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The Hydrolysis of Phenyl Phosphate by Mouse-Liver Acid Phosphatase

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To determine acid-phosphatase activities of liver homogenates, with phenyl phosphate as substrate, previous workers have usually adopted methods similar to the Robinson, Gutman & Gutman (1939) adaptation of the King & Armstrong (1934) method for serum phosphatase (see, for example, Greenstein, 1942; Kochakian & Fox, 1944; Abul-Fadl & King, 1949; Goodlad & Mills, 1957). However, results obtained by Tsuboi (1952), in studies of the hydrolysis of phenyl phosphate by mouse-liver acid phosphatase, have indicated that the assay conditions of the Robinson *et al.* (1939) method, and of similar methods, allow only partial expression of the capacity of liver homogenate to hydrolyse phenyl phosphate in acid media.

In the present work, a re-examination has been made of the hydrolysis of phenyl phosphate by mouse-liver acid phosphatase and, although the results have differed from those of Tsuboi (1952), it has been confirmed that the Robinson *et al.* (1939) procedure is unsuitable for quantitative measurement of liver acid-phosphatase activity. From the results of these studies a simple and rapid method for quantitative measurement of mouse-liver acid-phosphatase activity with phenyl phosphate has been developed.

MATERIALS AND METHODS

Experimental animals were normal, well-fed, adult male mice of mixed laboratory strains. Chemicals were of A.R. grade whenever possible. Spectrophotometric measure-

ments were made on a Unicam spectrophotometer (SP. 500) and pH measurements on a model C pH electrometer (N. L. Jones, Melbourne). Enzyme assays and homogenate incubation were made at 37° with shaking racks fitted to a Braun model V rotary Warburg bath.

Phenol solution. A stock phenol soln. (1 g./l.), prepared by dissolving distilled phenol in *N*-HCl, was standardized by iodometric titration (Cameron & White, 1940). $E_{290} - E_{320}$ and $E_{295} - E_{320}$ values, for *mm*-phenol in *N*-NaOH, were 2.46 and 1.89 respectively. Corresponding values of 2.45 and 1.92 were reported by Brawerman & Chargaff (1954).

Phenyl phosphate. Commercial disodium phenyl phosphate was purified by the method of Tsuboi (1952). Total phosphorus of product, 14.3%; theoretical for $\text{Na}_2\text{C}_6\text{H}_5\text{PO}_4$, 14.2%.

Ethylenediaminetetra-acetate. The commercial disodium salt of ethylenediaminetetra-acetic acid (EDTA) was purified by the method of Blaedel & Knight (1954).

Sulphydryl compounds. *p*-Chloromercuribenzoic acid was prepared according to Whitmore & Woodward (1941). Phenyl mercuric acetate was obtained from British Drug Houses Ltd., and *o*-iodosobenzoic acid from L. Light and Co. Ltd. Aqueous solutions of these compounds were prepared with minimal NaOH.

Fresh solutions of L-cysteine hydrochloride (British Drug Houses Ltd.) were adjusted to pH 5.9 before use.

Preparation of liver homogenates. Preliminary studies of the intracellular distribution of mouse-liver acid phosphatase, with phenyl phosphate as substrate, indicated that mitochondria contained unreactive enzyme (equivalent to approx. 40% of the total enzyme activity) and that suspension of the mitochondria in water led to activation of this bound enzyme. These results were in general agreement with those obtained by Berthet & de Duve (1951) in studies of the hydrolysis of β -glycerophosphate by rat-liver acid

phosphatase. Water homogenates were therefore used to measure total liver acid-phosphatase activity.

Freshly excised liver, freed from gall-bladder, was homogenized in previously boiled and cooled glass-distilled water in a Potter & Elvehjem (1936) homogenizer. The homogenate (usually 0.8%, w/v) was stood in an ice bath for 15–30 min. and samples of the supernatant were used for enzyme determinations.

Total nitrogen was measured by the method of Francis, Mulligan & Wormald (1954).

