Chromatography of Ribonuclease on Carboxymethyl Cellulose Columns*

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In the course of an investigation of the chemical phosphorylation of ribonuclease1 (cf. (1)), it became necessary to develop a chromatographic procedure for the fractionation of the phosphorylated protein. The method of Hirs et al. (2), employing the weak cation exchanger Amberlite XE-64, is an extremely valuable method for the chromatographic purification of pancreatic ribonuclease, but the use of phosphate buffer, pH values below 7, and the rapid movement of the protein through the column rendered the method unsuitable for a study of acid-labile, phosphorylated ribonuclease. In the method described in this paper carboxymethyl cellulose ion exchange columns and a gradient elution technique were employed, based on the procedures of Peterson and Sober (3). Quantitative measurements were also made of the affinity of the exchanger for ribonuclease as a function of pH and ionic strength. The CM-cellulose method offers a number of advantages over the method employing the Amberlite resin. Furthermore, by this chromatographic procedure crystalline bovine pancreatic ribonuclease is fractionated into at least four enzymatically active components, in contrast to the two active components separated on the Amberlite column.

After this work had been completed, a report by Åqvist and Anfinsen appeared (4) describing an ion exchange chromatographic fractionation of ovine pancreatic ribonuclease on CM-cellulose columns. These authors also describe an experiment in which crystalline bovine pancreatic ribonuclease was subjected to CM-cellulose chromatography with sodium phosphate as the eluting buffer between the gradient limits 0.01 to 0.1 in which crystalline bovine pancreatic ribonuclease was fractionated into at least four enzymatically active components, in contrast to the two active components separated on the Amberlite column.

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EXPERIMENTAL

Materials—Ribonuclease was an Armour product (crystalline bovine pancreatic, Lot No. 381.050). It was stored over CaCl2 at 2°C, in a vacuum. Material stored under these conditions lost no weight when heated at 100° over P2O5 in a vacuum for 10 hours. Ribonuclease acid was a Schwarz preparation (yeast, Lot No. NH 5732). It was dialyzed against several changes of distilled water for 3 days and then lyophilized. CM-cellulose was prepared by the method of Peterson and Sober (3), from standard grade Whatman Ashless Cellulose Powder which passed through a 200-mesh sieve. The air-dried product contained 0.7 meq of carboxyl groups per gm and the apparent pK of the ionizable group was 3.6. These estimates were made from a titration curve obtained with a suspension of the material in 0.5 M NaCl. Used ion exchanger was regenerated as described by Peterson and Sober (3). All other reagents were commercial products of reagent grade purity.

Equilibration of Ion Exchanger—Buffer solutions (0.005 M) were prepared with Tris* (Sigma, primary standard grade) adjusted to the required pH values with HCl. All pH values were measured with a glass electrode (Beckman model G pH meter) at room temperature. The equilibration of CM-cellulose with buffer was carried out batch-wise at 2°C, by stirring for 30 minutes, then centrifuging at 2,000 × g, 10 minutes. The pH of the supernatant fluid was checked at 0.04 pH unit of the pH of the buffer. After overnight storage of the supernatant in buffer, followed by a few more washings, the ion exchanger was ready for use. Storage of the exchanger suspended in buffer under toluene, at 2°C, for a few weeks was without harmful effects on the chromatographic patterns. Before use, toluene was removed by 4 washings with buffer. A small peak was observed in the effluent pattern in such cases. Its size and position were reproducible and independent of the column charge. It was absent if toluene was not used. In the figures it is shown by a dotted line (peak T).

Ribonuclease Adsorption by CM-Cellulose. Effect of pH and Ionic Strength—In a set of centrifuge tubes, ion exchanger equilibrated with a given buffer was mixed with ribonuclease dissolved in the same buffer (pH adjusted to the pH of the buffer after solution of the protein). The mixtures were placed on a shaker for 30 minutes, then centrifuged (International Clinical Centrifuge, top speed, 15 minutes), and the optical density of the supernatant fluid was determined at 280 nm. Blanks not containing protein were used (the optical density of such blank solutions when read against buffer was about 0.010). Optical density values were expressed in percentages of the values obtained with

1 The abbreviation used is: Tris, tris(hydroxymethyl)aminomethane.
corresponding protein solutions which contained no ion exchanger. Shaking for more than 30 minutes (up to 12 hours) resulted in no significant change of the optical density readings indicating that equilibrium had been reached within 30 minutes. To such mixtures increments of a concentrated NaCl solution were added, followed by shaking, centrifugation, and measurement of the optical density of the supernatant fluid. The salt solution was concentrated enough to keep the dilution factor below 1.02. This small dilution was ignored. The capacity of CM-cellulose for ribonuclease adsorption was determined in a similar manner, varying the amount of ribonuclease in buffered mixtures containing a constant amount of the ion exchanger.

