Affinity Purification and Some Molecular Properties of Human Liver Alkaline Phosphatase

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Alkaline phosphatase from human liver was purified to homogeneity. The purification procedure included solubilization with butanol, fractionation with acetone, and chromatography on concanavalin A-Sepharose, DEAE-cellulose, Sephadex G-200 and DEAE-Sephadex. Purity was established by standard and sodium dodecyl sulphate/polyacrylamide-gel electrophoresis. The isoelectric point of the protein was determined to be 4.0. Sephadex-gel filtration gave a mol.wt. of 146000, although a higher value was obtained in the presence of 100mM-NaCl. The subunit mol.wt. 76700, was determined by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis. Neuraminidase treatment resulted in two enzyme-activity bands on isoelectric-focused gels with isoelectric points of 6.6 and 6.8. The desialylated enzyme gave only one protein band on sodium dodecyl sulphate/polyacrylamide-gel electrophoresis with a subunit molecular weight indistinguishable from that of the non-neuraminidase-treated protein. The desialylated enzyme was more readily denatured by sodium dodecyl sulphate in the presence of mercaptoethanol than was the native enzyme.

The classical procedure of extracting alkaline phosphatase (orthophosphoric monoester phosphohydrolase, EC 3.1.3.1) from animal tissue was introduced by Morton (1950), and modified by Moss *et al.* (1967) to include fractionation with acetone and $(\text{NH}_4)_2\text{SO}_4$, Sephadex-gel permeation and DEAE-cellulose anion-exchange chromatography. In the present paper we describe a further modification to this purification procedure that involves the use of affinity chromatography on concanavalin A-Sepharose, and ion-exchange chromatography with both DEAE-Sephadex and DEAE-cellulose. Concanavalin A is a haemagglutinating protein isolated from jack bean (*Canavalia ensiformis*), which forms insoluble complexes with polysaccharides (Goldstein *et al.*, 1965) and glycoproteins (Clarke & Denborough, 1971). It is bound to Sepharose 4B by the CNBr method of Axén *et al.* (1967). Concanavalin A-Sepharose has been used to purify several glycoproteins: α -foetoprotein (Caron *et al.*, 1973), very-low-density lipoprotein (Shore & Shore, 1973) and more recently γ -glutamyltransferase (EC 2.3.2.2) (Takahashi *et al.*, 1974). Human alkaline phosphatases isolated from placenta, liver, bone and kidney are glycoproteins that contain sialic acid residues (Ghosh, 1969; Robinson & Pierce, 1964) and more specifically the enzyme as isolated from placenta contains glucose, galactose, mannose and fucose (Ghosh *et al.*, 1974). It seems reasonable therefore that liver alkaline phosphatase should bind to,

and be selectively eluted from, concanavalin A-Sepharose and that a significant purification of the enzyme would result thereby.

We are interested in a comparison of the multiple molecular forms of alkaline phosphatase and particularly those from liver and bone. To this end the enzyme from human liver was purified for study. This report describes the purification, determination of the isoelectric point and the molecular weights of native and desialylated liver alkaline phosphatase. Suitable long-term storage conditions for the enzyme are suggested.

Experimental

Materials

Routine chemicals were of reagent quality and for the most part were supplied by Fisher Scientific Co. Ltd., Fair Lawn, NJ, U.S.A. Biochemicals were generally of the highest purity available and were from the following sources: *p*-nitrophenyl phosphate, β -naphthyl acid phosphate, Fast Blue BB salt, 2-amino-2-methylpropan-1-ol, rabbit muscle phosphorylase α , naphthol AS-MX phosphoric acid (3-xylididyl-2-naphthol phosphoric acid) as the disodium salt and *Escherichia coli* alkaline phosphatase from Sigma Chemical Co., St. Louis, MO, U.S.A.; acrylamide, Coomassie Blue R-250 and Coomassie Blue G-250 from Bio-Rad Laboratories, Richmond, CA, U.S.A.; Ampholine from LKB,

