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# Alkaline Phosphatase from Pig Kidney

## METHOD OF PURIFICATION AND MOLECULAR PROPERTIES

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Alkaline phosphatase (EC 3.1.3.1) from pig kidney brush-border membranes was solubilized from membrane precipitates by butan-1-ol at a critical pH of 7.0. The 12000-fold purification procedure included $(NH_4)_2SO_4$ precipitation, DEAE- and TEAE-cellulose chromatography, Sephadex G-200 gel filtration and neuraminidase digestion followed by DEAE-cellulose chromatography. The purified protein contained 20% (w/w) carbohydrate and had mol.wt. 150000–156000 as estimated by Sephadex filtration and ultracentrifuge analysis. It was a tetrameric glycoprotein consisting of identical subunits, and it had a molecular activity at 25°C of $2600 s^{-1}$ per tetramer. Its concentration in kidney was estimated to be 8.5–8.8 mg/kg.

Microvillar membranes appear in cells at sites that have a high secretory and resorptive activity. It is well known from light- and electron-microscope investigations (Clark, 1961; Ito, 1969; Goldfischer *et al.*, 1964; Reale & Luciano, 1967; Scarpelli & Kanczak, 1965; Hugon & Borgers, 1969) and membrane separations (Porteous & Clark, 1965; Wilfong & Neville, 1970; Pockrandt-Hemstedt *et al.*, 1972) that these membranes contain a high alkaline phosphatase activity (orthophosphoric monoester phosphohydrolase, EC 3.1.3.1). The enzyme from pig kidney is readily available and suitable for large-scale preparations. Isolation of the pure enzyme is described in preparation for a study of its properties in the membrane.

Mammalian alkaline phosphatase has been isolated from various organs, especially from human placenta (Harkness, 1968; Sussman *et al.*, 1968; Ghosh & Fishman, 1968), calf intestine (Morton, 1954; Portman *et al.*, 1960; Engström, 1961), bovine liver (Engström, 1964) and pig kidney (Mathies, 1958; Binkley, 1961). However, the information about its structural properties is very limited (Gottlieb & Sussman, 1968; Sussman & Gottlieb, 1969), because the purity of the preparations was not ascertained. This information is desirable for comparisons between the membrane-bound enzymes and between these enzymes and the bacterial enzyme (Schlesinger *et al.*, 1969). In the present paper we describe an improved method for the preparation of alkaline phosphatase and the biochemical and physical properties of the enzyme from pig kidney, especially of its monomer unit.

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## Experimental

### *Materials*

All reagents were of analytical grade. Sephadex G-100 and G-200 were purchased from Pharmacia, Uppsala, Sweden. TEAE-cellulose was from Sigma Chemical Co., St. Louis, Mo., U.S.A.; DEAE-cellulose (DE-52) was from Whatman Biochemicals Ltd., Maidstone, Kent, U.K.; *p*-nitrophenyl phosphate (disodium salt) and Amido Black 10B were from Merck, Darmstadt, Germany; Fast Blue BB salt, sodium 1-naphthyl phosphate, sodium dodecyl sulphate, acrylamide, *NN*-methylenebisacrylamide, *NNN'N'*-tetramethylethylenediamine, trypsin (twice recrystallized; 160 $\mu$mol of *p*-tosyl-L-arginine methyl ester/min per mg of protein) and lipase from wheat (5–7 $\mu$mol of acetic acid from triacetin hydrolysed/min per mg of protein at 37°C) were from Serva, Heidelberg, Germany; neuraminidase (from *Clostridium perfringens*; 0.6 $\mu$mol of mucin hydrolysed/min per mg at 37°C) was from Boehringer, Mannheim, Germany; cellulose–Avicell for t.l.c. was from Schleicher und Schüll, Dassel, Germany.

### *Methods*

*Optical measurements.* These were carried out with an Eppendorf photometer (Netheler and Hinz, Hamburg, Germany) fitted with a thermostatically controlled cuvette holder. The enzyme was assayed as follows: 10–50 $\mu$l of enzyme solution was added to 0.9 ml of buffer (50 mM-Tris–HCl buffer, containing 10 mM-$MgCl_2$), incubated for 5 min and then 0.1 ml of substrate was added. The enzyme solution was diluted to give a change in absorbance at 405 nm of

0.1 within 10–60s after addition of substrate. Unless otherwise stated, the rates of hydrolysis in 1ml of 0.1mM-*p*-nitrophenyl phosphate at pH7.5 or at 1mM-*p*-nitrophenyl phosphate and pH9.5 were calculated from the absorption changes at 37°C. The molar extinction coefficients used were $E_{405}^{1cm}$ at pH7.5 = 11800 or $E_{405}^{1cm}$ at pH9.5 = 18500 for *p*-nitrophenol. The reaction rate was linear up to 10% of substrate consumption. Enzyme units are given as μmol of the *p*-nitrophenyl phosphate hydrolysed/min at 37°C at substrate saturation, i.e. $V_{max.}$ (Hiwada & Wachsmuth, 1974). Protein concentrations were determined by the biuret method (Gornall *et al.*, 1949) with bovine serum albumin as standard.

*Total carbohydrate concentrations.* These were determined by the orcinol-$H_2SO_4$ method (François *et al.*, 1962) by using enzyme solutions (20–100μg of protein in 0.5ml of water).

