Electrophoresis on Cellulose Acetate of Insulin and Insulin Derivatives: Correlation with Behavior on Countercurrent Distribution and Partition-Column Chromatography*

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The technique of Sundby (1962) for paper electrophoresis of insulin derivatives in urea-containing buffers has been modified by the substitution of cellulose acetate strips for paper and by the application of relatively high voltages (40 v/cm). At *p*H 6.5 in 0.065 M phosphate buffer which is 7 M in urea, good separation of various charged forms of insulin were observed in 3 hours of electrophoresis. The electrophoresis procedure was standardized on insulin A and on desamido-insulin fractions which had been separated by countercurrent distribution and partition-column chromatography and characterized by amide analysis. The electrophoretic technique revealed that such samples were contaminated with small amounts of species differing in electrophoretic mobility from the main components. The desamido forms of insulin which are formed in the acid-catalyzed transformation reaction (Dickinson, 1956; Chrambach and Carpenter, 1960; Sundby, 1962) were characterized by electrophoretic mobility and partition-column chromatography. The response of various modified insulins, including desalanine-desasparagine-insulin, desalanine-desamido-insulin, and desoctapeptide-insulin to electrophoresis and partition-column chromatography or countercurrent distribution were compared. Of all the samples investigated only one sample, desalanine-desasparagine-insulin from a 2300-transfer countercurrent distribution experiment, behaved as a homogeneous substance on electrophoresis.

During the past few years, a number of techniques have revealed heterogeneity in many preparations of crystalline insulin of bovine origin. Included in these techniques are countercurrent distribution (Harfenist and Craig, 1952), electrophoresis-convection (Timasheff *et al.*, 1953), partition-column chromatography (Carpenter and Hess, 1956), ion-exchange chromatography (Cole, 1960; Thompson and O'Donnell, 1960), and zone electrophoresis (Barrett *et al.*, 1962). In a few instances, the heterogeneity of the samples has been traced to the presence of forms with a different number of amide groups (Harfenist, 1953; Carpenter and Chrambach, 1962). Related to this type of heterogeneity is an acid-catalyzed transformation of insulin which gives rise to several biologically active components. Dickinson (1956) detected the transformation by chromatography on a column of kieselguhr–calcium phosphate. Chrambach and Carpenter (1960) reported the separation from the transformation mixture of up to four components by partition-column chromatography. The major component formed early in the transformation was shown to be a desamido-insulin (Carpenter and Chrambach, 1962). This desamido-insulin was composed largely (80–90%) of a form in which the amide had been lost from the carboxyl terminal asparagine of the A chain (Slobin and Carpenter, 1963b). Recently, Sundby (1962) has reported on the use of paper electrophoresis to follow the acid transformation of insulin. Various acid treatments resulted in the production of up to six components which were separated by electrophoresis. The technique made use of the urea-containing buffers which were introduced by Cole (1960) for the ion-exchange chromatography of insulin.

The present work was undertaken in order that previous results obtained by the use of countercurrent distribution and partition-column chromatography could be correlated with those obtained by the electrophoretic technique. The technique has proved to be a very sensitive and rapid method for detecting insulins of differing charge and has revealed the presence of heterogeneity in many insulin preparations which had been isolated by partition-column chromatography or countercurrent distribution.

In view of the results on the desamido forms, it was desirable to investigate the electrophoretic behavior of a number of insulin derivatives which have been prepared in recent years by the action of various enzymes on insulin. These include desalanine-desasparagine-insulin and desalanine-desamido-insulin obtained by the action of carboxypeptidase A on insulin A, and desamido-insulin, respectively (Slobin and Carpenter, 1963a; 1963b), and desoctapeptide-insulin obtained by the action of trypsin on insulin (Young and Carpenter, 1961).

During the course of the work, the original procedure of Sundby (1962) was modified in an attempt to decrease the time of electrophoresis by increasing the applied voltage. Very poor results were obtained when filter paper was used as a support with the high voltage. The substitution of cellulose acetate strips for filter paper resulted in a marked improvement in separation. However, the presence of a high concentration of urea in the buffer was still an important factor in obtaining good resolution.

EXPERIMENTAL

Buffer.—The buffer used in electrophoresis was 0.065 M in phosphate and 7 M in urea (deionized) at *p*H 6.5. It was prepared according to the procedure of Cole (1960) and was stored at 4° when not in use. One batch of buffer was used for as many as five runs over a period of 12 days.

