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.

Acknowledgments. The authors wish to express their appreciation to Miss Karen Scheibe for the amino acid analyses and enzymatic analyses and to Miss Roberta Klimaski for the biological assays. They also wish to thank Eli Lilly and Co. for generous gifts of crystalline bovine insulin.

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

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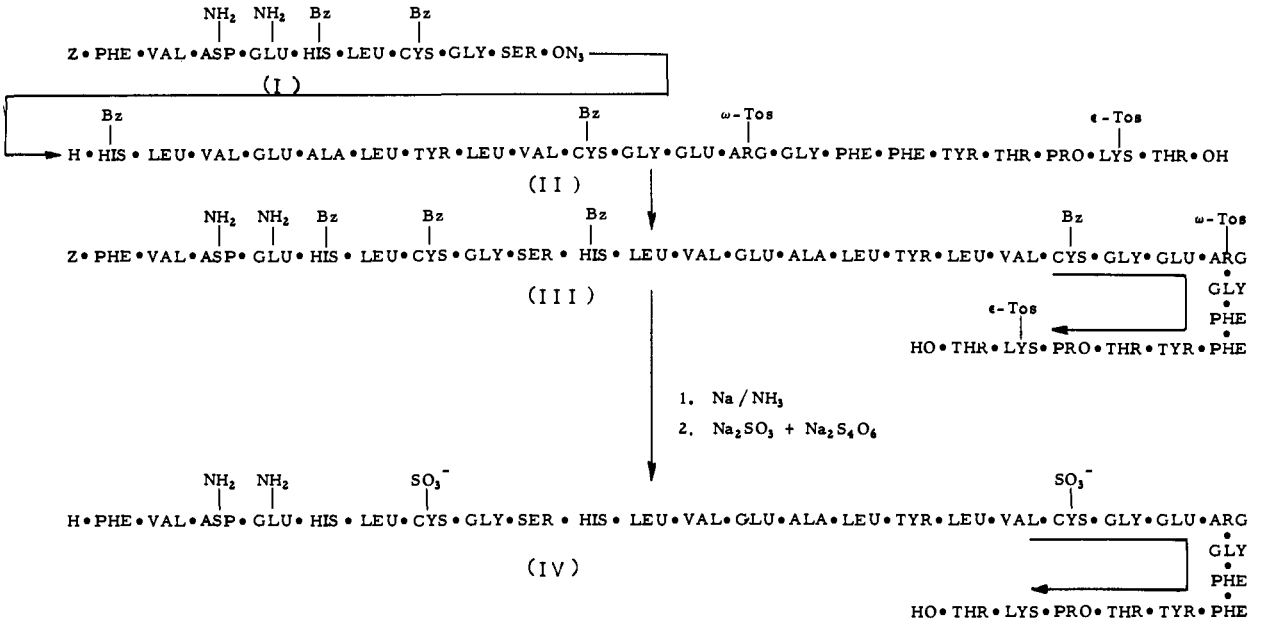
Abstract: A synthesis of the human insulin B chain and its isolation in a highly purified form as the S-sulfonated derivative are described. The key intermediate in this synthesis was the partially protected triacontapeptide which contains the entire amino acid sequence of the human insulin B chain. 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

(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, *Amer. J. Med.*, 40, 652 (1966).
(3) P. G. Katsoyannis, *Science*, **154**, 1509 (1966).
(4) P. G. Katsoyannis, A. Tometsko, J. Ginos, and M. Tilak, *J. Amer. Chem. Soc.*, **88**, 164 (1966).
(5) P. G. Katsoyannis, A. C. Trakatellis, C. Zalut, S. Johnson, A. Tometsko, G. Schwartz, and J. Ginos, *Biochemistry*, **6**, 2656 (1967).
(6) P. G. Katsoyannis, A. Tometsko, and C. Zalut, *J. Amer. Chem. Soc.*, **89**, 4505 (1967).

