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Studies on the Synthesis of Insulin from Natural and Synthetic A and B Chains. II. Isolation of Insulin from Recombination Mixtures of Natural A and B Chains*

Panayotis G. Katsoyannis, Anthony C. Trakatellis, Stanley Johnson, Clyde Zalut, and Gerald Schwartz

ABSTRACT: The isolation of insulin from recombination mixtures of natural A and B chains is described. Chromatography of recombination mixtures on carboxymethylcellulose columns with urea–acetate buffer (pH 4.0) resulted in the resolution of the various components of such mixtures. Insulin activity was distributed in two fractions, tentatively designated insulin I and insulin II. Insulin II was compared with the natural hormone as to amino acid composition, specific activity, mobility on thin layer electrophoresis and column chromatography, and infrared spectrum. All of these comparisons afforded convincing evidence of the identity of insulin II with the natural hormone. Insulin I was eventually converted to insulin II. Furthermore, natural insulin, under certain conditions, could give rise to insulin I. The nature of this insulin is not known. The relative amounts of insulins I and II present in the chromatogram greatly depended on the conditions used during the isolation processes. The insulin activity present in a recombination mixture was exclusively recovered as insulin II upon treatment of this mixture with picric acid prior to chromatography. Chromatography of the picric acid treated recombination mixtures on carboxymethylcellulose columns at pH 3.3 with an exponential sodium chloride gradient resulted in the isolation of insulin in highly purified form and in 50–65% over-all yield. This insulin was identical with the natural hormone by all criteria employed.

It has been established that synthesis of insulin can be accomplished by combination of its individual chains, natural (Dixon and Wardlaw, 1960; Du *et al.*, 1961; Katsoyannis and Tometsko, 1966; Zahn *et al.*, 1966) or synthetic (Katsoyannis *et al.*, 1963, 1964, 1966a,b; Katsoyannis, 1964a,b, 1966a,b, 1967; Katsoyannis and Tometsko, 1966; Meienhofer *et al.*, 1963; Zahn *et al.*, 1965a,b; Wang *et al.*, 1965; Niu *et al.*, 1966; Kung *et al.*, 1966). However, the isolation of insulin thus synthesized presents considerable difficulties and is indeed a problem of a different order of magnitude than isolation of this hormone from natural sources. Whereas, in the former case, a protein is to be separated from closely related polypeptide chains, in the latter instance such problems, to our knowledge, do not exist. Furthermore, because of the nature of the reactants and products of the combination mixture (*i.e.*, thiol, reduced and oxidized insulin chains, and insulin) it is possible that interactions might occur which could lead to insulin alteration or even destruction. Indeed, such complications were encountered during this investigation. Finally, although recovery of insulin in pure form, and in good yields, from recombination mixtures of the A and B chains is the primary aim of any isolation procedure,

* From the Division of Biochemistry, Medical Research Center, Brookhaven National Laboratory, Upton, New York 11973. *Received May 26, 1967.* This work was supported by the U. S. Atomic Energy Commission and the U. S. Public Health Service.

another interesting aspect of the isolation problem is the identification of other products of the recombination reaction.

Isolation of insulin produced by recombination of natural chains through extraction by acidified alcohol (Banting *et al.*, 1922), as modified by Du *et al.* (1961), is unsatisfactory, since the over-all recovery of pure insulin is low. Especially, this procedure is unsatisfactory and of no practical value in the isolation of synthetic insulin, where enormous losses of the hormone, up to 95%, are encountered (Kung *et al.*, 1966). Furthermore, the method of Du *et al.* (1961) contributes little to the understanding of the nature of the recombination products.

Systematic studies were, therefore, undertaken in this laboratory directed toward the development of effective methods for the isolation of insulin from recombination mixtures of the A and B chains and for the identification of the by-products of the recombination reaction. This communication describes such methods of isolation of insulin in highly purified form as well as the identification of other products produced by the recombination of the insulin chains. Valuable information for the successful outcome of this work was provided by some recent methods (Randall, 1964; Smith, 1964) reported in the literature for the isolation of insulin from natural sources.

Materials and Methods

S-Sulfonated Derivatives of the A and B Chains of Bovine Insulin (A-SSO_3^-, B-SSO_3^-)[1]

The methods for the preparation of these compounds from natural bovine insulin were described previously (Katsoyannis *et al.*, 1967a).

Insulin Synthesis by Recombination of the A and B Chains

Insulin Synthesis by Interaction of the Sulfhydryl Form of the A Chain with B-SSO_3^-. REDUCTION OF A-SSO_3^- WITH 2-MERCAPTOETHANOL. This method has been described in detail in a previous publication (Katsoyannis and Tometsko, 1966). Briefly, an aqueous solution of 20 mg of A-SSO_3^- was treated at pH 5.0 with 2-mercaptoethanol for 6 min at 100°. The reaction mixture was then extracted with ethyl acetate to remove the 2-mercaptoethanol and the resulting reduced A chain was allowed to react with 5 mg of B-SSO_3^- at pH 9.6. After 18–22 hr at 2° the solution of the recombination mixture was assayed for insulin activity. As was reported previously, under these conditions, the yield of insulin formed ranged from 60 to 80% of theory, based on the amount of B-SSO_3^- used.

REDUCTION OF A-SSO_3^- WITH DITHIOTHREITOL. A solution of 20 mg of A-SSO_2^- in 6 ml of 0.1 M Tris-HCl (pH 8.0) was adjusted to pH 8.0 with 0.1 N NaOH, deaerated by flushing with nitrogen, and mixed with 25 mg of dithiothreitol (Cleland's reagent). After 24 hr at room temperature under nitrogen, the solution was extracted four times with 40-ml portions of ethyl acetate. Following the last extraction, the residual ethyl acetate was removed by flushing the reaction vessel with nitrogen. The resulting jelly-like product was mixed with 5 mg of B-SSO_3^- and 7.8 ml of water was deaerated previously by boiling under nitrogen. The pH of the reaction mixture was brought to 10.2 with 1 N NaOH and the ensuing clear solution was finally adjusted to pH 9.6 with 1 N acetic acid. This solution was diluted with 1 ml of 0.1 M glycine buffer (pH 9.6), stirred for 24 hr at 2° in contact with CO_2-free air, and submitted to insulin assays. The yield of insulin formed under these conditions ranged from 55 to 65% of theory, based on the amount of B-SSO_3^- used.

Insulin Synthesis by Interaction of the Sulfhydryl Forms of the A and B Chains. REDUCTION OF THE S-SULFONATED A AND B CHAINS WITH 2-MERCAPTOETHANOL. This method has been described in detail previously (Katsoyannis and Tometsko, 1966). A mixture of 10 mg of B-SSO_3^- and 42 mg of A-SSO_3^- was treated at pH 5 with 2-mercaptoethanol at 100° and the reduced chains were isolated by centrifugation at pH 3.8. A suspension of these chains in water was adjusted to pH 10.6 and the resulting solution was air oxidized for 24 hr at 2°. Under these conditions, the yield of insulin formed was 50% of theory.

REDUCTION OF THE S-SULFONATED A AND B CHAINS WITH THIOGLYCOLIC ACID. The method of Du *et al.* (1965) was followed. Briefly, 5.1 mg of A-SSO_3^- and 4.9 mg of B-SSO_3^- were reduced with thioglycolic acid at pH 5 and at room temperature. The sulfhydryl forms of the chains thus obtained were precipitated at pH 3.8, isolated by centrifugation, and recombined under the conditions described in the previous method. The yield of insulin formed ranged from 15 to 22% of theory based on the amount of B-SSO_3^- used.

Column Chromatography

Carboxymethylcellulose Columns. CM-CELLULOSE COLUMN (0.9 × 23 cm). Preswollen microgranular CM-cellulose (Whatman CM52/1) was used. CM-cellulose was suspended in 0.5 N NaOH, stirred gently for 20–30 min, filtered, and washed with 0.5 N NaOH and water. The CM-cellulose was subsequently suspended in water and allowed to settle. Fines were removed by decanting the supernatant fluid, and this process was repeated four to five times. The aqueous suspension of the resin was then made acidic with HCl and the CM-cellulose cake obtained by filtration, after washing with water, was resuspended in an acetate buffer (sodium acetate (0.024 M) brought to pH 3.3 with glacial acetic acid). The resin was filtered and suspended again in the same buffer. After filtration, the ion exchanger was poured into the column as a thick slurry in the same acetate buffer. Acetate buffer was pumped through the column at 35–40 ml/hr (Sigmamotor kinetic pump) until the conductivity of the effluent was stabilized (1.56 mmhos). During chromatography the effluent was monitored by a Gilford recording spectrophotometer and a conductivity meter (Radiometer, Copen-

[1] Abbreviations used: A-SSO_3^-, S-sulfonated derivative of the A chain; B-SSO_3^-, S-sulfonated derivative of the B chain.

