transfer reaction (Morton, 1952), and hence it is unlikely that the observed reactions with PC could be due to specific phosphoamidases.

These findings do not preclude the possibility that true phosphoamidases may exist in certain tissues, but they clearly show that the present classification of phosphoamidases as distinct from orthophosphate monoesterases (Roche, 1950), especially as this relates to activity at alkaline pH values, is not justified by experimental findings. True substrate-specific phosphoamidases have yet to be isolated and described.

Since phosphoamides are hydrolysed by both alkaline phosphatases, it is likely that thiophosphates, which have recently been synthesized (Walsh, 1952), are also hydrolysed by these enzymes. It is to be expected that the -S-P bond in thiophosphates is even more analogous to the -O-P bond in orthophosphates than is the -N-P bond in phosphoamides. Although no true acyl phosphate has been tested, the somewhat related PEP is rapidly hydrolysed by both enzymes, and there is no reason for doubting that the true acyl phosphates would also be hydrolysed. Lipmann (1946) has suggested that acyl phosphates are hydrolysed at alkaline pH by a non-specific phosphatase, as well as by a more specific enzyme.

It should be noted that most of the compounds which were not hydrolysed by the alkaline phosphatases were examined over a wide range of pH conditions. Large amounts of enzymes were used together with extended incubation periods. This seemed particularly necessary in order to establish a negative finding, in view of the dependence of the rate of hydrolysis by alkaline phosphatase on both the pH and the substrate concentration (Morton, 1952).

The results shown in Table 1 and Fig. 2 establish that the milk and intestinal phosphatases have

similar substrate specificities. However, these enzymes are not identical, as has been shown in another paper (Morton, 1955a).

*The action of some esterase inhibitors
on alkaline phosphatases*

The known inhibitors of alkaline phosphatases (see Roche & Thoai, 1950; Roche, 1950) are relatively unspecific. They fall into the following classes: (a) metal-binding agents such as $\alpha\alpha'$ -dipyridyl and ethylenediaminetetraacetic acid; (b) inhibitory metal salts such as zinc and beryllium chlorides; (c) amino-binding agents such as keten, phenyl isocyanate, nitrous acid and formaldehyde, and (d) competitive inhibitors of hydrolysis, such as inorganic phosphate. Sizer (1942) also showed that oxidizing agents such as potassium permanganate and iodine inhibit alkaline phosphatase activity. This inhibition was partly reversible.

It was hoped that it would be possible to find specific reagents which would non-competitively inhibit alkaline phosphatases. Since the study of the substrate specificity as already discussed clearly indicated that both the milk and intestinal enzymes are true orthophosphate monoesterases, the investigation of certain esterase inhibitors for this purpose was suggested by Dr M. Dixon.

However, as shown by the results in Table 3, none of the organic phosphates, polyphosphates, phosphonates, phosphites or fluorophosphonates investigated had any marked inhibitory action on either the intestinal or milk enzyme. It is noteworthy, however, that all of the organic phosphites and phosphonates investigated were secondary or tertiary compounds. It is possible that certain primary phosphites or phosphonates may inhibit more strongly.

Many of the organophosphorus compounds listed in Table 3 are extremely toxic, being powerful inhibitors of carboxylic esterases, especially of cholinesterases (McCombie & Saunders, 1946; Webb, 1948; Mackworth & Webb, 1948). Hence the phosphoric esterases, which are only slightly inhibited by these compounds, must differ considerably from the carboxylic esterases. The organophosphorus compounds which are inhibitory to carboxylic esterases are apparently hydrolysed by these enzymes, giving rise to inactive phosphorylated enzyme derivatives (see Davies, 1954, for review). However, the phosphoric esterases do not appear to hydrolyse, or combine with, organophosphorus compounds other than the primary esters of orthophosphoric acid, and hence are not inhibited by the organophosphorus compounds listed in Table 3. It should be noted that Webb (1948) had previously shown that crude kidney alkaline phosphatase was only slightly inhibited by relatively high concentrations of DFP.

