

Purification and Properties of Molecular-Weight Variants of Human Placental Alkaline Phosphatase

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1. Alkaline phosphatase of human placenta was purified by a procedure involving homogenization with tris buffer, pH 8.6, extraction with butanol, ammonium sulphate fractionation, exposure to heat, ethanol fractionation, gel filtration, triethylaminoethylcellulose anion-exchange chromatography, continuous curtain electrophoresis on paper and equilibrium dialysis. Methods for both laboratory-scale and large-scale preparation were devised. 2. Two major molecular-weight variants designated A and B were separated by molecular sieving with Sephadex G-200 and variant A was purified 4000-fold. 3. Variant B, which comes off the Sephadex G-200 column before variant A, is the electrophoretically slower-moving species on starch gel and is quite heterogeneous. 4. Purified variant A was fairly homogeneous on the basis of electrophoretic studies on starch gel and Sephadex gel, ultracentrifugation and immunodiffusion. 5. The respective molecular weights for variants A and B were 70 000 and over 200 000 on the basis of sucrose-density-gradient ultracentrifugation. Variant A exhibited a sedimentation coefficient of 4.2s. 6. Crystalline variant B could be converted into fast-moving variant A and vice versa. 7. Kinetic studies indicated no difference between the two variants. These include linear rates of hydrolysis, pH optimum, Michaelis constants and uncompetitive stereospecific L-phenylalanine inhibition. 8. The amino acid compositions of variants A and B and of placental albumin were determined.

One reason to undertake the purification of human placental alkaline phosphatase was the need to investigate with a homogeneous enzyme preparation the nature of the inhibition of placental alkaline phosphatase by L-phenylalanine (Fishman & Green, 1967), an amino acid that had been recognized earlier as a stereospecific organ-specific inhibitor of the intestinal alkaline phosphatase of man (Fishman, Green & Inglis, 1963) and of rat (Fishman, Green & Inglis, 1962; Ghosh & Fishman, 1966).

Purification of human placental alkaline phosphatase (Anagnostopoulos & Matsudaira, 1957; Ahmed & King, 1960) occurred before the existence of polymorphic forms of enzymes was widely known. Consequently, the products of purification were not homogeneous preparations.

The present investigation was therefore aimed at obtaining pure human placental alkaline phosphatase. Two species of enzyme were obtained that are indistinguishable enzymically, are both inhibited uncompetitively by L-phenylalanine, but differ in their molecular weights. A preliminary report of this work has been presented (Ghosh & Fishman, 1967).

MATERIALS AND METHODS

Materials. The chemicals used in this study and their sources are: tris (Trizma Base, reagent grade), Sephadex G-200, TEAE-cellulose* anion-exchanger (capacity 0.89m-equiv./g.) and disodium phenyl phosphate from Sigma Chemical Co. (St Louis, Mo., U.S.A.); butanol (reagent grade), MgCl₂·6H₂O and KCl from Fisher Scientific Co. (Pittsburgh, Pa., U.S.A.); (NH₄)₂SO₄ (enzyme grade) from Mann Research Laboratories (New York, N.Y., U.S.A.); sodium α -naphthyl acid phosphate from the Borden Chemical Co. (Philadelphia, Pa., U.S.A.); magnesium acetate from J. T. Baker Chemical Co. (Phillipsburg, N.J., U.S.A.); hydrolysed starch for gel electrophoresis from Connaught Medical Research Laboratories (Toronto, Ont., Canada); Fast Blue RR Salt from Edward Gurr Ltd. (London, S.W. 14).

D- and L-Phenylalanine were purchased from Calbiochem (Los Angeles, Calif., U.S.A.) and dissolved in hot water to prepare a 0.05M stock solution. Crystalline catalase and bovine serum albumin were obtained from Worthington Chemical Corp. (Freehold, N.J., U.S.A.) and Sigma Chemical Co. respectively.

* Abbreviation: TEAE-cellulose, triethylaminoethyl-cellulose.

The tris buffer referred to below was 0.05 M-tris-HCl solution, pH 8.6, in all experiments.

Assay for alkaline phosphatase. The assay mixture (2 ml.) contained 72 mM-disodium phenyl phosphate in 50 mM- Na_2CO_3 - NaHCO_3 buffer, pH 10.7, and 10 mM- MgCl_2 . Enzyme solution (0.1 ml.) was added to the preheated (5 min.) digest to initiate the reaction. The amount of phenol liberated in 15 min. at 37° was measured by the method of Stolbach, Nisselbaum & Fishman (1958) as applied by us in earlier studies on intestinal alkaline phosphatase (Ghosh & Fishman, 1966; Fishman & Ghosh, 1967a).

The unit of the enzyme is defined as that amount which liberates 1 μ mole of phenol/ml./min. from 72 mM-disodium phenyl phosphate containing 10 mM- MgCl_2 in 50 mM- Na_2CO_3 - NaHCO_3 buffer, pH 10.7, at 37°.

Measurement of protein. This was done by the micro-Kjeldahl method, by the method of Lowry, Rosebrough, Farr & Randall (1951) or by measuring the extinction at 280 μ and at 260 μ in a Beckman model DU spectrophotometer.

Starch-gel electrophoresis. This was carried out by the method of Smithies (1959) as modified in this Laboratory (Fishman, Inglis & Ghosh, 1968; Fishman & Ghosh, 1967a) for 16 hr. at 6 v/cm. at 4–6°.

Sucrose-density-gradient centrifugation. The sucrose-density-gradient centrifugation of the different purified specimens was carried out by applying 1 ml. of the diluted enzyme specimens to a 31 ml. linear sucrose gradient (5–20%, w/v) in tris-HCl buffer containing 1 mM- MgCl_2 . The reference standards for molecular weight were ^{14}C -labelled RNA at pH 7.0, and bovine serum albumin and crystalline catalase at pH 7.0 and pH 8.3, and these were applied in a volume of 1 ml. to a companion sucrose gradient. After centrifugation of the gradients in a Beckman Spinco model L ultracentrifuge at 4° for 17 hr. at 25000 rev./min. in the no. SW 25.1 rotor, 1 ml. fractions were collected in graduated centrifuge tubes for assay.

The radioactivity of ^{14}C -labelled RNA was measured in a Nuclear-Chicago mark I liquid-scintillation counter by using the channels-ratio counting technique.

Ultracentrifugal analysis of purified placental alkaline phosphatase (variant A). The sedimentation velocity of the purified isoenzyme (variant A) of placental alkaline phosphatase was measured in a Beckman Spinco model E analytical ultracentrifuge with a double-sector quartz cell at 6–8° and a maximum speed of 59780 rev./min. The protein concentrations were 3.8, 3.9 and 1.5 mg./ml. The enzyme was dissolved in and dialysed against 0.1 M-tris-HCl buffer, pH 8.6. The photographs of the protein boundary of purified variant A were taken in the operating ultracentrifuge at 16 min. intervals with a 70° diaphragm angle for the first photograph and 60° for the remainder, the exposure time being 2 sec. in each case.

On one occasion the enzyme specimen was dissolved in 0.1 M-veronal-HCl buffer, pH 8.6, containing 0.5 M-NaCl and dialysed against this buffer and then centrifuged as described.

The logarithm of the distance of the boundary from the axis of rotation was plotted against time. The slope of the straight line obtained was used in calculating the sedimentation coefficient, *S*, of the placental isoenzyme, the coefficient being determined by applying the relation of Schachman (1957).

Immunochemical study. Ouchterlony double-diffusion tests of a number of preparations of human placental alkaline phosphatase obtained at different stages of purification were done in 1% agarose gel in 0.05 M-veronal-HCl buffer, pH 8.6, for 40 hr. at room temperature. The preparation of the specific antibody against placental alkaline phosphatase was made by a modified method of Reif & Norris (1960).