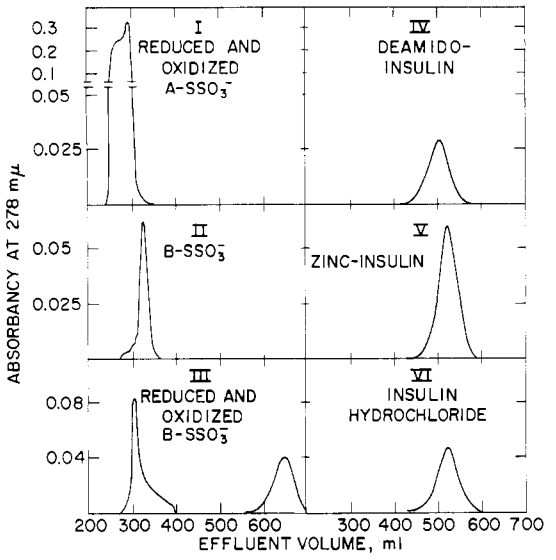

FIGURE 1: Chromatography of insulin and A and B chains on the 2 × 100 cm CM-cellulose column with urea–acetate buffer. (I) A-SSO_3^- (10 mg) was reduced, oxidized, and chromatographed; (II) B-SSO_3^- (4 mg); (III) B-SSO_3^- (10 mg) was reduced, oxidized, and chromatographed; (IV) monodeamido-insulin (3 mg); (V) crystalline bovine zinc insulin (6 mg); and (VI) bovine insulin hydrochloride (4 mg).

hagen). For elution of insulin an exponential salt gradient was used, obtained with the following technique. A Mariotte-type flask was used as a reservoir, containing 0.35 M NaCl in the same acetate buffer. This reservoir was connected with a constant volume mixing chamber containing 130 ml of the acetate buffer. From the mixing chamber the buffer was withdrawn and brought to the column with the aid of the pump.

CM-CELLULOSE COLUMN (2 × 100 cm). CM-cellulose (Mannex lot N2263; high capacity, 0.9 ± 0.1 mequiv/g) was used. The ion exchanger was washed according to Peterson and Sober (1962) and then suspended in urea–acetate buffer prepared as described in the preceding publication (Katsoyannis *et al.*, 1967a). The column was packed under pressure which was raised during the packing by 1 psi/10 cm of packed bed, *i.e.*, when the bed height was 10 cm the pressure was 1 psi, when 20 cm, 2 psi, up to a final pressure of 10 psi. This column was operated at 1 psi and the flow rate was approximately 120 ml/hr.

Sephadex Columns. SEPHADEX G-15 (4 × 60 cm). Sephadex G-15 (Pharmacia Uppsala) was suspended in distilled water and the suspension was heated to 80° for 1 hr and then allowed to settle at room temperature. Fines were removed by decanting the supernatant fluid. The resin was finally suspended in 5% acetic acid (v/v), deaerated with an aspirator, and poured into the column. The effluent was monitored by a Gilford recording spectrophotometer. Samples to be applied to this column were adjusted to pH 3.0 with concentrated HCl.

SEPHADEX G-15 (1.2 × 50 cm). The Sephadex resin was treated as described previously and was equilibrated with 0.05 M ammonium bicarbonate solution. The effluent was monitored with a Zeiss recording spectrophotometer.

Additional Techniques

Insulin picrates and insulin hydrochlorides were used repeatedly throughout this investigation. Formation of these derivatives was carried out as described by Randall (1964). Crystallization of insulin was carried out either as described by Randall (1964) or by Epstein and Anfinsen (1963). Protein determinations were performed by the method of Lowry *et al.* (1951) and thin layer electrophoresis as described by Tometsko and Delihas (1967). Amino acid analyses of acid hydrolysates were performed in a Beckman-Spinco amino acid analyzer (Model 120B) equipped with a digital readout system (Model CRS-10AB. Infotronics Corp., Houston, Texas) according to the method of Spackman *et al.* (1958). For taking infrared spectra, the following procedure was followed. A sample (1 mg) was thoroughly mixed with 250 mg of KBr and converted to a pellet under 18,000 lbs of total load pressure with a Carver laboratory press, Model B. The infrared spectrum was taken with a Perkin-Elmer Infracord, Model 337. All operations were performed at 25°. Determination of insulin activity was carried out by the mouse convulsion method as it has been described previously (Katsoyannis and Tometsko, 1966).

Results

Isolation of Insulin from Recombination Mixtures of A and B Chains by CM-cellulose Chromatography with Urea-Containing Buffer

Preliminary experiments indicated that a satisfactory resolution of the components of a recombination mixture of A and B insulin chains could be accomplished by chromatography of the mixture on a 2 × 100 cm CM-cellulose column with urea–acetate buffer. It is obvious that, after completion of the recombination reaction, the mixture will predominately contain regenerated insulin and chain components produced from the non-recombined A and B chains. Consequently, to facilitate the identification of the different peaks obtained upon chromatography of the recombination mixture, the behavior of insulin and of the A and B chains on the same chromatographic system was examined.

Chromatography of Insulin and of A and B Chains. A CHAIN. The chromatographic behavior of reduced and oxidized A chain S-sulfonate is shown in Figure 1-I. A-SSO_3^- was reduced with mercaptoethanol and treated as described in Materials and Methods with the only difference that B-SSO_3^- was not added to the reaction mixture. After the air-oxidation step glacial acetic acid was added to a final concentration of 10% (v/v) and the solution was lyophilized. The material obtained was dissolved in 2 ml of urea-acetate buffer (pH 4.0) and placed on the 2 × 100 cm CM-cellulose column. The chromatogram (Figure 1-I) of the reduced and oxidized

A-SSO_3^- shows two overlapping peaks. The position of the first peak was at 265–270 ml, whereas the position of the second peak was at about 280–290 ml of effluent. The presence of more than one component in the oxidized mixture of the A chain is to be expected because of the presence of four sulfhydryl groups in the molecule of the reduced chain.

B CHAIN. Chromatography of the B-SSO_3^- on the 2 × 100 cm CM-cellulose column gave the pattern shown in Figure 1-II. The chromatographic behavior of the B-SSO_3^- which has been reduced and oxidized is depicted in Figure 1-III. Briefly, a suspension of 10 mg of B-SSO_3^- in 3 ml of water was solubilized with the addition of 5 N methylamine. This solution was adjusted to pH 5 with acetic acid and the resulting suspension was treated with 1 ml of 1 N thioglycolic acid which was adjusted to pH 5 with methylamine. After standing for 18 hr at room temperature under nitrogen the reaction mixture was brought to pH 3.8 with acetic acid and cooled for 15 min in an ice bath, and the precipitated product was isolated by centrifugation. A suspension of the precipitate in 2 ml of water was solubilized by adjusting the pH to 10.6 with 1 N NaOH. The resulting solution was diluted with 0.3 ml of 0.1 M glycine–NaOH buffer (pH 10.6) and stirred at 2° for 48 hr in contact with CO_2-free air. The reaction mixture was subsequently adjusted to pH 4.0 with acetic acid made 8 M in urea by the addition of crystalline urea and placed on the 2 × 100 cm CM-cellulose column. The chromatogram (Figure 1-III) showed a major component eluted at the same position where the B-SSO_3^- is eluted (Figure 1-II). Presumably this component is unreacted B-SSO_3^- and indicates that this compound is not reduced quantitatively under the conditions used, in agreement with data reported by Du *et al.* (1965). Another component present has a peak position at approximately 650 ml of effluent and traveled more slowly than insulin (see below, Figure 1-IV). Minor components emerge at approximately 350–400 ml of effluent.

INSULIN. The chromatographic behavior of bovine deamido-insulin, crystalline bovine zinc insulin, and bovine insulin hydrochloride is illustrated in Figure 1-IV–VI. The peak positions of the latter two materials were the same (about 520 ml). The peak position of deamido-insulin was at about 500 ml of effluent. It is apparent that this chromatographic procedure will not significantly separate deamido-insulin from insulin.

