Insulin Peptides. XXI. A Synthesis of the B Chain of Sheep (Bovine, Porcine) Insulin and Its Isolation as the S-Sulfonated Derivative[1]

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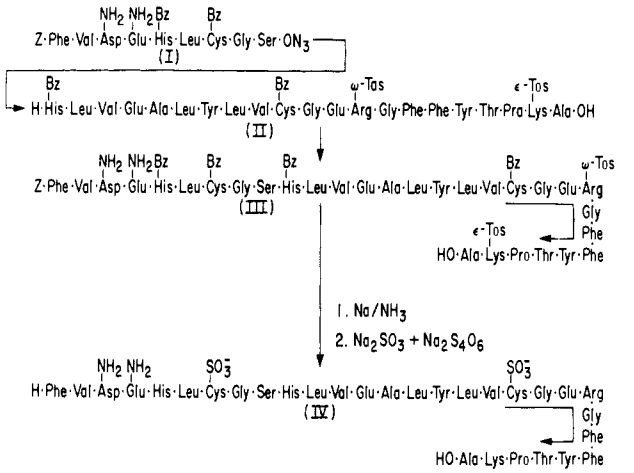
Abstract: The synthesis and isolation in a highly purified form of the S-sulfonated B chain of sheep insulin, which is identical with the B chain of bovine and porcine insulins, are described. This was accomplished by first preparing the partially protected triacontapeptide derivative that embodies the amino acid sequence of the insulin B chain and then removing the protecting groups and sulfitolyzing the ensuing product. The triacontapeptide derivative was prepared by the azide coupling of the N-terminal nonapeptide subunit with the C-terminal heneicosapeptide subunit. Removal of the protecting groups from the triacontapeptide derivative was accomplished upon treatment with sodium in liquid ammonia. Cleavage of the chain at the amino terminal side of the proline residue, a well-documented event which for all practical purposes takes place almost quantitatively during the sodium in liquid ammonia step, has been completely avoided. This was accomplished by carrying out the sodium–liquid ammonia reaction in the presence of sodium amide. Purification of the synthetic B-chain *S*-sulfonate was accomplished by chromatography on a CM-cellulose column with a urea–acetate buffer, pH 4.0. The chemical purity of the synthetic material was established by amino acid analysis of acid and enzymatic hydrolysates, by column chromatography, and by high-voltage thin-layer electrophoresis in two pH values. Its stereochemical homogeneity was established by complete digestion with aminopeptidase M. The synthetic B-chain *S*-sulfonate was compared with its natural counterpart as to amino acid composition, electrophoretic mobility on thin layer in two pH values, chromatographic pattern on CM-cellulose, and specific rotation. In all of these comparisons, the synthetic B-chain *S*-sulfonate and the natural B-chain *S*-sulfonate exhibited an identical behavior. This synthetic chain, as was already reported, upon combination with synthetic or natural A chain produced insulin identical with the naturally occurring protein.

In previous papers we have reported in detail the total synthesis of sheep insulin and its isolation in crystalline form.[2] In that synthesis the final step consisted of the interaction of the sulfhydryl form of the A chain with the S-sulfonated derivative of the B chain; the insulin thus produced was isolated in pure form, crystallized, and found to be identical with the natural protein by all criteria employed. The combination of the synthetic A and B chains to produce insulin and the isolation of the synthetic protein in crystalline form has been accomplished by procedures developed in this laboratory and already reported.[2-4] Similarly, a detailed account of the synthesis of the S-sulfonated form of the A chain and a brief account of the original synthesis of the S-sulfonated form of the B chain have also been recorded.[5,6] In the present paper we wish to give in detail experimental procedures for an improved synthesis of the S-sulfonated form of the B chain of sheep insulin and its isolation in highly purified form. The B chain of sheep insulin is identical with the B chain of bovine and porcine insulins.[7,8] This synthetic B chain was employed in the total synthesis of sheep insulin and in the preparation of half-synthetic bovine insulin as described previously.[2]

The synthesis of the S-sulfonated form of the B chain followed the pattern used in this laboratory for the synthesis of the insulin chains in that it involved the preparation of the protected form of the B chain III (Chart I),

Chart I



cleavage of the protecting groups with sodium in liquid ammonia, and conversion of the resulting sulfhydryl form of the B chain to its S-sulfonated derivative IV by oxidative sulfitolysis.[6,9]

(1) This work was supported by the National Institute for Arthritis and Metabolic Diseases, U. S. Public Health Service (AM 12925).
(2) P. G. Katsoyannis, A. C. Trakatellis, C. Zalut, S. Johnson, A. Tometsko, G. Schwartz, and J. Ginos, *Biochemistry*, **6**, 2656 (1967).
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The key intermediate, the protected triacontapeptide III, was prepared by the azide coupling of the C-terminal heneicosapeptide subunit II with an excess of the N-terminal nonapeptide fragment I. The latter compound was used with the imidazole moiety of the histidine residue at position 5 either free or protected with a benzyl group. Its synthesis by the stepwise method or by coupling the N-terminal pentapeptide subunit with the C-terminal tetrapeptide fragment was described in preceding papers.[6,10] The heneicosapeptide derivative II was prepared as described previously by the condensation of the C-terminal hexadecapeptide fragment with the N-terminal pentapeptide subunit.[11]

The protected nonapeptide azide I was prepared in the usual way from the corresponding hydrazide. As was the case with the peptide azides used throughout our synthetic work on insulin the stability of the protected azide I was studied by infrared spectroscopy.[5] This azide was stable if stored at 0° for several hours as was ascertained by the presence of the characteristic band at 4.75 μ. There was no evidence for the presence of an isocyanate band at 4.50 μ, the principal decomposition product of a peptide azide, or of an oxazolidone band at 5.70 μ, a probable decomposition product of serine azides.[12] The solid azide I was allowed to react with the partially protected heneicosapeptide II, which was obtained from its carbobenzoxy derivative upon treatment with hydrogen bromide in trifluoroacetic acid, to give the protected triacontapeptide III. The crude product was subsequently exposed to sodium in liquid ammonia[13] whereupon the carbobenzoxy, benzyl, and *p*-toluenesulfonyl protecting groups were removed.

