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Human Kidney and Urinary Alkaline Phosphatases

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1. Four fractions of kidney alkaline phosphatase were prepared by chromatography on DEAE-Sephadex. An investigation of their properties suggests that the fractions represent modifications of a single kidney enzyme. 2. Urinary alkaline phosphatase resembles kidney enzyme in most of its properties, but differs in K_m and in the degree by which it is activated by Mg^{2+} ions. 3. Estimates of the molecular weights of kidney and urinary alkaline phosphatase gave values of 150000–170000 for kidney phosphatase and 75000 for the urinary enzyme. 4. It is suggested that urinary alkaline phosphatase is a sub-unit of kidney phosphatase, but it has not been possible to simulate the formation of urinary enzyme by treating kidney enzyme with urea or H^+ ions.

Urine from patients suffering from various diseases of the kidney often contains high concentrations of alkaline phosphatase, which appears to be identical with the enzyme found in normal urines (Wacker, Dorfman & Amador, 1964; Butterworth, Moss, Pitkanen & Pringle, 1965*a*,*b*). From previous work, however, it would appear that urinary alkaline phosphatase differs from kidney phosphatase in the way it migrates on starch-gel electrophoresis and separates during gel filtration (Butterworth *et al.* 1965*b*), These differences indicate that the kidney enzyme may undergo some modification during the excretion process. The present investigation was undertaken in an attempt to show how the kidney enzyme may give rise to the urinary form.

EXPERIMENTAL

Enzyme preparations. Human kidneys were obtained *post mortem* from patients in whom there was no evidence of kidney disease. Extracts of kidney alkaline phosphatase were prepared by the modification described by Ahmed & King (1960) of Morton's (1950) method by using butan-1-ol. Further purification of the crude extracts consisted of gel filtration on Sephadex G-200 followed by chromatography on DEAE-Sephadex (Butterworth & Moss, 1966). Chromatographic peaks of enzyme activity were concentrated by dialysis against a 30% (w/v) solution of Carbowax 20M at 4°, followed by dialysis against glass-distilled water for 3hr. at 4°. About 70–80% of the activity present in the original crude extracts was recovered. The protein content of the concentrated fractions was determined by a biuret method (Wootton, 1964) and the concentrated DEAE-Sephadex-chromatographic fractions contained 5–14 King & Armstrong (1934) units/mg. of protein N. This represented a 10–15-fold increase in specific activity over that of the crude extracts.

Overnight specimens of urine from healthy adult male subjects were collected in polythene containers without the addition of a preservative. The volumes were measured and any sediment, if present, was removed by centrifugation. The urine samples were enclosed in Visking 36/32 tubing bags and concentrated to 2ml. by dialysis against 30% Carbowax 20M for 24hr. at 4°. Urinary alkaline phosphatase was not inactivated by the dialysis procedure and the concentrated preparations contained 70–100 King–Armstrong units/100ml. Because of the low activity, further purification was limited to a single step involving gel filtration on Sephadex G-200. Concentrated urine containing 15–20mg. of protein was loaded on to a column (50cm. × 1cm.) of Sephadex G-200 and the phosphatase eluted with 0·9% NaCl solution. The single peak of activity was concentrated by dialysis against 30% Carbowax 20M. The recovery of alkaline phosphatase from gel filtration was 80–90%; the concentrated fractions had specific activities of 1–2 King–Armstrong units/mg. of protein N, representing a twofold increase over the starting material.

Assay of alkaline phosphatase. Phosphatase activities of effluents from Sephadex and DEAE-Sephadex chromatography columns were determined by the method of Kind & King (1954). In all experiments, except when mentioned, the substrate was 3mM-*p*-nitrophenyl phosphate (Koch–Light Laboratories Ltd., Colnbrook, Bucks.) in 0·1M-Na_2CO_3–$NaHCO_3$ buffer, pH10 and I 0·22, and phosphatase activity was determined by following the increase in E_{400} in a Uvispek spectrophotometer fitted with a Gilford Kinetic Unit. Values for the Michaelis constants, K_m, of the enzymes studied were obtained from experiments at various substrate concentrations by plotting data by the procedure of Lineweaver & Burk (1934). Lines were fitted to the experimental points by the method of least squares.