Measurement of enzyme activities. The substrate phenyl phosphate (pK_a , 5.72; King & Delory, 1939) was also used as buffer (pH 5.9) except as indicated in the text. Thus freshly prepared solutions of disodium phenyl phosphate were adjusted to pH 5.9 with HCl.

To tubes containing 5.0 ml. of buffered substrate solution (at 37°), samples of homogenate (0.5 ml.) were added and incubation was continued, with shaking, at 37°. Enzyme action was arrested by addition of 1 ml. of N -NaOH. In control tubes, homogenate was added after NaOH. All determinations were run in duplicate. Phenol values were determined from measurements of E at 320, 295 and 290 $m\mu$ (Brawerman & Chargaff, 1954). This method of phenol measurement offered advantages over the colorimetric method of King & Armstrong (1934) in being simpler and in being free from interference by cysteine and by EDTA.

Measurements were also made of the inorganic orthophosphate released by phosphatase action on phenyl phosphate. After spectrophotometric measurement of phenol, 2.5 ml. of 30% trichloroacetic acid was added to each tube, the tube contents were centrifuged and inorganic phosphate in the supernatant was determined by the method of King (1932). In all experiments (except in studies of the effects of inorganic phosphate and pyrophosphate on acid-phosphatase activity) phosphate values, corrected for inorganic phosphate in controls, agreed closely with values expected from phenol determinations. However, phosphate values were used only to confirm phenol values and are not reported in this paper.

Measurement of the acid-phosphatase activity of mouse blood indicated that, under the conditions used, blood in the preparations made negligible contributions to hydrolysis of phenyl phosphate by homogenates of mouse liver.

RESULTS

Optimum pH

The effect of pH on enzyme activity was determined in experiments in which liberated phenol was measured by reaction with Folin & Ciocalteu (1927) reagent (Cameron & White, 1940). A single well-defined peak of enzyme activity was obtained (Fig. 1). The optimum pH range was subsequently confirmed in experiments in which phenol was measured by ultraviolet spectrophotometry (Brawerman & Chargaff, 1954) and in which inorganic phosphate was determined by the method of King (1932).

Since the hydrolysis of phenyl phosphate tends to increase the pH of the solution (Schönheyder, 1952), substrate solutions were subsequently adjusted to pH 5.9.

Effect of substrate concentration

With homogenate samples containing 1–20 mg. (wet wt.) of liver, maximum rates of hydrolysis were obtained at pH 5.9 with phenyl phosphate concentrations of approximately 0.1M. Increasing the substrate concentration to 0.25M did not cause enzyme inhibition.

With 0.15M-phenyl phosphate (pH 5.9) and incubation for 5 min. periods, a linear relationship between rate of hydrolysis and quantity of liver (1–20 mg. wet wt.) was obtained.

Instability of liver acid phosphatase in dilute homogenates

In the absence of substrate. In preliminary studies of the relationships between liver acid-phosphatase activity and incubation period it was observed that short incubation periods (5–10 min.) generally resulted in significantly higher activity values than were obtained with longer incubation periods. Since it appeared that these differences were due to enzyme inactivation during incubation at 37°, the stability of liver acid phosphatase, in both the

Fig. 1. pH-Activity curve of phenyl phosphate hydrolysis by mouse-liver acid phosphatase. Reaction mixtures (5.5 ml.) contained 3.5 mg. of liver and 0.14M-sodium phenyl phosphate and were incubated for 5 min. at 37°. Buffers used: pH 3.4–4.5, 0.10M-sodium acetate-HCl; pH 4.8–6.4, 0.14M-disodium phenyl phosphate-HCl; pH 7.0–9.0, 0.10M-sodium veronal-HCl. Enzyme activity was arrested with 2.5 ml. of diluted (1:3) Folin & Ciocalteu (1927) reagent and phenol measured colorimetrically (Cameron & White, 1940).

absence and the presence of substrate, was examined. Dilute, untreated liver homogenate (0.6%, w/v; pH 6.8) showed slow loss of enzyme activity when stored in an ice bath, and preincubation of the same homogenate at 37°, with shaking, resulted in increased enzyme inactivation. Adjustment of the homogenate to pH 5.9, the pH of enzyme assay, caused a further increase in the rate of inactivation at 37° (Fig. 2).

