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Properties of Alkaline-Phosphatase Fractions Separated by Starch-Gel Electrophoresis

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The availability of refined techniques for the resolution of protein mixtures, e.g. chromatography on substituted cellulose ion-exchangers and zone electrophoresis on a variety of supporting media, has resulted in the separation of several enzymes that had previously been regarded as single entities into a number of active components (see Wróblewski, 1961). These enzyme fractions, which catalyse similar reactions and have similar (but not necessarily identical) substrate specificities, but which differ in chemical, electrophoretic or immunological properties, have been termed isoenzymes. Starch-gel electrophoresis (Smithies, 1955), which combines electrophoretic separation with a molecular sieving effect, has proved particularly effective in resolving isoenzyme mixtures, and the heterogeneity of alkaline phosphatases from several human organs has been demonstrated in this way (Boyer, 1961).

Moss, Campbell, Anagnostou-Kakaras & King (1961) reported the Michaelis constants, K_m, of the major fractions of alkaline-phosphatase activity from human liver, bone, kidney and small intestine after starch-gel electrophoresis. One or more minor phosphatase components are present after electrophoresis of extracts of each of these tissues, and the K_m values of these have now been determined. Certain other properties of the alkaline-phosphatase fractions from these organs are also reported.

A preliminary account of part of this work has been published (Moss & King, 1962).

EXPERIMENTAL

Enzyme preparations. Extracts of recent-post-mortem human bone, liver, kidney and small intestine were prepared by the modification described by Ahmed & King (1960) of Morton's (1950) butan-1-ol procedure. The bone specimen was from a stillborn full-term foetus; the other tissues were normal adult organs. The whole aqueous extract from each was concentrated to small volume (about 1 ml.) by dialysis against polyvinylpyrrolidone powder (average mol.wt. 11 000). The concentrates contained 400–500 King & Armstrong (1934) units of alkaline-phosphatase activity/100 ml.

Starch-gel electrophoresis of the concentrated extracts. This was carried out on horizontal gels by the method of Smithies (1955) in the discontinuous citrate–borate buffer of Poulik (1957) at pH 8·65. The enzyme zones were located after electrophoresis as described by Moss *et al.* (1961). The enzyme activity was recovered either by macerating the segments of gel with pH 10 carbonate–bicarbonate buffer (Delory & King, 1945) and centrifuging off the starch debris, or by freezing and thawing the gel and expressing the solution from the thawed sections by squeezing them against a sintered-glass plate in a glass syringe. The recoveries of enzyme activity and the properties of a given enzyme fraction were similar whichever method was adopted. Overall recovery of enzyme was up to 30%. The relative proportions of the individual phosphatase bands within a given tissue reported below depend on the assumption that the recoveries of each are similar; since the fractions from a single tissue lose activity at the same rate on heating (see below) this assumption is probably justified.

Determination of Michaelis constants. A spectrofluorimetric assay of enzyme activity was used, with disodium β-naphthyl phosphate as substrate (Moss, 1960). The reaction velocity at optimum pH for each substrate concentration was used in determining K_m (Motzok, 1959); experimental conditions were as described by Moss *et al.* (1961). Apparent K_m values were obtained from Lineweaver & Burk (1934) plots of $1/v$ against $1/s$, lines being fitted to the experimental points by the method of least squares.

Effect of pH, Mg^{2+} *ions and heating at* 55° *on activity of enzyme fractions.* Spectrofluorimetric estimation of enzyme activity with β-naphthyl phosphate as substrate was used in each case. The optimum pH at a substrate concentration of 5 mM was determined for each phosphatase fraction, and the effect of varying the Mg^{2+} ion concentration at this optimum pH and substrate concentration was investigated. Carbonate–bicarbonate buffers [0·1 M (Delory & King, 1945)] were used, and the pH of the reaction mixtures was determined with the glass electrode. The solution of each tissue-phosphatase fraction recovered from the gel was adjusted to pH 7 by addition of 0·1 N-hydrochloric acid; portions were then incubated for various times at 55° and their activities were compared with that of an unincubated control at a substrate concentration of 5 mM and optimum pH.