Amino acid composition. The purified enzyme protein was treated with 6 N-HCl, transferred to a Pyrex flask and the air was replaced by N_2 . After being sealed under vacuum, the tube was heated at 114° for 20 hr. Then the hydrolysate was evaporated to dryness *in vacuo* in a Ruchii Rotovapor to remove HCl and was analysed in the JLC-3BC amino acid analyser.

RESULTS

After a satisfactory procedure had been developed in the laboratory, it became evident that a large-scale process was needed to prepare the enzyme in the desired quantity. Such a large-scale method was successfully devised. Both procedures are described as they differ in certain important respects.

Laboratory-scale method of purification of placental alkaline phosphatase

The human placentas were collected at St Margaret's Hospital (Dorchester, Mass., U.S.A.) just after delivery and placed in the deep-freeze. All adherent blood clots were removed with a jet of cold water. The vascular mesoderm covering the foetal surface of the placenta and the umbilical cord were dissected away, the thoroughly washed placentas, each weighing 200–500 g., becoming the starting material.

Step 1: preparation of the homogenate. Washed placenta was homogenized (500 g.) in 4.5 l. of cold tris buffer, pH 8.6, in the Osterizer (John Oster Manufacturing Co., Milwaukee, Wis., U.S.A.) at top speed for 4 min. in batches of 50 g. of placenta and 450 ml. of tris buffer.

Step 2: butanol treatment. To the pooled homogenate (5000 ml.) from step 1, 1975 ml. of cold butanol (5°) was added slowly while the mixture was being magnetically stirred at 5°. A total of 1 hr. stirring produced a stable emulsion, the temperature of which was then raised to and maintained at 37° for 10 min. The thoroughly emulsified homogenate was poured into transparent 250 ml. polycarbonate cups and then centrifuged in a Servall centrifuge (model RC-2) with rotor no. GSA at 9500 rev./min. (14600 g) for 30 min. at 4°. The top butanol layer was carefully siphoned off. The yellowish aqueous phase was then passed through glass wool to remove any suspended particles and again centrifuged for 30 min. at 17000 rev./min. (35000 g) in the refrigerated Servall

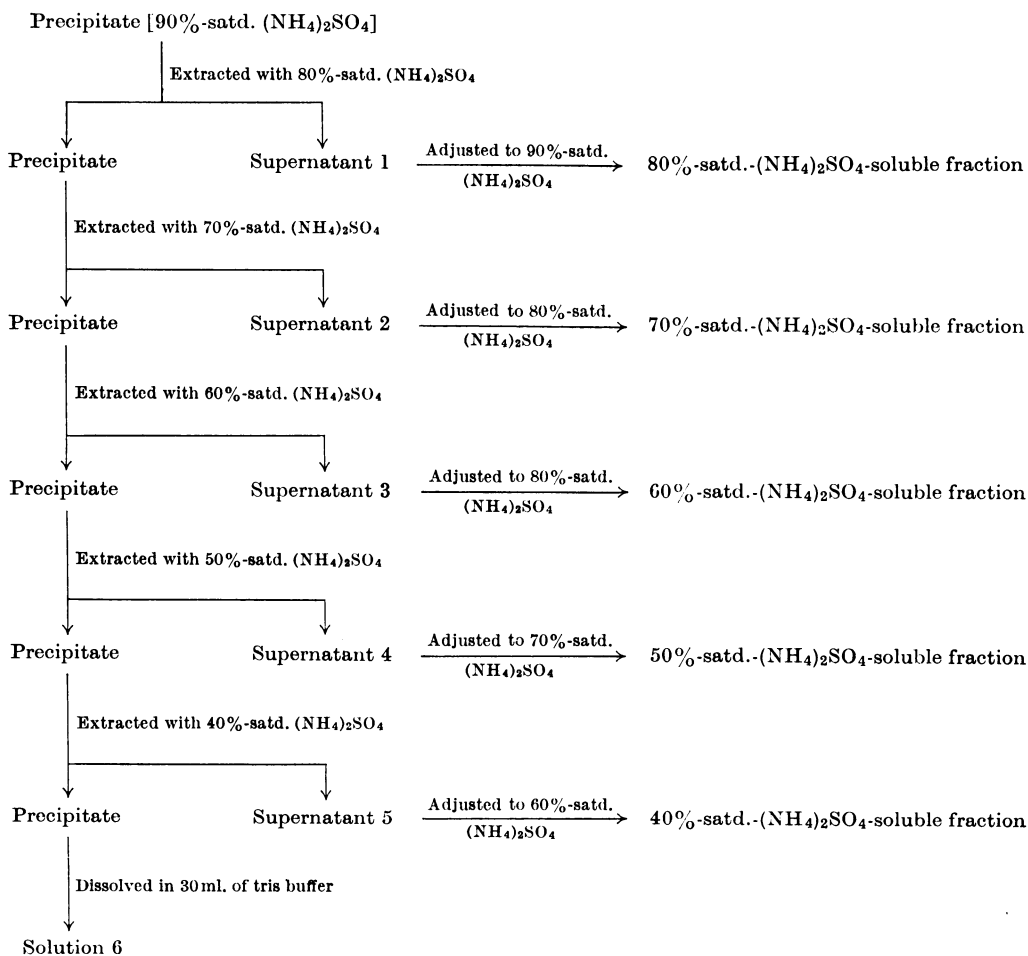
centrifuge with rotor no. SS-34 and a batch capacity of 400ml. divided between eight 50ml. polyethylene cups. The supernatants were pooled into large dialysis bags and dialysed overnight at 5-6° against two changes (10 vol. each) of tris buffer. This dialysed solution, designated the 'butanol supernatant', was used in the subsequent steps.

Step 3: precipitation with ammonium sulphate. The dialysed butanol supernatant (5l.) from step 2 cooled to 7° was treated with 3110g. of solid enzyme-grade ammonium sulphate and stirred vigorously for 30 min., and the resulting precipitate was allowed to settle in the cold for 2 hr. and spun in the Servall centrifuge at 35 000g for 30 min. The solid residue so obtained was dissolved in 500ml. of tris buffer, the supernatant being discarded.

Step 4: heat treatment. The enzyme solution

(500ml.) in tris buffer (step 3) was transferred to a 1l. flask, and this was immersed in a bath thermostatically maintained at 55° for 1 hr. with occasional agitation. The turbid solution was cooled in ice for 30 min. and then was subjected to high-speed centrifugation (37 000g) for 20 min. at 4°. The residue (suspended in tris buffer) exhibited a specific activity of 0.02 and was discarded. The supernatant was preserved for the next step. Two such supernatant preparations are required for the ammonium sulphate fractionation described in the next step.

Step 5: ammonium sulphate fractionation. The supernatant (600ml.) from step 4 was treated with 397g. of solid ammonium sulphate and the resulting precipitate was collected by centrifugation as described in step 3 and was subjected to further fractionation as outlined in Scheme 1.



Scheme 1.

The fractionation was done by extracting the solid precipitate with a variety of ammonium sulphate solutions of decreasing saturations. The enzyme in each fraction was recovered by raising the ammonium sulphate concentration of the mixture to a value at which these proteins become insoluble.

To justify the choice of this extraction rather than precipitation, the enzyme solution of step 4 prepared from a separate batch of 100 g. of placenta was subjected to ammonium sulphate fractionation in the conventional method. That this 'extraction method' is more efficient than the 'precipitation method' is evident from Table 1.

Each of the enzyme specimens was dialysed against tris buffer at 5–7° until free from ammonium ion as determined by Nessler's reagent. The complete removal of ammonium sulphate (an inhibitor of alkaline phosphatase) by dialysis is mandatory before the next step.

