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STUDIES ON HUMAN PLACENTAL ALKALINE PHOSPHATASE

PURIFICATION BY IMMUNOABSORPTION AND COMPARISON OF THE "A" AND "B" FORMS OF THE ENZYME

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Summary

Human placental alkaline phosphatase (orthophosphoric-monoester phosphohydrolase (alkaline optimum), EC 3.1.3.1) was purified from 150 000 × *g* (max.) pellets of pooled placental extracts by butanol extraction, immunoabsorbent chromatography, gel filtration, ion exchange chromatography and salt-mediated hydrophobic chromatography. The "A" (fast electrophoretic mobility) and "B" (slow electrophoretic mobility) forms were compared in several chromatographic and electrophoretic systems for their ability to raise monospecific antiserum, and by immunoquantitation. It was found that (1) the "A" form enzyme is able to enter polyacrylamide gels even if no Triton X-100 detergent is included in the sample and gel matrix; the "B" form requires detergent, (2) the "A" form has 3 times the specific activity and 3 times more of a specific protein subunit than the "B" form, detected by sodium dodecyl sulfate polyacrylamide electrophoresis, (3) the "A" form is readily resolved into distinct peaks on ion-exchange and salt-mediated hydrophobic chromatography systems; the "B" form is poorly resolved on both systems in the absence of detergent, (4) monospecific antisera can be raised against the "A" form enzyme up to one year after primary immunization, with repeated boosters; the antisera raised against the "B" form enzyme have trace antibody contaminants, and (5) immunoquantitation by radial immunodiffusion followed by enzyme staining demonstrated both enzyme forms had the same immunoreactivity of active enzyme per unit of catalytic activity. These data, in conjunction with previous studies which demonstrated that the "A" and "B" form enzymes differed in molecular weight and isoelectric pH, support

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the conclusion that the "R" form enzyme is complexed with non-alkaline phosphatase protein and is not a polymer of the same protein species in an altered conformational state. The significance of the two enzyme forms in relation to the membrane localization of alkaline phosphatase is discussed.

Introduction

Human placental alkaline phosphatase (orthophosphoric-monoester phosphohydrolase (alkaline optimum), EC 3.1.3.1) has attracted interest because of its tissue specificity [1,2], its genetic polymorphism [3—7], and the appearance in some cancers of an isoenzyme, the "Regan isoenzyme," which is apparently identical to it by a number of criteria [1,8,9].

Studies on the structure of placental alkaline phosphatase have been complicated by the fact that the enzyme exists in several forms of different molecular weights [4,5,10] and its chromatographic resolution in adsorption chromatography systems is poor [11], as found for other membrane-associated proteins [12]. Despite these difficulties, we were able to purify the "B" form (higher molecular weight, slower electrophoretic mobility) enzyme using a combination of salt-mediated hydrophobic chromatography and several ion-exchange chromatography steps with and without Triton X-100 detergent in the eluting buffer [11].

In attempting to simplify the purification of human placental alkaline phosphatase for the isolation of the enzyme from individual placentae and its counterpart from cancer tissues, we further extended the observations of Pitarra et al. [13] and Hoag et al. [14] that immunoabsorbent chromatography could be used to purify the human placental enzyme if antisera collected shortly after immunization were used, and if alkaline pH buffers were used to elute the enzyme.

In applying the method of immunoabsorbent chromatography, we found that significant amounts of "A" form (lower molecular weight, faster electrophoretic mobility) enzyme were released by butanol extraction of placental membrane preparations and that this form differed significantly from the "B" form enzyme in its chromatographic resolution. These differences were useful in that the "A" form enzyme was resolved easily into well-defined, narrow peaks which greatly facilitated the purification of the enzyme.

This work describes the preparation of homogeneous "A" form enzyme by a combination of immunoabsorbent, gel filtration, ion-exchange and salt-mediated hydrophobic chromatography techniques. The enzyme produced in this way is homogeneous by the criteria of disc-gel, SDS gel, and the ability to raise monospecific antisera one year after primary immunization.

Evidence is also presented that the "A" and "B" form enzymes share the same catalytic subunit but that the "B" form enzyme is associated with hydrophobic and alkaline residues and that the differences in the properties of these two enzyme forms is not due to polymerization of subunits alone.

Experimental Procedures

Materials

Chemicals used in this study and their suppliers were: phenyl phosphate,

disodium salt and L-phenylalanine: Sigma; Tris and ammonium sulphate (ultrapure): Mann; Sephadex G-200, Sepharose 4B and DEAE-Sephadex A-25: Pharmacia; cyanogen bromide: Matheson, Coleman and Bell; complete and incomplete Freund's adjuvant: Difco; human placental alkaline phosphatase (grade AP): New England Enzyme Center; protein standard: Sigma (Sigma catalogue No. 540-10); agarose: Marine Colloids, Inc., Rockland, Maine.

Methods

Immunization protocol. Rabbits were immunized with an emulsion of 10 units ($\mu mol \cdot min^{-1}$, see below) of enzyme in 1 ml saline and 1 ml complete Freund's adjuvant at multiple sites along the back. After 8 weeks, booster injections of an emulsion of 10 units in saline and incomplete Freund's adjuvant were adminstered subcutaneously at monthly intervals. Rabbits were bled from an ear artery. Generally, 50 ml of blood was collected at each bleeding.

Preparation of antibody-Sepharose. Antibody was precipitated from serum in 40% saturated ammonium sulfate solution, and the precipitated antibody was exhaustively dialyzed against multiple changes of 0.05 M sodium bicarbonate, pH 8.3. 10 mg of antibody was coupled per gram of Sepharose 4B, activated by the procedure as described previously [11] and incubated for 18 h at 4°C with mixing. Effciency of coupling was 95—98% as judged by recovery of protein (A_{280}) in the washings of the antibody-Sepharose preparations. For the experiments described in this work, serum from 6 rabbits obtained at each bleeding date was pooled.

Preparation of butanol-extracted enzyme. In a previous work [15], aliquots of placentae were homogenized in saline (2 ml/g of tissue) and were centrifuged at 6000 × *g* for 10 min; the supernatant was used to assay for rare D-variants. After screening, the common, non-variant placental extracts were pooled and centrifuged at 150 000 × *g* (max.) in a Beckman ultracentrifuge using a 60 T_i rotor. Under these conditions, 60—80% of the enzyme activity was found in the pellet. These pellets were washed twice in 5 mM Tris acetate-buffered saline, pH 8.0, and then stored frozen at −20°C until they were used.

The preparative experiments described in this work represent butanol extracts of pellets prepared from 1000 placental aliquots. The washed pellets were suspended in 10 vol. (ml/g) of 5 mM Tris acetate-buffered saline, pH 8.0 (uniform suspensions required approximately 8 h of vigorous mixing on a magnetic stirrer), and an amount of butanol equal to one half the buffer volume was added to the suspended pellets at 4°C. The butanol/aqueous emulsion was stirred on a magnetic stirrer at 4°C for 24 h, then centrifuged at 40 000 × *g* (max.) in a Sorvall refrigerated centrifuge for 30 min. The butanol layer was removed by suction, and the aqueous layer was dialyzed against 2 changes of 10 vol. of 0.05 M Tris/Acetate, pH 8.0 and concentrated in an Amicon ultrafiltration cell using a PM-30 membrane.