*Immunoelectrophoretic analysis.* This was carried out in 1.5% (w/v) agar gels in 0.06M-veronal–HCl buffer, pH8.6. After electrophoresis goat antisera raised against an impure alkaline phosphatase fraction with a specific activity of 50μmol/min per mg of protein were diffused for 48h against antigen solution. The slides were washed with phosphate-buffered saline(0.137M-NaCl, 2.68mM-KCl, 8.1mM-$Na_2HPO_4$, 1.45mM-$KH_2PO_4$, pH7.4) and water (each time for 24h), dried and stained for enzymic activity or protein. Enzyme activity was detected by incubation at 25°C for 10min in α-naphthyl phosphate (0.1mg/ml) and Fast Blue BB salt (0.5mg/ml). The slides were then dipped in 0.1M-sodium phosphate buffer, pH7.2, and washed in water before being dried. Protein was stained with 1% Amido Black or 0.25% Coomassie Brilliant Blue in acetic acid–methanol–water (1:5:4, by vol.) for 10min, the slides were destained in 7% (v/v) acetic acid and dried.

*Measurement of dry weight.* For this 1ml of enzyme preparation was dialysed against three changes of 1litre of distilled water for 48h. A sample (0.1ml) was then dried in a platinum boat for 24h *in vacuo* and then over $P_2O_5$ *in vacuo* at 70°C for another 24h. This dry material was weighed and analysed for H, C and N. In parallel, the original sample was analysed for biuret reaction at 546nm, for the absorption spectrum and for carbohydrate content. All measurements were made at least in duplicate.

*Amino acid analysis.* Protein samples (0.15mg) were analysed with a Technicon TSM 1 Amino Acid AutoAnalyzer by the method of Moore & Stein (1963) after hydrolysis for 24h and 72h in 0.5ml of 6M-HCl.

*Peptide 'mapping'.* Alkaline phosphatase (1.6mg) was boiled for 10min in 20% (w/v) trichloroacetic acid. After cooling, the precipitate was washed three times with ether and dried. The precipitate was dissolved in performic acid and kept for 4h at 4°C for oxidation, then diluted with water and freeze-dried. The powder was twice dissolved in water and freeze-dried and finally was dissolved in 100μl of 0.2M-triethylammonia, adjusted to pH8.0 by bubbling with $CO_2$. Protein hydrolysis was done by two subsequent incubations with 10μg of trypsin for 2h at 37°C and at pH8.0. The solution was then boiled for 3min, centrifuged and the supernatant freeze-dried. The dry material was twice dissolved in water and freeze-dried. This material served for two-dimensional chromatography on cellulose–Avicell plates at pH6.4 in either pyridine–acetic acid–water (25:1:225, by vol.) or at pH1.9 in acetic acid–formic acid–water (19:5.3:175.7, by vol.) in one dimension, followed by ascending chromatography in butan-1-ol–pyridine–acetic acid–water (19:12:4:15, by vol.) at right angles. The peptides were located with the chlorine reagent (Greig & Leaback, 1960).

*Polyacrylamide-gel electrophoresis.* This was carried out in 7.5% (w/v) polyacrylamide–0.2% *NN*-methylenebisacrylamide, in 0.05M-Tris–HCl buffer, pH8.3, for 12h at 2.5mA/gel, in tubes of 0.5cm diameter (Davies, 1964). The gels were stained for enzyme activity, protein or carbohydrate (with periodic acid–Schiff reagent). Homogeneity studies and molecular-weight analysis were done in 0.1% sodium dodecyl sulphate–0.1M-sodium phosphate buffer, pH7.2, by using 10% (w/v) polyacrylamide gels and electrophoresis for 8h at 8mA and 40V/tube. The protein solutions were applied to the gel in 10% (v/v) glycerol. Alternatively, the protein was denatured by heating for 15min in 1% sodium dodecyl sulphate at 96°C in 0.1M-sodium phosphate buffer, pH7.2, containing 0.1M-mercaptoethanol (Vinuela *et al.*, 1967). After electrophoresis and staining, the gels were analysed densitometrically. The standards were phosphorylase *a* (mol.wt. 95000), catalase (mol.wt. 60000), bovine serum albumin (mol.wt. 67000), aspartate transcarbamoylase (mol.wt. 33500 and 17000) and lysozyme (mol.wt. 14300).

*Molecular weights.* These were determined in the Beckman model E analytical ultracentrifuge at 19°C by using either schlieren or u.v.-absorption optics equipped with a monochromator and a photoelectric scanner at 280nm. Diffusion was measured in the ultracentrifuge at 8000rev./min in a synthetic-boundary cell. The partial specific volume was measured in a digital density precision instrument DMA 02/c (Anton Paar K.G., Graz, Austria). Equilibrium sedimentation was done in a Kel-F double-sector cell with fluorocarbon FC-43 as the bottom layer and the molecular weight was calculated from eqn. (10) of Chervenka (1969).

Sephadex G-200 filtration experiments were carried out by the method of Andrews (1965) on a column (2.5cm×87cm) in 0.01M-Tris–HCl buffer, pH8.0, at 15°C, with an upward flow rate of 25ml/h. Dextran Blue (mol.wt. 2000000), pig kidney

aminopeptidase (mol.wt. 280000; Wachsmuth *et al.*, 1966), purified human $\gamma_1$ immunoglobulin G (mol.wt. 160000) and bovine serum albumin (mol.wt. 68000) were used to calibrate the column.

*Small-scale preparation of pig kidney alkaline phosphatase.* The preparation of the enzyme in all steps was followed by measurement of specific activity ($\mu$mol of *p*-nitrophenyl phosphate hydrolysed/min per mg of protein). All procedures were carried out at 4°C, except where stated (Table 1).

