

THE EFFECT OF TEMPERATURE ON THE CHROMATOGRAPHY OF INSULIN ON DEAE-CELLULOSE

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Summary

The effect of ionic strength (range 0.15–0.3), pH (range 7–9), and temperature (range 1–25°C) on the chromatographic behaviour of three samples of insulin on diethylaminoethyl (DEAE)-cellulose columns has been studied. These three factors have a similar effect, a decrease of temperature or pH and an increase in ionic strength lowering the elution volume of the protein. The marked effect of temperature is not due to aggregation–disaggregation of the insulin since bovine plasma albumin which does not aggregate reversibly also showed this effect. The desamido component of insulin could not be detected in commercial insulin under the conditions studied but a minor component varying from 2–6 per cent. of the insulin was separated, as well as various amounts of bound ammonia. Removal of zinc from the insulin did not affect the elution curve.

I. INTRODUCTION

The effect of aggregation of an acidic protein on its chromatographic behaviour is not known. Since we wished to study the possibility of separating extracted wool proteins by chromatography on diethylaminoethyl (DEAE)-cellulose and it is known that at least one of the components, α -keratose, is aggregated in solution (O'Donnell and Woods 1956) we have been studying the chromatography of insulin on this basic ion-exchanger. Insulin seemed a suitable model protein since it is an acidic aggregating protein which has been extensively studied by countercurrent distribution (Harfenist and Craig 1952), partition chromatography (Porter 1953), and chromatography on "Amberlite IRC-50" in 8M urea (Cole 1959). This communication describes the pronounced effect of temperature on the chromatography and elution volume of insulin on DEAE-cellulose and also on the non-aggregating system of bovine plasma albumin.

II. MATERIALS AND METHODS

DEAE-cellulose (Eastern Chemical Corporation) was freed of soluble material absorbing light at 276 m μ by successive washings on a wrist-action shaker with 1N aqueous potassium chloride solution containing 0.001M "Versene", ethanol (optically pure), 50 per cent. ethanol–water, and 1N potassium chloride until the absorption at 276 m μ was low and reproducible. For easy filtration on a Buchner it was necessary to remove some of the "fines". Shaking 200 ml of 1N potassium chloride

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with the original DEAE-cellulose (10 g) gave an $E_{276\text{ m}\mu}^{1\text{ cm}} = 0.55$ but after conditioning as described the $E_{276\text{ m}\mu}^{1\text{ cm}}$ was only 0.01. The resin was stored in the refrigerator under 1N potassium chloride solution.

The insulins used were the International sample No. 2189 (Anon. 1957) and Boots sample No. 9011G (Boots Pure Drug Co., Nottingham, England) which have been analysed by countercurrent methods (Harfenist and Craig 1952), the latter having been also chromatographed on sulphonated polystyrene resin by Boardman (1955) and the former with column partition chromatography by Porter (1953). Lilly insulin sample No. 535664 (Eli Lilly and Co., Indianapolis, U.S.A.)

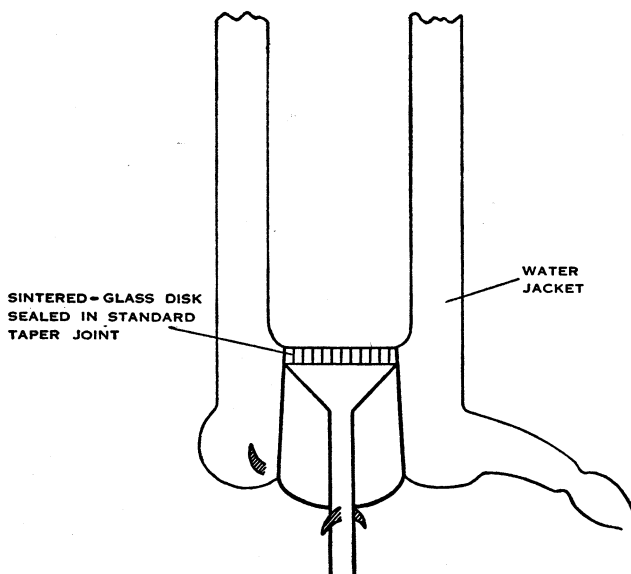


Fig. 1.—Construction of the lower part of the water-jacketed chromatographic columns. A sintered-glass disk was sealed in the top of a standard taper joint with a small built-in funnel to collect the effluent.

and a sample (batch No. A1) from the Commonwealth Serum Laboratories, Parkville, Vic., were also compared. The bovine plasma albumin was obtained from Armour and Co., Chicago, lot No. CA2140.