Chromatography of enzyme

1. Immunoabsorption. a. Small scale. To test the different bleeding dates for efficiency of association and dissociation, the following protocol was used:

Association of the enzyme: A small column (0.9 × 8 cm) of immunoglobulin-Sepharose was washed with 50 ml of Tris/base (0.5 M) and equilibrated with 50 mM sodium bicarbonate, 100 μM $ZnAc_2$ pH 8.3 buffer. 2 ml of

enzyme (NEEC grade AP) containing 226 μmol · min^{-1} · ml^{-1} of enzyme were applied directly to the column and recycled using a peristaltic pump overnight (17 h) in buffer with 0.1% sodium azide at room temperature to achieve maximum binding. The 150 000 × g (max.) supernatant required a larger volume of sample to contain the same number of enzyme units (552), therefore, it was circulated through the column from a reservoir of 61.6 ml. The amount of association was calculated from the amount of activity recovered in 50 ml wash with association buffer, compared with the 552 units of enzyme applied.

Dissociation of the enzyme. Elution was carried out at 4°C (because of enzyme denaturation in pH 12 at room temperature) on an LKB fraction collector set at 30 drops (1.9 ml) per fraction and at a flow rate of 0.6 ml per minute. The elution buffers were 50 mM sodium carbonate/bicarbonate pH 10.5 and 5 mM sodium phosphate pH 12. (Sodium phosphate was used because its high pK_3 produced pH 12.0 at a low buffer concentration which facilitated subsequent lowering of the pH with a small amount of more concentrated neutral buffer.) The total elution profile consisted of 40 fractions, 10 fractions at pH 10.5, 10 fractions immediately at pH 12, 10 fractions after 2 h and another 10 after 24 h of incubation in pH 12 at 4°C. In order to reduce the pH of the column eluate, each tube into which eluate was collected contained 0.5 ml of association buffer (50 mM sodium bicarbonate, 100 μM $ZnAc_2$, pH 8.3). Thus, the pH 10.5 eluate was brought to pH 10.0 and the pH 12 eluate to pH 8.7.

b. Large scale. In preparation of larger amounts of immunoabsorbent-purified enzyme, the following protocol was used:

Association of the enzyme: 200 g of antibody-Sepharose was added to 6000—9000 units of enzyme in 1.5 l of 10 mM Tris acetate-buffered saline, pH 8.0. Solid sodium azide was added to a final concentration of 0.1%, and the mixture was stirred on a motor-driven stirrer overnight at room temperature. The suspended antibody-Sepharose with bound enzyme was then poured into a 2.6 × 40 cm Pharmacia column, and the settled gel was washed with 5 column volumes of the Tris-buffered saline and one column volume of 0.05 M Tris/acetate, 100 μM $ZnAc_2$, pH 8.0. Under these conditions, 80—95% of the enzyme was bound to the immunoabsorbent.

Dissociation of the enzyme. The column was removed to the cold room (4°) and was washed with 200 ml of cold 50 mM Tris/Acetate buffer, 100 μM $ZnAc_2$, pH 8.0. The enzyme was then eluted with 5 mM sodium phosphate, pH 12, and 10 ml fractions were collected into tubes containing 1 ml of 0.05 M Tris/acetate, 100 μM $ZnAc_2$, pH 8.0. In order to optimize the flow rate of the pH 12 buffer through the column, the reservoir was held at a height of 2 m above the outlet tube. Under this pressure, elution was effected in 2—3 h. After the enzyme had been eluted, the column was flushed with 1 vol. of 0.05 M Tris/acetate, pH 8.0, 100 μM $ZnAc_2$, and the gel was then ready for the next cycle of association and dissociation. Gel used in this way has been carried through 20 cycles of association and dissociation with good retention of adsorption capacity. The dissociated enzyme can be concentrated in 200 ml Amicon stirred-cell apparatus using a PM-30 membrane; after concentration, the buffer is changed to 0.05 M Tris/acetic, pH 8.0, by adding 150 ml of this buffer to the chamber and concentrating three times to a volume of less than 20 ml.

2. Gel filtration. This was performed in 0.05 M Tris/acetate buffer pH 8.0 containing 100 μM $ZnAc_2$ on Sephadex G-200. Column dimensions were 2.6 × 65 cm (345 ml); sample size was 15 ml, and 150 drops (approximately 10 ml) per fraction were collected.

3. DEAE-Sephadex. Samples were applied to an A-25 gel which had been equilibrated with 0.05 M Tris/acetate buffer, pH 8.0 containing 100 μM $ZnAc_2$. Enzyme eluted from the immunoabsorbent in pH 12 buffer and collected in the Tris/acetate buffer can be applied directly to this DEAE-Sephadex gel, with efficient binding. Elution can then be effected using a gradient of 0.00—0.20 M NaAc in the Tris/acetate buffer, followed by 0.1% Triton X-100 detergent in 0.2 M NaAc to elute the "B" form (see Results).

4. L-phenylalanine-Sepharose (salt-mediated hydrophobic chromatography): Enzyme in 0.05 M Tris/acetate, pH 8.0, was mixed with an equal volume of 2.5 M ammonium sulfate, 0.05 M Tris/acetate, pH 8.0, to give a final ammonium sulfate concentration of 1.25 M. The enzyme was then applied to L-phenylalanine-Sepharose which had prevously been equilibrated with 1.25 M ammonium sulfate, 0.05 M Tris/acetate, pH 8.0. Elution was achieved using a gradient of 1.00 ammonium sulfate, 0.05 M Tris/acetate, pH 8, in the mixing chamber and 0.5 M sodium chloride, 0.05 M Tris/acetate, pH 8, in the addition chamber. (The higher (1.25 M) ammonium sulfate concentration was used to ensure efficient binding to the column, but 1.0 M ammonium sulfate solution was an adequate starting concentration for the gradient (see Results)). Concentration of the column eluate was achieved using vacuum dialysis.

Enzyme assay

The assay for enzyme activity of placental alkaline phosphatase according to the method of Fishman et al. [16] was performed at 37° C with 18 mM phenyl phosphate in 50 mM sodium carbonate/sodium bicarbonate buffer (pH 9.8) 10 mM $MgCl_2$, or with 72 mM phenyl phosphate in 50 mM sodium carbonate-sodium bicarbonate buffer (pH 10.7) without added $MgCl_2$. The unit of enzyme activity is defined as that amount of enzyme which liberates 1 μM of phenol/min per ml at pH 9.8 unless otherwise specified.

Enzyme staining

This was by the method of D. Angellis et al. [17] except that neither zinc nor magnesium was included in the buffer. These were omitted since their inclusion appeared to interfere with the visualization of the "A" (fast-moving) form of the enzyme after electrophoresis.

Protein assay

Protein concentration (mg/ml) was estimated by A_{280} and by the method of Lowry et al. [18] with Sigma protein standard (80 mg/ml stock).

Sodium dodecylsulfate polyacrylamide gel electrophoresis

The method of Fairbankds et al. [19] was used except that the final concentration of sodium dodecyl sulfate was 0.1% in the gel and buffers. Gel scanning was carried out in a Gilford gel scanner (2410-S) set at 520 nm.

