CHROMATOGRAPHY OF PROSTATIC PHOSPHATASE

by

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INTRODUCTION

Many attempts have been made to apply chromatography to proteins and enzymes, and in a recent review ZITTLE[1] gives more than 250 references.

Up to now, it is, however, only in two types of chromatography and in a very limited number of cases, where it has been possible to separate proteins on columns. One of these types involves the use of phase systems with inorganic salt, water and cellosolve for partition chromatography. Since ZITTLE's review PORTER[2] has in this way investigated the chromatographical behavior of insulin and some other proteins.

The other type is ion-exchange chromatography which has proved successful in the isolation of some basic proteins (for earlier references see[1]). With this technique BOARDMAN AND PARTRIDGE[3] have also been able to separate different hemoglobins and more recently ion-exchange resins have been utilized by JOLLÈS AND FROMAGEOT[4] for the purification of a lysozyme from rat liver and by HIRS[5] for a chromatographic investigation of chymotrypsinogen.

The present communication describes a method for chromatographic purification of prostatic phosphatase on ionic exchange columns using pH gradient elution. The paper also reports a study of some purified enzyme samples. A preliminary report has earlier been published[6] but the technique has since that time been modified in several ways.

MATERIALS AND METHODS

The enzyme determinations

These were carried out with *p*-nitrophenyl phosphate as substrate according to BESSEY *et al.*[7]. The substrate solutions were 0.02 *M* in concentration and were made up from the disodium salt (Sigma Chemical Company) dissolved in a Sørensen's citrate buffer of pH 5.0 or 5.9. Of this solution 200 μl was incubated with 10 μl from the appropriate fractions for a period of 30 minutes at 37°. Carlsberg micro pipettes were used in pipetting. The reaction was started as well as stopped in an ice-bath[7]. After cooling 2 ml of 0.1 *N* NaOH was added. The amount of nitrophenol liberated was measured at 400 mμ directly in the test tubes (external diameter 1.1 cm) using a Beckman B spectrophotometer. A substrate solution treated as the test samples was used as a blank. One blank was included with each series of determinations. Only test tubes with the same blank extinction were used. The enzyme containing fractions were always diluted, 10–200 times in order to give a straight line curve for the liberation of nitrophenol for different enzyme concentrations. This dilution was ordinarily made with a citrate-phosphate buffer of pH 5.2 but in the case of the purest fractions it was necessary to stabilize the enzyme with serum albumin as found by LONDON AND HUDSON[8]. The amount of nitrophenol liberated was read off a standard curve constructed from measurements with pure *p*-nitrophenol (E. MERCK, Darmstadt). The final activities were always expressed as millimoles of substrate per liter of incubation mixture split during 30 minutes at 37° using the earlier mentioned proportions of enzyme and substrate.

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The total amount of protein material in the effluent was followed by measuring the ultraviolet extinction at 280 mμ in a Beckman DU spectrophotometer.

The citrate phosphate buffers used always had an extinction at 280 mμ of 0.01 to 0.03 and it has not been possible to find any citric acid or $Na_2HPO_4 \cdot 12\ H_2O$ without extinction. To some extent this light absorption must be due to impurities since buffer passed through a column sometimes but not always shows a lowered extinction. For this reason the starting buffer was used as the blank except in cases where the extinction had been lowered, in which buffer already passed through the column was employed. In spite of this arrangement the extinction curves often show some background absorption.

The risk of interference from low-molecular-weight resin material has been mentioned earlier[6]. This was only observed when the column was left for some weeks; the buffer standing in the column then had a fairly high ultraviolet extinction. As the active fractions after dialysis only showed decrease in extinction of about 4%, the enzyme seems to be nearly free from ultraviolet absorbing impurities of low molecular weight.

The nitrogen content of both the crude extracts and the purified enzyme fractions were followed by Kjeldahl analysis.