The jacketed columns (Fig. 1) 0.9 and 3 cm in diameter were maintained at constant temperature, $\pm 0.1^\circ\text{C}$, by circulation of water. The area ratio of the cross sections is 11 : 1 and provided this ratio was maintained in scaling up experiments from the smaller to the larger column results from the two columns were comparable. The larger column was used for preparative experiments.

A volumetric fraction dispenser (Simmonds 1958) was used for collecting 3–7-ml fractions but drop counting was found to be the most precise method for collecting fraction sizes of 0.2–0.5 ml when using the smaller column.

III. EXPERIMENTAL

(a) Preparation of the Columns

The DEAE-cellulose was washed on a Buchner funnel with the appropriate buffer and after suspending in the buffer the flask was evacuated to remove air bubbles. The slurry was then poured into the column and compacted under 10 lb/sq. in. nitrogen pressure (Sober *et al.* 1956). The columns varied from 12 to 15 cm in length.

(b) Operation and Loading of the Columns

The columns were eluted with solutions of constant ionic strength and pH, there being no advantage in using gradient elution in this work (Alm, Williams, and Tiselius 1952; Moore and Stein 1956). For the smaller column 0.5 ml of protein solution was added and washed in with 2×0.25 ml of buffer. For the larger column these amounts were increased by a factor of 10. During the loading of the column the flow was stopped by putting a finger over the bottom end of the column until the protein or buffer was added. The aliquots were then washed into the cellulose column at the same or a slower rate than was subsequently used for development of the column. This loading flow rate could be controlled by immersing the column in a measuring cylinder of water (Boardman and Partridge 1955), by a rubber tube and clip over the end of the column, or by connecting the buffer reservoir using polythene tubing so that only a small positive head was established before removal of the finger.

Experiments were carried out at either 1, 13, 18, or 25°C. Fractions were collected from the time of application of protein to the column.

The rates of flow of effluent from the columns were varied from 3 to 30 ml/sq.cm/hr with a normal flow rate of 12 ml/sq.cm/hr.

The hold-up volume of a column was determined from the position of the ammonia peak in the insulin samples or by passing arginine through the column since this was not retarded by the resin.

To obtain constant drop size when using the drop counter for collecting fractions it was found that the addition of 0.5 per cent. of a non-ionic detergent solution, "Brij 35" (Moore and Stein 1954), was necessary. Although over the course of an elution the fraction size may have had an overall weight variation of 6 per cent. the difference between any two successive fractions was never more than 1 per cent.

Columns were run without regeneration as long as it was certain all protein had been removed by the eluting buffer. They were equilibrated with new buffer before changing a buffer system. Periodically the DEAE-cellulose was removed, regenerated with 1N potassium chloride, treated with 50 per cent. ethanol-water to eliminate contamination by bacteria or bacterial proteases, and repacked in buffer.

(c) Buffers

The pH range 7-9 was used and the buffer solutions consisted of 0.001M "Versene", 0.005M tris (tris(hydroxymethyl)aminomethane) plus hydrochloric acid to the required pH, and 0.1-0.3M potassium chloride or other salt. In some

experiments merthiolate (1 in 20,000) was added as a preservative but since this absorbs light strongly at 276 $m\mu$ absorption must be determined by difference from a control tube containing no protein.

