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Studies on the Synthesis of Insulin from Natural and Synthetic A and B Chains. I. Splitting of Insulin and Isolation of the S-Sulfonated Derivatives of the A and B Chains*

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ABSTRACT: The S-sulfonated derivatives of the A and B chains (A-SSO_3^- and B-SSO_3^-) of bovine and porcine insulins were prepared by sulfitolysis of the respective natural hormones. The separation and purification of the so-produced A-SSO_3^- and B-SSO_3^- were accomplished by three different methods: (a) continuous-flow electrophoresis, (b) chromatography on carboxymethylcellulose columns, and (c) chromatography on aminoethylcellulose columns. Identical material was obtained by all three methods as judged by amino acid analyses of acid and enzymatic hydrolysates, paper and thin layer electrophoresis, and infrared spectroscopic analysis. The over-all yield of the purified chains, based on the amount of insulin sulfitolyzed, was 60–70% for the A chain and 60–73% for the B chain. Amino acid analyses of acid hydrolysates of the purified chains gave data in excellent agreement with the theoretically expected values. On paper and thin layer electrophoresis at two pH values, the isolated chains exhibited single Pauly-positive spots. Complete digestion of the A-SSO_3^- by leucine aminopeptidase and of the B-SSO_3^- by aminopeptidase M indicated that the stereochemical homogeneity of the chains was preserved during the preparation and isolation processes. The A-SSO_3^- and B-SSO_3^- thus prepared and the products obtained by reduction of each chain followed by air oxidation were devoid of insulin activity as judged by the mouse convulsion assay method.

The molecule of insulin is composed of two polypeptide chains, A and B, linked together by two disulfide bridges. In addition, there is an intrachain disulfide bond in the A chain (Sanger and Tuppy, 1951a,b; Sanger and Thompson 1953a,b). Upon treatment of insulin with sodium sulfite in the presence of a mild oxidizing agent (sulfitolysis), its disulfide bonds are broken and the A and B chains are converted to the corresponding S-sulfonated derivatives (Swan, 1957; Bailey and Cole, 1959). The separation and purification of the S-sulfonated derivatives of the A and B (A-SSO_3^-, B-SSO_3^-)[1] chains can be accomplished by ion-exchange chromatography (Dixon and Wardlaw, 1960; Du *et al.*, 1961) and gel filtration (Varandani, 1966). The separated chains can, under certain conditions, recombine and regenerate insulin (Dixon and Wardlaw, 1960; Du *et al.*, 1961; Katsoyannis and Tometsko, 1966; Zahn *et al.*, 1966).

Systematic studies in this laboratory for the past seven years resulted in the synthesis of the A and B chains of sheep and human insulins and subsequently in the synthesis of these insulins by combination of the corresponding synthetic chains (Katsoyannis, 1964a,b, 1966a,b; Katsoyannis *et al.*, 1963, 1967a,b). From the

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

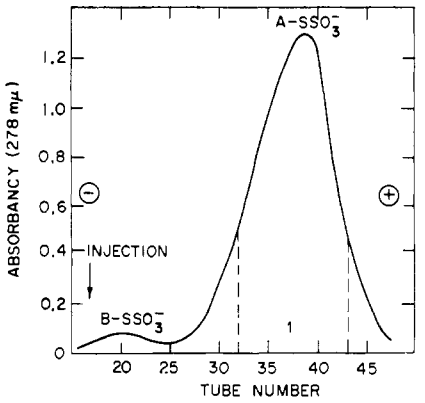

FIGURE 1: Continuous-flow electrophoresis of a preparation enriched in A-SSO_3^-. This preparation (150 mg) was placed on the continuous-flow separator (2400 v and 100–130 ma) with a dosing rate of 3.2 ml/hr and a flow rate of the supporting buffer of 150 ml/hr. Tubes within the dotted lines were pooled and lyophilized to give 94 mg of A-SSO_3^-. The amount of B-SSO_3^- obtained from the corresponding peak was 6 mg.

