with the assay system of the method of Karmen (1955).

2. L-Glutamate–oxaloacetate transaminase has been shown to be present in rat-liver mitochondria in latent form. After activation the activity of transaminase in mitochondria is three to four times that in the supernatant fraction.

3. Activation has been achieved by using hypo-osmotic media, incubation at 37°, storage at 4°, treatment with Triton X-100, mechanical disintegration and ultrasonic disintegration. With ultrasonic and mechanical disintegration, but not with hypo-osmotic treatment, this activation is accompanied by release of the enzyme in soluble form.

4. It has been shown that mitochondrial transaminase differs from supernatant transaminase in its substrate affinities, pH-dependence and electrophoretic mobility.

5. Two L-glutamate–oxaloacetate transaminases separated electrophoretically from rat-liver extract appear to correspond to the mitochondrial and supernatant enzymes respectively.

6. Transamination from L-aspartate is inhibited by the oxaloacetate produced.

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# Characterization of Tissue Alkaline Phosphatases and their Partial Purification by Starch-Gel Electrophoresis

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Attempts have been made to assign the alkaline phosphatase present in abnormal amounts in human blood plasma in certain diseases to an origin in a given tissue by a study of the properties of the enzyme increment. These attempts have yielded only inconclusive results. [References may be found in the recent review by Gutman (1959).] Studies of this nature are impeded by the presence in serum of inhibitors such as albumin (Henneman, Rourke & Jackson, 1955) and bile acids (Bodansky, 1937), of activators, such as metal cations, and of a factor of an unknown nature which appears to be present in icteric serum (Cantarow, 1940). Amino acids may inhibit or activate depending on their concentration (Bodansky, 1946). The properties of alkaline phosphatase in serum may reflect therefore not so much the properties of the enzyme as those of its environment.

Electrophoresis on starch gel (Smithies, 1955) permits a refined separation of the serum proteins

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to be made, although the amounts of protein separated are small. The resolving power of electrophoretograms run in the conventional manner on horizontal gels declines if the sample volume exceeds about 0·1 ml. of serum/cm.$^2$ of gel cross-section, and this limits the use of the method for preparative purposes. However, this paper shows that if a sufficiently sensitive technique for measuring enzyme activity is available, reproducible values for the Michaelis constant of alkaline phosphatase purified from serum by starch-gel electrophoresis can be obtained. By these means it has been possible to isolate in a semi-pure state the alkaline phosphatases from tissue preparations and from blood plasma and to study their properties.

## EXPERIMENTAL

*Enzyme preparations.* Recent post-mortem human tissues, bone, liver, kidney and small intestine were used as sources of alkaline phosphatase. After cleaning and grinding, the tissues were kept in 5 vol. of chloroform–water. When activity had reached a maximum (2–5 days) each solution was cleared of solid matter by centrifuging, and the supernatant was poured into 5 vol. of ethanol–ether (3 : 2, v/v). The precipitate was collected by centrifuging and was immediately redissolved in normal human blood serum or in 0·9% NaCl solution. The precipitate from bone was further purified by grinding in a mortar with a small volume of 50% ethanol. The filtered or centrifuged 50% ethanol solution was treated with ethanol–ether to give a final composition of 3 vol. of ethanol, 2 vol. of ether and 1 vol. of water. The precipitate that formed was recovered by centrifuging and was similarly redissolved, either in normal serum or in 0·9% NaCl solution.

Serum from a case of Paget's disease with alkaline-phosphatase activity 65 King–Armstrong (1934) units/100 ml. was also used as a source of bone alkaline phosphatase.

