PURIFICATION OF PLACENTAL ALKALINE PHOSPHATASE

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SUMMARY

1. Alkaline phosphatase has been prepared in a highly-purified form from human placental tissue.

2. Two methods have been used for extracting the enzyme, *i.e.* the butanol extraction procedure of MORTON and the acetone–toluene–ethylacetate autolysis procedure of ALBERS.

3. The specific activity of alkaline phosphatase prepared by the butanol extraction method was 1150 KING-ARMSTRONG units/mg N, and that prepared by the autolysis method was 990 units.

4. The highly concentrated alkaline phosphatase was further purified by paper electrophoresis. The resulting enzyme preparation had a phosphatase activity of 1750 KING-ARMSTRONG units/mg N. This was less than was calculated to be present on the paper electrophoretic strip. But it was comparable with the highest activities obtained by other workers, when converted to their units by calculation. Placental tissue thus seems to have yielded as highly active a phosphatase as other tissues previously studied.

5. An attempt to purify alkaline phosphatase with an ion exchange resin (Dowex-2) was not successful.

INTRODUCTION

The preparation and purification of alkaline phosphatase has been the subject of much investigation. Sources rich in the enzyme have usually been used, such as kidney, whole intestine, intestinal mucosa, bone (normal and rickitic), and faeces. The methods used vary from simple water extraction followed by precipitation with ethanol and ether[1], to autolysis of the tissue with water and ethyl acetate[2] followed by fractionation with acetone[3] and (in other studies, ABUL-FADL AND KING[4]) by tryptic digestion and further fractionation. MORTON[5,6] introduced butanol extraction which he found to disrupt the lipoprotein, without denaturing the enzyme. He obtained a highly active preparation from both milk and calf intestinal mucosa. Preparation and concentration of placental alkaline phosphatase has been described in two previous papers[7,8]. Its further purification, by fractional precipitation and paper electrophoresis, is described here.

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EXPERIMENTAL

Collection and cleaning of placentas

The placentas were collected from the maternity ward as fresh as possible. The cord and membrane were removed, and the placenta freed from blood before clotting occurred: it was otherwise very difficult to get rid of clots and sometimes impossible. Perfusion with saline or saline containing heparin was of value only if the placenta was quite fresh. The best way to free the placenta of blood is to use a strong jet of water, cannulating one artery (or vein) after another until the whole organ is blanched. The cleaning of one placenta took about 1.5 h. Whenever it was not possible to begin the isolation and purification procedures immediately, the organ was stored at -15° after cleaning.

Preparation of aqueous placental extract

The placenta, cleaned as described above, was cut into small pieces, dried with blotting paper and weighed. It was then minced in a mechanical mincer, mixed with an equal volume of water and homogenized in a waring blender for 2 min. The homogenate was divided into 4 equal parts; one was left untreated, to the second an equal volume of butanol was added, the third was treated with 10 % of its volume of toluene, and to the fourth was added 10 % of its volume of toluene–ethyl acetate (1:1). All were stirred continuously for 30 min at room temperature. A sample of each mixture was centrifuged at 2000 rev./min for 15 min. The enzyme activity was determined in the aqueous layers at different pH values, using the phenyl phosphate method of KIND AND KING[9] (amino-antipyrine–ferricyanide) and expressing the result as KING-ARMSTRONG[10] (K-A)units, *i.e.* mg phenol liberated in 15 min.

The remainder of each of the mixtures was allowed to stand for 24 h at room temperature, and the enzyme activity then determined at the optimum alkaline pH. (The acid phosphatase was destroyed in all the mixtures except that containing only water.) Liberation of alkaline phosphatase in the solution was best with butanol, whether the tests were performed immediately or after 24 h. Results are shown in Table I.