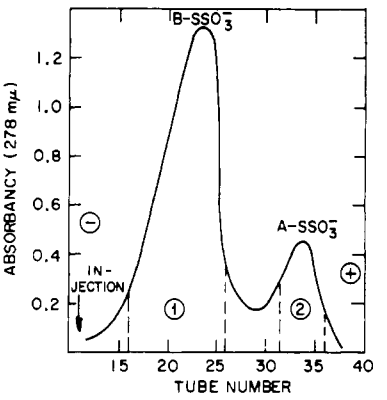

FIGURE 2: Continuous-flow electrophoresis of a preparation enriched in B-SSO_3^-. This preparation (150 mg) was placed on the continuous-flow separator under the conditions described in Figure 1. After lyophilization 98 mg of B-SSO_3^- and 15 mg of A-SSO_3^- were obtained.

early stages of this work, it became apparent that substantial amounts of natural A-SSO_3^- and B-SSO_3^- were necessary for comparison with the synthetic chains and for studying the recombination reaction. The present investigation was undertaken to establish efficient methods for the sulfitolysis of insulin and the separation of the A-SSO_3^- and B-SSO_3^- in pure chemical and stereochemical form.

Experimental Procedures and Results

Materials. Preswollen microgranular CM-cellulose (Whatman CM52/1), AE-cellulose (Whatman AE-11,186), and Sephadex G-15 (Pharmacia Uppsala) were used in this investigation. Crystalline LAP was purchased from Worthington and aminopeptidase M from Henley and Co., New York, N. Y. Crystalline bovine and porcine zinc insulins were generously provided by Eli Lilly and Co.

Preparation of the S-Sulfonated Derivatives of the A and B Chains of Bovine Insulin. To a solution of crystalline bovine zinc insulin (1 g) in 100 ml of 8 M guanidine hydrochloride were added 3.0 g of sodium sulfite and 1.4 g of freshly prepared sodium tetrathionate (Gilman *et al.*, 1946). This solution was adjusted to pH 8.9–9.2 with concentrated NH_4OH, stirred for 24 hr at room temperature, and then placed in $^{13}/_{32}$ Visking dialysis tubing and dialyzed at 4° for 24 hr against 16 l. of distilled water (four 4-l. portions). During the dialysis a white precipitate was formed. The dialysate was transferred to a centrifuge tube and after the pH was adjusted to 5.0 with dilute acetic acid the precipitate was separated by centrifugation. This precipitate was suspended in water and lyophilized to a fluffy white material (560 mg) which contains predominantly B-SSO_3^-. The supernatant fluid, upon lyophilization, gave 420 mg of fluffy white material composed mostly of A-SSO_3^-. Throughout this paper, these preparations of the crude chains are referred to as enriched B-SSO_3^- and ASSO_3^-, respectively. Purification of the crude chains was accomplished either by continuous-flow electrophoresis (Model "FF," Brinkmann Instruments) or by column chromatography.

Isolation of A-SSO_3^- and B-SSO_3^- by Continuous-Flow Electrophoresis. The separation and purification of the S-sulfonated chains were accomplished with a continuous-flow separator at 2400 v, 100–130 ma, and 5° using as a supporting buffer a 0.12% NH_4HCO_3 solution adjusted to pH 7.8 with concentrated NH_4OH. A solution of 0.315% NH_4HCO_3 was used as the electrode rinse. The lyophilized preparation enriched in A-SSO_3^- (or B-SSO_3^-) was suspended in 9.5 ml of supporting buffer, brought into solution by adjusting the pH to 10 with concentrated NH_4OH, and then placed in the continuous-flow separator. The absorbancy of the collected effluent was measured at 278 mμ in a Beckman DU spectrophotometer. Figure 1 shows the electrophoretic profile obtained with the preparation enriched in A-SSO_3^-, while Figure 2 illustrates the electrophoretic pattern of the preparation enriched in B-SSO_3^-. The tubes containing the A-SSO_3^- or B-SSO_3^- were then pooled and lyophilized to yield the S-sulfonated chains as white fluffy materials. Based on the amount of insulin used, the purified A and B chains were obtained in 70 and 63% over-all yields, respectively.