The sodium in liquid ammonia treatment of the protected B chain, however, besides removing the aforementioned blocking groups, also caused cleavage of the chain between the threonine and proline residues at positions 27 and 28. The cleavage was for all practical purposes almost quantitative, as can be seen from the amino acid analysis of an acid hydrolysate of the deblocked chain (see Experimental Section). Indeed the recoveries of the amino acids proline, lysine, and alanine occupying the carboxyl terminal sequence of the B chain indicate that the C-terminal tripeptide sequence has been almost completely eliminated. This cleavage occurs even when the light blue color, indicating an excess of sodium at the end point of the sodium in liquid ammonia reaction, was allowed to persist for only 30 sec. The near-quantitative cleavage of the threonine–proline bond upon treatment of the insulin B chain with sodium in liquid ammonia was employed in our laboratory in the synthesis of an insulin analog which lacks the C-terminal tripeptide sequence from the B chain and which possesses the full biological activity of insulin.[14,15]

Cleavage of peptide bonds, and particularly those involving proline, by sodium in liquid ammonia has been observed by many investigators and it is well documented in the literature. Hofmann and Yajima, for example, originally reported cleavage of the lysyl–proline bond by the sodium in liquid ammonia deblocking of peptides during the synthesis of α-MSH derivatives.[16] Later Guttmann described cases in which lysyl–proline bonds or other acyl–proline bonds were cleaved at the N-terminal side of the proline residue, on exposure to sodium in liquid ammonia.[17] A comprehensive study of the behavior of proline containing peptides on exposure to sodium in liquid ammonia was carried out more recently by Benisek and Cole.[18,19] These investigators, using a variety of proline-containing peptides, including the B chain of insulin, and changing the conditions of the sodium in liquid ammonia treatment, reported cleavage of the acyl–proline bond at the N-terminal side of the proline residue ranging from 56 to 90%. Obviously, then, the use of the sodium in liquid ammonia procedure, under the usual conditions, for the synthesis of the B chain of insulin or other proline-containing polypeptides, is prohibitive. Consequently, reported syntheses of B chain and of insulin where sodium in liquid ammonia, under the standard conditions, was used as the deblocking method must be reevaluated. This is particularly true since insulin that lacks the C-terminal tripeptide sequence from the B chain behaves as natural insulin in many physical and biological properties.[14,15]

We were able to overcome the problems connected with the sodium in liquid ammonia reaction and we have prepared the S-sulfonated form of the B chain without any indication of cleavage.[2,6,9] This was accomplished by carrying out the sodium in liquid ammonia deblocking step in the presence of an approximately 50-fold *M* excess of sodium amide. Presumably the sodium amide, as a strong base, reacts with proton donors which are present or are formed during the sodium in liquid ammonia reaction and thus prevents the reductive cleavage of the threonyl–proline bond. It might be pointed out that Benisek, *et al.*,[19] found that sodium amide does not prevent the cleavage of *N*-acetyl-L-proline when the latter compound was exposed to sodium in liquid ammonia. In this case, however, only onefold *M* excess of sodium amide was used.

The deblocked material was dissolved in 8 *M* guanidine hydrochloride and subjected to oxidative sulfitolysis upon treatment with sodium sulfite and freshly prepared sodium tetrathionate at pH 8.9 upon which the sulfhydryl functions were converted to the *S*-sulfonates. All inorganic salts, guanidine hydrochloride, and low molecular weight by-products formed during the sodium in liquid ammonia reaction were removed by dialysis. During dialysis and particularly when the pH of the dialyzate was adjusted to 5.0, a heavy precipitate was formed. Amino acid analysis, after acid hydrolysis of the precipitated material, gave a composition in molar ratios similar to that theoretically expected for the B chain. When the whole dialyzate, including the pH 5.0 precipitated product, was lyophilized, the material obtained gave also after hydrolysis an amino acid com-

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(11) P. G. Katsoyannis, M. Tilak and K. Fukuda, *ibid.*, **93**, 5857 (1971).
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(16) K. Hofmann and H. Yajima, *J. Amer. Chem. Soc.*, **83**, 2289 (1961).
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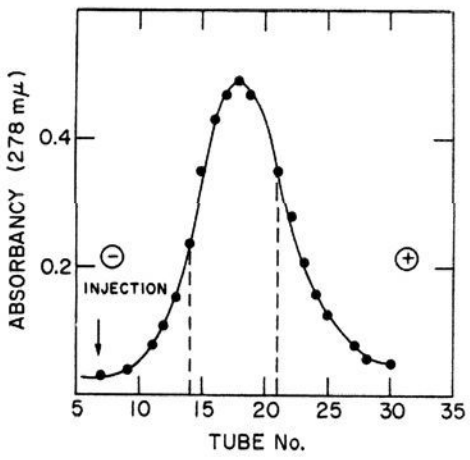

Figure 1. Continuous-flow electrophoresis of crude B-chain *S*-sulfonate. This preparation (140 mg) was placed on the continuous-flow separator (2400 V and 105–115 mA) with a dosing rate of 3.2 ml/hr and a flow rate of the supporting buffer of 150 ml/hr. Tubes within dotted lines were pooled and lyophilized to give 25 mg of material.