The reported peak positions of the various compounds chromatographed on the 2 × 100 cm CM-cellulose column represent the most frequently observed values in several chromatographic runs. It should be noted that small changes of the pH and ionic strength of the urea–acetate buffer strongly affected the R_F of the compounds chromatographed. Since the pH of this buffer tended to drift upward, freshly prepared buffers were used. For the same reason the column was thoroughly equilibrated before each run.

We have further observed that over a period of several weeks of use of the CM-cellulose column, there was a shift of the relative positions of the peaks of the various compounds chromatographed, apparently due to loss of capacity of the resin. For this reason, calibration runs with authentic samples of insulin and B-SSO_3^- were repeated from time to time with a given column.

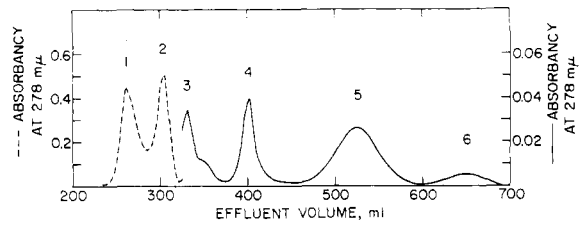

FIGURE 2: Chromatography of a recombination mixture on the 2 × 100 cm CM-cellulose column. The recombination mixture (9.1 ml) (total insulin activity 145 IU) was lyophilized and the material obtained was dissolved in 1 ml of urea–acetate buffer and placed on the column. Peak 4 is insulin I (16 IU or 9% of the original activity) and peak 5 is insulin II (65 IU or 45% of the original activity). The specific activity of insulin II at the hydrochloride form was found to be 24–26 IU/mg.

RECOVERY OF THE INSULIN FROM CM-CELLULOSE CHROMATOGRAPHY. Insulin was recovered from the effluent of the CM-cellulose column by the following procedure. The effluent was titrated to pH 3.0 with concentrated HCl and immediately placed on a 4 × 60 cm Sephadex G-15 column equilibrated with 5% (v/v) acetic acid and the protein was eluted with the same solvent. The effluent containing the insulin peak was concentrated in a rotary evaporator to approximately 10 ml. The concentrated solution was placed in a centrifuge tube mixed with an equal volume of saturated aqueous picric acid solution and stored at 0° overnight. The insulin picrate thus obtained was converted to insulin hydrochloride and, if desirable, to crystalline zinc insulin as noted in the Materials and Methods. Starting with zinc insulin (Eli Lilly), after chromatography on CM-cellulose and Sephadex and picrate formation, about 86% of the original amount of insulin was recovered as insulin hydrochloride and 73% as crystalline zinc insulin.

Chromatography of Recombination Mixtures. The recombination mixture produced by interaction of B-SSO_3^- with the sulfhydryl form of A chain (see Materials and Methods) was directly lyophilized and the resulting white solid was dissolved in 1 ml of urea–acetate buffer and placed on a 2 × 100 cm CM-cellulose column. A typical chromatogram is presented in Figure 2.

The first two peaks (1 and 2) of the chromatogram of Figure 2 were eluted at approximately the same position as the reduced and oxidized A-SSO_3^- (Figure 1-I). After dialysis and lyophilization, amino acid analysis (Table I) showed that these two components have an amino acid composition corresponding to the A chain. Peak 2 appears to be slightly contaminated with B chain; this is to be expected since B-SSO_3^- is eluted (Figure 1-II) in a position very close to that of peak 2. Biological assays of

TABLE I: Amino Acid Analysis[a] of Acid Hydrolysates of Peaks 1, 2, 3, and 6 Obtained from the CM-Cellulose Chromatography with Urea–Acetate Buffer.

Amino Acid	A Chain Theory	Peak 1 Found	Peak 2 Found	Peak 3 Found	Peak 6 Found	B Chain Theory
Lysine			tr	0.8	1.0	1
Histidine			tr	1.8	2.0	2
Arginine			tr	0.9	0.7	1
Aspartic acid	2	2.0	1.9	1.2	0.9	1
Threonine			tr	1.0	0.7	1
Serine	2	1.8	1.7	0.9	0.5	1
Glutamic acid	4	4.2	3.8	3.3	3.2	3
Proline				1.1	1.1	1
Glycine	1	1.1	1.3	3.0	2.9	3
Alanine	1	1.0	1.2	2.1	1.8	2
Valine	2	1.7	1.7	2.8	2.9	3
Isoleucine	1	0.7	0.7	tr		
Leucine	2	2.0	2.0	4.3	4.4	4
Tyrosine	2	*b*	*b*	*b*	*b*	2
Phenylalanine			tr	2.8	2.9	3
Half-cystine	4	*b*	*b*	*b*	*b*	2

[a] Data given as number of amino acid residues per molecule. [b] Not determined.

peaks 1 and 2 by the mouse convulsion method gave no indication of insulin activity.

Peak 3 was eluted at approximately the same position as was B-SSO_3^- (Figure 1-II). Removal of urea was performed with chromatography on Sephadex G-15 as has been described in the Materials and Methods. Amino acid analysis of an acid hydrolysate (Table I) showed that the material obtained from this peak was predominately B chain contaminated with A chain. Biological assays gave no indication of insulin activity.

Peak 4 was shown to possess insulin activity and was designated as insulin I. This insulin which had a peak position at 400 ml was eluted earlier than insulin or deamido-insulin (Figure 1-IV–VI). Insulin I was recovered as the hydrochloride salt as described in the Results. The amount of this insulin varied from preparation to preparation and greatly depended, as will be discussed below, upon the conditions used for the recombination of the chains and the handling of the recombination mixture.

The next component (peak 5) was eluted at the same position as zinc insulin (Figure 1-V) and insulin hydrochloride (Figure 1-VI) and was designated as insulin II. The amount of insulin II varied from preparation to preparation, being most frequently approximately 50% of the insulin activity present in the recombination mixture.

The last component (peak 6) traveled more slowly than insulin II and was eluted at the same position (650 ml of effluent) as one of the components formed upon reduction and oxidation of B-SSO_3^- (Figure 1-III). The material obtained from peak 6, after removal of the urea upon chromatography on Sephadex, is readily converted to picrate and hydrochloride salt. Amino acid analysis of an acid hydrolysate of this material (Table I) was similar to that of the B chain. On thin layer electrophoresis in 0.5 N acetic acid (Figure 3), and upon rechromatography on the same column, the hydrochloride of this compound behaved as a single component.

Chromatographic patterns which differed only quantitatively from the one shown in Figure 2 were obtained when the recombination mixture was treated, prior to chromatography, in a different way than that described previously. Figure 4-I illustrates a typical chromatogram obtained when the recombination mixture (approximate volume, 10 ml) was directly dialyzed at 2° against four 2-l. portions of water, lyophilized, and then placed on the column. Figure 4-II shows the chromatographic pattern obtained when the recombination mixture was treated as follows. Glacial acetic acid was added to the reaction mixture to give a 10% final concentration (v/v) and the resulting solution was mixed with an equal volume of saturated picric acid solution. The entire procedure was carried out at 2°. After 24 hr at 2° the precipitate was collected by centrifugation and washed twice with half-saturated picric acid solution. This solid was dissolved in a few drops of acetone–water (4:1) and the resulting solution was mixed with 40 ml of ice-cold dry acetone containing 4 drops of concentrated hydrochloric acid. A white precipitate was formed immediately. After 2 hr in the cold the precipitate was collected by centrifugation and washed once with dry acetone and once with dry ether. This material was dissolved in 2 ml of urea–acetate buffer and placed on the CM-cellulose column. The precipitation of insulin from the recombination mixture as the picric acid salt de-

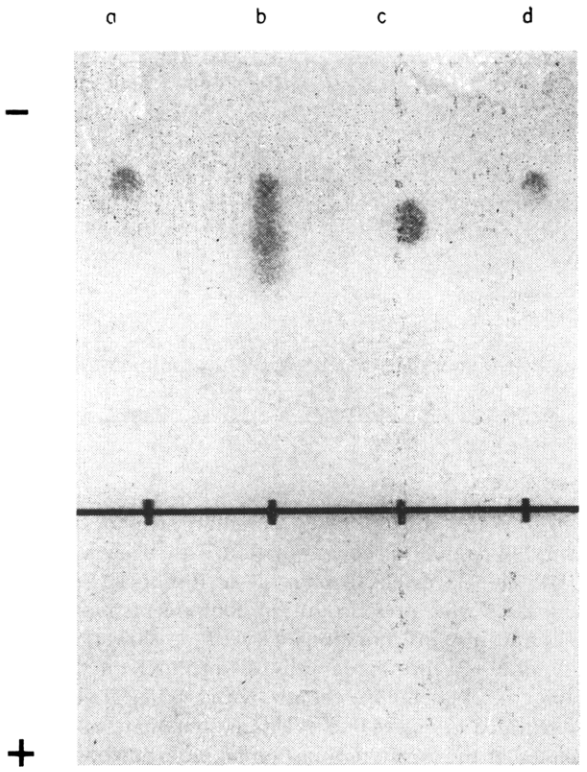

FIGURE 3: Thin layer electrophoresis of natural bovine insulin hydrochloride (a), insulin I (b), peak 6 (c), and insulin II (d); 0.5 N acetic acid, 3400 v, 15 min.