Enzyme solutions

When human semen was used as starting material, it was diluted five times with citrate-phosphate buffer of pH 5.2 and then centrifuged at 2,000 *g* for 20 minutes in order to remove all the spermatozoa and large cell fragments. The pH of the supernatant fluid was checked and it was usually necessary to add some 0.1 *M* citric acid in order to bring the pH down to 5.2. The adjusted solution of the seminal plasma was applied directly to the column. The dialysis employed earlier[6] can thus be left out if the pH is adjusted as mentioned.

If too concentrated solutions of seminal plasma are applied directly to the column, the buffer will not be able to control the pH during the run. Probably by some displacement procedure, some alkaline compounds in the first peak will give rise to a zone of pH 5.7–5.9. At this pH the phosphatase is only partly adsorbed and the run will thus show two enzyme peaks, one in the beginning and the other when the eluting agent has reached the pH of elution.

When prostatic tissue was used the enzyme was extracted from slices as recommended by LONDON AND HUDSON[8]. The frozen glands were cut into thin slices and left for 24 hours at 3°, in five times their weight of citrate-phosphate buffer of pH 5.2. After adjustment of the pH to 5.2 the extract was centrifuged at 2000 *g* for 20 min and finally filtered through Pyrex glass wool.

The enzyme solutions were always stored in the frozen state. In spite of this, precipitation of some inactive material often occurred after some time. Such precipitates were always removed by centrifugation before the solution was used.

Chromatographic method

The gradient elution method of chromatography was used. Its general principles have been outlined by ALM *et al.*[9] and the theory has recently been discussed by DRAKE[10]. The method was used in such a way that a higher pH buffer (in the following referred to as the elution buffer) continuously replaced a lower pH buffer (in the following referred to as the starting buffer), thus producing a pH gradient in the eluting agent.

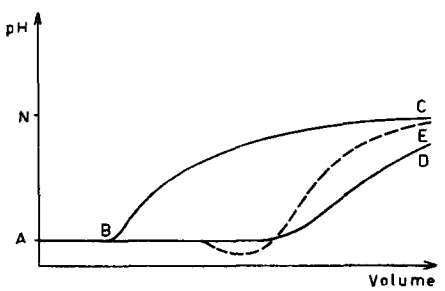

Fig. 1. Three different types of pH curves in gradient elution. A is the pH of the starting buffer and N that of the elution buffer.

The shape of a gradient can be varied in several ways by varying the sections of the vessels according to DESREUX[11], by regulating the in and out flow of the mixing chamber according to LAKSHMANAN AND LIEBERMAN[12], or by having two mixing chambers according to DRAKE[10].

When a closed mixing chamber is used as by ALM *et al.*[9] the outcoming gradient will have the shape of the curve ABC in Fig. 1 where the distance AB corresponds to the volume between the mixing chamber and the end of the column. There is, however, as pointed out by LAKSHMANAN AND LIEBERMAN[12], sometimes a disadvantage of having the steepest part of the gradient emerging almost immediately after the dead volume. This is particularly true when analysing a crude extract where there is material which passes right through the column as well as material that is eluted in the beginning of the gradient. In these cases the separation efficiency can be improved by using a pH gradient of the form ABD (Fig. 1). Such a curve is produced by two simple arrangements. In the first one the start of the gradient is delayed by eluting with the pure starting buffer until about twice the dead volume has

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passed through the column. The second one involves the use of an open mixing chamber and a vessel with a conical section for the elution buffer.

Fig. 2 shows one arrangement for work with a pH curve of the form ABD. The mixing chamber, F, is a piece of glasstubing 4.6 cm internal diameter and 20 cm long, with a rubber stopper as bottom. It has two outlets, one to the column and the other to the conical vessel, G. This consists of an 500-ml Erlenmeyer flask (Pyrex), connected to the mixing chamber by polyvinyl tubing. The dimensions of the columns used were about 3.1 cm × 25 cm. The bottom of the columns was a glass filter plate (Pyrex, number 1). It is convenient to have a ground and greased glass stopper at the top of the column as a rubber stopper gets slippery and loose during the regeneration of the resin with the 2 *N* NaOH.

