CHROMATOGRAPHY OF PROTEINS ON CELITE ION-EXCHANGE RESINS

II. CHROMATOGRAPHY OF INSULIN ON A CELITE-SULPHONIC ACID ION-EXCHANGE RESIN

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INTRODUCTION

The preparation of a sulphonic acid ion-exchange resin with a high capacity for protein is described in the preceding paper[1]. Particles of the diatomaceous earth, Celite 545, were coated with a thin film of cross-linked sulphonated polystyrene to produce a resin with a large surface area.

This paper describes the application of columns of the Celite–cross-linked sulphonated polystyrene (Cel-SPX) to a study of the homogeneity of crystallized insulin. A preliminary account of the work was published previously[2].

MATERIALS AND METHODS

Ion-exchange resin

The Cel-SPX ion-exchange resin was prepared as described in the preceding paper[1].

Dowex 50

For comparison with its behaviour on columns of Cel-SPX, 6 times crystallized insulin also was chromatographed on a column consisting of a mixture of Celite 545 and the sulphonic acid ion-exchange resin, Dowex 50, in a finely divided state. Beads of Dowex 50 (X8) were ground in a laboratory hammer mill. Material which passed through a 200 mesh/in. sieve was treated several times with hot 2 *N* NaOH, washed with water and sieved again in the wet condition. Material which passed a 200 mesh/in. sieve was dried and mixed with Celite 545 in the ratio 1:20.

Insulin

Most of the insulin samples, either crude or 6 times crystallized, were generously provided by Boots Pure Drug Co. A sample of crystallized insulin which had been prepared by Dr. LENS[3] was kindly supplied by Dr. F. SANGER.

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Preparation and operation of the columns

The procedures employed in packing and operating the columns were the same as described previously[4]. Most of the experiments were carried out with columns of Cel-SPX, 0.9 cm in diameter, and 12 cm high. 0.4 ml samples containing 3–4 mg of insulin were added to the columns and the effluent collected in 1.0 ml fractions. The rate of flow of buffer through the column was 1.0 ml/h. The choice of buffers for the chromatography of insulin was limited by the low solubility of insulin. Insulin is insoluble in the pH range 4–7 and even in the pH range 2–4 it is not soluble in all buffers. An acetate buffer of the following composition was used to elute insulin from the columns: 65.3 g $CH_3COONa \cdot 3H_2O$, 30.5 g NaCl and 300 ml glacial acetic acid/l. The pH of the buffer was 3.39 and the Na^+ concentration 1.0 g ions/l.

Protein concentration in the effluent was measured photometrically at 276 mμ in a Hilger "Uvispec" spectrophotometer. A correction for extraneous absorption was made from simultaneous measurements of the optical density at 320 and 360 mμ, according to the method of BEAVEN AND HOLIDAY[5]. The line through the points at 320 and 360 mμ was extrapolated to 276 mμ and this formed a base line from which the optical density was measured.

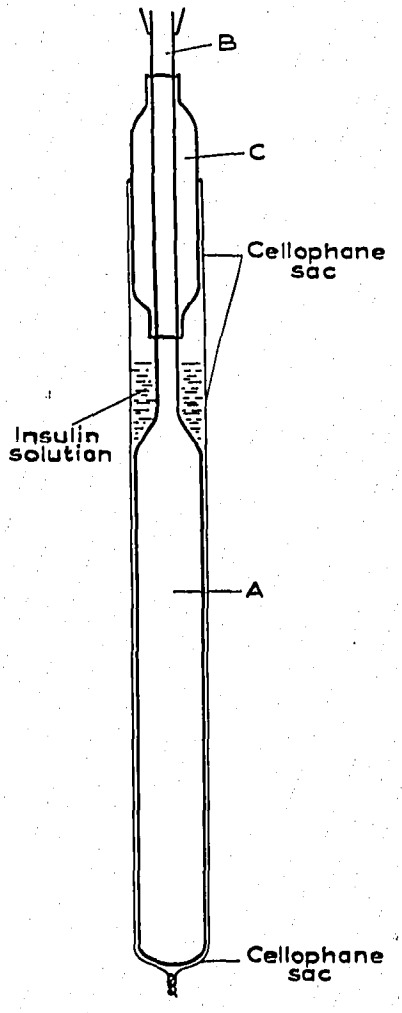

Fig. 1. Apparatus for the rapid dialysis of insulin solutions (see text).