*Starch-gel electrophoresis.* This was carried out in horizontal trays measuring 17 cm. × 10·5 cm. with a gel thickness of about 0·4 cm. essentially by the method of Smithies (1955). The gels were prepared in tris–citrate buffer (Poulik, 1957), except when specific activities of enzymes recovered from the gels were determined, when the gels were made in dilute $H_3BO_3$–NaOH buffer (Smithies, 1955). In each case, concentrated $H_3BO_3$–NaOH buffer (Smithies, 1955) was used in the electrode chambers. The sample was applied on a filter-paper support; when nearly the full width of the gel was used, it was possible to run samples of about 0·2 ml. After electrophoresis at 20 v/cm. for 5–6 hr. the alkaline-phosphatase band was located in the following way: strips of filter paper, moistened with a solution of disodium α-naphthyl phosphate (5 mM) in $Na_2CO_3$–$NaHCO_3$ buffer, pH 10 (Delory & King, 1945), containing 5 mM-$MgCl_2$, were laid along the longitudinal edges of the two cut surfaces of the gel so that the papers just overlapped the edges of the electrophoresis zones. After incubation at 37° (usually 5–10 min. was sufficient) the papers were removed and the gels were viewed under an ultraviolet lamp. The ends of the alkaline-phosphatase zones were then visible as areas of pale-blue fluorescence in the dark-blue fluorescent margins. The narrow strip across the gel between the two ends of the phosphatase zone was removed for elution of the enzyme. The ends of the zones where α-naphthyl phosphate hydrolysis had taken place were avoided in cutting because of their contamination with α-naphthol and its phosphate and consequent marked fluorescence. α-Naphthyl phosphate was preferred to the β-isomer for the visual location of phosphatase, since the fluorescence of α-naphthol (emission max. 455 mμ) is more apparent to the eye than is that of β-naphthol.

Phosphatase was recovered from the gel by macerating the enzyme-containing strips with $Na_2CO_3$–$NaHCO_3$ buffer, pH 10, and then centrifuging (for the two strips of gel obtained from one electrophoresis, 2 ml. of buffer was used and yielded 0·7–1 ml. of supernatant). The enzyme solution was used immediately. This method of recovering the enzyme gave approximately the same activity in the volume of solution needed for a Michaelis-constant determination (1·25 ml.) as did freezing, thawing and centrifuging the gel, which necessitated greater subsequent dilution of the enzyme solution expressed. Overall enzyme recoveries of up to 30% were obtained, allowance being made for the volume of buffer retained by the gel after centrifuging.

For specific-activity determinations, nitrogen was estimated in the phosphatase solution recovered from gels made in $H_3BO_3$–NaOH buffer by Kjeldahl digestion followed by nesslerization (a piece of gel from a protein-free region of the same weight as the phosphatase strip was treated with buffer to provide a control). Phosphatase activity was estimated in the same solution by the Kind & King (1954) modification of the King & Armstrong (1934) method to enable comparison to be made with other purified enzyme preparations.

*Determination of Michaelis constant.* Disodium β-naphthyl phosphate was used as substrate. This was prepared by the method of Friedman & Seligman (1950), and was recrystallized (Found: C, 43·8; H, 3·2; P, 11·0; Na, 17·4. Calc. for $C_{10}H_7O_4PNa_2$: C, 44·8; H, 2·6; P, 11·6; Na, 17·1%). This ester is hydrolysed by serum alkaline phosphatase at a rate approximately 0·75 that of phenyl phosphate, with liberation of free β-naphthol. The fluorescence excitation and emission spectra (Fig. 1) of β-naphthol and of its orthophosphate ester differ sufficiently in alkaline solution to permit the measurement of traces of β-naphthol in the presence of a large excess of the ester, by using a spectrofluorimeter with activation wavelength set at 350 mμ and emission measurement at 420 mμ (Moss, 1960*a*).

The spectrofluorimeter used in the present experiments employs quartz-prism monochromators, xenon-arc light-source and photomultiplier with pulse-counting circuits as detector. The output of the sample is measured in terms of the output of a fluorescent screen which monitors the activating beam (Moss, 1960*b*).

For measurement of β-naphthol an activation band width of 5 mμ was used, with emission band width of either 5 or 15 mμ. Calibration curves at the two settings in terms of μmoles of β-naphthol in a volume of 3 ml. are given in Fig. 2. With the 15 mμ emission band width the mean value of nine determinations of 0·2 μm-mole of β-naphthol, expressed as a percentage, was 102 ± 3% (S.D.).