position, expressed in molar ratios, comparable to that theoretically expected for the B chain. Both the aforementioned materials, however, on thin-layer electrophoresis appeared to contain five components. Finally, comparable amounts of insulin were produced when each of the above-mentioned materials was combined with A chain according to the procedure reported previously from this laboratory.[3] It is therefore apparent that no purification of the synthetic S-sulfonated B chain is achieved by precipitation at pH 5.0 as was suggested by other investigators.[20] A partial purification of the crude B-chain *S*-sulfonate obtained by lyophilization of the aforementioned dialyzate was accomplished by continuous flow electrophoresis, a procedure that we have used previously for the isolation of natural B-chain *S*-sulfonate.[21] Figure 1 illustrates the electrophoretic pattern obtained with the crude B-chain *S*-sulfonate. The fractions corresponding to the positions at which the natural B-chain *S*-sulfonate was found to migrate under similar conditions were pooled and lyophilized. The material thus obtained after acid hydrolysis was found to have an amino acid composition expressed in molar ratios closely related to that theoretically expected for B-chain, but on thin-layer electrophoresis (Figure 2) was found to consist of three major components, one of which had the same mobility as natural B-chain *S*-sulfonate. This material produced twice as much insulin as the material obtained by precipitation at pH 5.0 upon combination with natural A chain. Isolation of the synthetic B-chain *S*-sulfonate in a highly purified form was finally accomplished by ion exchange chromatography of the crude material obtained by lyophilization of the previously mentioned dialyzate. For this purpose a CM-cellulose column with urea–acetate buffer at pH 4.0 as the eluting solvent was employed. This chromatographic system was used previously in our laboratory for the separation and purification of natural A- and B-chain *S*-sulfonates and for the isolation of regenerated insulins.[4,21] From the elution pattern of this column, shown in Figure 3-II, it is apparent that in the crude synthetic preparation at least four major components (1, 2, 3, and 4) are present and fairly well separated. For

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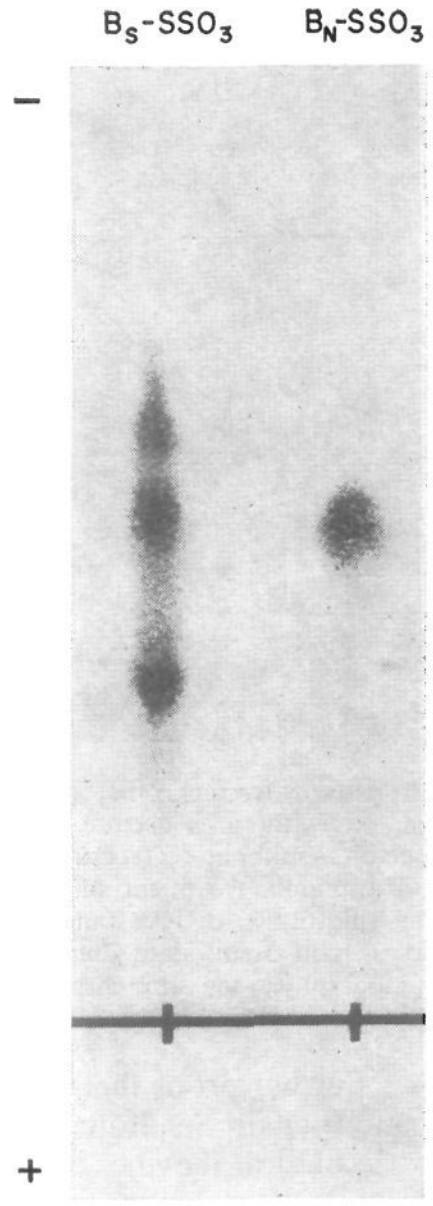

Figure 2. High-voltage thin-layer electrophoresis of natural bovine B-chain *S*-sulfonate (B_NSSO_3) and of the synthetic (B_SSSO_3) which was partially purified by continuous-flow electrophoresis: 1 *M* acetic acid, 3300 V, 20 mA, 30 min.

the isolation of each of these components from the urea containing chromatographic effluent, the fractions under each peak were individually pooled and passed through a Sephadex G-15 column equilibrated with 5% acetic acid. The effluent containing each component was concentrated to a small volume and the peptide material present was converted to the corresponding picric acid salt. Chromatography of these picrates on a Sephadex G-15 column equilibrated with 0.05 *M* ammonium bicarbonate buffer resulted in the separation of the picric acid from the peptide material which was obtained as a white fluffy powder upon lyophilization of the effluent.

As can be seen from Figure 3-II, component 3 is eluted at the same position where the natural B-chain *S*-sulfonate emerges in this chromatographic system (Figure 3-I) and upon rechromatography in the same system it exhibits a pattern (Figure 3-III) identical with that of natural B chain. Similarly, on thin-layer electrophoresis, component 3 exhibits the same mobility as natural B-chain *S*-sulfonate (Figure 4), whereas components 1, 2, and 4 migrate differently.

Amino acid analysis of an acid hydrolysate of component 3 gave a composition in molar ratios in excellent agreement with that theoretically expected for B-chain *S*-sulfonate. Digestion of this material with aminopeptidase M (APM) and amino acid analysis of the digest showed that the constituent amino acids were present in the theoretically expected ratios for B chain and thus established the stereochemical homogeneity of the syn-

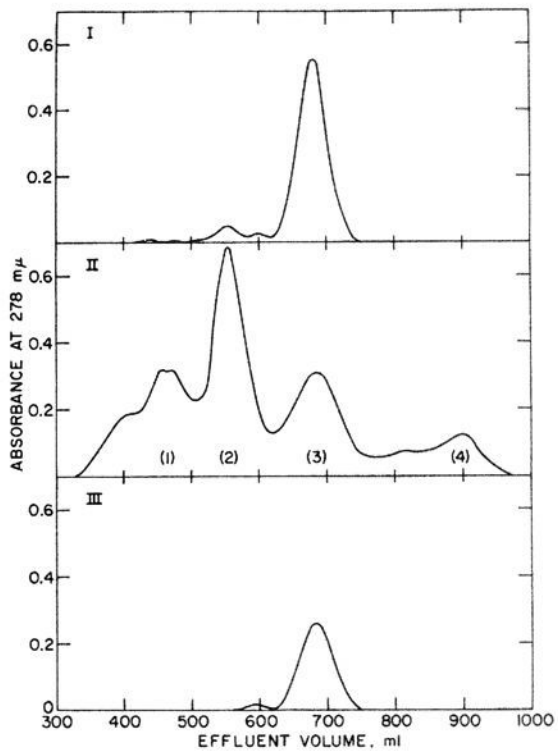

Figure 3. Elution patterns from chromatography on a 4 × 60 cm CM-cellulose column with urea–acetate buffer, pH 4.0: (I) natural bovine B-chain *S*-sulfonate; (II) crude mixture obtained by the sodium–liquid ammonia treatment of synthetic protected B chain followed by sulfitolysis, dialysis, and lyophilization; (III) purified synthetic B-chain *S*-sulfonate obtained from crude material upon chromatography in the same chromatographic system.