Step 1. Fresh pig kidney (6.5kg) was minced and then homogenized in a mixer (MX32; Braun, Frankfurt, Germany) in 13litres of 0.05M-Tris–HCl buffer, pH8.0. The homogenate was stirred for 2h, then spun at 3000*g* for 1h.

Step 2. The supernatant was adjusted to pH5.0 with acetic acid, stirred for 3–4h, centrifuged at 8000*g* for 30min and the sediment suspended in 0.05M-Tris–HCl buffer, to a volume of 6.3 litres and at pH7.0 (i.e. one-third of the starting volume).

Step 3. To this suspension 3.1litres of butan-1-ol at 4°C was added slowly with vigorous stirring by a top stirrer. After 1h of stirring the temperature was raised to 37°C for 10min, then the emulsion was centrifuged at 8000*g* for 30min; the top layer of butan-1-ol was siphoned off and the dark-yellowish aqueous phase passed through glass wool for removal of flocculated material. The remaining butan-1-ol was removed in a rotary evaporator at 25°C and the solution was concentrated to 2.2litres. This solution was dialysed against 5litres of 0.01M-Tris–HCl buffer, pH8.0, for 24h with three changes of the buffer.

Step 4. Solid $(NH_4)_2SO_4$ was added to the dialysed solution to give 45% (w/v) saturation at 0°C. After being stirred for 2h the protein precipitate was removed by centrifuging at 12000*g* for 30min. The supernatant fluid was adjusted to 75% saturation with $(NH_4)_2SO_4$ at 0°C, stirred for at least 2h and centrifuged. The sediment was dissolved in 250ml of 0.01M-Tris–HCl buffer, pH8.2, and dialysed extensively against the same buffer.

Step 5. The dialysed solution was applied to a column (10cm×15cm) of DEAE-cellulose, equilibrated with 0.01M-Tris–HCl buffer, pH8.2. The column was developed with 1.5litres of 0.01M-Tris–HCl, pH8.2, 1.5litres of 0.04M-NaCl in this buffer and finally with 2litres of 0.1M-NaCl in this buffer. The three pooled fractions (each about 600ml) corresponding to the three protein peaks contained 4.2% ($D_1$), 73% ($D_2$) and 7% ($D_3$) of the applied activity respectively.

Step 6. After dialysis against 5mM-Tris–HCl buffer, pH8.0, the solution ($D_2$) was applied to a column (6.0cm×20cm) of TEAE-cellulose, equilibrated with the same buffer, then washed with 0.5litre of starting buffer. Elution was performed with a linear gradient with 500ml of buffer in the mixing flask and 500ml of 45mM-NaCl in buffer in the reservoir flask. The

Table 1. *Purification of alkaline phosphatase*

Purification after each fractionation step is expressed in terms of specific activity and the yield in terms of enzymic activity at pH7.5 and 9.5.

| Fractionation step | Total activity (μmol/min) | | Total amount of protein (mg) | Specific activity (μmol/min per mg of protein) | | Yield (%) | | Purification factor | |
|---|---|---|---|---|---|---|---|---|---|
| | pH7.5 | pH9.5 | | pH7.5 | pH9.5 | pH7.5 | pH9.5 | pH7.5 | pH9.5 |
| 1. Whole homogenate from 6.5kg of kidney | 7450 | 47900 | 1125000 | 0.0066 | 0.043 | | | | |
| 2. pH5.0 sediment resuspended in Tris–HCl buffer, pH8.2 | 4620 | 45900 | 409400 | 0.011 | 0.112 | 62.0 | 95.9 | 1.67 | 2.6 |
| 3. Butanol extraction at pH7.0 | 3860 | 57900 | 86000 | 0.045 | 0.673 | 51.8 | 121.0 | 6.82 | 15.6 |
| 4. $(NH_4)_2SO_4$ precipitation between 45 and 75% saturation | 3320 | 48100 | 21900 | 0.152 | 2.200 | 44.5 | 100.4 | 23.00 | 51.2 |
| 5. DEAE-cellulose column chromatography (0.04M-NaCl) | 2220 | 33600 | 3600 | 0.605 | 9.180 | 29.6 | 70.2 | 91.60 | 213.5 |
| 6. TEAE-cellulose column chromatography (7–12mM-NaCl) | 1350 | 21000 | 318 | 4.250 | 66.04 | 18.1 | 43.8 | 644.00 | 1536.0 |
| 7. Gel filtration on Sephadex G-200 | 1120 | 17600 | 18.3 | 61.200 | 960.00 | 15.0 | 36.5 | 9270.00 | 22300.0 |
| 8. DEAE-cellulose column chromatography after treatment with neuraminidase | 887 | 14000 | 10.6 | 83.70 | 320.00 | 11.9 | 29.2 | 12700.00 | 30700.0 |

Table 2. *Large-scale purification of alkaline phosphatase from pig kidney (45 kg)*

The purification is described in detail in the text. The initial steps of the large-scale preparation are designed to minimize the large volume of homogenates involved, yet retain a high enzymic activity.

| | Total volume (litres) | Total activity at 37°C, pH9.5 (mmol/min) | Total amount of protein (g) | Specific activity at 37°C, pH9.5 (μmol/min per mg) | Recovery of enzymic activity (%) |
|---|---|---|---|---|---|
| Whole homogenate | 135 | 335 | 6655 | 0.050 | — |
| Acetone precipitate | 85 | 278 | 3672 | 0.075 | 83 |
| Butanol-1-ol extraction | 20 | 262 | 188 | 1.390 | 78 |
| $(NH_4)_2SO_4$ precipitation (42.5–70% satn.) | 2.36 | 242 | 36.1 | 6.700 | 72 |

active enzyme was eluted at 7–12mM-NaCl. Recovery for this step was 74%. The pooled enzymically active fraction (100ml) was then concentrated by ultrafiltration in an Amicon apparatus with a Diaflo UM-10 membrane.

