Insulin Peptides. XVII. The Synthesis of the A Chain of Human (Porcine) Insulin and Its Isolation as the S-Sulfonated Derivative[1,2]

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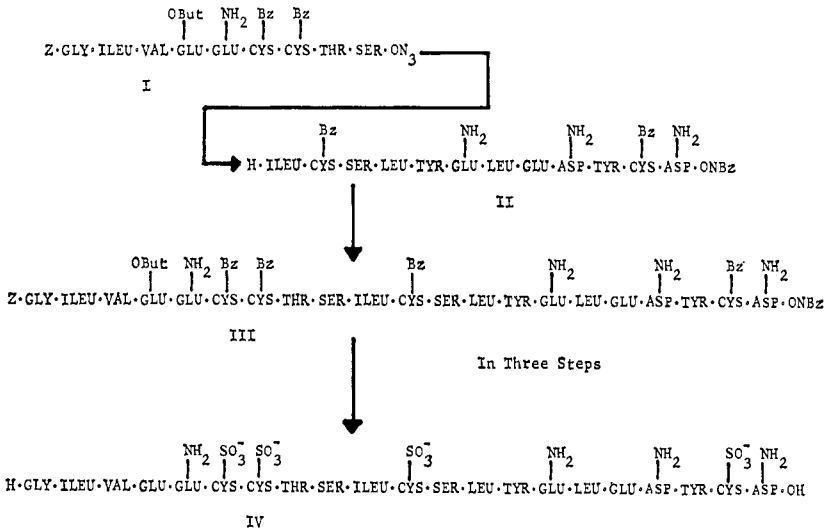
Abstract: The A chain of human insulin, which is identical with the A chain of porcine insulin, has been synthesized and isolated as the S-sulfonated derivative in a highly purified form. This was accomplished by first preparing the protected heneicosapeptide which contains the entire amino acid sequence of the A chain and then removing the protecting groups and sulfitolyzing the ensuing product. The protected heneicosapeptide intermediate was prepared by two routes: (a) the azide coupling of the N-terminal nonapeptide subunit with the C-terminal dodecapeptide subunit and (b) most efficiently by the azide coupling of the N-terminal tetrapeptide fragment with the C-terminal heptadecapeptide fragment. Purification of the A chain S-sulfonate prepared by either one of these procedures was accomplished by chromatography on Sephadex. The chemical purity of the synthetic product was established by paper chromatography in two solvent systems, paper electrophoresis at two pH values, thin layer electrophoresis at two pH values, and amino acid analysis. Its stereochemical homogeneity was established by complete digestion with leucine aminopeptidase. The synthetic human A chain S-sulfonate was compared with natural porcine (human) A chain S-sulfonate as to amino acid composition, electrophoretic mobility on paper, thin layer electrophoresis at two pH values, chromatographic pattern on Sephadex G-50, specific rotation, and infrared pattern. In all of these comparisons, the synthetic human A chain S-sulfonate and the natural porcine (human) A chain S-sulfonate exhibited an identical behavior. Furthermore, the synthetic chain was compared with natural bovine A chain S-sulfonate as to electrophoretic mobility on paper at two pH values, paper chromatography in two solvent systems, and thin layer electrophoresis at two pH values. Both compounds behaved similarly in all of these tests. The synthetic product was converted to its sulfhydryl form and combined with natural bovine B chain S-sulfonate. Hybrid insulin (one chain synthetic, the other chain natural) was thus produced in yields ranging from 30 to 42% of theory based on the amount of the B chain S-sulfonate used. When natural porcine (human) A chain was combined with natural bovine B chain under the same conditions, all natural insulin (porcine) was produced in yields ranging from 35 to 50% of theory.

In previous communications we have reported the synthesis of the A and B chains of sheep insulin and their isolation in the S-sulfonated form.[3-5] Conversion of the S-sulfonated derivative of the A chain to its sulfyhydryl form and combination of the latter product with natural or synthetic B chain in the S-sulfonated or the sulfhydryl form led to the production of the respective insulins.[6,7] Subsequently, we reported in preliminary communications[2,8] the synthesis of the A and B chains of human insulin according to the structure postulated by Nicol and Smith[9] (Figure 1) and their combination to generate this protein.[6,7] In the present report, we wish to give detailed experimental procedures

Chart I



(1) This work was supported by the U. S. Atomic Energy Commission.

(2) Preliminary report of part of the results described in this paper has appeared: P. G. Katsoyannis, A. Tometsko, and C. Zalut, *J. Am. Chem. Soc.*, **88**, 166 (1966).

