Human Liver Alkaline Phosphatase Purified by Affinity Chromatography, Ultracentrifugation and Polyacrylamide-Gel Electrophoresis

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A method is presented for the preparation of human liver alkaline phosphatase (orthophosphoric monoester phosphohydrolase, EC 3.1.3.1). The method gives a purification factor of 12.5×10^3 over the initial aq. butan-1-ol extract, a recovery of 6.0% and a specific activity for the preparation of 1450–1550 units/mg of protein, 1 unit being defined as the amount of enzyme catalysing the hydrolysis of $1 \mu\text{mol}$ of *p*-nitrophenyl phosphate/min at 35°C in 0.1 M -2-amino-2-methylpropan-1-ol/HCl buffer, pH 10.5, containing 10 mM -*p*-nitrophenyl phosphate. Homogeneity was studied by ultracentrifugation, by immunoelectrophoresis and by polyacrylamide-gel electrophoresis. A single contaminating protein was present which was less than 5% of the total. Ultracentrifugation and equilibrium-gradient-pore electrophoresis techniques indicated a mol.wt. of 156000 and 160000 respectively. Equilibrium-gradient-pore electrophoresis indicated that the alkaline phosphatase molecule is possibly a dimer, comprising two subunits of about 80000 mol.wt. Amino acid analysis proved remarkably similar to that for alkaline phosphatase from other sources, regardless of species.

Alkaline phosphatase (orthophosphoric monoester phosphohydrolase, EC 3.1.3.1) obtained from different tissues can be shown by various means to be composed of a number of isoenzymes having similar functions (Hodson *et al.*, 1962; Fishman *et al.*, 1963; Sussman *et al.*, 1968), and an understanding of their most important differences may only be possible when all are available in pure form. Although there are many methods described in the literature for the purification of mammalian alkaline phosphatase, only the human placental (Ghosh *et al.*, 1966; Lehmann, 1975a) and intestinal (Narayanan & Appleton, 1972; Lehmann, 1975a) enzymes have been made sufficiently pure for crystallization to occur. There have been reports of the preparation of partially purified human liver phosphatase (Smith *et al.*, 1968; Hodson & Latner, 1971; Lehmann, 1975b; Sugiura *et al.*, 1975) as well as that from bovine liver (Engström, 1964).

The glycoprotein nature of liver alkaline phosphatase has been established (Moss *et al.*, 1966) and affinity chromatography under suitable conditions has been shown to be an adaptable technique for the purification of macromolecules (Cuatrecasas, 1972). Sialoproteins react with concanavalin A. This substance has been used bound to agarose to purify alkaline phosphatases partially (Chang *et al.*, 1975). We have now used a similar technique, as well as preparative polyacrylamide-gel electrophoresis (Hodson & Latner, 1971), to prepare human liver

alkaline phosphatase from material initially purified by a procedure based on techniques of extraction and fractionation previously developed (Morton, 1954; Smith *et al.*, 1968).

Experimental

Materials

α -Methyl D-glucoside and Tris were obtained from Sigma (London) Chemical Co., Kingston-upon-Thames, Surrey, U.K.; DEAE-cellulose (Whatman type DE11) was from H. Reeve Angel and Co. Ltd., London E.C.4, U.K.; cellulose tubing (Visking) was from Scientific Instrument Centre Ltd., London W.C.1, U.K.; Coomassie Brilliant Blue G was from Raymond A. Lamb, North Acton, London NW10 6JL, U.K.; concanavalin A-Sepharose and Sephadex G-200 were from Pharmacia AB, Uppsala, Sweden. $(\text{NH}_4)_2\text{SO}_4$, specially low in heavy metals, and other reagents, usually of AnalaR grade, were from BDH Chemicals Ltd., Poole, Dorset, U.K. The agar used was a purified preparation, code no. L28, obtained from the Oxoid Division of Oxo Ltd., London S.E.1, U.K. Catalase and alcohol dehydrogenase were from Boehringer Corp. (London) Ltd., London W.5, U.K.