Paper Electrophoresis.—The apparatus was similar to that described by Crestfield and Allen (1955) in which the glass plate which supports the paper was cooled by a water sprinkler. The power supply was a 2-kv instrument made by Servonuclear Corporation, Long Island, New York. Three strips of cellulose acetate (36 × 5 cm) (Oxoid, available from Colab

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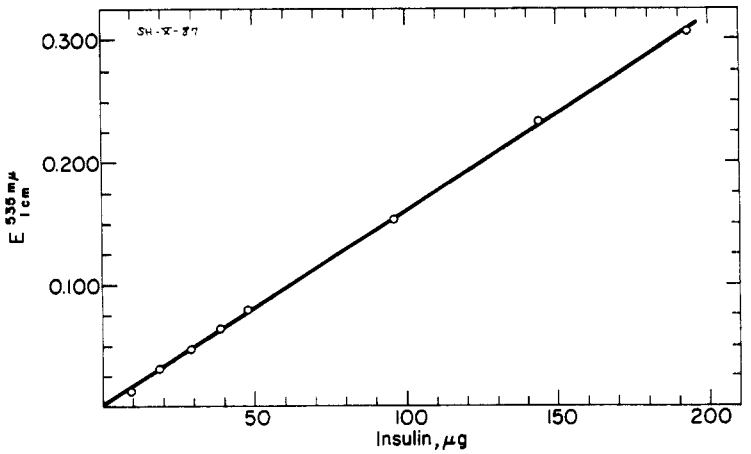

FIG. 1.—Amount of absorbancy eluted from the dyed strips as a function of the amount of insulin hydrochloride (prepared from PJ-3371) deposited on the cellulose acetate strip. The dyeing with Ponceau S, elution, and determination of absorption were as described in the experimental section.

Laboratories, Chicago Heights, Illinois) were used in each run. The strips were wet from the underside by flotation on the buffer and were then immersed. The strips were placed on the glass plate and connected with the buffer reservoirs (1500-ml capacity) by filter paper wicks (Whatman No. 1). The strips were rapidly but thoroughly blotted with cellulose tissues and then immediately equilibrated for 15–20 minutes at the voltage to be used in the experiment. The samples were dissolved in the buffer at a concentration of 20–80 mg/ml and a volume of 5 μl of the solution was applied to the center of the strip in a narrow line. The electrophoresis was run for 3 hours at an applied potential of 40 v/cm of length. The total distance between the surfaces of the buffer reservoirs was about 48 cm which resulted in an applied voltage of 1900 v. The total current carried by the three strips was about 15 ma. Caffeine was used as an indicator for electroosmosis (Crestfield and Allen, 1955).

At the completion of the run, the strips were placed between sheets of filter paper (Whatman No. 1) and dried in an oven at either 80 or 110°. The lower temperature was used in the more recent work. The dried strips were floated on a 0.2% solution of Ponceau S (National Aniline Division, Allied Chemical Corp.) in 3% acetic acid. After the dye had penetrated them the strips were immersed for 5–10 minutes. The dyed strips were washed with 5% acetic acid until they ceased to shed color (four to six washings) and were dried between sheets of filter paper at room temperature.

The strips were photographed by back-lighting, using Kodak Gravure Copy film (4 × 5–in. sheets) which was developed in Kodak DK-50 developer.

Quantitative Procedure.—Each colored band of each strip was cut into four to six pieces and placed in a test tube with 4 ml of 0.1 N sodium hydroxide. An area of approximately equal size was cut from a nonstained portion of the strip to serve as a blank. The tubes were shaken gently for 5 minutes and allowed to stand at room temperature for 30 minutes. Just before determining the absorbancy of the solution, 0.4 ml of 40% acetic acid was added and the absorbancy at 535 mμ was measured immediately in a Zeiss PMQII spectrophotometer. The absorbancy of each band is expressed as the percentage of the total absorbancy eluted from the strip. In order to establish the linearity of the response, various amounts of insulin (10–200 μg) were deposited as streaks on the cellulose acetate strips and subjected to the same procedure of drying, staining, and eluting as was used for the experimental samples (Fig. 1).