Stockholm, Sweden; human serum albumin and Mes [2-(*N*-morpholino)ethanesulphonic acid] from Calbiochem, Los Angeles, CA, U.S.A.; bovine liver glutamate dehydrogenase from Boehringer, Mannheim, Germany; egg-white ovalbumin and neuraminidase (*Clostridium perfringens*; catalogue no. 4775) from Worthington Biochemical, Corp., Freehold, NJ, U.S.A.; rabbit muscle glyceraldehyde 3-phosphate dehydrogenase prepared by the method of Ferdinand (1964); pig muscle lactate dehydrogenase prepared as described by Stinson & Gutfreund (1971); rabbit muscle pyruvate kinase, a gift of Dr. J. J. Holbrook, University of Bristol; yeast phosphoglycerate kinase prepared as described by Stinson (1974); DEAE-cellulose (DE-52) from Whatman Biochemicals Ltd., Maidstone, Kent, U.K.; concanavalin A-Sepharose, Sephadex G-200 and DEAE-Sephadex A-50 from Pharmacia, Uppsala, Sweden. All water was deionized and glass-distilled and contained less than 2 p.p.m. of ionizable impurities.

Methods

Optical measurements. Alkaline phosphatase was assayed at 30°C in a Beckman Acta CIII or a Unicam SP.800 spectrophotometer equipped with scale expansion, external recorder, and a temperature-controlled cuvette holder. The assay medium contained 10 mM-*p*-nitrophenyl phosphate and 1.5 mM-MgCl₂ in 0.782 M-2-amino-2-methylpropan-1-ol, pH 10.3. The rate of the reaction was monitored continuously at 404 nm and the calculations were based on a molar extinction coefficient of 16.7 for *p*-nitrophenol (Halford, 1970). To obtain linear reaction-rate curves, the reaction was initiated with substrate after a 10 min preincubation of the other components of the medium plus 5–50 µl of enzyme solution in 1.0 ml. Activity was expressed as units/ml at 30°C, where 1 unit corresponds to 1 µmol of *p*-nitrophenyl phosphate hydrolysed/min.

Protein concentrations were determined by the method of Schacterle & Pollack (1973), with human serum albumin as standard. Samples for protein determination were dialysed against 50 mM-sodium phosphate buffer, pH 7.6, to avoid interference from the buffers. In the chromatographic steps, protein was monitored at 280 nm with a Pharmacia u.v. monitor.

Preparation of human liver alkaline phosphatase. The preparation of the enzyme was followed in all steps by the measurement of specific activity (µmol of *p*-nitrophenyl phosphate hydrolysed/min per mg of protein). All procedures were carried out at 4°C unless stated otherwise.

Step 1. Human liver obtained from autopsy specimens within 12 h of death was stripped of any vessels and the outer membrane and sliced into 1-in (2.5 cm) cubes. After washing with 0.9% NaCl to remove blood, the cubes were frozen at –70°C until required. A sample (500 g) was thawed and homo-

genized in a Waring Blendor in 1 litre of 10 mM-Tris/HCl buffer (pH 7.6)/0.1 mM-MgCl₂/0.02 mM-ZnCl₂, for 5 min at low speed followed by 2 min at high speed.

Step 2. Butanol (750 ml) cooled to –20°C was added slowly to this suspension with vigorous overhead stirring. The mixture was stirred for a further 30 min and then centrifuged at 9000g for 30 min in a refrigerated Sorvall centrifuge; 1 litre of aqueous layer was recovered.

Step 3. Acetone (430 ml) at –20°C was added to the aqueous solution at 0°C while stirring with an overhead mixer to give a final concentration of 30% (v/v) acetone. The mixture was gently stirred for an additional 15 min, and then centrifuged at 9000g for 20 min. The supernatant (1.4 litres), which contained the alkaline phosphatase activity, was made 50% (v/v) in acetone by the slow addition, with stirring, of 550 ml of acetone at –20°C. After centrifugation at 9000g for 20 min, the pellet, which contained the alkaline phosphatase activity, was recovered and suspended in 60 ml of 100 mM-Tris/HCl (pH 7.6)/100 mM-NaCl/1 mM-MgCl₂/1 mM-MnCl₂/1 mM-CaCl₂/0.02 mM-ZnCl₂. The mixture was centrifuged at 9000g for 20 min to remove undissolved residue.