Methods

Alkaline phosphatase activity. The substrate solution used was 10 mM -*p*-nitrophenyl phosphate tetra-

hydrate in 0.1 M-2-amino-2-methylpropan-1-ol/HCl buffer, pH 10.5, containing 0.1 mM-MgCl₂ and 1 μ M-ZnSO₄. Activity was tested with 0.1 ml of an appropriate dilution of the enzyme preparation added to 1 ml of the buffered substrate. After incubation at 35°C for 30 min, the reaction was stopped by the addition of 10 ml of 0.02 M-NaOH. The enzyme solutions were previously diluted to give a final E_{410} of 0.3–0.9. The molar absorbance of the *p*-nitrophenol in 20 mM-NaOH was assumed to be 1.853×10^4 at 410 nm. When many measurements were made, this method was adapted for use with a reaction-rate analyser (Ultralab System; LKB Instruments Ltd., South Croydon, Surrey, U.K.). Both methods gave identical results.

ATPase* and pyrophosphatase activity. These were determined at 35°C in 0.1 M-Tris/HCl buffer containing 0.1 mM-MgCl₂ and 1 μ M-ZnSO₄ by modification of methods of Felix & Fleisch (1974), and the phosphate subsequently released was measured as described by Hørdér (1972). The ATPase activity was measured at pH 10.0 with 2.5 mM-ATP; pyrophosphatase was measured at pH 9.0 in 2 mM-sodium pyrophosphate. One unit of enzyme activity is defined as the amount of enzyme catalysing the hydrolysis of 1 μ mol of substrate/min at 35°C. For pyrophosphatase activity, 2 μ mol of P_i released is equal to 1 μ mol of substrate hydrolysed.

Protein assay. Solutions were concentrated by ultrafiltration in cellulose tubing (Gregory, 1954) and protein content was determined by measuring E_{280} and assuming an $E_{1\text{cm}}^{1\%}$ of 10. This gives a result closely similar to that shown by the technique of Lowry *et al.* (1951). On occasions, absorbance was measured at 210 nm.

Detection of haem pigments. Haem pigments were detected by measuring their absorbance at 413 nm, an absorption maximum, in the Soret-band range, present in the initial crude extracts.

Electrophoresis in starch gel. Starch-gel electrophoresis was performed in vertical trays as described by Smithies (1959) with minor modifications (Hodson & Latner, 1975).

Localization of alkaline phosphatase. Alkaline phosphatase activity in polyacrylamide, starch and agar gels was located as described by Hodson *et al.* (1962), modified by substituting 1-naphthyl disodium orthophosphate for the calcium salt and by the addition of 1 mM-MgCl₂ and 1 μ M-ZnSO₄ to the staining solution.

Localization of protein. Protein in the various gels was located by staining with Coomassie Brilliant Blue G (Diezel *et al.*, 1972).

Gradient-pore polyacrylamide-gel electrophoresis. Polyacrylamide-gel gradients were formed by using the gradient mixer (Margolis, 1969), gel tower (Margolis & Kenrick, 1968) and associated equipment

available from Universal Scientific Ltd., London E13 0QU, U.K. The gels were made so that a 4–24% (w/v) concave acrylamide gradient was formed (Margolis & Kenrick, 1969) in Tris buffer, pH 8.28, containing (per litre) 10.75 g of Tris, 0.93 g of EDTA (disodium salt) and 5.04 g of boric acid. This buffer was also used in the electrode compartments of the electrophoresis apparatus. After electrophoresis, the gel was sliced horizontally to obtain two slices with the flat outer surfaces intact. One of these was stained for protein and the other for enzyme activity as described above.

Ultracentrifuge observations. Analytical work was undertaken in the Beckman model E analytical ultracentrifuge by using an AnD rotor double-sector 12 mm cell and a u.v. scanner at 280 nm. The rotor was centrifuged at 52000 rev./min (160000g; r_{av} , 6.5 cm) with the temperature controlled at 4°C. Concentration of protein in the cell was 0.9 mg/ml in 0.01 M-Tris/HCl buffer, pH 7.0, containing 0.1 M-KCl.

Other analytical and preparative work involved the MSE Super-Speed 65 ultracentrifuge and a swing-out rotor (type 4 3127-102) holding 3 $\times$ 6.5 ml tubes. Protein samples were centrifuged in a stepped gradient made up of 10% (w/v) sucrose layered on to an equal volume of 20% (w/v) sucrose. The sucrose was dissolved in 0.01 M-Tris/HCl buffer, pH 7.0, containing 0.1 M-KCl. The tubes were centrifuged at 45000 rev./min (160000g; r_{av} , 7.33 cm) for 16 h with the temperature controlled at 4°C. At the end of the centrifuge run the tubes were removed so that the gradient could be fractionated. In order to do this a glass capillary with plastic tubing (Technicon Auto-Analyzer pump tube, internal diam. 1.14 mm) attached to the upper end was inserted downwards to the bottom of each tube. About 36 fractions were obtained from each tube by pumping out the contents with a peristaltic pump (type 4912, LKB Instruments Ltd.). The individual fractions were assayed for enzyme and protein content.