Step 7. Protein (26.5mg; 4ml) was applied to a column (2.5cm×87cm) of Sephadex G-200, equilibrated with 0.01M-Tris–HCl buffer (pH8.0)–0.1% $NaN_3$, and washed for 3 days at a flow rate of 25ml/h at 15°C. The enzymically active fraction (50ml) was concentrated in an Amicon apparatus, as in step 6, to about 5.0ml.

Step 8. Solution containing 17.5mg of enzyme was treated with 5.5mg of neuraminidase in 6.0ml of 0.01M-Tris–HCl buffer, pH7.5, containing 2mM-$CaCl_2$ and 30mM-NaCl, for 5h at 37°C, then filtered through a column (4.5cm×90cm) of Sephadex G-100 to remove neuraminidase and *N*-acetylneuraminic acid. The enzyme appeared in the exclusion peak as shown by the absorption at 280nm and enzymic activity. After concentration in an Amicon apparatus (see step 6) the enzyme solution was applied to a DEAE-cellulose column (2.5cm×15cm, equilibrated with 0.01M-Tris–HCl buffer, pH8.2), which was then washed with 300ml of starting buffer. The enzymic was eluted at a concentration of 6mM-NaCl with a linear gradient of NaCl (from 0 to 10mM) in 300ml of starting buffer. The enzyme fraction was concentrated in an Amicon apparatus (see step 6) to about 5mg/ml and dialysed against 0.01M-Tris–HCl buffer (pH8.0)–0.1% $NaN_3$ and stored at 4°C. Such a preparation did not change its activity for at least 8 months.

*Large-scale purification.* To prepare a sufficient amount of enzyme the first four steps in purification had to be altered so that commercially available equipment holding up to 100kg could be used (Table 2). Kidney (45kg) was minced and stored at −40°C. It was thawed at room temperature, homogenized in 90 litres of deionized water at 4°C at pH6.8, in a Dispax–Reaktor type 3-6/6 (Janke and Kunkel KG, Staufen, Germany) with a flow rate of 500litres/h, stirred for 3h at about 15°C, centrifuged (connected in series) in a Decantor type Z 1 Lg (Flottweg, Vilsbiburg, Germany) and in a Self-Cleaning Separator type SAMR-3036 (Westfalia Separator A.G., Oelde/Westf., Germany). The sediment was discarded. To the 130 litres of supernatant, which had been cooled by stirring in baths of tap water and then ice-cold water, 65 litres of acetone at 3°C was added slowly, with stirring. The suspension was left overnight and then centrifuged in the Self-Cleaning Separator type SAMR-3036 with a flow speed of 180litres/h at 81kPa and with removal of sediment every 3min. The supernatant was discarded. The sediment (40litres) was carefully suspended in 45litres of 0.05M-Tris–HCl buffer, pH7.5, at 3°C, then 45litres of butan-1-ol cooled to 3°C were added to it and the suspension passed through the Dispax–Reaktor type 3-6/6. The homogeneous suspension (85litres) was heated to 37°C for 15min, then cooled to tap-water temperature and the two phases were separated in a Self-Cleaning Separator type SAOH 3036 (Westfalia Separator AG) with a flow rate of 160litres/h. The lower aqueous phase (80litres) was left overnight. The supernatant was decanted and the sediment was passed three times through the Self-Cleaning Separator type SAMR 3036. The combined supernatant fluids were then concentrated in a circular vacuum evaporator from 65 litres to 20 litres at 35–40°C and adjusted to 45% saturation with $(NH_4)_2SO_4$. The suspension was centrifuged and the supernatant was adjusted at 4°C to 80% saturation by addition of 250g of $(NH_4)_2SO_4$/litre. After centrifugation the sediment was dissolved in 0.01M-Tris–HCl buffer, pH8.2, and dialysed extensively against the same buffer. Further purification was by the scheme for small-scale preparation starting at step 5.

## Results

### *Enzyme preparation*

By comparison of the percentage recovery of enzymic activity and of specific activity over the pH range 6–10, pH7 was found to be the optimum pH for the extraction of alkaline phosphatase with

butan-1-ol (84% recovery of initial enzymic activity). Extensive trypsin, lipase and Triton X-100 treatment also had an extraction optimum at pH7, but generally gave much lower yields of enzyme. Neuraminidase did not solubilize the alkaline phosphatase.

Table 1 shows the purification of the enzyme assayed at two pH values. The pH9.5/pH7.5 activity ratio for the membrane-bound enzyme was 9.93. After solubilization it changed to, and remained at, 15. For large-scale preparations an acetone precipitation had to be used (Table 2). This had the advantage of producing a precipitate that sedimented faster than the pH5 sediment, but it involved larger volumes. The specific activity was lower than in the pH5 sediment. However, after butanol treatment the specific activity was higher, but the recovery was lower than with the small-scale preparation method (Table 2).