Step 4. The material from the dissolved acetone pellet was applied to a column (2 cm diam. × 13 cm long) of concanavalin A-Sepharose equilibrated with 100 mM-Tris/HCl (pH 7.6)/100 mM-NaCl/1 mM-MgCl₂/1 mM-MnCl₂/1 mM-CaCl₂/0.02 mM-ZnCl₂, at 4°C. The column was washed with the column buffer until the *E*₂₈₀ began to fall and was then eluted with 500 ml of the same buffer containing mannose in a linear gradient from 0 to 0.1 M at a flow rate of 85 ml/h.

Step 5. The fractions with the highest specific activity from the concanavalin A-Sepharose column were pooled and concentrated by ultrafiltration in an Amicon stirred cell (Amicon Corp., Lexington, MA, U.S.A.) by using a PM-10 membrane. The concentrate was equilibrated with 10 mM-Tris/HCl (pH 7.6)/0.1 mM-MgCl₂/0.02 mM-ZnCl₂ by repeated dilution and concentration in the cell. This material was applied to a column (1.5 cm diam. × 90 cm long) of DEAE-cellulose equilibrated with the same buffer at 4°C. The column was washed with 2 column volumes of buffer and eluted with 1 litre of buffer that contained NaCl in a linear gradient from 0 to 0.2 M at a flow rate of 40 ml/h.

Step 6. The fractions with the highest specific activity from the DEAE-cellulose column were concentrated to 5 ml in an Amicon stirred cell with PM-10 membrane and applied to a column (2.5 cm diam. × 90 cm long) of Sephadex G-200 equilibrated with 100 mM-Tris/HCl (pH 7.6)/100 mM-NaCl/0.1 mM-MgCl₂/0.02 mM-ZnCl₂.

Step 7. The most active fractions from the peak of material that contained the alkaline phosphatase were

pooled and equilibrated with 10mM-Mes/100mM-NaCl/0.1mM-MgCl₂/0.02mM-ZnCl₂, pH 6.0, by repeated dilution and concentration in an Amicon stirred cell with a PM-10 membrane. The material was applied to a column (1.5cm diam. × 25cm long) of DEAE-Sephadex equilibrated in the same buffer, washed with 1 column volume of buffer and eluted with 200ml of the buffer that contained NaCl in a linear gradient from 0.1 to 0.2M at a flow rate of 20ml/h. The highest-activity fractions were pooled so that 50% of the eluted activity was recovered. The pooled fractions were again concentrated by ultrafiltration and dialysed for 24h against 200ml of 10mM-Tris / HCl (pH 7.6) / 100mM-NaCl / 0.1mM-MgCl₂ / 0.02mM-ZnCl₂ / 10% (v/v) glycerol / 0.02% NaN₃, and stored at 4°C.