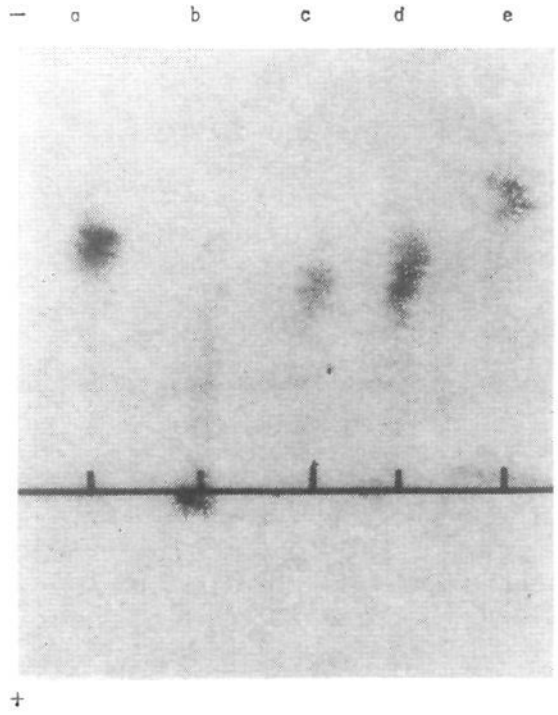

Figure 4. High-voltage thin-layer electrophoresis of natural bovine B-chain *S*-sulfonate (a) and of the four components obtained from CM-cellulose chromatography of synthetic crude B-chain *S*-sulfonate: (b) component 1; (c) component 2; (d) component 3 (purified B-chain *S*-sulfonate); and (e) component 4; 0.5 *N* acetic acid, pH 2.9, 3400 V, 15 mA, 13 min.

thetic product. Further proof that component 3 is indeed the synthetic B-chain *S*-sulfonate was provided by comparison with natural bovine B-chain S-sulfonate prepared by sulfitolysis of bovine insulin as described previously.[21] On thin-layer electrophoresis at two pH values the synthetic B-chain *S*-sulfonate exhibited a single Pauly-positive spot and behaved in an identical manner with the natural chain. On column chromatography the synthetic B-chain *S*-sulfonate and its natural counterpart exhibited identical elution patterns. The synthetic chain possessed the specific rotation $[\alpha]^{25}D$ $-91.9 \pm 1.2°$ (*c* 0.1, 0.5 *N* acetic acid) compared to $[\alpha]^{25}D$ $-95.8 \pm 4.3°$ (*c* 0.1, 0.5 *N* acetic acid) which we found for the natural bovine B-chain *S*-sulfonate. The most decisive proof, however, that the synthetic material is indeed identical with the natural B chain was provided by the fact that when the synthetic B chain was combined with synthetic sheep insulin A chain or with natural bovine A chain the all-synthetic and half-synthetic insulins produced were isolated in crystalline form and found to be identical with the respective naturally occurring proteins.[2,22]

Components 1, 2, and 4 which were separated from the crude B chain upon chromatography on the CM-cellulose column (Figure 3-II) were also isolated and obtained as white fluffy solids. All three materials on thin-layer electrophoresis exhibited single Pauly-positive spots (Figure 4) and had mobilities different from that of the synthetic (or natural) B-chain *S*-sulfonate. Amino acid analysis after acid hydrolysis or after APM digestion of component 4 gave a composition expressed in molar ratios in very good agreement with the theoretically expected values for the C-terminal heneicosapeptide of the B chain. Obviously then, the component 4 is unreacted heneicosapeptide derivative from the final synthetic step in the synthesis of the protected B chain (Chart I). The identification of components 1 and 2 was not pursued. Amino acid analysis after acid hydrolysis of both of these components gave compositions expressed in molar ratios somewhat different from that theoretically expected for the B chain. The most striking difference between amino acid compositions of components 1 and 2 and the B chain was the low histidine content of the former materials. In spite of these differences, however, upon combination with insulin A chain both components 1 and 2 produced a small but definite insulin activity. It seems likely that components 1 and 2 are by-products formed from the B chain during the final steps in the synthesis and are different structurally from that chain.

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Experimental Section

The general analytical procedures used were described in paper XVIII of this series.

The amino acid analyses were performed in a Beckman-Spinco amino acid analyzer Model 120B, equipped with a digital readout system (Model CRS-10AB, Infotronics Corp., Houston, Tex.) according to the method of Spackman, *et al.*[23] Thin-layer electrophoresis was carried out according to a method developed in this laboratory[24] and was performed with a Wieland-Pfleiderer pherograph (Brinkman Instruments). Continuous-flow electrophoresis was carried out on a Model FF continuous-flow separator (Brinkman Instruments) using an ammonium bicarbonate buffer at pH 7.8 as described previously.[21] For column chromatography preswollen microgranular CM-cellulose (Whatman CM 52/1) and Sephadex G-15 (Pharmacia Uppsala) were used. The washing of the resin and the preparation of the columns and the buffers used were described in a previous report.[21] For the enzymatic digestion with aminopeptidase M (APM) the method of Pfleiderer, *et al.*, was employed.[25] APM was purchased from Henley and Co., New York, N. Y. Optical rotations were taken with a Zeiss photoelectric precision polarimeter. The following abbrevations were used: Z, carbobenzoxy; Bz, benzyl; N_3, azide; Tos, *p*-toluenesulfonyl (tosyl).