When adjustment of the pH of preincubated liver homogenate was made with sodium malonate-HCl buffer, pH 5.9, the rate of enzyme inactivation varied with malonate concentration. As shown in Fig. 3, increasing the concentration of malonate in preincubated homogenate (1 hr. at 37°) led to increased protection of enzyme until, at 0.15M-malonate, enzyme inactivation was completely prevented.

This effect was not due simply to increase in ion content since added NaCl (0.1M) failed to influence enzyme inactivation in the presence of 0.01M-sodium malonate-HCl buffer and marked variations in enzyme inactivation occurred in the presence of other buffer systems. Comparisons of the effects produced by sodium malonate-HCl, sodium citrate-HCl, sodium succinate-HCl, disodium EDTA-NaOH (pK_a of EDTA, 6.16; Martell & Calvin,

1952) and sodium acetate-HCl buffer systems (all 0.1M, pH 5.9) showed that citrate was nearly as efficient as malonate in protecting acid phosphatase, that EDTA and succinate were noticeably less efficient than malonate and that acetate apparently had no protective action (Table 1). In assessing the results of Table 1, allowance was made for direct inhibition of enzyme activity by the tested buffers. With the fresh, buffered homogenates malonate was non-inhibitory whereas succinate caused 20%, citrate 9%, acetate 8% and EDTA 6% inhibition.

Possible participation of O_2 , in the process of enzyme inactivation, was indicated by the finding that enzyme inactivation was slightly reduced when N_2 was used as gas phase (Table 1). Addition of cysteine to preincubated homogenates also gave slight protection of enzyme (Table 1).

In the presence of substrate. Evidence of inactivation of acid phosphatase during incubation of liver homogenate with phenyl phosphate at 37° is presented in Fig. 4.

With 0.15M-phenyl phosphate, and with the substrate as buffer (pH 5.9), 4 mg. of liver showed maximum enzyme activity during incubation for 10 min. and this activity was not influenced by the

Fig. 2. Acid-phosphatase activities of 0.6% liver homogenate (containing 0.01M-sodium malonate-HCl buffer, pH 5.9) when kept in an ice bath (O), and when incubated, with shaking, at 37° (●). At specified times, samples (0.5 ml.) of homogenate were used for enzyme assays. Assay conditions: 0.10M-sodium phenyl phosphate, pH 5.9; total vol., 5.5 ml.; incubation for 10 min. at 37°.

Fig. 3. Effects of sodium malonate-HCl buffer (pH 5.9) on inactivation of acid phosphatase in preincubated liver homogenate. Samples (20 ml.) of 0.6% liver homogenate, containing malonate buffer as indicated, were incubated in stoppered 50 ml. conical flasks for 1 hr. at 37° (with shaking) and portions (0.5 ml.) used for enzyme assays. Conditions of enzyme assay: 0.15M-sodium phenyl phosphate, pH 5.9; total vol., 5.5 ml.; incubation for 10 min. at 37°. O, Activities of fresh homogenates; ●, activities after preincubation for 1 hr.

Table 1. *Acid-phosphatase activities of mouse-liver homogenate preincubated with various buffer systems*

Samples (20 ml.) of 0.6% liver homogenate, containing indicated buffers (pH 5.9), were incubated, with shaking, in stoppered 50 ml. conical flasks (or Thunberg tubes when nitrogen was used as gas phase) for 1 hr. at 37° and portions (0.5 ml.) were used for enzyme assays. Residual enzyme activities are expressed as percentages of the activity of fresh 0.6% homogenate containing 0.10M-sodium malonate-HCl buffer (pH 5.9). Conditions of enzyme assay: 0.15M-sodium phenyl phosphate, pH 5.9; total vol., 5.5 ml.; incubation for 10 min. at 37°.