Isoelectric focusing on polyacrylamide gels. This procedure was carried out in 4.0% (w/v) polyacrylamide, 0.2% (v/v) NNN'-N'-tetramethylethylenediamine, 2% (w/v) Ampholine, 10% (v/v) glycerol and 0.25mM-ZnCl₂ (R. A. Stinson, unpublished work). For native alkaline phosphatase, 2% Ampholine was made up of 1% pH 4-6 Ampholine and 1% pH 3-10 Ampholine, whereas for the neuraminidase-treated samples the Ampholine was made up of 1% pH 5-8 and 1% pH 7-9 Ampholine. The gels were 3mm diam. × 93mm long. The upper or cathodal buffer was 0.02M-NaOH/0.25mM-ZnCl₂ and the lower or anodal buffer was 0.01M-H₃PO₄/0.25mM-ZnCl₂. These buffers were precooled to 4°C and the electrofocusing apparatus (MRA electrofocusing apparatus; Metaloglass Inc. Boston, MA, U.S.A.) was precooled to 0°C. The gels were prefocused at 0.5mA/gel until the voltage reached 400 V and then at 400 V constant for a total prefocusing time of 1h. The samples (0.003 unit of alkaline phosphatase activity or 5μg of protein) were mixed 1:1 (v/v) with a 50% (w/v) sucrose solution which contained 8% (w/v) Ampholine pH 6-8 and were applied to the gels under a layer of 15μl of 20% (w/v) sucrose solution containing 2% (w/v) Ampholine pH 6-8. The samples were then focused for 16-18h at 400 V and the gels removed from the apparatus and stained. The stain contained 0.006mM-naphthol AS-MX phosphoric acid, 6mM-MgCl₂, 0.1mM-ZnCl₂ and 0.1% (w/v) sucrose in 1.8M-2-amino-2-methylpropan-1-ol buffer, adjusted to pH 9.8 with HCl. Each gel was stained with 0.85ml of the staining solution for 10-15 min, the stain was decanted from the gel stain tubes and fluorescent bands were photographed under u.v. light with a Polaroid camera.

To determine the pH gradients, a gel run under the same conditions as the sample gels but not stained was cut into 3.1mm segments with a razor blade. The segments (26 for the average 78mm gel) were placed in 0.5ml of de-gassed water and the pH was read on a Radiometer model 26 pH meter after 1h. The isoelectric points of the stained bands could be deter-

mined by measuring the distance from one end of the gel and noting the pH of the particular segment in the gradient gel.

Polyacrylamide-gel electrophoresis. Analytical polyacrylamide-disc-gel electrophoresis were run in 7.0% gels in Tris/borate buffer, pH 9.5, by the method of Green *et al.* (1972). Alkaline phosphatase samples that contained 10% (v/v) glycerol (0.003 unit of activity or 5μg of protein) were applied to gels (0.5cm × 6.5cm) and they were electrophoresed at 2.0mA/gel for 45 min. The gels were removed from the running tubes and stained for alkaline phosphatase activity either fluorimetrically as described above or colorimetrically by the method of I. Smith *et al.* (1968). This latter method involved incubation at room temperature (25°C) in the dark in a 60mM-borate buffer, pH 9.7, that contained β-naphthyl acid phosphate (2mg/ml) and Fast Blue BB salt (1 mg/ml). When the bands were sufficiently developed, the stain solution was replaced with 7% (v/v) acetic acid to stop further colour development and prevent diffusion of the stained bands. Protein was stained by the method of Diezel *et al.* (1972) with Coomassie Brilliant Blue G-250.

Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis. The minimum molecular weight of alkaline phosphatase was determined by the method of Segrest & Jackson (1972). A series of gels with acrylamide concentrations from 6.5% to 10.5% (w/v) were prepared and electrophoresed by the procedure of Weber & Osborn (1969). Because incubation in 1% (w/v) sodium dodecyl sulphate or 1% sodium dodecyl sulphate/1% 2-mercaptoethanol at 37°C failed to denature alkaline phosphatase completely, the enzyme was incubated at 100°C for 2min. The gels were stained for protein with Coomassie Blue G-250 (Diezel *et al.*, 1972) and the bands intensified in 5% (v/v) acetic acid. The standards and their subunit mol.wts. were: rabbit muscle phosphorylase *a*, 92000; human serum albumin, 68000; bovine liver glutamate dehydrogenase, 57500; egg-white ovalbumin, 43000; rabbit muscle glyceraldehyde 3-phosphate dehydrogenase, 37000; pig muscle lactate dehydrogenase, 35000.

Molecular-weight determinations on Sephadex G-200. Gel-filtration experiments on Sephadex G-200 were carried out by the method of Determann (1968) on a column (2.5cm diam. × 90cm long) equilibrated with 100mM-Tris / HCl buffer (pH 7.6) / 100mM-NaCl/0.1mM-MgCl₂. Chromatography was by upward flow at 18ml/h. The standard proteins and their mol.wts. were: rabbit muscle pyruvate kinase, 237000; rabbit muscle aldolase, 160000; *E. coli* alkaline phosphatase, 86000; yeast phosphoglycerate kinase, 47000.