Isolation of A-SSO_3^- and B-SSO_3^- by Column Chromatography on CM-cellulose. The separation of the A-SSO_3^- and B-SSO_3^- was accomplished with a 4 × 60 cm CM-cellulose column. 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 resin was subsequently suspended in water and allowed to settle.

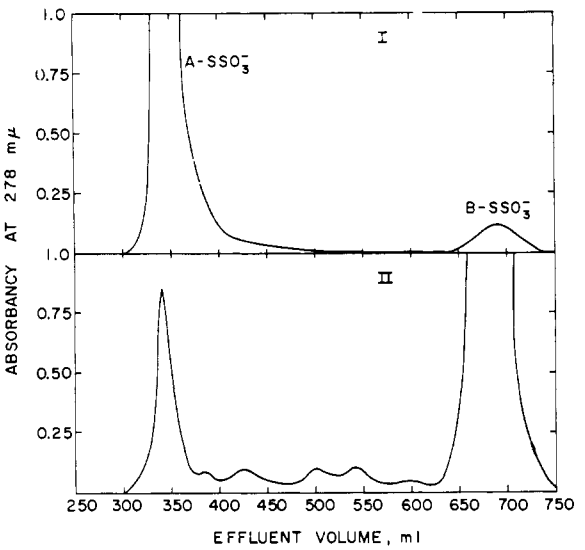

FIGURE 3: Separation of A-SSO_3^- and B-SSO_3^- by chromatography on a 4 × 60 cm CM-cellulose column with urea–acetate buffer (pH 4.0). The column effluent was monitored by a Zeiss recording spectrophotometer. (I) A preparation (125 mg) enriched in A-SSO_3^- was placed on the column. A-SSO_3^- (60 mg) was recovered. No attempt was made to isolate the B-SSO_3^-. (II) A preparation (150 mg) enriched in B-SSO_3^- was placed on the column. B-SSO_3^- (100 mg) and 26 mg of A-SSO_3^- were recovered.

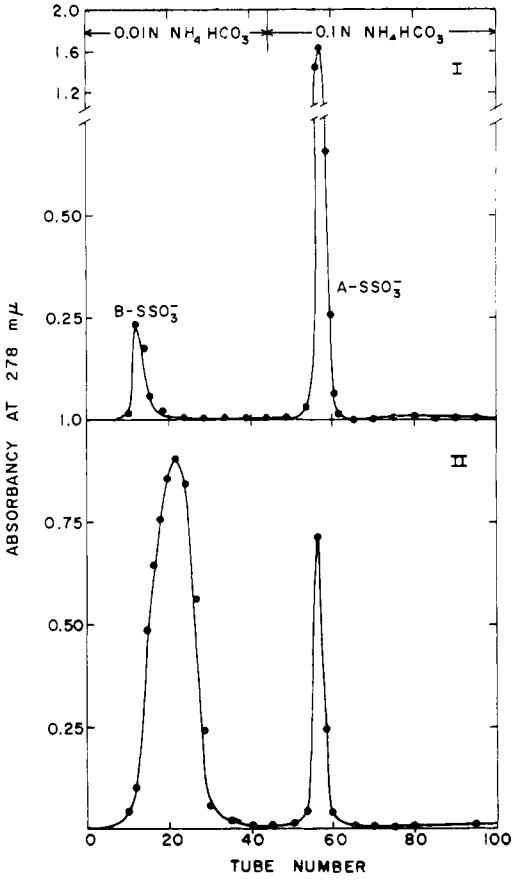

FIGURE 4: Separation of A-SSO_3^- and B-SSO_3^- by chromatography on a 1.7 × 42 cm AE-cellulose column with ammonium bicarbonate buffer. Fractions of 7 ml each were collected at a flow rate of 18 ml/hr. (I) A suspension of 70 mg of a preparation enriched in A-SSO_3^- in 2 ml of water was solubilized by adjusting the pH to 9.7 with 1 N NH_4OH. The solution was adjusted to pH 9.0 with 1 N CH_3COOH and chromatographed. A-SSO_3^- (40 mg) was recovered. No attempt was made to isolate the B-SSO_3^-. (II) A preparation (115 mg) enriched in B-SSO_3^- was dissolved in 2 ml of 0.01 N NH_4HCO_3 (pH 9.0); this solution was adjusted to pH 9.0 with 1 N NH_4OH and placed on the column. B-SSO_3^- (92 mg) and 12 mg of A-SSO_3^- were recovered.