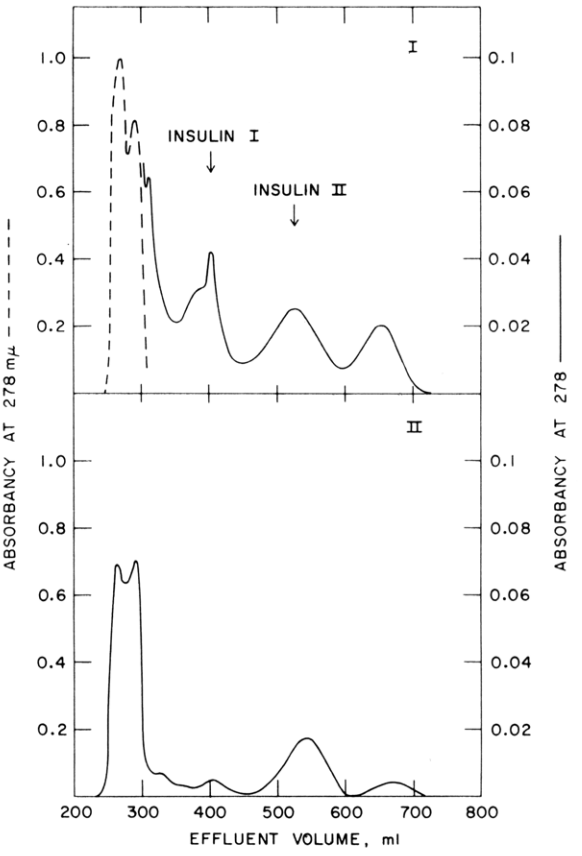

FIGURE 4: Chromatography of recombination mixtures on a 2 × 100 cm CM-cellulose column with urea–acetate buffer. (I) A recombination mixture with a total insulin activity, 144 IU, was dialyzed for 24 hr in the cold against four 4-l. portions of glass-distilled water and then lyophilized. The material obtained was dissolved in 1 ml of urea–acetate buffer and chromatographed. A quantity of 14 IU was recovered as insulin I (sp act. 18 IU/mg) and 63 IU was recovered as insulin II (sp act. 25 IU/mg). (II) A recombination mixture with a total insulin activity of 85 IU was processed through the picric acid treatment. Insulin II (45 IU) was recovered (sp act. 25 IU/mg).

scribed above was almost quantitative as judged from the following experiment. The precipitated picrate was dissolved in a few drops of 1 N NH_4OH and 2 ml of 0.05 M ammonium bicarbonate and placed on a 1.2 × 50 cm Sephadex G-15 column equilibrated with 0.05 M ammonium bicarbonate solution. Under these conditions the protein material was separated completely from the picric acid salts which traveled extremely slowly on this column. In several experiments the amount of insulin activity recovered from the Sephadex column was approximately 90% of the activity present in the recombination mixture.

Quantitatively different chromatographic patterns than the one shown in Figure 2 were obtained also when insulin synthesis was achieved by interaction of the sulfhydryl forms of the A and B chains (Figure 5-I, II) and the recombination mixture was lyophilized before chromatography. In Figure 5-I, the conversion of the sulfonated chains to their sulfhydryl forms was carried out upon treatment with 2-mercaptoethanol (see Material and Methods), whereas in Figure 5-II this conversion was accomplished with thioglycolic acid (see Materials and Methods).

A quite different chromatogram of the recombination mixture from the CM-cellulose column was obtained when the conversion of the A-SSO_3^- to its sulfhydryl form prior to its interaction with B-SSO_3^- was carried out on exposure to dithiothreitol (see Materials and Methods). Figure 5-III represents a chromatogram of a recombination mixture obtained this way which was lyophilized and then chromatographed. As can be seen in this case, insulin II is not present, whereas insulin I is greatly increased. However, when a recombination mixture obtained as described above was subjected to the picric acid treatment instead of lyophilization, it upon chromatography exhibited the pattern shown in Figure 5-IV. In contrast to Figure 5-III, in Figure 5-IV insulin II is now the major component, whereas insulin I is only present in trace. It is, therefore, apparent from the results just described that picric acid treatment of the recombination mixture is the preferred route to process the reaction mixture prior to chromatography. Table II

TABLE II: Insulin Recovery from Combination Mixtures of A and B Chains.

Method of Recombination	Type of Treatment of Recombination Mixtures	Per Cent of the Original Activity[a]	
		Insulin I[b]	Insulin II[b]
A-SSO_3^- reduced with β-mercaptoethanol and allowed to react with B-SSO_3^-	Lyophilization	8–25	25–42
	Dialysis and lyophilization	10–25	25–45
	Picric acid		50–65
A-SSO_3^- reduced with dithiothreitol and allowed to react with B-SSO_3^-	Lyophilization	25–35	
	Picric acid		50–55

[a] Insulin activity determined by the mouse convulsion method. [b] Insulin activity determined on the effluent obtained from the Sephadex G-15 column.

summarizes the recoveries of insulins I and II obtained by the procedures described previously in a considerable number of experiments.

Insulin I. This material was isolated from the effluent after removal of the urea by Sephadex chromatography and was recovered *via* picrate as the hydrochloride according to the procedure described in the Results.

In eight preparations the specific activity of the hydrochloride of insulin I ranged from 16 to 22 IU when assayed by the mouse convulsion method. Amino acid analysis after acid hydrolysis showed a composition in molar ratios corresponding to natural insulin, but with varying degrees of contamination with B chain (Table III). Thin layer electrophoresis of insulin I (Figure 3) indicated the presence of a component moving as natural insulin contaminated with a slower moving material. Rechromatography of insulin I on the 2 × 100 cm CM-cellulose column with urea–acetate buffer illustrated in Figure 6 revealed that this material is now eluted at the position of insulin II (peak position at 520

TABLE III: Amino Acid[a] Analysis of Insulin I.

Amino Acid	Bovine Insulin Theory	Insulin I (from Figure 5-II) Found	Insulin I (from Figure 5-III) Found
Lysine	1	1.3	1.0
Histidine	2	2.6	2.2
Arginine	1	1.4	1.2
Aspartic acid	3	2.4	2.8
Threonine	1	1.1	1.1
Serine	3	2.1	2.4
Glutamic acid	7	6.4	6.8
Proline	1	1.5	1.2
Glycine	4	4.0	4.5
Alanine	3	2.7	3.2
Valine	5	4.0	5.1
Isoleucine	1	0.5	0.8
Leucine	6	6.4	6.2
Tyrosine	4	*b*	*b*
Phenylalanine	3	3.5	3.4
Half-cystine	6	*b*	*b*
Specific activity (IU/mg)	25	16	20

[a] Data given as number of amino acid residues per molecule. [b] Not determined.

TABLE IV: Amino Acid Analysis[a] of Insulin Obtained upon Rechromatography of Insulin I.

Amino Acid	Bovine Insulin Theory	On CM-cellulose with Urea Buffer	On CM-cellulose with NaCl Gradient
Lysine	1	0.9	0.9
Histidine	2	1.9	1.8
Arginine	1	1.0	0.9
Aspartic acid	3	3.1	2.9
Threonine	1	1.0	1.0
Serine	3	2.7	2.8
Glutamic acid	7	7.1	6.8
Proline	1	0.9	0.9
Glycine	4	4.1	4.0
Alanine	3	3.0	2.9
Valine	5	4.7	4.5
Isoleucine	1	0.7	0.7
Leucine	6	6.1	5.7
Tyrosine	4	*b*	*b*
Phenylalanine	3	3.0	2.8
Half-cystine	6	*b*	*b*
Specific activity (IU/mg)	25	23	24

[a] Data given as number of amino acid residues per molecule. [b] Not determined.