Neuraminidase treatments. Neuraminidase treatments were carried out at 30°C in 10mM-Mes buffer (pH 6.0) / 30mM-NaCl / 1mM-CaCl₂ / 0.1mM-MgCl₂, which contained 0.02% NaN₃ as a preservative. The

specific activity of the neuraminidase was 6 units/mg at 37°C. Neuraminidase solution was added to the alkaline phosphatase preparations so that the amount of alkaline phosphatase protein was 25 times greater than that of neuraminidase. The progress of the desialylation was monitored with electrofocused gels and judged complete when the pattern of the gels stained for enzyme became constant.

Results and Discussion

Enzyme preparation

Table 1 is a summary for a typical purification of human liver alkaline phosphatase. The recovered enzyme represented 6% of the original activity and had a specific activity of 650 units/mg of protein; 500g wet wt. of liver yielded approx. 250 µg of

alkaline phosphatase protein. This represents a purification of 12900 times over that of the crude homogenate. Figs. 1–4 show the elution profiles from the concanavalin A-Sepharose, DEAE-cellulose, Sephadex G-200 and DEAE-Sephadex columns respectively. The concanavalin A-Sepharose column yielded the largest increase in purity of any step in the purification procedure, 17.5 times. Affinity chromatography on concanavalin A-Sepharose has several major advantages in the purification of alkaline phosphatase. Since concanavalin A has a specific affinity for glycoproteins, all non-glycoproteins will be discarded at this stage. The alkaline phosphatase is relatively weakly bound to the column and is eluted early in the mannose gradient. As shown in Fig. 1, other proteins are eluted with severe trailing, so that the phosphatase is at least partially resolved from

Table 1. *Purification of human liver alkaline phosphatase*

Procedure	Total protein (mg)	Total activity (units)	Specific activity (units/mg)	Yield (%)	Purification factor
Crude homogenate	42000	2233	0.05	100	1
Butan-1-ol extract	7200	2110	0.29	95	6
30–50% Acetone	3650	1410	0.39	63	8
Concavalin A-Sepharose	164	1110	6.8	50	136
DEAE-cellulose	4.5	356	79	16	1580
Sephadex G-200	1.2	279	234	13	4720
DEAE-Sephadex	0.21	136	650	6	12900

Fig. 1. *Elution profile of alkaline phosphatase from concanavalin A-Sepharose*

Enzymic activity (●) and protein (E_{280} ; ○) were monitored throughout a linear gradient of mannose from 0 to 0.1M in 100mM-Tris/HCl (pH 7.6)/100mM-NaCl/1mM-MgCl₂/1mM-CaCl₂/1mM-MnCl₂. The start of the sample, buffer wash, and mannose gradient are indicated by 1, 2 and 3 respectively.

Fig. 2. *Elution profile of alkaline phosphatase from DEAE-cellulose*

Enzymic activity (●) and protein (E_{280} ; ○) were monitored throughout a linear gradient of NaCl from 0 to 0.2M in 10mM-Tris/HCl buffer (pH 7.6)/0.1mM-MgCl₂. The gradient was started after a buffer wash (320ml) subsequent to sample application.

Fig. 3. Gel filtration of alkaline phosphatase on Sephadex G-200

Enzymic activity (●) and protein (E_{280} ; ○) were monitored during elution of the proteins with 100 mM-Tris/HCl (pH 7.6)/100 mM-NaCl/0.1 mM-MgCl₂ from a column of Sephadex G-200 equilibrated with the same buffer.

some of the more tightly bound glycoproteins. Other affinity-chromatography steps that were tried proved unsuccessful. Dean *et al.* (1971) used cellulose phosphate to retain phosphatases from bacterial sources, but we were unable to achieve adequate retention with the enzyme from human liver.

