Partition Column Chromatography of Insulin: Production and Separation of Transformation Products

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As a result of a general study on the partition column chromatography of insulin, it was noted that a number of commercial samples of crystalline insulin gave elution curves which indicated the presence of at least two components in the eluted fractions (3, 4). The solvent systems used in these studies were similar to those that had been reported by Harfenist and Craig in their countercurrent distribution studies on insulin (5–8). The latter workers had reported the fractionation of bovine insulin into two components (A and B) which appeared to differ from one another in the number of amide groups (8).

The present report describes improvements in the technique of partition column chromatography of insulin that resulted in the separation of up to four components from some samples of commercial crystalline insulin. Several of these components were found to be formed from the main component when it was treated with acid and are designated transformation products. The preparation and separation of these components are described.

In recent years several other chromatographic fractionations of insulin have been reported. These reports include the work of Dickinson (9), who detected an acid transformation of insulin by the use of chromatography on calcium phosphate gel, of Boardman (10, 11), who used resin-coated diatomaceous earth, of Cole (12, 13), who used a cation exchange resin and eluted with buffers containing urea, of O’Donnell and Thompson (14, 15), who used diethylaminoethyl-cellulose along with urea-containing buffers, and of Volini and Mitz (16), who also used diethylaminoethyl-celluloses. The chemical differences between the fractions revealed by these chromatographic procedures have not been reported. Probably some of the procedures are detecting the same type of heterogeneities revealed in partition column chromatography.

EXPERIMENTAL PROCEDURE

Materials—The insulin samples were all crystalline zinc-insulin of bovine origin. Most of the experiments were performed on lot No. 693502, T-2842, PJ-3026, PJ-3369, PJ-3370, and PJ-3371 of Eli Lilly and Company and No. 9011-G of Boots Drug Company. All the samples were converted to their hydrochlorides by precipitation from 0.1 N HCl solution with acetone (4) before being subjected to chromatographic analysis. As judged by the results on chromatography, acetone precipitation at 3° gave a better product than the lyophilization procedure used in early experiments (3). Precipitation of the insulin hydrochloride with acetone removes most of the zinc.

The 2-butanol and n-butanol were purified by refluxing over and distillation from calcium hydride (4). Only that portion of the distillate was used which had an optical density of 0.2 or less when read against distilled water at 275 μm in a 1-cm cuvette.

The inert support for the aqueous (stationary) phase was a mixture of Micro-cell and Celite 545.1 Both materials were purified as follows. A thick suspension in 2 water and then subjected again to the acid treatment. The acid-free residue was suspended in 2-butanol, the suspension was stirred overnight, and the residue was collected on a filter and washed on the funnel with distilled water and then filtered by suction through Whatman No. 1 paper. The residue was washed on the funnel with distilled water and then subjected again to the acid treatment. The acid-free residue was suspended in 2-butanol, the suspension was stirred overnight, and the residue was collected on a filter and washed with fresh butanol. The residue was dried in air at temperatures not exceeding 50° until the odor of butanol had disappeared.

Solvent Systems—The solvent systems are designated according to their composition before mixing. A mixture of butanols was equilibrated with an equal volume of 0.1 N HCl. The composition of the butanol mixture was varied according to the temperature at which chromatography was being performed and the partition factor of the insulin fraction being chromatographed. For chromatography at 25°, 2-butanol was used. For chromatography of commercial samples or rechromatography of Fraction I at 3°, 12 parts of n-butanol were mixed with 18 parts (volume per volume) of 2-butanol. For rechromatography of Fractions III and IV at 3°, 18 or 24 parts of n-butanol were mixed with 52 or 76 parts (volume per volume) of 2-butanol, respectively. The phases were equilibrated at the temperatures at which the chromatography was to be performed.

Preparation of Columns—Jacketed columns of the type previously described (3) were used. The cross-sectional area of the columns was varied according to the load: 0.83 cm² (designated analytical) for loads up to 15 mg; 3.64 cm² (designated semipreparative) for loads of 40 to 75 mg; 11.1 cm² (designated preparative) for loads of 200 to 250 mg. An intimate mixture of 4 parts lot Nos. 693502, T-2842, PJ-3026, PJ-3369, PJ-3370, and PJ-3371 of Eli Lilly and Company and No. 9011-G of Boots Drug Company.