Fines were removed by decanting the supernatant fluid and this process was repeated four to five times. The water 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–urea buffer prepared as follows. Urea and sodium acetate were dissolved in water and the pH of the solution was adjusted to 4.4 with glacial acetic acid. This solution was treated with Norit, filtered through a filter paper layered with Celite, and brought to pH 4.0 and to the final volume by adding water and glacial acetic acid. The amounts of urea and sodium acetate were calculated so that their concentrations in the final solution were 8 and 0.04 M, respectively. Freshly prepared buffers were always used. 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 buffer. During chromatography, the effluent was monitored with a Zeiss recording spectrophotometer. The lyophilized preparation enriched in A-SSO_3^- (or B-SSO_3^-) was dissolved in 4–5 ml of urea–acetate buffer and placed on the 4 × 60 cm CM-cellulose column. Figure 3-I shows the chromatogram obtained with the enriched A-SSO_3^- preparation, whereas Figure 3-II illustrates the chromatographic pattern of the preparation enriched in B-SSO_3^-. A-SSO_3^- was eluted between 300 and 375 ml while the B-SSO_3^- was eluted between 625 and 725 ml of effluent. For removing the urea, the fractions containing the A-SSO_3^- and B-SSO_3^- were titrated with concentrated HCl to pH 3.0 and then placed on a 4 × 60 cm G-15 Sephadex column equilibrated with 5% (v/v) acetic acid. This column was made as follows. Sephadex G-15 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 with a Gilford recording spectrophotometer. The pooled fractions containing the A-SSO_3^- were

TABLE I: Amino Acid Analyses[a] of Acid and Enzymatic Hydrolysates of Bovine A-SSO_3^-.

Amino Acid	Acid Hydrolysis		Enzymatic Hydrolysis (LAP)	
	Theory	Found	Theory	Found
Aspartic acid	2	2.0		
Glutamine			2	Emerge at the same position; not determined
Asparagine			2	
Serine	2	1.8	2	
Glutamic acid	4	4.1	2	2.0
Glycine	1	1.0	1	1.0
Alanine	1	1.0	1	1.0
Valine	2	1.7	2	2.0
Isoleucine	1	0.7	1	1.0
Leucine	2	2.0	2	2.0
Tyrosine	2	*b*	2	2.0
Cysteine	4	*b*	4	*b*

[a] Number of amino acid residues per molecule. [b] Not determined.

TABLE II: Amino Acid Analyses[a] of Acid and Enzymatic Hydrolysates of Bovine B-SSO_3^-.

Amino Acid	Acid Hydrolysis		Enzymatic Hydrolysis (aminopeptidase M)	
	Theory	Found	Theory	Found
Lysine	1	1.0	1	1.2
Histidine	2	2.0	2	2.1
Arginine	1	1.0	1	1.1
Aspartic acid	1	1.0		
Glutamine			1	Emerge at the same position; not determined
Asparagine			1	
Serine	1	0.9	1	0.8[b]
Threonine	1	0.9	1	1.1
Glutamic acid	3	3.1	2	1.5
Proline	1	1.2	1	0.8
Glycine	3	3.0	3	3.1
Alanine	2	2.0	2	1.9
Valine	3	3.0	3	3.2
Leucine	4	3.9	4	4.2
Tyrosine	2	*c*	2	2.1
Phenylalanine	3	3.0	3	3.1
Cysteine	2	*c*	2	*c*