For work with pH curves of the form ABC a gas washing bottle (Quickfit number MF 29/3) was used as mixing chamber. It had a volume of 315 ml but it can as well be only partly filled and in the experiment shown on Fig. 5 it did not contain more than 100 ml (stirring was effected magnetically). As pointed out earlier by ALM *et al.*[9] variations in the volume of the mixing chamber, here produced by only partly filling the gas washing bottle, will change the steepness but not the shape of the gradient.

When using gradient elution chromatography it is desirable to have an adsorbent to which the gradient producing substance is not adsorbed. Thus work with pH gradients requires an adsorbent without buffering capacity in the pH region of the gradient. The resin used was the sulfonated polystyrene ion exchanger Dowex 50 with 8% cross-linking (200–400 mesh). According to BAUMAN AND EICHHORN[13] the acid form of Dowex 50 is completely titrated at pH 4.0 and the resin is thus suitable for work with pH gradients above this value.

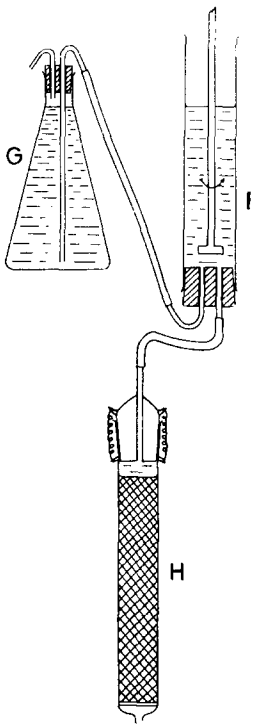

Fig. 2. Schematic drawing of the arrangements for work with pH gradients of the form ABD in Fig. 1. H is the column with Dowex 50, F is the mixing chamber (with stirrer) and G the container for the elution buffer. When beginning an experiment, F is filled with the starting (pH 5.2 or 5.5) and G with the elution buffer (pH 6.8). During the run, continuously increasing amounts of elution buffer are mixed with the starting buffer, thus producing a pH gradient in the eluting agent.

The commercial product must be pretreated according to MOORE AND STEIN[14]. If this is omitted no phosphatase activity can be eluted. After an experiment the column may be regenerated by washing with 2 *N* NaOH which hydrolyses and removes most of the irreversibly adsorbed material resting on the column. The regeneration is more effective if carried out at or above room temperature.

The rate of flow through the column is influenced by the proteins and with a fraction collector working on time basis, as the one used here, the volumes of the fractions will vary. This is, however, not of importance as the gradient elution should not give rise to symmetrical peaks even with a constant volume plot.

The rate of flow was regulated by adjusting the pressure of the eluting buffers. With a purified enzyme solution on the column a pressure of 30 cm water will give a rate of flow of 20–24 ml per hour. With the raw extracts a pressure of about 40 cm will give the same rate of flow. This high flow velocity does not seem to have any disadvantage and in the cases where only the first part of the gradient is used an experiment can thus be completed in 12–15 hours.

All experiments described here have been run in cold room (4°). It is possible to work at room temperature, with or without a water-cooling jacket, but both recovery and separation efficiency are better if the temperature is kept low.

An ordinary experiment is started in the following way. Of a prostatic extract of pH 5.2 prepared as described above, 10 ml is applied to the top of the column (3.1 × 25 cm) and allowed to sink into the resin by its own pressure. During that time the Erlenmeyer flask (G in Fig. 2) and its connection to the mixing chamber are filled with the elution buffer. The tubing between F and G is then closed with a screw clamp. It is important that this connection does not contain any bubbles as these often temporarily stop the free communication between the two vessels. When the extract has run into the resin, the mixing chamber F, its connection to the column and the volume between the resin and the glass stopper are filled with the starting buffer and the first part of the elution is begun. While the extract is entering into the column, fractions are shifted every 30 min. When the elution is started, the pressure of the buffer increases the flow so that a shift of fraction every 18 min gives volumes of around 7 ml. After about three hours the amount of starting buffer in the

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mixing chamber is adjusted to the same height as that of the elution buffer in the flask. The gradient is then started by opening the connection between the two vessels (F and G).