Effect of pH, Mg^{2+} *ions and heat on kidney and urinary alkaline phosphatase.* The pH optima were determined with a range of 0·1M-Na_2CO_3–$NaHCO_3$ buffers and the effect of $MgCl_2$ on enzymic activity was investigated at the pH optimum. The stability to heat of the enzymes under study was tested by heating at 55°, 57·5° and 60° in 0·05M-tris–HCl buffer, pH7·7, in the presence or in the absence of Mg^{2+} ions (5mM).

Rates of hydrolysis of three phosphate esters. The rates of hydrolysis at 37° of phenyl phosphate, β-glycerophosphate and α-naphthyl phosphate by kidney and urinary alkaline phosphatases were compared by measuring the rate of release of inorganic phosphate. Phosphate was determined by the method of Delsal & Manhouri (1958). The concentration of substrate was 5mM in each case. Phenyl phosphate and β-glycerophosphate were obtained as the disodium salts from Hopkin and Williams Ltd. (Chadwell Heath, Essex) and disodium α-naphthyl phosphate was from British Drug Houses Ltd. (Poole, Dorset).

Inorganic pyrophosphatase activity. This was determined by the method of Moss, Eaton, Smith & Whitby (1966). Pyrophosphatase activity at pH 8·6 was compared with phenylphosphatase activity at pH 10.

Gel filtration. Kidney and urinary alkaline phosphatases and a number of protein markers were eluted from a column (68cm. × 2·1cm.) of Sephadex G-200 with 0·9% NaCl solution. Phosphatase activities of effluent portions were determined by the *p*-nitrophenyl phosphate method, protein concentrations from measurements of E_{280} and Blue Dextran from E_{560}.

Starch-gel electrophoresis. This was carried out in horizontal trays by the method of Smithies (1955) with the discontinuous buffer system of Poulik (1957) at pH 8·6. A potential difference of 10 v/cm. was applied for 18hr., after which zones of alkaline phosphatase were located on cut surfaces of the gel by the method of Estborn (1959). When gels containing 3M-urea and methylurea were required, the amount of starch was increased by 50% and gels were kept for 18hr. at 4°, after which time they had reached a suitable consistency for use.

Effect of urea. Inhibition studies with urea and methylurea were carried out as described by Butterworth & Moss (1967).

Inactivation by H^+ ions. Phosphatases were mixed with 0·1M-citrate buffers, pH range 3–5, at 0°. At timed intervals, acid inactivation was stopped by the addition of alkaline buffer and residual enzyme activities were determined at pH 10 in reaction mixtures containing 5mM-Mg^{2+} ion.

Incubation with neuraminidase. Concentrated chromatographic fractions and whole kidney extract were incubated with 50 units of neuraminidase (Koch–Light Laboratories Ltd.) at 37° for 20hr. in 0·05M-tris–HCl buffer. The pH optima, activation by Mg^{2+} ions and stability to heat of the treated fractions was investigated by the methods described above and the K_m for the hydrolysis of *p*-nitrophenyl phosphate at pH 10 was determined.

RESULTS

There was some variation in the appearance of DEAE-Sephadex chromatograms obtained with extracts from different kidneys, but four fractions (designated A, B, C and D) were usually separable (Butterworth & Moss, 1966). Fraction A was eluted by the starting buffer and represented 40–60% of the total phosphatase in all extracts studied. Fractions B, C and D were eluted at higher chloride concentrations. A peak in E_{280} that coincided with fraction A may have represented phosphatase protein, but no direct relationship appeared to exist between the E_{280} and phosphatase activity of fractions prepared from a number of different extracts, and enzyme and protein peaks did not coincide on all chromatograms. Fractions A, B, C and D differed in electrophoretic mobility on starch gel, but all fractions could be converted into slowly migrating components, which were identical electrophoretically, by incubation with neuraminidase (Butterworth & Moss, 1966).