Disc-gel electrophoresis

A modification of the method of Fishman [20] was used in that the gels were 0.5 × 6.5 cm, and the current was 1.5 mA per gel for 45 min and then constant voltage (250 V) for an additional 45 min. 250 ml of electrophoresis buffer was used in each chamber in the Shandon apparatus.

Radial immunodiffusion experiments

These were carried out in 1.5% agarose gels as follows: 20 ml of hot gel solution in 0.04 M veronal/HCl, pH 8.4, was poured into 3.5-inch square plastic petri dishes on a level surface. When the gel had set, 20 ml of 30 $\mu g \cdot ml^{-1}$ solution of lyophilized antiserum to placental alkaline phosphatase in 1.0% Triton X-100, 0.04 M veronal/HCl, pH 8.4, containing 0.1% sodium azide, was placed over the gel. After 48 h, the overlay was poured off, and the gel was used for diffusion experiments.

Wells (3 mm diameter) were cut and the agarose was removed by suction. Samples (10 microliters) of diluted enzyme were applied to the wells using a Hamilton syringe. After 96 h of diffusion, the gels were stained and the diameters of the stained precipitin rings were measured. Area of the precipitin rings is expressed as the square of the measured diameter in millimeters minus the square of the 3 mm diameter of the well (d^2—9).

Double-diffusion experiments

Antiserum specificity was tested in 2.5 mm thick 1.5% agarose gels in 0.04 M veronal/HCl, pH 8.4, using a gel punch that provides a broad range of proportions of antiserum to antigen [21]. Diffusion was allowed to proceed for 48 h at 24°C after which the plate was washed exhaustively with multiple changes of 0.01 M Tris/acetate buffered saline, pH 8.0, before being stained for enzyme or protein.

Results

Effect of bleeding date of rabbit antiserum upon binding and elution patterns

Fig. 1 shows typical A_{280} and enzyme activity elution profiles for a series of Sepharose-antibody preparations that differ in the bleeding date after primary immunization at which the serum was obtained. Several points are evident from these profiles. (1) The amount of enzyme recovered in the pH 10.5 and pH 12 eluates increases with duration of the interval after primary immunization, reflecting the increased titer of the antiserum. (2) The relative amount of enzyme recovered in the pH 12 eluate compared to the pH 10.5 eluate increases with prolongation of immunization, reflecting the increasing avidity of the antiserum preparations. (3) Although the yield of additional enzyme after incubation in the pH 12 buffer for 2 and 24 h was not great, this amount also increased with time of immunization. (4) Upon incubation at pH 12 for 2 and 24 h, more protein was eluted from the columns. This amount of protein was approximately the same for all antibody preparations, irrespective of bleeding date and recovery of enzyme in these later elutions.

Recovery of the bound enzyme from antibody-Sepharoses was optimal for 2- and 3-week antisera (100%), but binding to immunoabsorbents prepared

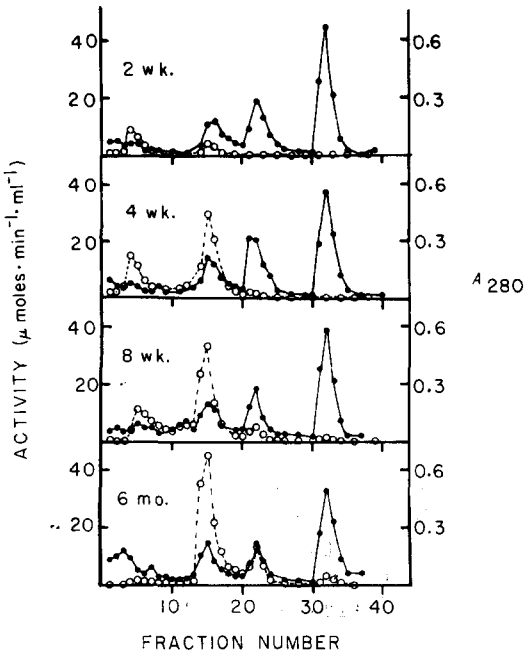


Fig. 1. Elution of human placental alkaline phophatase from antibody-Sepharose columns differing in the bleeding date after primary immunization from which the antibody was prepared. For details, see Methods. Tubes 1—10, elution in 0.05 M sodium carbonate/bicarbonate, pH 10.5; tubes 11—20, elution in 0.005 M sodium phosphate, pH 12; tubes 21—30, incubation in pH 12 buffer for 2 h after the first pH 12 elution; tubes 31—40, incubation in pH 12 buffer for 24 h after the second pH 12 elution; 1.9 ml per tube. (●———●), protein; (○———○), enzyme.

from these bleeding dates of antisera was weak (15 and 35% of total enzyme applied, compared with 80—99% for later bleedings). These very early antisera did, however, dissociate more of the bound antigen under the milder condition of treatment at pH 10.5 (68% and 50% for the 2- and 3-week antisera compared with 28% for the 8-week and 7% for the 6-month antisera) indicating some advantage in using these very early antisera for more labile antigens.

To test the potential of the immunoabsorbent technique for isolation of enzyme from crude extracts, the purification which could be achieved using a New England Enzyme Center preparation (specific activity 26 μmol · min^{-1} · mg^{-1}) and a 150 000 × g (max.) supernatant of a crude placental homogenate (specific activity 0.2 μmol · min^{-1} · mg^{-1}) were compared. The results are given in Table I.

Despite the 130-fold difference in starting specific activity, the final specific activity was reasonably similar for the two sources. The result of performing immunoabsorption on the 150 000 × g (max.) supernatant was the purification of the enzyme by 800—850 fold with 92% yield. The method is therefore useful for isolation of enzyme from crude mixtures.

Purification of the enzyme

Fig. 2 gives a typical elution profile for a large scale preparation of enzyme eluted from an antibody-Sepharose column in 0.005 M sodium phosphate pH

TABLE I

PURIFICATION OF HUMAN PLACENTAL ALKALINE PHOSPHATASE BY IMMUNOABSORPTION USING TWO DIFFERENT ENZYME PREPARATIONS DIFFERING GREATLY IN SPECIFIC ACTIVITY

Enzyme prep.	Initial S.A. **	Association *		Dissociation *		S.A. ** pH 10.5 eluate	S.A. ** pH 12 eluate
		units	%	units	%		
NEEC Grade AP	26	467	85	315	68	324(12.5) ***	228(8.8) ***
150 000 × *g* supernatant	0.2	472	85	436	92	160(800) ***	170(850) ***

* 5 week immunization antiserum
** S.A. (specific activity) μmol · min^{-1} · mg^{-1}.
*** fold purification

12. The peak of enzyme shows a marked asymmetry and is very broad while two peaks of protein were contained within the peak elution of enzyme. Elution on this scale required 2—3 h because of limitations of flow rate so that the second peak of protein may correspond to the additional protein peaks observed after 2 h of incubation of the smaller-scale columns at pH 12 (see Fig. 1).

In order to further free the enzyme from contaminating proteins, gel filtration was first used to separate the molecular weight variants of the enzyme. As suggested previously [10], G-200 Sephadex was used for this purpose. The result of such a separation is seen in Fig. 3. Most of the protein and about 40% of the activity is in the void volume of G-200; 60% of the activity is eluted later, accompanied by a very small protein peak.