Preincubated homogenate			Residual enzyme activity (%)
Buffer system (0.10M)	Gas phase	Cysteine (0.015M)	
Sodium malonate-HCl	Air	-	88
Sodium citrate-HCl	Air	-	77
Disodium EDTA-NaOH	Air	-	60
Sodium succinate-HCl	Air	-	47
Sodium acetate-HCl	Air	-	23
Sodium acetate-HCl	Air	+	33
Sodium acetate-HCl	Nitrogen	-	33
Sodium acetate-HCl	Nitrogen	+	49

Fig. 4. Time course of hydrolysis of phenyl phosphate by mouse-liver acid phosphatase. Reaction mixtures (5.5 ml.; pH 5.9) contained 4 mg. of liver and: ○, 0.15M-sodium phenyl phosphate; ●, 0.15M-sodium phenyl phosphate + 0.05M-sodium malonate; □, 0.04M-sodium phenyl phosphate; ■, 0.04M-sodium phenyl phosphate + 0.05M-sodium malonate. Mixtures were incubated, with shaking, at 37°. Each point is the mean value of duplicate tubes. Broken lines are straight-line extrapolations of the maximum initial rates of hydrolysis obtained with the two concentrations of substrate.

presence of malonate (0.05M). However, when the incubation period was increased to 60 min., the presence of malonate resulted in greater hydrolysis of phenyl phosphate over this period. Similar patterns of enzyme activity were obtained with 0.04M-phenyl phosphate and prolonged incubation,

Table 2. *Effects of ethylenediaminetetra-acetic acid and metal ions on mouse-liver acid-phosphatase activity*

Conditions of enzyme assay: 7 mg. of liver; 0.10M-sodium phenyl phosphate, pH 5.9 (adjusted with acetic acid); additions as shown in Table; total vol., 5.5 ml.; incubation for 6 min. at 37°. Enzyme activity was arrested by addition of 1.0 ml. of N-NaOH (0.325M with respect to tetrasodium salt of EDTA).

Addition	Concn. (mm)	Percentage inhibition of acid-phosphatase activity
Sodium EDTA (pH 5.9)	50	0
Magnesium acetate	10	0
Calcium acetate	10	0
Barium chloride	10	0
Manganese chloride	0.1	0
Stannous chloride	0.1	10
Aluminium chloride	1.0	45
	0.1	10
Zinc sulphate	1.0	75
	0.1	15
Ferric sulphate	0.1	50
Lead nitrate	0.1	0
Silver nitrate	0.1	65
Copper sulphate	0.1	85
Mercuric acetate	0.1	90

though enzyme inactivation in the absence of malonate, and degree of malonate protection, were obviously greater than when 0.15M-phenyl phosphate was used. With 0.04M-phenyl phosphate, significant enzyme inactivation occurred during incubation for 10 min. Measurements of the pH of the reaction mixtures, before and after incubation at 37°, showed that losses of activity in the absence of malonate buffer were not due to inadequate buffering by the substrate.

It was concluded that the mechanism of enzyme inactivation is the same as that previously observed in preincubated homogenates (Fig. 2) and that although phenyl phosphate has a protective

Table 3. *Effects of some added compounds on inhibition of mouse-liver acid phosphatase by Fe³⁺, Cu²⁺ and Hg²⁺ ions*

Conditions of enzyme assay: 4 mg. of liver; 0.10M-sodium phenyl phosphate, pH 5.9; inhibitory metal salts, 0.1 mM; added compounds (pH 5.9) as shown in Table; total vol., 5.5 ml.; incubation for 10 min. at 37°. Inhibitions are expressed as percentages of enzyme activities in the absence of inhibitory metal ions.