(d) *Preparation of Protein Solutions*

At the pH values used the insulin would not spontaneously dissolve to give a water-clear solution. It was found preferable to add the insulin to the buffer and then take the pH to 2.3 with 1-6N hydrochloric acid; here the insulin dissolved completely. The pH was then immediately brought back to 7, 8, or 9 with concentrated potassium hydroxide. The various insulin preparations differed in the pH at which they became water-clear on the addition of this alkali. The insulin dissolved between pH 6.8-7.2, the exact value depending on the sample of insulin and this was not altered by the removal of zinc from the insulin (Onoley *et al.* 1952). Furthermore, these solutions were not stable for they often developed cloudiness on standing overnight. For this reason not all samples could be studied at pH 7.

The insulin solution was then either loaded directly onto the column or dialysed against buffer overnight at 2°C on a shaker. 18/32 Visking "Cellophane" tubing was used to dialyse the insulin since Craig, King, and Stracker (1957) have shown that insulin does not pass through this tubing. The concentration of the insulin solution added to the column varied from 5 to 16 mg per ml buffer with a usual working value of 10 mg per ml.

Bovine plasma albumin was dissolved directly in the buffer at a concentration of 4 mg per ml. This caused the pH to fall a little and it was adjusted back to the value of the original buffer with potassium hydroxide solution.

(e) *Analysis of Effluent Fractions*

The protein concentration of the effluent from the large column was measured either by absorption of light at 276 $m\mu$ or by taking 0.5-ml aliquots of each fraction and using the ninhydrin technique of Moore and Stein (1948) after adjusting to approximately pH 5 with one or two drops of hydrochloric acid (0.035N). The effluent from the smaller column was analysed by the ninhydrin technique only.

(f) *Re-chromatography of Insulin Fractions*

The bulk fractions from the larger column were dialysed on a rocking dialyser at 2°C to free them from salt. The protein solution was concentrated either by freeze-drying or by isoelectric precipitation at pH 5.4. The latter method was preferable in some respects but recovery of the protein was not complete. The recovered protein was then treated in the same manner as the original for chromatography.

(g) *Titration Curves of DEAE-cellulose*

Air-dried DEAE-cellulose (0.2 g) was stirred under nitrogen in 20 ml 0.5M potassium chloride (Peterson and Sober 1956), titrated to pH 11 with 1N alkali, and back-titrated in intervals of 0.25 pH units to pH 3 with 1N hydrochloric acid. These titration curves at 10, 20, 30, and 40°C were kindly performed by Dr. H. Lindley on

a pH-stat described by Wood (1959). Equilibrium readings were taken after consumption of acid had ceased for 1 min. No correction was applied for the titration of water.