Chart I



in detail improved experimental procedures for the synthesis and isolation of the S-sulfonated form of the B chain of human insulin. The mapping and implementation of the synthesis of the human B chain were patterned after that of the sheep B chain[4,7,8] since both chains have the same amino acid sequence, differing only in the C-terminal amino acid residue; instead of the alanine present in the sheep (bovine, porcine) chain, the human chain contains threonine.[9]

As was the case with the sheep derivative, the synthesis of the S-sulfonated human B chain involved the construction of the protected triacontapeptide III (Chart I), removal of the blocking groups with sodium in liquid ammonia, and conversion of the resulting sulfhydryl derivative to the S-sulfonated form.[4]

The protected triacontapeptide III was prepared by the interaction of an excess of the azide of the N-terminal nonapeptide fragment I with the C-terminal heneicosapeptide derivative II. The syntheses of the fragments I and II are described in preceding papers.[10,11] Removal of benzyloxycarbonyl, toluenesulfonyl, and benzyl blocking groups from the protected triacontapeptide III was accomplished by sodium in liquid ammonia.[12] As was the case in the synthesis of the S-sulfonated sheep insulin B chain,[7] the presence of sodium amide during the sodium in liquid ammonia reaction inhibited effectively the cleavage of the human B chain between the threonine and proline residues at positions 27 and 28.

In order to ensure better control of reaction conditions and to avoid high local concentrations of sodium, which could lead to undesirable side reactions,[13] a glass apparatus was devised. This apparatus, shown in Figure 1, permits the dropwise addition of liquid ammonia

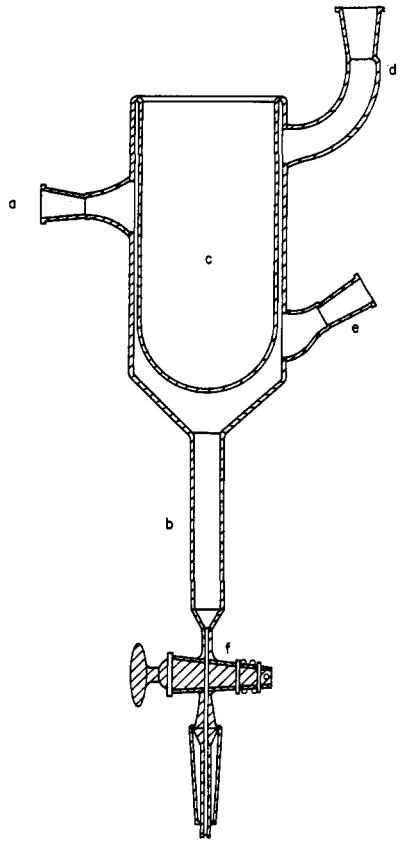


Figure 1. Glass apparatus for sodium in liquid ammonia reaction. Anhydrous liquid ammonia is introduced through a and is collected in the graduated tube b after condensation with the aid of Dry Ice–acetone contained in c. The system is vented through a sodium hydroxide containing drying tube at d. When the desired amount of ammonia is collected, a piece of sodium metal is introduced through e which is kept stoppered. The addition of the liquid ammonia solution of the sodium from b, into a suitable glass flask containing the liquid ammonia solution of the compound to be reduced, is controlled by manipulation of stopcock f.

(7) P. G. Katsoyannis, C. Zalut, A. Tometsko, M. Tilak, S. Johnson, and A. C. Trakatellis, *ibid.*, **93**, 5871 (1971).
(8) P. G. Katsoyannis, K. Fukuda, A. Tometsko, K. Suzuki, and M. Tilak, *ibid.*, **86**, 930 (1964).
(9) D. S. H. W. Nicol and L. F. Smith, *Nature (London)*, **187**, 483 (1960).
(10) P. G. Katsoyannis, M. Tilak, J. Ginos, and K. Suzuki, *J. Amer. Chem. Soc.*, **93**, 5862 (1971).
(11) P. G. Katsoyannis, J. Ginos, and M. Tilak, *ibid.*, **93**, 5866 (1971).
(12) R. H. Sifferd and V. du Vigneaud, *J. Biol. Chem.*, **108**, 753 (1935).
(13) W. F. Benisek, M. A. Raftery, and R. D. Cole, *Biochemistry*, **6**, 3780 (1967).

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

Number of amino acid residues per molecule					
	Acid hydrolysate			APM digest	
Amino acid	Calcd	Found	Amino acid	Calcd	Found
Lysine	1.00	0.90	Lysine	1.00	1.00
Histidine	2.00	1.70	Histidine	2.00	1.50
Arginine	1.00	1.00	Arginine	1.00	1.00
Aspartic acid	1.00	0.70	Asparagine	1.00	Emerge on the
Threonine	2.00	1.60[a]	Glutamine	1.00	same position
Serine	1.00	0.60[a]	Serine	1.00	N.d.
Glutamic acid	3.00	2.90	Glutamic acid	2.00	2.00
Proline	1.00	0.70	*S*-Sulfocysteine	2.00	2.00
Glycine	3.00	3.00	Threonine	2.00	2.10
Alanine	1.00	1.10	Proline	1.00	1.00
Cysteine	2.00	1.50	Glycine	3.00	2.70
Valine	3.00	2.70	Alanine	1.00	0.90
Leucine	4.00	4.00	Valine	3.00	2.80
Tyrosine	2.00	2.00	Leucine	4.00	3.70
Phenylalanine	3.00	3.00	Tyrosine	2.00	2.20
			Phenylalanine	3.00	3.10

[a] Uncorrected for destruction.

solution of sodium into the reaction mixture and can be used as a titrimeter if so desired.