(3) P. G. Katsoyannis, A. Tometsko, and K. Fukuda, *ibid.*, **85**, 2863 (1963).

(4) P. G. Katsoyannis, K. Fukuda, A. Tometsko, K. Suzuki, and M. Tilak, *ibid.*, **86**, 930 (1964).

(5) P. G. Katsoyannis, A. Tometsko, C. Zalut, and K. Fukuka, *ibid.*, **88**, 5625 (1966).

(6) P. G. Katsoyannis, *Am. J. Med.*, **40**, 652 (1966).

(7) P. G. Katsoyannis, *Science*, **154**, 1509 (1966).

(8) P. G. Katsoyannis, A. Tometsko, J. Ginos, and M. Tilak, *J. Am. Chem. Soc.*, **88**, 164 (1966).

(9) D. S. H. W. Nicol and L. F. Smith, *Nature*, **187**, 483 (1960).

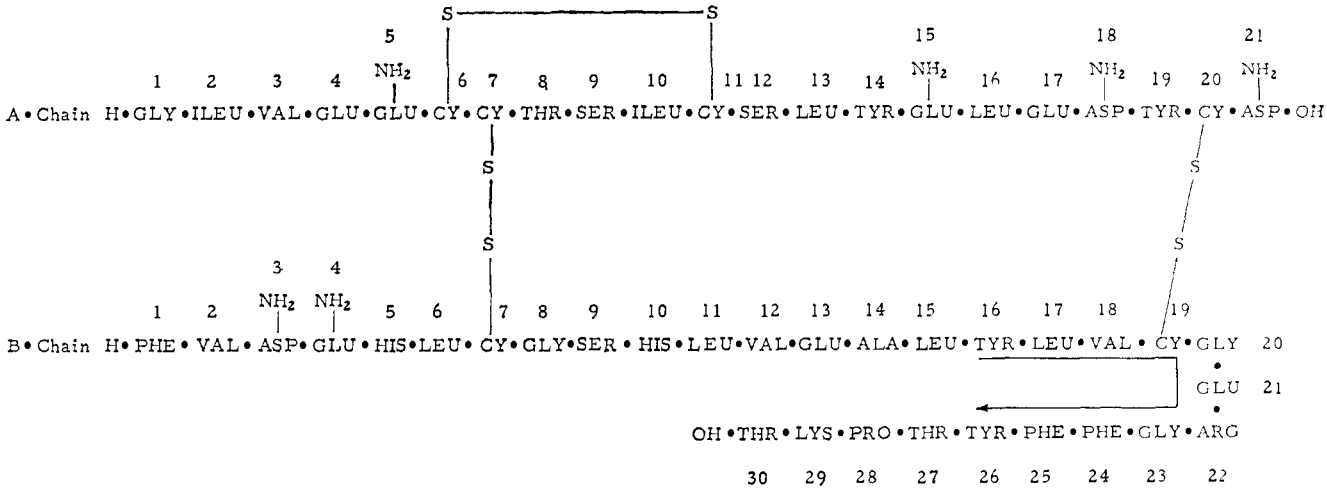


Figure 1. Structure of human insulin.

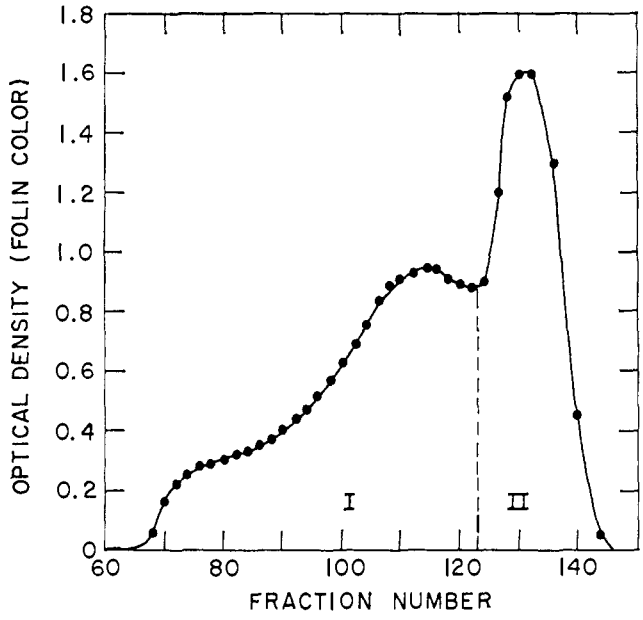


Figure 2. Chromatographic pattern of crude synthetic human A chain S-sulfonate on a Sephadex G-25 column equilibrated with 1 *M* pyridine.

for the synthesis and isolation in the S-sulfonated form of the A chain of human insulin.