Results and Discussion

General.—In Figure 2 the symbol *s* indicates the starting point where the samples were applied to the paper. The symbol *O* indicates the position to which caffeine migrated under these experimental conditions and is used to measure the electroosmosis effect (Crestfield and Allen, 1955). Presumably, components with a zero net charge would migrate similarly to caffeine. The other numbers (−1, −2, etc.) of Figure 2 are used to identify the various components by their approximate net charge at *p*H 6.5. Insulin A with six carboxyl groups, two amino groups of the amino-terminal residues, one ε-amino of lysine, one guanidinium group of arginine, and two imidazole groups of histidine would be expected to bear a net charge of about −1 at *p*H 6.5. Progressive deamidation of insulin would give rise to species with an increasing number of negative charges. Despite the net charge of −1 for insulin A, it moves slightly toward the negative pole owing to electroosmosis effects. This effect is largely attributable to the brand of cellulose acetate used in these experiments. In preliminary experiments performed on a sample of cellulose acetate from another manufacturer (Sepraphore III, Gelman Instrument Company, Chelsea, Michigan) the electroosomotic effect was greatly diminished.

The electrophoretic experiments of Sundby (1962) were performed on filter paper strips (Whatman No. 1), using a 0.13 M phosphate–7 M urea buffer at *p*H 6.0 at relatively low voltage for 23–25 hours. At the higher voltages used in the present work, the components of a mixture were very poorly separated on filter paper strips (Whatman No. 1). However, when cellulose acetate was substituted for filter paper, the resolution was considerably improved. If the urea were omitted in the electrophoresis on cellulose acetate, the separation again became very poor. Good resolution was obtained only where both cellulose acetate and buffers containing urea were used. The decrease in the molarity of phosphate (from 0.13 to 0.065 M) and the increase in *p*H (from 6.0 to 6.5) over that of Sundby gave increased resolution of the components in a shorter time.

In some of the experiments there was an apparent doubling of certain bands (see Figure 2, D and E at −2). In other instances a "halo" was revealed (see Figure 2,