[a] Number of amino acid residues per molecule. [b] Separated from glutamine and asparagine in a 30° chromatographic run. [c] Not determined.

concentrated in a rotary evaporator (30°) to a small volume, transferred to $^{18}/_{32}$ Visking dialysis tubing, and dialyzed against 4 l. of water at 2°. Upon lyophilization of the dialysate, the A-SSO_3^- was obtained as a white fluffy material. The fractions containing the B-SSO_3^- were pooled, concentrated in a rotary evaporator to 10 ml, and mixed with 12 ml of saturated picric acid solution. After 24 hr at 2° the precipitated picrate of the B-SSO_3^- was isolated by centrifugation and washed with half-saturated picric acid solution. This precipitate was dissolved in 2 ml of 0.05 M NH_4HCO_3 containing a few drops of 1 N NH_4OH and placed on a 1.2 × 50 cm Sephadex G-15 column equilibrated with 0.05 M NH_4HCO_3. The chromatogram was developed with the same solvent and the effluent of the column was monitored with a Zeiss recording spectrophotometer. Under these conditions the B-SSO_3^- was separated completely from the picric acid salts which trav-

TABLE III: Amino Acid Analyses[a] of Acid Hydrolysates of Porcine A-SSO_3^- and B-SSO_3^-.

Amino Acid	A-SSO_3^-		B-SSO_3^-	
	Theory	Found	Theory	Found
Lysine			1	0.9
Histidine			2	1.9
Arginine			1	0.9
Aspartic acid	2	2.0	1	1.0
Threonine	1	0.9	1	0.9
Serine	2	1.7	1	0.9
Glutamic acid	4	4.1	3	3.1
Proline			1	1.0
Glycine	1	1.0	3	3.1
Alanine			2	2.0
Valine	1	0.8	3	3.0
Isoleucine	2	1.7		
Leucine	2	2.0	4	4.0
Tyrosine	2	*b*	2	*b*
Phenylalanine			3	3.0
Cysteine	4	*b*	2	*b*

[a] Number of amino acid residues per molecule. [b] Not determined.

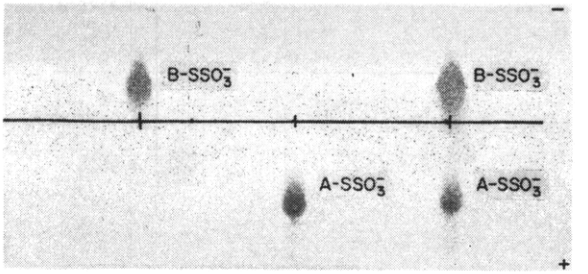

FIGURE 5: High-voltage paper electrophoresis of A-SSO_3^- and B-SSO_3^- and of a mixture of these chains; pyridine–acetate–8 M urea buffer, pH 4.2, 1700 v, 2 hr.

elled extremely slowly on this column. The B-SSO_3^- was recovered as a white fluffy material upon lyophilization of the effluent. The over-all yield of purified chains thus obtained, based on the amount of insulin used, was 65% for the $ASSO_3^-$ and 60% for the B-SSO_3^-.