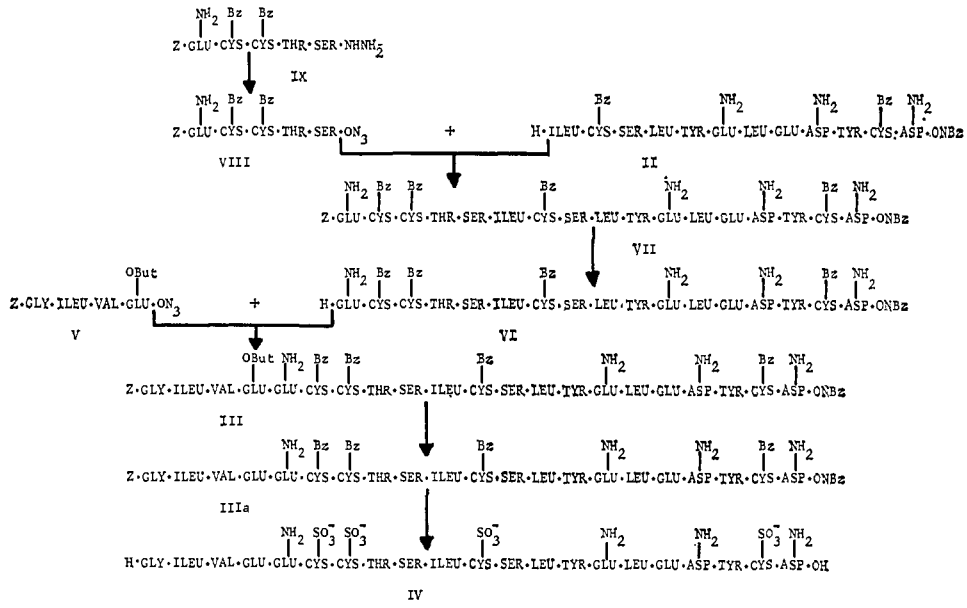
On the basis of considerations similar to those discussed previously in the synthesis of sheep insulin A chain,[5] we proceeded with the assumption that, if we could prepare the protected human A chain III (Chart I), the removal of the protecting groups should give the reduced form of that chain, and this in turn, on treatment with sodium sulfite and sodium tetrathionate, should yield the S-sulfonated derivative IV.

The key intermediate, the protected heneicosapeptide III, was originally prepared[2] (procedure A) by the azide coupling of the N-terminal nonapeptide subunit with the C-terminal dodecapeptide derivative. Thus, interaction of N-carbobenzoxyglycyl-L-isoleucyl-L-valyl-γ-*t*-butyl-L-glutamyl-L-glutaminyl-S-benzyl-L-cysteinyl-S-benzyl-L-cysteinyl-L-threonyl-L-serine azide (I) with L-isoleucyl-S-benzyl-L-cysteinyl-L-seryl-L-leucyl-L-tyrosyl-L-glutaminyl-L-leucyl-L-glutamyl-L-asparaginyl-L-tyrosyl-S-benzyl-L-cysteinyl-L-asparagine *p*-nitrobenzyl ester (II) yielded the desired product III. The protected nonapeptide azide I was prepared in the usual way from the corresponding hydrazide.[10] Infrared spectroscopic analysis indicated that this azide was quite stable if stored at 0° for at least 72 hr and only after several hours at room temperature did the characteristic azide band at 4.75 μ decrease.[5] It is interesting to note that the decomposition product of this azide, unlike other peptide azides we have encountered in our work, is not an isocyanate (absorption band at 4.50 μ) but rather a product with an absorption band at 5.70 μ. Since oxazolidone derivatives have been isolated as decomposition products of serine azides[11] and have absorption bands at approximately 5.70 μ,[12] it is most probable that the decomposition product of the nonapeptide azide I is an oxazolidone derivative. The stable azide I was allowed to react with the amino component II for 72 hr at 5°, and we thus obtained the protected heneicosapeptide III in satisfactory yield. The partially protected dodecapeptide II was prepared from the respective N-carbobenzoxylated derivative[10] on exposure to hydrogen bromide in trifluoroacetic acid.