Other organic solvents were compared with butanol for their efficiency in

TABLE I

ENZYME ACTIVITY AT DIFFERENT pH VALUES OF PLACENTAL EXTRACTS PREPARED BY DIFFERENT METHODS

Enzyme	*Enzyme activity in* KING-ARMSTRONG *units/g wet tissue in extracts separated at 30 min (and at 24 h)*									
	pH 3.6	*5*	*5.6*	*6.5*	*7.4*	*8.4*	*9.4*	*9.7*	*10*	*10.5*
Fresh water extract	0	3	2	0	5	7	35	48	60 (59)	57
Butanol extract	0	0	0	0	3	11	62	85	107 (105)	103
Toluene extract	0	0	0	0	1	6	34	44	56 (60)	54
Toluene + ethyl acetate extract	0	0	0	0	3	8	20	30	40 (50)	38

bringing alkaline phosphatase into solution. They are as follows, and the relative activities of their extract, compared with butanol, are given as numbers in parentheses: butanol (1), amyl alcohol (0.8), isopropyl ether (0.7), toluene (0.7), water alone (0.7), toluene + ethyl acetate (0.6), carbon tetrachloride (0.5), cychlohexanol (0.5), chloroform (0.4), propanol (0.2). The results emphasize the superiority of butanol.

Comparison of ALBERS' *autolysis method with* MORTON'S *butanol extraction method*

ALBERS' *autolysis method*

Extraction of enzyme: The mixed clean placentas were homogenized with an equal volume of water for 2 min. Pure acetone was added slowly with continuous stirring until a final concentration of 25 % was obtained. Toluene–ethyl acetate mixture (1:1) was then added (10 %). The whole mixture was thoroughly stirred for 1 h and then allowed to stand at room temperature for 3 days with occasional stirring. The mixture was squeezed through muslin, and centrifuged at 2000 rev./min for 30 min. If the supernatant solution was turbid, it was filtered through fluted filter paper. The specific phosphatase activity at this stage ranged from 9 to 13 K-A units/mg N. The clear aqueous solution showed no precipitate when adjusted to pH 4.9 with acetic acid. It was brought to pH 6.5 with NaOH.

Fractionation with acetone: The enzyme solution was chilled to —5°, and acetone at the same temperature added to a final concentration of 55 %. After 30 min at —5°, the solution was centrifuged and the precipitate washed 3 times with cold acetone and dried under vacuum over $CaCl_2$. The crude acetone powder was thus collected from 16 placentas. The specific activity of the powder was 80 K-A units/mg N. Attempts at further purification with acetone were not successful.

Ammonium sulphate fractionation: A 2 % solution of the enzyme powder was prepared, any insoluble material being removed by centrifugation. To the clear solution $(NH_4)_2SO_4$ powder (previously finely ground) was added until 0.4 saturation was obtained, the formula of KUNITZ[11] being used to calculate the amount of $(NH_4)_2SO_4$. The precipitate was centrifuged off and discarded. To the clear supernatant more $(NH_4)_2SO_4$ was added to raise its concentration to 0.5 saturation. The precipitate formed was separated by centrifugation, dissolved in water, and dialysed in a cellophane bag against running tap water overnight and then against frequent changes of distilled water. The specific activity of the enzyme solution was now 225 units/mg N.

Refractionation with acetone: The pH of the enzyme solution was adjusted to 6.2 with dilute acetic acid. The solution was chilled and acetone added to 35 %. The small precipitate was removed and more acetone added to 50 %. The precipitate was centrifuged, washed with chilled acetone and dried under vacuum over $CaCl_2$. This powder had a specific activity of 550 K-A units/mg N.

Refractionation with acetone between 35 and 45 % gave a preparation of 990 units/mg N. No further purification could be achieved by further refractionation with acetone.

MORTON'S *butanol method*

Conditions for butanol extraction: The effectiveness of extraction is influenced by the amount of butanol used: 1.5 ml/g is the minimum favourable amount. It is

necessary to stir vigorously for at least half an hour in order to obtain the thorough emulsification necessary for an enzyme-rich aqueous layer. Warming the butanol emulsion to 35° for 5 min before centrifuging was found very effective in causing the colloidal particles to coalesce, so that a clear aqueous layer was obtained after centrifuging. This treatment did not affect the enzyme activity.

The optimum pH for both the extraction of the enzyme and for its maintenance was 6.5. In the presence of butanol the enzyme solution was stable in the cold room (+ 5°) for about 7 days. Inactive protein can best be precipitated at pH 4.9. Acetone precipitation of enzyme-rich material is best carried out at —5° at pH 6.5.