The difference in electrophoretic mobility of these two forms has been well-documented and will be considered further below. More important, for purposes of deriving purification protocols, is the difference in chromatographic behavior of these two enzyme species. In a previous work [11], it was demonstrated that Triton X-100 detergent was important for the elution of the "B"

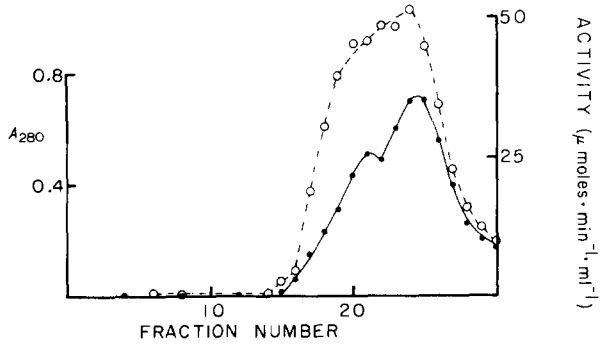


Fig. 2. Elution profile for the large-scale elution of human placental alkaline phosphatase from an antibody-Sepharose column. 5300 units of enzyme were bound to the column, and the enzyme was eluted at pH 12. See Method. Fractions were 10 ml; column dimensions 2.6 × 30 cm. (●———●), protein; (○———○), enzyme.

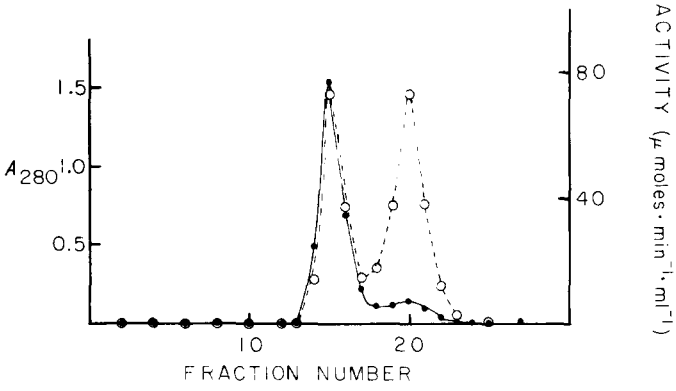


Fig. 3. G-200 Sephadex elution profile of the enzyme prepared by immunoabsorbent chromatography. 3700 units contained in 13 ml applied to a 2.6 X 64 cm column of Sephadex G-200, equilibrated with 0.05 M Tris/acetate, 0.1 mM $ZnAc_2$, pH 8.0. See Method. 10 ml per fraction. (●———●), protein; (○———○), enzyme.

form enzyme as a well-defined peak from DEAE-Sephadex. In the case of the "A" form, however, no such requirement for detergent was observed. Fig. 4 shows the DEAE-Sephadex elution profiles for the two forms separated by gel-filtration chromatography. The "A" form enzyme elutes as a well-defined peak in 0.06—0.07 M sodium acetate; the "B" form enzyme has a very broad profile without a defined peak, except for the elution of enzyme in 0.1% Triton X-100, 0.2 M sodium acetate.

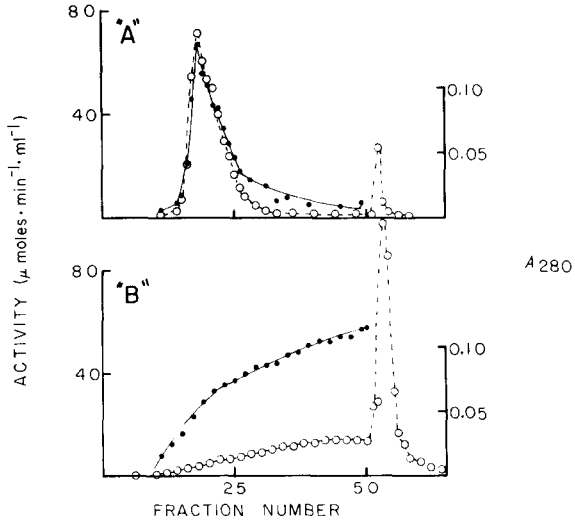


Fig. 4. Elution profiles of the "A" and "B" enzyme forms separated by G-200 chromatography and rechromatographed on DEAE-Sephadex A-25. Column dimensions 0.9 X 25 cm. 1800 units of "A" form and 1300 units of "B" form were applied to these columns and were washed with 50 ml of 0.05 M Tris/acetate, 0.1 mM $ZnAc_2$, pH 8.0, and were eluted with a gradient of 50 ml each of 0.05 M Tris/acetate, pH 8.0, in the addition chamber. Tubes 1—50, gradient, 2 ml per tube, tubes 51—60, stepwise elution with 0.2 M sodium acetate, 0.05 M Tris/acetate, pH 8.0, containing 0.1% Triton X-100 detergent (●———●), protein; (○———○), enzyme.

Salt-mediated chromatography, a procedure by which enzyme is bound to hydrophobic supports (e.g. phenylalanine Sepharose) at high ammonium sulfate concentrations and eluted at lower, was used previously in the isolation of "B" form enzyme [11]. This same method was used as a last step for purification of the "A" form enzyme.

The "A" form enzyme separated by DEAE-Sephadex chromatography (Fig. 4) was applied to a column of L-phenylalanine-Sepharose in 1.25 M ammonium sulfate (see Methods). As seen in Fig. 5, the enzyme elutes as a peak at 0.7 M ammonium sulfate with an apparent "shoulder" at 0.8 M ammonium sulfate. The protein and activity profiles showed excellent correspondence considering the background A_{280} value of 0.005—0.012 for this column eluate.

The elution of this highly-purified "A" form enzyme as a well-defined peak over a narrow range of ammonium sulfate concentrations differs significantly from the extremely broad elution profile of the crude "B" form enzyme on L-phenylalanine-Sepharose. In separate experiments of "B" form enzyme separated by immunoabsorbent chromatography, the elution profiles have also been less well-defined than that for the purified "A" form (data not shown).

A representative purification of human placental alkaline phosphatase is summarized in Table II. In this case, the immunoabsorption step represents two elutions of enzyme from the immunoabsorbent.

In comparing the results to previously published ones, it must be recognized that the use of twice-washed 150 000 $\times g$ (max.) pellets represents a 10-fold purification of enzyme relative to crude extract and that the butanol extraction procedure eliminates 90% of the non-alkaline phosphatase protein with excellent recovery (90—95%) of the enzyme in the aqueous phase. Thus, the enzyme is approximately 100-fold purified before immunoabsorption.

Also, the G-200 chromatography step separates the "A" and "B" forms with only the "A" form being carried through the subsequent ion-exchange and salt-mediated hydrophobic chromatography steps.