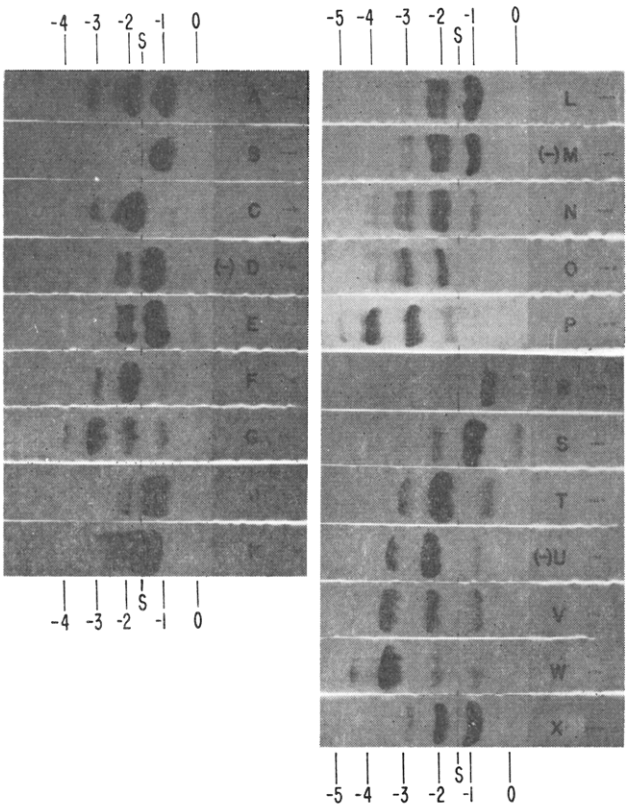

FIG. 2.—Electrophoresis of various insulin samples on cellulose acetate strips under conditions described in experimental section. Samples were placed on strip at *s*. The numbers (0, −1, −2, etc.) indicate the approximate net charge on the species. (A) Bovine insulin hydrochloride (Eli Lilly and Company, lot PJ-3371) which was composed about equally of insulin A and desamido-insulin with small amounts of other components as revealed by countercurrent distribution and partition-column chromatography (Figs. 1 and 3 of Slobin and Carpenter, 1963b). (B) Insulin A hydrochloride isolated from a 1650-transfer countercurrent distribution of sample PJ-3371 (see Fig. 1 of Slobin and Carpenter, 1963b). (C) Desamido-insulin hydrochloride isolated from a countercurrent distribution of sample PJ-3371 (see Fig. 1 of Slobin and Carpenter, 1963b). (D–G) Fractions isolated from the partition-column chromatography of bovine insulin (Eli Lilly and Company, lot PJ-5279) (see Fig. 3). (J) Main component (insulin A hydrochloride) isolated from partition-column chromatography of bovine insulin (Eli Lilly and Company, PJ-4812) (see Fig. 4, upper, of Slobin and Carpenter, 1963b). (K) Crystalline zinc-insulin (bovine) (Eli Lilly and Company, PJ-4812) before being subjected to acid transformation reaction. (L–P) Fractions isolated by partition-column chromatography of acid-transformed insulin (PJ-4812) (see Fig. 4, lower, of Slobin and Carpenter, 1963b). (R) Desalanine-desasparagine-insulin which had been isolated from 2350-transfer countercurrent distribution experiment (see component A of Fig. 5 in Slobin and Carpenter, 1963a). (S) Desalanine-desasparagine-insulin which had been isolated by partition-column chromatography (see Fig. 4, upper). (T) Desalanine-desamido-insulin which had been isolated by partition-column chromatography (see Fig. 4, lower). (U–W) Fractions of desoctapeptide-insulin which had been isolated from 2350-transfer countercurrent distribution (see Fig. 5). (X) Desalanine–desasparagine–insulin hydrochloride which had been incubated at 30° in 0.1 N hydrochloric acid for 12 days, solubilized by brief alkali treatment, and precipitated as the hydrochloride according to the procedure of Slobin and Carpenter (1963b).

A at −2). The "double-banding" or "halo" effects may be caused by partial separation of different species with the same net charge. However, the possibility that they are artifacts arising from the method of introducing the sample on the strips has not been eliminated. In any event, when the strips were cut in pieces to measure the amount of compound present in each area, the double bands or halos were included within the main component of the band.

TABLE I

AMOUNT OF MATERIAL IN EACH BAND OF ELECTROPHORETIC STRIP

Strip No.	Percentage of Total Material on Strip Moving with Net Charge of:						
	−5	−4	−3	−2	S	−1	0
A			10	53		37	
B				11	16	73	
C			4	90	5	1	
D			0.5	13		86	0.5
E			2	19		77	2
F			8	90		2	
G		4	60	26	1	9	
J				10		90	
K			1	11		88	
L			8	34		58	
M			4	35		61	
N		3	26	60		11	
O		2	42	49	7		
P	3	41	53	4			
R					4	96	
S				3		94	3
T			7	86		7	
U			12	86		2	
V		1	53	31	2	12	
W		4	87	6	2	1	
X			2	26	3	69	

Quantitative Results.—The method used to obtain a quantitative estimate of the various components presumes that the several derivatives exhibit the same affinity for the dye and that the amount of dye absorbed is a linear function of the amount of protein present in the band. This latter point has been subjected to experimental test with the results shown in Figure 1. At least over the range of 10–200 μg of protein per band, the amount of dye eluted from the stained strips was a linear function of the amount of protein applied. In a few instances (see strips B, C, and O of Figure 2) noticeable amounts of stainable material remained at the starting line. This irreversibly bound material (denatured?) might be expected to stain more intensely than the other fractions. Nevertheless, the color eluted from the starting area was included in the total color yield from the strip. Fortunately, in most samples a negligible amount of material remained at the origin (Table I).

Insulin-A and Desamido-Insulins from Countercurrent Distribution.—Strip A of Figure 2 shows the results obtained on electrophoresis of a sample of insulin (PJ-3371) which has been studied by several techniques in this laboratory. From the amount of color eluted from the various areas, the mixture contained 37% of a component with net charge of −1, 53% with a net charge of −2, and 10% with net charge of −3 (Table I). This same sample of insulin had been subjected to a 1650-transfer countercurrent distribution. The results (Figure 1 of Slobin and Carpenter, 1963b) showed the separation into three main components in the same ratio as noted by electrophoresis. The two main fractions isolated from countercurrent distribution have been characterized previously (Carpenter and Chrambach, 1962) to be composed primarily of insulin A (containing 6 amides) and desamido-insulin (containing 5 amides). Strip B of Figure 2 shows the results obtained on electrophoresis of the sample of insulin A isolated by countercurrent distribution.

