THE CHROMATOGRAPHY OF INSULIN ON DEAE-CELLULOSE IN BUFFERS CONTAINING 8M UREA*

By E. O. P. THOMPSON† and I. J. O'DONNELL†

[*Manuscript received March* 2, 1960]

Summary

The chromatography of four samples of insulin by elution analysis at constant pH and ionic strength on a diethylaminoethyl (DEAE)-cellulose column has been studied. The presence of three components was apparent at pH 7·4 in a buffer containing 8M urea. The results are compared with those obtained on the same samples of insulin by other workers using countercurrent and chromatographic techniques. The experimental and theoretical curves for the major peak of the International standard insulin sample coincide.

I. INTRODUCTION

In a previous paper (O'Donnell and Thompson 1960) it was shown that when various insulin preparations were chromatographed on diethylaminoethyl (DEAE)-cellulose in the pH range 7–9 they could be separated into a major and a minor protein component. It was not found possible under a variety of conditions of pH, ionic strength, and temperature to separate from the main peak the desamido insulin, varying amounts of which exist in most insulin preparations (Harfenist and Craig 1952). To enable chromatography to be carried out at lower pH values near the isoelectric point, where insulin is normally insoluble, and under disaggregating conditions, buffers containing 8M urea have been used. Under these conditions four samples of insulin have each been separated into at least three components. Comparison has been made with countercurrent separations on the same insulins (Harfenist and Craig 1952; Human and Leach 1960).

Cole (1959) has given a preliminary account of the separation of the commercial insulin preparations into three components by chromatography on an acidic resin, "Amberlite IRC-50", with buffers containing 8M urea.

II. EXPERIMENTAL

(*a*) *Columns*

The preparation and operation of the DEAE-cellulose columns were as described in a previous paper (O'Donnell and Thompson 1960). The DEAE-cellulose was equilibrated with buffer containing 8M urea and packed under 10 lb/sq.in. nitrogen pressure in a 13–17 by 0·9 cm (dia.) column kept at constant temperature. The column was then further equilibrated overnight by flowing buffer through it until the influent and effluent pH values were identical. The column was loaded with 0·5 ml of a 2 per cent. solution of insulin, which was washed in with filtered

* A preliminary account of this work was presented to the Australian Biochemical Society at Canberra in January 1960.

† Division of Protein Chemistry, C.S.I.R.O. Wool Research Laboratories, Parkville, Vic.

buffer ($2 \times 0 \cdot 5$ ml) and then eluted at a flow rate of 12–20 ml/sq.cm/hr with buffer which had passed through a subsidiary 5 cm column of DEAE-cellulose. This smaller column was connected by a ground-glass joint to the top of the main column to filter the urea buffer. This filter, which was designed to remove ultraviolet-absorbing material and dark impurity, also collected carbonate ions. If, after use, the filter was washed with 1N potassium chloride, coloured bands were eluted together with material absorbing strongly at 276 mμ.

Approximately $0 \cdot 7$ ml fractions of the eluate were collected by a drop-counting fraction collector and the exact size of the fraction determined by weighing. The density of the buffers was $1 \cdot 11$.

A detergent for minimizing drop-size variation was not added to the buffers and the fraction size (12 drops) showed a marked decrease on emergence of protein (see Fig. 2).

(*b*) *Buffer and Insulin Solutions*

The buffer used contained 8M urea (B.D.H. "Analar"), 0·01M Tris (tris-(hydroxymethyl)aminomethane), and 0·001M "Versene" and it was adjusted to the required pH with hydrochloric acid. The insulin spontaneously dissolved in the buffer and this solution was adjusted to the correct pH with 1N Tris solution.

The insulin samples chromatographed were International sample No. 2189 (Anon 1957), Boots sample No. 9011G (Boots Pure Drug Co., Nottingham, England), Lilly sample No. 535664 (Eli Lilly and Co., Indianapolis, U.S.A.), and a sample from the Commonwealth Serum Laboratories (C.S.L.), Parkville, Vic.