**Column Chromatography of Ribonuclease**—The equilibrated ion exchanger was suspended in buffer (0.005 M Tris-HCl, pH 8.00 ± 0.02) and fines not sedimenting by gravity within 15 minutes were removed. A column, 0.9 cm in diameter and 21 cm in height, was poured in 6 to 7 portions with a slurry of the ion exchanger which was allowed to settle by gravity. The column (containing 1.5 gm of ion exchanger, dry weight) was then washed with 50 to 100 ml of buffer, with a hydrostatic head of about 50 cm, at a flow rate of 15 to 20 ml per hour. During this washing the column shrank to a height of 20 cm which it maintained throughout an experiment. The optical density of this wash fluid (read against fresh buffer at 280 mp) was 0.010 to 0.025. The column was charged with a protein solution (less than 10 ml in the same buffer, or in an aqueous solution of low salt content the pH of which had been adjusted to that of the buffer). Gradient elution was carried out as described by Alm et al. (6), from a 500-ml mixing chamber containing 0.005 M Tris-HCl buffer, pH 8.00, and a separatory funnel equipped with a Mariotte tube and containing a NaCl-buffer solution (0.10 or 0.15 M NaCl in buffer). The gradients shown in the figures were calculated according to Alm et al. (6). An initial flow rate of less than 10 ml per hour increased during elution by as much as 50%. Fractions of 1.5 to 4.5 ml were collected. All operations were carried out at 22°C. The volume and the optical density at 280 mp (buffer blank) of each fraction were measured. Fractions containing light-absorbing material were assayed for enzymatic activity by the Kunitz spectrophotometric assay (7).

**RESULTS**

**Effect of pH and Ionic Strength on Adsorption of Ribonuclease by CM-Cellulose**—In Fig. 1A the results of those experiments are shown in which the extent of binding of ribonuclease by CM-cellulose was tested under conditions of varying pH and salt concentration. All mixtures contained 2.0 mg of ribonuclease and 2.0 mg of CM-cellulose per ml of 0.005 M Tris-HCl buffer. The pH of the mixtures was varied between 7.10 and 8.80. The NaCl concentration was varied between zero and 0.110 M. All these experiments were carried out at room temperature (22°C). Above 0.1 M NaCl essentially no ribonuclease is bound by the exchanger between pH 7 and 9. In the absence of NaCl about one-fifth of the light-absorbing material (at 280 mp) was free in solution. A test for the effect of the CM-cellulose treatment on the enzymatic activity of ribonuclease revealed no inactivation of the enzyme at any of the pH values used in the adsorption experiments.

The capacity of CM-cellulose for ribonuclease was determined at pH 8.00, 22°C. In 5.00 ml of 0.005 M Tris-HCl buffer, pH 8.00, containing 3.34 mg of CM-cellulose (2.3 µ eq of carboxyl groups) the amount of ribonuclease was varied between 1.0 and 50.0 mg (0.07 to 3.57 µmoles of protein) (see Fig. 1B). After equilibration and centrifugation the amount of ribonuclease bound by the exchanger was estimated on the basis of the optical density of the supernatant solution. CM-cellulose was saturated by more than its own weight of ribonuclease (1.2 mg of protein per 1.0 mg of ion exchanger, or 0.13 µmole of protein per 1.00 µ eq of ion exchanger carboxyl groups). The reversibility of the adsorption was tested at each level of the total amount of ribonuclease used. Addition of a NaCl solution to the mixtures to yield 0.10 M solutions with respect to NaCl, resulted in the recovery of 91 to 98% of the ribonuclease in the supernatant solution after removal of the CM-cellulose by centrifugation.