All enzymic reactions were carried out at 37°. The range of substrate concentration used was 0·05–1 mM, and the

reaction mixtures were buffered with $Na_2CO_3$–$NaHCO_3$ buffers (Delory & King, 1945), except in a few cases (indicated in the results) in which the $Na_2CO_3$–veronal buffers (King & Delory, 1940) were used.

The solutions were made 5 mM with respect to $Mg^{2+}$ ions by addition of an appropriate volume of 0·1 M-$MgCl_2$. Before addition of enzyme the volume of the buffered reaction mixture was 2 ml.; the reaction was started by addition of 0·05 ml. of enzyme solution from a constriction pipette. With the crude tissue extracts the reaction was stopped after 5 min. by addition of 1 ml. of a mixture of 8 vol. of 0·5 N-NaOH and 2 vol. 0·1 M-EDTA; for the enzyme recovered from the gels hydrolysis was allowed to proceed for 15 min. Addition of NaOH brings the pH to about 12, at which alkaline phosphatase is inactive and β-naphthol is strongly fluorescent (the fluorescence of β-naphthol measured at the chosen wavelengths reaches a maximum, constant value at pH 10 and above). The EDTA keeps $Mg^{2+}$ ions in solution; in the absence of EDTA a gelatinous precipitate of $Mg(OH)_2$ forms. Controls were set up in which buffered substrate at each concentration used was incubated for the same time as the tests, enzyme solution being added at the end of incubation immediately after addition of the NaOH–EDTA. The blank fluorescence depends almost entirely on substrate concentration, being due partly to traces of free β-naphthol present in the phosphate, but also to the inherent fluorescence of the phosphate itself. Non-enzymic hydrolysis during the 5 or 15 min. incubation was in fact negligible. A pH-optimum curve was determined for each substrate concentration with each enzyme preparation, and the optimum velocities at each concentration were used in constructing the Lineweaver–Burk (1934) reciprocal plot (Motzok, 1959). Straight lines were fitted to the experimental points by the method of least squares. The term $K_m$ is used in this paper to denote the parameters derived from the intercepts of these lines on the abscissae.

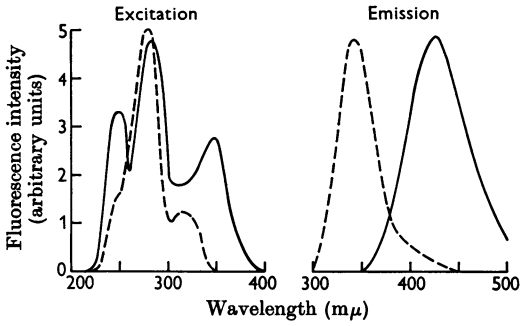


Fig. 1. Fluorescence excitation and emission spectra of β-naphthol (——) and β-naphthyl phosphate (- - -), at pH 10. The excitation spectra have been corrected to constant quantum intensity. Excitation maxima: β-naphthol, 250, 285 and 350 mμ; β-naphthyl phosphate, 280 and 320 mμ. Emission maxima: β-naphthol 425 mμ; β-naphthyl phosphate, 340 mμ.