L-Phenylalanyl-L-valyl-L-asparaginyl-L-glutaminyl-L-histidyl-L-leucyl-*S*-sulfo-L-cysteinylglycyl-L-seryl-L-histidyl-L-leucyl-L-valyl-L-

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glutamyl-L-alanyl-L-leucyl-L-tyrosyl-L-leucyl-L-valyl-*S*-sulfo-L-cysteinylglycyl-L-glutamyl-L-arginylglycyl-L-phenylalanyl-L-phenylalanyl-L-tyrosyl-L-threonyl-L-prolyl-L-lysyl-L-alanine (Sheep Insulin B-Chain *S*-Sulfonate) (IV). The synthesis of N^{α}-benzyloxycarbonyl-*im*-benzyl-L-histidyl-L-leucyl-L-valyl-γ-butyl-L-glutamyl-L-alanyl-L-leucyl-L-tyrosyl-L-leucyl-L-valyl-*S*-benzyl-L-cysteinylglycyl-L-glutamyl-N^{ω}-tosyl-L-arginylglycyl-L-phenylalanyl-L-phenylalanyl-L-tyrosyl-L-threonyl-L-prolyl-N^{ϵ}-tosyl-L-lysyl-L-alanine by the coupling of the N-terminal pentapeptide subunit with the C-terminal hexadecapeptide fragment and its conversion to the hydrobromide of the partially protected derivative II (Chart I) on exposure to hydrogen bromide in trifluoroacetic acid were described in a preceding paper.[11] To a solution of the hydrobromide of compound II (400 mg) in dimethylformamide (30 ml) containing triethylamine (0.1 ml) and cooled to 0° was added the nonapeptide azide I prepared as follows. A solution of *N*-benzyloxycarbonyl-L-phenylalanyl-L-valyl-L-asparginyl-L-glutaminyl-*im*-benzyl-L-histidyl-L-leucyl-*S*-benzyl-L-cysteinylglycyl-L-serine hydrazide[10] (400 mg) or its derivative having the imidazole moiety unprotected[10] in dimethylformamide (20 ml) containing 2 *N* HCl (2 ml) and cooled to −10° was mixed with $NaNO_2$ (30 mg) dissolved in cold water (0.5 ml). After stirring for 5 min at −10° the reaction mixture was poured into cold, half-saturated NaCl solution (100 ml), and the precipitated azide was filtered, washed with cold water, and dried for 1.5 hr over P_2O_5 at 0° *in vacuo*. The infrared spectrum of the dry product exhibited the characteristic azide band at 4.75 μ without any trace of the isocyanate or oxazolidone bands at 4.50 and 5.70 μ, respectively. The dry nonapeptide azide was then added to the solution of the heneicosapeptide derivative prepared as described previously. After stirring at 5° for 48 hr the reaction mixture was poured into cold methanol (200 ml) containing concentrated HCl (0.1 ml) and water (1 ml). The precipitate was filtered, washed successively with absolute methanol, 50% aqueous methanol, and water, and dried. This product was further triturated with boiling MeOH and dried: 225 mg. Amino acid analysis after acid hydrolysis gave the following amino acid composition expressed in molar ratios: $Lys_{0.7}Arg_{1.1}Asp_{0.7}Thr_{1.0}Ser_{0.3}Glu_{2.9}$*S*-benzylcysteine$_{1.3}Gly_{3.3}Pro_{1.1}Ala_{2.0}Val_{2.8}Leu_{3.7}Tyr_{2.0}Phe_{3.3}$ (*im*-benzylhistidine and uncleaved tosyllysine were not determined).

This material was converted to the B-chain *S*-sulfonate by reduction with sodium in liquid ammonia, followed by oxidative sulfitolysis with sodium sulfite and sodium tetrathionate, as follows. The thoroughly dry, partially protected crude sheep B chain (200 mg) was dissolved in anhydrous liquid ammonia (200 ml) in a 250-ml round-bottomed flask fitted for magnetic stirring. To this solution was added solid sodium amide (*ca.* 100 mg) followed by the addition of small pieces of sodium until a faint blue color appeared throughout. The entire reaction was carried out at the boiling point of liquid ammonia. The blue color was allowed to persist for 30 sec and then discharged by the addition of two drops of glacial acetic acid. The resulting solution was concentrated at atmospheric pressure to about 10 ml and dried from the frozen state. The residue was dissolved in 8 *M* guanidine hydrochloride (35 ml), and to this solution, adjusted to pH 8.9 with acetic acid or dilute NH_4OH (depending on the pH of the solution), was added sodium sulfite (1.2 g) and freshly prepared sodium tetrathionate[26] (0.6 g). The reaction mixture was stirred for 24 hr at room temperature and then placed in an 18/32 Visking dialysis tubing and then dialyzed against four changes of distilled water (4 l. each) at 4° for 20 hr. During the dialysis a white precipitate was formed. Isolation of the S-sulfonated B chain from this *dialyzate* was attempted by precipitation at pH 5.0, by lyophilization, by continuous-flow electrophoresis, and by column chromatography.

Isolation of Synthetic Sheep B-Chain *S*-Sulfonate. A. Precipitation at pH 5.0. The above dialyzate was transferred to a centrifuge tube and after the pH was adjusted to 5.0 with acetic acid, the precipitate was separated by centrifugation, resuspended in water, and lyophilized; 100 mg. Amino acid analysis of an acid hydrolysate of this material showed the following composition expressed in molar ratios: $Lys_{0.8}His_{1.7}Arg_{1.0}Asp_{0.7}Thr_{0.7}Ser_{0.6}Glu_{2.9}Pro_{1.1}Gly_{3.1}Ala_{1.6}Cys_{1.5}Val_{3.0}Leu_{4.1}Tyr_{2.0}Phe_{2.8}$. Thin-layer electrophoresis (1 *M* acetic acid; 3300 V) indicated the presence of five major components. In five experiments the combination of this crude material with natural bovine A chain gave insulin in yields ranging from 2 to 5% of theory based on the amount of B chain used.[3]

B. Direct Lyophilization. The dialyzate, obtained from the sulfitolysis reaction, was lyophilized to give a fluffy powder; 140 mg. Amino acid analysis, after acid hydrolysis of this material, showed the following composition expressed in molar ratios: $Lys_{0.8}His_{2.1}Arg_{0.8}Asp_{1.0}Thr_{0.7}Ser_{0.8}Glu_{3.2}Pro_{0.8}Gly_{2.9}Ala_{2.0}Cys_{1.4}Val_{3.2}Leu_{4.1}Tyr_{1.1}Phe_{2.6}$. Thin-layer electrophoresis of this material (1 *M* acetic acid; 3300 V) indicated also the presence of five components as in A. In four experiments the combination of this crude material with natural bovine A chain gave insulin in yields ranging from 2 to 5% of theory based on the amount of B chain used.[3] From these data it is apparent that the material isolated by direct lyophilization of the dialyzate and the material precipitated by adjusting the pH of the dialyzate to 5.0 are essentially similar in regard to heterogeneity, amino acid composition, and the ability to combine with the A chain to form insulin.