(*c*) *Analysis of Effluent Fractions*

The effluent fractions were diluted with 3 ml water and if necessary the pH adjusted to about $7 \cdot 5$ with a drop of hydrochloric acid of suitable normality. The absorption of light was measured at 276 mμ in 1 cm cells using a Beckmann DU spectrophotometer. Following the procedure of Goodwin and Morton (1946) the absorption was also measured at 320 and 360 mμ and corrections for scattering made to the 276 mμ value. No activities of the separated insulin components were determined.

(*d*) *Characteristics of the Column*

The method of Mayer and Tompkins (1947) was used to calculate the theoretical elution curve using the parameters obtained with insulin on the DEAE-cellulose column. For this purpose it was necessary to know the hold-up volume (volume of mobile liquid held between the swollen cellulose particles) of the column. This was determined by a method based on that of Pepper, Reichenberg, and Hale (1952); a small column, 5 cm long and fitted with a sintered disk as described by these authors, was packed in buffer with DEAE-cellulose under the same conditions as those used for the larger column. After allowing the liquid to drain to the level of the cellulose the tube plus cellulose was placed in a graduated centrifuge tube, centrifuged at 400 *g* for 20 min and the volume of emerging liquid measured. Centrifugation at 500 *g* did not increase this volume which was close to two-thirds of the total volume of the packed column. The centrifuged pellet was washed free

of buffer on a Buchner funnel, dried at 100°C, and weighed. This gave a value of 6·2 ml (5·9, 6·6, 6·0) as the hold-up volume per gram of dry DEAE-cellulose for a column operated under these conditions. Thus our columns had a hold-up volume of 8–9 effluent fractions, depending on the length of the column, and this agreed with the tube number in which the fraction size suddenly decreased (see Fig. 2) and often with the emergence of a small amount of front-running impurity.