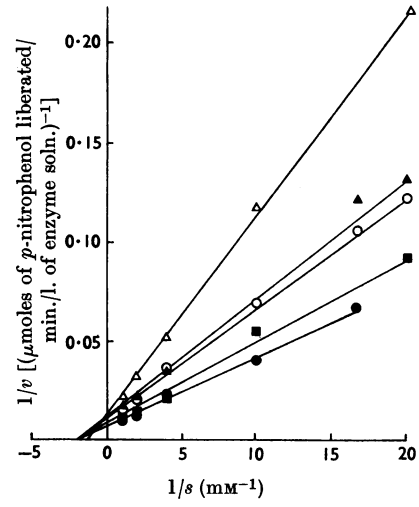

Fig. 1. Plots of $1/v$ against $1/s$ for the hydrolysis of *p*-nitrophenyl phosphate at pH 10 by kidney and urinary alkaline phosphatases. ○, Fraction A; ▲, fraction B; ■, fraction C; ●, fraction D; △, urinary enzyme. K_m for all kidney fractions, $5{\cdot}6 \times 10^{-4}$M; K_m for urinary enzyme, $8{\cdot}2 \times 10^{-4}$M.

Kidney phosphatase fractions A, B, C and D gave similar K_m values of $5{\cdot}6 \times 10^{-4}$M (S.D. $\pm 0{\cdot}08 \times 10^{-4}$M, calculated from three determinations on each fraction) for the hydrolysis of *p*-nitrophenyl phosphate at pH 10, whereas the value obtained from two determinations with the urinary enzyme was $8{\cdot}2 \times 10^{-4}$M (Fig. 1). At a concentration of 3mM-*p*-nitrophenyl phosphate the pH optimum of each kidney fraction and the urinary enzyme was the same (pH 9·9–10) and at this pH all enzymes studied were activated maximally by a Mg^{2+} ion concentration of 5mM. The degree of activation of kidney alkaline phosphatase varied from two- to nine-fold, but the urinary enzyme was activated to a smaller extent: a doubling of activity by the addition of 5mM-Mg^{2+} ion was the greatest effect that could be demonstrated. At pH 10, and in reaction mixtures containing 5mM-Mg^{2+} ion, the enzyme preparations studied had similar substrate specificities; phenyl phosphate was hydrolysed more rapidly than were α-naphthyl phosphate and β-glycerophosphate (Table 1). The enzyme preparations also possessed similar pyrophosphatase activity (Table 1).

Table 1. *Relative rates of hydrolysis of phosphate esters and sodium pyrophosphate*

The rates for the hydrolysis of α-naphthyl phosphate and β-glycerophosphate at pH 10 and of sodium pyrophosphate at pH 8·6 are expressed as percentages of the rate for the hydrolysis of phenyl phosphate at pH 10.

	Relative rate of hydrolysis (%)		
Kidney alkaline phosphatase	α-Naphthyl phosphate	β-Glycerophosphate	Sodium pyrophosphate
Fraction A	86	12	6
Fraction B	87	12	8
Fraction C	80	9	7·5
Fraction D	85	11	10
Urinary alkaline phosphatase	85	13	8·5

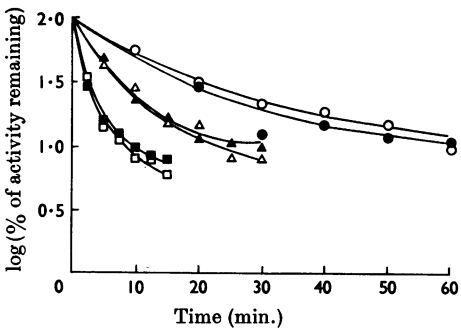

Fig. 2. Inactivation of urinary alkaline phosphatase by heat carried out in the absence (△, ○ and □) and presence (▲, ● and ■) of 5mM-Mg^{2+} ion. ○ and ●, Inactivation at 55°; △ and ▲, inactivation at 57·5°; □ and ■, inactivation at 60°.

A comparison of the rates of inactivation at 55°, 57·5° and 60° showed that all fractions of the kidney enzyme were identical in their stability to heat. Urinary enzyme was similar to kidney phosphatase fractions in its stability, and the presence of 5mM-Mg^{2+} ion did not protect kidney or urinary enzyme against inactivation by heat (Fig. 2).