It has been reported that the sodium in liquid ammonia deblocking of the protected synthetic human insulin B chain entails the destruction of the C-terminal amino acid residue, threonine.[14] It was further stated that the destruction of the human B chain during the sodium in liquid ammonia reaction was so extensive that experiments in regenerating insulin by combining such a chain with insulin A chain were not successful.[15] We did not encounter such problems in our laboratory. The S-sulfonated B chain, which was produced by the sodium in liquid ammonia deblocking of the protected triacontapeptide III, had an amino acid composition in excellent agreement with the theoretically expected values. As is shown in Table I, the molar ratio of Thr:Pro:Lys, which occupy the C-terminal tripeptide sequence in the B chain, is 1.6:0.7:0.9 after acid hydrolysis and 2.1:1:1 after enzymatic hydrolysis. Furthermore, the all-synthetic human insulin and a hybrid insulin (one chain from one species and the other chain from another species), which were obtained by combining the synthetic human B chain with the respective A chains, had amino acid compositions in excellent agreement with the theoretically expected values.[5]

The reduced material which was obtained from the sodium–liquid ammonia treatment of the triacontapeptide III, after removal of the ammonia, was dissolved in 8 *M* guanidine hydrochloride and subjected to oxidative sulfitolysis on exposure to sodium sulfite and sodium tetrathionate at pH 8.9 upon which the sulfhydryl functions were converted to the *S*-sulfonates. Dialysis of the reaction mixture followed by lyophilization afforded the crude human insulin B-chain *S*-sulfonate. Amino acid analysis of this product after acid hydrolysis gave a composition expressed in molar ratios closely related to the theoretically expected values for the B chain. On high-voltage thin-layer electrophoresis, however, this material was heterogeneous, consisting of at least five components, one of which had the same mobility as the natural bovine insulin B-chain *S*-sulfonate. It might be pointed out that a similar situation was encountered in the synthesis of the sheep insulin B-chain *S*-sulfonate.[7]

For purification the crude material was chromatographed on a CM-cellulose column equilibrated and eluted with a urea–acetate buffer, pH 4.0.[7] From the elution pattern of this column shown in Figure 2-II, it is apparent that, as in the case of synthetic sheep B chain,[7] at least four major components (1, 2, 3, and 4) are present and fairly well separated. The isolation of these components from the urea containing chromatographic effluent was accomplished by the procedure employed previously in the synthesis of the sheep B chain.[7] This consists of chromatography of the pooled fractions under each peak on Sephadex G-15 columns using 5% acetic acid as the eluting solvent, precipitation of the peptide material from this effluent as the picric acid salt, followed by chromatography of the picrates on Sephadex G-15 columns equilibrated with ammonium bicarbonate, and recovery of the peptide material by lyophilization of the effluent.

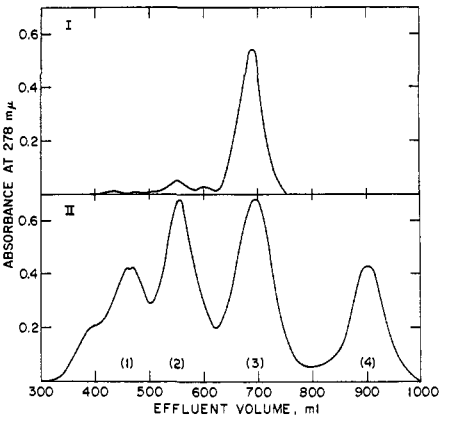


Figure 2. Elution patterns from a CM-cellulose chromatography on a 4 × 60 cm column with a urea–acetate buffer, pH 4.0: (I) natural bovine B-chain *S*-sulfonate; (II) crude synthetic human B-chain *S*-sulfonate obtained by the sodium–liquid ammonia reduction of the protected triacontapeptide, followed by oxidative sulfitolysis, dialysis, and lyophilization.

(14) H. Zahn and G. Schmidt, *Tetrahedron Lett.*, 50, 5095 (1967).
(15) H. Zahn, T. Okuda, and Y. Shimonish, *Peptides, Proc. Eur. Symp., 8th, 1966*, 108 (1967).