*Purity and homogeneity*

Chromatography on DEAE-cellulose and Sephadex G-200 gave only one protein peak, which coincided with the enzymic activity. In 7.5% (w/v) polyacrylamide gel at pH8.3 only one band was revealed with Coomassie Blue. It was in the same position as the Schiff–periodic acid-positive band and the band detected by incubation with the specific substrate of the enzyme. In the immunoelectrophoretic analysis, however, at antigen concentrations of 10mg/ml two arcs were stained with Coomassie Blue, but only the strong one stained for enzymic activity. The weaker arc was not detectable at an antigen concentration lower than 5mg/ml. Sodium dodecyl sulphate–polyacrylamide-gel electrophoresis with 30–100$\mu$g of protein gave two bands that were always detectable with Coomassie Blue. The major band, 90% of the protein, had an apparent mol.wt. of 79000 and also stained with Schiff's reagent. The minor band had an apparent mol.wt. of 45000, but did not stain with Schiff's reagent. Another less purified enzyme preparation, which had a specific activity of 1200$\mu$mol/min per mg of protein instead of 1320$\mu$mol/min per mg, contained 20% of the same minor band and on immunoelectrophoretic analysis gave an enzymically inactive precipitation arc as above, which was stronger than in the purer preparation. Therefore the most purified preparation of alkaline phosphatase still contained 10% of another protein, as an impurity which had very similar charge and size as the enzyme before denaturation, but which was not a glycoprotein. The most purified enzyme preparation had a specific activity of 1320$\mu$mol/min per mg of protein at 37°C and pH9.5, with *p*-nitrophenyl phosphate as substrate. The specific activity at 25°C and pH9.5 was 1000$\mu$mol/min per mg of protein, which corresponds to a molecular activity of 2560$s^{-1}$.

*Biochemical analysis*

For the following measurements the protein concentration was estimated by dry weight. At a protein concentration of 1mg/ml the solution gave a typical protein spectrum: $E_{1cm}^{0.1\%}$ at 280nm = 0.74; $E_{1cm}^{0.1\%}$ at 260nm = 0.409; $E_{1cm}^{0.1\%}$ at 250nm = 0.295. With the biuret reagent in 2ml we determined an absorption $E_{1cm}^{1mg}$ at 546nm of 0.12. From this value a conversion factor of 8.3 can be calculated, whereas this conversion factor is 6.8 for human serum albumin. This difference can be attributed to the amount of carbohydrate in the enzyme preparation.

The carbohydrate content in the purified enzyme preparation before treatment with neuraminidase was 0.31mg of carbohydrate/mg of dry protein, and after treatment was 0.204–0.206mg. The dried protein samples of 1.413–1.425mg contained 6.7% H and 49.6% C as estimated by elementary analysis and 14.3% N as estimated by the Kjeldahl method. The conversion factor on the basis of N content was therefore 7.

The amino acid analysis is given in Table 3. No tryptophan and cysteine could be detected after 24h or 72h of hydrolysis. The values for threonine and serine were extrapolated to zero time from the 24h and 72h values.

Tryptic peptide 'maps' (see the Experimental section) showed that no ninhydrin- or chlorine-reagent-positive substance remained at the origin after electrophoresis or ascending chromatography. About 33 peptides were detectable at pH6.4 and 31 at pH1.9, which was about one-quarter of that expected from the lysine plus arginine content (Table 3) had the protein been a single polypeptide chain.

Table 3. *Amino acid composition of alkaline phosphatase*

Results are expressed as mol of amino acid in 156000g of enzyme, ±S.D. of five determinations, after acid hydrolysis.

| | |
|---|---|
| Glycine | 101.5±3.1 |
| Alanine | 115.3±5.8 |
| Serine | 61.6±2.6 |
| Threonine | 74.7±5.7 |
| Proline | 56.6±3.3 |
| Valine | 87.4±4.7 |
| Isoleucine | 53.3±2.1 |
| Leucine | 83.7±0.6 |
| Phenylalanine | 31.2±1.2 |
| Tyrosine | 45.8±3.3 |
| Methionine | 34.8±2.8 |
| Aspartic acid | 111.8±5.0 |
| Glutamic acid | 109.2±2.6 |
| Arginine | 64.9±1.7 |
| Histidine | 48.4±5.4 |
| Lysine | 63.1±1.9 |

Table 4. *Temperature stability of alkaline phosphatase*

Enzyme (4 $\mu$g/ml) was incubated in 50mM-Tris–HCl buffer, pH8.0, at given temperatures. Samples were removed after different times, preincubated in a cuvette in 50mM-Tris–HCl (pH9.5)–5mM-$MgCl_2$ for 5min at 37°C and then measured for enzymic activity by addition of *p*-nitrophenyl phosphate to 1mM. There was no difference between the values obtained by inactivation without or with 5mM-$MgCl_2$.

| Temperature (°C) | First-order rate constant ($s^{-1}$) |
|---|---|
| 47 | $\infty$ |
| 51 | $2.3\times10^{-4}$ |
| 55 | $4.0\times10^{-4}$ |
| 57 | $7.6\times10^{-4}$ |
| 60 | $30.0\times10^{-4}$ |

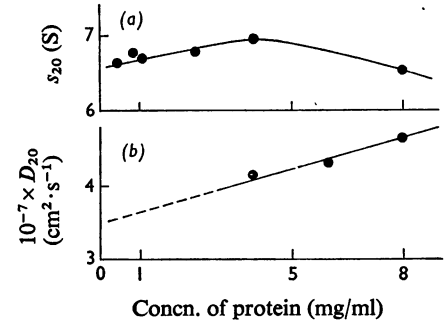


Fig. 1. *Concentration dependence of the sedimentation coefficients and the diffusion coefficient (D) for alkaline phosphatase*

Experiments were carried out in 0.1M-Tris–HCl–10mM-$MgCl_2$, pH7.5. For details see the Experimental section.