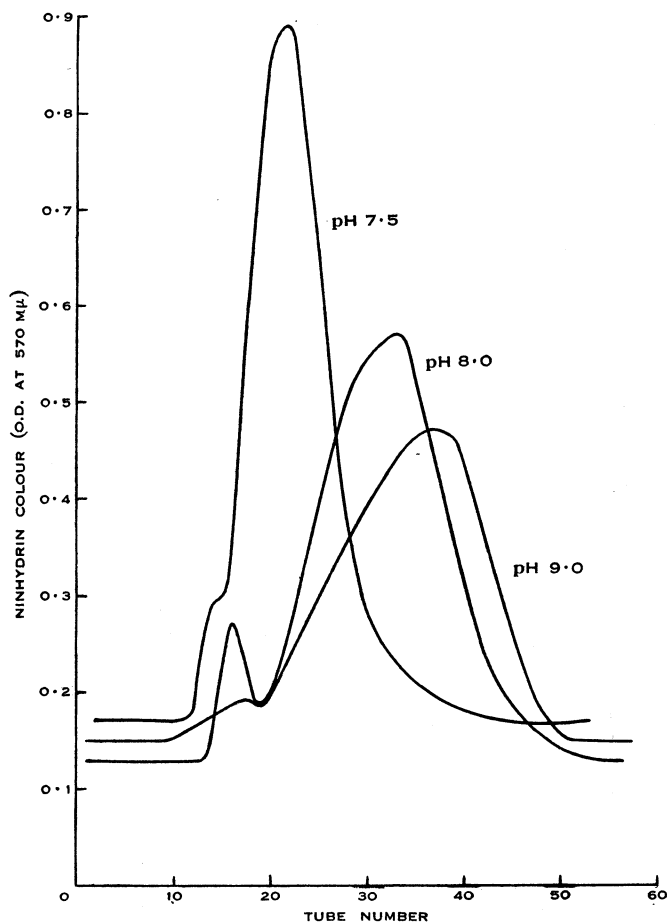


Fig. 2.—Effect of variation of pH at 18°C on the elution curve of International insulin (No. 2189) chromatographed on a 0.9×13 cm column of DEAE-cellulose. About 5 mg of insulin was chromatographed in each case, with $0.005M$ tris buffer containing $0.3M$ KCl. The effluent was collected in 0.5-ml fractions.

IV. RESULTS

(a) *Effect of Variation of pH*

The general effect of the variation of pH on the chromatography of insulin on DEAE-cellulose can be predicted from the titration curve of the DEAE-cellulose (Peterson and Sober 1956) and the protein (Cohn and Edsall 1943). Thus at fixed ionic strength a rise in the pH from 7 to 9 increases the charge on the protein causing stronger binding between the protein and cellulose; this is manifest in an increasing elution volume with increase in pH as shown in Figure 2.

(b) *Effect of Variation of Ionic Strength and Nature of Ions*

As shown in Figure 3 the effect of increasing the ionic strength at a fixed pH value is to lower the elution volume. With 0.1M potassium chloride the insulin was retained by the column.

In the chromatography of proteins on negatively charged resins such as "Amberlite IRC-50" multivalent buffer anions such as phosphate have been particularly useful (Hirs, Moore, and Stein 1953; Boardman and Partridge 1955; Moore and Stein 1956) because of combination with the protein and concomitant lowering of

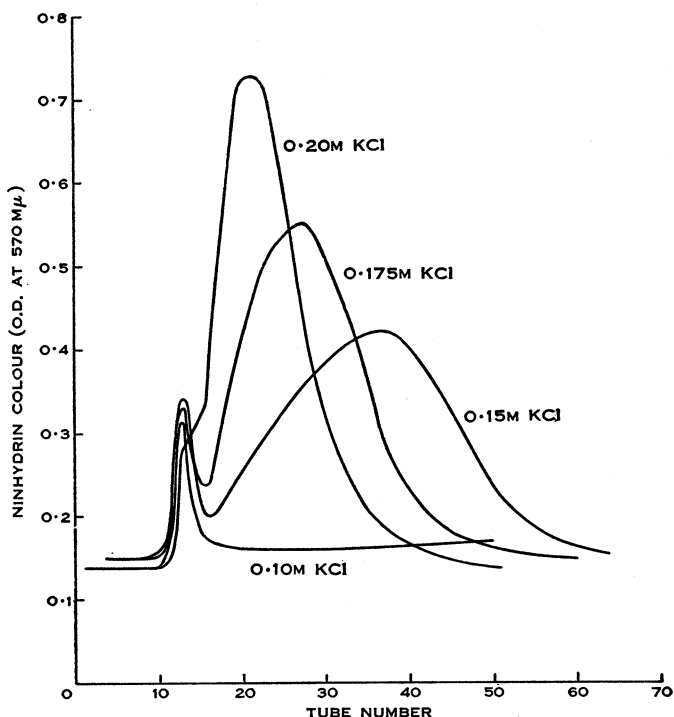


Fig. 3.—Effect of ionic strength on the elution curve of International insulin (No. 2189) chromatographed on a 0.9×13 cm column of DEAE-cellulose. About 5 mg of insulin was chromatographed in each case, with 0.005M tris buffer containing varying concentrations of KCl at pH 7.5 and 1°C. The effluent was collected in 0.5-ml fractions.

the isoelectric point. With insulin at pH 8 and 18°C in buffer containing 0.1M dipotassium hydrogen phosphate (ionic strength approx. 0.3) insulin was not eluted from the DEAE-cellulose. When sulphate or acetate (of equal ionic strength) were substituted for 0.3M chloride ion at pH 8 and 18°C the elution curve of the insulin was very spread. For our purposes the combination of chloride ions and tris buffer cations, which have advantages for chromatography on anion exchange resins (Boman and Westlund 1956) proved the most satisfactory.