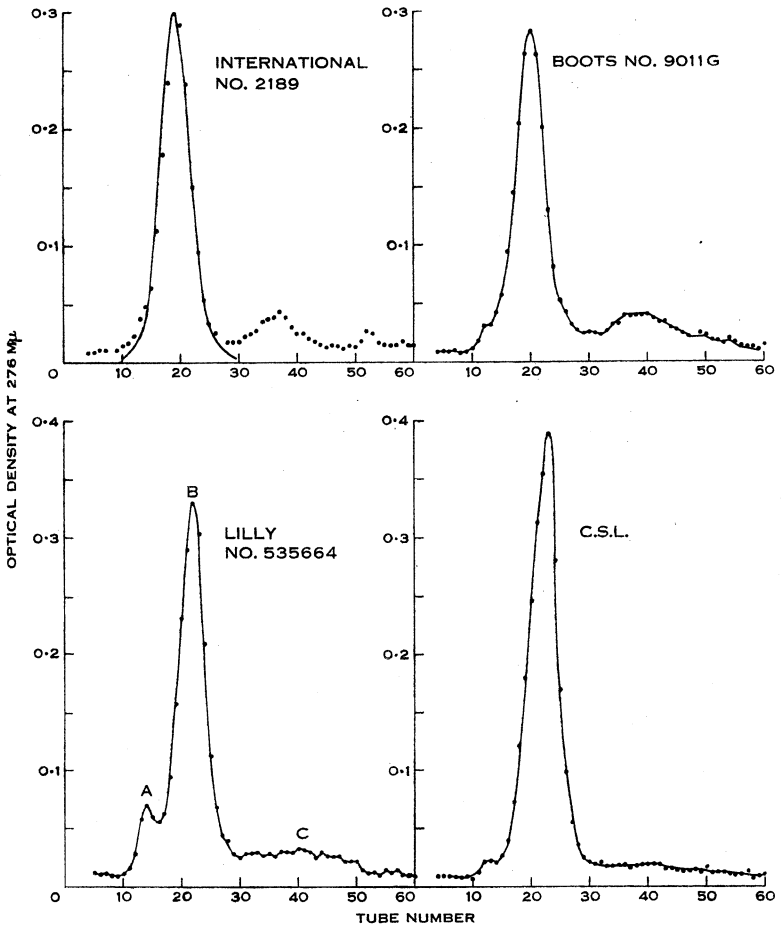

Fig. 1.—Chromatography of crystallized insulin samples on a 0·9 by 17 cm column of DEAE-cellulose at 25°C. About 10 mg of insulin was chromatographed in each case with 8M urea containing 0·01M Tris buffer and 0·001M "Versene" at pH 7·4. The effluent was collected in *c.* 0·7-ml fractions. Solid circles are experimental points. For International insulin No. 2189 only, the continuous line is the calculated theoretical curve.

III. Results and Discussion

Figure 1 shows the elution curves obtained by chromatographing four samples of insulin on DEAE-cellulose in buffers containing 8M urea at pH 7·4. The shoulders on the curves were reproducible. All the samples have separated into three

components, the percentages of each being given in Table 1. The values for the front A peak are only approximate due to difficulty in assessing the complete curve of the first component and hence are not identical with previous estimates (O'Donnell and Thompson 1960) where separation was more complete.

In the presence of 8M urea much lower concentrations of salt are required to elute the insulin from the DEAE-cellulose; for example, at 18°C and pH 7·4 in the absence of urea an ionic strength of approximately 0·31 was necessary to produce an R_F of 0·5 (O'Donnell and Thompson 1960) whereas in buffer containing 8M urea less than 0·014 ionic strength is required. The recovery of protein from the column was quantitative (90–100 per cent.) within the limits of error in fixing the base line.

TABLE 1

PERCENTAGE OF VARIOUS COMPONENTS IN COMMERCIAL INSULINS

Insulin chromatographed on DEAE-cellulose at pH 7·4 in 8M urea containing 0·01M Tris and 0·001M "Versene"

Component	Percentages of Components in Insulin			
	International No. 2189	Boots No. 9011G	C.S.L.	Lilly No. 535664
A	6	3	2	9
B	80	76	92	75
C	14	21	6	16

Our results are in general agreement with those of Harfenist and Craig (1952) who first showed that crystallized insulins could be resolved into native and desamido fractions by countercurrent distribution between *n*-butanol and aqueous dichloroacetic acid. Boardman (1959*b*) succeeded in partially resolving crystallized insulin by chromatography on a specially prepared sulphonated polystyrene stationary phase whereas Porter (1953) could not separate a desamido fraction by liquid–liquid chromatography on kieselguhr columns.

The separations by chromatography on DEAE-cellulose in buffers containing 8M urea are better than those obtained by either Porter (1953) or Boardman (1959*b*). Boots insulin No. 9011G has been examined by countercurrent distribution (Harfenist and Craig 1952) and chromatographically by Boardman (1959*b*), the latter reporting non-quantitative recoveries indicative of irreversible binding. The International standard insulin No. 2189 we have examined is supplied with sample curves obtained by the techniques of countercurrent distribution (Harfenist and Craig 1952) and liquid–liquid chromatography (Porter 1953) and the resolution we have obtained is superior to that shown by either of these techniques.

It is apparent that, of the insulins we have chromatographed, the C.S.L. insulin contains least of the minor components. However, the main peak shows a skewness not obvious in the other samples. Countercurrent distribution studies (Human and Leach 1960) by the method of Harfenist and Craig (1952) also showed less of the desamido component in a sample of C.S.L. insulin compared with either the Lilly or the International samples of insulin.