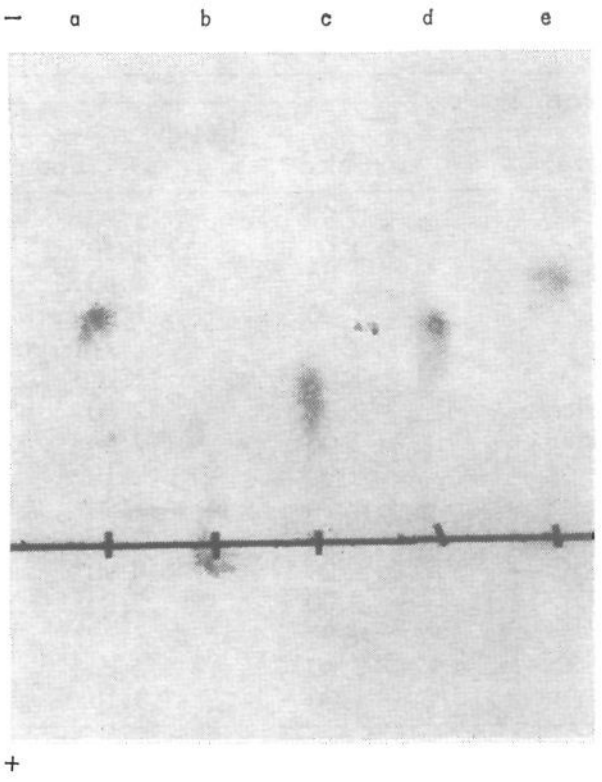


Figure 3. High-voltage thin-layer electrophoresis of natural bovine B-chain *S*-sulfonate (a) and of the four components obtained by the CM-cellulose chromatography of crude synthetic human B-chain *S*-sulfonate: (b) component 1; (c) component 2; (d) component 3 (human B-chain *S*-sulfonate;) and (e)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

From Figure 2-II, it is apparent that component 3 is eluted at the same position where the natural bovine B-chain *S*-sulfonate, which is very similar structurally with the human B chain,[16] emerges in this chromatographic system (Figure 2-I). Furthermore, on high-voltage thin-layer electrophoresis, component 3 had the same mobility as the natural bovine B-chain *S*-sulfonate, whereas components 1, 2, and 4 exhibit different mobilites (Figure 3). Amino acid analysis of an acid hydrolysate of component 3 gave a composition expressed in molar ratios in excellent agreement with the theoretically expected values for human B chain. Similarly, digestion of this material with aminopeptidase M[17] (APM) and amino acid analysis of the digest showed that the constituent amino acids were present in the theoretically expected ratios for human B-chain *S*-sulfonate and, thus, established the stereochemical homogeneity of the synthetic product. Further proof of the chemical homogeneity of the synthetic human B-chain *S*-sulfonate was provided by comparing this material with natural B-chain *S*-sulfonate.

Natural human insulin is not available in enough quantity to permit splitting and isolation of its individual chains to be used for comparison purposes with synthetic chains. We have, therefore, compared the synthetic human B-chain *S*-sulfonate (component 3) with natural bovine B-chain *S*-sulfonate, which, as was indicated previously, is very similar structurally with the human chain, and which we can readily prepare from the commercially available bovine insulin.[18] On high-voltage thin-layer electrophoresis at two pH values, the synthetic human B-chain *S*-sulfonate exhibited a single Pauly-positive spot and had the same mobility as the natural chain. On CM chromatography the synthetic material was eluted at the same position as the natural product. The synthetic human B-chain *S*-sulfonate possessed the specific rotation $[\alpha]^{24}D$ $-96.1 \pm 2°$ (*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. Direct proof, however, that the synthetic material is identical with the natural human B chain was provided by the fact that the insulin produced by combining the synthetic human B chain with synthetic human A chain was identical with the naturally occurring human insulin.[5]

The other components (1, 2, and 4), which were separated by the CM-cellulose chromatography of the crude human B-chain *S*-sulfonate, were also isolated by the procedure described previously.[7] All three compounds appear homogeneous of thin-layer electrophoresis and exhibited mobilities different from those of the synthetic human and natural bovine B-chain *S*-sulfonates (Figure 3). The amino acid composition of component 4, as was determined by amino acid analysis of an acid hydrolysate and an APM digest, indicates that this material is a derivative (*S*-sulfonate) of unreacted heneicosapeptide II used in the final coupling step in the synthesis of the protected B chain (Chart I). As was the case in the synthesis of the sheep B chain,[7] components 1 and 2 were not identified. Their amino acid composition, however, and their behavior on CM-cellulose chromatography (Figure 2-II) and thin-layer electrophoresis (Figure 3) suggest that these materials are structurally different from the B chain.