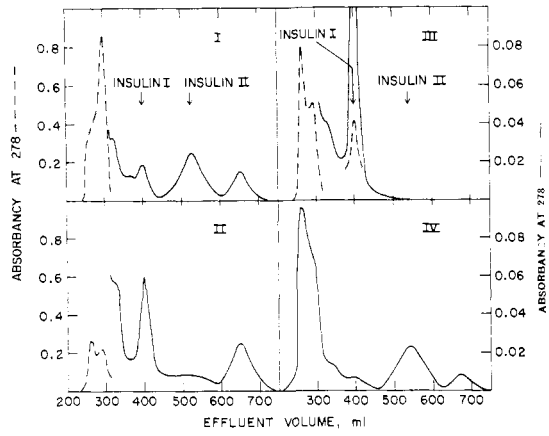

FIGURE 5: Chromatography of recombination mixtures on a 2 × 100 cm CM-cellulose column with urea–acetate buffer. (I) Synthesis of insulin was achieved by interaction of the sulfhydryl forms (mercaptoethanol) of the A and B chains (see Materials and Methods) and the recombination mixture (total activity 118 IU) was lyophilized. The material obtained was dissolved in 1 ml of urea–acetate buffer and chromatographed. A quantity of 45 IU was recovered as insulin II and 8 IU as insulin I. (II) Synthesis of insulin was achieved by interaction of the sulfhydryl forms (thioglycolic acid) of the A and B chains (see Materials and Methods) and the recombination mixture (total activity 90 IU) was lyophilized. The material obtained was dissolved in 1 ml of urea–acetate buffer and chromatographed. A quantity of 26 IU was recovered as insulin I (sp act. 16 IU/mg) and 10 IU as insulin II. The peak after insulin II gave a material with an amino acid composition similar to that of the B chain. (III) Synthesis of insulin was achieved by interaction of the sulfhydryl form of the A chain (reduction with dithiothreitol) with the B-SSO_3^-. Two recombination mixtures (total activity 202 IU) were lyophilized. The material obtained was dissolved in 1 ml of urea–acetate buffer and chromatographed. A quantity of 44 IU was recovered as insulin I (sp. act. 20 IU/mg). (IV) Synthesis of insulin was achieved as in III. The recombination mixture (total activity 103 IU) was treated with picric acid. The hydrochloride obtained was dissolved in 1 ml of urea–acetate buffer and chromatographed. A quantity of 52 IU was recovered as insulin II (sp act. 25 IU/mg).

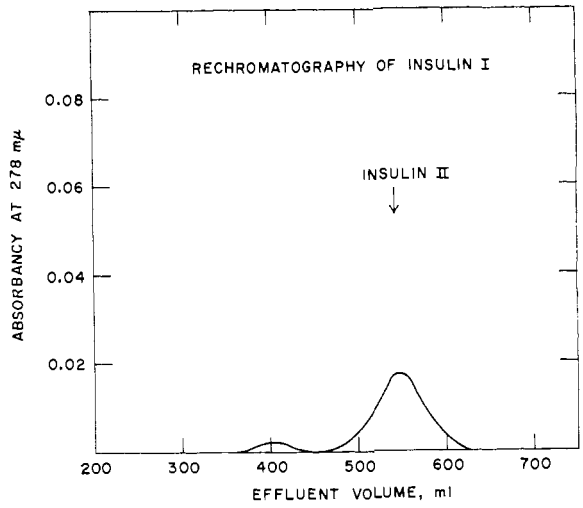

FIGURE 6: Rechromatography of insulin I on a 2 × 100 cm CM-cellulose column with urea–acetate buffer. Insulin I from Figure 5-III was converted to hydrochloride (35 IU, sp act. 20 IU/mg), dissolved in 1 ml of urea–acetate buffer, and placed on the column. A quantity of 25 IU was recovered as insulin II (sp act. 23 IU/mg).

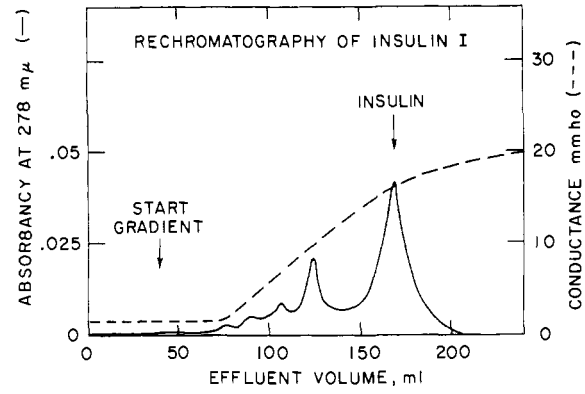

FIGURE 7: Rechromatography of insulin I on a 0.9 × 23 cm CM-cellulose column with an exponential sodium chloride gradient. Insulin I, obtained from a chromatographic separation of a recombination mixture similar to that illustrated in Figure 5-III, was converted to hydrochloride (sp act. 20 IU/mg), dissolved in 2 ml of acetate buffer (pH 3.3), and placed on a 0.9 × 23 cm CM-cellulose column. The insulin recovered (sp act. 24 IU/mg) gave the amino acid analysis listed in Table IV.

ml of effluent) and only traces of material appear to be present at the position where insulin I was originally eluted (370–430 ml of effluent). In the particular experiment depicted in Figure 6 insulin I with a total activity of 35 IU was placed on the column and 25 IU was recovered from the peak eluted at the position of insulin II. The insulin from the effluent was isolated *via* picrate as the hydrochloride and crystallized. The crystalline material was identical with insulin II as far as specific activity (23 IU/mg), amino acid composition (Table IV), and mobility on thin layer electrophoresis is concerned.

Furthermore, chromatography of insulin I (Figure 7) on a CM-cellulose column with an exponential NaCl gradient (see Material and Methods) revealed also the presence of a major component eluted at the same position as natural insulin. The specific activity (24 IU/mg) and amino acid composition (Table IV) of this in-

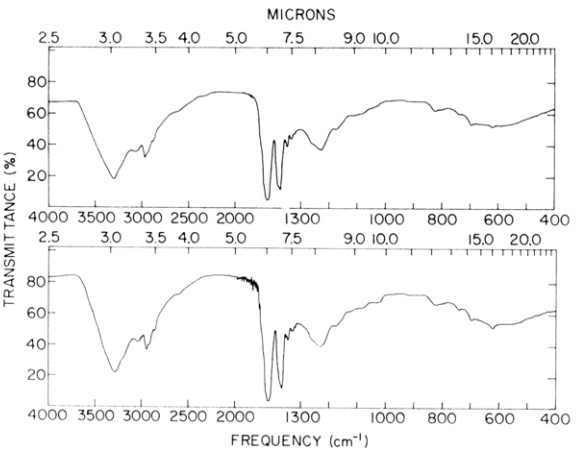

FIGURE 8: Infrared spectra of natural bovine insulin hydrochloride (top) and insulin II hydrochloride (bottom).

sulin was similar to that of the natural protein. The other component of the chromatogram, the amount of which varies with different batches of insulin, gave the following amino acid composition, expressed in molar ratios: $Lys_{1.0}His_{2.0}Arg_{1.0}Asp_{1.0}Thr_{0.9}Ser_{0.9}Glu_{3.0}Pro_{1.0}$-$Gly_{3.0}Ala_{2.0}Val_{3.0}Leu_{4.0}Phe_{2.9}$ (tyrosine and cysteine were not determined), which corresponds to that of B chain.

TABLE V: Amino Acid Analysis[a] of Acid Hydrolysates of Natural Bovine Insulin and of Insulin II Recovered from Recombination Mixtures.