In the previous paper (O'Donnell and Thompson 1960) it was concluded that, in the absence of urea, the desamido fraction was inseparable from the main peak. From comparison with countercurrent distribution curves of samples of insulin (Harfenist and Craig 1952; Human and Leach 1960) it is probable that the C component reported here is the desamido fraction characterized by Harfenist and Craig (1952) and Harfenist (1953). The A component is the same as that previously separated by O'Donnell and Thompson (1960). A sample of desamido insulin isolated by countercurrent distribution from Lilly insulin (Human and Leach 1960) split, in the absence of urea, into component A and a component which ran in the same position as the major insulin fraction. With the buffer containing 8M urea at pH 7·4 a small sample of the desamido insulin split into only A and C components but insufficient material was available to give large unequivocal peaks.

The binding of proteins to ion-exchange resins is due to a combination of ionic, van der Waal's forces, and hydrogen bonds and the forces involved are usually multiple (Moore and Stein 1956). In the absence of urea relatively high concentrations of salt were required to elute the insulin but the presence of urea with its strong disaggregating properties and high dielectric constant reduced the binding of the insulin to such an extent that very little salt was required to elute the protein. Moreover, at pH 7·4 in the presence of urea, resolution of the desamido component (C) was achieved which was not possible in the absence of urea. Either the isoelectric points of the insulin components have been raised in 8M urea or the weaker binding of the proteins to the DEAE-cellulose is responsible for the improved resolution.

A theoretical distribution curve (Mayer and Tompkins 1947) agreed well with the experimental curve for the main peak (B) of insulin No. 2189 in Figure 1 which chromatographed with an R_F of 0·5. In order to compare the practical and theoretical insulin curves at a lower R_F value the effect of pH and temperature on the chromatography of insulin was investigated. A change of temperature between 10 and 35°C had no effect on the R_F in contrast to the marked effect in the absence of urea (O'Donnell and Thompson 1960). Increase in pH at 25°C to 8·3 lowered the R_F to 0·3 while at pH 8·9 it was 0·25. A theoretical curve for $R_F = 0·3$ is given in Figure 2 (for insulin No. 2189) and it is seen that the chromatographic behaviour of insulin under these conditions is close to ideal. As the pH of the urea buffer was increased to pH 8·9 the separation of the minor components became scarcely detectable. This is because the desamido component (C) is not eluted at the higher pH value, while the minor A component becomes too diffuse for accurate measurement. Presumably the desamido component of insulin could be prepared by

adsorption on a DEAE-cellulose column at pH 9 under the conditions given here and subsequently eluted at a lower pH value.

At pH 6·4 the main peak is comprised of the A and B components and has shifted back towards the break-through point while the desamido component (C) appears as a pronounced shoulder on the elution curve (Fig. 3).

This trailing desamido component of the various insulins studied does not chromatograph the same in all cases (Fig. 1). For example, the Lilly insulin does

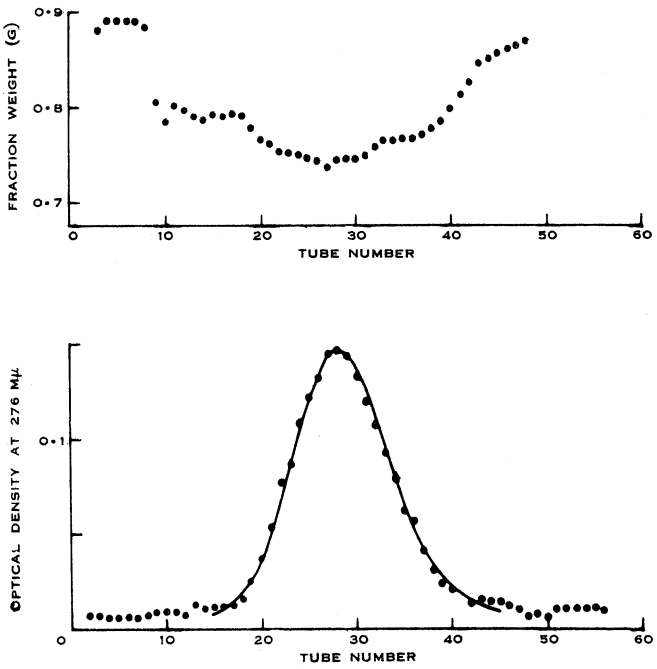

Fig. 2.—Chromatography of International insulin No. 2189 on a 0·9 by 14 cm column of DEAE-cellulose at 25°C. About 10 mg of insulin was chromatographed at pH 8·3 in a buffer containing 8M urea, 0·01M Tris, and 0·001M "Versene". The effluent was collected in *c.* 0·7-ml fractions. Solid circles are experimental points and the continuous line is the calculated theoretical curve. Weights of fractions (12 drops) are given in the upper curve.