In some preparations, gel filtration on Sephadex G-200 was repeated before the final ion-exchange step. The second filtration step enabled somewhat higher yields to be taken in the final step without sacrificing the purity of the preparation.

The enzyme was treated in various ways to determine the most suitable storage form for alkaline phosphatase. Full activity was retained over several months with the enzyme at 4° or -20°C in 10 mM-Tris/HCl (pH 7.6)/100 mM-NaCl/0.1 mM-MgCl₂/0.02 mM-ZnCl₂/10% glycerol/0.02% NaN₃.

Purity and homogeneity. The purified enzyme was shown to be better than 95% homogeneous when subjected to electrophoresis on standard polyacrylamide gels or gels containing sodium dodecyl sulphate (Plate 1a). Standard polyacrylamide gels, stained with periodic acid/Schiff reagent for carbohydrate (Kapitany & Zebrowski, 1973) or for activity as described above, showed bands in the position identical with that of the protein-stained band. When fractions from the other purification steps were electrophoresed and stained for enzyme activity, only one band was found which had the same mobility as the band obtained with the purified enzyme. The absence of other bands suggests that there is only one molecular form present in normal human liver.

Alkaline phosphatase has been reported to be relatively resistant to denaturation with sodium dodecyl sulphate in the presence of mercaptoethanol (Mather & Keenan, 1974; Cathala *et al.*, 1975; Ohkubo *et al.*, 1974). Plate 1(b) demonstrates that the conversion of 'dimer' human liver alkaline phosphatase into the monomer by incubation at 37°C in 1% sodium dodecyl sulphate/1% 2-mercaptoethanol is relatively slow. After 24 h the conversion is still not complete. Although the dimeric form was enzymically active after 24 h in sodium dodecyl sulphate and mercaptoethanol, it is probable that only a part of the activity was expressed. Sufficient enzyme remained in the dimeric form after 24 h incubation to be stained for protein, but only a trace of activity, relative to a 2 h incubation in sodium dodecyl sulphate, could be demonstrated. The desialylated enzyme, however, was much more readily denatured under these conditions and conversion into the inactive monomer was judged to be complete within 2 h. This evidence suggests that the sialic acid residues protect the enzyme from denaturation by sodium dodecyl sulphate. Kawahara *et al.* (1973) have shown that some desialated glycoproteins have a relatively low content of water of hydration compared with their native counterparts, and this may partly explain the

Fig. 4. Elution profile of alkaline phosphatase from DEAE-Sephadex

Enzymic activity (●) and protein (E_{280} ; ○) were monitored throughout a linear gradient of NaCl from 0.1 to 0.2 M in 10 mM-Mes buffer, pH 6.0, that contained 0.1 mM-MgCl₂. The gradient was started after a buffer wash (40 ml) after sample application.

resistance of native alkaline phosphatase to denaturation by sodium dodecyl sulphate.

The specific activities determined on several preparations that gave a single protein band on standard polyacrylamide gels or sodium dodecyl sulphate-containing gels ranged from 650 to 850 units/mg.

This is the first report to our knowledge that demonstrates the purity of a preparation of human liver alkaline phosphatase.

Isoelectric focusing

Plate 2(a) shows purified human liver alkaline phosphatase focused in a 4% polyacrylamide gel containing 1% pH3-10/1% pH4-6 Ampholine. The purified preparation had only one enzymically active band with an isoelectric point of 4.0.

Studies on human serum alkaline phosphatases (R. A. Stinson, unpublished work) have shown that the predominant band found in 'normal' sera has an isoelectric point of 4.2 whereas sera demonstrating a 'fast' liver band (Rhone *et al.*, 1973) on electrophoresis have an additional band focusing at pH4.0.

Isoelectric focusing is a powerful tool for the separation of proteins (Vesterberg & Svensson, 1966). Smith *et al.* (1971), using 5.5% polyacrylamide gels and pH3-10 Ampholine, found that human liver alkaline phosphatase focused in the pH range 4-6. More recently workers have obtained more precise isoelectric points: pH4.3-4.6 for baboon liver (Hammond *et al.*, 1973), 3.9 for human liver (Usategui-Gomez *et al.*, 1974) and 3.8 for human liver (Greene & Sussman, 1973).