Experimental Section

The general analytical procedures used were described in papers XVIII and XXI of this series. The following abbreviations were used: Z, benzyloxycarbonyl; Bz, benzyl; N_3, azide; Tos, *p*-toluenesulfonyl (tosyl). APM was purchased from Henley and Co., New York, N. Y.

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-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-threonine (Human Insulin B-Chain *S*-Sulfonate) (IV). To a solution of *im*-benzyl-L-histidyl-L-leucyl-L-valyl-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-threonine trihydrobromide[11] (845 mg), in dimethylformamide (65 ml) containing triethylamine (0.24 ml) and cooled to 0°, was added the protected nonapeptide azide I prepared as follows. A solution of *N*-benzyloxycarbonyl-L-phenylalanyl-L-valyl-L-asparaginyl-L-glutaminyl-L-histidyl-L-leucyl-*S*-benzyl-L-cysteinylglycyl-L-serine hydrazide (1.03 g) (or its derivative having the imidazole moiety benzylated[10]) in dimethylformamide (30 ml) containing 2 *N* HCl (5.1 ml) and cooled to −10° was mixed with a solution of $NaNO_2$ (64 mg) in water (0.5 ml). After stirring for 5 min at −10°, the reaction mixture was poured into cold half-saturated NaCl solution (200 ml) and the precipitated solid azide was isolated by filtration, washed with cold water, and dried for 3 hr over P_2O_5 at 0° *in vacuo*. Infrared spectroscopic[19] studies on the stability of this azide showed that this compound was stable over several hours when stored at 5° in the solid state. However, on exposure to room temperature, the characteristic azide band at 4.75 μ gradually disappeared as rearrangement to the isocyanate and eventually oxazolidone derivative gave rise to the absorption bands at 4.50 and 5.70 μ, respectively.[6] The dry nonapeptide azide was then added to the solution of the heneicosapeptide derivative prepared as described previously. The reaction mixture was stirred at 5° for 48 hr and then poured into methanol (800 ml). The precipitated prod-

(16) F. Sanger and H. Tuppy, *Biochem. J.*, **49**, 463, 481 (1951).
(17) G. Pfleiderer, P. G. Celliers, M. Stanulovic, E. D. Wachsmuth, H. Determann, and G. Braunitzer, *Biochem. Z.*, **340**, 552 (1964).
(18) P. G. Katsoyannis, A. Tometsko, C. Zalut, S. Johnson, and A. C. Trakatellis, *Biochemistry*, **6**, 2635 (1967).
(19) P. G. Katsoyannis, A. Tometsko, C. Zalut, and K. Fukuda, *J. Amer. Chem. Soc.*, **88**, 5615 (1966).

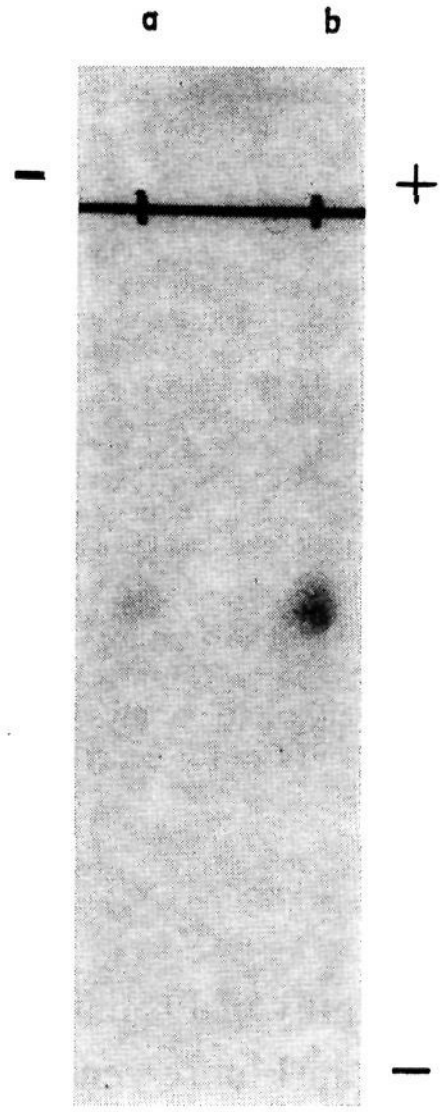


Figure 4. High-voltage thin-layer electrophoresis of synthetic human B-chain *S*-sulfonate (a) and natural bovine B-chain *S*-sulfonate (b); 0.5 *N* acetic acid, pH 2.9, 3400 V, 15 mA, 22 min.