not show a pronounced peak as do the Boots and International insulins and maybe more than one type of deamidated molecule is present. It is possible that the deamidation of insulin has occurred at different positions on the molecule. There are six amide groups in insulin and some deamidation of the terminal asparagine residue has been reported (Sanger and Thompson 1953; Harris 1955). In addition to peptides containing the terminal asparagine in a deamidated form some peptides have been isolated, indicative of deamidation of a glutamine residue (Sanger, Thompson, and Kitai 1955). If a mixture of various deamidated components is present this might not be expected to chromatograph as a single substance.

The presence of a desamido fraction in commercial insulins suggests that either deamidation has resulted from manufacturing processes or that synthesis of a proportion of deamidated molecules has occurred in the pancreas (Vaughan and Steinberg 1959). The variation in percentage of the desamido fraction between the different insulins might suggest that the former explanation is more likely. However, it is

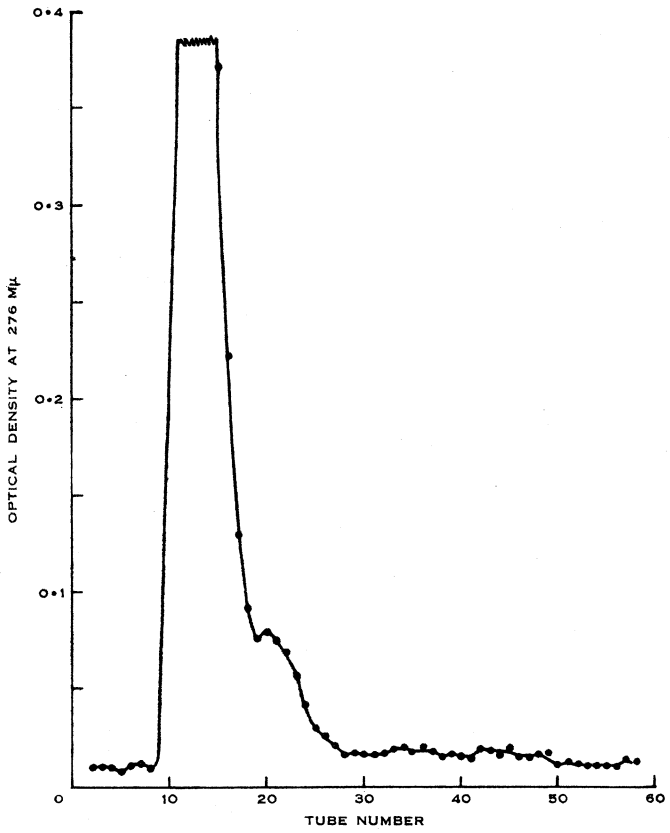

Fig. 3.—Chromatography of International insulin No. 2189 on a 0·9 by 17 cm column of DEAE-cellulose at 25°C. About 10 mg of insulin was chromatographed at pH 6·4 in a buffer containing 8M urea, 0·01M Tris, and 0·001M "Versene". The effluent was collected in *c.* 0·7-ml fractions.

also possible that the various manufacturing processes fractionate the desamido component from the bulk of the insulin to different extents. Moreover, studies on ribonuclease (Martin and Porter 1951; Hirs, Moore, and Stein 1953; Aquist and Anfinsen 1959) have shown the presence of at least two components, differing probably by only one carboxyl group (Tanford and Hauenstein 1956), in crude extracts of pancreas under conditions which did not seem to favour deamidation of one molecule into the other.