Thus, of some 5530 units separated in the "A" form on G-200 chromatography, 3840 units (or 69%) were recovered in the final concentrated prepara-

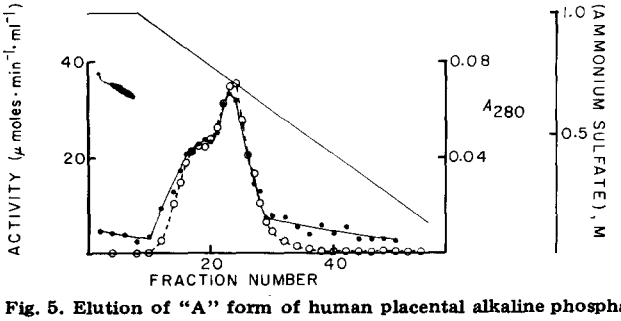


Fig. 5. Elution of "A" form of human placental alkaline phosphatase from L-phenylalanine Sepharose in a gradient of decreasing ammonium sulfate concentration. See Methods. 800 units of "A" form enzyme, purified by G-200 chromatography and DEAE-Sephadex chromatography, were applied to a 0.9 X 25 cm column of L-phenylalanine Sepharose equilibrated with 1.25 M ammonium sulfate, 0.05 M Tris/acetate, pH 8.0, and washed with 50 ml of the same buffer. Elution was with 50 ml of 1.00 M ammonium sulfate, 0.05 M Tris acetate in the mixing chamber and 50 ml of 0.5 M sodium chloride, 0.05 M Tris/acetate, pH 8.0, in the addition chamber. Each fraction continaed 2 ml. (●———●), protein; (○———○), enzyme.

TABLE II

PURIFICATION OF HUMAN PLACENTAL ALKALINE PHOSPHATASE

Step	Activity (μmol $\cdot$ min^{-1})	Protein (mg)	Specific activity (μmol $\cdot$ min^{-1} $\cdot$ mg^{-1})	Recovery (%)	Fold purification
Butanol extract 150 000 × *g* (max.) pellet	12 980	455	28.5	100	
Immunoabsorption, pH 12 eluate	9700	52.7	184	74.7	6.5
G-200 DEAE-Sephadex, L-phenylalanine-Sepharose chromatography	3838	8.6	446	29.6	15.6

tion. Most importantly, enzyme prepared by this method is homogeneous by a number of different criteria, as documented below.

Comparison of "A" and "B" forms of human placental alkaline phosphatase

Fig. 6 shows the polyacrylamide disc-gel electrophoresis of placental enzyme at various stages of purification stained for enzyme activity and protein. Samples were electrophoresed in the absence (—) and in the presence (+) of 0.5% Triton X-100 detergent included in the gel and the sample buffer.

Several points can be made for this experiment. First, the immunoabsorption step, although providing only 6.5 fold purification (see Table II), removes most

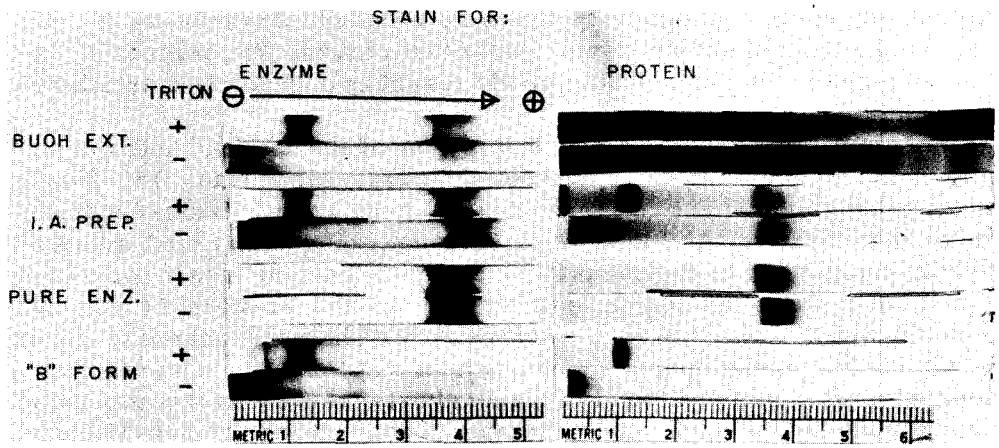


Fig. 6. Disc-gel electrophoresis of different enzyme preparations, electrophoresed in the absence (—) and the presence (+) of Triton X-100 detergent included in the gel matrix and sample solution. For enzyme staining, 0.006 units of enzyme were applied per gel. For protein staining, 4.5 units of enzyme were applied per gel, except in the case of the "B" form enzyme where 1.3 units per gel were applied. "B" form enzyme was prepared as described previously [11]. Arrow indicated the direction of migration.

of the detectable non-alkaline phosphatase protein which can enter the polyacrylamide gel. Second, no matter what the extent of purification of the "B" form, it does not enter the polyacrylamide gel unless Triton X-100 detergent is present. Third, the "A" form enzyme purified by immunoabsorbent, gel filtration, ion-exchange and salt-mediated hydrophobic chromatographies shows complete correspondence of stainable enzyme with stainable protein, and therefore is electrophoretically homogeneous.

SDS polyacrylamide gel electrophoresis

The technique of SDS polyacrylamide gel electrophoresis was used to determine whether the differences in specific activities of the "A" and "B" forms represent conformational isomers of the same protein species or an association of alkaline phosphatase protein with other non-alkaline phosphatase moieties. SDS gel electrophoresis dissociates and separates protein subunits; therefore, the amount of protein subunit in the position of alkaline phosphatase would either be proportional to the amount of enzyme activity applied (if there is association with other species in the "B" form) or proportional to the amount of protein applied (if the "A" and "B" forms are conformational isomers).

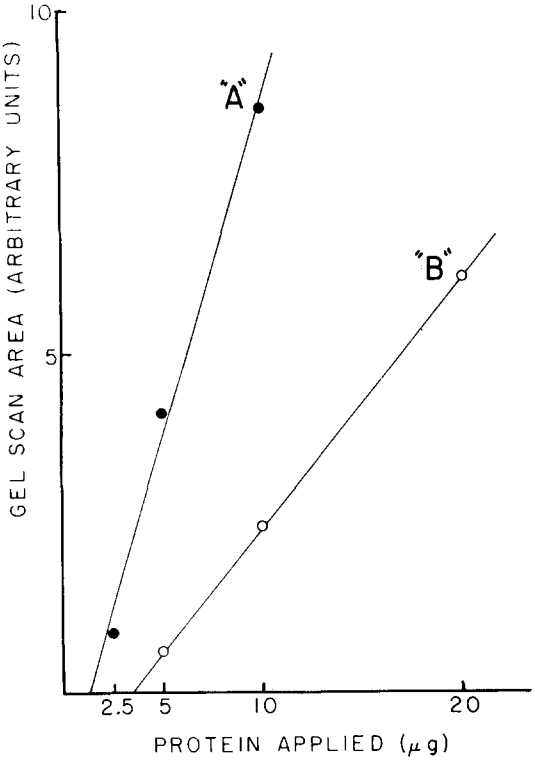
Fig. 7 shows the result for application of equivalent amounts of enzyme protein (Lowry). Both the "A" and "B" form enzymes yielded a single band of protein with a R_F of 0.40 (3 cm mobility), but there was apparently much less of this protein subunit in the "B" form enzyme. Gel scanning provided a quantitative measure of the amount of stainable protein in this band for the different amounts applied. This measure yielded a linear relationship for 2.5—10 μg of "A" form enzyme and for 5—20 μg of "B" form enzyme. The ratios of the slopes was 3.05 for "A"/"B" which is very nearly the ratio of their specific activities (Table III).