Isolation of the A-SSO_3^- and B-SSO_3^- by Column Chromatography on AE-cellulose. The separation of the S-sulfonated derivatives of the A and B chains was accomplished with a 1.7 × 42 cm AE-cellulose column. AE-cellulose was suspended in 0.5 N HCl, stirred gently for 30 min, filtered, and washed with water. The resin was subsequently suspended in 0.5 N NaOH, stirred gently for 30 min, filtered, and washed with water. The cellulose was then suspended in 0.01 N NH_4HCO_3 which was adjusted to pH 9.0 with NH_4OH, homogenized for 30 sec in a Waring Blendor, and allowed to settle. Fines were removed by decanting the supernatant fluid and this process was repeated four to five times. The ion exchanger was then poured into the column as a thick slurry in 0.01 N NH_4HCO_3 (pH 9.0). A suspension of the lyophilized preparation enriched in A-SSO_3^- in water was brought to pH 9.7 with 1 N NH_4OH. The resulting solution was adjusted to pH 9.0 with 1 N acetic acid and placed on the column. For the stepwise elution of the B-SSO_3^- and the A-SSO_3^- 0.01 N NH_4HCO_3 (pH 9.0) and 0.1 N NH_4HCO_3 (pH 9.0) were used, respectively, and Figure 4-I illustrates the chromatographic pattern obtained. The lyophilized preparation enriched in B-SSO_3^- was suspended in 0.01 N NH_4HCO_3 (pH 9.0) and the solution obtained after adjusting the pH to 9.0 with 1 N NH_4OH was placed on the AE-cellulose column. The stepwise elution of the chain material was accomplished as described previously and the chromatographic pattern obtained is shown in Figure 4-II. The A-SSO_3^- and B-SSO_3^- were finally obtained as white fluffy powders by lyophilizing the respective pooled fractions containing the chain material. Based on the amount of insulin used, the purified A and B chains were obtained in 60 and 73% over-all yields, respectively.

Criteria of Chemical and Stereochemical Homogeneity of the Separated Chains. Identical material was obtained by all three separation procedures as judged by amino acid analysis, paper electrophoresis, LAP and aminopeptidase M digestibility, and infrared spectroscopic analysis.

Amino acid analyses of the purified chains were performed according to the method of Spackman *et al.* (1958) in a Beckman-Spinco amino acid analyzer, Model 120B, equipped with a digital readout system (Model CRS-10AB, Infotronics Corp., Houston, Texas). Acid hydroysis was performed in constant-boiling HCl under nitrogen at 108° for 24 hr. To calculate the molar ratios given in this and the following two communications, the average micromoles of glutamic acid, glycine, and leucine found were assumed to be equal to the theoretical number of residues in accordance with the known number of each of these residues in the compound to be analyzed. The molar ratios obtained are shown in Tables I–III. Incubation of the A-SSO_3^- with LAP according to the method of Hill and Smith (1957) and of the B-SSO_3^- with aminopeptidase M by the method of Pfleiderer *et al.* (1964) gave the amino acid composition expressed in molar ratios shown in Tables I–III.

On paper and thin layer electrophoresis at two pH values, the A-SSO_3^- and B-SSO_3^- exhibited single Pauly-positive spots (Figures 5–8).

The infrared spectra of the S-sulfonated chains are shown in Figure 9. For taking the 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 lb 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°.

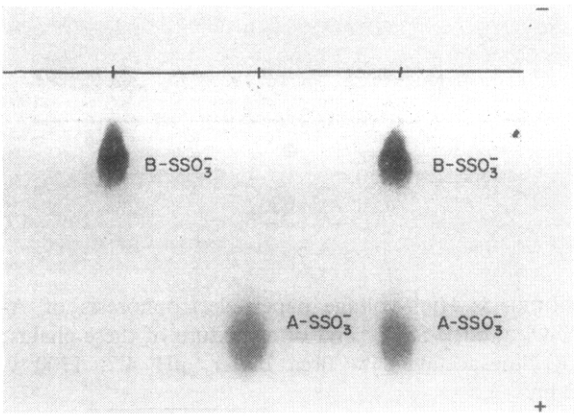

FIGURE 6: High-voltage paper electrophoresis of A-SSO_3^- and B-SSO_3^- and of a mixture of these chains; Tris buffer, pH 9.0, 1800 v, 1 hr.