Preparation of enzyme extract with butanol: One or more placentas were cleaned, weighed, cut into small pieces, minced and homogenized with an equal volume of water for 2 min. The pH was adjusted to 6.5 with *N* acetic acid. Butanol (1.5 ml/g placenta) was added gradually with continuous vigorous mechanical stirring for at least 30 min. The homogenate became cream-coloured and well emulsified. It was squeezed through muslin, and heated in a water bath to 35° for 5 min, when it was poured quickly into previously cooled 250 ml cups and centrifuged for 45 min at 1800 rev./min at —4°. The mixture separated into four layers, *i.e.* a heavy precipitate, a clear yellowish aqueous zone, over-laid by light particles, and a surface layer of excess butanol containing dissolved material. The aqueous layer was sucked off carefully, but it was not usually obtained free from some suspended material. It was therefore filtered through a thick layer of Hyflo supercel on Whatman No. 1 filter paper on 100 ml Buchner funnels. The filtration did not affect the enzyme activity, which was 15 K-A units/mg N at this stage.

Acidification of aqueous extract: The solution was brought to pH 4.9 by the careful addition of dilute acetic acid. It turned cloudy and was left at room temperature for 30 min to allow for complete precipitation of inactive material. The precipitate was removed by filtration. (There was no loss of enzyme activity during filtration.) The clear filtrate was adjusted to pH 6.5 with 0.1 *N* NaOH. The specific activity was now 40 K-A units/mg N.

Fractional precipitation with acetone: The enzyme solution was cooled to —1° and chilled acetone (—5°) was added carefully with continuous stirring to 30 %, maintaining the temperature at —0°. After 1 h at —5° the solution was centrifuged at 1800 rev./min for 20 min. The supernatant (still at —5°) was treated with more chilled acetone to 40 %. The precipitate was allowed to settle and then centrifuged, and washed thrice with chilled acetone. The powder was dried *in vacuo* over $CaCl_2$, and stored at —15°. Several batches of placentas, 36 in all, were worked up in this way. The pooled acetone-dried powder had a specific activity of 135 K-A units/mg N. Further fractionation with acetone was unprofitable at this stage.

Fractional precipitation with ammonium sulphate: A 2 % solution of the enzyme powder was prepared, any insoluble material being removed by centrifugation. Powdered $(NH_4)_2SO_4$ was added to 0.4 saturation, a precipitate removed and discarded and more $(NH_4)_2SO_4$ added to 0.5 saturation. The precipitate was separated by centrifugation, dissolved in water, and dialysed in a cellophane bag against running tap water overnight and then against distilled water. The specific activity of the enzyme solution was 475 units/mg N.

Refractionation with acetone: The enzyme solution was adjusted with 0.05 *N* NaOH to pH 6.2, cooled and treated with acetone. The fraction obtained between

TABLE II

ENZYME ACTIVITY AT DIFFERENT STEPS OF PURIFICATION OF PLACENTAL ALKALINE PHOSPHATASE

Procedure	*Total units* × 10^3*	*Units*/mg N*	*Yield (%)*
ALBERS' *autolysis method*			
Original homogenate		1	
Aqueous extract	90	3	100
Filtered autolysate	75	10	83
Acetone fractionation	70	80	78
Ammonium sulphate fractionation 0.4–0.5	35	225	39
Acetone fractionation (35–50%)	20	550	22
Acetone refractionation (35–45%)	12	990	13
MORTON'S *butanol method*			
Original homogenate		1	
Aqueous extract	190	3	100
Butanol aqueous layer	170	16	89
Butanol aqueous layer after acidification	165	40	87
Acetone fractionation (30–40%)	150	135	79
Ammonium sulphate fractionation 0.4–0.5	80	475	42
Acetone refractionation (35–45%)	35	1150	18

* Units = KING-ARMSTRONG units.

35 and 45% was collected, washed carefully thrice with cold acetone and dried *in vacuo* over $CaCl_2$. The specific activity of the powder was now 1150 K-A units/mg N. Further refractionation did not increase the specific activity; nor did treatment with activated charcoal.

Table II shows the results of the attempted purification by both the ALBERS and MORTON methods.