RESULTS

The location of the alkaline-phosphatase fractions after electrophoresis of the tissue extract, compared with that of the principal protein bands of normal blood serum, is shown in Fig. 1. In each extract one fraction predominates; in liver and bone this fraction moves slightly more slowly than the transferrin (β-globulin) fraction, but in in-

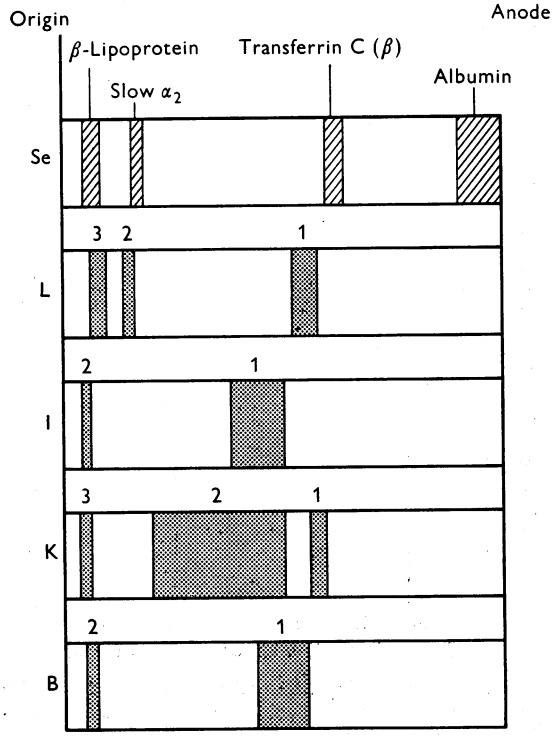

Fig. 1. Location of alkaline-phosphatase fractions after starch-gel electrophoresis of concentrated butan-1-ol extracts of bone (B), liver (L), kidney (K) and small intestine (I), compared with that of principal protein fractions of normal blood serum (Se).

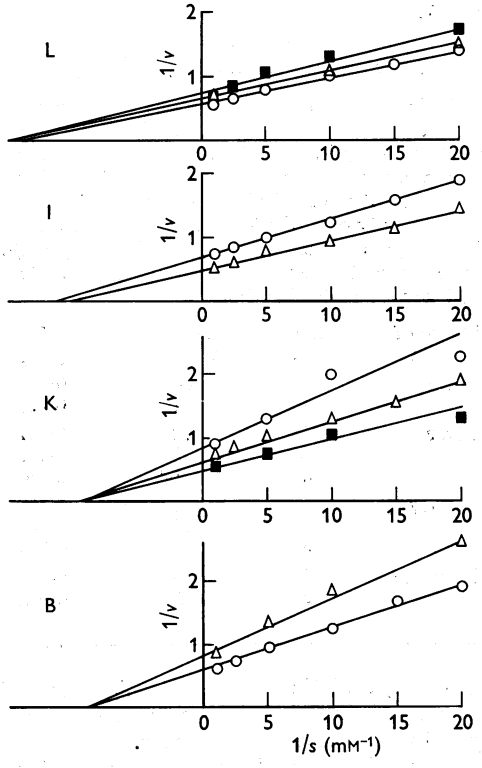

Fig. 2. Reciprocal plots ($1/v$, at optimum pH, against $1/s$) for individual enzyme bands of each tissue extract after starch-gel electrophoresis. B, bone; L, liver; K, kidney; I, intestine. Symbols for each extract correspond with the band numbering in Fig. 1: ○, band 1; △, band 2; ■, band 3. The ordinate scale is in arbitrary units: actual values of $V_{max.}$ for solutions of individual bands (μmoles of substrate hydrolysed/min./l. of enzyme solution) were: B(1) 20·0, B(2) 4·7; L(1) 7·3, L(2) 0·7, L(3) 1·0; K(1) 0·6, K(2) 6·1, K(3) 6·7; I(1) 13·0, I(2) 1·3.

testine and kidney it migrates in the haptoglobin region. Additional minor bands are present in the β-lipoprotein region in all four tissues, with faint bands in the slow α_2-globulin zone in liver and ahead of the main enzyme band, close to the transferrin-protein fraction, in kidney. The slowest enzyme zone in liver appears to be double in some preparations, but has been treated as a single band for determination of K_m etc. The main kidney-phosphatase band, which shows some evidence of fine structure, has similarly been treated as a single zone.