Added compound	Concn. (M)	Enzyme inhibition (%)		
		In the presence of ferric chloride	In the presence of copper sulphate	In the presence of mercuric acetate
None	—	44	89	95
Sodium malonate	0.05	9	53	60
Sodium citrate	0.05	8	—	—
Sodium EDTA	0.05	29	3	43
L-Cysteine	0.003	0	0	3

action on liver acid phosphatase, the presence of another protective system, such as malonate, is required when incubation periods longer than 10 min., or substrate concentrations less than 0.1 M, are used.

Effects of ethylenediaminetetra-acetic acid and metal ions

The results of studies of the effects of EDTA and selected metal ions on liver acid-phosphatase activity are presented in Table 2. In these experiments, pH adjustments of substrate and disodium EDTA were made with acetic acid and enzyme action was arrested by addition of 1 ml. of N-NaOH containing tetrasodium EDTA (0.325 M). This was added to the NaOH to dissolve metal hydroxides and thereby reduce their interference in spectrophotometric determinations of phenol. As in previous experiments, phenol values were confirmed by measurements of the inorganic phosphate released by enzyme action on phenyl phosphate.

EDTA was not inhibitory and none of the metal ions tested stimulated phenyl phosphate hydrolysis. It therefore appears that metal ions are not required for enzyme activity. This is consistent with the previous observation of linearity between acid-phosphatase activity and quantity of liver in the absence of added metal ions.

Marked inhibition of phenyl phosphate hydrolysis was obtained with Hg²⁺, Cu²⁺, Ag⁺ and Fe³⁺ ions (0.1 mM). Inhibition by Fe³⁺ ions was of special interest as it raised the possibility that enzyme inactivation in preincubated homogenates (Fig. 2), and during incubation with substrate (Fig. 4), was due to Fe³⁺ ions contained in the homogenate. To test this possibility, comparisons were made of the effects of malonate, citrate and EDTA on enzyme inhibitions by Fe³⁺, Hg²⁺ and Cu²⁺ ions (Table 3). The efficiencies of these organic compounds in preventing enzyme inhibition by Fe³⁺ ions closely paralleled their efficiencies in preventing enzyme inactivation in preincubated

Table 4. *Effects of some sulphhydryl inhibitors on mouse-liver acid-phosphatase activity*

Conditions of enzyme assay: 4 mg. of liver; 0.10M-sodium phenyl phosphate, pH 5.9; additions as shown in Table; total vol., 5.5 ml.; incubation for 10 min. at 37°. Inhibitions are expressed as percentages of enzyme activities obtained in the absence of sulphhydryl inhibitors.

Additions	Concn. (mM)	Enzyme inhibition (%)
<i>p</i> -Chloromercuribenzoate	0.1	61
<i>p</i> -Chloromercuribenzoate + L-cysteine	0.1 3.0	0
<i>p</i> -Chloromercuribenzoate + sodium malonate (pH 5.9)	0.1 50	57
Phenyl mercuric acetate	0.1	61
Phenyl mercuric acetate + L-cysteine	0.1 3.0	6
<i>o</i> -Iodosobenzoate	0.1	17
<i>o</i> -Iodosobenzoate + L-cysteine	0.1 3.0	4

homogenates. Thus malonate and citrate afforded almost complete protection against Fe³⁺ ions, whereas EDTA gave only slight protection. By contrast, EDTA was more efficient than malonate in protecting liver acid phosphatase against inhibitions by Hg²⁺ and Cu²⁺ ions. Addition of cysteine to liver homogenate gave complete protection against Fe³⁺, Hg²⁺ and Cu²⁺ ions (Table 3) and this provided evidence that the inhibitory actions of these metal ions were due to reaction with sulphhydryl groups.

Effects of some typical sulphhydryl inhibitors

In view of the sensitivity of liver acid phosphatase to traces of Hg²⁺, Cu²⁺ and Fe³⁺ ions (Table 2), studies were made of the effects of some other typical SH inhibitors. *p*-Chloromercuri-

benzoate, phenyl mercuric acetate and *o*-iodosobenzoate inhibited acid-phosphatase activity and these inhibitions were prevented by the presence of cysteine (Table 4). Malonate had no significant effect on enzyme inhibition by *p*-chloromercuribenzoate (Table 4).