As Moore and Stein (1956) have pointed out, the number of proteins that have been chromatographed successfully by elution analysis at constant pH and ionic strength is limited to a comparative few, e.g. cytochrome *c*, ribonuclease, lysozyme, chymotrypsin, and chymotrypsinogen and most of these have been run on columns of "Amberlite IRC-50", where a reversible distribution coefficient giving an R_F between 0·7 and 0·1 has been obtained. There are some proteins such as the haemoglobins studied by Boardman and Partridge (1955) which can be chromatographed at constant pH and ionic strength but only at relatively high R_F values ($>0{\cdot}5$). At lower R_F values the adsorption is not truly reversible as it is in the case of other proteins such as cytochrome *c* (Boardman 1959*a*) which can be chromatographed at low R_F values where the test for homogeneity of a protein by comparison of experimental and theoretical curves is more exacting. Insulin in buffers containing 8M urea appears to undergo a true reversible adsorption on DEAE-cellulose at an R_F of 0·3 as shown by the close agreement between the theoretical and practical curves (Fig. 2).

IV. Acknowledgments

The authors are indebted to Drs. J. P. E. Human and S. J. Leach for a sample of desamido insulin and to the Commonwealth Serum Laboratories, Parkville, Vic., and Eli Lilly Laboratories, Indianapolis, U.S.A., for gifts of insulin. It is also a pleasure to acknowledge several helpful discussions with Dr. N. K. Boardman on aspects of elution chromatography.

V. References

Anon (1957).—*Nature* **179**: 567.

Aquist, S. E. G., and Anfinsen, C. B. (1959).—*J. Biol. Chem.* **234**: 1112.

Boardman, N. K. (1959*a*).—*J. Chromatogr.* **2**: 388.

Boardman, N. K. (1959*b*).—*J. Chromatogr.* **2**: 398.

Boardman, N. K., and Partridge, S. M. (1955).—*Biochem. J.* **59**: 543.

Cole, R. D. (1959).—*Fed. Proc.* **18**: 206.

Goodwin, T. W., and Morton, R. A. (1946).—*Biochem. J.* **40**: 628.

Harfenist, E. J. (1953).—*J. Amer. Chem. Soc.* **75**: 5528.

Harfenist, E. J., and Craig, L. C. (1952).—*J. Amer. Chem. Soc.* **74**: 3083.

Harris, J. I. (1955).—"Symposium on Peptide Chemistry." Special Publication No. 2. p. 71. (The Chemical Society: London.)

Hirs, C. H. W., Moore, S., and Stein, W. H. (1953).—*J. Biol. Chem.* **200**: 493.

Human, J. P. E., and Leach, S. J. (1960).—*Aust. J. Chem.* **13**(4): (in press).

Martin, A. J. P., and Porter, R. R. (1951).—*Biochem. J.* **49**: 215.

Mayer, S. W., and Tompkins, E. R. (1947).—*J. Amer. Chem. Soc.* **69**: 2866.

Moore, S., and Stein, W. H. (1956).—*Advanc. Protein Chem.* **11**: 191.

O'Donnell, I. J., and Thompson, E. O. P. (1960).—*Aust. J. Biol. Sci.* **13**: 69.

Pepper, K. W., Reichenberg, D., and Hale, D. K. (1952).—*J. Chem. Soc.* **1952**: 3129.

Porter, R. R. (1953).—*Biochem. J.* **53**: 320.

Sanger, F., and Thompson, E. O. P. (1953).—*Biochem. J.* **53**: 366.

Sanger, F., Thompson, E. O. P., and Kitai, R. (1955).—*Biochem. J.* **59**: 509.

Tanford, C., and Hauenstein, J. D. (1956).—*Biochim. Biophys. Acta* **19**: 535.

Vaughan, M., and Steinberg, D. (1959).—*Advanc. Protein Chem.* **14**: 115.