Step 6: ethanol fractionation. The dialysed 50–60% saturated ammonium sulphate fraction of the previous step was subjected to ethanol fractionation in ice by starting with 30% (v/v) ethanol and raising the concentration in 10% (v/v) steps to 90% (v/v). The precipitates obtained between 30 and 80% ethanol were collected, dissolved in water and subjected to a second ethanol fractionation, increments of 5% (v/v) being used. The precipitate obtained with 65% (v/v) ethanol showed the highest enzyme activity and was dissolved in water for the following step.

Step 7: Sephadex-G-200-gel filtration. Sephadex G-200 (particle size 40–120 μ) was allowed to swell in tris buffer, pH 8.6, at 5–7°. The buffer was changed at 16 hr. intervals for 5 days to remove the lighter particles by decantation. The gel was poured as a thin slurry into a closed vertical column

(60 cm. \times 2.5 cm.) and was then allowed to settle to 25% of the desired column height. Next, additional Sephadex slurry was added until the desired bed height was obtained with the drain outlet open. Finally, the bed was washed for at least 24 hr. with tris buffer in the cold (4–5°), to ensure the equilibrium and stabilization of the column height.

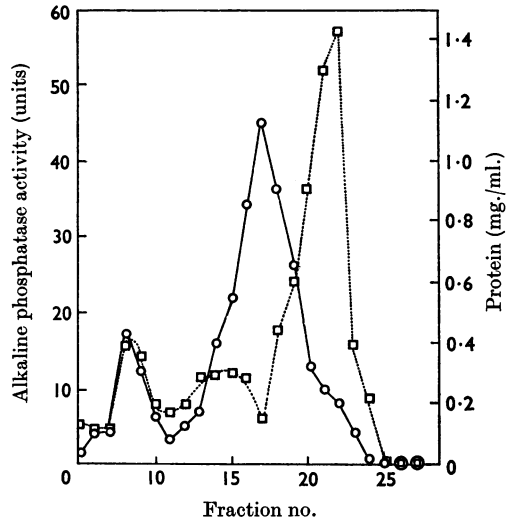


Fig. 1. Typical elution profile of enzyme and protein on Sephadex-G-200-gel filtration carried out by the large-scale method. Fractions (160 ml.) 1–11 contain variant B; fractions 12–24 contain variant A. \circ , Enzyme activity; \square , protein.

Table 1. Specific activities of human placental alkaline phosphatase obtained by ammonium sulphate fractionation

The precipitation method involved precipitation of enzyme protein with increasing concentrations of $(\text{NH}_4)_2\text{SO}_4$, starting with the lowest one. The fractionation method was initiated from the highest concentration of $(\text{NH}_4)_2\text{SO}_4$, the details of the procedure being outlined in Scheme 1.

Concn. of $(\text{NH}_4)_2\text{SO}_4$ (% saturation)	Specific activity (units/mg. of protein)	
	Precipitation method	Extraction method
0–40	1.344	1.680
40–50	1.876	14.84
50–60	2.968	28.98
60–70	6.216	2.324
70–80	4.116	1.680
80–90	2.800	0.700

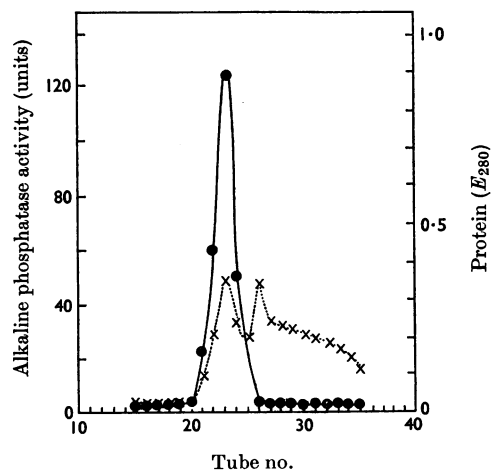


Fig. 2. TEAE-cellulose anion-exchange chromatograms (step 8). —, Enzyme activity; $\times \dots \times$, protein.

The enzyme solution was layered dropwise on the top of the column over 5 hr. by a siphon arrangement from a reservoir and then was eluted with the same tris buffer with a well-controlled gravity flow. Fractions (20 ml.) were monitored for protein and enzyme activity. Starch-gel-electrophoresis patterns of eluates from a typical filtration indicate slow-moving (B) and fast-moving (A) variants of human placental alkaline phosphatase. The separation of variants A and B is more efficient with the larger column (Fig. 1).

Variant A contains appreciable amounts of placental albumin, which can be removed by either step 8 (TEAE-cellulose anion-exchange chromatography) or step 9 (continuous paper curtain electrophoresis) or by both steps.

Step 8: TEAE-cellulose anion-exchange chromatography. The best enzyme samples from step 7 were concentrated and dialysed against eluting buffer. The concentrated enzyme solution was applied dropwise on the top of a TEAE-cellulose column (2 cm. \times 80 cm.) equilibrated with tris buffer. The column was washed with the same buffer and then

gradient elution was performed with 500 ml. of tris buffer in the mixing chamber and 500 ml. of 50 mM-magnesium chloride in tris buffer in the reservoir flask. Fractions (3 ml.) were collected every 20 min. in the Technicon automatic fraction collector. The extinction at 280 m μ and enzyme activity of each of the fractions were determined and the values are plotted in Fig. 2.

Step 9: continuous curtain electrophoresis. This was carried out in a Beckman Spinco model CP unit at 4–5° in the presence of 20 mM-veronal-hydrochloric acid buffer, pH 8.6, with a current of 70 ma at 800 v (constant voltage). The enzyme sample was first concentrated to one-third volume in a cellophane bag covered with dry Sephadex G-200 at 4–5° for 48 hr. or more and then equilibrated by dialysing against 20 mM-veronal buffer, pH 8.6, overnight at 4°.

The paper curtain was equilibrated with buffer until smooth vertical movement of the protein dye spots was obtained. The speed of the sample delivery was regulated to 3–4 ml./hr. and fractions were collected automatically in a Beckman Spinco model CPF fraction collector at 12 hr. intervals starting from the time of first appearance of protein at the drip points (usually 6 hr. after the feeding of the sample) until about 12 hr. after the enzyme solution had been completely introduced into the system. All tubes were assayed for alkaline phosphatase activity, and protein and the total activity for each tube were computed.

After completion of the run, the position of protein remaining on the paper was ascertained as follows. The paper was dried at 100° for 30 min., washed with methanol and stained for 1 hr. with methanolic 0.1% bromophenol blue solution, washed four to six times with 5% (v/v) acetic acid and dried again at 100°. The stained areas on the paper of a typical run are shown in Fig. 3. The values for total enzyme activity and the protein concentration per tube are plotted against the corresponding fraction numbers, which are shown to originate from the stained areas of the paper.

In another experiment, each of the 32 fractions obtained by continuous curtain electrophoresis was subjected to starch-gel electrophoresis. The starch-gel-electrophoresis patterns of protein of the fractions and a typical zymogram showing the enzyme protein band (variant A) are presented in Fig. 4 along with the curtain-electrophoresis curves for enzyme activity and protein concentration.

The results presented in Figs. 3 and 4 indicate the presence of enzyme in fractions 9–17, whereas fractions 22–32 contained very little enzyme but considerable amounts of albumin.

Variant B purified by Sephadex-gel filtration could be subjected to continuous curtain electrophoresis in the same manner as variant A.