Effects of inorganic phosphate and other anions

Previous workers have reported that certain anions, e.g. oxalate (Belfanti, Contardi & Ercoli,

1935), fluoride (Belfanti *et al.* 1935; Goodlad & Mills, 1957) and tartrate (Goodlad & Mills, 1957), inhibit liver acid-phosphatase activity. The effects of a number of different anions were tested under the assay conditions used in the present work (Table 5). Under these conditions fluoride and arsenate (0.01 M) acted as strong inhibitors (approx. 60% inhibition). Phosphate, pyrophosphate and cyanide caused significant inhibition (20–30%), whereas citrate, β -glycerophosphate, oxalate and tartrate had little effect. Determinations of enzyme activities in the presence of phosphate and pyrophosphate were based solely on measurements of phenol released.

Inhibition by inorganic phosphate was competitive. This is illustrated in Fig. 5, in which experimental results have been recorded as double-reciprocal plots (Lineweaver & Burk, 1934). In the experiments of Fig. 5 the enzyme reaction mixtures also contained 0.05 M-malonate (pH 5.9). This ensured reliable values for enzyme activities over the substrate concentration range used (0.01–0.10 M) and eliminated the complicating factor of substrate protection of enzyme. Even in the presence of malonate, substrate concentrations of approximately 0.1 M were required for enzyme saturation. The Michaelis constant, calculated from Fig. 5, was 13 mM.

Comparison of liver acid-phosphatase activities of well-fed and of starved mice

To determine whether starvation affected liver acid-phosphatase activities and thus accounted for the lower values obtained by Tsuboi (1952), measurements were made of the liver-enzyme activities of mice starved for 22–28 hr. Mean values [$\pm$ s.d. (no. of animals)] of 3.5 ± 0.5 (10) μ g. of phenol/mg. of liver/min. and 102 ± 14 (10) μ g. of phenol/mg. of liver nitrogen/min. were obtained. Corresponding values for well-fed mice were 3.4 ± 0.4 (9) μ g. of phenol/mg. of liver/min. and 91 ± 11 (9) μ g. of phenol/mg. of liver nitrogen/min.

DISCUSSION

The association of liver acid phosphatase with a special class of cytoplasmic granules, the lysosomes, has already been clearly demonstrated by de Duve, Pressman, Gianetto, Wattiaux & Appelmans (1955) in experiments with rat liver and with β -glycerophosphate as substrate. It follows that, to obtain maximum values for liver acid-phosphatase activity, the selected method must ensure (a) complete liberation of active enzyme from intracellular particles, (b) prevention of enzyme inactivation in the preparation of liver homogenates, and (c) prevention of enzyme inactivation during incubation of homogenate sample with substrate. The

Table 5. *Effects of some anions on mouse-liver acid-phosphatase activity*

Conditions of enzyme assay: 4 mg. of liver; 0.1 M-sodium phenyl phosphate, pH 5.9; additions (previously adjusted to pH 5.9 with HCl) as shown in Table; total vol., 5.5 ml.; incubation for 10 min. at 37°.

Addition (0.01 M)	Enzyme inhibition (%)
Sodium fluoride	66
Sodium arsenate	58
Sodium phosphate	29
Sodium cyanide	26
Potassium pyrophosphate	20
Sodium citrate	12
Sodium β -glycerophosphate	7
Potassium oxalate	7
Sodium L-tartrate	6

Fig. 5. Double-reciprocal plots (Lineweaver & Burk, 1934) of hydrolysis of phenyl phosphate by mouse-liver acid phosphatase acting in the absence (O) and the presence (●) of 0.01 M-sodium phosphate. Reaction mixtures (5.5 ml.) contained 4 mg. of liver and 0.05 M-sodium malonate and were incubated at pH 5.9 for 10 min. at 37°. Values for reaction velocity (*v*) are expressed as μ g. of phenol/10 min.

described method appears to satisfy these requirements and has led to higher values for mouse-liver acid-phosphatase activity than have been reported by previous workers (Kochakian & Fox, 1944; Tsuboi, 1952). By virtue of this high enzyme activity, and the permissible dilution of liver homogenates for enzyme assays, the spectrophotometric procedure of Brawerman & Chargaff (1954) has been adopted for measurement of phenyl phosphate hydrolysis by liver homogenates.