Without any purification, the protected heneicosapeptide III was exposed successively to trifluoroacetic acid and to sodium in liquid ammonia,[13] whereupon the *t*-butyl,[14] carbobenzoxy, benzyl, and *p*-nitrobenzyl protecting groups were removed.[15] The sodium in liquid ammonia reduction was carried out as was described in the synthesis of the sheep insulin A chain.[5] The reduced material obtained after evaporation 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 fol-

(10) P. G. Katsoyannis, C. Zalut, and A. Tometsko, *J. Am. Chem. Soc.*, **89**, 4502 (1967).
(11) J. S. Fruton, *J. Biol. Chem.*, **146**, 463 (1942).
(12) S. Pinchas and D. Ben-Ishai, *J. Am. Chem. Soc.*, **79**, 4099 (1957).
(13) R. H. Sifferd and V. du Vigneaud, *J. Biol. Chem.*, **108**, 753 (1935).
(14) (a) L. A. Carpino, *J. Am. Chem. Soc.*, **79**, 98 (1957); (b) R. Schwyzer, W. Rittel, H. Kappeler, and B. Iselin, *Angew. Chem.*, **72**, 915 (1960).
(15) For a review see: K. Hofmann and P. G. Katsoyannis, *Proteins*, **1**, 53 (1963); E. Schroder and K. Lubke, "The Peptides," Vol. II, Academic Press Inc., New York, N. Y., 1966.

Chart II



lowed by lyophilization afforded the crude human insulin A chain S-sulfonate.

For purification the crude material was chromatographed on a Sephadex G-25 column equilibrated with 1 *M* pyridine. The elution pattern shown in Figure 2 reveals the presence of at least two components (I and II) which were partly separated. Amino acid analysis of these components after acid hydrolysis indicated that I is mostly A chain S-sulfonate contaminated with unreacted nonapeptide, and II is mostly unreacted dodecapeptide contaminated with A chain S-sulfonate. The material obtained by lyophilization of the fractions under peak I was subsequently placed on a second Sephadex G-25 column and chromatographed using 1% acetic acid as the eluting solvent. The chromatogram is illustrated in Figure 3. Pooling and lyophilization of the fractions under peak II yielded the human insulin A chain S-sulfonate in highly purified form. Based on the amount of the dodecapeptide used, the over-all yield in purified material is about 25% of theory. The fractions under peak I were pooled and lyophilized to yield a material which by amino acid analysis was shown to be unreacted nonapeptide.

As was the case with the sheep insulin A chain,[5] the best method to prepare the protected heneicosapeptide III and hence the human insulin A chain S-sulfonate is by coupling the N-terminal tetrapeptide fragment with the C-terminal heptadecapeptide subunit. This approach is illustrated in Chart II. The pentapeptide N-carbobenzoxy-L-glutaminyl-S-benzyl-L-cysteinyl-S-benzyl-L-cysteinyl-L-threonyl-L-serine hydrazide (IX) which was prepared from the corresponding ester derivative[10] by treatment with hydrazine was converted in the usual way to the respective solid azide VIII. This material exhibited a behavior similar to that of the nonapeptide azide I. Infrared spectroscopic analysis indicated that the azide was stable at 0° for an extended period of time as judged by the characteristic azide absorption band at 4.75 μ, with no trace of the band at 4.50 μ (Figure 4) of the isocyanate, which is the principal decomposition product of many azides. It was further noted that by allowing the azide to stand at room temperature the band at 4.50 μ was progressively decreased, whereas a band at 5.70 μ, presumably of an oxazolidone derivative, was increased proportionally. Consequently, the azide VIII was allowed to react for 3 days at 0° with the dodecapeptide subunit II to yield the protected hepta-

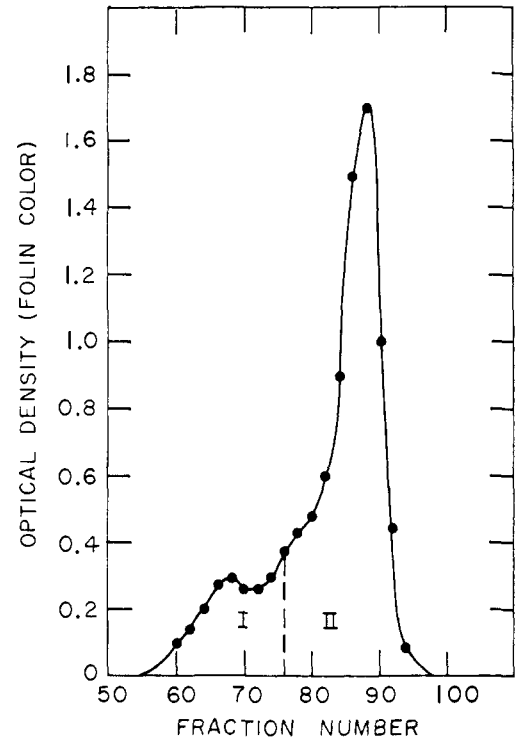


Figure 3. Elution pattern of partially purified (Sephadex G-25, 1 *M* pyridine) synthetic human A chain S-sulfonate from a Sephadex G-25 column equilibrated with 1% acetic acid.