The most effective inhibitors among the compounds investigated were iodine and cysteine, both of which caused strong inhibition of the milk and intestinal enzymes at 10^{-3} M inhibitor. The inhibition by cysteine is complicated by the possibility of metal chelation and by the fact that, since the enzyme and cysteine were held at pH 7.8 for 20 min. before addition of the substrate, much of the cysteine may have been oxidized to cystine.

The inhibitory action of iodine is particularly interesting. It is very probably due to the iodination of phenolic hydroxyl groups of the enzyme, as suggested by Sizer (1942). It seems likely that certain tyrosine groups of intestinal phosphatase may be directly involved in the formation of an enzyme-substrate complex. Some evidence in support of this hypothesis has been obtained by using the method of Dixon (1953b) to examine the pK_m versus pH relationship for hydrolysis of phenyl phosphate by intestinal phosphatase (Morton, 1952; Dixon, 1953b). This work will be presented in detail in a subsequent paper.

Sodium arsenate, at 10^{-3} M, caused a much greater inhibition of the intestinal phosphatase than did the same concentration of sodium phosphate. Since sodium phosphate is a competitive inhibitor of hydrolysis of organic phosphates (Jacobsen, 1932, 1933), it is probable that sodium arsenate is also a competitive inhibitor. In this regard, it should be noted that the activity test was carried out with very high substrate concentration and at highly alkaline pH in order to reduce competitive inhibition to a minimum. Under other experimental conditions, inhibition by arsenate may be much greater. It is probable that the phosphatases would have a greater affinity for organic derivatives of arsenic acid than for inorganic arsenate. Thus, organic derivatives of arsenic acid would seem worthy of investigation as useful competitive inhibitors of alkaline (and acid) phosphatases.

SUMMARY

1. The substrate specificity of alkaline phosphatases, purified from cow's milk and calf intestinal mucosa, has been investigated, using a wide range of compounds under several different experimental conditions.

2. All true orthophosphate monoesters were rapidly hydrolysed by both enzymes, as well as the orthophosphoamide, phosphocreatine, and the enolic phosphate, phosphoenolpyruvate.

3. Sodium pyrophosphate, sodium hexametaphosphate, diphenyl pyrophosphate, ATP, ADP and DPN were not hydrolysed by either enzyme, showing absence of any pyrophosphatase activity. Absence of diesterase activity was shown from studies with partially degraded yeast nucleic acid,

both in the presence and absence of added ribonuclease.

4. A number of organic polyphosphates, phosphates, phosphites, phosphonates and fluorophosphonates were found to cause only slight inhibition of milk or intestinal phosphatase at 10^{-2} M concentration under the experimental conditions used. However, iodine and cysteine at 10^{-3} M concentration both caused strong inhibition of the two phosphatases. A possible mechanism for the inhibition by iodine is discussed.