*Stability of the enzyme*

In the absence of $MgCl_2$ the enzymic activity decreased on storage of the enzyme in 10mM-Tris–HCl buffer, pH7.5, at protein concentrations below 50 $\mu$g/ml at 20°C. It was completely restored within 2min at 20°C on preincubation with 5mM-$MgCl_2$. This inactivation did not occur with 50 $\mu$M-$MgCl_2$, even at very low enzyme concentrations such as 0.17 $\mu$g/ml.

At 47°C the enzyme was completely stable with and without $MgCl_2$. It lost 50% of its activity after 12min at 57°C when tested for hydrolysis of *p*-nitrophenyl phosphate. $Mg^{2+}$ had no stabilizing effect at these temperatures (Table 4). $Mg^{2+}$ (5mM) had no effect on the pH stability of the enzyme. Between pH6.0 and 11.3 at 20°C the enzyme was stable for at least 6h. It lost 50% of the activity within 10min at pH5.0 and within 20min at pH11.8. On dialysis in 50mM-Tris–HCl (pH8.0)–5mM-$MgCl_2$ for 24h after neutralization no reactivation of the enzyme was detectable.

*Molecular-weight estimation*

An apparent mol.wt. of 155000 was measured for alkaline phosphatase by the method by Andrews (1965) on Sephadex G-200 columns.

The molecular weight was also estimated by sedimentation-velocity and sedimentation-equilibrium techniques. In 0.1M-Tris–HCl–10mM-$MgCl_2$, pH7.5, the partial specific volume was found to be 0.731ml$\cdot g^{-1}$ by using concentrations from 0.937mg of protein/ml to zero. From sedimentation-velocity experiments the following values were obtained at 4mg of protein/ml of the above buffer system: $D_{20,w} = 4.18\times10^{-7}cm^2\cdot s^{-1}$ and $s_{20,w} = 6.96S$. With these values the molecular weight of alkaline phosphatase was calculated to be 150000. By measuring *s* and *D* coefficients at different concentrations (Fig. 1) and extrapolating to zero concentration a maximal mol.wt. of 156000 was calculated. The results from low-speed sedimentation equilibrium confirmed this molecular weight. Sedimentation equilibrium at 6400rev./min at a protein concentration of 0.6mg of protein/ml showed two components that had apparent mol.wts. of 140000 and 39000 respectively (Fig. 2*a*). This latter finding and the decrease of the sedimentation coefficient at low protein concentration (Fig. 1) indicated a dissociation of the protein into subunits.

*Dissociation into subunits*

Since sodium dodecyl sulphate–polyacrylamide-gel electrophoresis and the peptide 'maps' indicated that there were proteins in the enzyme preparations with apparent mol. wts. less than 80000, it seemed clear that alkaline phosphatase is an oligomer. To determine the size of the subunits, analytical ultracentrifugation was carried out under conditions enhancing dissociation, i.e. in guanidine or at acid pH values.

Enzyme (500 $\mu$l) was dialysed for 48h against 500ml of 6M-guanidine hydrochloride–0.1M-mercaptoethanol–0.1M-Tris–HCl, pH8.0. The sedimentation velocity and the diffusion were measured with schlieren optics at a protein concentration of 2mg/ml and were $s_{20,guanidine} = 0.87S$, which is $s_{20,w} = 2.23S$, and $D_{20,guanidine} = 2.29\times10^{-7}cm^2\cdot s^{-1}$, which is $D_{20,w} = 3.5\times10^{-7}cm^2\cdot s^{-1}$. With those data and the partial specific volume of 0.731ml$\cdot g^{-1}$, as determined in Tris–HCl buffer, and the solution density of 1.141g$\cdot l^{-1}$ (Kawahara & Tanford, 1966), the molecular weight was calculated to be 54000. Sedimentation-equilibrium studies at 18000rev./min, as measured with schlieren optics, also indicated a decrease of the molecular weight in guanidine hydrochloride. When the results were plotted two slopes were seen, from

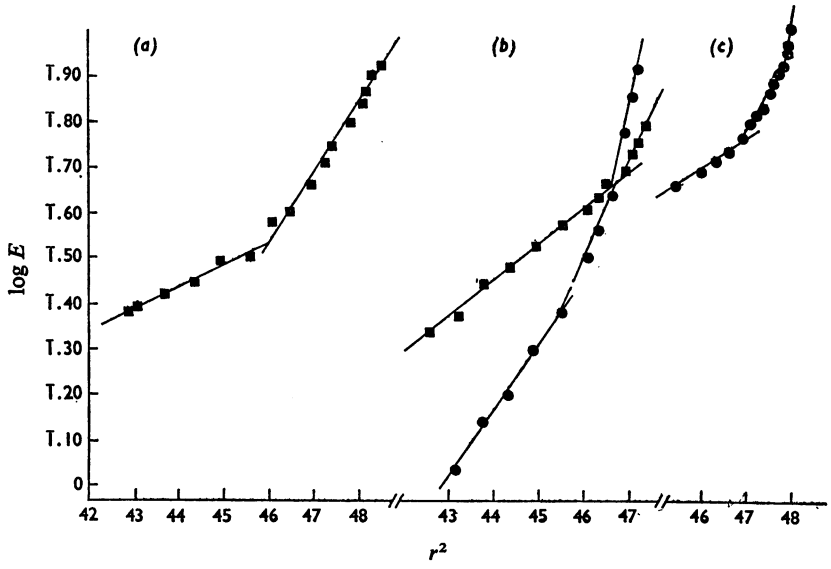


Fig. 2. *Equilibrium centrifugation of alkaline phosphatase at low* [6400*rev./min* (■)] *and high* [12000*rev./min* (●)] *speed*