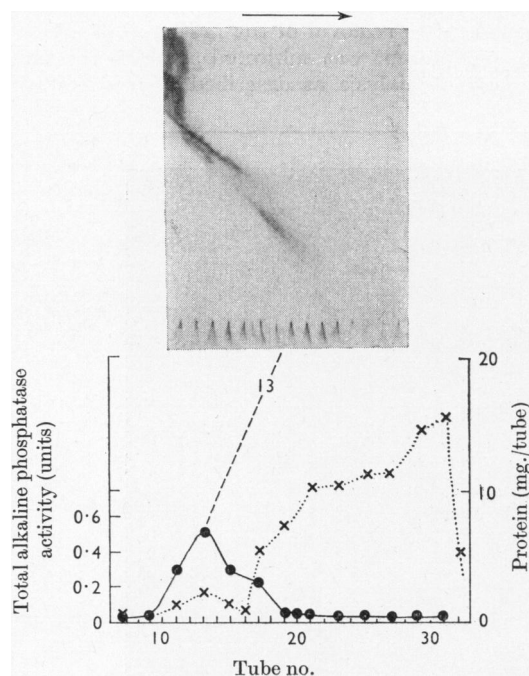


Fig. 3. Continuous-curtain-electrophoretic pattern (step 9). The paper has been stained for protein with bromophenol blue. Enzyme activity (●—●) and protein concentration (×...×) were measured in eluates of each paper segment. The arrow indicates the direction of current. The relation between position on the curtain and the tube number is given at tube 13.

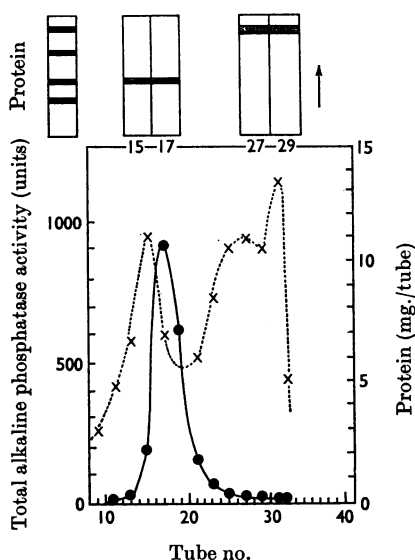


Fig. 4. Starch-gel zymogram of protein in fractions collected during paper curtain electrophoresis. This curtain-electrophoretic run was done on a different batch of enzyme from that illustrated in Fig. 3. Enzyme activity (●—●) and protein concentration (×...×) were measured in eluates of each paper segment. The protein pattern on the far left is that of the preparation applied on the curtain. The other patterns are identified by tube number.

Step 10: conversion of variant A into a crystalline product by equilibrium dialysis. The enzyme specimens (variant A or a mixture of variants A and B) of highest specific activity in the previous step were precipitated with ethanol and dissolved in a minimum volume of 0.1M-magnesium chloride and dialysed. The dialysis of this enzyme solution, which exhibited a conductance of about 8600 μ mhos, was carried out in cellophan tubing in a self-agitating Oxford multiple dialyser (model B) (from Arthur H. Thomas Co., Philadelphia, Pa., U.S.A.) constructed according to the design of Durrum, Smith & Jetton (1954). The sample was rotated electrically in the dialysing bath at constant rate of 10 rev./min. for 2-5 days at 5-7° against 400 vol. of a solution of pH near 5.6 containing 2mM-potassium chloride and 1mM-magnesium chloride (conductance 200 μ mhos). The initial pH 7.0 of the enzyme specimen slowly changed to pH 5.6 at the end when the equilibrium was reached. The dialysis was continued until the conductance of the enzyme solution inside the cellophan bag and of the outside solution both were about 300 μ mhos. Crystals with characteristic 'silky shimmer' appeared after 1-2 days of dialysis but completion of the crystallization required several weeks. After removal of the first crop of crystals the supernatant was subjected again to the same method of dialysis as described above after the

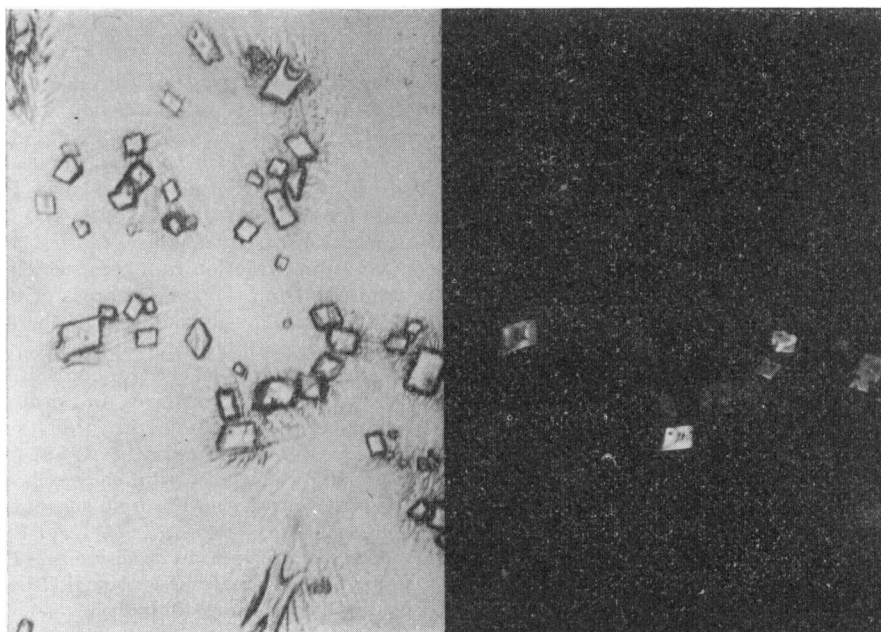


Fig. 5. Crystalline human placental alkaline phosphatase (variant B), photographed in bright light (left) and in polarized light (right). Magnification $\times 450$.

addition of seed crystals. On microscopic examination the crystals appeared as colourless hexagonal prisms capped by pyramids (Fig. 5). The crystals, which were insoluble in distilled water at 20–25° and sparingly soluble in buffer, were washed with water five or six times to remove adhering impurities and were reconstituted in 50mM-magnesium acetate. Crystalline variant B exhibited an activity of nearly 400 units/mg. of protein. Starch-gel electrophoresis of the crystalline enzyme specimen prepared by this method demonstrated only one broad enzyme band near the origin (Fig. 8).

Large-scale procedure

The equipment and facilities of the Tufts Enzyme Center were employed and the direction followed was that of the laboratory-scale purification except that it was necessary to simplify the extraction of the enzyme from the ammonium sulphate precipitate and to omit the ethanol step.

All operations described in steps 1–3 were carried out at 5–7°.

Step 1: preparation of the homogenate. Washed placentas (18kg.) were ground in an electrical meat grinder and then were introduced, 500g. at a time, into a precooled Gifford–Wood colloid mill to make the 10% homogenate with 170l. of tris buffer, pH 8.6, homogenization being carried out for 4 min. The homogenate (approx. 180l.) was then put in a glass-lined thermostatically controlled tank (300l. capacity). Cold butanol (112l.) was then added in a slow stream while the whole mixture was mechanically agitated and the stirring was continued for 1 hr. After thorough emulsification of the tissue-butanol mixture, the temperature of the tank was increased to 37° and maintained at that temperature for 10 min. and then cooled to 4°.

Step 2: preparation of the butanol supernatant. The mixture (246 l.) from step 1 was centrifuged in a precooled De Laval centrifuge (De Laval Separator Co., Poughkeepsie, N.Y., U.S.A.) at 50000g. The supernatant from the De Laval centrifuge was then centrifuged twice in the Sharples centrifuge at 60000g (in the cold) (30lb./in.² air pressure) to remove as much solid matter as possible. The turbid supernatant from the Sharples centrifuge, which contained butanol and some particulate matter, was then centrifuged at 5–7° in a small De Laval centrifuge equipped to separate the fatty butanol layer and the aqueous layer into two different streams. The removal of the last traces of fine solid particles was done at 4° in the preparative Beckman Spinco zonal ultracentrifuge (model L). The centrifuged solution was dialysed against two changes of 300l. of tris buffer for 24 hr. at 5–7°.