Concentration of insulin in effluent

Before attempting to concentrate the insulin in the effluent, it was necessary to dialyse away most of the buffer salts. Normal dialysis against water, using a Visking cellophane sac, but without agitating the liquid inside or outside the sac proved unsatisfactory because of heavy losses of insulin from inside the sac during the time taken to remove 95% of the buffer salts. However, it was observed that most of the salt could be removed, without loss of insulin, by increasing the rate of dialysis. This was done by increasing the effective area of the sac in relation to the volume of liquid it contained, and by agitating the solution inside the sac.

The protein solution was agitated by a rotating glass tube, A (Fig. 1), which was sealed at one end and joined at the other to a length of glass tubing of narrower bore (B). B was connected to the shaft of a stirrer motor. A small length of glass tubing (C), drawn down at both ends, acted as a guide for the rotating tube as well as a support to which the cellophane sac was tied.

10 ml of effluent was placed in the sac, tube A was inserted and then connected

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to the motor. The sac was tied to C which was then lightly clamped. The sac was lowered into 5 l of distilled water and stirring commenced. The water outside the sac also was agitated by a magnetic stirrer. Dialysis was allowed to continue for 30 min with one change of water and during this period 95% of the salt was removed with negligible loss of insulin. The solution of insulin was concentrated by allowing it to stand over calcium chloride in a desiccator.

RESULTS

Chromatography of crystallized insulin

Distribution experiments were carried out in graduated centrifuge tubes[6] in order to select conditions under which the adsorption of insulin to Cel-SPX was reversible. It was found that the crystallized insulin was reversibly adsorbed on Cel-SPX from an acetate buffer of pH 3.39 and Na^+ concentration 1.0 g ions/l and under these conditions the distribution coefficient was favourable for chromatography.

The continuous line in Fig. 2 shows the chromatogram obtained from a sample of 6 times crystallized insulin (Boots Insulin, Batch 9011G), using a column of Cel-SPX, 12.3 × 0.9 cm. The shape of the elution curve indicates that the sample of crystallized insulin contained at least two components. Furthermore, the recovery of protein from the column was 73%, showing that there was some adsorption of material on the column. Attempts were made to resolve the components shown in Fig. 2, but without success. The dotted line in Fig. 2 shows the chromatogram obtained with a sample of crystallized insulin which had been prepared by Dr. LENS[3]. In this case,

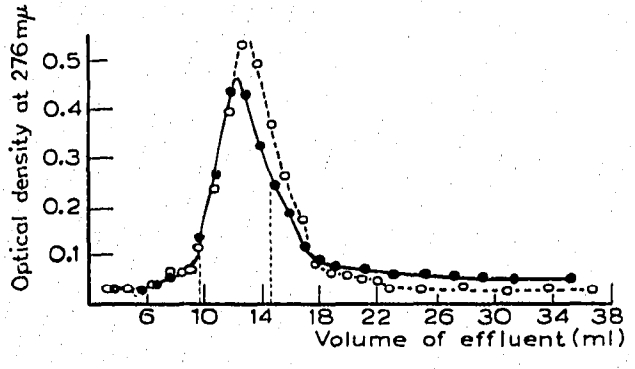

Fig. 2. Chromatogram of crystallized insulin on a column of Cel-SPX, 12.3 × 0.9 cm. Buffer: sodium acetate, pH 3.39, Na^+ concn. 1.0 g ions/l. Temp. 2°. –•–•–•– 6 × crystallized insulin obtained from Boots Pure Drug Co. Amount on column 3.3 mg. ···o···o···o··· Insulin crystallized by LENS. Amount on column 3.5 mg (for the main peak $R = 0.62$, $R_F = 0.34$).