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EXPERIMENTAL PROCEDURE

Materials—The insulin samples were all crystalline zinc-insulin of bovine origin. Most of the experiments were performed on lot No. 535664 of Eli Lilly and Company or No. 2189 of British Drug Houses, Ltd. A few experiments were performed on

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of Micro-cel to 5 parts of Celite 545 (weight per weight) was made. Eleven grams of the mixture were used for each square centimeter of cross-sectional area. The inert support was stirred with 3 to 5 volumes (volume per g) of upper (moving) phase until it was uniformly suspended. Then 1.1 ml of lower phase per g of support were added, and the mixture was stirred vigorously for about 30 minutes. Thereafter, slow stirring was continued for another hour in order to allow for the escape of entrained air. The suspension was poured into the column through a funnel, with care to direct the flow against the wall of the column. The column was intermittently rotated about its vertical axis while the elution of solvent proceeded for a few hours. Analytical columns were left to stand overnight and again eluted for 1 to 2 hours before application of the sample. Semipreparative columns were eluted overnight before the run. Columns prepared in such a manner had heights of about 40 cm, a hold-up volume (V_s) of about 2.5 ml per g of support, and a ratio of hold-up volume to stationary phase volume (V_H to V_s) of about 2.2 (3).

**Chromatography**—Insulin hydrochloride was dissolved in the moving phase to give a concentration of 0.5% (analytical columns) or 1.5% (semi- and preparative columns). The solution was applied to the column by a bent-tip pipette, with care to direct the flow against the wall of the column just above the surface of the packing. The sample was washed into the column with two washes, each of a volume equal to one-half the volume of the sample solution. Finally, the column was filled with upper phase and the constant-head-reservoir (3) was attached. The collection of eluate fractions of 1.5, 5.0, or 15 ml for the analytical, semi- or preparative columns, respectively, was started immediately after the application of the sample. The rate of flow was generally the maximal obtainable but did not exceed 0.1 ml per cm² cross-sectional area per minute. The insulin was detected in the eluate fractions, after addition of ethanol (10% of the volume of the fraction) to insure a single phase, by absorption measurements at 275 μm. The estimation was based on an optical density of 1.0 for an insulin solution containing 1 mg per ml and having a length of 1 cm (3). 

**Isolation**—The contents of the tubes comprising the fraction under investigation were pooled and shaken with 5 volumes of U.S.P. ether from a newly opened can. The insulin hydrochloride was precipitated from the aqueous phase by the addition of 18 volumes of acetone and isolated as described above. By this procedure 70 to 80% of the material present in the fraction, as determined by the absorption at 275 μm, was obtained in solid form as the hydrochloride.

**Calculations**—The distribution constant (K), the partition factor (K'), and the number of theoretical plates (N) were calculated from the following formulas (3):

$$K = V_B/(V_B - V_H) \quad (1)$$

$$K' = V_B/(V_B - V_H) \quad (2)$$

$$N = 5.55 [(V_B)^2 - V_s V_H]/(V_B)^2 \quad (3)$$

where V_B is the volume of stationary phase used in preparing the column, V_H is the hold-up volume of the column, V_s is the volume of solvent eluted from the time the sample was introduced until the fraction of an elution peak of maximal concentration has emerged, and V_B is the width of the elution band in volume units at one-half the maximal concentration.

**RESULTS**

**Improvements in Chromatography**—In the earlier work reported from this laboratory, insulin had been subjected to partition column chromatography in a solvent system obtained by equilibrating 2-butanol with 0.5% dichloroacetic acid in 0.1 n HCl (3). Investigations (4, 17) of the effect of various acids on the distribution constant of insulin in 2-butanol-water mixtures showed that in the presence of HCl the dichloroacetic acid had little effect on the distribution constant of insulin. Consequently, the dichloroacetic acid was omitted from the solvent system. On theoretical grounds (3) the optimal conditions for the separation of closely related insulins would be approached in a chromatographic system which would yield a value for the partition factor (K') of 0.1 for the slowest moving component. The partition factor (K') is a function of the distribution constant (K) and the ratio of the hold-up volume (V_H) to the stationary phase volume (V_s):

$$K' = KV_B/V_s \quad (4)$$

Insulin possesses a distribution constant (K) of about 0.1 in the 2-butanol-0.1 n HCl system at 25°. In the early work (3) in which Hyflo Super-Cel was used as a support for the stationary phase, the maximal amount of stationary phase that could be held on the column was about 0.6 ml per g of Hyflo Super-Cel; the corresponding V_H to V_s ratios of these columns were about 4. As a consequence, the partition factor (K') was about 0.4, a value somewhat too high for a critical separation of closely related components. In order to approach optimal conditions, a systematic study of the several factors that control the K' value was made.