The differences between certain of the molecular forms of alkaline phosphatase may be due to the number of sialic acid residues on the enzyme molecule. This possibility arises on examination of Plate 2, gels (b)-(e), which shows that as neuraminidase cleaves the terminal sialic acid residues from human liver alkaline phosphatase, the isoelectric point is increased in a series of discrete steps from 4.0 to a constant pattern of two bands of approximately equal enzyme activity with isoelectric points of 6.8 and 6.6. These values are very close to those obtained with neuraminidase-treated bone alkaline phosphatase (R. A. Stinson, unpublished work). This suggests the possibility of a basic similarity in the structure of alkaline phosphatase from these two tissue sources.

Determination of subunit molecular weight in sodium dodecyl sulphate

The apparent subunit mol.wt. of human liver alkaline phosphatase was found to be 76700 (s.d. 2800, $n = 7$) by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis against standard proteins (Fig. 5). Seven determinations of the subunit mol.wt. were performed in polyacrylamide concentrations varying from 6.5 to 10.5%. Segrest & Jackson (1972) showed that glycoproteins that

Fig. 5. Subunit-molecular-weight analysis of alkaline phosphatase

The enzyme was electrophoresed in sodium dodecyl sulphate gels at polyacrylamide concentrations of 6.5% (●), and 10.5% (○). The arrows mark the relative mobility and subunit molecular weight of the enzyme.

contain more than 10% carbohydrate behave inconsistently during sodium dodecyl sulphate/polyacrylamide-gel electrophoresis owing to decreased sodium dodecyl sulphate binding to the glycoprotein compared with the standard proteins. Therefore an increase in the polyacrylamide concentration in the gels makes molecular sieving rather than charge the predominant factor that determines electrophoretic mobility, and hence the higher apparent molecular weights obtained in more concentrated gels approach

EXPLANATION OF PLATE I

Gel electrophoresis of purified human liver alkaline phosphatase (a) and subunit-molecular-weight analysis (b)

(a) Gel (A), polyacrylamide-gel electrophoresis of the pure enzyme in the presence of (0.1%) sodium dodecyl sulphate and subsequent protein staining; gel (B), polyacrylamide-gel electrophoresis of the pure enzyme and subsequent protein staining; gels (C) and (D), fluorescent and colorimetric localization of the enzyme activity after polyacrylamide-gel electrophoresis. (b) Gels (A), (B), and (C), protein-stained bands obtained when native enzyme was incubated with 1% (w/v) sodium dodecyl sulphate and 1% (w/v) 2-mercaptoethanol for 0.5, 2 and 24 h respectively followed by electrophoresis in 7.5% polyacrylamide gels that contained 0.1% sodium dodecyl sulphate. The slower band was enzymically active (see the text). Gel (D) shows the single band obtained when the desialylated enzyme was similarly treated for 2 h. The molecular-weight markers shown in gels (E)–(J) were phosphorylase α , human serum albumin, glutamate dehydrogenase, ovalbumin, glyceraldehyde 3-phosphate dehydrogenase and lactate dehydrogenase respectively. Experimental details are outlined under 'Methods'.

EXPLANATION OF PLATE 2

Isoelectric focusing of human liver alkaline phosphatase

(a) Human liver alkaline phosphatase was focused on 4% (w/v) polyacrylamide gels containing 1% pH4–6 Ampholine and 1% pH3–10 Ampholine; neuraminidase treatment of the enzyme after (b) 24 h, (c) 36 h, (d) 48 h and (e) 72 h was followed by focusing on 4% polyacrylamide gels containing 1% pH5–8 Ampholine and 1% pH7–9 Ampholine.

the true molecular weight asymptotically. This phenomenon was not seen with the glycoprotein alkaline phosphatase, whose molecular weight varied randomly over the polyacrylamide concentration range tested. Perhaps the disparity in behaviour of alkaline phosphatase from that shown by certain other glycoproteins is that either alkaline phosphatase has a low carbohydrate content or its nature is such that sodium dodecyl sulphate binding is not decreased. The value of 76700 obtained for the subunit mol.wt. of human liver alkaline phosphatase compares well with the value of 75000 obtained for rat liver alkaline phosphatase (Ohkubo *et al.*, 1974).