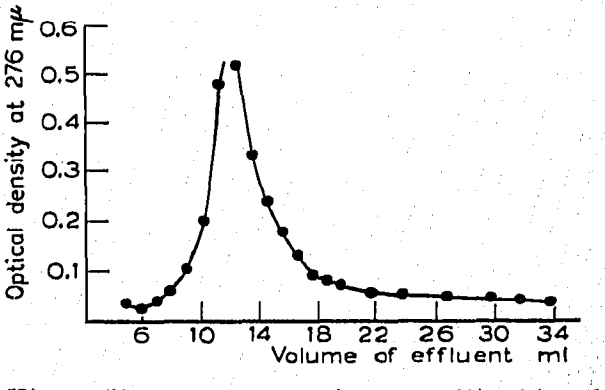

Fig. 3. Chromatogram of a crystallized insulin on a column of Cel-SPX, 12.3 × 0.9 cm. Buffer: sodium acetate pH 3.39, Na^+ concn. 1.0 g ions/l. Temp. 2°. Amount on column 3.6 mg.

the elution peak was almost symmetrical, showing that the material eluted from the column was essentially homogeneous; the recovery of protein from the column was 78%. Other samples of crystallized insulin, kindly supplied by Dr. DICKINSON, were chromatographed. Most samples showed the presence of a second component, but

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one sample which had been purified on columns of calcium phosphate[7] gave an almost symmetrical peak (Fig. 3).

Rechromatography of insulin

In order to obtain sufficient material for rechromatography, 35 mg of 6 times crystallized insulin (Boots) were chromatographed on a column 7.6 × 1.8 cm. A cut which corresponded to the dotted vertical lines in Fig. 2 was made and the insulin concentrated by rapid dialysis, followed by freezing and allowing to stand over calcium chloride. Rechromatography on a fresh column gave an elution curve which still showed the presence of two components (Fig. 4), but the recovery of protein (85%)

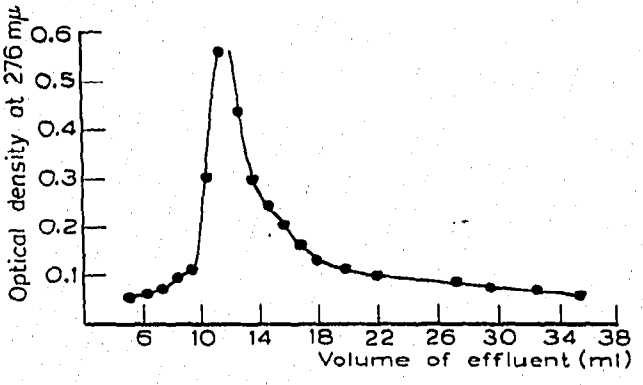

Fig. 4. Rechromatography of 6 × crystallized insulin on Cel-SPX. Column 12.3 × 0.9 cm. Buffer: sodium acetate, pH 3.39, Na^+ concn. 1.0 g ions/l. Temp. 2°. Amount on column 3.3 mg.

was higher than in the original chromatogram. This inhomogeneity of the insulin on rechromatography was not surprising in view of the small degree of separation obtained in the first elution and the width of the cut.

Assay of insulin

Biological assay of insulin which had been eluted from a column of Cel-SPX was kindly carried out by Boots Pure Drug Co. The activity of the eluted insulin was 23.1 I.U./mg (P = 0.95, 20.16–26.68), a value not significantly different either from the activity of the crystallized insulin before chromatography (22.2 I.U./mg) or from the International Standard.

Chromatography of a preparation of crude insulin

A preparation of crude insulin was supplied by Dr. DICKINSON. This preparation was obtained at an intermediate stage of the manufacturing process and it represented the protein contained in the liquor for isoelectric precipitation. Fig. 5a shows the result obtained when the crude insulin was subjected to chromatography on a column of Cel-SPX, 12.3 × 0.9 cm under the same conditions as was used for the chromatography of crystallized insulin. A protein band emerged from the column before the insulin, but the separation of this protein from the insulin was incomplete. An im-

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proved separation was obtained on a longer column of Cel-SPX, 20.0 × 0.9 cm (Fig. 5b).

Attempts also were made to purify the crude preparation of insulin by chromatography on columns of Cel-SPX with stepwise changes in the eluting buffer. Stepwise elution of proteins from a column offers particular advantages for preparative work, provided conditions can be found for the complete separation of the protein being purified from the other proteins in the mixture, while retaining the particular protein