(c) Effect of Temperature

It was found (Fig. 4(a)) that lowering the temperature at which chromatography is carried out has the same effect on the chromatographic behaviour of insulin as increasing the ionic strength or lowering the pH, i.e. the elution volume is lowered. Furthermore, as can be seen from the figure the skewness was changed. The same behaviour was found with bovine plasma albumin (Fig. 4(b)).

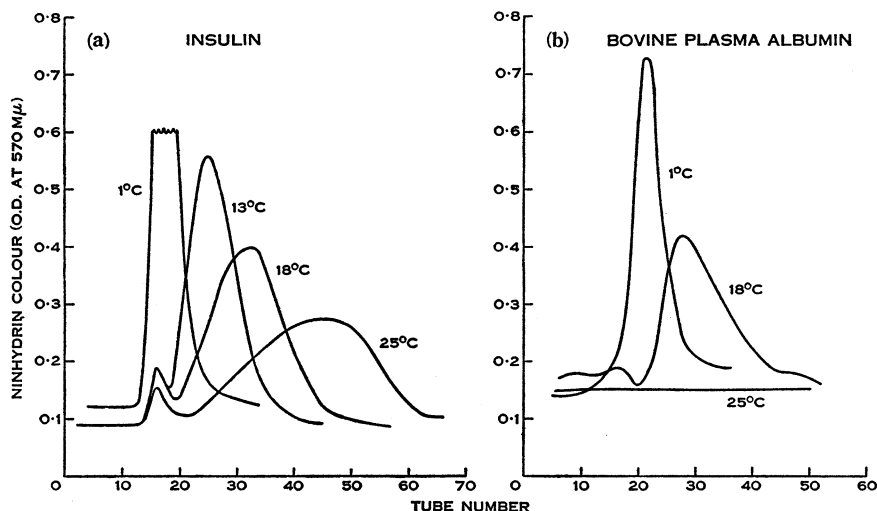


Fig. 4.—(a) Effect of temperature on the elution curve of International insulin (No. 2189) chromatographed on a 0.9×13 cm column of DEAE-cellulose. About 5 mg of insulin was chromatographed in each case, with $0.005M$ tris buffer containing $0.3M$ KCl at pH 8 as eluent. (b) Elution curves of bovine plasma albumin chromatographed on a 0.9×14 cm column of DEAE-cellulose at 1, 18, and $25^\circ C$. About 2 mg of albumin was chromatographed in each experiment, with $0.005M$ tris buffer containing $0.11M$ KCl at pH 8. The effluents were collected in 0.5-ml fractions.

(d) Effect of Flow Rate

The elution curves of insulin developed at the normal flow rate of 12 ml/sq. cm/hr with buffer at pH 8 containing $0.3M$ potassium chloride at $18^\circ C$ had a trailing edge which was steeper than the leading edge so that the curves were slightly asymmetric (Fig. 5(b)). This effect was not due to lack of equilibration with the resin since reducing the flow rate to 3 ml/sq. cm/hr increased the skewness (Fig. 5(a)) while increasing the flow rate to 30 ml/sq. cm/hr increased the symmetry with no change in the elution volume.

(e) Comparison of Insulin Preparations

Chromatography over the pH range 6.5–9 of the insulin samples studied revealed the presence of three components. The first of these, which was not absorbed on the column, was ammonia. This varied in amount among various insulin preparations. The presence of the ammonia peak on the elution curve could be avoided by (1) measurement of the ultraviolet absorption curve at $276 m\mu$ on the 5-ml

fractions from the larger column: ninhydrin determinations confirmed the presence of ammonia; (2) dialysis overnight of the insulin sample against the eluting buffer