**Chromatography of Ribonuclease**—Fig. 2 shows the elution patterns obtained with 40.6 mg and 200.0 mg of ribonuclease, respectively. A gradient with an upper limit of 0.15 M NaCl was used. It is evident that as much as 200.0 mg of ribonuclease may be effectively fractionated on the standard size (0.9 x 20 cm) column. For the preparation of large amounts of the major component (D) of crystalline pancreatic ribonuclease the resolution afforded under the given conditions is satisfactory, but for the separation of the minor components (B and C) less steep gradients may be preferred. When a gradient with an upper limit of 0.10 M NaCl was used in a complete separation of components B and C could be effected. Under these conditions the maxima of each peak had the following positions in the effluent diagram: A, 11 ml; B, 296 ml; C, 348 ml; and D, 412 ml. The corresponding NaCl concentrations were 0.092, 0.044, 0.050, and 0.056 M, respectively. Chromatography of large amounts of ribonuclease resulted in the appearance of a broad band (E) after the elution of the major component (Fig. 2). This band is hardly distinguishable from the base line if small amounts of ribonuclease preparations are chromatographed. When fractions containing the major component of 200 mg of unfractionated ribonuclease were combined, dialyzed against water (30% loss of protein on dialysis), 92.5 mg of protein were obtained as a lyophilized powder. Rechromatography of this material resulted in the elution of a single, symmetrical band as shown in Fig. 3.
Analysis of chromatographic fractions of ribonuclease

The data shown represent average values obtained in 5 to 7 experiments. The range of individual data is indicated in each case in parentheses. The components are identified by letters as used in the chromatographic effluent patterns (e.g. Fig. 2).

| Effluent fractions | Recovery* | Enzymatic activity | Specific enzymatic activity
<table>
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<tbody>
<tr>
<td></td>
<td>OD (280 mp)</td>
<td>%</td>
<td>%</td>
</tr>
<tr>
<td>A</td>
<td>7.6 (7.3-7.7)</td>
<td>0 (less than 0.07)</td>
<td>0</td>
</tr>
<tr>
<td>B</td>
<td>4.5 (3.6-5.1)</td>
<td>4.4 (3.9-5.2)</td>
<td>98</td>
</tr>
<tr>
<td>C</td>
<td>7.7 (7.2-8.5)</td>
<td>8.2 (7.5-8.5)</td>
<td>106</td>
</tr>
<tr>
<td>D</td>
<td>64.8 (63.0-67.0)</td>
<td>71.3 (67.0-78.4)</td>
<td>110</td>
</tr>
<tr>
<td>E</td>
<td>3.7 (3.5-3.9)</td>
<td>2.9 (2.8-2.9)</td>
<td>78</td>
</tr>
<tr>
<td>All peaks</td>
<td>88.3 (85.6-92.3)</td>
<td>86.8 (83.1-94.5)</td>
<td>98</td>
</tr>
<tr>
<td>All fractions</td>
<td>95.1 (92.5-104.0)</td>
<td>†</td>
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* 100.0% is the amount placed on the column.
† Specific enzymatic activity of fresh aqueous ribonuclease solution is taken as 100.
‡ Not determined.

The composition of crystalline ribonuclease in terms of the individual chromatographically distinct components was estimated by integration of the area under each peak of the effluent diagram. The results, in percentages of the original column charges, are summarized in Table I. They are all corrected for "base line" optical density, which in fact was the optical density of the column wash fluid collected before charging the column with protein. That this correction procedure was a fair one was shown by a blank chromatography run. The column was eluted with the usual gradient in the absence of protein and it was found that the optical density readings did not vary significantly throughout the elution process. The artifact peak T did appear if toluene was employed. This, of course, was also corrected for in the recovery calculations. The table also shows the results of enzymatic activity measurements. Specific enzymatic activity values are expressed in terms of percentages of the specific activity of freshly prepared aqueous solutions of crystalline ribonuclease. Protein concentrations were estimated from optical density data with the molar extinction coefficient of ribonuclease component D at 280 mp (9.63 X 10³).