Investigations of K_m, pH optima, heat inactivation and substrate specificities of kidney enzyme fractions after digestion with neuraminidase showed that these parameters were unaffected by incubation with neuraminidase.

Estimates of the molecular weights of the phosphatases by Sephadex-gel filtration (Andrews, 1964) gave values of 150000–170000 for kidney phosphatase or fractions A, B, C and D determined separately, and 75000 for the urinary phosphatase.

At low concentrations urea and related compounds inhibit kidney alkaline phosphatase reversibly, but at concentrations of approx. 3M and above there is a change in the nature of the inhibition; it is no longer reversible and proceeds by a mechanism that is time- and temperature-dependent (Butterworth & Moss, 1967). Fig. 3 shows the effect of urea on kidney and urinary alkaline phosphatases. The results are presented in the form of plots of v/v_i against i, where v is the velocity of the non-inhibited reaction, v_i is the velocity in the presence of inhibitor and i is the inhibitor concentration. Urea inhibition of urinary

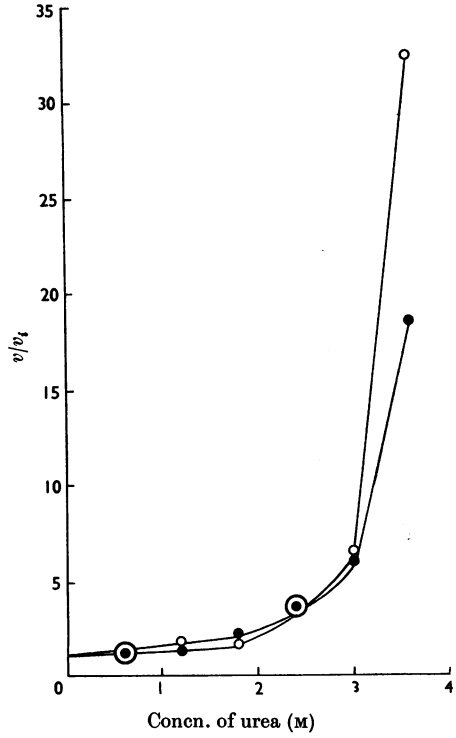

Fig. 3. Relationship between ratio of velocity of *p*-nitrophenyl phosphate hydrolysis at zero time (v) to velocity after 15min. incubation with urea at 37° (v_i) and urea concentration. ○, Kidney phosphatase fraction A; ●, urinary phosphatase. The results obtained with kidney fractions B, C and D were identical with those of fraction A.

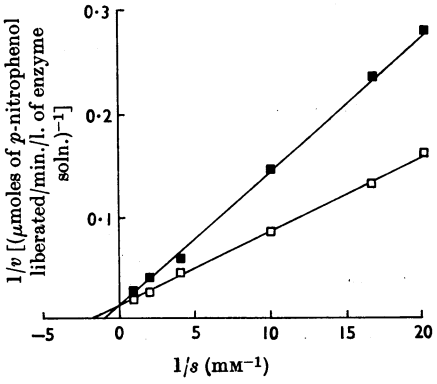

Fig. 4. Plots of $1/v$ against $1/s$ for the hydrolysis of *p*-nitrophenyl phosphate. □, Kidney phosphatase fraction A; ■, kidney fraction A in reaction mixtures containing 1 M-urea. K_i for all kidney fractions, 1·6 M.

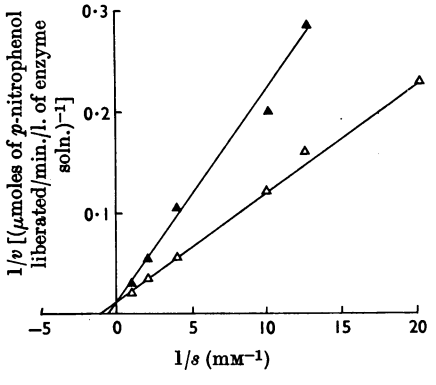

Fig. 5. Plots of $1/v$ against $1/s$ for the hydrolysis of *p*-nitrophenyl phosphate. △, Urinary enzyme; ▲, urinary enzyme in reaction mixtures containing 1 M-urea. K_i for urinary enzyme, 1·3 M.