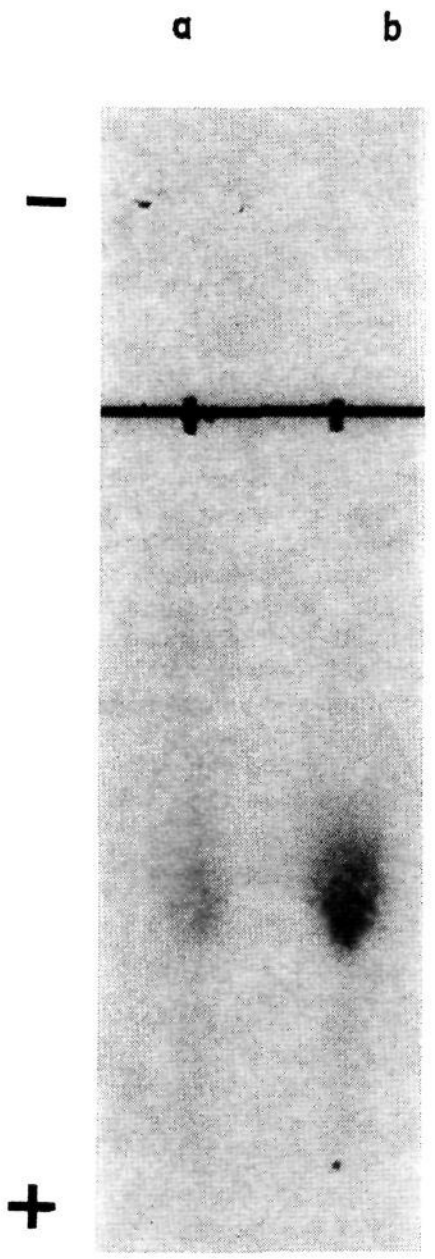


Figure 5. High-voltage thin-layer electrophoresis of synthetic human B-chain *S*-sulfonate (a) and natural bovine B-chain *S*-sulfonate (b); 0.01 *M* NH_4HCO_3 adjusted to pH 10.0 with NH_4OH, 3100 V, 15–20 mA, 25 min.

uct was isolated by centrifugation, washed successively with methanol and ether, and dried to constant weight; 735 mg. An acid hydrolysate of this material showed the following composition in molar ratios:[20] $Lys_{0.6}His_{0.7}Arg_{1.1}Asp_{0.7}Thr_{2.3}Ser_{0.5}Glu_{3.0}Pro_{1.3}$-$Gly_{3.2}Ala_{0.9}Val_{2.9}Leu_{4.0}Tyr_{2.1}Phe_{3.3}$*S*-benzylcysteine$_{1.6}$ (*im*-benzylhistidine and uncleaved tosyllysine were not determined).

This crude protected triacontapeptide was converted to the B-chain *S*-sulfonate by deblocking with sodium in liquid ammonia in the presence of sodium amide, followed by oxidative sulfitolysis with sodium sulfite and sodium tetrathionate. The reduction procedure was essentially that described in the synthesis of the sheep B-chain *S*-sulfonate and was carried out in the glass apparatus illustrated in Figure 1. The thoroughly dry crude protected triacontapeptide (300 mg) was dissolved in anhydrous liquid ammonia (300 ml) in a 500-ml round-bottomed flask fitted for magnetic stirring. To this solution was then added solid sodium amide (*ca.* 120 mg). The reaction was carried out at the boiling point of the solution. Reduction was accomplished by the dropwise addition of a liquid ammonia solution of sodium into the reaction system. The faint blue color was allowed to persist for 30 sec and then discharged by the addition of 2–3 drops of glacial acetic acid. The 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 (40 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 (3 g) and freshly prepared sodium tetrathionate[21] (1.5 g). After stirring for 24 hr at room temperature, the reaction mixture was placed in an 18/32 Visking dialysis tubing and dialyzed against four changes of distilled water (4 l. each) at 4° for 20 hr. Upon lyophilization of the dialyzate the crude human B-chain *S*-sulfonate was obtained as a white powder; 130 mg. Amino acid analysis of this material after acid hydrolysis gave the following composition expressed in molar ratios: $Lys_{1.1}His_{1.6}Arg_{1.4}Asp_{0.5}Thr_{1.9}Ser_{0.4}Glu_{3.0}Pro_{1.0}$-$Gly_{3.1}Ala_{1.1}Cys_{1.5}Val_{2.7}Leu_{4.1}Tyr_{2.4}Phe_{2.8}$. On high-voltage thin-layer electrophoresis[22] (0.5 *N* acetic acid, pH 2.9, 3400 V, 15 mA) this material was heterogeneous and exhibited five Pauly-positive spots, one of which had the same mobility as the natural bovine B-chain *S*-sulfonate (Figure 3).