Preparative polyacrylamide-gel electrophoresis. The method used was essentially the same as that previously described (Hodson & Latner, 1971).

Amino acid analysis. Samples of the most purified preparation were hydrolysed in 6 M-HCl at 110°C in a sealed evacuated tube for 20 h. Afterwards the hydrolysate was dried by rotary evaporation, the residue was redissolved in water and evaporation was repeated and the residue dissolved in 0.2 M-HCl. A sample containing approx. 50 μ g of hydrolysed protein was applied to an ion-exchange column (55 cm long $\times$ 0.9 cm diam) of Aminex A6 resin. Analysis was performed by a modification of the Moore *et al.* (1958) technique, described in the instruction manual of the Biocal BC100 amino acid analyser (LKB Instruments Ltd.) used. Insufficient material was available for repeat analysis after a longer hydrolysis period.

* Abbreviation: ATPase, adenosine triphosphatase.

Antibody preparation. Rabbits were immunized by an initial intramuscular injection of 0.5ml of butan-1-ol extract from human liver containing 1 unit of enzyme activity and 20mg of protein. After 7, 14 and 28 days the animals were injected with 0.5ml of a partially purified extract (ethanol/chloroform fractionation step described below) containing 6 units of enzyme and 3mg of protein.

Serum was collected 2 weeks after the final injection. The antigen solutions used were sterilized by filtration and injected together with an equal volume of Freund's complete adjuvant.

Immunoelectrophoresis. The method was essentially that described by Bodman (1963), with 1% (w/v) agarose in 0.05M-barbitone buffer, pH 8.6, containing 1.8g of diethylbarbituric acid, 10.3g of sodium diethylbarbiturate and 500mg of NaN_3 /litre. Precipitation time was 72h at room temperature (23°C) in a humid compartment. Before staining for protein the gels were washed for 72h with six changes of 0.9% NaCl in 0.05M-Tris/HCl buffer, pH 7.6, containing 500mg of NaN_3 /litre.

Immunodiffusion experiments. Ouchterlony (1967) double-diffusion experiments were carried out in gels prepared in the same way as for immunoelectrophoresis. Diffusion, precipitation and washing conditions were also similar.

Preparation of human liver alkaline phosphatase. Whenever possible, or unless otherwise stated, all the steps of extraction and purification were carried out at 4°C. All the buffers used contained $1.0\mu\text{M}$ - ZnSO_4 and 1.0mM- MgCl_2 unless otherwise stated. Measurement of buffer pH was made with the buffer as well as the reference and glass electrodes at 25°C.

Step 1: crude extract. Livers were obtained from the post-mortem room within 48h after death, freed of the gall bladder and stored at -20°C. For extraction of alkaline phosphatase, the livers were left to thaw at 4°C overnight, then cut up into slices of approx. 5mm × 30mm × 50mm. The sliced liver tissue (12.4kg) in 0.9% NaCl (6.2 litres), cooled to 4°C, was homogenized in batches of 1.5kg for 15s at the lowest speed setting of a blender (Waring model CB5; Waring Products Co., Winsted, CO, U.S.A.). The homogenate was immediately centrifuged at 2000g for 4h at 4°C. The deposit was mixed with water and butan-1-ol, pre-cooled to 4°C (4 parts of deposit: 4 parts of water: 1 part of butan-1-ol, by vol.) and homogenized for 1min in the blender at its high-speed setting, then left overnight at 4°C before being centrifuged at 6000g for 30min. A clear aqueous supernatant free of excess of butan-1-ol was obtained, decanted and adjusted to pH 7 at 4°C with 1.0M-NaOH.