C. Continuous-Flow Electrophoresis. The aforementioned dialyzate was lyophilized to give a fluffy powder; 140 mg. This powder (50 mg) was dissolved in 5 ml of NH_4HCO_3 buffer[21] (pH 7.8) with the aid of a few drops of NH_4OH (10% v/v) and placed on a Model "FF" continuous-flow separator and subjected to electrophoresis at 2400 V and 105–115 mA. The dosing rate was 3.2 ml/hr and the buffer rate approximately 150 ml/hr. Figure 1 gives the pattern obtained by measuring the absorbance of the collected effluent with a Beckman DU spectrophotometer at 278 mμ. The liquid in tubes 14–21, corresponding to the position at which the natural B-chain *S*-sulfonate was found to migrate under similar conditions,[21] was pooled and lyophilized to give a white, fluffy solid; 25 mg. The following amino acid composition in molar ratios was found by amino acid analysis of an acid hydrolysate of this material: $Lys_{0.8}His_{2.0}Arg_{1.0}Asp_{0.8}Thr_{0.7}Ser_{0.8}Glu_{3.1}Pro_{0.9}Gly_{3.0}Ala_{1.9}Cys_{1.5}Val_{3.1}Leu_{4.2}Tyr_{1.8}Phe_{2.8}$, in agreement with the expected values for natural sheep B-chain *S*-sulfonate. In spite of the fairly good amino acid composition, this material was shown to be heterogeneous by thin-layer electrophoresis (Figure 2) and chromatography on CM-cellulose as described in the following section (D). In the latter case, the elution patterns obtained by chromatography of the material before and after continuous-flow electrophoresis exhibited only a quantitative difference in peak height but appeared qualitatively identical. Combination of the material isolated by continuous-flow electrophoresis with natural A chain produced insulin in a yield ranging from 7 to 10% of theory based on the amount of B chain used.[3]

D. Column Chromatography. The material obtained from direct lyophilization of the aforementioned dialyzate (140 mg) was dissolved in approximately 6 ml of the urea–acetate buffer (0.04 *M* sodium acetate, 8 *M* urea, pH 4.0) and placed on a CM-cellulose column (4 × 60 cm) equilibrated with the same buffer. Fractions of 10 ml each were collected at a flow rate of 50 ml/hr. The eluted material was monitored continuously with a Zeiss recording spectrophotometer at 278 mμ. The chromatographic pattern obtained (Figure 3-II) indicated the presence of four main components 1, 2, 3, and 4. Fractions 42–52 were pooled to eventually yield component 1, fractions 53–61 component 2, fractions 63–73 component 3, and fractions 85–93 component 4. The isolation of the peptide material from the aforementioned fractions was accomplished by the following procedures. Each fraction, after being adjusted to pH 3.0 with concentrated HCl, was placed on a Sephadex G-15 column (4 × 60 cm) using 5% (v/v) acetic acid as an eluting buffer. Peptide material was detected with a Gilford recording spectrophotometer at 278 mμ and collected. The effluent containing the peptide material was then concentrated in a rotary evaporator (25–30°) to small volume (10 ml) and mixed with an equal volume of saturated picric acid solution. The precipitated picrate was allowed to settle overnight at 4° and isolated by centrifugation. This precipitate was then dissolved in 0.05 *N* NH_4HCO_3 (2 ml) with the aid of a few drops of dilute NH_4OH (10% v/v) and placed on a Sephadex G-15 column (1.2 × 50 cm) equilibrated with 0.05 *N* NH_4HCO_3. A complete and facile separation was obtained in this chromatographic system, since the peptide component moved well in front of the picric acid boundary. The peptide material located by continuous scanning of the effluent with a Zeiss recording spectrophotometer at 278 mμ was recovered by lyophilization giving the respective component as a white, fluffy powder. Component 1 (10 mg) after acid hydrolysis gave the following amino acid composition expressed in molar ratios: $Lys_{0.8}His_{1.2}Arg_{0.7}Asp_{0.6}Thr_{0.8}Ser_{0.6}Glu_{2.8}Pro_{0.9}Gly_{3.0}Ala_{2.0}Cys_{1.7}Val_{3.1}Leu_{3.9}Tyr_{2.0}Phe_{2.7}$. Component 2 (25 mg) isolated by the same procedure gave the following amino acid composition: $Lys_{1.1}His_{0.8}Arg_{0.8}Asp_{0.8}Thr_{1.1}Ser_{0.3}Glu_{3.0}Pro_{1.2}Gly_{3.3}Ala_{2.2}Cys_{1.4}Val_{2.5}Leu_{3.7}Tyr_{2.6}Phe_{2.5}$. Although the amino acid compositions

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Table I. *Amino Acid Composition of the Synthetic S-Sulfonate of the B Chain of Sheep Insulin Determined by the Stein–Moore Procedure*