phosphatase resembled that of kidney; an inflexion occurred in v/v_i plots at a urea concentration of approx. 3 M. At urea concentrations above 3 M the inhibition was irreversible and time- and temperature-dependent. A rise in temperature of 10° increased the rates of denaturation of both kidney and urinary enzymes by 6 M-urea 1·6-fold. Methylurea was a less potent inhibitor of kidney and urinary alkaline phosphatases, but the general shape of the inhibitor curves resembled those obtained with urea.

Figs. 4 and 5 show the effect of 1 M-urea on double-reciprocal Lineweaver & Burk (1934) plots. At this concentration reversible inhibition by urea appeared

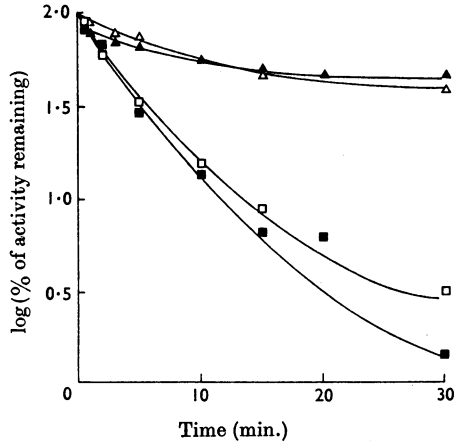

Fig. 6. Inactivation of kidney and urinary alkaline phosphatases at pH 3·3. □, Kidney fraction A; ■, kidney fraction D; △ and ▲, urinary enzyme. Fractions B and C of kidney phosphatase resembled fractions A and D in stability.

to be competitive in type (K_i for kidney enzyme, 1·6 M; K_i for urinary enzyme, 1·3 M), and a similar finding was obtained with methylurea (K_i for kidney enzyme, 1·3 M; K_i for urinary enzyme, 0·7 M).

Urinary alkaline phosphatase migrated as a single band on starch-gel electrophoresis, and its rate of migration towards the anode was faster than that of any of the kidney phosphatase components. Starch-gel electrophoresis in 3 M-urea- and methylurea-containing gels failed to show any new electrophoretic bands of phosphatase activity, and phosphatase incubated at 37° with 6 M-urea for various times up to 15 min. did not exhibit any change in electrophoretic behaviour. The only change in properties seen was a progressive loss of activity as a result of the incubation with urea.

At 0°, alkaline phosphatase was resistant to acid inactivation between pH 4 and 7. Below pH 4 there was a loss of enzymic activity that was progressive with time. Fig. 6 shows inactivation curves at pH 3·3. The urinary phosphatase preparation was more stable than the kidney preparation under these conditions, but after treatment with the acid buffer for 30 min. the inactivation of both enzymes was irreversible. Fractions A, B, C and D of kidney enzyme were identical in stability. Incubation of the partially inactivated enzymes at 37° for 48 hr. in tris buffer, pH 7·7, containing 5 mM-Mg^{2+} ion did not restore the activity, and when incubation was repeated in tris buffer containing 0·01 mM-Zn^{2+} ion for prolonged periods there was no change in its electrophoretic mobility.

DISCUSSION

The results of the investigation of K_m values, pH optima, activation by Mg^{2+} ions, denaturation by heat, urea and H^+ ions and the rates of hydrolysis of phosphate esters show that the several fractions of kidney alkaline phosphatase have very similar properties, and suggest that they represent modification of a single enzyme species rather than distinct isoenzymes. Digestion with neuraminidase has been shown to abolish the chromatographic and electrophoretic heterogeneity of kidney alkaline phosphatase, and it seems likely that the heterogeneity of crude preparations is due to the presence of different proportions of sialic acid covalently bound to the enzyme molecules (Butterworth & Moss, 1966). No changes in properties other than in net molecular charge can be detected after incubation with neuraminidase, so that, if it is true that fractions of kidney alkaline phosphatase differ only in sialic acid content, it follows that the presence of the carbohydrate component has a negligible effect on phosphatase properties.