The first factor studied was the effect of the nature of the acid in the solvent system on the distribution constant of insulin. The results (4, 17) indicated that the distribution constant of the insulin was related by a relatively simple equation to the distribution constant of the strong mineral acid in the system. Several solvent systems were discovered which gave K values below 0.1 for insulin at 25° and which have proved useful in separating tryptic digests of insulin (18, 19).

Another approach to lowering the K' value was to change the ratio of V_B to V_s. This could presumably be achieved by finding inert supports which would hold a larger volume of stationary phase relative to that held by the Hyflo Super-Cel. Accordingly an investigation was made of a number of commercially available diatomaceous earths in search of an improved supporting material. A calcined diatomaceous earth, Micro-cel, was found to be superior to all others investigated in its ability to hold the stationary phase. It retained about 1.7 ml of lower layer per g of Micro-cel; the corresponding columns had ratios of V_H to V_s of about 2. However, columns made with Micro-cel as support often exhibited rates of flow that were too slow for practical use. In order to improve the flow rate while retaining some of the desirable properties of the Micro-cel, various mixtures of Micro-cel with the coarse diatomaceous earth, Celite 545, were made and tested. On the basis of this study, the ratio 4 parts Micro-cel to 5 parts Celite 545 (weight per weight) was adopted. This mixture retained about 1.1 ml of stationary phase per g of support and gave columns which had a V_B to V_s ratio of about 2.2.

The use of the above mixture of diatomaceous earths produced columns in which the K' value for insulin was about 0.22 in the 2-butanol-0.1 n HCl system. Although this is still not the optimal K' value, the results obtained with it were much superior
Transformation of Insulin — From the anomalous behavior of Fraction I on rechromatography, it was apparent that some transformation of the insulin was taking place either during chromatography or during the procedure of reisolation. As studies of the isolation procedure indicated that it was not at fault, we decided to see whether the transformation could be avoided by performing the chromatography at 3°. This decision required another change in the chromatographic systems. The K value of the main component of insulin in 2-butanol-0.1 N HCl at 3° was about 0.3, corresponding to a partition factor (K') of about 0.7, a value much too high for good separation of closely related components. A study was made on the effect of the admixture of various amounts of n-butanol with 2-butanol on the distribution constant of water and HCl at 3°. This study revealed that a mixture of 12 parts of n-butanol with 88 parts of 2-butanol when equilibrated with 0.1 N HCl at 3° gave a solvent system in which the water and the HCl had about the same distribution constants as they possessed in the 2-butanol-0.1 N HCl system at 25°. The use of this mixture of butanols resulted in the same elution curve on the chromatography of insulin at 3° as had been obtained at 25° (cf. Fig. 1 with Fig. 2A). Furthermore, when the material in Fraction I was isolated and subjected to chromatography a second and third time at 3°, it behaved as a nearly homogeneous component (Figs. 2B and 2C). Although material from Fraction III also appeared fairly homogeneous on a second chromatography (Fig. 2D), it moves too rapidly in this system for good separation from neighboring materials (Fractions II and IV). These results demonstrated that the transformation observed during chromatography at 25° could be avoided by performing the chromatography at 3°. Only the semipreparative and analytical size columns gave separations at 3° which were comparable to those of Fig. 24. Difficulties were encountered with the preparative size columns, and conditions have yet to be elaborated that will give comparable separations with the large columns. These difficulties have limited our ability to obtain materials for further characterization.