Step 3: precipitation and fractionation with

ammonium sulphate. The butanol supernatant (57 l.) from step 2 was treated and stirred with 37.7kg. of solid enzyme-grade ammonium sulphate in a 300l. tank. The powdered ammonium sulphate was added slowly while the mixture was stirred vigorously and the stirring was continued for another 30min. The mixture was kept at 5–7° for 1 hr., whereupon the enzyme precipitate rose to the top. The aqueous layer (65l.) was drained out through the outlet at the bottom. The remaining mixture (27l.) was either centrifuged in the Sharples supercentrifuge at 60000g or filtered through a Buchner funnel under vacuum.

The residue obtained was stored at 5–7° until it could be subjected to a simplified fractionation procedure outlined in Scheme 2.

Step 4: exposure to heat. The three enzyme solutions (i), (ii) and (iii) from step 3 (Scheme 2) were each heated at 55° for 60min. and cooled in an ice bath to 4°. They were centrifuged, the precipitates were discarded and the three supernatants dialysed overnight against 150l. of tris buffer, pH 8.6, and then centrifuged again.

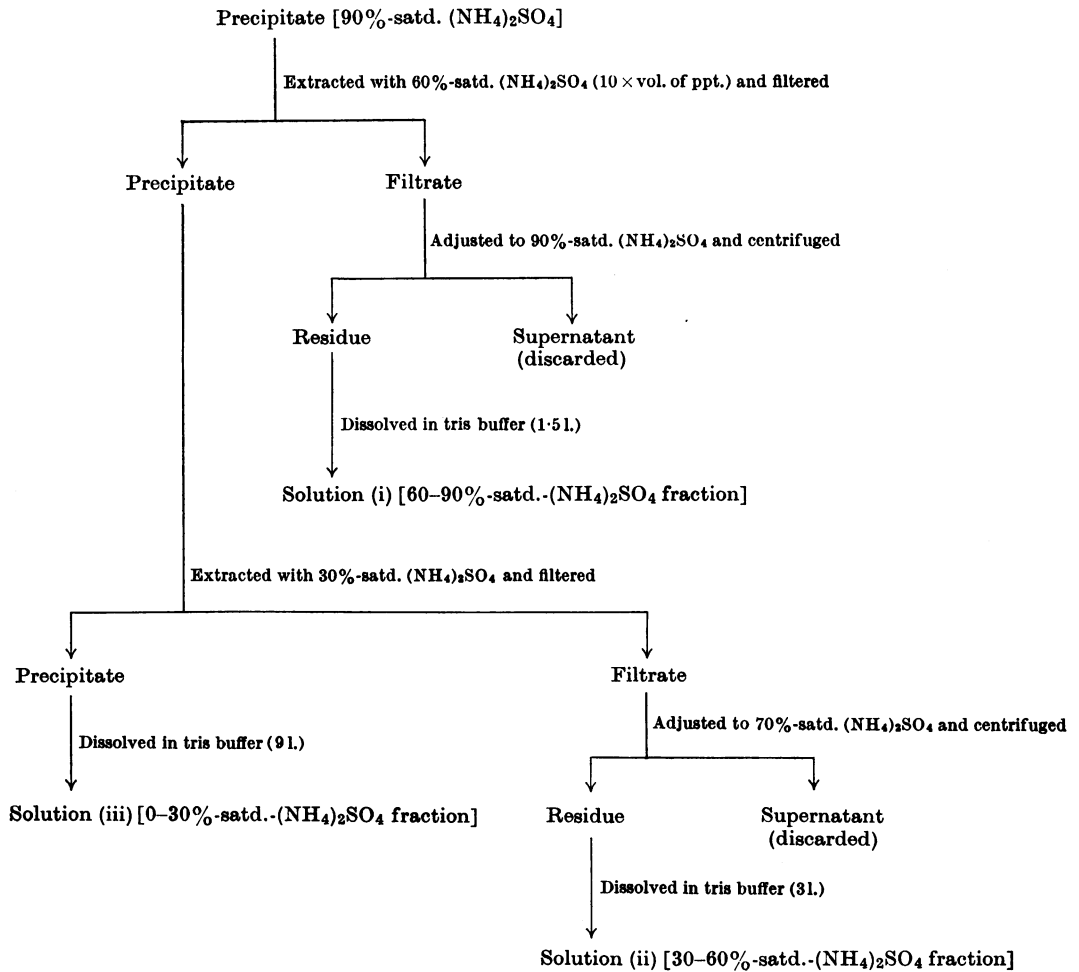
Step 5: Sephadex-G-200-gel filtration. Gel filtration was carried out in a 100 cm. × 10 cm. vertical column after freeze-concentration of specimens (ii) and (iii) of step 4 to nearly one-quarter of their original volume. The elution profile for the gel filtration of specimen (ii) is presented (Fig. 1). During molecular sieving of the enzyme two distinct pigmented protein bands could be seen inside the column. From the lower zone, one variant form of alkaline phosphatase appeared (variant B); variant A was eluted later with a larger volume of the eluent (upper zone). Thus variant B is a heavier molecular species than variant A.

The three remaining steps are conducted as outlined under the description of the laboratory-scale method for steps 8, 9 and 10.

The typical protocols of purification by the laboratory-scale and large-scale methods are presented in Tables 2 and 3 respectively. An analysis of the two fractionations indicates that a major loss recurred during large-scale purification in the activity of the 60–90% and 30–60% saturated ammonium sulphate fractions; this may now reasonably be attributed to the denaturation produced by employing the large-scale heavy-duty mixers.

Physical properties of molecular variants A and B of placental alkaline phosphatase

Sucrose-density-gradient centrifugation. The rates of centrifugation of variants A and B of placental alkaline phosphatase were compared in Fig. 6 with those of three standard markers, crystalline bovine serum albumin, 4s, 16s and 23s ¹⁴C-labelled



Scheme 2.

RNA samples and crystalline bovine liver catalase. The alkaline phosphatase activities were measured in the fractions; the bovine serum albumin was determined spectrophotometrically by recording the extinction at $280m\mu$ and the catalase was assayed by noting the rate of diminution of the extinction at $240m\mu$ ($\Delta E_{240}/\text{min.}$) in the Beckman Spinco DK-1 automatic recording spectrophotometer with hydrogen peroxide as the substrate at pH 7.1 (50mM-phosphate buffer). Molecular variant B is more rapidly sedimented than variant A, which, in turn, sediments like bovine serum albumin. Variants A and B are found to have molecular weights of 70000 and >200000 respectively when these sedimentation data are computed according to the method of Martin & Ames (1961).

The sucrose-density-gradient centrifugation profile (Fig. 6) indicates that variant B of placental alkaline phosphatase exists as a heterogeneous mixture of the heavier forms of placental alkaline phosphatase.

Starch-gel zymograms of the alkaline phosphatase fractions in the less-dense region of the sucrose density gradient showed a fast-moving zone in contrast with the slow-moving zone from fractions representing the more-dense region of the sucrose density gradient.

Ultracentrifugation. Fig. 7(b) shows the ultracentrifugal pattern of purified molecular variant A in 0.1 M-veronal buffer, pH 8.6, taken 73 min. after attaining 59780 rev./min. with the phase-plate angle 50° . Fig. 7(a) illustrates the results obtained in 0.1 M-tris buffer, pH 8.6, 128 min. after attaining

Table 2. *Purification of human placental alkaline phosphatase by the laboratory-scale method*

The values given for steps 5, 6 and 7 represent the fractions of highest specific activity selected for the next step of the purification. Step 8 (TEAE-cellulose anion-exchange chromatography) is illustrated in Fig. 2 and step 9 (continuous curtain electrophoresis) in Fig. 3.