In view of the sensitivity of liver acid phosphatase to Hg^{2+} , Cu^{2+} , Ag^{+} and Fe^{3+} ions (Table 2) and to *p*-chloromercuribenzoate, phenyl mercuric acetate and *o*-iodosobenzoate (Table 4), it is concluded that liver acid phosphatase is a sulphhydryl enzyme. In studies of acid phosphatases of other mammalian tissues, namely erythrocytes and prostate, Abul-Fadl & King (1949) obtained evidence that these enzymes are sulphhydryl enzymes and this conclusion has been confirmed by later studies of purified enzymes from these tissues (Tsuboi & Hudson, 1953, 1955).

The apparent dependence of liver acid phosphatase upon sulphhydryl groups may explain the enzyme inactivation which occurs in liver homogenate incubated at 37° either alone (Fig. 2) or with phenyl phosphate (Fig. 4). The possibility that such inactivation is due to oxidation of sulphhydryl groups is supported by the higher enzyme activity shown by liver homogenate pre-incubated with nitrogen as gas phase and in the presence of cysteine (Table 1). Acid-phosphatase inactivation by proteolytic enzymes contained in homogenates appears unlikely in view of the short incubation periods (1 hr.) used in inactivation studies and in view of the protective actions of malonate and citrate (Figs. 3 and 4, and Table 1).

The technique of using substrate as buffer (pH 5.9) has permitted assessment of the effects of added buffer systems on phenyl phosphate hydrolysis by liver acid phosphatase. Of the various buffers tested (malonate, citrate, EDTA, succinate, acetate), malonate (pK_a 5.68; Umbreit, Burris & Stauffer, 1951) appears to be the most suitable in that, although without obvious effect on the enzyme reaction by itself, it provides greatest protection of acid phosphatase in liver homogenate incubated at 37°. Citrate is almost as efficient as malonate in preventing enzyme inactivation but has the undesirable property of causing enzyme inhibition during assays, particularly when low substrate concentrations are used.

Studies of the effects produced by malonate, citrate and EDTA have revealed close parallels in the efficiencies of these compounds in preventing enzyme inactivation in preincubated homogenate (Table 1) and in preventing enzyme inhibition by added Fe^{3+} ions. These results suggest that traces of

Fe^{3+} ions in liver homogenates are responsible for the observed enzyme inactivations and that enzyme protection by malonate and citrate is due to conversion of Fe^{3+} ions into non-inhibitory metal complexes (see Martell & Calvin, 1952). Inhibition of acid phosphatases of erythrocytes and prostate by added Fe^{3+} ions, in the presence of acetate buffer, pH 5.0, has been observed by Abul-Fadl & King (1949) and these authors have also shown that such inhibition is prevented by citrate buffer of the same pH. However, the lack of suitable methods for determining Fe^{3+} ion contents of liver homogenates at pH 5.9 has prevented further studies of the possible inactivation of liver acid phosphatase by such ions.

In these experiments, enzyme inactivation has also been prevented by phenyl phosphate concentrations of 0.1 M or greater. The protection of liver acid phosphatase by phenyl phosphate has been shown to vary with the concentration of phenyl phosphate used (Fig. 4). The mechanism of phenyl phosphate protection is uncertain. Possible mechanisms are (a) union between phenyl phosphate and sulphhydryl groups of acid phosphatase and consequent prevention of their oxidation and (b) combination of phenyl phosphate with inactivating agent and prevention of enzyme inactivation by this agent.