Isolation of Synthetic Human B-Chain *S*-Sulfonate. The lyophilized material (130 mg) was dissolved in 6 ml of urea–acetate buffer (0.04 *M* sodium acetate, 8 *M* urea, pH 4.0) and applied to a CM-cellulose column (preswollen microgranular, Whatman CM 52/1; 4 × 60 cm) equilibrated with the same buffer.[7] The chromatogram was developed with the aforementioned buffer at a flow rate of 50 ml/hr. After 120 fractions of 10 ml each were collected, four major components were detected (1, 2, 3, and 4) by continuous monitoring of the effluent with a Zeiss recording spectrophotometer at 278 mμ as illustrated in Figure 2-II. Fractions 40–49 were pooled to eventually give component 1, fractions 50–61, component 2, fractions 62–78, component 3, and fractions 82–99, component 4. The isolation of the peptide material from each of these four fractions was accomplished by the method described previously in the synthesis of sheep B-chain *S*-sulfonate.[7] Component 1 (12 mg) after acid hydrolysis gave the following amino acid composition expressed in molar ratios: $Lys_{0.8}His_{1.2}Arg_{0.9}Asp_{0.6}Thr_{1.6}Ser_{0.5}$-$Glu_{2.8}Pro_{0.8}Gly_{3.0}Ala_{1.1}Cys_{1.5}Val_{2.9}Leu_{4.0}Tyr_{2.0}Phe_{2.5}$. On thin-layer electrophoresis at pH 2.9 (Figure 3) this component did not migrate from the origin. Component 2 (23 mg) gave the following amino acid composition after acid hydrolysis: $Lys_{1.1}His_{0.9}Arg_{1.3}$-$Asp_{0.5}Thr_{2.3}Ser_{0.4}Glu_{3.0}Pro_{1.2}Gly_{3.3}Ala_{1.1}Cys_{1.5}Val_{3.0}Leu_{3.8}Tyr_{2.5}$-$Phe_{3.3}$. This material as shown in Figure 3 on thin-layer electrophoresis moved slower toward the cathode than natural bovine B-chain *S*-sulfonate. Component 4 (17 mg) which on thin-layer electrophoresis shows a higher mobility toward the cathode than

(20) D. H. Spackman, W. H. Stein, and S. Moore, *Anal. Chem.*, **30**, 1190 (1958).

(21) A. Gilman, F. S. Philips, and E. S. Koelle, *Amer. J. Physiol.*, **164**, 348 (1946).

(22) A. Tometsko and N. Delihas, *Anal. Biochem.*, **18**, 72 (1967).

the natural bovine B-chain *S*-sulfonate (Figure 3) gave the following amino acid composition expressed in molar ratios after acid hydrolysis: $Lys_{1.0}His_{1.1}Arg_{1.0}Thr_{1.7}Glu_{2.0}Pro_{1.0}Gly_{2.2}Ala_{0.8}Cys_{0.8}Val_{1.8}Leu_{2.8}Tyr_{1.8}Phe_{2.1}$. This material also showed the following amino acid composition after digestion with APM: $Lys_{0.9}His_{0.9}Arg_{0.9}Thr_{1.9}Glu_{1.9}Pro_{0.9}Gly_{2.0}Ala_{1.0}Val_{2.0}Leu_{3.0}Tyr_{1.9}Phe_{2.0}$*S*-sulfocysteine$_{0.9}$. This composition is in excellent agreement with the theoretically expected values for the C-terminal heneicosapeptide derivative of the human B chain. Finally component 3 which is the major component (27 mg) is eluted (Figure 2-II) from the CM-cellulose column at the same position as the natural bovine B-chain *S*-sulfonate (Figure 2-I). The evidence presented below demonstrates that this component is purified human insulin B-chain *S*-sulfonate.