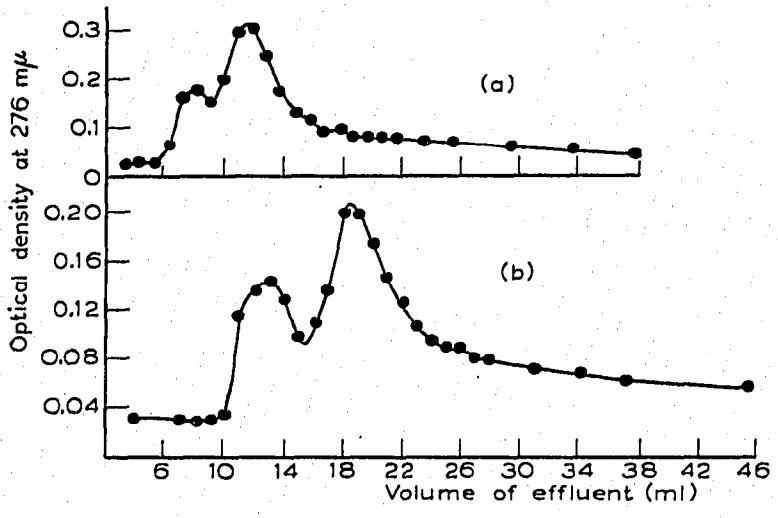

Fig. 5. Chromatograms of a preparation of crude insulin on Cel-SPX. (a) Column 12.3 × 0.9 cm. (b) 20.0 × 0.9 cm. Buffer: sodium acetate pH 3.39, Na^+ concn. 1.0 g ions/l. Temp. 2°. Amounts on column: approx. 3.5 mg.

or enzyme in a fully active state. Stepwise elution was particularly successful in the preparation of cytochrome *c*[8].

In the experiments with the crude insulin the aim was to apply the protein mixture to the column in a buffer such that the insulin remained as a stationary band at the top of the column, while the contaminating proteins passed through the column. After washing the latter proteins from the column, it was hoped to elute the insulin in a fully active state by changing to a buffer of higher eluting power. Unfortunately, we were unable to find a buffer which would quantitatively elute the contaminating proteins from the column and, at the same time, leave the insulin completely adsorbed on the resin.

In one experiment, the preparation of crude insulin was applied to the column of Cel-SPX in a buffer of Na^+ concentration 0.60 g ions/l and pH 3.39, and the same buffer was passed through a column until 40 ml of effluent was collected. The buffer was then changed to one of Na^+ concentration 1.5 g ions/l and pH 3.39. At first sight, the result appeared satisfactory; two distinct peaks were obtained with a good base line between them. However, the amount of protein eluted by the first buffer was low, suggesting that a proportion of the contaminating proteins was adsorbed to the resin from the first buffer and eluted with the insulin when the buffer was changed. The protein peak eluted by the first buffer showed a small amount of tailing, which might

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suggest that some of the contaminating protein was spread out over the column.

When the starting buffer was increased in Na^+ concentration to 0.80 g ions/l, it was found that part of the insulin was eluted in a second broad band by the starting buffer. The remainder of the insulin was eluted by the second buffer of Na^+ concentration 1.5 g ions/l. The results of these two experiments show that great care must be exercised in interpreting the results of protein chromatography if stepwise elution is used.

Chromatography of insulin on the carboxylic acid ion-exchange resin

Insulin was irreversibly adsorbed on a column of Celite, coated with cross-linked methacrylic acid (Cel-MX) from an acetate buffer of pH 3.4 and Na^+ concentration 1.0 g ions/l.

Chromatography of insulin on Dowex 50 and Celite

Finely ground Dowex 50 was mixed with Celite 545 in the ratio 1:20 (w/w). The exchange capacity/g of the mixture for Na^+ was similar to the exchange capacity/g of Cel-SPX. The open circles in Fig. 6 show the result obtained when a sample of 6 times crystallized insulin was passed through a column of Dowex 50, mixed with Celite, with an acetate buffer of pH 3.4 and Na^+ concentration 1.0 g ions/l. The closed circles show the result obtained when insulin was passed through a column of Celite 545. There was no significant retardation of the insulin band in either experiment, and the yield of insulin from the columns was quantitative. Comparison of the two curves in Fig. 6 shows that Dowex 50 even in a very finely divided form has a negligible capacity to adsorb insulin. The success of Cel-SPX as a resin for the chromatography of insulin is probably due to the large surface area of ion-exchange material presented