Purification by paper electrophoresis

LEVY AND MAZIA[12] achieved a three-fold increase in activity of renal alkaline phosphatase by paper electrophoresis. Elongated tracks were obtained, and the enzyme was only partially separated from accompanying protein. ROCHE AND BOUCHILLOUX[13] electrophoresed intestinal phosphatase, which separated into three components only one of which was active. BAMANN AND TIETZ[14] employed continuous electrophoresis successfully for the separation of lipase.

Apparatus: The electrophoresis boxes used were similar to those described by GRASSMAN AND HANNIG[15] and by TISELIUS AND FLODIN[16]. The filter paper strips were of Whatman No. 1, and were 28 × 4 cm.

Reagents: The buffer was phosphate of pH 8.0. The staining solution was 1% lissamine green in 15% acetic acid. The washing solution was 2% acetic acid. For oiling, a mixture was made of 34 ml liquid paraffin, 17 ml bromonaphthalene, 20 ml xylene and 1 ml sorbitol monooleate. In order to reveal the presence of the alkaline phosphatase on the filter paper, a spraying solution was made of 0.01 *M* *p*-nitrophenyl phosphate in 0.1 *M* sodium carbonate–bicarbonate buffer[17] (1:1) of pH 9.9. This solution was prepared in small amounts and kept in the refrigerator.

Method: The enzyme preparation of specific activity 1150 K-A units/mg N was used. 50 mg were dissolved in 1 ml distilled water. The filter paper was immersed

in the buffer, spread over a glass sheet and blotted lightly with dry filter paper, and then applied to the holder. 0.02 ml of the enzyme solution was run on to the edge of a glass slide, which was then applied across the strip about 9 cm from the cathodic end. A second strip was similarly treated, and the holder with its two strips placed in the apparatus. Care was taken that the horizontal part of the filter paper was well stretched and that the vertical parts dipped in the buffers were of equal length.

Six boxes were used at a time, thus running 12 strips simultaneously. After 20 min to attain equilibration, the current was switched on and maintained for 16 h (*i.e.* overnight).

One strip was dried at 95° for 20 min, stained with lissamine green for 15 min, washed in 2 % acetic acid until excess dye was removed, and finally dried at 95° for 10 min. It was then oiled and allowed to stand upright on a filter paper sheet to drain. After 20 min it was scanned in the automatic recording densitometer of LAURENCE[18] using a narrow-cut red light filter (Ilford 205) combined with a neutral filter of density 0.5 (*cf.* Fig. 2).

Another strip was left at room temperature until almost dry and then in a 37° oven until quite dry. It was sprayed lightly with *p*-nitrophenyl phosphate solution. A yellow band appeared almost immediately, due to the *p*-nitrophenol liberated at the site where the phosphatase activity resided. Having thus identified the segment of the filter paper on which the phosphatase activity appeared, the corresponding segments in the remaining ten sheets were cut out and collected. They were stored in a jar in the deep freeze. The operation was repeated with twelve more papers.

Elution: A Whatman No. 1 filter paper (28 × 4 cm) was folded double, and the closed end trimmed so as to form a blunt wedge, a triangle (1.5 × 3–4 cm) being cut from either side of the folded end (see Fig. 1*). The effect of this was to leave a blunt

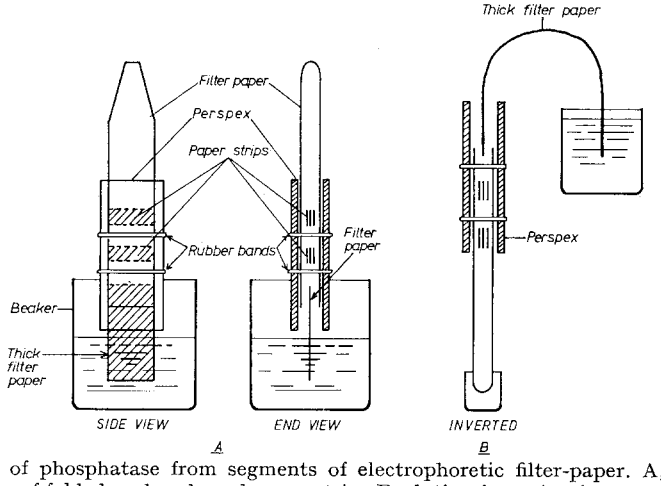

Fig. 1. Elution of phosphatase from segments of electrophoretic filter-paper. A, elution from segments into tip of folded wedge-shaped paper strip; B, elution from tip of the wedge into water.