Step 2: acetone fractionation. The aq.-butan-1-ol extract was ultrafiltered (Gregory, 1954) using a 2-litre thick-walled Erlenmeyer flask with a side arm and a 50cm length of Visking tubing for each

500ml sample of the extract. In all subsequent ultrafiltration procedures described below a similar technique was used. When the total volume of the aq.-butan-1-ol extract had been decreased to 9.6 litres, ultrafiltration was terminated and the material divided into four portions. Each portion was initially cooled to 0°C on a cold plate (Fryka Kaltetechnik type KP250TR; Camlab, Cambridge, U.K.), and acetone, previously cooled to -20°C, was added. After the addition of some acetone to prevent freezing the temperature could be lowered to -10°C. An appropriate volume of acetone was then added until the concentration was 33% (v/v). Precipitated protein was removed by centrifugation at 2000g for 20min at -10°C. A further appropriate volume of acetone was added to the supernatant until the concentration was 40% (v/v). The protein precipitate, which contained most of the enzyme, was removed by centrifugation as above and dissolved in 0.1M-sodium phosphate buffer, pH 7.6. This buffer was also used to remove residual acetone by dialysis and the solution was concentrated by ultrafiltration to a volume of 310ml (170mg of protein/ml).

Step 3: ethanol/chloroform fractionation. A modification of the method of Herbert & Pinsent (1948) was used, which involved a 3:1 (v/v) mixture of ethanol and chloroform. This was pre-cooled to -20°C and added with stirring to the combined concentrated enzyme solution from the previous step until the concentration of the ethanol/chloroform was 44% (v/v). The temperature of the protein solution was maintained at 0°C in an ice bath. After removal of the precipitate by centrifugation at 9000g for 30min at 0°C the aqueous supernatant was dialysed extensively against 0.1M-sodium phosphate buffer, pH 7.6.

Step 4: $(\text{NH}_4)_2\text{SO}_4$ fractionation. The dialysed solution from the previous step was concentrated by ultrafiltration until the protein content was approx. 170mg/ml. Water at 0°C, containing $1.0\mu\text{M}$ - ZnSO_4 and 1.0mM- MgCl_2 , was saturated with $(\text{NH}_4)_2\text{SO}_4$ and adjusted to pH 7.2 with 1.0M-NaOH. An appropriate volume was added to the concentrated protein solution to give 50% saturation at 0°C, and after stirring the protein precipitate was removed by centrifuging at 9000g for 30min at 0°C. The supernatant was adjusted similarly to 70% saturation with $(\text{NH}_4)_2\text{SO}_4$ at 0°C, stirred and centrifuged as above. Most of the enzyme was precipitated at 50-70% saturation. This fraction was dissolved in 50ml of 0.1M-sodium phosphate buffer, pH 7.6, then dialysed against 4mM-sodium phosphate buffer, pH 7.6, until free of NH_4^+ ion. All of this material was used for the next step.

Step 5: DEAE-cellulose chromatography. The DEAE-cellulose was prepared by first washing with 0.5M-NaCl, then sequentially with ethanol, 0.5M-HCl and 0.5M-NaOH (Peterson & Sober, 1962). After

equilibration with the starting buffer (4mM-sodium phosphate, pH 7.6), the DEAE-cellulose was used to pack a column (22cm long $\times$ 4cm diam.). The enzyme sample was applied in 71 ml of the starting buffer and subsequent elution was carried out with various concentrations of NaCl in the same buffer. The volume of each fraction was 7.5ml. Initially 0.04M-NaCl was passed until the E_{280} was decreased to almost zero. Afterwards a linear gradient of 0.04–0.07M-NaCl was passed and a concentration of 0.07M-NaCl then maintained until almost all the enzyme had been removed. Fig. 1 shows the gradient used and the chromatography profile of 31000 units of the enzyme fraction collected after this fractionation procedure. Fractions 211–271 inclusive were pooled and subjected to the next fractionation step.

Step 6: affinity chromatography. Concanavalin A-Sepharose was prepared by washing first with starting buffer, pH 7.0, which was 0.1 M-sodium acetate containing 1.0mM-MnSO₄ and 1.0mM-CaCl₂ in addition to the Mg²⁺ and Zn²⁺ salts. This was followed by a further washing with the starting buffer, which also contained 50mM- α -methyl D-glucoside. Material from the peak of the alkaline phosphatase activity eluted from the DEAE-cellulose column was concentrated by ultrafiltration to 8.5ml, dialysed extensively against the starting buffer and divided into two portions. One portion, containing one-seventh of the total activity, was used as a preliminary trial to test the efficiency of the method, and the remaining six-sevenths was chromatographed afterwards, but the method was essentially the same in both cases. The dialysed sample was applied to a column (7.5cm long $\times$ 1.5cm diam.) of concanavalin A-Sepharose which had been thoroughly eluted with 0.1M-sodium acetate buffer, pH 7.0, to remove the α -methyl D-glucoside and then equilibrated with the starting buffer. After a 2h interval at 4°C, to allow maximum

binding of the alkaline phosphatase to occur, elution of unbound substances was carried out by continued passage of the starting buffer, until the E_{280} was zero. The volume of each fraction was 2ml. Next, bound substances were eluted by passing starting buffer containing 50mM- α -methyl D-glucoside. The separation obtained with the larger sample is shown in Fig. 2. Fractions 103–125 inclusive were pooled and subjected to the next fractionation step. The separation profile obtained with the smaller sample was almost identical, except that it was on a smaller scale.