Fig. 2 shows examples of double-reciprocal plots for the individual enzyme zones of the four tissues. Numerical values of K_m derived from such results are collected in Table 1, which also gives the relative activities of individual phosphatase bands.

Bands 1 and 2 of bone, 1 and 3 of liver and 2 and 3 of kidney were maximally activated by a Mg^{2+} ion concentration of 5 mM, but for bands 1 and 2 of intestine the optimum concentration was 6 mM. The pH optimum at a substrate concentration of 5 mM was 10·1 for bands 1 and 2 of bone and intestine and bands 1 and 3 of liver, and 10·2 for bands 2 and 3 of kidney. The results of incubation of the enzyme solutions at 55° and pH 7 are presented in Fig. 3.

DISCUSSION

Moss *et al.* (1961) showed slight differences in K_m between the major phosphatase fractions from bone, liver, kidney and intestine: the greatest difference was between liver and the other tissues. The present results show that these differences are reflected in the minor enzyme components. Within a given tissue, the K_m values of the minor bands are not significantly different from that of the major component (the standard deviation of the method of estimating K_m is ± 0·004 mM, based on 13 results). In the other properties studied also, the enzyme fractions from a given tissue resemble

Table 1. *Michaelis constants for hydrolysis of β-naphthyl phosphate by fractions of tissue alkaline phosphatases obtained by starch-gel electrophoresis*

Individual enzyme bands are numbered to correspond with Fig. 1. The temperature was 37° and the Mg^{2+} ion concentration 5 mM. Velocity at optimum pH for each substrate concentration was used in determining K_m. Relative activities of individual bands are given as multiples of activity of weakest band in each tissue. Absolute activities (μmoles of substrate hydrolysed/min./l. of enzyme solution) for weakest bands were: bone, 4·7; liver, 0·7; kidney, 0·6; intestine, 1·3.

Tissue of origin	Band no.	Relative activity	K_m (mM)
Bone	1	34	0·110
	2	1	0·118
Liver	1	42	0·067
	2	1	0·067
	3	1·4	0·070
Kidney	1	1	0·105
	2	34	0·103
	3	6	0·096
Intestine	1	26	0·090
	2	1	0·098

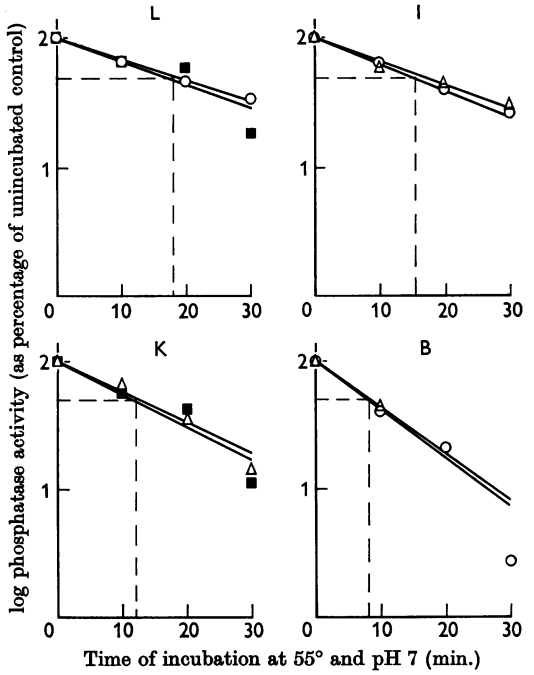

Fig. 3. Plots of the logarithm of phosphatase activity (as percentage of unincubated control) against time of incubation of individual enzyme bands at 55° and pH 7. B, Bone; L, liver; K, kidney; I, intestine. Symbols for each extract correspond with the band numbering in Fig. 1: ○, band 1; △, band 2; ■, band 3. The incubation periods required to reduce activity to half original value (min.) were: bone, 8; liver, 18; kidney, 12; intestine, 15. The initial concentrations of enzyme activity (μmoles/min./l.) were: B(1) 5·4, B(2) 1·3; L(1) 17·1, L(3) 3·6; K(2) 20·8, K(3) 7·2; I(1) 13·5, I(2) 5·0.

each other, and there are some slight variations between alkaline phosphatases from different organs in some of these properties.