Purification step	Volume (ml.)	Total protein (mg.)	Specific activity (units/mg. of protein)	Total activity (units)	Yield (%)	Purification
1. Tris homogenate supernatant	5000	120000	0.25	29900	—	× 1
2. Dialysed 'butanol supernatant'	5000	37500	2.80	105000	100	× 11
3. (NH ₄) ₂ SO ₄ -precipitated proteins (dissolved in 50 mM-tris buffer, pH 8.6)	500	30000	3.36	100800	96	× 13
4. Supernatant after exposure to heat	300	14000	4.39	61550	58	× 17
5. (NH ₄) ₂ SO ₄ extract (50–60% saturation)	7.5	524	29.79	15490	16	× 119
6. (a) First ethanol fractionation (60–70%, v/v)	2.0	52	62.44	3250	3	× 250
(b) Second ethanol fractionation (60–65%, v/v)	1.0	15	84.84	1275	0.8	× 340
7. Sephadex-G-200-gel filtration	2.0	1.0	254.5	255	0.08	× 1020

Table 3. *Purification of human placental alkaline phosphatase variants A and B by the large-scale method*

The values given for steps 5, 6 and 7 represent the fractions of highest specific activity selected for the next step of the purification. Steps 6, 7 and 8 correspond respectively to steps 8, 9 and 10 of the laboratory-scale method.

Purification step	Volume (ml.)	Activity (units/ml.)	Protein (mg./ml.)	Specific activity (units/mg.)	Total activity (units)	Yield (%)	Purification
1. Tris homogenate	180000	12.4	35.0	0.28	2230000	100.0	× 1
2. Dialysed 'butanol' supernatant (50000g)	186000	10.4	4.0	2.60	1932000	86.0	× 7
3. (NH ₄) ₂ SO ₄ extract (30–60% saturation)	3000	40.0	5.2	7.7	120000	5.4	× 27
4. Exposure to heat (55°)	2330	45.6	2.8	16.3	106300	4.7	× 59
5. Sephadex-G-200-gel filtration							
Variant A	800	32.1	0.29	110.7	25680	2.0	× 395
Variant B	800	9.5	0.25	38.0	7600	1.3	× 134
6. TEAE-cellulose anion-exchange chromatography*							
Variant A	25	250.0	1.0	250.0	6250	0.5	× 892
7. Continuous curtain electrophoresis							
Variant A	20	16.3	0.014	1171	320.6	0.27	× 4192
Variant B	20	10.3	0.05	212.0	206.0	0.17	× 757
8. Crystals							
Variant B	1.0	78.5	0.10	220.0	22.0	0.02	× 785

* The material from step 5 underwent a single anion-exchange chromatographic separation.

the maximum speed, the bar angle being 60°. The observed sedimentation coefficients were converted into $S_{20,w}$ by applying the viscosity correction and the corrected values in three instances with 3.8, 3.9 and 1.5 mg. of protein/ml. concentrations were 4.10, 4.30 and 4.24 s respectively.

Starch-gel and Sephadex-gel electrophoresis. Starch-gel electrophoresis (Fishman *et al.* 1968) of

purified variant A showed one protein band, at the same location as the single enzyme band; albumin was absent. When the same specimen was subjected to Sephadex-G-200-gel electrophoresis (Inglis, Ghosh & Fishman, 1968) it emerged as a single protein.

Conversion of variant B into A. The method of equilibrium dialysis was successfully applied to

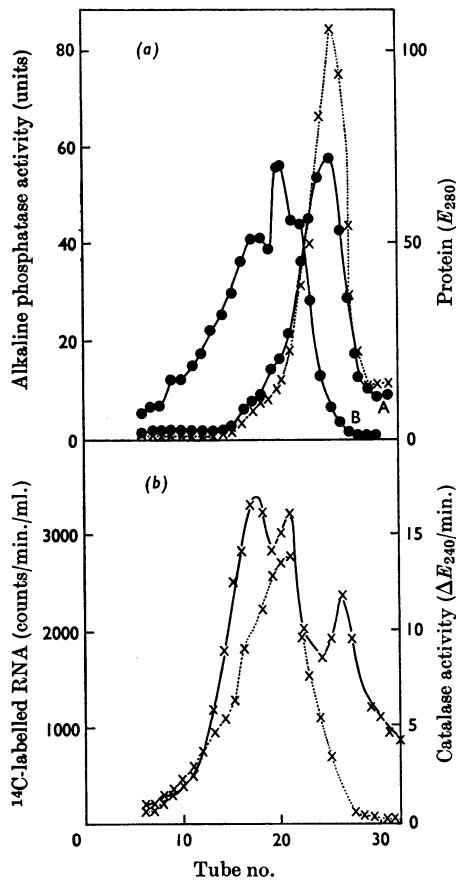


Fig. 6. Characterization of variants A and B by sucrose-density-gradient centrifugation. (a) ●—●, Enzyme activity of variant A (A) and of variant B (B); ×...×, protein values for crystalline bovine serum albumin. (b) ×...×, Activity of crystalline bovine liver catalase; ×—×, ^{14}C -labelled RNA. The ^{14}C -labelled RNA shows three peaks in ascending order from right to left, 4s, 16s and 23s. Tube 32 is the top of the gradient with 5% sucrose.

convert molecular variant A into crystalline variant B (Fig. 5). The starch-gel-electrophoretic patterns of variant A and of variant B obtained by crystallization (Fig. 8) show that fast-moving variant A becomes variant B. Crystalline variant B of placental alkaline phosphatase when kept in 50mm-carbonate-bicarbonate buffer, pH 9.2, at 5–6° for about 4 months showed the formation of electrophoretically fast-moving variant A. These results were corroborated by ultracentrifugal studies, which indicated that the supernatant of variant B after long storage contained the electrophoretically fast-moving variant A with its

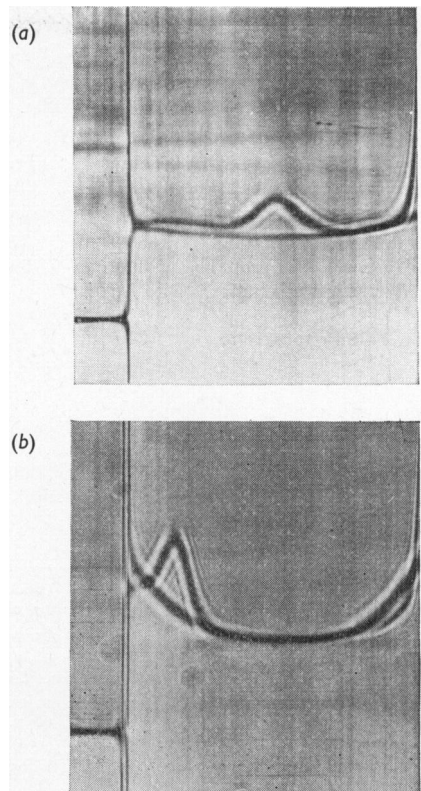


Fig. 7. Ultracentrifugation pattern of variant A of placental alkaline phosphatase in (a) tris buffer and (b) veronal buffer.

characteristic sedimentation peak of 4s enzyme protein. The comparatively low electrophoretic mobility of variant B can likewise be explained by the heavier mass of the molecular aggregate.

Immunodiffusion. Immunodiffusion patterns of purified placental alkaline phosphatase at different steps of purification are presented in Fig. 9. The final purified product of variant A gave rise to a single precipitin line that fused completely with one line of the other preparations. Variant A thus shows immunochemical homogeneity.