Comparison of Chromatography of Ribonuclease on Amberlite XE-64 and on CM-Cellulose—The correspondence of chromatographically distinct fractions obtained by the present procedure and by the method of Hirs et al. (2) was also determined. A sample of "ribonuclease B" (40.6 mg) obtained from an Amberlite XE-64 column (cf. (2)) was dissolved in 5.00 ml of 0.005 M Tris-HCl buffer, pH 8.0, dialyzed against this buffer for 8 hours at 2°, and this solution (containing 33.3 mg of protein, rest lost on dialysis) was chromatographed as usual on CM cellulose. Fig. 4 shows that "ribonuclease B" contains in addition to some inactive component A also the active components B and C. Total recovery from this column was 98.9% of the material placed on the column. Of the original material 17.4% was present in peak A, 20.2% in peak B, and 30.2% in peak C (total

I am very grateful to Dr. Frederic M. Richards for a generous gift of dialyzed and lyophilized "ribonuclease B."
of three peaks was 73.8%). The ratio of material in peaks B and C (C/B, 1.79) agrees well with the distribution of material between these peaks when obtained by direct chromatography of crystalline ribonuclease on CM-cellulose (C/B from Table I, 1.71). The reverse test, chromatography of material on Amberlite XE-64, after prior chromatography on CM-cellulose was also carried out. Suitable fractions obtained from a large scale experiment were combined, their pH was adjusted to 6.48, and they were then rechromatographed on an Amberlite XE-64 column (0.2 M Na-phosphate buffer, pH 6.48; cf. (2)). The effluent patterns obtained with unfractionated enzyme and with the CM-cellulose column fractions are shown in Fig. 5. Component E when rechromatographed on Amberlite XE-64 did not appear as a peak but only as an irregular base line. In 70 ml of effluent volume only 56% of the material was recovered.

### Ultraviolet Absorption Spectra of Chromatographic Components of Crystalline Ribonuclease—The ultraviolet absorption spectra (in 0.005 M Tris-HCl buffer, pH 8.0) of the chromatographically distinct fractions of crystalline ribonuclease revealed that the enzymatically active components were identical in the positions of their maxima (278 µm), minima (256 µm), and of a shoulder on the long wave length side of the principal absorption band (at about 284 µm). The inactive component A however exhibited a non-protein absorption spectrum under the same conditions. A minimum at 238 µm and a maximum at 255 µm (with a broad shoulder in the region 266 to 276 µm) suggested nucleotide material (290 µm/290 µm optical density ratio, 1.47). That this material may be of relatively large molecular size is inferred from the fact that on dialysis at 2º in Visking 26/32" cellophane tubing, against several changes of distilled water, only about 30% of the material was lost in 10 hours, and about 50% in 43 hours (losses estimated on the basis of the drop in the optical density of the solution during dialysis). Under the same conditions, the enzymatically active components passed through the dialysis membrane at a faster rate (30 to 50% in 10 hours, and 70 to 80% in 43 hours).

### DISCUSSION

The results presented above show that chromatography of crystalline bovine pancreatic ribonuclease on a CM-cellulose column leads to the resolution of the protein into four components all of which possess enzymatic activity. All chromatograms shown (cf. Figs. 2 and 4) suggest that component B may actually represent a mixture of at least two species since the shoulder on the leading side of the elution band was invariably present. Rechromatography of individual components on CM-cellulose or Amberlite XE-64 columns (Figs. 3 and 5) resulted in the elution of single peaks suggesting that the original fractionation was indeed a reflection of the heterogeneity of the crystalline enzyme preparation and did not merely represent an artifact, possibly due to some modification or cleavage occurring on the surface of the ion exchanger or during storage of the protein, dry or in solution. This view is further strengthened by the fact that "ribonuclease B" as obtained from an Amberlite column was shown by the CM-cellulose procedure to contain two active components which are eluted at effluent volumes identical with those of components B and C of previously unfractionated ribonuclease (cf. Figs. 2 and 4). In addition, the close agreement of the ratios of C/B in the two preparations (unfractionated ribonuclease, 1.71; "ribonuclease B", 1.79) and the presence of the shoulder on the leading side of component B also in the "ribonuclease B" chromatogram make it even more likely that the heterogeneity of crystalline ribonuclease is a real one. It may be noted that the "ribonuclease B" sample (derived from the same lot but a different shipment of Armour ribonuclease, as compared with the protein preparation used in all other experiments) had been obtained 1 year before the CM-cellulose preparations. This fact, when considered together with the surprising constancy of the C/B ratios supports the view that the CM-cellulose procedure reflects a heterogeneity which is characteristic of crystalline ribonuclease preparations as such, independently of treatment or storage.