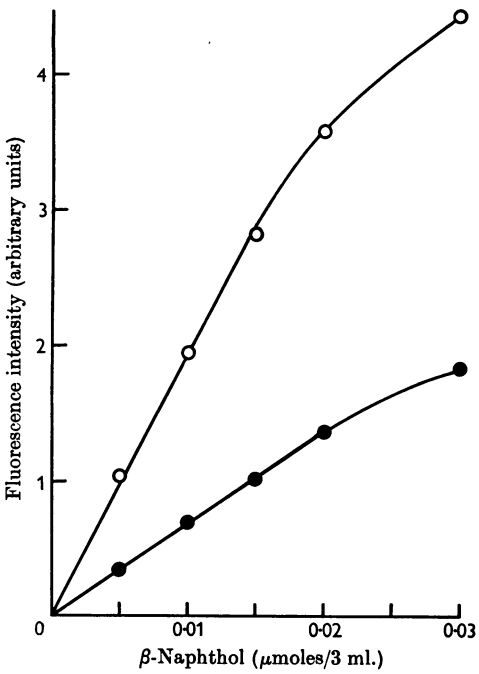


Fig. 2. Relation between concentration of β-naphthol and fluorescence, at pH 12. Fluorescence excitation, 350 mμ; emission measured at 420 mμ. Excitation band width, 5 mμ; emission band width: ●, 5 mμ; ○, 15 mμ.

For the enzyme solutions recovered from the gels only the points on each pH–activity curve needed to define the optima were determined, since the volume of solution available was limited.

*Inhibition by sodium deoxycholate.* The effect of sodium deoxycholate on the hydrolysis of β-naphthyl phosphate by alkaline phosphatase extracts from bone, liver and intestine was investigated as a possible additional means of differentiating between the enzymes from different tissues. A 1 mM-substrate concentration and 5 mM-deoxycholate were used, $Mg^{2+}$ ions being 5 mM and pH 9·7 at 37°. The velocity of hydrolysis in the presence of deoxycholate was compared with that in controls from which deoxycholate was omitted.

## RESULTS

The major portion of human serum alkaline phosphatase has a mobility on starch-gel electrophoresis which is slightly less than that of the transferrin-C (β-globulin) protein fraction (Kowlessar, Pert, Haeffner & Sleisenger, 1959; Estborn, 1959). The tissue phosphatases prepared in the present study gave a main band of activity in this region of the gel, whether dissolved in serum or 0·9% sodium chloride solution, and the Michaelis constants reported here of electrophoretically purified enzymes refer to phosphatases recovered from this zone of the gels. A phosphatase fraction, moving rather more slowly than the slow $\alpha_2$-protein fraction, that was present in certain preparations, notably from intestine, has not yet been investigated fully.

Table 1 shows that partial purification by starch-gel electrophoresis considerably changed the Michaelis constants of the liver and kidney enzymes. The values for the bone and intestinal enzymes were less affected. The $K_m$ values were in good agreement whether the tissue phosphatase was submitted to electrophoresis as a solution in serum or in 0·9 % sodium chloride solution. Bone

Table 1. *Michaelis constants for hydrolysis of β-naphthyl phosphate by tissue alkaline phosphatases, before and after partial purification by starch-gel electrophoresis*

Temp. 37°; $Mg^{2+}$, 5 mM. Velocity at optimum pH for each substrate concn. used in determining $K_m$. c, $Na_2CO_3$–$NaHCO_3$ buffers; v, veronal-$Na_2CO_3$ buffers; sa, submitted to electrophoresis in solution in 0·9 % NaCl; se, submitted to electrophoresis in solution in normal human serum; p, Paget's-disease serum.

| | Before electrophoresis | | | After starch-gel electrophoresis | | |
|---|---|---|---|---|---|---|
| Tissue of origin of phosphatase | $K_m$ (mM) | $V_{max}$ for enzyme solution used in $K_m$ determination (μmoles/min./l.) | Mean $K_m$ (mM) | $K_m$ (mM) | $V_{max.}$ for enzyme solution used in $K_m$ determination (μmoles/min./l.) | Mean $K_m$ (mM) |
| Bone | 0·101 (v) | 48 | 0·103 | 0·110 (c, sa) | 3·5 | 0·110 |
| | 0·091 (v) | 98 | | 0·110 (c, se) | 1·2 | |
| | 0·116 (c) | 84 | | 0·110 (c, p) | 9·0 | |
| Liver | 0·149 (v) | 40 | 0·139 | 0·075 (c, sa) | 2·7 | 0·067 |
| | 0·137 (v) | 30 | | 0·071 (c, se) | 18 | |
| | 0·132 (c) | 82 | | 0·063 (c, se) | 17 | |
| | | | | 0·060 (c, se) | 10 | |
| Intestine | 0·097 (c) | 96 | 0·099 | 0·088 (c, sa) | 26 | 0·090 |
| | 0·100 (c) | 112 | | 0·095 (c, sa) | 13 | |
| | | | | 0·087 (c, se) | 2·9 | |
| Kidney | 0·133 (c) | 98 | 0·167 | 0·100 (c, sa) | 6·1 | 0·103 |
| | 0·200 (c) | 125 | | 0·105 (c, se) | 3·3 | |