Although it cannot be concluded unequivocally on the basis of results obtained with a single substrate that the phosphatase bands in a given tissue extract are fractions of the same enzyme, these findings may indicate the presence of a single alkaline phosphatase in each tissue, with properties characteristic of the tissue of origin. If each organ does contain only one alkaline phosphatase, the main phosphatase zone probably corresponds to the free enzyme protein: the subsidiary zones seen on starch-gel electrophoresis may then represent proportions of the enzyme of which the electrophoretic mobility has been modified in some way. Factors which may influence the mobility of the enzyme protein molecules may include aggregation or disaggregation, modification of the molecule by environmental factors or by post-mortem autolysis (e.g. removal of a charged residue), or attachment of the enzyme protein to indifferent protein fractions. There is some support for the last of these hypotheses with one of the alkaline-phosphatase fractions, namely that migrating in the β-lipoprotein region, which occurs in all the tissue extracts. Alkaline phosphatase is known to occur in association with lipid material in the tissues, particularly with microsomes (Dixon & Webb, 1958), and Morton (1950) introduced the use of butan-1-ol to break this lipid–phosphatase association. The activity in the β-lipoprotein region may represent extracted, but undissociated, lipoprotein–phosphatase complex. The method of preparation of the tissue extracts influences the amount of activity in this region: there is proportionately less activity in extracts made by autolysis at room temperature for 4 days (Moss, 1962), which may be accounted for by the instability of lipoprotein during the autolysis period. Recovery of the phosphatase activity from the β-lipoprotein region of the starch gel by freezing and thawing followed by concentration and re-electrophoresis fractionates the band into a portion with the original mobility, together with a faster-moving component which may result from the dissociation or degradation of an enzyme–lipoprotein complex.

The enzyme of which the component isoenzymes have been most thoroughly studied so far is probably lactate dehydrogenase. The isoenzymes of this enzyme occurring in a single mammalian tissue differ considerably in substrate preference,

K_m values towards a given substrate and inactivation by heat, as well as in electrophoretic mobility (Wilkinson, Cooke, Elliott & Plummer, 1961; Plagemann, Gregory & Wróblewski, 1960). These lactate-dehydrogenase isoenzymes thus appear to be distinct proteins, with closely similar, but not identical, enzyme activities, and probably occur as such *in vivo*. Further, homologous isoenzyme zones from different tissues appear to resemble each other. The findings for the alkaline-phosphatase fractions do not appear, therefore, to parallel those for lactate dehydrogenase. In the present study the only difference found between the phosphatase fractions from a single tissue was in electrophoretic mobility, and homologous enzyme bands from different tissues, e.g. the main zones from liver and bone, were dissimilar in properties.

SUMMARY

1. Starch-gel electrophoresis of concentrated butan-1-ol extracts of human bone, liver, kidney and small intestine reveals a number of active alkaline-phosphatase fractions in each.

2. The apparent K_m values (substrate, β-naphthyl phosphate) of the individual enzyme bands from these tissues have been determined. Other properties of the bands, namely pH optimum, activation by Mg^{2+} ions and inactivation by heating at 55° and pH 7, have also been studied.

3. The phosphatase fractions from a given organ are similar in these properties and in K_m values, although there are some differences between phosphatases from different tissues.

4. The significance of these findings is discussed.

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The Oxidation of Steroids with *tert.*-Butyl Chromate

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The oxidation of steroidal secondary alcohols to ketones is readily effected by a variety of agents, often with almost quantitative yields. The relevant literature (see Fieser & Fieser, 1959) largely concerns preparative experiments necessitating the use of only a slight excess of the oxidant. The application of this conversion to the analysis of complex mixtures of steroids seems promising, since in general it is easier to detect and measure very small quantities of ketones than of the corresponding alcohols. To perform the reaction effectively on an analytical sample of unknown composition an oxidant is required which, even when used in large excess, acts in a predictable manner, so that measurement or identification of the reaction products will give information about the amounts or identities of the compounds under investigation.

Oppenauer & Oberrauch (1949) introduced *tert.*-butyl chromate as an oxidizing agent and employed it in a mixture of 2-methylpropan-2-ol and an inert non-polar organic solvent, in the non-polar solvent alone and in a mixture of the solvent and acetic acid. They observed that increased acidity made the oxidation more vigorous and less selective. We hoped to increase the selectivity of the reagent by performing the oxidation in the presence of an organic base. The chromic anhydride–pyridine complex (Poos, Arth,