Choice of buffer system

When working with pH gradients it is valuable to have a buffer system with about the same buffering capacity over the entire region of the gradient. Further, to get gradients of the theoretical form, it appears to be necessary to have exclusively the same ions in the different buffers. In the experiments with closed mixing chamber published earlier[6] acetate was used as starting buffer and borate* as elution buffer. However, this always produced pH curves of the form ABE in Fig. 1 and similar results were obtained also with acetate and phosphate buffers. A constant sodium concentration did not alter the form of the curve.

The buffer system used throughout this investigation was the McIlvaine's citrate-phosphate buffer[15] which has several advantages. It has a buffer capacity between pH 2.5 and 7.5, it only contains two anions and in this particular case both citrate and phosphate seem to stabilise the prostatic phosphatase. These effects will, however, be further discussed later in this paper.

The stock solutions were 0.1 *M* citric acid and 0.2 *M* disodium orthophosphate. For work with raw extracts the starting-buffer was made from 4.64 parts citric acid and 5.36 parts phosphate solution giving a pH of 5.2, and for work with the purified enzyme it was made from 4.32 parts citric acid and 5.68 parts phosphate solution giving a pH of 5.5. The elution buffer had a pH of 6.8 and was made from 2.27 parts citric acid and 7.73 parts phosphate solution.

RESULTS

Two different types of phosphatase solutions have been studied chromatographically, raw extracts and partly purified enzyme fractions. Fig 3 shows the results from an experiment with seminal extracts. Here, and in the other figures the solid circles represent the phosphatase activities, the open circles the extinctions at 280 mμ, and the squares the pH values for the different fractions. The total amount of nitrogen applied to the column was 19.4 mg. When the gradient was started, the mixing chamber (here somewhat smaller, internal diameter 3.8 cm) contained 130 ml starting-buffer. As can be seen the enzyme is eluted between pH 5.5 and 6.0 as one single peak. The extinction values show that there is at least one inactive component just beginning to separate from the active material. The peak coming after the enzyme is probably the same substance which with a borate gradient[6] appeared as a tail after the enzyme. A large part of the nitrogen stays on the resin and about 30% of the amount applied can be found in the sodium hydroxide passed through the column during the regeneration.

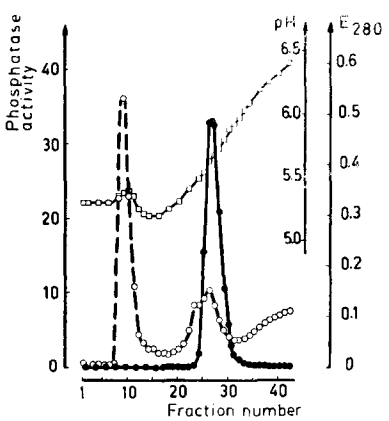

Fig. 3. Chromatographic experiment with seminal extract. Solid circles represent the phosphatase activity in millimoles of substrate split during 30 minutes at 37°, open circles the extinction at 280 mμ, and squares the pH values.

Fig. 4 gives the results of an experiment with a prostatic extract. As with the seminal extract, a zone with high extinction comes directly after the dead volume. The second peak corresponds to the active material. In contrast to the results obtained with the seminal extracts, the rest of the extinction curve varies rather much from one experiment to another. A possible explanation of these variations is the presence of some pH-labile products (compare the precipitations mentioned under *Enzyme Solutions*).

* There is probably a slow inhibition of the prostatic phosphatase from the borate but the activity can be reversed by dialysing.