Specificity of antisera and immunoquantitation

When antisera were raised previously in rabbits to highly-purified "B" form enzyme, the specific antibody species were invariably accompanied by contaminating antibodies which reacted with other proteins. Preparations of "A" form enzyme, by contrast, elicit antibody responses only to placental alkaline phosphatase.

Fig. 7 shows the results for double-diffusion of the anti-"A" and anti-"B" antisera against preparations of 150 000 $\times g$ (max.) supernatant, 150 000 $\times g$ (max.) pellet (butanol extract), and 6000 $\times g$ (max.) pellet (butanol extract). The antiserum to the "A" form enzyme is monospecific when tested against all these preparations (two enzyme positive precipitin lines are seen in some cases in the antibody excess region possibly due to the presence of the two molecular-weight variants in these crude extracts). The antiserum raised against the "B" form enzyme, therefore, demonstrates that this enzyme is in fact associated with other proteins found in placental extracts and that this association is not completely reversible, using a number of chromatography steps to purify the enzyme.

Radial immunodiffusion (RID) has been used elsewhere for immunoquantitation of enzymes [22,23]. In comparison of the "A" and "B" forms, it provides a useful alternative to SDS electrophoresis to identify the catalytically-



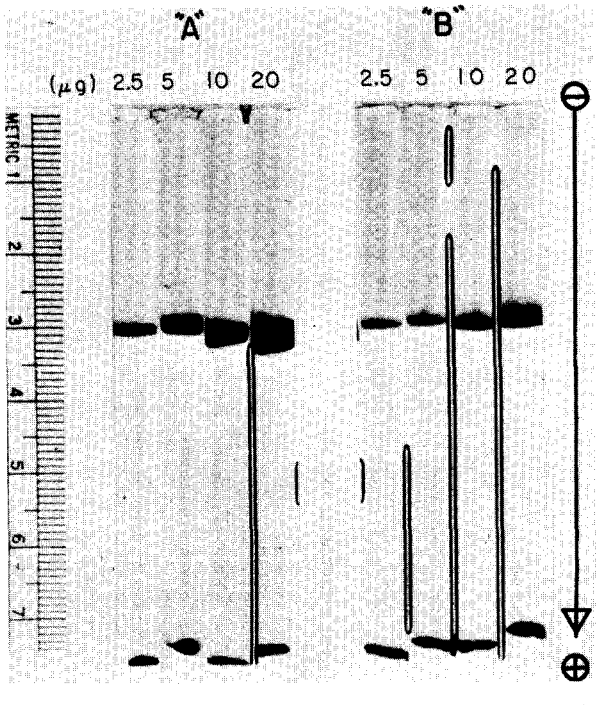


Fig. 7. SDS polyacrylamide gel electrophoresis of the purified "A" form enzyme (this work) and "B" form enzyme [11] of human placental alkaline phosphatase, stained for protein. Arrow indicates direction of migration.

TABLE III

COMPARISON OF THE PROPERTIES OF "A" AND "B" FORMS OF HUMAN PLACENTAL ALKALINE PHOSPHATASE

Property	"A" form	"B" form	Reference (this paper *)
Specific activity			
pH 10.7	1177	384	11 *
pH 9.8	446	155	11 *
pH 10.7	1171	220	10
Isoelectric pH	4.2—4.3	6.9—7.0	15
Gel-filtration (Sephadex G-200 chromatography)	Retained	Eluated near void volume	10 *
Polyacrylamide gel electrophoresis	Fast-moving, no detergent required	Slow-moving, Triton X-100 required	20 *
SDS gel electrophoresis	Single band	Band in same location as the "A" form; 1/3 the amount of protein in this band per mg protein applied as the "A" form	11 *
Ion-exchange chromatography (DEAE-Sephadex)	Distinct peak elution at 0.06—0.07 M sodium acetate in 0.05 M Tris acetate pH 8.0	Distinct peak elution at 0.03—0.1 M sodium acetate when Triton X-100 detergent is present; broad profile in the absence of Triton	11 *
Salt-mediated hydrophobic chromatography	Distinct peak solution at 0.7 M, shoulder at 0.8 M ammonium sulfate	Broad elution frm 0.75 ammonium sulfate to 0.25 M Tris base	11 *
Immune specificity of antisera raised against it by double diffusion	Monospecific	Trace reaction with other proteins	24, 25 *
Immunoquantitation by radial immunodiffusion	Ring size per unit enzyme activity applied identical for both forms		