Amino Acid	Natural Bovine Insulin		Insulin II
	Theory	Found	Found
Lysine	1	1.0	0.9
Histidine	2	1.9	1.8
Ammonia	6	6.1	5.5
Arginine	1	0.9	1.0
Aspartic acid	3	3.0	3.0
Threonine	1	0.9	1.2
Serine	3	2.6	2.6
Glutamic acid	7	7.3	7.1
Proline	1	1.0	1.0
Glycine	4	4.0	3.8
Alanine	3	3.0	3.2
Valine	5	5.3	4.6
Isoleucine	1	0.7	0.8
Leucine	6	5.9	5.9
Tyrosine	4	*b*	*b*
Phenylalanine	3	2.9	3.1
Half-cystine	6	*b*	*b*

[a] Data given as number of amino acid residues per molecule. [b] Not determined.

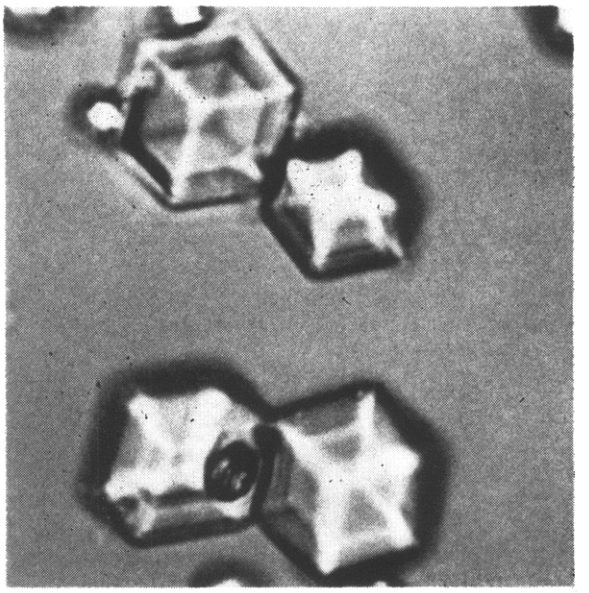

FIGURE 9: Crystalline bovine zinc insulin II.

Insulin II. The isolation of this component and its recovery as the hydrochloride was accomplished as described in Results. In ten preparations the specific activity of insulin II hydrochloride ranged from 23 to 25 IU/mg when assayed by the mouse convulsion method. Amino acid analysis after acid hydrolysis showed a composition in molar ratios in excellent agreement with the theoretically expected values for bovine insulin (Table V). Thin layer electrophoresis of insulin II indicated the presence of a single component with the same mobility as natural insulin (Figure 3). No differences

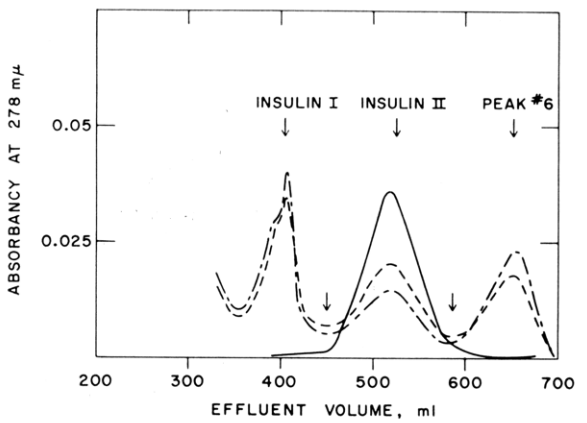

FIGURE 10: Rechromatography of insulin II on a 2 × 100 cm CM-cellulose column with urea–acetate buffer. Two preparations of insulin II recovered as hydrochlorides from two chromatographic runs (broken lines) were pooled, dissolved in 1 ml of urea–acetate buffer, and placed on the column. Upon chromatography (solid line) the material emerged as a single peak in the position of insulin II.

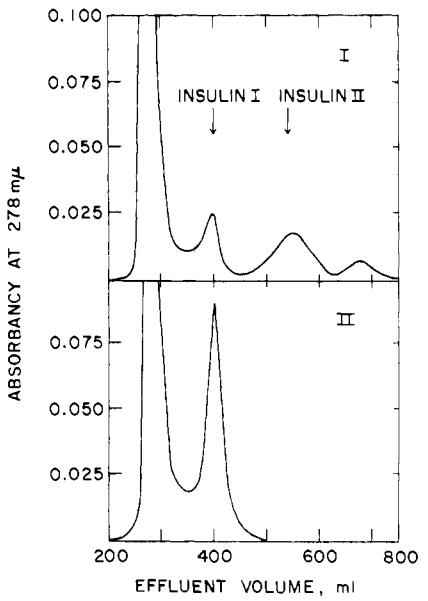

FIGURE 11: Effect of reduced and oxidized A-SSO_3^- on natural bovine insulin. (I) A-SSO_3^- (20 mg) was reduced with 2-mercaptoethanol, mixed with insulin (5 mg), and air oxidized for 24 hr at pH 9.6 and at 2°. Upon chromatography of the lyophilized mixture on a 2 × 100 cm CM-cellulose column with urea–acetate buffer 50 IU was recovered as insulin II and 12 IU as insulin I. (II) A-SSO_3^- (20 mg) was reduced with dithiothreitol, incubated with insulin (8 mg), and treated as in I. Upon chromatography 50 IU was recovered as insulin I.

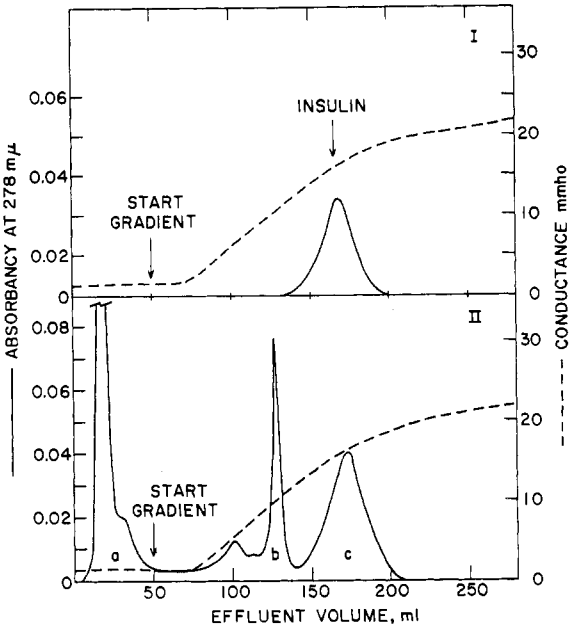

FIGURE 12: Chromatography of natural bovine zinc insulin and of a combination mixture of A and B chains on a 0.9 × 23 cm CM-cellulose column with an exponential NaCl gradient. (I) Bovine zinc insulin (2 mg) was dissolved in 2 ml of sodium acetate buffer (pH 3.3; Na^+, 0.04 M) and placed on the column. The insulin (1.5 mg) was recovered as the hydrochloride. (II) A combination mixture containing 107 IU of regenerated bovine insulin was processed. The insulin (2.1 mg) was recovered as the hydrochloride (sp act. 25 IU/mg).

were detected in the infrared spectra of insulin II and of natural insulin (Figure 8). Insulin II was readily crystallized by Randall's (1964) or Epstein and Anfinsen's (1963) method. The shape and specific activity of the crystalline material was identical with that of natural bovine zinc insulin (Figure 9). Rechromatography of insulin II hydrochloride on the 2 × 100 cm CM-cellulose column with urea–acetate buffer (Figure 10) gave an elution pattern identical with that of natural insulin (Figure 1-V–VI).

Effects of the Conditions Used in the Recombination and Isolation Procedures on Insulin

The appearance of insulin I and the occurrence of variable losses of insulin during the isolation steps prompted us to examine the stability of this protein under the conditions prevailing in the recombination reaction. In these series of experiments insulin was subjected to the influence of all the components of the recombination reaction. B-SSO_3^-, however, was excluded to prevent regeneration of insulin.

A-SSO_3^- was reduced with mercaptoethanol as described in Materials and Methods. A suspension of the resulting jelly-like reduced product in 7.8 ml of water was solubilized by adjusting the pH to 9.6 with 1 N NaOH and then mixed with 5 mg of zinc insulin. After stirring for 24 hr at 2° in contact with CO_2-free air, the reaction mixture was lyophilized and the resulting fluffy material was dissolved in urea–acetate buffer and placed on the 2 × 100 cm CM-cellulose column. The chromatogram (Figure 11-I) showed, in addition to the expected A chain (250–325 ml) and insulin II (475–610 ml), the presence of insulin I (375–420 ml) and of a component which was eluted after insulin II (625–725 ml). This last component, after acid hydrolysis, gave the following amino acid composition, expressed in molar ratios: $Lys_{1.0}His_{1.9}Arg_{1.0}Asp_{1.0}Thr_{0.9}Ser_{0.8}Glu_{3.0}Pro_{1.1}Gly_{3.1}$-$Ala_{1.9}Val_{2.9}Leu_{3.8}Phe_{2.9}$ (cysteine and tyrosine were not determined), in excellent agreement with the theoretically expected values for B chain. Since B chain was not added to the original incubation mixture, its presence indicates that insulin is split under these conditions. The insulin activity recovered from the insulin I and II fractions was only about 50% of the insulin originally added to the incubation mixture.