Urinary alkaline phosphatase resembles kidney enzyme in most of its properties, but differs in K_m and in the degree by which it is activated by Mg^{2+} ions. Non-activation of urinary alkaline phosphatase has been reported (Amador, Zimmerman & Wacker, 1963), and the relatively small activation by Mg^{2+} ions reported in the present paper suggests that the ion is firmly bound to the phosphatase molecule and prolonged dialysis is unable to remove it. Alternatively, it might be supposed that Mg^{2+} ions are less important for urinary alkaline phosphatase activity than for most other phosphatases studied.

Competitive inhibition of lactate dehydrogenase by 1 M-urea was described by Withycombe, Plummer & Wilkinson (1965), who suggested that a displacement of substrate from the active site occurs in the presence of urea. A similar explanation may apply with alkaline phosphatase, but, because of the unrelated chemical structures of *p*-nitrophenyl phosphate and urea, a more likely explanation would be to assume that the binding of the inhibitor may bring about a conformational change in the enzyme molecule leading to distortion of the catalytic site. Competitive kinetics implies that the distortion can be resisted by bound substrate.

Kidney alkaline phosphatase has a molecular weight about twice that of urinary enzyme and it is possible that the latter enzyme is a sub-unit of kidney phosphatase. If such a relationship exists it implies that dissociation of the kidney enzyme is not accompanied by a total loss of activity, but K_m values suggest that the affinity for *p*-nitrophenyl phosphate is decreased. Many proteins have been dissociated into sub-units by treatment with urea, but though the effect of urea on alkaline phosphatases may be indicative of changes in protein secondary and tertiary structure (Butterworth & Moss, 1967) no active sub-units are produced. Sub-units may be formed that do not possess enzyme activity, so that their presence cannot be detected. Urinary alkaline phosphatase itself is readily inactivated by urea and would probably not be detected if formed from kidney enzyme by disruption with urea.

Kidney alkaline phosphatase is inactivated by H^+ ions. The loss of activity could result from the removal of essential activating cations, but since reactivation was not achieved by addition of Mg^{2+} and Zn^{2+} ions it seems unlikely that loss of ions alone could account for the effects of acid on phosphatases. Schlesinger & Barrett (1965) dissociated *Escherichia coli* alkaline phosphatase by exposure to low pH. Acid-inactivation curves of kidney alkaline phosphatase resemble those of *E. coli* phosphatase described by Schlesinger & Barrett (1965), and the results obtained with kidney enzyme may represent dissociation of this enzyme also.

It would seem that the urinary enzyme is more stable to acid than that of the kidney and, had it been formed in these experiments, it should have been demonstrable. Proof of a dissociation of kidney alkaline phosphatase in these experiments and in those with urea is difficult to obtain because the enzyme is not present in sufficient quantity to enable it to be detected by usual protein reagents. Schlesinger & Barrett (1965) found that acid-prepared sub-units of *E. coli* alkaline phosphatase readily dimerized in cation-containing buffers and full enzyme activity was restored. Urinary alkaline phosphatase cannot be induced to associate to an enzyme form resembling kidney phosphatase, and it must be assumed that urinary enzyme is not produced from kidney by dissociation alone. Dissociation may be accompanied by changes in molecular conformation that it has not been possible to reproduce in the laboratory.

The inability to detect active sub-units of kidney alkaline phosphatase after treatment with urea and H^+ ions might appear to invalidate the theory that the urinary enzyme is a sub-unit of kidney phosphatase. Sub-units of *E. coli* alkaline phosphatase, however, differ in type according to the way in which they are prepared (Levinthal, Signer & Fetherolf, 1962; Schlesinger & Barrett, 1965), and kidney alkaline phosphatase may be similar in that the nature of the products of dissociation may be critically dependent on the conditions under which they are formed. It is possible that in the present experiments the conditions were unfavourable for the dissociation of kidney phosphatase into an enzymically active sub-unit that could be identified conclusively with urinary alkaline phosphatase.

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