Step 7: gel filtration. The peaks of alkaline phosphatase activity recovered from both the concanavalin A-Sepharose chromatography columns were combined, concentrated by ultrafiltration to a volume of 13ml and dialysed extensively against 0.1M-sodium phosphate buffer, pH 7.6, containing 0.1M-NaCl in addition to the Mg²⁺ and Zn²⁺ salts. The preparation was divided into two portions. One portion contained one-seventh of the total activity, the other contained the remainder, and each was chromatographed separately on a column (90cm long $\times$ 3.2cm diam.) of Sephadex G-200. The 0.1 M-sodium phosphate buffer, pH 7.6, containing 0.1M-NaCl was used to equilibrate the Sephadex G-200 and for elution. Fraction volume was 3.2ml. The results for both experiments were similar, and the elution profiles showed two overlapping peaks of protein, the first coinciding with the single peak of alkaline phosphatase. The fractions containing the leading half of the enzyme peak from both portions were pooled and subjected to the next fractionation step.

Step 8: preparative ultracentrifugation. The enzyme solution recovered from Sephadex G-200 gel chromatography was concentrated by ultrafiltration and dialysed extensively against 0.01M-Tris/HCl buffer, pH 7.0, containing 0.1M-KCl in addition to the

Fig. 1. Elution profile of alkaline phosphatase from DEAE-cellulose

The volume of each fraction was 7.5ml. Fractions 211–271 inclusive were pooled, concentrated and subjected to the next fractionation step. ●, Alkaline phosphatase activity; ○, E_{280} ; ▲, E_{413} ; —, NaCl concentration in elution buffer.

EXPLANATION OF PLATE I

Gradient-pore polyacrylamide-gel electrophoresis of final preparation (a) and results of Ouchterlony technique (b)

(a) Gel slice (A) stained for protein; gel slice (B) stained for alkaline phosphatase activity. The arrow in gel slice (A) indicates the position of a minor band of protein. (b) shows the pattern of wells on the Ouchterlony plate of which six were peripheral and one central. The two upper wells contained enzyme solution at a concentration of 1 g of protein/litre. Of the three middle wells, the outside two contained enzyme at a concentration of 0.5 g of protein/litre and the central well contained anti-serum. The lowest two wells contained enzyme at a concentration of 0.25 g of protein/litre. The plate was cut vertically so that one half could be stained for protein and the other half for alkaline phosphatase activity. The two halves were then assembled so as to reconstruct the original situation. A precipitin arc can be seen below the two upper wells. The left half of this is stained for protein and the right half for alkaline phosphatase activity.

Fig. 2. Elution profile of alkaline phosphatase from concanavalin A-Sepharose

The volume of each fraction was 2 ml. Fractions 103–125 inclusive were pooled, concentrated and subjected to the next fractionation step. Elution with 50 mM- α -methyl D-glucoside added to the buffer was started after fraction 98. ●, Alkaline phosphatase activity; ○, E_{280} .

Fig. 3. Sucrose-gradient-ultracentrifugation separation of alkaline phosphatase

E_{280} and enzyme content refer to 0.16 ml fractions of the gradient diluted to 1.16 ml. Fractions 11–19 inclusive were pooled, concentrated and subjected to the next fractionation step. ●, Alkaline phosphatase activity; ○, E_{280} . The arrow indicates the direction of sedimentation.

Mg^{2+} and Zn^{2+} salts. The volume of the preparation was 1.7 ml and contained 7200 units of enzyme activity. This was divided into three portions and each subjected to ultracentrifugation on a stepped sucrose gradient. Each 0.16 ml fraction obtained from the gradient was diluted to 1.16 ml and protein and enzyme were assayed. A typical separation profile is shown in Fig. 3. Fractions 11–19 inclusive were pooled in each case, combined and subjected to the next fractionation step.