A more dramatic effect was observed when the A-SSO_3^- used for incubation with insulin under the conditions described previously was reduced with dithiothreitol (see Materials and Methods). Figure 11-II depicts the chromatographic pattern obtained from such an experiment. Insulin appears exclusively as in-

TABLE VI: Amino Acid Analysis[a] of Acid Hydrolysates of Peaks a–c Obtained from the CM-Cellulose Chromatography with an Exponential Sodium Chloride Gradient.

Amino Acid	Bovine A Chain Theory	Peak a Found	Bovine B Chain Theory	Peak b Found	Bovine Insulin Theory	Peak c Found
Lysine			1	1.0	1	0.9
Histidine			2	2.0	2	1.9
Arginine			1	1.0	1	1.0
Aspartic acid	2	2.0	1	1.0	3	3.0
Threonine			1	0.8	1	1.0
Serine	2	1.8	1	0.9	3	2.8
Glutamic acid	4	4.1	3	3.2	7	6.9
Proline		tr	1	0.9	1	0.8
Glycine	1	1.1	3	3.0	4	4.0
Alanine	1	1.0	2	2.1	3	3.0
Valine	2	1.6	3	3.1	5	4.8
Isoleucine	1	0.7			1	0.8
Leucine	2	2.0	4	4.0	6	6.1
Tyrosine	2	*b*	2	*b*	4	*b*
Phenylalanine			3	2.9	3	3.0
Half-cystine	4	*b*	2	*b*	6	*b*

[a] Data given as number of amino acid residues per molecule. [b] Not determined.

sulin I and from the 8 mg of insulin used in this experiment only 25% was recovered.

Isolation of Insulin through Chromatography on CM-Cellulose with a Sodium Chloride Gradient

Zinc insulin (2 mg) was dissolved in 2 ml of acetate buffer (pH 3.3; Na^+, 0.04 M), placed on a 0.9 × 23 cm CM-cellulose column, and eluted with an exponential NaCl gradient (see Materials and Methods). Figure 12-I presents the chromatographic pattern obtained. The effluent containing the insulin was concentrated in a rotary evaporator to approximately 10 ml and mixed with an equal volume of saturated picric acid solution. The insulin picrate formed was converted to the hydrochloride and subsequently was crystallized. The over-all recovery of insulin at the hydrochloride stage was 80–85% and at the crystalline stage 70–75% of theory. Recombination mixtures to be chromatographed by this technique were treated as follows. To the reaction mixture (approximately 10 ml) glacial acetic acid was added to give a 10% final concentration (v/v) and the resulting solution was mixed with an equal volume of saturated picric acid solution. The entire procedure was carried out at 2°. After 24 hr at 2° the precipitate was collected by centrifugation and washed twice with half-saturated picric acid solution. This solid was dissolved in a few drops of acetone–water (4:1) and the resulting solution was mixed with 40 ml of ice-cold dry acetone containing 4 drops of concentrated hydrochloric acid. A white precipitate was formed immediately. After 2 hr in the cold the precipitate was collected by centrifugation and washed once with dry acetone and once with dry ether. A suspension of the precipitate in 3 ml of acetate buffer (pH 3.3; Na^+, 0.04 M) was stirred vigorously for a few minutes and the remaining insoluble material was removed by centrifugation and washed with 2 ml of the same buffer. The combined supernatants were placed on the 0.9 × 23 cm CM-cellulose column. Figure 12-II illustrates the chromatographic pattern obtained. Dialysis and lyophilization of the fractions under the first peak (peak a, 14–40 ml of effluent) yielded a material which did not possess any insulin activity as judged by the mouse convulsion method and which, upon amino acid analysis, showed an amino acid composition corresponding to that of the A chain (Table VI). The material present in the effluent under peak b was converted in the usual way to picrate and finally to the hydrochloride. Amino acid analysis of an acid hydrolysate of this product indicated a composition corresponding to that of the B chain (Table VI). Rechromatography on the 2 × 100 cm CM-cellulose column with urea–acetate buffer gave the pattern shown in Figure 13-I, indicating that this material is eluted at the same position where the previously described peak 6 was located (see Figure 2). The last component of the chromatogram (peak c) which was eluted at the same position as natural insulin (Figure 12-I) was isolated as the hydrochloride *via* picrate and found to be identical with the natural hormone. Its specific activity ranged from 23 to 25 IU/mg when assayed by the mouse convulsion method. Amino acid analysis after acid hydrolysis showed a composition in molar ratios in excellent agreement with the theoretically expected values for bovine insulin (Table VI). On thin layer electrophoresis

in 0.5 N acetic acid it behaved as a single component and exhibited the same mobility as bovine insulin. Finally, this regenerated insulin was readily crystallized by Randall's (1964) or Epstein and Anfinsen's (1963) method. The shape and specific activity of the crystalline material was identical with that of natural bovine zinc insulin. Rechromatography of the regenerated insulin hydrochloride on 2 × 100 cm CM-cellulose column, with urea–acetate buffer, gave the chromatogram shown in Figure 13-II indicating that this insulin was eluted at the same position as the previously described insulin II (Figure 2).

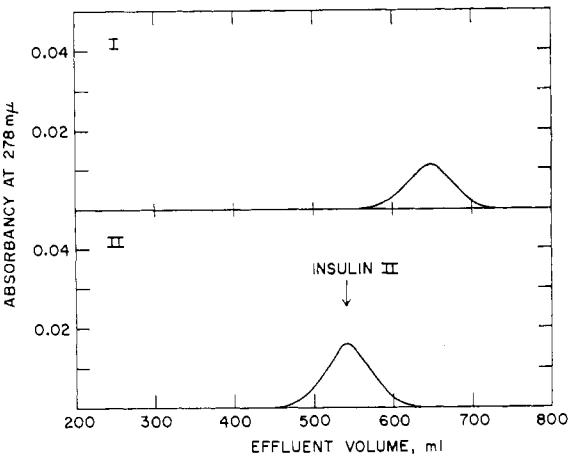

FIGURE 13: Rechromatography of peaks b (I) and c (II) recovered from the corresponding peaks of Figure 12-II on a 2 × 100 cm CM-cellulose column with urea–acetate buffer.

Discussion

The part of this investigation described in the present communication has dealt with three phases of the problem of insulin isolation: (1) to separate and possibly identify the products formed by recombination of A and B chains of insulin; (2) to study possible interactions of the reactants and products of the recombination reaction and their effect on insulin; and (3) to establish procedures for isolation of insulin from recombination mixtures of its individual chains and find the optimum conditions required for a most effective application of these procedures.

Chromatography of the recombination mixture of the A and B chains of insulin on a CM-cellulose column with urea–acetate buffer showed the presence of six major components (Figure 2). The first two components (peaks 1 and 2) have an amino acid composition corresponding to the A chain whereas the components corresponding to peaks 3 and 6 have an amino acid composition comparable to that of the B chain. These A and B chain components do not possess any insulin activity. The remaining two components, peaks 4 and 5, were found to possess insulin activity and were tentatively designated as insulins I and II, respectively. Insulin II was identical with the natural hormone with respect to amino acid composition, specific activity, electrophoretic mobility on thin layer electrophoresis, and chromatographic mobility on CM-cellulose columns eluted with urea–acetate buffer or with an exponential NaCl gradient and infrared spectrum. Insulin I, which is eluted very close to the position where B chain components emerge on the 2 × 100 cm CM-cellulose column, has an amino acid composition comparable to that of the natural insulin. However, this insulin, because of its location on the chromatogram, was often contaminated with varying amounts of B chain, and, therefore, its potency ranged from 16 to 22 IU/mg.