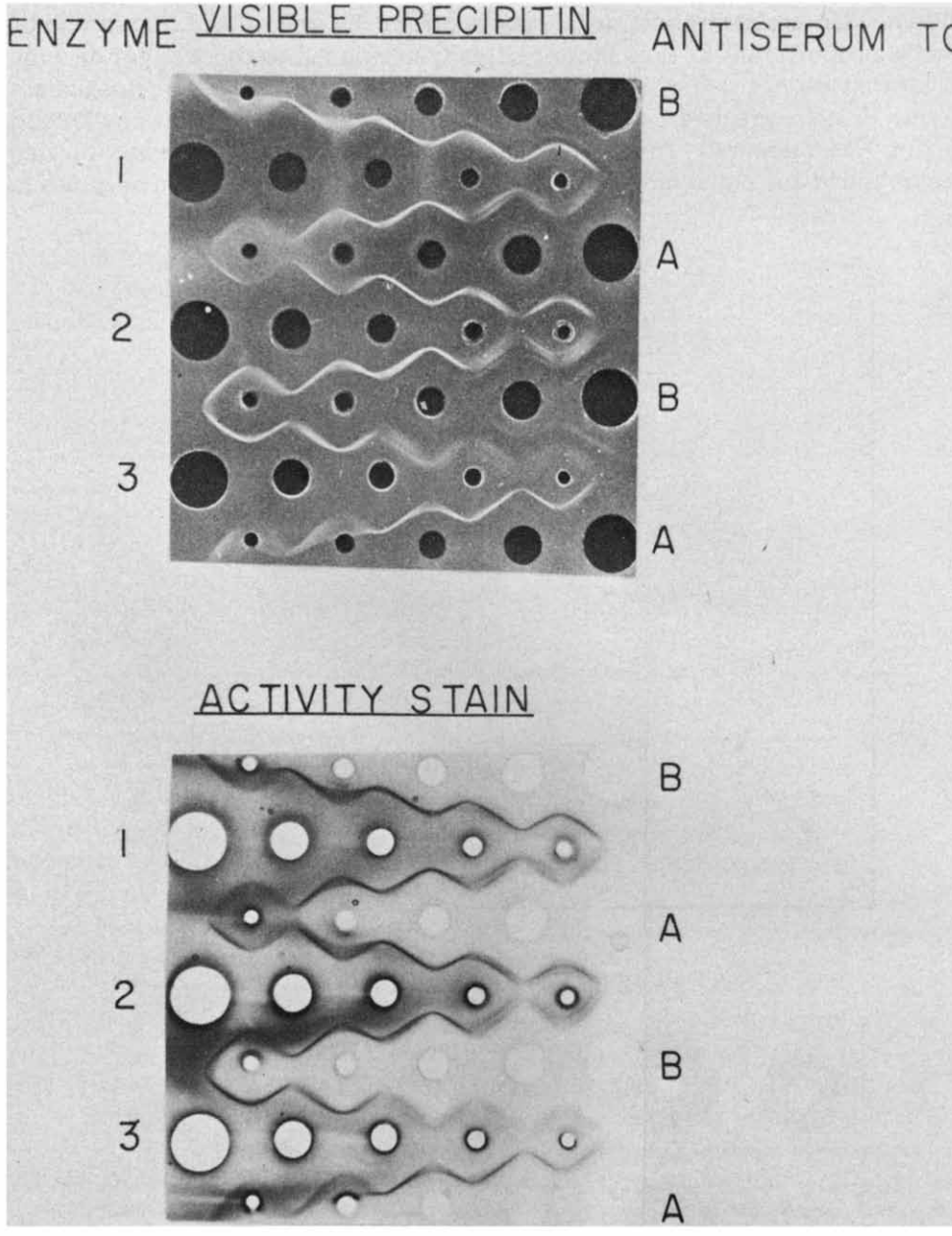


Fig. 8. Double diffusion of antisera raised against the "B" and "A" form preparations of human placental alkaline phosphatase tested against various preparations of enzyme. Antiserum to "B" form was obtained 3 months after primary immunization; antiserum to "A" form was obtained 9 months after primary immunization. Enzyme preparations represent (1) 150 000 × *g* (max.) supernatant of 100 placentas, 40 units/ml, specific activity 0.2 μmol · min^{-1} · mg^{-1}, (2) 150 000 × *g* (max.) pellet, butanol extract from 1000 placentas, 40 units/ml, specific activity 28 μmol · min^{-1} · mg^{-1}, (3) 6000 × *g* (max.) pellet, butanol extract from 400 placentas, 40 units/ml, specific activity 4 units. Diffusion proceeded for 48 h at room temperature (24°C) after which the plate was exhaustively washed with multiple changes of saline over a 3-day period.

active protein concentration since at equlibrium the area of the ring should be directly proportional to the amount of enzyme applied to the well for enzyme species that do not differ in specific activity. The specific alkaline phosphatase enzyme protein precipitin can then be identified by staining for enzyme and the ring size measured. The result of such an experiment, comparing the ring sizes obtained for equal amounts of activity of "A" and "B" form enzymes in

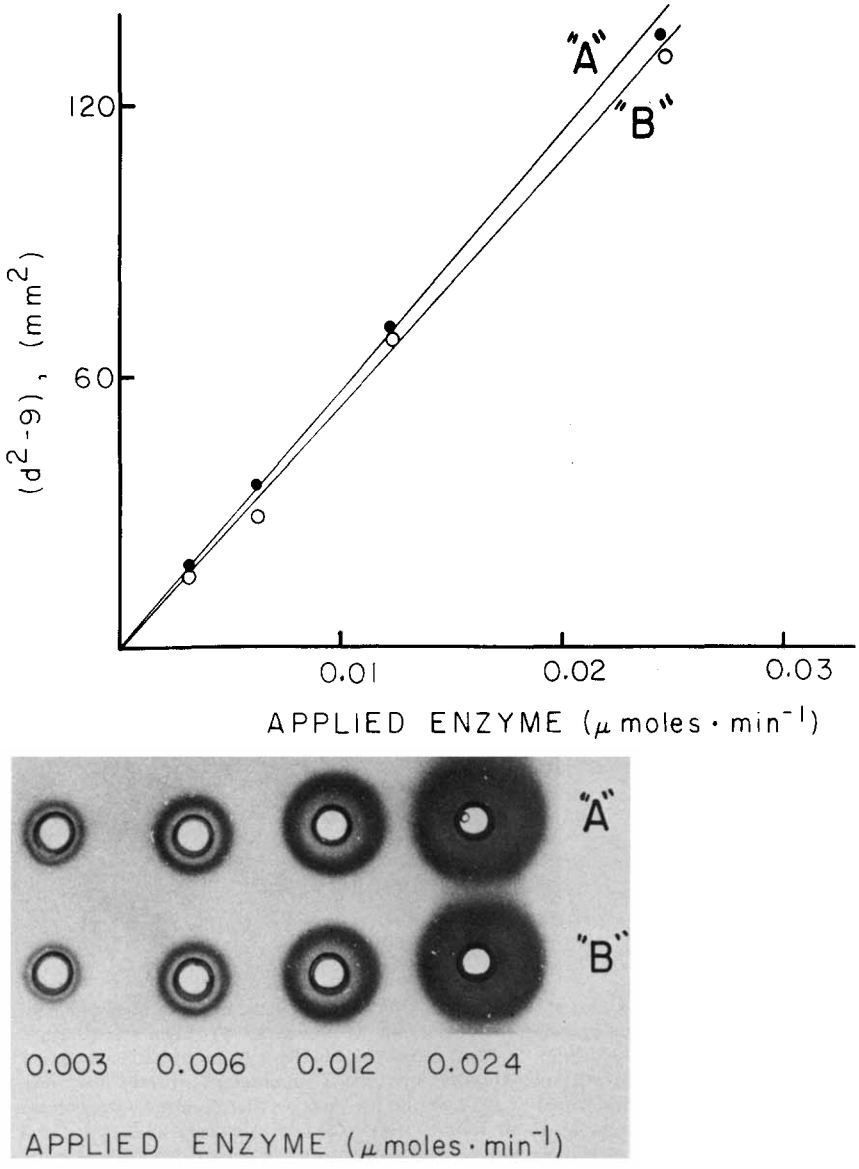


Fig. 9. Radial immunodiffusion of chromatographically separated human placental alkaline phosphatase preparations (Fig. 3). Plates were prepared as described in Methods, and the indicated amounts of enzyme were placed in the well. Diffusion was allowed to proceed for 96 h at room temperature (24°C) after which the plates were stained for enzyme and the ring sizes measured.

gels containing 0.5% Triton X-100 detergent, is shown in Fig. 8. Clearly, the ring size obtained is directly proportional to the amount of enzyme activity applied to the well and so the amount of enzyme protein measured by immunoquantitation is identical for the two enzyme forms. This indicates, as does the SDS electrophoresis data, that two-thirds of the "B" form protein determined by the Lowry procedure, is not alkaline phosphatase. The inability to see this non-phosphatase protein on SDS electrophoresis is perhaps due to its not being fixed in SDS electrophoresis during the staining procedure, or to its being a Lowry-positive, non-protein moiety attached to the enzyme.

Discussion

The properties of the "A" and "B" enzyme forms are summarized in Table III. The "B" form enzyme is lower in specific activity, higher in molecular weight, has less protein separable by SDS gel electrophoresis identical with the "A" form subunit and is difficult to electrophorese and chromatograph in the absence of non-ionic detergent. Antisera raised against the "B" form are not monospecific, and the "B" form is identical to the "A" form in the number of immunoreactive sites per unit enzyme activity as measured by radial immunodiffusion.