Step 9: preparative polyacrylamide-gel electro-

phoresis. The enzyme solution separated by ultracentrifugation was concentrated by ultrafiltration to 1.3 ml, of which 1 ml containing 3000 units was subjected to preparative electrophoresis. The electrophoresis buffer was 0.05 M-sodium borate, pH 8.6. Fractions were collected at 30 min intervals during 60 h and a constant d.c. potential of 50 V was applied across the electrodes. This gave an initial current of 18 mA, which decreased progressively to 15 mA at the end of the electrophoresis period. The volume of each fraction collected was 8 ml. Enzyme activity was present in a single peak, of which the fractions in the leading two-thirds were pooled and concentrated by ultrafiltration to a volume of approx. 1 ml.

After concentration, the alkaline phosphatase was dialysed against 70% satd. $(NH_4)_2SO_4$, adjusted to pH 7.2 with a few drops of 1 M-NaOH, and containing 0.1 mM- $MgCl_2$ and 1.0 μ M- $ZnSO_4$. The enzyme material precipitated in this fashion and kept in the precipitating solution was stable at 4°C. For amino acid analysis and other investigations a portion of the suspension was dialysed against a large volume of 0.01 M-Tris/HCl, pH 7.0, containing 0.1 M-KCl, until free of $(NH_4)_2SO_4$.

Results

Purity and homogeneity

Purification yield. A summary of the purification of human liver alkaline phosphatase is presented in Table 1. The final recovery would undoubtedly have been higher had we used all of the material from Step 8 for Step 9. This would have given an expected recovery of 6%.

Electrophoresis. Preparative polyacrylamide-gel electrophoresis, the last step in the purification method, gave a single peak of protein which coincided with the enzyme activity. After electrophoresis in gradient-pore polyacrylamide gel, one major and one faint minor band were detected by staining with Coomassie Blue G. A sample of 0.5 μ g of protein is quite clearly detected by this method, and it could be assessed by studying appropriate dilutions that the minor band amounted to less than 5% of the total protein present. The major band of protein was in the same position as the enzyme activity, as demonstrated by the respective staining techniques (Plate 1a).

Ultracentrifugation. A single peak of protein, which coincided exactly with the enzyme activity, was obtained when material from the preparative polyacrylamide-gel electrophoresis was subjected to ultracentrifugation in a sucrose gradient in the MSE Super-Speed 65 ultracentrifuge.

Immunological. Double-diffusion experiments showed a single precipitation band after staining with Coomassie Blue G. The band also had alkaline phosphatase activity (Plate 1b).

Table 1. *Purification data for human liver alkaline phosphatase*

	Total activity (units)	Specific activity (units/mg)	Step purification	Step recovery (%)	Purification factor	Yield (%)
1. Butan-1-ol extract	51500	0.12	1.0	100	1.0	100
2. 33–40% acetone	42000	0.8	6.5	82	6.5	82
3. Chloroform/ethanol	35700	2.9	3.6	83	24	67
4. 50–70%-satd. (NH ₄) ₂ SO ₄	31000	6.5	2.2	89	53	60
5. DEAE-cellulose	16700	100	15	54	820	33
6. Concanavalin A–Sephadex	12775	413	4.1	85	3400	25
7. Sephadex G-200	9100	920	2.2	71	7500	17.8
8. Preparative ultracentrifugation	4720	1350	1.5	66	11100	14
9. Preparative polyacrylamide-gel electrophoresis	2300	1450	1.1	77	12500	4.5

Table 2. *Amino acid composition of human liver alkaline phosphatase*

The values are means of six observations and are uncorrected for hydrolytic losses.

	Content (mol/100 mol of glycine)
Aspartic acid	133
Threonine	76
Serine	64
Glutamic acid	106
Proline	59
Glycine	100
Alanine	102
Cysteine	13
Valine	77
Methionine	11
Isoleucine	40
Leucine	87
Tyrosine	35
Phenylalanine	33
Histidine	51
Lysine	73
Arginine	47

A previously obtained enzyme preparation purified by a very similar method but with a slightly higher activity of 1550 units/mg of protein instead of 1450 units/mg of protein showed only one band after gradient-pore polyacrylamide-gel electrophoresis and immunoelectrophoresis as well as homogeneity after ultracentrifugation.

Amino acid analysis

The amino acid composition of the purified human liver alkaline phosphatase is presented in Table 2. The results are the mean of six observations and no correction has been made for hydrolytic losses.