* The apparatus used to elute the electrophoretic strips was a modification of one used by our colleague, Dr. J. L. REIS, to whom we are grateful. It was found desirable to use a folded tip because a large amount of material could then be transferred during one operation. A single tip proved to be too thin and insufficiently rigid. Drying of the tip would occur if the transfer were carried out at room temperature, but was avoided by conducting the operation in the refrigerator. This apparatus and procedure are very efficient for dealing with small volumes of solution, and nearly 100 % recoveries can be obtained.

tip of 1 cm in width. The segments of filter paper (22) containing the enzymically active material were placed between the fold in two adjacent lots, about one-third of the distance from the open end of the folded strip. A thick filter paper strip was now placed in contact and in between the folded filter strip. The other end of the thick filter paper was intended to dip into a vessel containing distilled water. A strip of transparent plastic (perspex, 8 cm × 4.5 cm), was placed on each side of the above arrangement, and the two held together by two stout rubber bands. The assembly was held in a upright position, the thick paper dipping into a beaker of distilled water, with the ends of the two plastic strips just clearing its surface. The wedge-shaped upper end of the folded strip, projecting in a vertical position, slowly became saturated with the enzymically most active material, which was eluted from the 22 pieces of filter paper used for electrophoresis contained between the folded strip and which passed slowly up the strip by osmosis to concentrate in its tip. The whole procedure was carried out in the cold room overnight. After 16 h the assembly was inverted, the filter paper wick bent over to dip into a fresh beaker of water, and another small beaker without water placed below the wedge-shaped end of the folded strip, now in the bottom position. The enzymically active material in the tip now eluted into the small bottom beaker, as water passed slowly up over and down the thick paper strip, through the folded paper to its tip and dripped into the small beaker. The volume of the eluate was usually about 1 ml.

Dialysis

The eluate contained besides the enzyme a considerable amount of buffer salts. These were eliminated by dialysing the eluate against distilled water in the refrigerator overnight. The cellophane bag containing the eluate was filled so as to contain no vacant space.

The specific activity of this isolated purified enzyme was 1750 K-A units/mg N. On paper electrophoresis, it now gave only a single band, and this was in the same position whether stained with lissamine green or with *p*-nitrophenyl phosphate.

Activity

If we convert the activity of our preparation of 1750 K-A units to MORTON units by multiplying by 57 (the average conversion figure for placental phosphatase in our hands) we get a specific activity of 99,750 Morton units/mg N. This is more than MORTON's highest activity (83,530) and more than double that of ROCHE *et al.*[3,19]. But such conversions are unreliable, and the most that can be said is that we appear to have obtained a highly-active placental alkaline phosphatase whose activity seems to be about that of the most active alkaline phosphatases previously prepared. When run in veronal buffer pH 8.6 and phosphate buffer pH 8, it was electrophoretically homogeneous, but the only sense in which it could be claimed to be "pure" is that it seemed to be free of extraneous protein.

Fig. 2 shows the proportion of the total, before elution, which was enzymically active, *i.e.* that which stained yellow with *p*-nitrophenyl phosphate. It was 22 % of the whole lissamine green-stained protein area. In another preparation it was 25 %. If it be assumed that this area corresponds to the pure enzyme, then it would have a specific activity of about 4,600 units/mg N, since the enzyme solution used in the electrophoresis had a specific activity of 1,150 units. The specific activity of the eluted

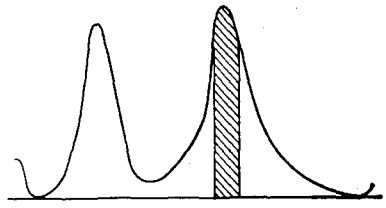

Fig. 2. Tracing of electrophoretic filter paper strip of purified phosphatase (1150 units/mg N) before elution. ———, total protein stained with lissamine-green; hatched area, phosphatase stained with *p*-nitrophenyl phosphate.