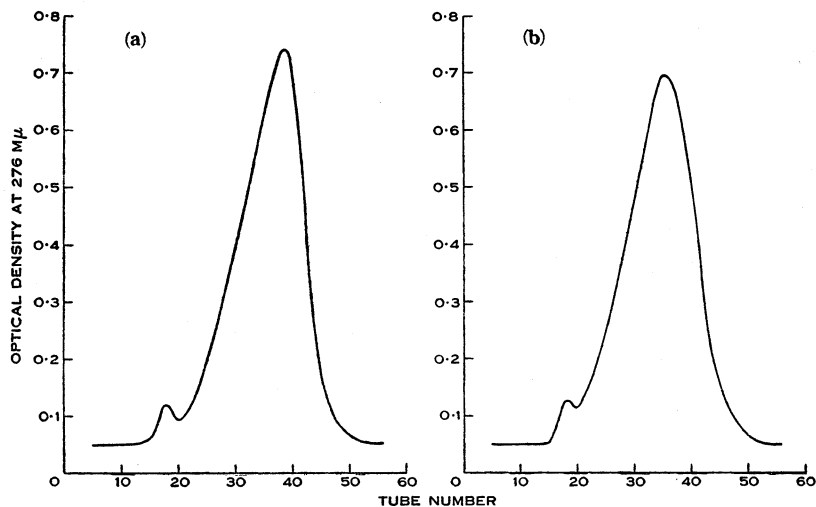


Fig. 5.—Effect of flow rate on the symmetry of the effluent curves of Commonwealth Serum Laboratories insulin chromatographed on a 3×15 cm column of DEAE-cellulose. About 60 mg of insulin was chromatographed in each case at 18°C , with 0.005M tris buffer containing 0.3M KCl at pH 8 as eluent. The effluent was collected in 5.5-ml fractions. The flow rates were (a) 3 ml/sq. cm/hr; (b) 12 ml/sq. cm/hr.

followed by ninhydrin assay of the tubes (the column was first equilibrated, and subsequently developed with, the dialysate); (3) isoelectric precipitation of the

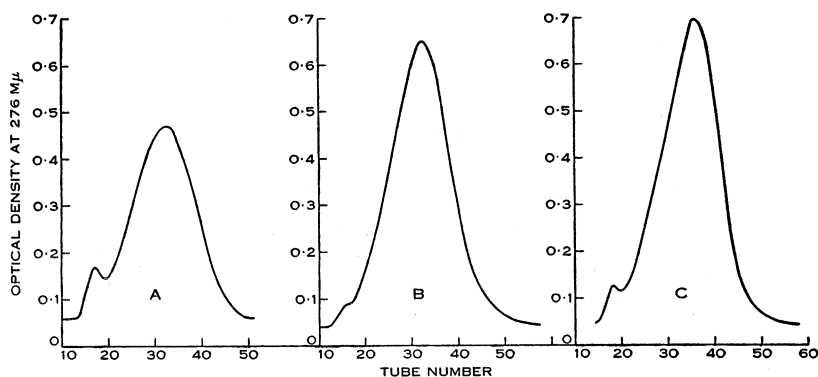


Fig. 6.—Comparison of insulins by chromatography on a 3×15 cm column of DEAE-cellulose at pH 8 and 18°C in 0.005M tris buffer containing 0.3M KCl. A, 50 mg Lilly insulin; B, 63 mg International insulin (No. 2189); C, 62 mg Commonwealth Serum Laboratories insulin. Effluents collected in 5.5-ml fractions.

insulin solution followed by centrifugation and re-dissolution of the precipitate in buffer, the elution being followed by ninhydrin assay; (4) alkaline hydrolysis of

the fractions before ninhydrin assay (Hirs, Moore, and Stein 1956). The second chromatographic component which overlapped the ammonia peak was a minor one and varied from 2 per cent. in the International sample to 6–7 per cent. in the Lilly sample (Fig. 6). The third and major component is insulin plus any desamido component present. Below pH 7·2–7·3 the tendency of the insulins to come out of solution was reflected in a trailing tail which was more apparent at the lower pH values.