Most of the material (73%) moves with a net charge of −1. However, some material (11%) moves with a net charge of −2, corresponding to desamido-insulin. This result is not unexpected since carboxyl-terminal analyses (Slobin and Carpenter, 1963b) had indicated that this preparation contained 10% of carboxyl-terminal aspartic acid. This amount of cross contamination of desamido-insulin with insulin A would not be expected from the separation curve of the countercurrent distribution experiment. Therefore it is highly probable that a gradual transformation of the insulin A to desamido-insulin takes place under the acidic conditions used in the countercurrent distribution experiment. A sizable portion (16% as judged by the color eluted from the paper, Table I) of the insulin A, isolated by countercurrent distribution, remained at the starting line.

Strip C of Figure 2 shows the results obtained on electrophoresis of the desamido-insulin which had been isolated by countercurrent distribution. It contained very little material (< 2%) with a net charge of −1 (insulin A). Most of the material (90%) moved with a net charge of −2 (desamido-insulin) with a small portion (4%) at −3 (des*di*amido-insulin). Carboxyl-terminal analysis of this same sample had shown the presence of 81% aspartic acid and 17% asparagine (Slobin and Carpenter, 1963b).

Electrophoresis showed that there was not enough insulin A (< 2%) in the sample to account for the 17% of carboxyl-terminal asparagine. Therefore, the desamido-insulin must be a mixture containing at least 15% of desamido-insulins in which the amide is missing from some location other than the carboxyl-terminal asparagine. This conclusion is also confirmed by comparison of the results of carboxyl-terminal analyses with the electrophoresis on the original sample (PJ-3371) before it was subjected to countercurrent distribution. Electrophoresis had indicated the presence of 37% insulin-A, 53% desamido-insulin, and 10% des*di*amido-insulin. Carboxyl-terminal analyses had indicated the presence of 51% asparagine. The difference between the value for carboxyl-terminal asparagine and the amount of insulin A indicates the presence of about 14% of desamido-insulins in which the amide groups have been lost from positions other than the carboxyl-terminal asparagine.

Insulin-A and Desamido-Insulins from Partition-Column Chromatography.—A sample of insulin (Eli Lilly and Company, lot PJ-5279) was subjected to partition-column chromatography at 4° in the *n*-butanol–2-butanol–0.1 N hydrochloric acid system (16:84:100) (Chrambach and Carpenter, 1960) with the results shown in Figure 3. The materials isolated from the various fractions were subjected to electrophoresis with the results shown on strips D-G of Figure 2 and Table I. The slowest-moving band in partition-column chromatography, corresponding to insulin A, was isolated in two parts (D and E).

Electrophoresis shows that neither one of these fractions of insulin A is homogeneous. Fraction D contains 13% of substance moving with a net charge of −2 (desamido-insulin) which is increased to 19% in fraction E. As with countercurrent distribution, it is very likely that the desamido forms present in fractions D and E were formed during partition-column chromatography owing to the acidic condition used in the separation procedure. Both D and E contain trace amounts of substance moving with a net charge of 0 and −3. The latter is undoubtedly des*di*amido-insulin. The substance moving with a net charge of 0 could be an insulin containing seven rather than the six amides of insulin A. However, an alternative

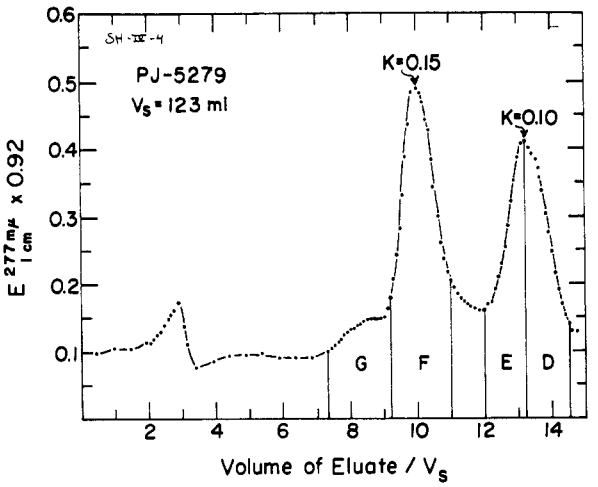

FIG. 3.—Partition-column chromatography of insulin hydrochloride (200 mg) prepared from lot PJ-5279 (Eli Lilly and Company). The chromatography was performed at 4° as described previously (Chrambach and Carpenter, 1960; Slobin and Carpenter, 1963b). Diatomaceous earths (54 g of Microcel-C and 74 g of Celite 545) were admixed with 123 ml (V_s) of the lower layer from *n*-butanol–2-butanol–0.1 N hydrochloric acid (16:84:100) and poured as a slurry in the upper layer into a tube with a cross-sectional area of 11.4 cm^2 to yield a column 48 cm high. The eluates were pooled for isolation of material as indicated in the figure.

explanation should be considered. This explanation supposes a partial esterification of the insulin in the acid-alcohol used in the partition-column chromatography (Fraenkel-Conrat and Olcott, 1945). Esterification would give rise to species with a lower net negative charge. Further evidence for the esterification hypothesis is the fact that the substances moving with a net charge of 0 at *p*H 6.5 were absent from the original insulin preparations and were detected only in those fractions which had been isolated by partition-column chromatography in the butanol–hydrochloric acid system.