Protein concentration was 0.6mg/ml. (*a*) In 0.1M-Tris–HCl–10mM-$MgCl_2$–0.1% $NaN_3$, pH7.5; (*b*) at pH3.5, conditions as in Table 5(*d*); (*c*) at pH3.5 in 24% (w/v) sucrose, conditions as in Table 5(f).

Table 5. *Apparent molecular weight determined by analytical ultracentrifugation*

The protein concentration of the samples was 0.6mg/ml. The samples contained 20% of the non-glycosylated protein contaminant as estimated by sodium dodecyl sulphate–polyacrylamide-gel electrophoresis. In experiment (a) identical values were obtained by using enzyme preparations contaminated with either 20% or 10% of the non-glycolysated impurity. In experiment (f) the enzyme preparation was contaminated with 10% of the non-glycolysated impurity.

| | $s_{20,w}$ | | $10^{-3}\times$ Molecular weight (sedimentation equilibrium) | |
|---|---|---|---|---|
| Buffer | S | % of applied protein sample | 6400rev./min | 12000rev./min |
| (a) 0.1M-Tris–HCl–0.2M-NaCl–0.1mM-EDTA, pH7.5 | 6.38 | | 157 | 116 |
| | | | 75 | 85 |
| | | | | 43 |
| (b) 0.1M-Sodium acetate–0.2M-NaCl–0.1% $NaN_3$, pH3.5 | 3.4 | 50 | 350 | 127 |
| | 5.2 | 50 | 153 | 117 |
| | | | 117 | 37 |
| | | | 41 | |
| (c) 0.1M-Sodium acetate–0.2M-NaCl–0.1% $NaN_3$–0.1mM-EDTA, pH3.5 | 3.42 | | 76 | 87 |
| | | | 46 | 35 |
| (d) 0.1M-Sodium acetate–0.2M-NaCl–0.1mM-EDTA–0.4mM-dithiothreitol, pH3.5 | 3.8 | Mean value | 170 | 137 |
| | | | 74 | 66 |
| (e) 0.1M-Sodium acetate–0.2M-NaCl–0.1mM-EDTA– | 7.1 | 25 | | 39 |
| 0.4mM-dithiothreitol–24% (w/v) sucrose, pH3.5 | 4.7 | 25 | — | — |
| | 3.2 | 25 | | |
| | 2.2 | 25 | | |
| (f) 0.1M-Sodium acetate–0.2M-NaCl–0.1mM-EDTA– | 7.1 | ~25 | | 167 |
| 0.4mM-dithiothreitol–24% (w/v) sucrose, pH3.5 | 4.7 | ~25 | — | 70 |
| | 3.2 | >23 | | 38 |
| | 2.2 | >23 | | |

which mol.wts. of 53000 and 35000 respectively were calculated from eqn. (14) of Chervenka (1969). Thus the guanidine seemed to increase dissociation of alkaline phosphatase into monomers.

Ultracentrifugation studies at low pH confirmed this assumption. A decrease in pH decreased the sedimentation coefficient (Table 5a and 5b), and this was more pronounced in the presence of EDTA. However, in no case was a sharp boundary seen, indicating a system of inhomogeneity in respect of molecular sizes. This could be very well demonstrated when sedimentation-equilibrium measurements were made for molecular-weight determination (Table 5a–5d, Figs. 2*a* and 2*b*). At least two protein species of different molecular weight were found in all experiments; the minimum mol.wt. was 35000–43000. The pattern of all those analyses did not detectably change when samples were analysed within 24h as usual, or 72–96h after pH adjustment by dialysis, or directly by dilution into the low pH buffer, thus excluding breakdown of the alkaline phosphatase by proteolytic digestion at low pH.

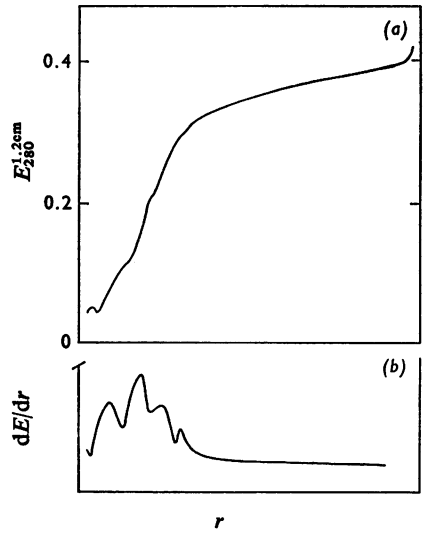


Fig. 3. *Sedimentation-velocity pattern of alkaline phosphatase in sucrose*

Enzyme solution [800$\mu$l in 20% (w/v) sucrose] was dialysed for 48h against 500ml of 0.1M-sodium acetate buffer (pH3.5)–0.771M-sucrose (24%)–0.2M-NaCl–0.1mM-EDTA–0.4mM-dithiothreitol, centrifuged and the absorption measured with this buffer as reference. Centrifugation was carried out in a 12mm double-sector cell (Kel-F) at 56000rev./min. The absorption ($E^{1,2cm}_{280}$) was 0.38 at 0min after maximal speed was reached. Pictures were taken at 15min intervals. (*a*) is a tracing of the absorption from the bottom of the cell (right) to the meniscus (left) and (*b*) is the derivative of this absorption tracing 224min after maximal speed had been attained.