The demonstration that the main component of insulin preparations (Fraction I) could be chromatographed repeatedly at 3° without substantial change made it possible to study the temperature dependence of the acid transformation of Fraction I. The results of such studies at 25° and 40° are shown in Figs. 3 and 4. Essentially homogeneous Fraction I (Fig. 3A) was allowed to stand in 0.1 N HCl at 25° for a period up to 12 days. Aliquots of the sample were removed and the material was precipitated with acetone after 6 and 12 days. The precipitated material was chromatographed at 3°. The results (Figs. 3B and 3C) show the slow transformation of Fraction I to Fractions II, III, and IV. The reaction is much more rapid at 40°, as shown in Fig. 4, which illustrates the results obtained on chromatography at 3° of a commercial sample of insulin which had been allowed to stand in 0.1 N HCl for 0 to 3 days at 40°. The materials labeled II, III, and IV which are present in small amounts in the original sample increase severalfold with a corresponding decrease in Fraction I during incubation at 40°.

We were not successful in converting all of Fraction I to the other components by prolonged incubation at 40°. Invariably after about 2 to 3 days of incubation in the acid at this temperature, the heat precipitate (20-22) designated fibril insulin by Waugh (23) would form and interrupt the studies. In this connection it is interesting to note that a sample of crystalline zinc-insulin (Lilly No. PJ-3371) which had been prepared from insulin fibrils contained a much larger percentage of Fraction III (Fig. 7) than was obtained in Fraction I. It is probable that the fibril insulin was a secondary transformation product of Fraction I which arose from continued exposure to acid from which the fibril insulin was not precipitated. This hypothesis is supported by the observation that material isolated from Fraction I and allowed to stand in 0.1 N HCl at 25° for 12 days elutes as a well-defined main peak on 3° chromatography. It is not directly related to any of the Fractions II, III, or IV. A second chromatography at 3° resulted in a separation similar to that obtained at 25° (cf. Fig. 7 with Fig. 8A). It is clear that further purification of the main component of Fraction I will require a temperature of 3° or lower. This conclusion is supported by the transformation of a commercial sample of insulin (Fig. 8B).
5) than that encountered in all other commercial preparations investigated (Fig. 6).

**Preparation and Separation of Components**—In order to obtain material for further study, commercial samples of insulin were maintained in 0.1 N HCl at either 25° or 40° until a large portion of the Fraction I material had been transformed (12 or 3 days, respectively). The material was then subjected to partition column chromatography for separation of the components. Most of the work was confined to Fractions I and III, and to a lesser extent to IV. The material designated as Fraction II was not present in amounts large enough to permit further work. The solvent system used for the separation of Fraction I at 3° (n-butanol-2-butanol, 12:88 equilibrated with 0.1 N HCl) was not satisfactory for a valid test of homogeneity of Fractions III and IV, since the latter components possessed K values (about 0.18 and 0.21) and K' values (about 0.40 and 0.46) which were too high for good separation from one another. Consequently Fractions III and IV were rechromatographed, each in a solvent system designed to yield more nearly optimal K and K' values. These systems were developed by incorporating increasing amounts of n-butanol in the solvent system: the ratios of n-butanol to 2-butanol were 18:82 and 24:76 for use on Fractions III and IV, respectively. The values of the distribution constants obtained for Fractions I, III, and IV in their respective systems were essentially the same (K about 0.12; K' about 0.26). After the second chromatography in their respective systems, following the initial chromatography in the system used to isolate Fraction I, Fractions III and IV gave essentially symmetrical elution curves indicative of single components (Fig. 7).

Bioassay of Fractions I, III, and IV in mice* indicated that they contained 24.5, 22.2, and 22.6 units per mg, respectively.