Fraction F was isolated from the eluates of partition-column chromatography in the area where desamido-insulin is found (Carpenter and Chrambach, 1962). At least 90% of the material (Table I) moves in electrophoresis with a net charge of −2, corresponding to desamido-insulin. A small portion (2%) moves with a net charge of −1 which could indicate a contamination with insulin A, but which again could also be due to the presence of some partially esterified form in the desamido-insulin. A portion of fraction F (8%) moves with a net charge of −3 and is presumably des*di*amido-insulin, formed in part by the acidic conditions used in the partition-column chromatography.

When fraction G from partition-column chromatog raphy (Figure 3) was subjected to electrophoresis, it was found (Figure 2, strip G) to consist to a large extent (60%) of material which moved with a net charge of −3. This charge would correspond to a des*di*amido-insulin. In agreement with this interpretation is the fact that amide analysis on similar materials isolated by partition-column chromatography had indicated the presence of four rather than six amides (Chrambach, 1960). As would be expected by the shape of the elution curve (Figure 3), fraction G is inhomogeneous and contains materials moving with a net charge of −2 (26%), −1 (9%), and −4 (4%). The last charge may be attributable to des*tri*amido-insulin. From the position of the elution curve (Figure 3), one would not expect to find any insulin A (net charge of −1) as a contaminant in fraction G.

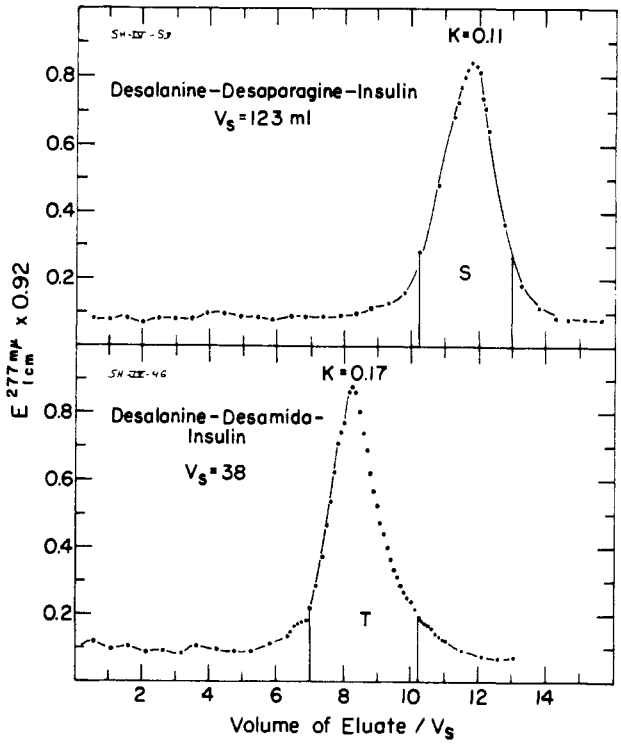

FIG. 4.—(Upper) Partition-column chromatography of desalanine-desasparagine-insulin (250 mg) prepared by the action of carboxypeptidase A on insulin A (Slobin and Carpenter, 1963a). The chromatography was performed at 4° as previously described (Chrambach and Carpenter, 1960; Slobin and Carpenter, 1963b) using the *n*-butanol–2-butanol–0.1 N hydrochloric acid (16:84:100) system. The eluates were pooled for isolation of components as indicated in the figure. (Lower) Partition-column chromatography of desalanine-desamido-insulin (74 mg) prepared by the action of carboxypeptidase A on desamido-insulin at *p*H 9.4 (Slobin and Carpenter, 1963b). The chromatography was performed at 4° using *n*-butanol–2-butanol–0.1 N hydrochloric acid (12:88:100). The eluates were pooled for isolation of components as indicated in the figure.