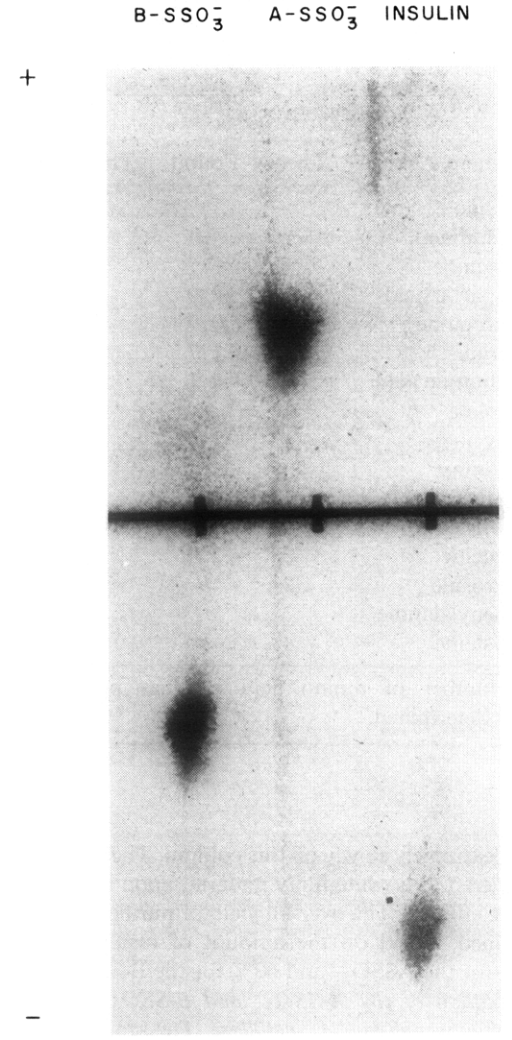

FIGURE 7: Thin layer electrophoresis of A-SSO_3^-, B-SSO_3^-, and bovine insulin; 0.5 N acetic acid, 3400 v, 12 min.

Biological Activities of the A and B Chains. The A-SSO_3^- and B-SSO_3^- are devoid of insulin activity as judged by the mouse convulsion assay method. This assay was carried out as has been described previously (Katsoyannis and Tometsko, 1966; Katsoyannis *et al.*, 1967a). Injections of the individual chains in doses up to 350 μg/mouse did not cause any convulsions. Similarly, no insulin response was produced with products obtained by reduction of each S-sulfonated chain followed by air oxidation. Thus, when A-SSO_3^- and B-SSO_3^- were reduced and subsequently air oxidized, as described by Katsoyannis *et al.* (1967a), and injected into mice in doses up to 200 μg each, neither caused any convulsion. This observation is in agreement with data obtained by Du *et al.* (1961) and Pruitt *et al.* (1966).

Isolation of A-SSO_3^- and B-SSO_3^- from Porcine Insulin. Sulfitolysis of crystalline porcine zinc insulin was carried out as described previously for bovine insulin. The separation of the S-sulfonated chains was accomplished by continuous-flow electrophoresis under the same conditions used for the bovine insulin chains. Based on the amount of porcine insulin used, the overall yields of the purified A-SSO_3^- and B-SSO_3^- were 70 and 63%, respectively.

The same criteria used to establish chemical and stereochemical homogeneity of the bovine chains were also applied to assess the chemical and optical purity of the porcine chains.

Discussion

Procedures for splitting insulin by sulfitolysis and for the separation of the S-sulfonated A and B chains have been described previously (Dixon and Wardlaw 1960; Du *et al.*, 1961, 1965). However, these methods could not be used for the production of large amounts of chains that are not contaminated with each other. In the procedure of Dixon and Wardlaw, it is difficult to free B-SSO_3^- from traces of A-SSO_3^- due to coprecipitation, unless it is vigorously purified by ion-exchange chromatography or electrophoresis in the presence of 8 M urea (Dixon, 1964). On the other hand, in the procedure of Du *et al.* (1965) in spite of the fact that the B-SSO_3^- was isoelectrically precipitated ten times, it appears that it is contaminated with about 2% of A-SSO_3^-. Application of gel filtration for the separation, in a small scale, of the A-SSO_3^- and B-SSO_3^- has been reported recently (Varandani, 1966). In all three procedures no data regarding the stereochemical homogeneity of the chains are provided.