No. of amino acid residues per molecule					
	Acid hydrolysate			APM digest	
Amino acid	Calcd	Found	Amino acid	Calcd	Found
Lysine	1.00	1.00	Lysine	1.00	1.00
Histidine	2.00	2.00	Histidine	2.00	1.50
Arginine	1.00	1.00	Arginine	1.00	1.00
Aspartic acid	1.00	1.00	Asparagine	1.00	Emerge on the
Threonine	1.00	0.90[a]	Glutamine	1.00	same position
Serine	1.00	0.80[a]	Serine	1.00	N.d.
Glutamic acid	3.00	3.00	Glutamic acid	2.00	2.20
Proline	1.00	0.90	*S*-Sulfocysteine	2.00	1.80
Glycine	3.00	3.10	Threonine	1.00	1.00
Alanine	2.00	1.80	Proline	1.00	0.90
Cysteine	2.00	1.80	Glycine	3.00	3.00
Valine	3.00	3.00	Alanine	2.00	2.00
Leucine	4.00	3.90	Valine	3.00	2.70
Tyrosine	2.00	1.70	Leucine	4.00	4.00
Phenylalanine	3.00	2.80	Tyrosine	2.00	2.20
			Phenylalanine	3.00	3.00

[a] Uncorrected for destruction.

of components 1 and 2 were not in close agreement with the amino acid composition theoretically expected for the sheep B-chain *S*-sulfonate, and although components 1 and 2 had an electrophoretic mobility different than that of the natural sheep B-chain *S*-sulfonate on thin-layer chromatography in 0.5 *N* acetic acid (Figure 4), a small but definite insulin activity (combination yield less than 1% of theory[3]) was detected upon combination of each component with natural bovine A-chain *S*-sulfonate (completely free of B chain *S*-sulfonate[21]). Component 4 (5 mg) isolated by the same procedure gave the following amino acid composition expressed in molar ratios after acid hydrolysis: $Lys_{1.2}His_{1.0}Arg_{0.7}Thr_{0.8}Glu_{2.1}$-$Pro_{0.9}Gly_{2.1}Ala_{1.9}Cys_{0.8}Val_{2.1}Leu_{2.9}Tyr_{1.7}Phe_{2.1}$. This material also showed the following amino acid composition expressed in molar ratios after APM digestion: $Lys_{1.2}His_{0.9}Arg_{0.6}Thr_{0.8}Glu_{1.9}Pro_{0.9}$-

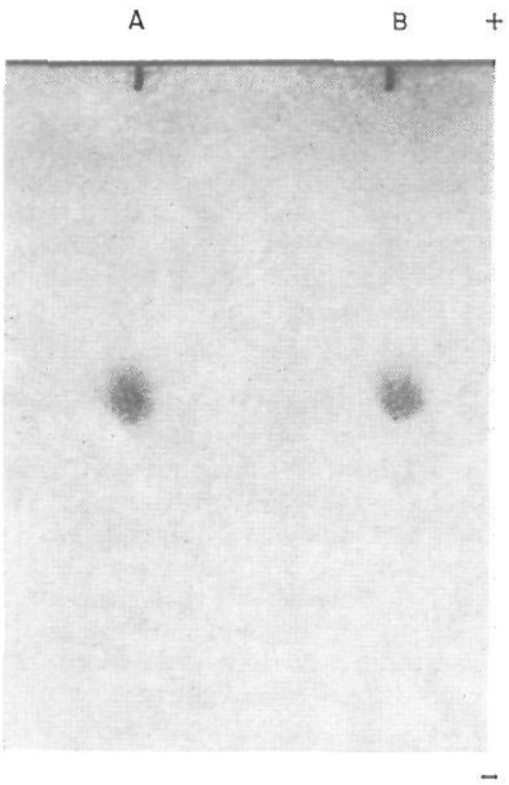

Figure 5. High-voltage thin-layer electrophoresis of synthetic B-chain *S*-sulfonate (A) and natural bovine B-chain *S*-sulfonate (B); 0.5 *N* acetic acid, pH 2.9, 3400 V, 15 mA, 17 min.

$Gly_{2.1}Ala_{1.9}Val_{2.1}Leu_{2.9}Tyr_{1.9}Phe_{2.2}$*S*-sulfocysteine$_{0.9}$. This composition is in good agreement with the theoretically expected values for the C-terminal heneicosapeptide of the sheep B chain. On thin-layer electrophoresis in 0.5 *N* acetic acid, component 4 exhibited one Pauly-positive spot moving faster toward the cathode than the natural sheep B-chain *S*-sulfonate (Figure 4). As shown in Figure 3-II, component 3 (12 mg) was eluted from the CM-cellulose column at the same position as natural B-chain *S*-sulfonate when chromatographed under identical conditions (Figure 3-I). Subsequent evidence (presented below) demonstrated that component 3 is identical with B-chain *S*-sulfonate.

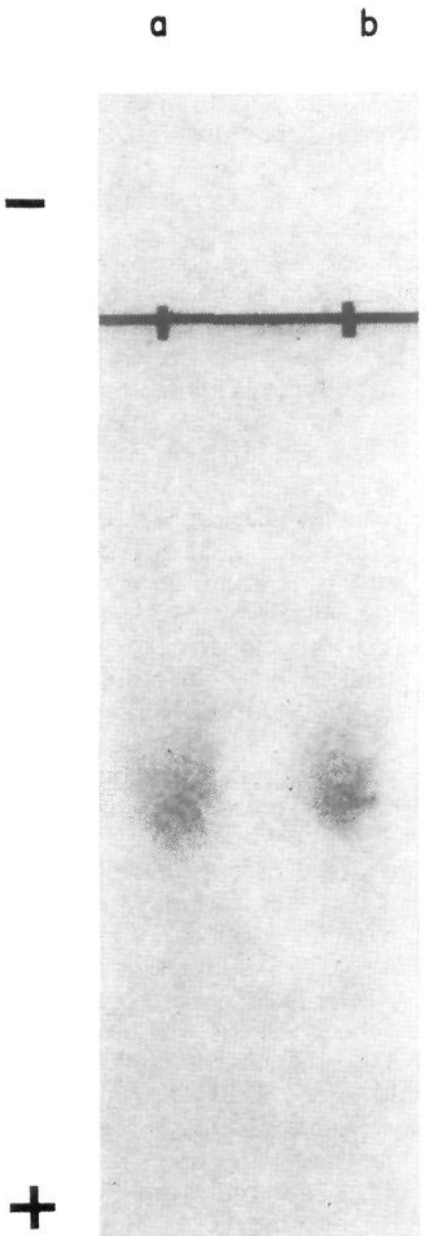

Figure 6. High-voltage thin-layer electrophoresis of synthetic B-chain *S*-sulfonate (a) and natural bovine B-chain *S*-sulfonate (b): 0.01 *M* NH_4HCO_3 adjusted to pH 10.0 with NH_4OH, 3100 V, 15–20 mA, 25 min.