The findings could be due either to an association–dissociation phenomenon of alkaline phosphatase at low pH or to protein contaminants such as those seen in sodium dodecyl sulphate–polyacrylamide-gel electrophoresis. It was therefore essential to estimate the amount of the different molecular-weight species during ultracentrifugation, for which the sedimentation-equilibrium technique is not suitable. By using the sedimentation-velocity technique in 24% (w/v) sucrose and optical scanning at 280nm we were able to show four different species and to estimate their concentrations from the $E_{280}$ (Fig. 3). The boundary of the extinction scans during the ultracentrifuge run showed steps and the derivative of this boundary had four peaks. The smallest component was 2.2S, corresponding to a mol.wt. of 38000 as measured by sedimentation equilibrium. To test whether this low-molecular-weight component was the contaminant in the enzyme preparation, two samples were analysed, one of which still had 20% of the non-glycosylated contaminant, the other (more purified) 10% of this impurity. In both cases the smallest-molecular-weight species was more than 23% of the total protein applied (Table 5e and 5f). This was much more than the percentage of the contaminants. Further, the sedimentation-velocity experiment in sucrose gave four peaks (Fig. 3).

Alkaline phosphatase is therefore a tetrameric enzyme. Its smallest subunit has mol.wt. 39000.

## Discussion

Our data on alkaline phosphatase from brush-border membranes of pig kidney demonstrate that the enzyme is a tetrameric glycoprotein of mol.wt. 150000–156000, which is tightly bound in the membrane. It could be released from the membrane by butan-1-ol extraction as described by Morton (1954) and a pH of 7 was optimal for this. Of the two well known marker enzymes of intestinal and kidney brush-border membranes, aminopeptidase (Eichholz, 1968; Kinne & Kinne-Saffran, 1969) and alkaline phosphatase (Ito, 1969; Wilfong & Neville, 1970; Eichholz, 1968; Kinne & Kinne-Saffran, 1969; Taylor *et al.*, 1971), 80% of aminopeptidase was released by trypsin and toluene treatment (Wachsmuth *et al.*, 1966) in contrast with, at most, 20% of alkaline phosphatase. Similar results have been obtained with isolated rat kidney brush-border membranes after papain digestion (Thomas & Kinne, 1972), and hamster (Eichholz, 1968) and rabbit (Oda *et al.*, 1969) intestinal brush-border membranes.

The molecular-weight analyses of the active enzyme by gel filtration, sedimentation velocity and the sedimentation-equilibrium technique gave similar results and agreed with the value of 150000–170000

(for the molecular weight) determined by Butterworth (1968) for human kidney alkaline phosphatase by gel filtration. Other workers have obtained lower molecular weights by using ultracentrifugation techniques, but they neglected the concentration- and pH-dependent dissociation of the enzyme shown here to give a minimum mol.wt. of 35000–43000. Ghosh & Fishman (1968) and Ghosh (1969) described a minimal mol.wt. of 70000 for human placenta alkaline phosphatase.

There appears to be an equilibrium distribution of oligomeric forms of the enzyme from monomer to tetramer that may not be shifted more than 25% in favour of the monomer. As the same distribution of the molecular-weight species was obtained with enzyme preparations of 10–20% impurity, we are confident that the real monomer has mol.wt. 35000–43000. Confirmation of tetrameric composition in this enzyme could not be obtained by using sodium dodecyl sulphate–polyacrylamide-gel electrophoresis, since the enzyme is a glycoprotein with 20% carbohydrate. Sodium dodecyl sulphate binding by the protein is presumably decreased in glycoproteins giving anomalously high subunit molecular weights, in this case 79000. If this represented the true molecular weight, complete tryptic hydrolysis would be expected to produce 64 peptides, yet only half this number can be detected. These findings are contributory evidence that alkaline phosphatase from pig kidney is a tetramer composed of four glycopeptide chains.

We have estimated a molecular activity of $2600\,s^{-1}$ at 25°C for the pig kidney alkaline phosphatase, in good agreement with the value of $2700\,s^{-1}$ determined for the enzyme from cow's milk (Barman & Gutfreund, 1966). Their estimate was based on a mol.wt. of 190000. Harkness (1968) quoted a specific activity of 750 $\mu$mol/min per mg for the human placenta enzyme and Ghosh & Fishman (1968) gave a value of 1171 $\mu$mol/min per mg of protein. Although the purification procedure established here results in a preparation of specific activity 1320 $\mu$mol/min per mg of protein, this may not represent a maximal activity, since the preparation contained 10% of a carbohydrate-free protein. This contaminant seemed to have no effect on measurements taken.

Pig kidney contains 8.5–8.8mg of alkaline phosphatase/kg of tissue on the basis of the total activity of pH5 sediments. This quantity (57nmol of enzyme/kg of tissue) is considerably greater than that calculated for human liver (9nmol/kg) and human intestine (6nmol/kg) (Moss *et al.*, 1968).

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