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The break on the pH curve (fractions 32–45) is not regular and was probably produced in this particular experiment by some air bubbles in the connection between the flask and the mixing chamber.

The most active fractions from the experiments with prostatic extracts have a specific actitivity on a nitrogen basis which is about 33.8 millimoles split/mg N. Compared to the specific activities of the raw extracts, a figure which varies from one gland to another, this indicates a 20- to 30-fold purification of the enzyme. The enzyme fractions from the experiments with seminal extracts only have a specific activity of 18.6 millimoles split/mg N.

The recovery of the enzyme from experiments with the seminal as well as the prostatic extracts is usually 70 to 80%.

The enzyme fractions from experiments like those just described have been reinvestigated under different conditions. Fig. 5 illustrates one experiment with a closed mixing chamber of 100 ml volume. The two most active fractions from a run with a seminal extract were dialysed against the starting buffer of pH 5.2 and then applied to the column. Like in the experiments with the raw extracts the enzyme was eluted between pH 5.5 and 6.0. No other peaks were observed indicating that the peaks in Fig. 3 correspond to real components and are not artefacts produced by the resin or impurities in the buffers.

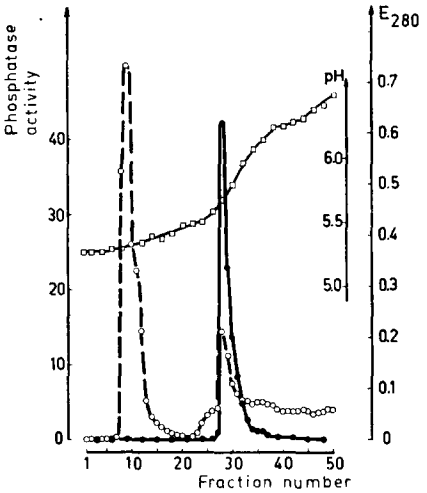

Fig. 4. Chromatographic experiment with prostatic extract. Solid circles represents the phosphatase activity given in millimoles of substrate split during 30 minutes at 37°, open circles the extinction at 280 mμ, and the squares the pH values.

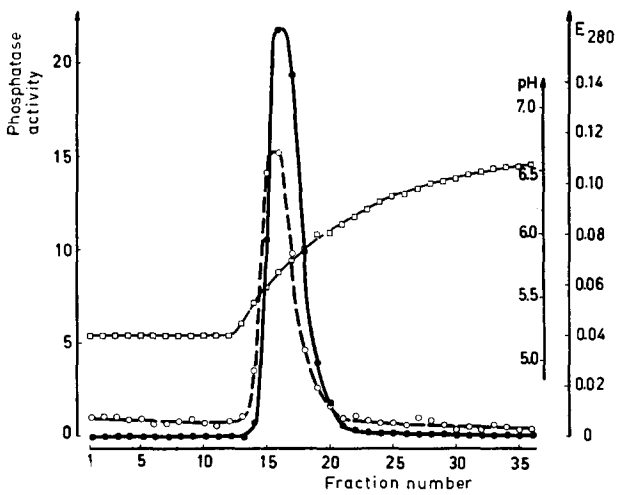

Fig. 5. Chromatography of a partially purified prostatic phosphatase obtained from an experiment with seminal extract. The ratio between activity and extinction increases three times between fractions 15 and 18 which indicates that the enzyme contains large amount of inactive material. Solid circles represent the phosphatase activity (millimoles of substrate split during 30 minutes at 37°), unfilled circles the extinction at 280 mμ, and squares the pH values.

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However, the figure shows that the zone with ultraviolet extinction travels somewhat faster than the active material. Also the ratio between activity and extinction at 280 mμ increases three times between fractions 15 and 18 (maximum activity in fraction 16) which indicates that the enzyme contains a large amount of inactive material.— In this experiment 70% of the activity was recovered.