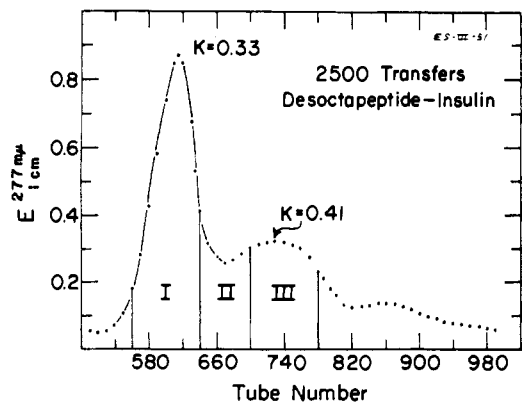

FIG. 5.—Twenty-five hundred-transfer countercurrent distribution of desoctapeptide-insulin (2.4 g) prepared by the action of trypsin or bovine insulin hydrochloride (British Drug House, lot 2189) according to procedure of Young and Carpenter (1961). The distribution was performed between 2-butanol aqueous dichloroacetic acid (1.56% w/v) at room temperature in an automatic 500-tube instrument. The distribution was interrupted after 500 transfers and the contents of tubes 0-110 and 220-340 were removed and replaced with new upper and lower layers. The instrument was set for recycle operation and an additional 965 transfers were performed. At this time the contents of tubes 20-140 were replaced with fresh solutions and the distribution was continued for a total of 2500 transfers. The contents of the various tubes were pooled as indicated in the figure and the desoctapeptide-insulin fractions were isolated as their hydrochlorides by a procedure similar to that described in Slobin and Carpenter (1963b).

Again, the fact that electrophoresis showed the presence of material (9%) with the same mobility as insulin A might be explained by partial esterification of desamido forms during the separation and isolation procedures to give material with the same net charge as insulin A.

Acid Transformation.—Earlier studies had shown that when insulin was subjected to mild acid conditions, it was transformed into a mixture of products which could be separated by partition-column chromatography (Chrambach and Carpenter, 1960). This transformation process was shown to be a deamidation reaction (Carpenter and Chrambach, 1962) which gave rise to a mixture of desamido-insulins. The principal product formed in the early stages of the reaction was a desamido form in which the amide group had been lost from the carboxyl-terminal asparagine of the A chain (Slobin and Carpenter, 1963b). Sundby (1962) has studied the acid-transformation reaction by the technique of paper electrophoresis in urea-containing buffers. In the present report, the products separated by partition-column chromatography from insulin which had been subjected to the acid-transformation reaction were subjected to electrophoresis on cellulose acetate.

Strip K of Figure 2 shows the behavior of the original sample of zinc-insulin (Eli Lilly and Company, PJ-4812) when subjected to electrophoresis. Judging from the amount of color eluted from the electrophoretic strip (Table I), the sample was composed primarily of insulin-A (88%) with a small amount of desamido-insulin (11%) and a trace of des*di*amido-insulin (1%). Partition-column chromatography of this sample also indicated that it was composed primarily of insulin A (see Figure 4, *upper*, of Slobin and Carpenter, 1963b). The sample was subjected to the acid-transformation reaction by being allowed to stand at 30° in 0.1 N hydrochloric acid for 14 days. The transformation mixture was subjected to partition-column chromatography and the eluate was divided into five fractions as shown in Figure 4, *lower*, of Slobin and Carpenter (1963b). Each fraction was subjected to electrophoresis with the results shown in strips L-P of Figure 2 and Table I. Comparison of the results shows that the slowest-moving fractions (I and Ia) of partition-column chromatography were composed to an amount of about 60% of material moving with a net charge of −1 (insulin A), of about 35% of material moving with a net charge of −2 (desamido-insulin), and of about 5% with a net charge of −3 (des*di*amido-insulin). The fastest-moving fraction (IV) of partition-column chromatography was composed to an amount of 4% of materials moving with a net charge of −2 (desamido-insulin), of 53% of materials moving with a net charge of −3 (des*di*amido-insulin), of 41% of materials moving with a net charge of −4 (des*tri*amido insulin), and of 3% of materials moving with a net charge of −5 (des*tetra*amido-insulin). The fractions (II and IV) from partition-column chromatography which were eluted in between Ib and III exhibited intermediate amounts of desamido-forms on electrophoresis.