The relative amounts of insulins I and II depend greatly on the method used for recombining the A and B chains and on the processing of the recombination mixture prior to the CM-cellulose chromatography. Thus, when the conversion of the A-SSO_3^- to its sulfhydryl form was carried out with dithiothreitol and the recombination mixture was directly lyophilized, insulin I was the sole insulin present in the chromatogram (Figure 5-III). However, when this recombination mixture was subjected to the picric acid treatment instead of lyophilization, the insulin recovered upon chromatography was almost exclusively insulin II (Figure 5-IV). Similarly, picric acid treatment of recombination mixtures, performed *via* the 2-mercaptoethanol route, gave, upon chromatography, mainly insulin II (Figure 4-II). On the other hand, both insulins I and II were present in the chromatogram obtained when such recombination mixtures were dialyzed and/or directly lyophilized (Figures 2 and 4-I). It was further observed that after Sephadex chromatography for removing the urea, the hydrochloride of insulin I obtained *via* the picrate route, upon rechromatography on the CM-cellulose column with urea–acetate buffer, emerged at the position of insulin II (Figure 6). Similarly, when the hydrochloride obtained from insulin I by the same route was chromatographed on a CM-cellulose column with an exponential NaCl gradient, it was also eluted at the same position where natural insulin emerges in this chromatographic system (Figure 7). The insulin obtained upon rechromatography of insulin I on the aforementioned CM-cellulose columns was compared with natural insulin as to potency, amino acid analysis, and crystalline form. In all these comparisons, both insulins exhibited an identical behavior. The implication, therefore, arises that insulin I, after its separation from the other components of the combination mixture by CM-cellulose chromatography with urea–acetate buffer and its subsequent isolation through Sephadex chromatography and hydrochloride formation *via* picrate, is converted to insulin II.

These results prompted us to study the effects of the conditions prevailing both during the recombination reaction and the isolation of the regenerated insulin upon the natural hormone. Of special importance was the study of the behavior of natural insulin under the conditions that favor insulin I formation. The data obtained from such studies indicate that natural insulin is indeed converted to some extent to insulin I

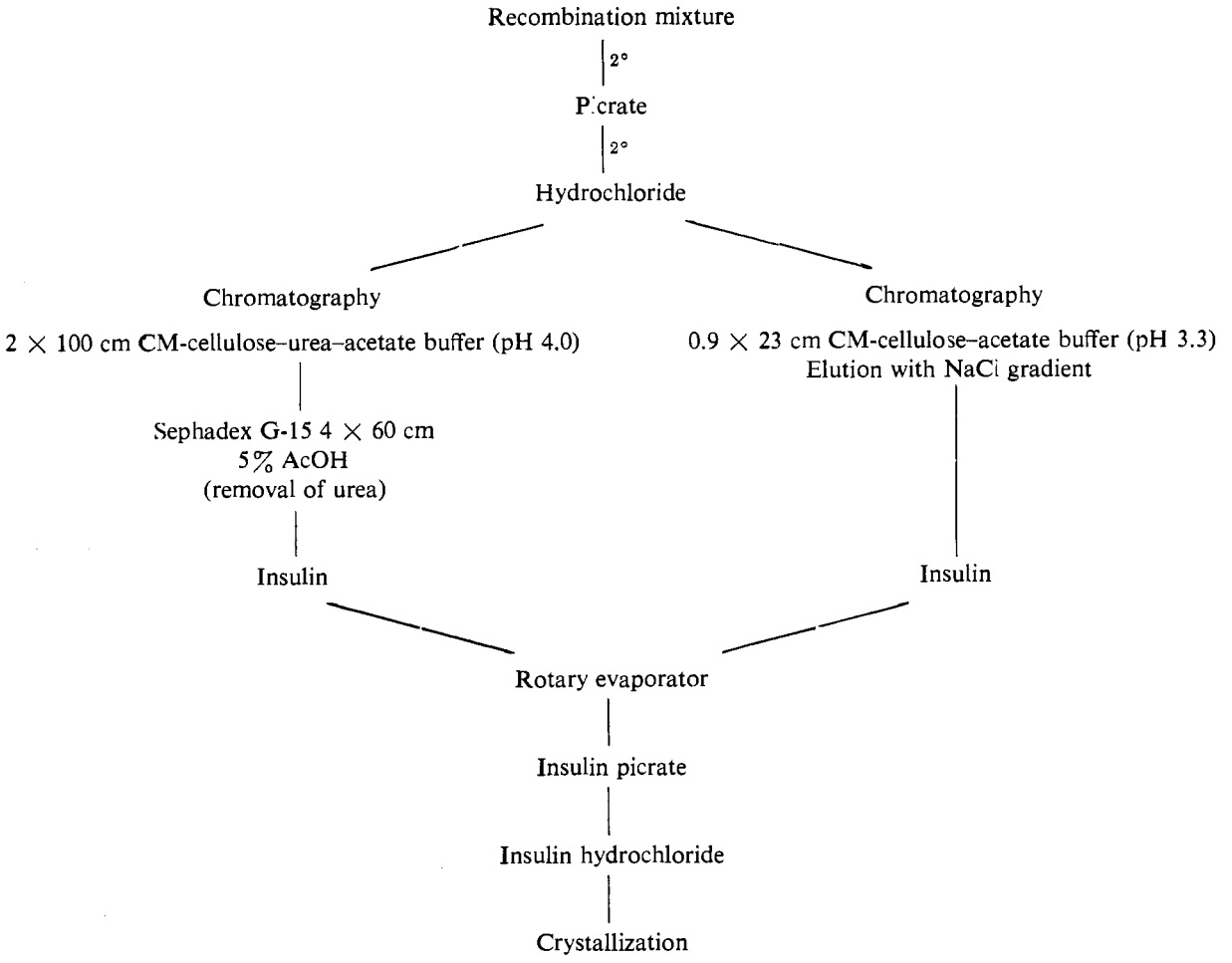

FIGURE 14.

and that the over-all recovery of insulin activity as insulins I and II was only about 50% of the original. It is, therefore, apparent that insulins I and II are closely related and under certain conditions are interconverted. The nature of insulin I and the mechanism underlying its interconversion with insulin II are not known.

Of more practical importance are the quantitative aspects of the chromatography of the recombination mixture on CM-cellulose with urea–acetate buffer. The over-all recovery of insulin activity (as insulins I and II), upon chromatography of a recombination mixture, which was directly lyophilized and then placed on the column, ranged from 30 to 50%. Of this amount of activity 8–25% was eluted as insulin I and 25–42% was eluted as insulin II (Table II). This over-all recovery was substantially lower than the recovery of natural insulin (86%) subjected to the same chromatographic processing. However, precipitation of insulin with picric acid from the recombination mixtures not only affords almost exclusively insulin II upon CM-cellulose chromatography, as was mentioned earlier, but also results in an over-all recovery of the hormone in yields ranging from 50 to 65% of theory.

These results show: (1) that undesirable interactions of the components of the recombination mixture do occur at acidic pH values which lead to insulin alterations or insulin destruction; and that (2) the picric acid treatment of the recombination mixture is the best way to avoid such interactions.

The CM-cellulose chromatography with urea–acetate buffer constitutes an efficient technique for the separation of the components of a recombination mixture and for the study of their interactions. Furthermore, a combination of this technique with the picric acid treatment provides an efficient method for the isolation of insulin from such mixtures. A simpler isolation procedure, which avoids the urea buffer and the additional step of removing the urea, is chromatography of the picric acid treated recombination mixture on CM-cellulose column with an exponential NaCl gradient. A similar chromatographic system has been described as a step of a method for the isolation of insulin from natural sources (Smith, 1964).

Chromatography of the picric acid treated recombination mixture on a CM-cellulose column with an exponential NaCl gradient effectively separated the regenerated insulin and in yields ranging from 50 to 65% of theory (Figure 12-II). The regenerated insulin was

eluted at the same position as natural bovine insulin and it was identical with the natural hormone in respect to amino acid composition, specific activity, electrophoretic mobility on thin layer electrophoresis, and crystalline form. Chromatography of the insulin thus isolated on the CM-cellulose column with urea–acetate buffer showed a single peak located at the same position as the previously described insulin II (Figure 13-II). A schematic presentation of the two isolation procedures established by the present investigation is shown in Figure 14.

The CM-cellulose chromatographic procedure using the NaCl gradient was successfully applied for the isolation of synthetic insulins (Katsoyannis *et al.*, 1967b) and is being used currently in our laboratory for the isolation of insulin analogs.

Acknowledgments

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