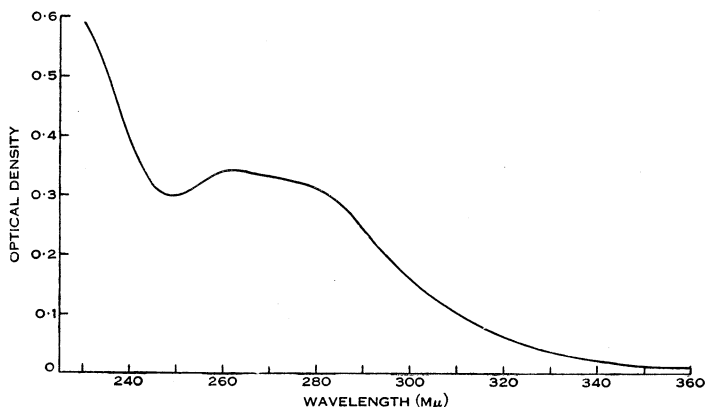


Fig. 7.—Ultraviolet absorption curve of an optically pure ethanol extract of Visking "Cellophane" measured with a Beckman DK-2 recording spectrophotometer.

Removal of the zinc from the Lilly and International samples did not affect their chromatographic behaviour.

The recovery of the ninhydrin-positive material was between 90 and 100 per cent. depending on the choice of base line. The fact that the curve returned to its original base line suggested that all material was removed from the column.

(f) *Re-chromatography of Insulin Fractions*

Cuts were made to separate the second and third components of the insulin chromatograms. These fractions were dialysed free of salt and freeze-dried before being dissolved and re-applied to the columns. Unfortunately this process of concentration produced a fast running artifact which was due to non-dialysable material dissolving from the "Cellophane" tubing. For example, 50 cm of "Cellophane" tubing when soaked in 50 ml optically pure ethanol gave the ethanol an optical density of 0.4 at 276 mμ (cf. Fig. 7). Preliminary washing of the "Cellophane" with optically pure ethanol followed by 50 per cent. ethanol–water and water at 50°C before using for dialysis did not completely eliminate the artifact. This artifact, in the case of the third or major component, could be avoided by isoelectric precipitation of the eluate fractions after dialysis followed by centrifugation and re-dissolution of the protein in buffer. It then emerged as a single peak. Such a treatment did not remove the second component from the original insulin.

The second component always re-chromatographed in the same position and was contaminated by this artifact but it did not contain any of the major third component thus eliminating the possibility that it was due to an aggregation equilibrium of the insulin. This second component could not be precipitated at pH 5.4.

V. DISCUSSION

Previous studies have shown that purified insulin may contain several components. The presence of varying amounts of bound ammonia was shown by Chibnall, Mangan, and Rees (1958) who also found that the only insulin examined by them which gave the theoretical six residues of amide nitrogen (Sanger, Thompson, and Kitai 1955) was a preparation isolated by Harfenist and Craig (1952) following countercurrent distribution. A de-amidated component of insulin was separated by Harfenist and Craig and analysed by Harfenist (1953). More of this component was found in a sample of Lilly insulin than in the Boots sample of insulin (No. 9011G) or International Batch No. 2189.

We have no evidence that we have succeeded in separating the desamido component of insulin on DEAE-cellulose since countercurrent studies by Human and Leach (unpublished data) of the Lilly insulin used revealed approximately 18 per cent. of the desamido component whereas we only found a small component of 6 per cent. separated from the main peak. Moreover, a sample of the desamido insulin isolated by Human and Leach according to the method of Harfenist and Craig (1952) when chromatographed on DEAE-cellulose at pH 8 and 18°C with buffer containing 0.3M potassium chloride gave a major peak in the same position as the original insulin and also a minor peak (12 per cent.) in the same position as the minor peak in the original insulin.

Another component which has been detected in insulin is glucagon (Porter 1953) which is present to the extent of no more than 1 per cent. Because the minor component obtained by us is much more than 1 per cent. of the total insulin it is unlikely to be glucagon. Harfenist and Craig (1952) showed the presence of a third component in insulins and it is possible that this is the component that we have isolated on DEAE-cellulose. We have made no measurements of activities of insulin or its fractions.