Any method designed for the preparation of the S-sulfonated derivatives of the A and B chains of insulin should fulfill the conditions. (a) It should afford complete separation of the A-SSO_3^- and B-SSO_3^-; (b) the stereochemical homogeneity of the chains should be preserved; and (c) the chains should be obtained in good yield. The methods for the preparation of the A-SSO_3^- and B-SSO_3^- reported in this investigation fulfill these

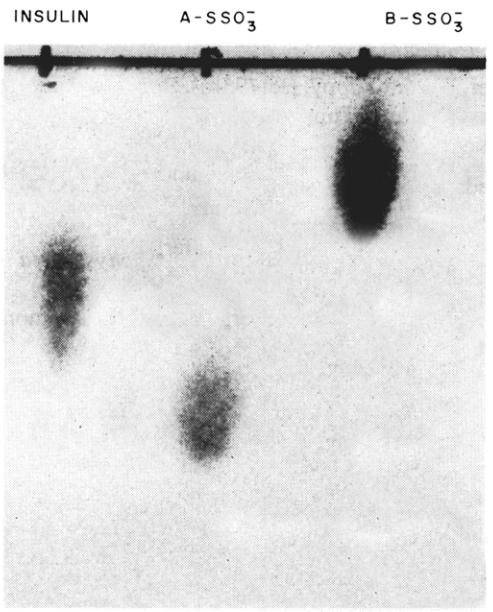

FIGURE 8: Thin layer electrophoresis of A-SSO_3^-, B-SSO_3^-, and bovine insulin; 0.01 N NH_4HCO_3 adjusted to pH 10.0 with NH_4OH, 2900 v, 13 min.

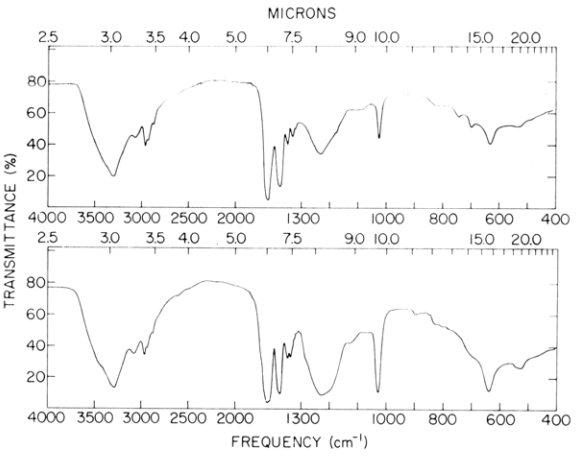

FIGURE 9: Infrared spectra of bovine B-SSO_3^- (top) and A-SSO_3^- (bottom).

conditions. Thus, amino acid analyses of acid hydrolysates of the purified chains gave amino acid compositions in molar ratios in excellent agreement with the theoretically expected values. Furthermore, amino acid residues characteristic for the one chain are completely absent in the hydrolysates of the other chain. On paper and thin layer electrophoresis at two pH values, the isolated chains behaved as homogenous components. The absence of insulin activity, as judged by the mouse convulsion method in the S-sulfonated chains, as well as in the reduced and oxidized products of these chains, provided further evidence that the chains were not contaminated with each other and with insulin. If the chains were contaminated with each other, to the extent of 2% under the conditions used for their reduction and oxidation, insulin would have been generated in measurable amounts (Du *et al.*, 1965).

Complete digestion of the A-SSO_3^- by LAP and of the B-SSO_3^- by aminopeptidase M, as judged by amino acid analysis of the digests, indicated that the stereochemical homogeneity of these chains was preserved during the isolation and purification processes.

It has been recently reported (Pruitt *et al.*, 1966) that none of the chain derivatives have a significant effect on the response of the assay systems to added insulin. Furthermore, in the present investigation, it was shown that A-SSO_3^- and B-SSO_3^- and their reduced and oxidized derivatives do not possess any insulin activity. However, interaction of these chains under conditions described in the subsequent publication (Katsoyannis *et al.*, 1967a) resulted in the regeneration of insulin as the exclusive product and in high yields. Consequently, the biological activity of a recombination mixture of the A and B chains is a reliable measure of the insulin formed by interaction of these chains.

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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*

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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,

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