Chemical composition

Amino acid composition. Table 4 summarizes the results of amino acid analyses of purified variants A and B of human placental alkaline phosphatase. These results are compared with the analyses of pure placental albumin, a by-product during curtain electrophoresis of placental alkaline phosphatase preparations. Lysine, serine and cysteine residues, all of which are implicated in the mechanism of catalysis (Fishman & Ghosh, 1967a,b), are

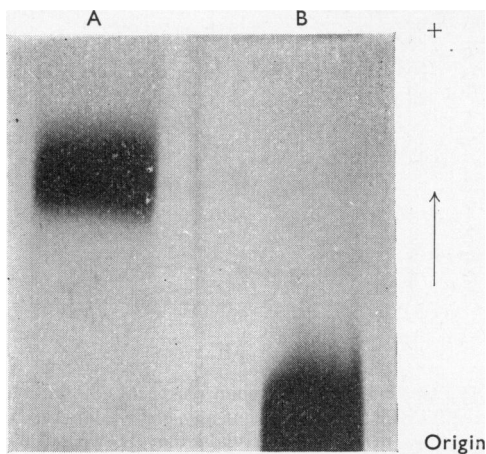


Fig. 8. Starch-gel zymograms of variant A and crystalline variant B. Anodal migration is towards the top. The methods of preparation of the two variants are described in the text.

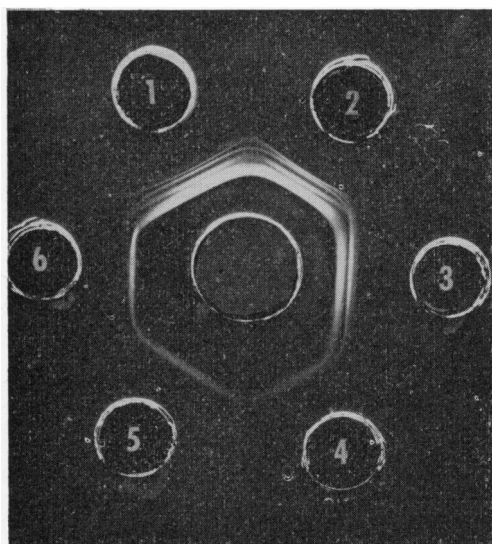


Fig. 9. Ouchterlony immunodiffusion patterns of placental alkaline phosphatase preparations purified by a modified method. 1, Best $(\text{NH}_4)_2\text{SO}_4$ fraction before heating (step 3); 2, after heating (at 55° for 1 hr.) and dialysis (step 4); 3, best fraction obtained by continuous curtain electrophoresis, which removes placental albumin (the main contaminant); bands for variants A and B can be seen; 4, 5 and 6, the three different fractions obtained by Sephadex-G-200-gel filtration of (3); no. 5 had the highest activity and is immunohemically homogeneous, showing one precipitin line. The antiserum against the best $(\text{NH}_4)_2\text{SO}_4$ fraction (2) of placental alkaline phosphatase was put in the central well.

Table 4. Amino acid compositions of purified variants A and B of human placental alkaline phosphatase compared with that of human placental albumin

The hydrolysis of protein was carried out as described in the Materials and Methods section. The values given in parentheses are the actual analytical values (averages of two determinations) in $\mu\text{moles/sample}$. Tryptophan was determined spectrophotometrically (Beaven & Holiday, 1952) and hexosamine by amino acid analysis with ninhydrin and also by the method of Yoshizawa (1961).

Amino acid	Amino acid residues (moles/70000 g. of protein)		
	Placental alkaline phosphatase		Placental albumin
	Variant A	Variant B	
Lysine	18 (0.263)	19 (0.273)	41 (0.589)
Histidine	8 (0.119)	8 (0.111)	12 (0.172)
Ammonia	92 (1.318)	185 (0.264)	215 (3.071)
Arginine	12 (0.179)	11 (0.154)	19 (0.273)
Aspartic acid	37 (0.591)	37 (0.467)	53 (0.762)
Threonine	20 (0.273)	21 (0.298)	25 (0.353)
Serine	23 (0.331)	22 (0.311)	23 (0.329)
Glutamic acid	43 (0.564)	40 (0.573)	83 (1.189)
Proline	17 (0.291)	21 (0.300)	49 (0.706)
Glycine	25 (0.355)	25 (0.366)	16 (0.232)
Alanine	32 (0.431)	30 (0.424)	48 (0.678)
Cystine (half)	20 (0.100)	20 (0.100)	20 (0.276)
Valine	23 (0.323)	24 (0.348)	32 (0.452)
Methionine	7 (0.098)	7 (0.098)	10 (0.144)
Isoleucine	12 (0.478)	13 (0.178)	16 (0.193)
Leucine	36 (0.199)	31 (0.447)	41 (0.582)
Tyrosine	12 (0.176)	12 (0.176)	12 (0.165)
Phenylalanine	17 (0.242)	17 (0.239)	20 (0.280)
Tryptophan	4 (0.100)	4 (0.100)	2 (0.100)
Hexosamine	4 (0.050)	4 (0.350)	0 (—)

present in the enzyme molecule. The unexpected peaks in the basic amino acid patterns were identified as glucosamine and galactosamine; the sum of both was determined by the method of Yoshizawa (1961).

Kinetic properties

Influence of time, substrate concentration and pH. The hydrolysis of phenyl phosphate was linear with both variants A and B up to 1 hr. Fig. 10 shows typical plots of v versus s and of $1/v$ versus $1/s$. The K_m values of these two enzyme species were identical (18 mm at pH 10.6). The two variants have optimum pH 10.6 and 10.7 with 18 mm- and 72 mm-phenyl phosphate respectively. A typical plot (Fig. 11) shows enzyme (variant A) activity as a function of pH at 72 mm substrate concentration. Fig. 12 illustrates that the optimum pH of

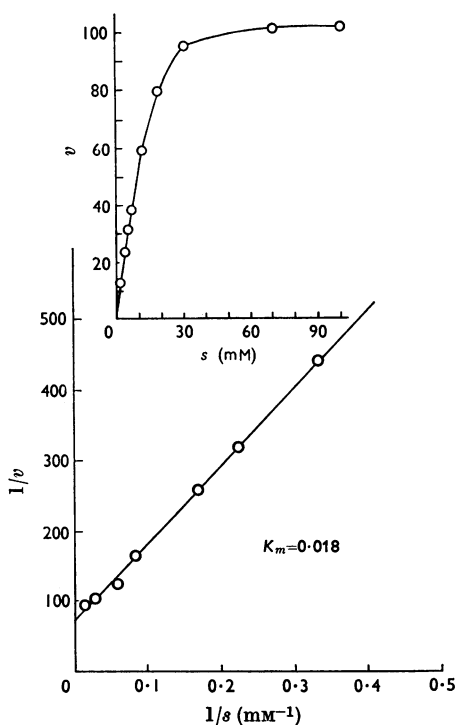


Fig. 10. Velocity of phenyl phosphate hydrolysis (by purified placental alkaline phosphatase, variant A) versus substrate concentration plot and Lineweaver-Burk plot at pH 10.6. The velocity, v , is expressed as $\mu\text{moles of phenol liberated/ml. min.}$ at 37° .

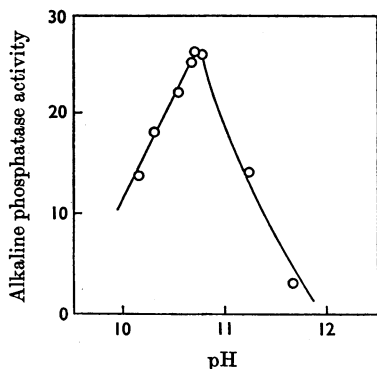


Fig. 11. pH-activity curve for variant A with 72mm-phenyl phosphate as substrate. Enzyme activity is expressed as $\mu\text{moles of phenol liberated/ml. in 15 min.}$ at 37° . 50mm- Na_2CO_3 - NaHCO_3 buffer was used.

the placental isoenzyme with 18mm-phenyl phosphate containing 5mm-D-phenylalanine is 10.6, which becomes 11.1 in digests containing the

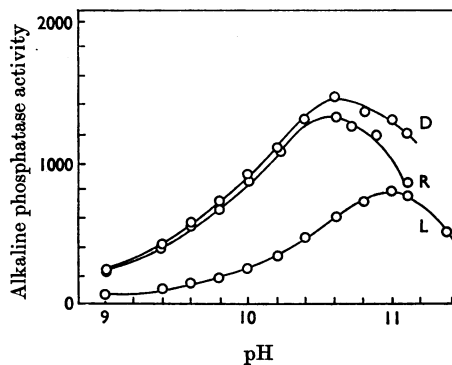


Fig. 12. Optimum pH of human placental alkaline phosphatase (variant A): R, without any phenylalanine; D, in the presence of 5mm-D-phenylalanine; L, with 5mm-L-phenylalanine in the digest. Enzyme activity is expressed in $\mu\text{moles of phenol liberated/ml. in 15 min.}$ at 37° . The substrate was 18mm-phenyl phosphate in 50mm- Na_2CO_3 - NaHCO_3 buffer.