Cole (1959) has recently reported the separation of commercial insulins into three components by chromatography in 8M urea at pH 6 on "Amberlite IRC-50" columns. Because of the insolubility of insulin in the pH range 4-7 we have not been able to work near the isoelectric point of insulin (in the absence of urea or dioxan) where minor differences in charge between various proteins might be expected to have their greatest effect in chromatographic separation. From the data available in the literature (Moore and Stein 1956; Hill, Kimmel, and Smith 1959) it appears that most successful chromatographic separations of proteins with similar isoelectric points have been achieved near the isoelectric point of the proteins.

The three variables—temperature, ionic strength, and pH—could be varied independently to produce similar effects on the chromatographic behaviour of insulin on DEAE-cellulose; for example, a variation of any one could be compensated by

adjustment of the other two. The effects of variation of pH and ionic strength are similar to those obtained during chromatography of proteins on this and other resins (Moore and Stein 1956; Sober *et al.* 1956) but to our knowledge the effect of temperature on chromatography on DEAE-cellulose has not previously been emphasized. Most chromatographic studies are conducted at constant temperature, e.g. at 4°C, to minimize denaturation effects. In our preliminary experiments conducted at room temperature a variation of 5°C during the course of the experiment led to breaks and spreading in the effluent curve.

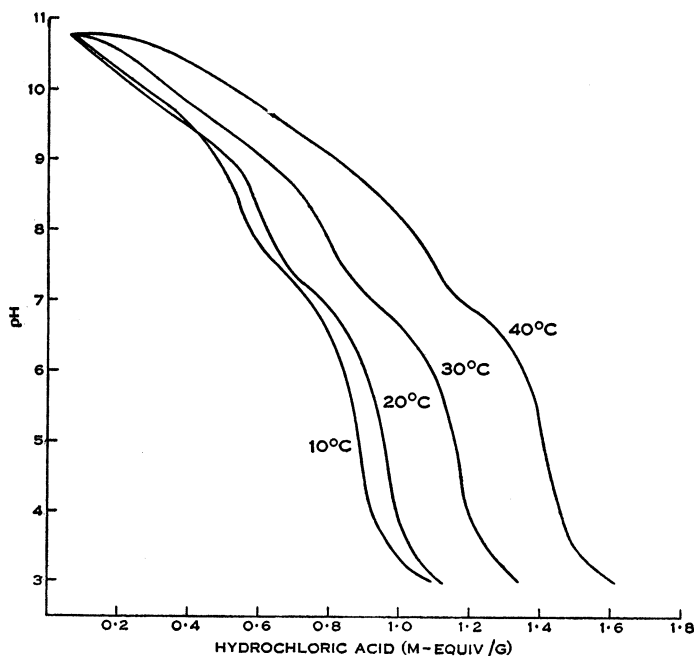


Fig. 8.—Titration curves of DEAE-cellulose in 0.5M KCl at temperatures of 10, 20, 30, and 40°C.

During the chromatography of histones on "Amberlite IRC-50", Crampton, Moore, and Stein (1955) found that a variation in temperature from 4 to 25°C did not affect the effluent pattern. Similarly Boardman and Partridge (1955) found their major peaks were in the same position at 2 and 25°C when chromatographing carbon monoxide haemoglobin on "Amberlite IRC-50" although at the higher temperature more denaturation occurred. The temperature effect observed with insulin on DEAE-cellulose is not peculiar to the aggregating insulin system since it also occurs with the non-aggregating bovine plasma albumin. It is known that the titration curves of insulin do not vary considerably between 4 and 25°C (Cohn and Edsall 1943) and we must consider the possibility of temperature affecting the pK 's of the amino groups on the DEAE-cellulose.

From the titration curves of DEAE-cellulose (Fig. 8) it is seen that there is a marked effect of temperature on the ionization of the basic groups of this insoluble

polyelectrolyte; as the temperature is increased the number of basic groups on the cellulose at any pH (range 3–11) usually increases. The increase is greater at higher temperatures and explains the marked increase with temperature in the elution volumes of proteins chromatographed on DEAE-cellulose. Moreover, the effect of temperature on the ionization of insulin (Cohn and Edsall 1943), though small, supplements this effect.

VI. ACKNOWLEDGMENTS

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