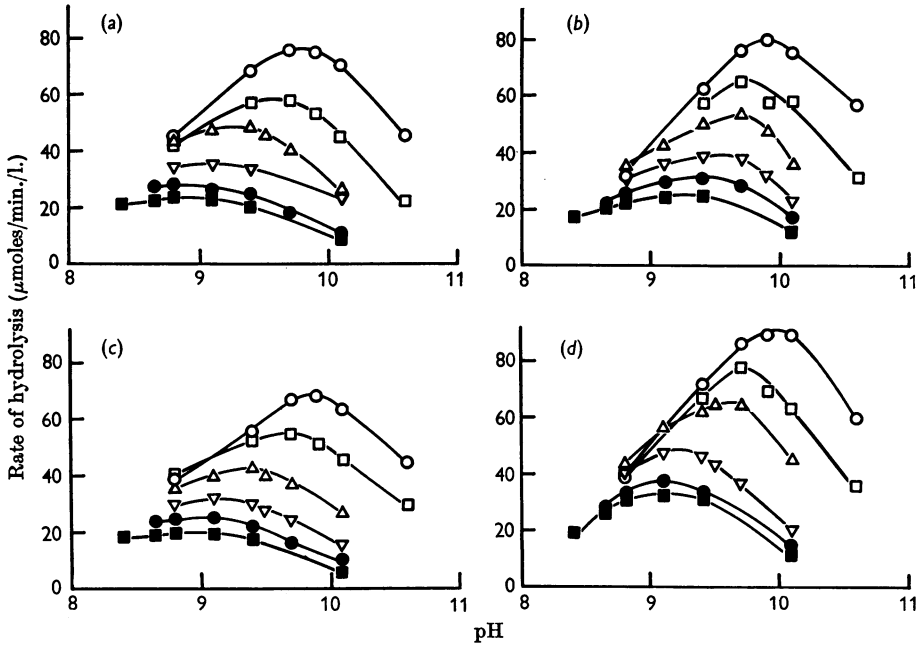


Fig. 3. pH–Activity curves for alkaline phosphatases of tissue extracts at various substrate concentrations. β-Naphthyl phosphate concn.: ○, 1 mM; □, 0·4 mM; △, 0·2 mM; ▽, 0·1 mM; ●, 0·067 mM; ■, 0·05 mM. (*a*), Bone; (*b*), kidney; (*c*), liver; (*d*), intestinal phosphatase. Temp. 37°; $Mg^{2+}$, 5 mM.

alkaline phosphatase recovered from Paget's-disease serum gave a $K_m$ identical with that of the tissue enzyme added to normal serum and recovered by electrophoresis. The possibility of obtaining reproducible $K_m$ values on enzymes recovered from serum by starch-gel electrophoresis as an aid to identification of their tissues of origin was an important reason for undertaking this work.

Examples of pH–activity curves for the saline solutions of tissue alkaline phosphatases before electrophoresis are given in Fig. 3 and reciprocal plots derived from these data in Fig. 4. The pH–activity curves for enzymes purified by starch-gel electrophoresis and the corresponding reciprocal plots are shown in Figs. 5 and 6.

The $K_m$ values obtained were independent of the concentration of enzyme used, as is shown by the results for bone enzyme recovered from serum, the same $K_m$ being given by two preparations, one of which had a value of $V_{max.}$ approximately eight times that of the other. Similarly, good agreement in $K_m$ was obtained when liver and intestinal preparations having $V_{max.}$ values differing by a factor of 2 were used. Examples of the increase in specific activity obtained by starch-gel electrophoresis of the enzyme preparations, both in solution in normal serum and in 0·9% sodium chloride solution, are given in Table 2. The nitrogen content of the eluates from the gel was very low, and the specific activities in Table 2 are regarded as approximations only.