If a starting buffer of pH 5.5 is used together with a weak pH gradient of the form ABD in Fig. 1 most of the ultraviolet absorbing material can be separated from the phosphatase activity. Fig. 6 and 7 shows the results of two such experiments performed in the same way but with slightly different materials. In Fig. 6 the starting material was the dialysed enzyme fractions from two experiments on prostatic extracts, run at room

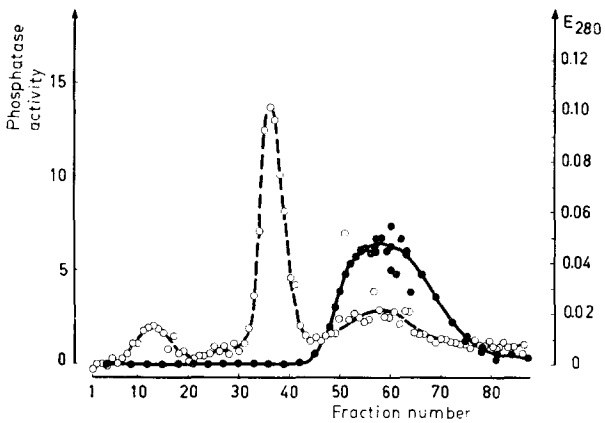

Fig. 6. Chromatography of partially purified prostatic phosphatase, obtained from experiments with prostatic extract. The starting buffer had a pH of 5.5 and the elution was achieved by a slow pH gradient. The recovery of the enzyme was 123%. Solid circles represent phosphatase activity (in millimoles of substrate split during 30 minutes at 37°), open circles the extinction at 280 mμ.

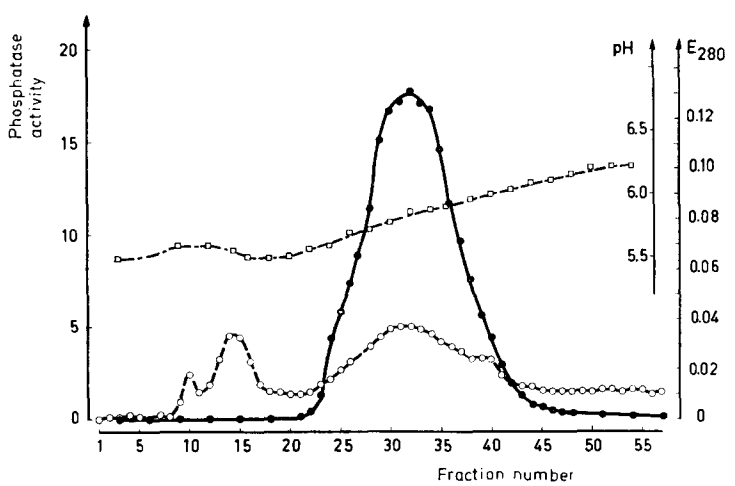

Fig. 7. Chromatography of purified prostatic phosphatase. The ratio between activity and extinction varies with less than 8% between fractions 29 and 35. Solid circles represent phosphatase activity (in millimoles of substrate split during 30 minutes at 37°), open circles the extinction at 280 mμ, and squares the pH values.

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temperature on a water-cooled column (temp. in the resin 9°). As can be seen a fairly sharp peak of inactive material (max. extinction 0.1 in fraction 36) has separated from the phosphatase activity. The extinction values for the enzyme fractions are here smaller than the differences often found between different buffers. In this experiment the tubes were shifted every 6 min, which gave fraction volumes of 2.7 ml. The spreading of the extinction values, not found elsewhere, shows, however, that such small fractions are inconvenient.

The dilutions for the activity measurements were made with buffer only. Judging from the following experiment and from the results of LONDON AND HUDSON[8], this gives activity values which are about 30% too low. In spite of this the recovery, as calculated from the area under the peak, is 123%.