Desalanine-Desasparagine-Insulin.—A sample of desalanine-desasparagine-insulin which had been prepared by the action of carboxypeptidase A on insulin and had been purified by a 2350-transfer countercurrent distribution (see Figure 5 of Slobin and Carpen-

ter, 1963a) was subjected to electrophoresis with the results shown in strip R of Figure 2. Except for a small portion (4%) which remained at the starting line, the rest (96%) moved as a single component with a net charge of −1. As would be expected, the electrophoretic mobility of desalanine-desasparagine-insulin is nearly identical to insulin A. When a sample of desalanine-desasparagine-insulin which had been purified by partition-column chromatography (Figure 4, *upper*) was subjected to electrophoresis, the results shown in strip S of Figure 2 were obtained. Most of the material (94%) moved with a net charge of −1, but small amounts moved with net charges of 0 (2%) and −2 (4%). The latter material must be a desamido-form arising from the acidic conditions used in the partition-column chromatography. Again the hypothesis of partial esterification may be invoked to explain the presence of the small amount of material moving with a net charge of 0.

Desalanine-Desamido-Insulin.—A portion of desamido-insulin which had been treated with carboxypeptidase at high *p*H, under conditions which favor the preferential removal of the carboxyl-terminal alanine from the B chain with little cleavage of the carboxyl-terminal aspartic acid from the A chain (Slobin and Carpenter, 1963b), was further purified by partition-column chromatography (Figure 4, *lower*). When the resulting material was subjected to electrophoresis, the results shown in strip T of Figure 2 were obtained. As would be predicted, most of the material (86%) moved with a net charge of −2 similar to that of desamido-insulin. However, the electrophoresis reveals the presence of a small amount (7%) of material moving with a net charge of −3 (desalanine-des*di*-amido-insulin) and a small amount moving with a net charge of −1. The latter may contain some desalanine-desaspartic acid-insulin formed by the partial cleavage of the carboxyl-terminal aspartic acid by the action of the enzyme.

Desoctapeptide-Insulin.—A sample of desoctapeptide-insulin which had been prepared by the action of trypsin on a commercial sample of insulin (Young and Carpenter, 1961) was purified by 2500-transfer countercurrent distribution as illustrated in Figure 5.

In countercurrent distribution the main component (I) moved with a distribution constant (K) of 0.33. The minor component (III) gave a broad band with a center corresponding to a K value of 0.41. The distribution was interrupted several times to remove materials moving with K values of insulin A (K = 0.66) and desamido-insulin (K = 0.86). Both components I and III contained less than 0.02 residue of lysine, threonine, and proline; the amino acids which are uniquely contained in the carboxyl-terminal octapeptide portion of the B chain of insulin.

The fractions isolated from countercurrent distribution were subjected to electrophoresis with the results shown on strips U-W of Figure 2. Owing to the loss of a positive charge with the cleavage of the lysine-containing peptide from insulin A, the resulting desoctapeptide-insulin A should move with a net charge of −2 at *p*H 6.5. The main component (I) isolated in the countercurrent distribution has the expected behavior in that 86% of the material moves with a net charge of −2 (strip U). There is also present a small amount (12%) of material with a net charge of −3. The latter can be attributed to desoctapeptide-desamido-insulin formed in the countercurrent distribution. When the minor component (III), isolated in the distribution experiment, was subjected to electrophoresis, most of the material (87%) moved with a net charge of −3 (strip W). This is the behavior expected for desoctapeptide-desamido-insulin. The intermediate fraction (II) of countercurrent distribution contained a mixture of components in which the major portion (53%) moved with a net charge of −3 (desoctapeptide-desamido-insulin) (strip V of Figure 2).

Acid Transformation of Desalanine-Desasparagine-Insulin.—Earlier work (Slobin and Carpenter, 1963b) has shown that the amide on the carboxyl-terminal asparagine of the A chain is the most labile to mild acid treatment. Accordingly, desalanine-desasparagine-insulin, which lacks this labile amide, should give rise to fewer desamido forms than those produced from insulin A on mild acid treatment. Strip X of Figure 2 shows the results obtained upon electrophoresis of a desalanine-desasparagine-insulin which had been subjected to the action of 0.1 N hydrochloric acid for 14 days at 30°. At the end of this time, approximately 70% of the material still moved as unchanged desalanine-desasparagine-insulin. A total of only 28% moved as desamido forms. This is in sharp contrast to the results illustrated in strips L-P of Figure 2 where insulin A gave rise to a much larger amount of desamido-insulins under the same conditions of acid transformation.

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