Sodium deoxycholate inhibited bone and liver phosphatase to the extent of 20–40% under the conditions used. Intestinal phosphatase was not inhibited, confirming the observation of Bodansky (1937).

## DISCUSSION

Considerable variation in the $K_m$ values of alkaline phosphatases from different tissues towards a given substrate has been reported, e.g. by Roche & Sarles (1948). These $K_m$ values were based on the change of velocity with substrate concentration at a constant pH. Motzok (1959) showed that if velocities at the optimum pH for each substrate concentration were used in deriving $K_m$ the data were resolved into two parts, giving a $K_m$ at low substrate concentrations and a second $K_m$ at high substrate concentrations; further, these $K_m$ values were independent of enzyme concentration. Motzok & Branion (1959) reported $K_m$

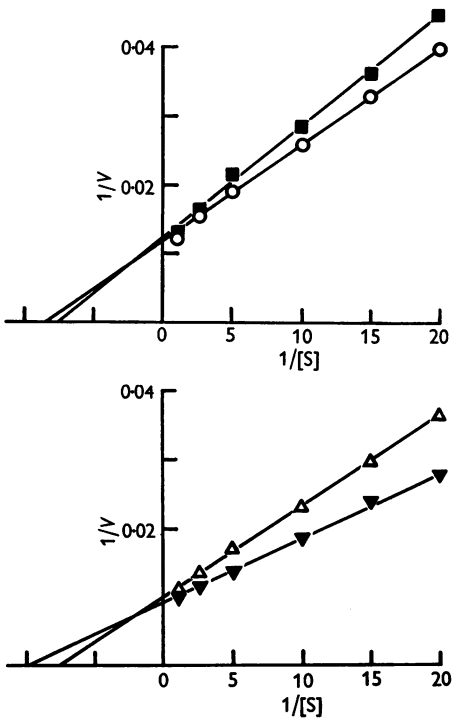


Fig. 4. Reciprocal plots ($1/V$, at optimum pH, against 1/[S]) of data from Fig. 3. ○, Bone ($K_m$ 0·11 mM); ■, liver ($K_m$ 0·13 mM); △, kidney ($K_m$ 0·13 mM); ▼, intestinal phosphatase ($K_m$ 0·10 mM).

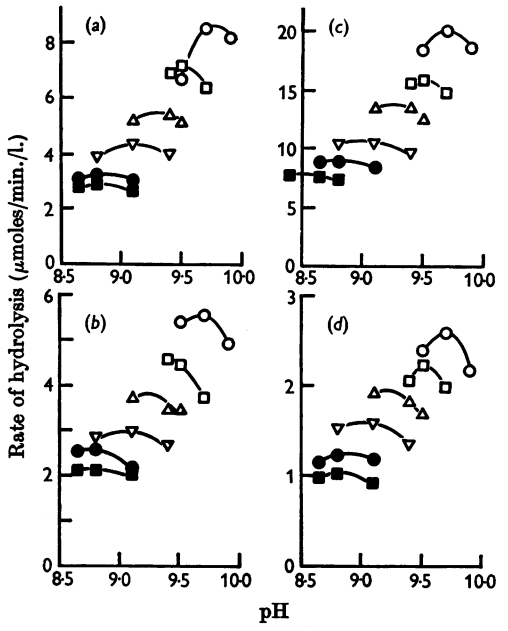


Fig. 5. pH–Activity curves for alkaline phosphatases partially purified by starch-gel electrophoresis. Substrate concentrations were as in Fig. 3. (*a*), Bone; (*b*), kidney; (*c*), liver; (*d*), intestinal phosphatase. Temp. 37°; $Mg^{2+}$, 5 mM.