Criteria of Chemical and Stereochemical Homogeneity of Synthetic Human B Chain *S*-Sulfonate and Comparison with Natural Bovine B-Chain *S*-Sulfonate. Amino acid analysis of the purified synthetic human B-chain *S*-sulfonate (component 3) 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 an automatic analyzer gave the amino acid ratios shown in Table I. As can be seen these ratios are almost identical with the theoretically expected values for human B-chain *S*-sulfonate. The APM digestion of the synthetic chain proves that the optical configuration of the constituent amino acids was preserved during the synthetic processes. On CM-cellulose chromatography the synthetic human B-chain *S*-sulfonate is eluted at the same position as the S-sulfonated natural bovine B chain (Figure 2-I, -II). The synthetic human B-chain *S*-sulfonate possessed the specific rotation of $[\alpha]^{24}D$ $-96.1 \pm 2°$ (*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 natural bovine B-chain *S*-sulfonate. 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 and 3100 V, the synthetic human B-chain *S*-sulfonate and the natural bovine B-chain *S*-sulfonate moved as single components (Pauly reaction) and had the same mobility as shown in Figures 4 and 5, respectively. The most decisive proof, however, as to the identity of the synthetic human B chain with its natural counterpart is provided by the fact that when the synthetic human B chain was combined with the synthetic human A chain, the all-synthetic human insulin produced was identical with the naturally occurring protein.[5]

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Studies of the Chymotrypsinogen Family of Proteins. XIII. Inhibitor-Induced Transient Change in Fluorescence of α-Chymotrypsin[1]

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Abstract: Further resolution of the substates of the "best-folded" state, state A, of α-chymotrypsin has been achieved in studies of the effects of inhibitors on the fluorescence behavior of this protein. An interpretation is proposed on the basis of which the substate diagram for the neutral to alkaline pH region is expanded to include effects of substrate-analog inhibitors (see Scheme I). In the presence of inhibitors similar to "good" substrates the single step $A_bH_2 \rightleftharpoons A_g + 2H^+$ of the free protein in which two protons are released cooperatively is resolved into two one-proton steps the first of which has an apparent pK_a of 8.1 at 25° and can take place as well when inhibitors are bound as absent. The second step with an apparent pK_a of 9.1 at 25° eliminates the ability of the protein to bind competitive inhibitors. The group of pK_a 8.1 is linked to the protein process responsible for the fluorescence change on inhibitor binding in such a way that only the protonated species, A_bH_2, undergoes the process which forms the A_eH_2I species. The data are consistent with a simple expansion of the formal mechanism previously found appropriate for fluorescence behavior of the free enzyme. As in the latter, the thermodynamic changes clearly suggest a significant conformational process for the step $A_bH_2 \rightleftharpoons A_fH_2$ only. Enthalpy and entropy changes for rates and equilibria of several steps have been determined. On the basis of the assumption that the step $A_jH \rightleftharpoons A_g$ involves breaking of the Ile-16 to Asp-194 ion pair, estimates of the thermodynamic importance of concomitant changes in protein conformation, charge arrangements, and solvation are estimated.

Studies of the pH dependence of α-chymotrypsin (α-CT)-catalyzed hydrolysis reactions[2-5] and interactions of α-CT with inhibitors[6-9] have provided information about the nature of the substrate and inhibitor binding sites and the substates of the free enzyme. The results of these studies show that an ionizable group with pK_a 8–9 controls the equilibrium between the catalytically active and the alkaline inactive forms of the enzyme in the neutral and alklaine pH ranges and

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(2) Y. Hayashi and W. B. Lawson, *J. Biol. Chem.*, **244**, 4159 (1969).

(3) M. L. Bender, G. E. Clement, F. J. Kezdy, and H. D. Heck, *J. Amer. Chem. Soc.*, **86**, 3680 (1964).

(4) A. Himoe, P. C. Parks, and G. P. Hess, *J. Biol. Chem.*, **242**, 919 (1967).

(5) H. Kaplan and K. J. Laidler, *Can. J. Chem.*, **45**, 547 (1967).

(6) F. Vaslow, *C. R. Lab. Carlsberg*, **31**, 29 (1958).

(7) D. Doherty and F. Vaslow, *J. Amer. Chem. Soc.*, **74**, 931 (1952); F. Vaslow and D. Doherty, *ibid.*, **75**, 928 (1953).

(8) C. H. Johnson and J. R. Knowles, *Biochem. J.*, **103**, 428 (1967).

(9) F. C. Wedler and M. L. Bender, *J. Amer. Chem. Soc.*, **91**, 3894 (1969).