Desialylation of the enzyme did not produce a detectable change in the subunit molecular weight determined by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis (Plate 1b, gel D).

Molecular-weight determination on Sephadex G-200

Two values for the apparent molecular weight were determined for the purified enzyme by the method of Determann (1968). The apparent mol.wt. was 146000 (S.D. 4500, $n = 8$) when determined in 10mM-Tris/HCl, pH 7.6, and 220000 (S.D. 2900, $n = 8$) when 100mM-Tris/HCl/100mM-NaCl, pH 7.6, was the column buffer. Although it is possible that 146000 is low as a result of a non-specific adsorption of the glycoprotein to the Sephadex matrix, this value is compatible with the subunit mol.wt. of 76700 obtained by electrophoresis in polyacrylamide gels containing sodium dodecyl sulphate. Subunit analysis predicts a mol.wt. of 153400 for the dimeric enzyme. On the other hand the mol.wt. of 220000 agrees well with the values of 220000 reported by Smith, J. K., *et al.* (1968) and 225000 by Moss (1970), who also included 100mM-NaCl in the column buffer. These values could be erroneously high owing to association of phospholipid with the protein or to effects of salt on the conformation of the enzyme. Bound phospholipid would be displaced by treatment with sodium dodecyl sulphate and therefore the molecular-weight determination in the presence of sodium dodecyl sulphate will represent the true subunit molecular weight of the protein. Because alkaline phosphatase is a tightly bound membrane glycoprotein it is likely to possess regions with high hydrophobicity. In the presence of NaCl, the protein might well become considerably less compact as some of the hydrophobic interactions are decreased giving rise to a higher apparent molecular weight. The purified phosphatase was treated with neuraminidase to remove sialic acid from the enzyme. Filtration on Sephadex G-200 in 100mM-Tris/HCl/100mM-NaCl, pH 7.6, gave a mol.-wt. of 188000. The change in mol.wt. from 220000 to 188000 is probably due to more than the weight of the sialic acid residues alone. Kawahara *et al.* (1973) have shown that some highly charged glycoproteins contain considerably more water of hydration in the

native than in the desialated forms. Owing to its membranous location, it is probable that the carbohydrate moieties on the native enzyme are distributed asymmetrically. Thus increased hydration, and therefore increased asymmetry, of the native protein could account for the rather large difference in apparent molecular weights of the native and desialylated enzyme. Subunit-molecular-weight studies showed that the native and desialylated proteins had very nearly the same subunit molecular weight. It seems unlikely that the decrease in molecular weight obtained by gel filtration as a result of desialylation is due to a decrease in the number of bound phospholipid molecules. Removal of tightly bound phospholipid is likely to cause major structural changes and loss of enzyme activity, and since the desialylated enzyme retains full activity this is probably not the case.

Studies on rat liver alkaline phosphatase (Ohkubo *et al.*, 1974) showed that the enzyme was a dimer with a mol.wt. as determined by sedimentation-equilibrium analysis of 154000. A subunit size of 75000 was obtained by sodium dodecyl sulphate/polyacrylamide gel electrophoresis.

Since this manuscript was submitted, two other papers on the purification of human liver alkaline phosphatase have appeared. Lehmann (1975) prepared a partially purified enzyme for immunological studies and Sugiura *et al.* (1975) reported the preparation of a 'homogeneous' human liver alkaline phosphatase. However, the polyacrylamide-disc-gel electrophoresis and immunodiffusion results of the latter authors have not provided convincing proof of the purity of their preparation.

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