Criteria of Chemical and Stereochemical Homogeneity of the Synthetic Sheep B-Chain *S*-Sulfonate and Comparison with the Natural Bovine B-Chain *S*-Sulfonate. The purified synthetic sheep

B-chain *S*-sulfonate (component 3) possessed the specific rotation $[\alpha]^{25}D$ $-91.9 \pm 1.2°$ (*c* 0.1, 0.5 *N* acetic acid) compared to $[\alpha]^{25}D$ $-95.8 \pm 4.3°$ (*c* 0.1, 0.5 *N* acetic acid) found for natural bovine B chain. Amino acid analysis by the method of Spackman, Stein, and Moore[23] of the synthetic chain after acid hydrolysis gave the molar ratios of amino acids shown in Table I, in excellent agreement with the theoretically expected values. Digestion of the synthetic material with APM and amino acid analysis of the digest with the automatic analyzer gave the amino acid molar ratios shown in Table I. As can be seen, these ratios are practically identical with the theoretically expected values. This proves that the synthetic B chain was completely digested by APM and consequently the optical configuration of the constituent amino acids was preserved during the synthetic processes. Chromatography of the synthetic B-chain *S*-sulfonate on CM-cellulose under the conditions described previously (section D above) showed that the synthetic material is eluted at the same position where the natural bovine B-chain *S*-sulfonate emerges in this chromatographic system (Figure 3-I and -III). On high-voltage thin-layer electrophoresis in 0.5 *N* acetic acid, pH 2.9 and 3400 V, and in 0.01 *M* NH_4HCO_3, pH 10.0 and 3100 V, the synthetic chain moved as a single component (Pauly-positive spot) and had the same mobility as the natural chain (Figures 5 and 6). Finally, when the synthetic B chain was combined with natural or synthetic A chain, insulin was produced which was identical with the natural protein by all criteria employed.[2,22]

Sodium in Liquid Ammonia Treatment of B Chain in the Absence of Sodium Amide. Several experiments were carried out in which the protected crude B chain was deblocked by sodium in liquid ammonia under exactly the same conditions as described previously, but in the absence of sodium amide. The product was isolated either by precipitation at pH 5.0 (section A) or by direct lyophilization (section B) of the dialyzate. A typical amino acid composition of the crude material thus obtained after acid hydrolysis expressed in molar ratios was as follows: $Lys_{0.2}His_{1.6}Arg_{1.0}Asp_{0.6}Thr_{0.1}Ser_{0.5}Glu_{2.7}Pro_{trace}Gly_{3.0}Ala_{1.1}Val_{2.8}Leu_{4.1}Tyr_{1.7}Phe_{3.1}$.

As can be seen, the molar ratios of Ala, Lys, and Pro are far below the expected values, while all the other amino acid ratios are in fairly good agreement with the theoretically expected values for the sheep B chain. It should be further noted that in the acid hydrolysate, threonine is also present in traces. Threonine is the acyl portion of the threonylproline segment which is cleaved on exposure to sodium in liquid ammonia and thus becomes the new C-terminal residue. It has been shown, however, that the sodium in liquid ammonia cleavage of acyl-proline peptides leads to the formation of multiple forms of the new C-terminal residue.[18,19] It appears that the prevalent forms of the new C terminus are the respective amino alcohol and amino aldehyde derivatives.[18,19] Upon acid hydrolysis, however, either form will be decomposed.

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Insulin Peptides. XXII. A Synthesis of the Human Insulin B-Chain *S*-Sulfonate[1]

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Abstract: A synthesis of the human insulin B chain and its isolation in a highly purified form as the S-sulfonated derivative are described. The key intermediate in this synthesis was the partially protected triacontapeptide which contains the entire amino acid sequence of the human insulin B chain. This intermediate was prepared by the interaction of the azide of the N-terminal nonapeptide fragment with the C-terminal heneicosapeptide fragment. The partially protected triacontapeptide obtained by this procedure was converted to the B-chain *S*-sulfonate by removing the protecting groups with sodium in liquid ammonia in the presence of sodium amide and sulfitolyzing the ensuing product. No destruction of the C-terminal amino acid threonine or cleavage of the chain at the amino terminal end of the proline residue was experienced during this deblocking step. An all-glass apparatus was devised for the sodium in liquid ammonia reaction in order to ensure better control of the addition of sodium. Purification of the crude B-chain *S*-sulfonate was accomplished by chromatography on a CM-cellulose column with a urea–acetate buffer at pH 4.0. The purified human B-chain *S*-sulfonate thus obtained was homogeneous as judged by high-voltage thin-layer electrophoresis in two pH values and amino acid analysis after acid hydrolysis, and optically pure as judged by complete digestion with aminopeptidase M. The synthetic product was compared with the structurally similar bovine B-chain *S*-sulfonate as to electrophoretic mobility in two pH values and chromatographic behavior on CM-cellulose chromatography. In these comparisons the synthetic human insulin B-chain *S*-sulfonate and the natural bovine insulin B-chain *S*-sulfonate exhibited an identical behavior. As was already reported, this synthetic human B chain upon combination with synthetic human A chain produced human insulin which was identical with the naturally occurring protein hormone.

The total synthesis of human insulin and its isolation in highly purified form have been described in previous papers from this laboratory.[2–5] Similarly, a detailed description of the synthesis of the human A-chain *S*-sulfonate and a brief account of the original synthesis of the *S*-sulfonated form of the B chain of the protein have also been recorded.[4,6] In this report, we describe

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