values for several tissue alkaline phosphatases of rabbit and pigeon with sodium β-glycerophosphate as substrate determined both at constant pH and at optimum pH for each substrate concentration. Differences between the values of $K_m$ for different tissue phosphatases within the same species were less marked when optimum pH conditions for each substrate concentration were used than when a constant pH was maintained throughout. These authors conclude that, even if the concentrations of enzymes used in different determinations of $K_m$ at constant pH were kept the same, values so obtained would not be comparable because of the effect of differences in pH optima existing between phosphatases from different sources. Phosphatase $K_m$ values in the literature may only be applicable therefore to the particular enzyme preparations and concentrations employed in their determination. In the present investigation marked differences between the pH optima at a given substrate concentration were not found for phosphatases from the several human tissues used. However, it would be difficult to maintain reasonably constant enzyme concentration, on the basis of approximately equal activities, when studying solutions recovered from starch gels and tissue extracts of widely varying enzyme content, so determination of $K_m$ from velocity at optimum pH for each concentration of substrate was preferred since this has been shown to be little affected by enzyme concentration (Motzok, 1959). The values reported here would correspond to those found by Motzok for the region of dilute substrate.

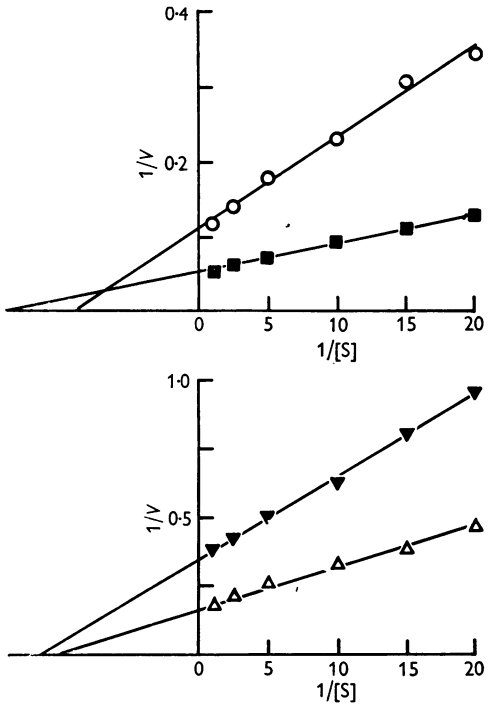


Fig. 6. Reciprocal plots ($1/V$, at optimum pH, against 1/[S]) of data from Fig. 5. ○, Bone ($K_m$ 0·11 mM); ■, liver ($K_m$ 0·07 mM); △, kidney ($K_m$ 0·10 mM); ▼, intestinal phosphatase ($K_m$ 0·09 mM).

The $K_m$ values reported for alkaline phosphatase from different tissues acting on β-naphthyl phosphate show only small differences in the enzymes partially purified by starch-gel electrophoresis. The most marked difference is between the enzyme obtained from liver and those from other sources. Purification by electrophoresis produced, in liver and kidney phosphatases, a marked fall in $K_m$. A similar fall in $K_m$ is seen when the value of 0·11 mM obtained for bone enzyme recovered from Paget's disease serum is compared with the value of 0·4 mM obtained for whole Paget's-disease serum by similar methods (Moss, 1960*a*); these changes in $K_m$ emphasize the need for at least partial purification of serum enzymes in studies of this nature, because of the uncontrolled effect of the activator and inhibitors previously mentioned. However, the fall in $K_m$ consequent on electrophoresis of the phosphatase fractions of tissues or sera cannot be ascribed without qualification to a removal of inhibitors. The possibilities that electrophoresis produces some change in the enzyme molecule itself, or that it selectively destroys some

Table 2. *Increase in specific activity of alkaline phosphatase resulting from starch-gel electrophoresis*

Intestinal and liver phosphatases were in solution in normal serum; bone phosphatase was in solution in 0·9% NaCl.