Another important feature of phenyl phosphate concentration is that approximately 0.1 M-solutions are required for enzyme saturation with substrate. A similar observation has been made by Tsuboi (1952). This concentration is much higher than that (5 mM) used by Robinson *et al.* (1939) and by the majority of workers with liver acid phosphatase.

The optimum pH for hydrolysis of phenyl phosphate by mouse-liver acid phosphatase is shown to be pH 5.9–6.1 (Fig. 1). This pH is higher than that (pH 4.9) used by Robinson *et al.* (1939) and by workers with liver acid phosphatase (e.g. pH 5.5, Tsuboi, 1952; pH 5.0, Abul-Fadl & King, 1949; pH 4.9, Kochakian & Fox, 1944; pH 4.6, Greenstein, 1942), but is almost identical with that reported by Schönheyder (1952) for seminal acid-phosphatase activity with phenyl phosphate. The possibility that the rate of inactivation of liver acid phosphatase varies with pH of incubation has not been examined in the present studies.

Inorganic phosphate acts as a competitive inhibitor of liver acid phosphatase. Inorganic phosphate behaves similarly in the hydrolysis of phenyl phosphate by seminal acid phosphatase (Schönheyder, 1952). This property of inorganic phosphate, considered together with the non-specificity of acid phosphatases, and the demonstration by Cohn (1949) of enzymic cleavage of P–O bonds, indicates that the union between substrate and acid phosphatase involves the phos-

phate moieties of phosphomonoester substrates. The failure of β -glycerophosphate to inhibit phenyl phosphate hydrolysis (Table 5) suggests that β -glycerophosphate has little affinity for the enzyme under the conditions used. This effect may be due to differences in the dissociations of the phosphate moieties of phenyl phosphate and β -glycerophosphate at pH 5.9 (Delory, Wiberg & Hetherington, 1955). Arsenate and pyrophosphate produce significant inhibition of phenyl phosphate hydrolysis (Table 5) and these inhibitions are presumed to be similar to that caused by inorganic phosphate. Fluoride and cyanide also inhibit liver acid phosphatase (Table 5), but, at present, no satisfactory explanations of these inhibitions can be given. Citrate, L-tartrate and oxalate resemble β -glycerophosphate in producing little enzyme inhibition under conditions suitable for maximum rates of hydrolysis of phenyl phosphate (Table 5).

The described method of measuring liver acid-phosphatase activity is simple and rapid and should prove useful as an assay method in the isolation of liver acid phosphatase. Awareness of the instability of this enzyme and of the prevention of enzyme inactivation by malonate should assist in obtaining high yields of the enzyme in isolation procedures.

SUMMARY

1. An examination has been made of the effects of variations in assay conditions on the rate of hydrolysis of phenyl phosphate by mouse-liver acid phosphatase at 37°.

2. Maximum rates of hydrolysis were obtained with 0.1M-phenyl phosphate (pH 5.9–6.1) and incubation periods of 5–10 min. There appeared to be no requirement for added metal ions.

3. Inactivation of acid phosphatase, shown to occur in liver homogenates incubated at pH 5.9 in both the absence and the presence of phenyl phosphate, was prevented by malonate. Evidence suggesting that enzyme inactivation is caused by traces of Fe^{3+} ions in liver homogenates is presented.

4. Hydrolysis of phenyl phosphate was inhibited by Hg^{2+} , Cu^{2+} and Fe^{3+} ions, *p*-chloromercuribenzoate, phenyl mercuric acetate and *o*-iodosobenzoate, and these inhibitions were prevented by cysteine. This suggests that liver acid phosphatase is a sulphydryl enzyme.

5. Under optimum conditions of phenyl phosphate hydrolysis, fluoride, arsenate, phosphate, cyanide and pyrophosphate caused significant

enzyme inhibition, whereas citrate, β -glycerophosphate, oxalate and L-tartrate had little effect. Inhibition by phosphate is shown to be competitive.

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