Molecular-weight determinations

By ultracentrifugation. Experiments carried out in

the Beckman analytical ultracentrifuge on the material recovered from the concanavalin A–Sephadex purification step showed that two components were present. Component 1 had an observed sedimentation coefficient ($s_{20,w}$) of 7.1S and component 2 had an observed sedimentation coefficient ($s_{20,w}$) of 4.2S. Assuming these were typical globular proteins, the mol.wt. of component 1 would be approx. 155000 and that of component 2 approx. 58000. It must be emphasized, however, that these deductions are based only on single observations. Component 1 contributed 65% and component 2 30–35% of the total protein present. Their relative proportions and the assumption that the major component contained the enzyme activity were confirmed by preparative sedimentation work in the MSE Super-Speed 65 ultracentrifuge by using a stepped sucrose gradient.

By gradient-electrophoresis. A mol.wt. of 160000 for the initial butan-1-ol-extracted enzyme as well as for the most purified preparation was estimated by electrophoretic mobility in gradient-pore polyacrylamide gel by prolonging the electrophoresis period until the alkaline phosphatase and protein markers were in equilibrium (Slater, 1969). The protein markers used were: human haemopexin, mol.wt. 80000; human transferrin, mol.wt. 90000; yeast alcohol dehydrogenase, mol.wt. 148000; bovine liver catalase, mol.wt. 240000. The gels were cut into slices and stained for protein or enzyme activity, as described under 'Methods', so that the protein band which coincided with the alkaline phosphatase could be identified.

Subunit structure

Incubation, at 35°C for 48h, of the native enzyme in a mixture of urea and mercaptoethanol resulted in complete inactivation. The incubation mixture contained 25 µg of enzyme, 330 µmol of urea and 50 µmol of mercaptoethanol in 100 µl of 0.01 M-sodium phos-

phate buffer, pH 7.8. The inactive material migrated as a diffuse band at a faster rate than the native enzyme in gradient-pore polyacrylamide-gel electrophoresis and had a calculated mol.wt. of not more than 9×10^4 and not less than 8×10^4 .

Catalytic properties of human liver alkaline phosphatase

For the most purified material, the pH optima, K_m values and relative activities for the substrates, *p*-nitrophenyl phosphate, ATP and PP_i were determined in 0.1M-Tris/HCl buffer containing 0.1mM- $MgCl_2$ and 1.0 μM - $ZnSO_4$ at the optimum pH for each substrate. The pH optima were determined with 10mM-*p*-nitrophenyl phosphate, 2.5mM-ATP and 2.0mM- PP_i . The activities compared were μmol of substrate hydrolysed/min at 35°C. The results are shown in Table 3.

Discussion

Electrophoresis, ultracentrifugation and immunological findings indicate that a high degree of homogeneity had been achieved.

Specific activity for comparison of the degree of homogeneity attained by purification techniques is of limited value because the methods and conditions for the determination of enzyme activity and protein nearly always vary for alkaline phosphatases derived from different tissue sources. However, a specific activity of 1450–1550 $\mu mol/min$ per mg gives a purification factor of 12.5×10^3 relative to the crude butanol extract, and this is the highest so far reported for human liver alkaline phosphatase.

One complicating factor in alkaline phosphatase preparation is the variation in the carbohydrate content (Ghosh & Fishman, 1968; Cathala *et al.*, 1975), which may be inherent or due to losses incurred during extraction and purification (Wachsmuth & Hiwada, 1974). We have observed that the electrophoretic mobility in starch gel as well as the molecular weight were unchanged during purification of our material. A loss of sialic acid would have shown as a lower mobility in the gel.

Table 3. *Catalytic properties of human liver alkaline phosphatase*

See the end of the Results section. Relative activity refers to *p*-nitrophenyl phosphate as substrate.

Substrate	Optimum pH (25°C)	K_m	Relative activity (%)
<i>p</i> -Nitrophenyl phosphate	10.5	0.5	100
ATP	10.0	0.8	14
PP_i	9.0	1.3	9

The overall amino acid composition found for human liver alkaline phosphatase is remarkably similar to published results for alkaline phosphatases derived from various mammalian tissues as well as to that from *Escherichia coli* (Rothman & Byrne, 1963). This is of interest and could possibly indicate that the evolutionary processes have not produced major changes in the protein portion of the alkaline phosphatase molecule.

Investigators studying the subunit structure of alkaline phosphatase from various sources (Rothman & Byrne, 1963; Harkness, 1968; Fosset *et al.*, 1974; Ohkubo *et al.*, 1974; Cathala *et al.*, 1975; Petitclerc *et al.*, 1975; Linden & Alais, 1976) have mainly concluded that it is dimeric. Our results indicate that human liver alkaline phosphatase could also be a dimer.