| | Before electrophoresis | | | After starch-gel electrophoresis | | |
|---|---|---|---|---|---|---|
| Source of phosphatase | Activity (King–Armstrong units/100 ml.) | Nitrogen (mg./100 ml.) | Specific activity (units/mg. of N) | Activity (King–Armstrong units/100 ml.) | Nitrogen (mg./100 ml.) | Specific activity (units/mg. of N) |
| Intestine | 1020 | 800 | 1·3 | 11·3 | 0·5 | 25 |
| Liver | 54 | 1330 | 0·04 | 1·0 | 0·2 | 5 |
| Bone | 180 | 51 | 3·5 | 2·0 | 0·2 | 10 |

isoenzyme fractions having different affinity constants, or separates minor isoenzyme fractions which are afterwards undetected, cannot at present be discounted.

Although the partially purified enzyme solutions obtained from the starch gels do not compare in specific activity with the very highly purified alkaline phosphatases (e.g. Ahmed & King, 1960; Morton, 1954), which had activities of 1750 and 1470 King–Armstrong units/mg. of N respectively, electrophoresis on starch gel offers a method of eliminating, in one simple operation, large quantities of plasma protein and of concentrating the enzyme in a protein-poor solution to an enzymic strength comparable with that obtained by extracting enzyme-rich tissues. The properties of these enzyme preparations can be studied with techniques of adequate sensitivity. An increase in sensitivity of one or two orders of magnitude compared with colorimetric or spectrophotometric procedures is obtained where fluorimetric or spectrofluorimetric techniques can be applied.

A knowledge of the organs of origin of enzymes circulating in blood is of biochemical interest and in pathological states may also be of diagnostic value. The problem of identifying the tissue sources of alkaline phosphatase by investigating the properties of the enzyme purified from serum by starch-gel electrophoresis is complicated by the similar mobilities of various tissue phosphatases on the gel. Thus the enzyme solution recovered from the transferrin-C–haptoglobin region of an electrophoretogram of a pathological serum may contain a mixture of enzymes derived from various organs. The $K_m$ value of this solution would give a curved reciprocal ($1/V$ against $1/[S]$) plot, tending to give at low substrate concentration the $K_m$ of the enzyme with the highest affinity for the substrate. It may be possible further to fractionate this mixture by studying the kinetics of activation or inhibition of its component enzymes. Although the observations of Bodansky (1937) that sodium deoxycholate inhibits bone but not intestinal phosphatase have been confirmed, liver enzyme is also inhibited; therefore the use of this particular inhibitor does not assist in the differentiation of the enzymes recovered from starch gel into those originating in bone and in liver, and this is the most interesting problem in the study of pathological sera. Application of the methods described in this paper to the identification of the tissues of origin of the serum alkaline phosphatase increments in disease is described by Moss, Campbell, Anagnostou-Kakaras & King (1961). $K_m$ values of 0·11 mM (2) and 0·095 mM were found in bone disease, 0·094 mM in a case of extrahepatic biliary obstruction, and a range of values from 0·061 to 0·083 in seven cases with liver involvement.

## SUMMARY

1. Alkaline phosphatases from human bone, liver, kidney and intestine have been partially purified by starch-gel electrophoresis. These enzymes have also been recovered from solution in blood serum by electrophoresis.

2. The $K_m$ values for the alkaline phosphatases have been compared before and after electrophoresis and after recovery of the enzymes from serum, by using a spectrofluorimetric enzyme assay with $\beta$-naphthyl phosphate as substrate.

3. Reproducible $K_m$ values can be obtained for the tissue enzymes submitted to electrophoresis, and, although the differences in substrate affinity between phosphatases from individual tissues are small, these differences can be demonstrated after electrophoresis. Mean $K_m$ values (mM-$\beta$-naphthyl phosphate) found after electrophoresis were: bone, 0·110; liver, 0·067; intestine, 0·090; kidney, 0·103.

4. By combining starch-gel electrophoresis with spectrofluorimetric determination of phosphatase activity, alkaline phosphatases in blood serum can be studied with reduced interference from activators and inhibitors present in the serum.

We thank Miss P. R. N. Kind for preparing the disodium $\beta$-naphthyl phosphate.

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