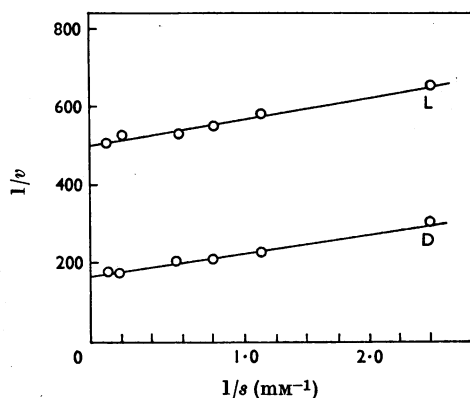


Fig. 13. Double-reciprocal plots of velocity versus substrate concentration with D-phenylalanine (2.5mm) (D) and L-phenylalanine (2.5mm) (L). The enzyme was variant A of human placental alkaline phosphatase. The velocity, v , is expressed as $\mu\text{moles of phenol liberated/ml. min.}$ at 37° . 50mm- Na_2CO_3 - NaHCO_3 buffer was used.

equivalent concentration of L-phenylalanine. Previous papers (Ghosh & Fishman, 1966; Fishman & Ghosh, 1967a) record similar shifts in optimum pH for rat and human intestinal alkaline phosphatases.

L-Phenylalanine inhibition of crystalline placental alkaline phosphatase (variant B) as function of phenyl phosphate concentration. The extent of L-phenylalanine inhibition of crystalline variant B was determined at various phenyl phosphate concentrations (0.9-27mm) and the results showed that the inhibition was dependent on substrate as

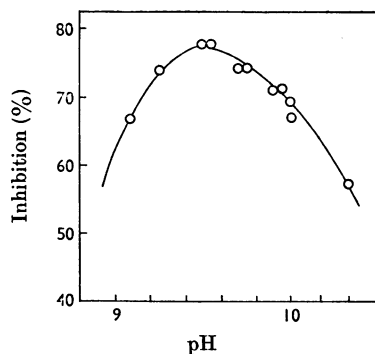


Fig. 14. pH-dependence of L-phenylalanine inhibition of placental alkaline phosphatase (variant A). The assay was done at 37° with 18 mM-phenyl phosphate as substrate.

Table 5. Kinetic parameters of variants A and B of human placental alkaline phosphatase

Sephadex-G-200-gel filtrates from step 7 (laboratory-scale preparation) were used.

Parameter	Variant A	Variant B
K_m (mM) at pH 10.3	9.50	8.30
Optimum pH with 72 mM-phenyl phosphate	10.75	10.70
L-Phenylalanine (5 mM) inhibition at pH 9.7 (%) (18 mM-phenyl phosphate)	75.0	73.0

well as inhibitor concentration. Lineweaver-Burk plots with fixed amounts of the inhibitor are shown in Fig. 13 and suggest that the inhibition is 'uncompetitive', as the reciprocal plots in the presence of L-phenylalanine are straight lines parallel to the one obtained in the presence of the equivalent concentration of the non-inhibitory D-phenylalanine, as with the rat (Ghosh & Fishman, 1966) and human (Fishman & Ghosh, 1967a) intestinal alkaline phosphatases. The extent of L-phenylalanine inhibition of human placental alkaline phosphatase was dependent on pH (Fig. 14), exhibiting optimum inhibition at pH 9.6.

Variants A and B of placental alkaline phosphatase are kinetically indistinguishable from each other. Some of the kinetic parameters of the isoenzyme variants are listed in Table 5.

DISCUSSION

A homogeneous preparation of placental alkaline phosphatase for studies of L-phenylalanine inhibition has been achieved and led to the discovery of forms with different molecular weights. As with the intestinal enzyme reported by Ghosh & Fishman

(1966) a type of pH- and substrate-dependent 'uncompetitive' inhibition was observed. Uncompetitive inhibition of intestinal and placental isoenzymes by L-phenylalanine is discussed by Fishman & Ghosh (1967a).

With regard to the purification process itself, the following features are of interest. The enzyme is firmly associated with membranes from which it can be released by butanol treatment. The thermal stability of the enzyme is a property that has been exploited to inactivate contaminating phosphatases and other enzymes. Placental albumin presented the most tenacious contaminant of the final preparations, from which it could be removed completely by TEAE-cellulose chromatography and by preparative paper curtain electrophoresis. The laboratory-scale and the large-scale purification methods have been tested with more than 15 batches of placenta and the purification has been found to be reproducible. The specific activity of the final product of variant A was 4000-fold that of the starting material and possessed the highest specific activity recorded so far for this enzyme. The crystalline product prepared by equilibrium dialysis (Liss, 1965; Yonetani, Chance & Kajiwara, 1966) was only slightly soluble in aqueous buffered solutions. Its lower specific activity is not unexpected on the basis of other reports (Womack & Colowick, 1967; Nyman, 1961; Rickli & Edsall, 1962) and the formation of crystalline enzyme with unusually low solubility and decreased specific activity has been noted by Störmer (1967).

The weight of experimental evidence supports the view that human placental alkaline phosphatase exists in variants that can be distinguished by their molecular weights. Physical methods that have successfully separated these forms include starch-gel and Sephadex-gel electrophoresis, gel filtration and sucrose-density-gradient centrifugation. Moreover, the analytical-ultracentrifuge data indicate that the lighter form has a molecular weight of 70 000. The heavier forms have molecular weights above 200 000 (sucrose-density-gradient centrifugation), and these two categories are now termed molecular-weight variants A and B respectively.

These two variants are interconvertible. Equilibrium dialysis yields a crystalline product that results from the association of enzyme proteins of low molecular weight (Craig, Chen & Printz, 1967). It travels as a slow-moving band on starch-gel electrophoresis. The crystalline material undergoes gradual disaggregation, yielding the lighter variant A as shown by ultracentrifugal and electrophoretic studies.

Variants A and B have very similar amino acid compositions except for the larger number of ammonia-forming amino acid residues in variant B.

The difference between the combined weight of the amino acids plus hexosamines and the molecular weight of 70000 is due to sialic acid (Ghosh, Goldman & Fishman, 1967) and other carbohydrates. Accordingly, the percentage of total carbohydrate residues in alkaline phosphatase of the placenta would then be 25–30%. Portmann (1957) also indicated the presence of protein-bound sugar and hexosamine in his rat intestinal alkaline phosphatase preparation.

Variants A and B do not differ enzymically as judged by studies of pH optima, Michaelis constants and of other criteria.

With regard to the general problem of electrophoretic variants of enzymes, the present data show clearly that protein aggregation can produce electrophoretic variants that are biochemically indistinguishable from each other.

Serum of pregnancy contains alkaline phosphatase whose electrophoretic properties agree with those of variant A. In late pregnancy, a slow-moving band near the origin often appears in addition. These two variants can be assumed to differ in molecular weight and this view should now be taken into consideration when starch-gel zymograms of pregnancy sera are being interpreted.

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