A human liver contains about 4mg of alkaline phosphatase, therefore methods capable of giving higher yields than those conventionally used are needed if useful amounts of the pure liver enzyme are to be prepared from a single liver. Polysaccharides form precipitates by combination with concanavalin A (Goldstein *et al.*, 1965), which suggests another possible method for isolation of liver alkaline phosphatase. In preliminary small-scale experiments, we have found that a precipitate of alkaline phosphatase bound to concanavalin A could be separated from unbound protein by centrifugation. Subsequent separation of concanavalin A from alkaline phosphatase was possible by dissolving the precipitate in a suitable buffer containing α -methyl D-glucoside, which dissolves the complex. Concanavalin A has a mol.wt. of 60000 (Kalb & Levitzki, 1968), which is considerably lower than that of human liver alkaline phosphatase. It therefore proved easily separable by gel filtration in an appropriate Sephadex gel. Starting with an aq.-butan-1-ol extract of human liver partially purified by DEAE-cellulose chromatography and fractionation with an ethanol/chloroform mixture, we have obtained material of high specific activity which closely approximates to the very pure material that we have described in the present paper. This method of preparation gave an overall recovery of 33%. We must, however, emphasize that we have not yet been successful in applying this technique on a scale corresponding to the extract of a whole human liver.

Since this work was completed, a paper has appeared (Trépanier *et al.*, 1976) in which similar affinity purification of human liver alkaline phosphatase has been described. Purity was said to be established by standard and sodium dodecyl sulphate/polyacrylamide-gel electrophoresis. We do not consider that this is sufficient evidence for homogeneity. In any case the specific activity of the product obtained was only 600 units/mg, compared with our 1450–1550 units/mg. The method used for the deter-

mination of enzyme activity was very similar to our own, except that a temperature of 30°C was used. During the course of our work we have studied the effect of temperature variations on our assay and have found that the increase in rate between 30° and 35°C is only 10%. We have already indicated that our method gives closely similar results to those with a reaction-rate analyser. It is significant that the units of enzyme extracted from 500g of liver by Trépanier *et al.* (1976) closely approximated to the number of units that we extracted from that amount. This must mean that both enzyme methods were giving values in units that were closely similar. The method of protein determination used by Trépanier *et al.* (1976) was a modification of the technique of Lowry *et al.* (1951). We have deliberately chosen an $E_{1\text{cm}}^{1\%}$ at 280nm of 10, since experience has shown that this gives us the same result as the technique of Lowry *et al.* (1951). This must mean that our material, in terms of specific activity, is at least twice as active as that described by Trépanier *et al.* (1976).

Moreover, we have obtained a purification factor of 12.5×10^3 when our final product is compared with the butan-1-ol extract. Trépanier *et al.* (1976) gave data that indicated that their purification factor in relation to their butan-1-ol extract was only 2.2×10^3 . It must be admitted that the specific activity of their butan-1-ol extract was presumably twice that of ours. This could not, however, account for the large differences in the two purification factors.

We have frequently obtained partially purified enzyme products that gave virtually a single band in various methods of polyacrylamide-gel electrophoresis. We therefore cannot agree that polyacrylamide-gel electrophoresis, in the presence or absence of sodium dodecyl sulphate, alone can be accepted as criteria of purity. In any case it is well recognized that alkaline phosphatase is a glycoprotein and that glycoproteins show impaired sodium dodecyl sulphate binding (Pitt-Rivers & Impiombato, 1968), which could affect electrophoretic mobility during this technique (Schubert, 1970).

We ourselves have used, as criteria of purity, not only gel-electrophoresis studies but also ultracentrifugal and immunological methods.

Sugiura *et al.* (1975) have claimed to have obtained a pure preparation of human liver alkaline phosphatase. We agree with the statement (Trépanier *et al.*, 1976) that the criteria for purity were insufficient.

Trépanier *et al.* (1976) encountered apparent changes in molecular weight with experimental conditions. They reported mol.wts. of 146000 and 220000. It is noteworthy that on one occasion after storage for 6 months in satd. $(\text{NH}_4)_2\text{SO}_4$ our own preparation showed a small amount of an alkaline phosphatase with mol.wt. approx. 300000. We have assumed this could be a tetrameric form of the molecule.

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