decapeptide N-carbobenzoxy-L-glutaminyl-S-benzyl-L-cysteinyl-S-benzyl-L-cysteinyl-L-threonyl-L-seryl-L-isoleucyl-S-benzyl-L-cysteinyl-L-seryl-L-leucyl-L-tyrosyl-L-glutaminyl-L-leucyl-L-glutamyl-L-asparaginyl-L-tyrosyl-S-benzyl-L-cysteinyl-L-asparagine *p*-nitrobenzyl ester (VII) in 70% yield. This peptide subunit which contains the C-terminal sequence of the A chain of

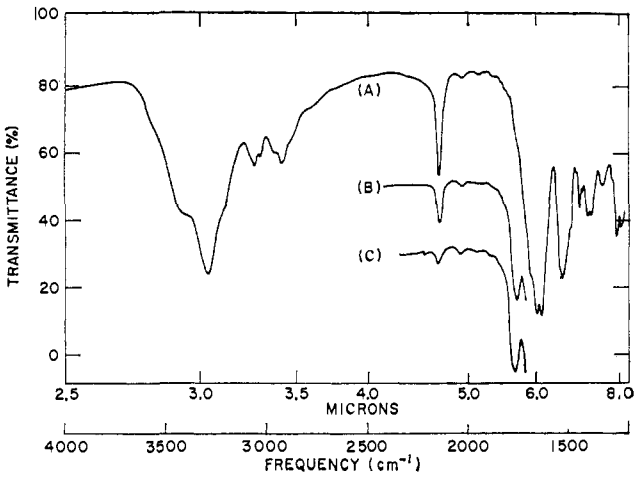


Figure 4. Infrared spectrum of the pentapeptide azide A_{5-9}: azide absorption 4.75 μ, "oxazolidone" absorption 5.7 μ. (A) 24 hr at 0°; (B) 16 hr at 25°; (C) 65 hr at 25°.

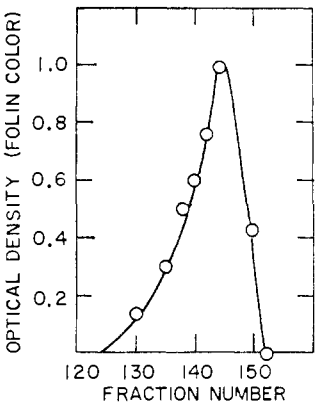


Figure 5. Chromatographic pattern of synthetic human A chain S-sulfonate on a Sephadex G-50 column equilibrated with 1 *M* pyridine.

human insulin was obtained in analytically pure form as judged by elemental analysis and amino acid analysis. On exposure to HBr in trifluoroacetic acid the protected heptadecapeptide VII was converted to the decarbobenzoxylated derivative VI bearing a free amino group. The latter product was subsequently allowed to react with an excess of N-carbobenzoxyglycyl-L-isoleucyl-L-valyl-γ-*t*-butyl-L-glutamic acid azide[16] (V), which embodies the N-terminal amino acid sequence of the A chain to yield, in analytically pure form, the protected heneicosapeptide III in 82% yield. The partially protected derivative IIIa was finally obtained in 95% yield upon treatment of III with trifluoroacetic acid. Elemental analysis and amino acid analysis established the chemical purity of this derivative.

Removal of all protecting groups from the partially protected heneicosapeptide IIIa was accomplished by treatment with sodium in liquid ammonia. The resulting reduced product was subsequently converted to the S-sulfonated derivative IV by oxidative sulfitolysis, as was described previously. For purification the crude product was chromatographed on Sephadex G-50 using 1 *M* pyridine as the eluting solvent. The chromatographic pattern obtained (Figure 5) indicated the presence of a single component. Lyophilization of the pooled fractions under the peak afforded the human A chain S-sulfonate in highly purified form and in 98% yield (based on IIIa used).