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REFERENCES

- Davidson, J. N. (1953). *The Biochemistry of the Nucleic Acids*, 2nd ed. London: Methuen and Co.
- Davies, D. R. (1954). *J. Pharm., Lond.*, **6**, 1.
- Delory, G. E. & King, E. J. (1943). *Biochem. J.* **37**, 547.
- Dixon, M. (1953a). *Biochem. J.* **55**, 170.
- Dixon, M. (1953b). *Biochem. J.* **55**, 161.
- Eggleton, P., Elsdon, S. R. & Gough, N. (1943). *Biochem. J.* **37**, 526.
- Ennor, A. H. & Stocken, L. A. (1948). *Biochem. J.* **43**, 190.
- Fiske, C. H. & Subbarow, Y. (1925). *J. biol. Chem.* **66**, 375.
- Folley, S. J. & Kay, H. D. (1936). *Ergebn. Enzymforsch.* **5**, 159.
- Gomori, G. (1948). *Proc. Soc. exp. Biol., N.Y.*, **69**, 407.
- Holter, H. & Li, S.-O. (1950). *Acta chem. scand.* **4**, 1321.
- Holter, H. & Li, S.-O. (1951). *C.R. Lab. Carlsberg, Sér. chim.* **27**, 393.
- Jacobsen, E. (1932). *Biochem. Z.* **249**, 21.
- Jacobsen, E. (1933). *Biochem. Z.* **267**, 89.
- King, E. J. (1951). *Micro-Analysis in Medical Biochemistry*, 2nd ed. London: Churchill.
- LePage, G. A. (1949). *Manometric Techniques and Tissue Metabolism*. Ed. by Umbreit, W. W., Burris, R. H. & Stauffer, J. F. Minneapolis: Burgess Publishing Co.
- Lineweaver, H. & Burk, D. (1934). *J. Amer. chem. Soc.* **56**, 658.
- Lipmann, F. (1946). *Advanc. Enzymol.* **6**, 231.
- McCombie, H. & Saunders, B. C. (1946). *Nature, Lond.*, **157**, 287.
- Mackworth, J. F. & Webb, E. C. (1948). *Biochem. J.* **42**, 91.
- Meyerhof, O. (1938). *Bull. Soc. Chim. biol., Paris*, **20**, 1345.
- Meyerhof, O. & Green, H. (1950). *J. biol. Chem.* **183**, 377.
- Meyerhof, O. & Oesper, P. (1949). *J. biol. Chem.* **179**, 1371.
- Morton, R. K. (1950). *Nature, Lond.*, **166**, 1092.
- Morton, R. K. (1952). Thesis, University of Cambridge.
- Morton, R. K. (1953a). *Biochem. J.* **55**, 795.
- Morton, R. K. (1953b). *Nature, Lond.*, **172**, 65.
- Morton, R. K. (1954). *Biochem. J.* **57**, 595.
- Morton, R. K. (1955a). *Biochem. J.* **60**, 573.
- Morton, R. K. (1955b). *Biochem. J.* **61**, 240.
- Needham, D. M. (1942). *Biochem. J.* **36**, 113.
- Novelli, G. D. (1953). *J. cell. comp. Physiol.* **41**, Suppl. 1, 67.
- Roche, J. (1950). *The Enzymes*, vol. 1, part 1, p. 473. Ed. by Sumner, J. B. & Myrback, K. New York: Academic Press.
- Roche, J. & Thoai, N.-v. (1950). *Advanc. Enzymol.* **10**, 83.
- Schmidt, G. & Thannhauser, S. J. (1943). *J. biol. Chem.* **149**, 369.
- Schramm, G. & Armbruster, D. (1954). *Z. Naturf.* **9b**, 114.
- Sizer, I. W. (1942). *J. biol. Chem.* **145**, 405.
- Walsh, F. O'F. (1952). *Nature, Lond.*, **169**, 546.
- Webb, E. C. (1948). *Biochem. J.* **42**, 96.
- Weil-Malherbe, H. & Green, R. H. (1951). *Biochem. J.* **49**, 286.
- Winnick, T. (1947). *Arch. Biochem.* **12**, 209.
- Zittle, C. A. (1946). *J. biol. Chem.* **166**, 491.
- Zittle, C. A. & Della Monica, E. S. (1952). *Arch. Biochem. Biophys.* **35**, 321.

The Action of Purified Alkaline Phosphatases on Di- and Tri-Phosphopyridine Nucleotides

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The conversion of triphosphopyridine nucleotide (TPN) into diphosphopyridine nucleotide (DPN) was first reported by von Euler & Adler (1938). The nature of this reaction was not apparent until Kornberg & Pricer (1950a) showed that the third phosphate group of TPN was esterified at either the 2'- or 3'-position of the ribose moiety attached to

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adenine. Hydrolytic cleavage of this esterified phosphate group could thus account for the conversion of TPN into DPN.

It seemed likely that such a reaction would be catalysed by alkaline phosphatases which had been purified from cow's milk and calf intestinal mucosa (Morton, 1950, 1953a, 1954). It had been established that these enzymes acted on monoesters and amides of orthophosphoric acid, but not on diesters