Component A is believed to be polynucleotide material on the basis of its ultraviolet spectrum and retention within the membrane sac on dialysis. Its presence in ribonuclease preparations appears to be quite general since its position in Amberlite column effluents is identical with that of the leading, enzymatically inactive band already shown by Hirs et al. (2) to appear in the elution pattern of unfractionated ribonuclease (cf. Fig. 5). The presence of this material may account for the repeatedly observed increase in enzyme specific activity on chromatography. Richards (8) has pointed out that such an increase in specific activity may be due to the removal of an inhibitor by the chromatographic procedure. This suggestion would be supported by the finding that nucleotide material is removed on chromatography in view of the reported inhibition of ribonuclease by mononucleotides (9) or ribonucleic acid substrate (10).
tages. The principal component (D) appears in the effluent at a salt concentration of 0.06 to 0.07 M NaCl (plus 0.005 M Tris-HCl buffer) or less, depending on the slope of the salt gradient, in contrast to the 0.2 M Na-phosphate buffer used with the Amberlite resin. The fact that in the present procedure the protein components appear in the effluent further removed from the solvent front makes it possible to apply this method to the fractionation of modified ribonuclease preparations so that a resolution of faster as well as slower moving components may be obtained. The large capacity of the CM-cellulose columns (cf. Fig. 1A) renders it possible to use 1 X 20-cm columns for preparative purposes. With a load of 200 mg of protein on such columns the principal component (D) has been obtained free from the other components in yields close to the theoretical yield after dialysis and lyophilization (after correction for loss on dialysis).  

Finally, the CM-cellulose columns permit an extension of the useful pH range of ribonuclease chromatography. The use of Amberlite XE-64 resin necessitates the use of buffers covering a narrow pH range below 7. The results with CM-cellulose, as shown in Fig. 1A, suggest that the pH region between 7 and 9 may also be suitable for the fractionation of ribonuclease on CM-cellulose columns. Acid-labile modifications of ribonuclease, such as phosphorylated derivatives, may be chromatographed (cf. (1)). Although by increasing the pH one is leaving the pH region in which ribonuclease is most stable, the enzymatic activity measurements have shown that under the conditions of these experiments no inactivation takes place.

The apparent reversal of the order in which the extent of ribonuclease adsorption to CM-cellulose varies with pH at about 0.005 M NaCl (cf. Fig. 1A) requires brief comment. The major portions of the curves above 0.005 M NaCl reflect the expected behavior of ribonuclease with respect to a fully dissociated cation exchanger. As the pH increases, that is ribonuclease loses more and more of its net positive charge (pI about 9.5; cf. (5)), so does its extent of binding by the ion exchanger decrease at any given salt concentration. Below 0.005 M NaCl, the unadsorbed material is greatly enriched by component A since little if any of this component is adsorbed by the ion exchanger. Almost half of the optical density of the supernatant fluid may be due to nucleotide materials in the absence of NaCl. Thus at low salt concentrations any effect that the pH may have on the spectral characteristics of component A would blur the significance of the optical density readings as a measure of unadsorbed protein. As the salt concentration is increased, however, the increase in optical density would duly reflect the release of protein by the ion exchanger into the supernatant fluid since the total amount of dissolved component A would remain essentially constant. Since increasing ionic strength will decrease the optical density of ribonucleic acid solutions as much as 50% at pH 5 and at 300 m\(\mu\) (9), and may do so also at 280 m\(\mu\) and somewhat higher pH values, it is possible that as the NaCl concentration is increased the contribution of component A to the total optical density of the supernatant solution may be diminished. In this case the real value of "per cent ribonuclease unbound" may be higher by an amount corresponding to not more than 4% of the optical density of the ribonuclease solution in the absence of ion exchanger. This may be the reason why at high salt concentrations the optical density values tend to level off at values slightly less than 100%.

**SUMMARY**

A method has been described for the chromatographic fractionation of crystalline pancreatic ribonuclease. The enzyme preparation is resolved into a minimum of four active components by ion exchange chromatography with carboxymethyl cellulose ion exchange columns buffered with tris(hydroxymethyl)aminomethane-hydrochloric acid at pH 8.00, and eluted with a sodium chloride concentration gradient. Columns of 20 cm height and 0.9 cm diameter are sufficient for preparative use, permitting the fractionation of as much as 200 mg of protein. The salt concentration in the effluent fractions containing the major ribonuclease component is less than 0.075 M. Batch-wise adsorption experiments indicate that the usefulness of the method may extend at least over the pH range between 7 and 9.

**REFERENCES**

Chromatography of Ribonuclease on Carboxymethyl Cellulose Columns
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