purified enzyme (1,750 units) was therefore much less than the amount conjectured from the assumption that the shaded area represented pure phosphatase. The discrepancy could be due to the conjecture being wrong or to a loss of enzyme activity in the elution and dialysis processes. Exposure of the enzyme to an alkaline pH for 16 h at room temperature may well have destroyed some of it. There may also have been a loss during the elution and dialysis, though these were carried out in the refrigerator and at approximate neutrality. Also, there may have been a loss of some co-factor, though there is no indication of this (compare ref. 8). Similar losses were experienced by ROCHE AND BOUCHILLOUX[13] who found that the specific activity of the active protein which they eluted after electrophoresis of intestinal phosphatase was less than the calculated one. CARLSON[20] used a vertical starch column for the electrophoretic purification of alkaline phosphatase. He found all the enzyme activity in a moving protein component which was 32% of the total protein. This should have given him a 3-fold enrichment of the enzyme, but his isolated phosphatase was only twice as active. Continuous electrophoresis might be a more profitable procedure to obtain further purification of alkaline phosphatase.

Ion exchange

BOMAN[21] used a Dowex-50 to purify crude prostatic acid phosphatase 60-fold. BOMAN AND WESTLUND[22] separated alkaline phosphatase from acid phosphatase in human serum with Dowex-2. They also reported a 200-fold purification of red cell acid phosphatase. In a personal communication, Dr. BOMAN advised us to use Dowex-2 in trying to purify placental alkaline phosphatase.

The apparatus used was a column 20 × 2 cm contained in a glass tube with a tap at its lower end. The lower end of the column was filled with glass wool. The column received a preliminary treatment with *N* NaCl, which served to convert it to the chloride form.

Tris buffer was used at pH 7.2, and at strengths 0.02, 0.1, 0.2 and 0.4 *M*.

Method: A purified enzyme powder, prepared by the butanol extraction procedure, was used. It had a specific activity of 130 K-A units/mg N. 0.1 g was dissolved in 2 ml Tris buffer (0.02 *M*) and the solution centrifuged. The clear supernatant was collected and applied to the top of the column (which had been previously washed through with 0.02 *M* Tris buffer). The column was treated successively with step-wise increases of molarity of buffer solution (0.02, 0.1, 02., 0.4 and 1 *M*). Fractions were collected every half-hour with an automatic fraction collector. The volume of each fraction was about 2.5 ml. The enzyme activity was determined in each fraction, and the total amount of protein by measuring the extinction at 280 mμ in 1-cm cells in a Beckman DU spectrophotometer.

Result: Fig. 3 shows the results obtained. One curve represents the phosphatase

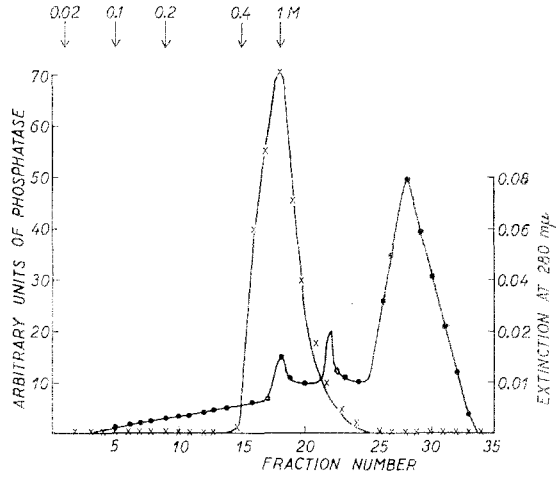

Fig. 3. Ion exchange separation of phosphatase. ×—×, Phosphatase; ●—●, Protein. Elution with 0.02–1 *M* Tris buffer.

activity, the other the extinction at 280 mμ. Most of the enzyme was eluted with the 0.4 *M* buffer. The extinction values showed that there are at least two inactive peaks in the solution. The recovery of the enzyme was about 50 %, and its specific activity increased from an initial 130 units to 230 units/mg N. The protein area under the phosphatase activity peak is about 23 % of the total. So one would expect a specific activity of about 570 units/mg N. Some loss of activity appears, therefore, to have taken place, as in the case of paper electrophoresis.

A further experiment was carried out with the highly-purified enzyme of 1,150 units/mg N, but no further increase of activity could be obtained.

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