These data point strongly to the conclusion that the "B" form enzyme is an aggregate of the "A" form enzyme, i.e. the protein species which bears the catalytic site, and some other ligand or ligands with strong affinity for DEAE-Sephadex, phenylalanine-Sepharose, and possibly acrylamide gel. Affinity for acrylamide would account for the requirement of Triton X-100 detergent in the gel matrix in order to effect electrophoretic resoluton of the "B" form especially since there is no such requirement for resolution by starch gel electrophoresis [3—7].

It is interesting to note that the isoelectric pH (p*I*) of the "B" form enzyme is more than 2.5 units higher than the "A" form. This would indicate that the moieties associated with the "B" form are alkaline. If they were highly charged in the acetic acid/isopropanol mixture, they might not have been fixed and hence would not be visible by this staining procedure.

Attempts to establish conditions for ready dissociation of the "A" form enzyme from "B" form enzyme under non-denaturing conditions have not met with success to date. This association is stable to precipitation with methanol, ethanol, propanol, dioxane and ethanol/ether (1:1) but not to storage for several months in 0.25 M Tris base, pH 10.5. This coincides with previous observations [10,26] that prolonged incubation changed the enzyme form. The forms are stable when stored in 20% glycerol, 0.05 M Tris/acetate, pH 8.0, containing 100 μM $ZnAc_2$, as seen in Fig. 6 for the "B" form enzyme which was stored at $-20°C$ for over 2 years.

Wallach and Winzler [12] have pointed out that attempts at the chromatographic separation of membrane-bound proteins have been frustrated by the poor resolution of these proteins in most chromatographic systems. In this work, we have described the properties of two forms of human placental alkaline phosphatase; the "B" form resists resolution by chromatographic techniques while the "A" form behaves like most soluble proteins and can readily

be purified to homogeneity. Perhaps identification of the contaminating species of non-alkaline phosphatase moieties associated with the "B" form would provide a clue as to the reason for the poor resolution of other membrane-associated proteins in chromatographic and electrophoretic systems. Such an analysis should probably await definition of the conditions for ready dissociation of "A" form enzyme from the "B" form under non-denaturing conditions.

It is interesting to compare the "A" and "B" forms of human placental alkaline phosphatase with the hexosaminidases "A" and "B" studied in relation to Tay-Sachs' and Sandhoff's diseases. First of all, the two forms of hexoaminidase 2—3-fold in their specific activities and by 2.5 units in their isoelectric pH values [27]. They have a similar subunit molecular weight [28], and the form with the lower specific activity (the "A" form in this case) has antigenic determinants which are not shared by the other form [29]. Significant differences are the ability of both forms to enter polyacrylamide gels [27] and the identity of their molecular weights by several criteria [27,28]. A model has been proposed which suggests that the similarity in molecular weights of these two enzymes is due to association in the "A" form of a catalytically active subunit with a subunit that regulates its activity or alters its substrate specificity toward GM_2 ganglioside [30].

A similar functional alteration may occur for alkaline phosphatase. Its physiological role has not yet been defined, but it is possible that this function is carried out by the electrophoretically slow-moving "B" form enzyme. This is the form that predominates in all tissues in its membrane-associated state [31]. By this logic, the "A" form enzyme would be the precursor of the membrane-associated "B" form enzyme. Studies on the sequence of incorporation of labeled amino acids into "A" and "B" form enzymes should determined whether this is the case. The immunoabsorbent chromatography procedure will be valuable for these studies.

It should be noted that the "B" form enzyme is retained at the origin using polyacrylamide gel electrophoresis in the absence of Triton X-100 detergent, but not using starch-gel or microzone technique [3—8,10]. It is distinct from the "high molecular weight" phosphatase found in some clinical samples (for review, see refs. 1 and 31) which is retained at the origin using all three techniques.

The "B" form enzyme is not membrane-associated after butanol extraction. It does, however, contain bound lipid (Doellgast, G. and Waite, M., unpublished observation), and may therefore be binding to acrylamide by a hydrophobic association, which can be reversed in the presence of Triton detergent. Experiments to test this possibility are currently underway.

Luduena and Sussman [32] have compared KB cell alkaline phosphatase with human placental alkaline phosphatase. It should be noted that their KB cell enzyme, which required Triton X-100 detergent to enter the polyacrylamide gel, resembled the "B" form enzyme while their placental phosphatase preparation resembled the "A" form enzyme. Further, their HeLa preparation was exclusively "A" form enzyme, whereas Singer and Fishman [33] have noted a predominance of "B" form phosphatase in HeLa cells. The data of Luduena and Sussman on KB cell alkaline phosphatase does support two subunits having [^{32}P]phosphate-binding sites and may well represent a hybrid

isoenzyme of two non-identical subunits. Several of the properties reported as unique for this phosphatase, however, such as decreased electrophoretic mobility, inability to enter polyacrylamide gels in the absence of detergent and appearance in the void volume of G-200 Sephadex, resemble those of the "B" form phosphatase found in every placental extract [3—7] and in HeLa cells monophenotypic for placental-type alkaline phosphatase [33].

The method of immunoaborption chromatography, introduced by Pitarra et al. [13] and Hoag et al. [14] and developed further in this work, is a simple method for purification of human placental alkaline phosphatase. The enzyme can be separated from soluble non-enzyme proteins very effectively, but preparation of enzyme by this technique is complicated by leaching of contaminant proteins from the Sepharose column at an alkaline pH. Also, the "B" and "A" forms differ in specific activity (Table III) and, therefore, contribute variably to the specific activity of immunoabsorbent-purified enzyme.

By a three-step procedure following immunoabsorbent chromatography consisting of gel filtration, ion-exchange chromatography and salt-mediated hydrophobic chromatography, a preparation of "A" form enzyme could be produced in good yield that was homogeneous by the criteria of disc-gel electrophoresis, SDS gel electrophoresis, and the ability to raise a monospecific antibody response in rabbits one year after primary immunization. These criteria satisfy the requirements of the techniques of radioimmunoassay and immunohistochemistry for reagent purity and allow us to avoid the costly and uncertain immunoabsorption against normal human serum and tissue extracts [24,25] to obtain antisera of sufficient specificity to be used for these techniques. Thus, although the last three chromatography steps provided only a slight increase in specific activity, they did accomplish the efficient removal of trace protein contaminants.

The order of the last three chromatography steps (i.e. (1) gel filtration, (2) ion exchange, and (3) salt-mediated hydrophobic chromatography) offers several advantages. First, the gel-filtration step efficiently separates the "A" and "B" forms of the enzyme [10]. This is important since there is some overlap of the elution of these two forms on ion exchange (Fig. 4) and salt-mediated hydrophobic chromatography. Second, 0.05 M Tris/acetate, pH 8.0, with which the G-200 Sephadex column is equilibrated, is the same buffer that is used to bind the enzyme to the ion-exchange column. Also, the peak eluate from the ion-exchange column can be brought to a higher ammonium sulfate concentration without first concentrating it and can then be applied to the L-phenylalanine-Sepharose column. Thus neither dialysis nor concentration is required except for concentration of enzyme after the immunoabsorbent chromatography step and the concentration of the final product from the salt-mediated hydrophobic chromatography step. By use of the three chromatography steps together, complete separation from trace contaminants was achieved.

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