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*Bioassays were performed at Eli Lilly and Company by the mouse convolution test with 80 mice per assay.
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possible exception of proline and cystine, the amino acid compositions of Fractions I and III were in good agreement with each other and with those already reported by Harfenist (8). In another study the optical rotatory dispersion at 25° between 320 and 690 mμ of an insulin solution in 0.1 N HCl was found not to change during incubation for 2 days at 40° (2). Under similar conditions, no change was noted in the ultraviolet absorption curve (2). The negative findings tend to eliminate large changes in the conformation of the molecule, hydrogen bonding, or state of oxidation of the phenolic groups during the transformation. Ryle and Sanger (25) have shown that disulfide interchange reactions in acid solution may be inhibited by the addition of thiol. The observation that the rate of transformation of Fraction I at 25° was not influenced by the presence of an equal molar amount of cysteine hydrochloride (2) is in contrast to what would be expected if disulfide interchange were involved in the transformation reaction. Fraction III was much more stable to the acid treatment than Fraction I; so much so that it could be rechromatographed with very little change at 25°. However, on prolonged exposure to 0.1 N HCl at 25°, it was converted in part to Fraction IV and other slower moving components, but it did not revert to Fraction I (2). This failure of Fraction III to revert to Fraction I was noted even when Fraction III was kept for 24 hours in slightly alkaline solution (pH 9) (2). The failure of III to revert to I under alkaline conditions may be considered as evidence against acyl migration from N → O during the formation of III from I (26, 27). The only positive result obtained in our studies on the transformation reaction was the observation that the reaction at 25° is accompanied by liberation of ammonia. Approximately 0.5 mole of ammonia per mole of insulin was formed concomitantly with the transformation of 29% of Fraction I to a mixture of the other components (2). Because of this and also because of the findings of Craig and Harfenist (7, 8) on the difference in amide content of insulin fractions obtained by countercurrent distribution, a comprehensive study of the amide content of these fractions has been undertaken and will form the subject matter of a separate communication.

Dr. R. D. Cole has subjected Fractions I, III, and IV to ion exchange chromatography in the buffered urea system (12, 15). Fraction I behaved as a single component and was eluted in the same position as the main component of insulin in this system. Fraction IV was eluted shortly after the hold-up volume in the same place as a sample of desamido-insulin (insulin B) secured from L. C. Craig. Fraction III was eluted between the other two components at the same location as the "urea transformation" product (12, 13). A cross-check in which the components obtained by ion exchange chromatography in buffered urea are subjected to partition column chromatography has yet to be performed, but should throw further light on the interrelations between the various components isolated by these chromatographic procedures.

SUMMARY

Improvements in the technique of partition column chromatography of insulin are reported. Use of the improved system revealed the presence of at least four components (Fractions I, II, III, and IV) in variable amounts in diverse insulin samples. Components II, III, and IV were formed in acid-catalyzed transformation(s) of the main component (Fraction I). Fractions I, III, and IV were prepared in apparently homogeneous form by

DISCUSSION

The demonstration of several biologically active components in commercial samples of crystalline insulin raises the question as to whether these components are produced by the pancreas or whether they arise under the acidic conditions used in isolating insulin from the gland. With the exception of the sample of insulin that had been derived from fibrils (Fig. 5), all the other insulin samples which we investigated contained Fraction I as the major component. This observation, along with the demonstration that Fractions II, III, and IV can arise from acid treatment of Fraction I, indicates that Fraction I is the main component produced in the gland and that the other components are artifacts arising during the isolation of insulin.

In connection with the structural relationships between the several fractions, some results should be summarized which are reported in detail elsewhere (2). The sedimentation constants of Fractions I and III were measured immediately after preparation of 1% solutions in 0.1 N HCl at 20° and were found to be 1.75 and 1.90.1 Dickinson has reported (9) an acid-catalyzed transformation of insulin in which the transformed products appear to differ in particle size. The similarity in sedimentation constants between I and III would appear to eliminate this possibility for these components. The distribution constants of Fractions I and III between 2 butanol and various 0.1 N acids were measured. Although the distribution constants of the two fractions differed in each of the systems, a plot of the logarithm of the distribution constant of the compound against the logarithm of the distribution constant of the acid in the systems gave straight lines with essentially the same slope for Fractions I and III (2). The result is interpreted to mean that there is no difference in the number of positive charges possessed by the two fractions in acid solution (17). A preliminary analysis of acid hydrolysates of Fractions I and III by the ion exchange method of Moore, Spackman, and Stein (24) was made (2).

Fig. 7. Second rechromatography at 34° (A) of Fraction III in the n-butanol-2-butanol(18:82)-0.1 N HCl and (B) of Fraction IV in the n-butanol-2-butanol(24:76)-0.1 N HCl solvent systems.

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the use of three different solvent systems; each fraction was subjected to chromatography in the solvent system most likely to separate it from closely related components. Fractions I, III, and IV have approximately the same biological activity in the mouse convulsion assay. The possible origin of the components, as well as their structural relationship, is discussed.

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