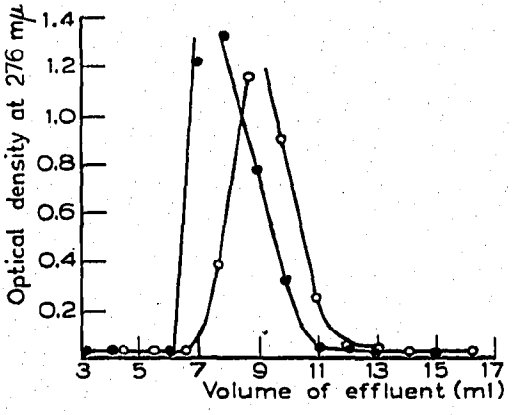

Fig. 6. -o-o-o- Chromatogram of 6 × crystallized insulin on a column of Dowex 50, mixed with Celite 545. Column 13.8 × 0.9 cm. -•-•-•- Chromatogram of 6 × crystallized insulin on a column of Celite 545, 12.0 × 0.9 cm. Buffer: sodium acetate pH 3.39, Na^+ conc. 1.0 g ions/l. Temp. 2°. Amount on columns 3.5 mg.

by the coated diatom skeletons. On the other hand, particles of Dowex 50 presumably have a relatively small surface area as they exhibit smooth glass-like surfaces when viewed under the microscope.

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DISCUSSION

There is evidence from the chromatograms for more than one component in all except one of the samples of insulin, which were investigated. With the samples of crystallized insulin, the separation of the components was insufficient to permit isolation of any component in a pure state. Therefore, no attempt was made to distinguish between the components, either by physical or chemical examination.

Our results are in general agreement with those of HARFENIST AND CRAIG[9], who showed that a number of samples of crystallized insulin from various sources could be split into two components by counter current distribution between *n*-butanol and dichloroacetic acid, although the proportion of the two components varied considerably among the samples investigated. Beef insulin from the Eli Lilly Co. contained a larger proportion of the minor component than did beef insulin obtained from Boots Pure Drug Co. HARFENIST[10] examined the amino acid composition of the two components of beef insulin from Eli Lilly Co. and it appeared that the only difference between the components was in their amide content. One component had 6 amide groups per molecule and the other 5. As HARFENIST points out, it is not improbable that one of the components is an artifact of the method of preparation.

Conversely, PORTER[11] in his studies on the partition chromatography of insulin on kieselguhr columns found that the insulin peak behaved as a single component. DICKINSON[7] used chromatography on calcium phosphate columns to distinguish between freshly prepared solutions of crystallized insulin and solutions which had been stored under various conditions. Freshly prepared solutions gave a single peak on the chromatograms with only slight tailing, while stored solutions showed the presence of a second component, which increased in amount with time of storage. DICKINSON suggested that the appearance of a second component may have been due to molecular weight changes in the insulin.

Recovery of insulin

The recovery of the crystallized insulins from the columns varied from 70 to 80%, and it was increased by a second passage of insulin through the column. This indicates that there were small amounts of material present in the crystallised insulin which were strongly adsorbed to the resin during the first passage through the column. However, the recoveries of insulin on rechromatography were still not quantitative, showing that there was some irreversible binding of insulin to the resin even on the second passage through the column.

Gradient elution analysis

For reasons which are outlined in the preceding paper[1], stepwise or gradient elution procedures may have practical advantages in protein chromatography over the method of elution with a buffer of constant composition. However, the experiments with insulin, reported in this paper, emphasize the difficulties of interpretation in experiments where the eluting buffer is not constant. Proteins which show marked

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tailing on the column may appear in several peaks, as each change of the eluting buffer may desorb protein from the column. The appearance of a protein in several peaks may occur even when a buffer of gradually increasing eluting power is applied to the column. It is also difficult to draw conclusions about the homogeneity of a protein preparation, when stepwise elution is used, as several proteins may be eluted together with a change in the eluting medium.

The stepwise and gradient elution methods suffer from a further disadvantage, particularly if labile proteins are being subjected to chromatography. In either method it is usual to select a starting buffer of very low eluting power, so that all the proteins in the mixture being chromatographed are strongly adsorbed on the column. Very strong adsorption may bring about irreversible changes, including hydrolytic ones in a protein, whereas in the method of elution at constant composition the buffer is usually chosen so that the particular protein being investigated moves down the column with an $R_F > 0.5$ (BOARDMAN AND PARTRIDGE[4]).

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SUMMARY

1. Several samples of crystallized insulin and a preparation of crude insulin were subjected to chromatography on columns of a sulphonic acid ion-exchange resin.

2. The chromatograms showed the presence of more than one component in all except one of the samples of insulin which were investigated.

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