In another experiment a prostatic extract was fractionated with ammonium sulphate[16]. The precipitate between 60 and 80% saturation was collected and dialysed. After removal of undissolved material by centrifugation it was found that the ratio between activity and UV-extinction had been doubled compared to the raw extract. The recovery was not more than about 10%. After dialysis against the starting-buffer of pH 5.2 the enzyme was adsorbed on a column but removed by stepwise elution (pH 6.8) which gives a less purified but more concentrated solution than that obtained with a gradient. The enzyme prepared in this way was dialysed against the starting-buffer of pH 5.5 and re-chromatographed with a slow pH gradient of the form ABD in Fig. 1 (when the gradient was started the mixing chamber contained 230 ml).

The results are shown in Fig. 7. In this experiment the dilutions for the activity measurements were made, both with and without serum albumin-containing buffer. In agreement with the results of LONDON AND HUDSON[8] the samples diluted with only buffer showed a poor reproducibility (compare the activity measurements in Fig. 6 and 7) and the activities found with only buffer were 30 to 40% lower than with buffer containing serum albumin. The slight abnormality on the front side of the activity curve caused by "too high" activities of fractions 24-26 may depend on the fact that other dilution proportions and another pipette were used for these fractions than for the rest of the curve. This can be expected since surface inactivation of the purified and unstabilized enzyme[8] is very marked during diluting and pipetting manipulations.

In this experiment the ratio between activity and extinction for fractions 29 to 35 (maximum activity in fraction 32) varies with less than 8% which must be regarded to be within the limits of error. On the side of the peak the ratio decreases due to the fact that the extinction never goes down to zero.

For the purified enzyme from this experiment the specific activity on a nitrogen basis was a little lower than that obtained after only one chromatographic run. If instead the specific activity is calculated on the basis of the extinction at 280 mμ, a value of 470 millimoles split per unit extinction is obtained which is 60 to 70 times higher than for the raw extract. It is thus likely that a large part of the nitrogen in this purified enzyme comes from the ammonium sulphate.

The behaviour of some other enzymes have been studied preliminary. From these experiments it appears that the acid phosphatases in both male and female urine can be purified in the same way as prostatic phosphatase. Also enolase can be chromatographed on Dowex 50 (to be published by BOMAN AND MALMSTRÖM).

As a large part of the proteins present in raw extracts appears to be adsorbed on Dowex 50, columns can also be utilized for purification when the material to be isolated

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is not adsorbed on the resin. In this way ANAGNOSTOPOULOS (personal communication) has reached a 3 fold purification of a partially purified acid phosphatase from white mustard seeds.

DISCUSSION

The chromatographic method. The use of a pH gradient for elution of proteins from an adsorbent may have some advantages compared to stepwise elution or elution at constant pH. The careful control of the pH and the ionic strength used by BOARDMAN AND PARTRIDGE[3] is not necessary here since the most favourable composition for elution will always be obtained during a gradient experiment. It is very probable that the best separation abilities are achieved with a constant pH, but there may be many natural mixtures which can be satisfactorily resolved with the more convenient gradient elution. Further, in those cases where it is necessary to use the maximum separation power of the adsorbent, the optimal conditions may be most easily found by gradient experiments. Also, the spreading of the zones, often known to cause trouble in chromatography of proteins, can usually be controlled with gradients.

The purified enzyme. In their study of the purification of prostatic phosphatase LONDON AND HUDSON[8] were able to obtain a 250-fold purification of the extract, counting on a nitrogen basis. Unfortunately lack of material has up to now limited the possibilities of obtaining larger amount of chromatographically purified enzyme. For this reason no figure can as yet be given for the purification on a nitrogen basis. There are also some difficulties in estimating the degree of purification on basis of the extinction at 280 mμ. The 60 fold purification calculated from the experiment shown in Fig. 7 may thus in reality be higher if a correction was made for the background extinction of the effluent before and after the enzyme zone. It can only be stated that the present chromatographic examination as such does not show any larger amounts of impurities in the enzyme.

Citrate has earlier been found to activate the prostatic phosphatase both in seminal plasma[17] and in partially purified prostatic extracts[16]. However, the action of citrate is at present rather obscure. It may act as an anti-inhibitor but it may as well have a stabilizing and protective effect. In any case it is possible that the experiment shown in Fig. 6 with an enzyme recovery of 123% reflects the same phenomenon as the citric acid effect.

In most cases when phosphatases are kept in phosphate solutions the activity (estimated without phosphate) is decreased due to the competitive inhibition. This effect has not been observed here with prostatic phosphatase which instead appears to have a higher activity and to be more stable in phosphate buffers than in acetate and citrate. The rate of inactivation in acetate and citrate must consequently dominate over the competitive inhibition by the phosphate.

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SUMMARY

1. A method for pH gradient elution chromatography of proteins is described.
2. The method has been used for the chromatographic examination of the raw extracts of human semen and prostatic glands as well as for chromatographically purified samples of prostatic phosphatase.
3. An enzyme preparation has been obtained which, judging from the chromatographic examination does not contain large amounts of impurities.

RÉSUMÉ

1. Une méthode de chromatographie des protéines avec élution par un gradient de pH est décrite.
2. La méthode a servi à l'examen chromatographique d'extraits bruts de sperme et de prostate humains et d'échantillons, purifiés par chromatographie, de phosphatase prostatique.
3. Les auteurs ont obtenu une préparation enzymatique qui, si l'on en juge par l'examen chromatographique, contient peu d'impuretés.

ZUSAMMENFASSUNG

1. Es wird eine Methode zur pH Gradienten-Elutions-Chromatographie von Proteinen beschrieben.
2. Die Methode wurde zur chromatographischen Prüfung von Rohextrakten aus menschlichem Samen und der Prostata als auch von chromatographisch gereinigter Prostataphosphatase verwendet.
3. Es wurde ein Enzympräparat erhalten, das beurteilt nach der chromatographischen Prüfung keine grösseren Mengen an Verunreinigungen enthielt.

REFERENCES

1 C. A. ZITTLE, *Adv. in Enzymology*, 14 (1953) 319.
2 R. R. PORTER, *Biochem. J.*, 53 (1953) 320.
3 N. K. BOARDMAN AND S. M. PARTRIDGE, *Nature*, 171 (1953) 208.
4 G. JOLLÈS AND C. FROMAGEOT, *Biochim. Biophys. Acta*, 11 (1953) 95.
5 C. H. W. HIRS, *J. Biol. Chem.*, 205 (1953) 93.
6 H. G. BOMAN, *Nature*, 173 (1954) 447.
7 O. A. BESSEY, O. H. LOWRY AND M. J. BROCK, *J. Biol. Chem.*, 164 (1946) 321.
8 M. LONDON AND P. B. HUDSON, *Arch. Biochem. Biophys.*, 46 (1953) 141.
9 R. S. ALM, R. J. P. WILLIAMS AND A. TISELIUS, *Acta Chem. Scand.*, 6 (1952) 826.
10 B. DRAKE, *Arkiv. f. Kemi*, 8 (1955) 1.
11 V. DESREUX, *Proceedings of the International Colloqium on Macromolecules, Amsterdam* 1949, p. 346.
12 T. K. LAKSHMANAN AND S. LIEBERMAN, *Arch. Biochem. Biophys.*, 45 (1953) 235.
13 W. C. BAUMAN AND J. EICHHORN, *J. Am. Chem. Soc.*, 69 (1947) 2830.
14 S. MOORE AND W. H. STEIN, *J. Biol. Chem.*, 192 (1951) 663.
15 *Handbook of Chemistry and Physics*, 1953–1954, p. 1617.
16 C. ANAGNOSTOPOULOS, *Bull. soc. chim. Biol.*, 35 (1953) 575.
17 F. LUNDQUIST, *Acta Physiol. Scand.*, 14 (1947) 263.

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