Identical material was obtained by any one of the procedures discussed previously, as judged by amino acid analysis, paper chromatography, thin layer electrophoresis, paper electrophoresis, and infrared spectroscopic analysis. The chemical purity of the synthetic A chain was established by a variety of tests. Amino acid analysis after acid hydrolysis gave a composition in molar ratios in good agreement with the theoretically expected. On paper chromatography in two solvent systems and on paper electrophoresis at two pH values, the synthetic material exhibited a single Pauly- and ninhydrin-positive spot, indicating the presence of a single component. Similarly, on thin layer electrophoresis, a highly sensitive analytical method developed in this laboratory,[17] at two pH values the synthetic material exhibited a single Pauly-positive spot. The stereochemical homogeneity of the synthetic chain was established by digestion with leucine aminopeptidase (LAP). Amino acid analysis of the digest by the automatic analyzer showed that the constituent amino acids were present in the theoretically expected ratios; this proved that the digestion was complete and hence that the optical purity of the constituent amino acids was preserved during all the synthetic processes.

Originally we compared the synthetic human A chain S-sulfonate with natural bovine A chain S-sulfonate which we had available.[6] The latter material differs from the human A chain in two amino acid residues. Instead of a threonine residue at position 8 and an isoleucine residue at position 10, the bovine A chain contains, in these positions, an alanine residue and a valine residue, respectively. On paper chromatography in two solvent systems, on high voltage paper electrophoresis at two pH values, and on thin layer electrophoresis at two pH values, the synthetic human A chain S-sulfonate and the natural bovine A chain S-sulfonate exhibited a similar behavior. The most convincing proof, however, that the synthetic material is identical with the natural human A chain would be derived only from direct comparison between these two compounds.

Since natural human insulin is not available in quantities enough to permit splitting and isolation of its individual chains to be used for comparison purposes with synthetic chains, we took advantage of the fact that human and porcine[18] insulins have identical A chains and differ only in their B chains. The porcine insulin has the same B chain as the bovine insulin. We have, therefore, cleaved porcine insulin by oxidative sulfitolysis and have isolated its individual chains in a highly purified form as the S-sulfonated derivatives.[19] This A chain was subsequently compared with the synthetic human A chain S-sulfonate. On thin layer electrophoresis at two pH values and on high-voltage paper electrophoresis the synthetic human A chain S-sulfonate and the natural porcine (human) A chain S-sulfonate behaved in an identical manner. No differ-

(16) P. G. Katsoyannis, A. Tometsko, and C. Zalut, *J. Am. Chem. Soc.*, **88**, 5622 (1966).

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

(18) J. I. Harris, F. Sanger, and M. A. Naughton, *Arch. Biochem. Biophys.*, **65**, 427 (1956).

(19) The sulfitolysis of porcine insulin and the isolation of its individual S-sulfonated chains were carried out by the procedure outlined in ref 6: P. G. Katsoyannis, A. Tometsko, C. Zalut, S. Johnson, and A. Trakatellis, *Biochemistry*, in press.

ences were detected in the infrared patterns of these compounds. The synthetic chain possessed the specific rotation $[\alpha]^{24}D$ $-91 \pm 1°$ (*c* 0.1, water), compared to $[\alpha]^{24}D$ $-93 \pm 1°$ (*c* 0.1, water) which we found for the natural porcine (human) A chain S-sulfonate. Similar elution patterns were obtained when the synthetic and natural chains were chromatographed on Sephadex G-50 using 1 *M* pyridine as the eluting solvent (Figures 5 and 6).

The synthetic S-sulfonated human A chain was converted to its sulfhydryl form and combined with the S-sulfonated derivative of the natural bovine insulin B chain according to the method developed in our laboratory.[20] As a result of this combination, hybrid insulin (half-synthetic porcine insulin) was produced in yields ranging from 30 to 42% of theory, based on the amount of the B chain S-sulfonate used. When natural porcine (human) A chain was combined with natural bovine (porcine) B chain under identical conditions, insulin (porcine) was produced in yields ranging from 35 to 50% of theory.

It is of interest to point out that when natural A and B chains of bovine insulin are prepared and recombined under the same conditions used for the preparation and recombination of the porcine insulin chains, insulin is generated, as was reported previously,[20] in yields ranging from 60 to 80% of theory. Whether the lower efficiency for recombination of the porcine insulin chains as compared to that of the bovine insulin chains is due to a species difference resulting from unfavorable folding of the porcine A chain (since both insulin species have identical B chains) or to enhanced instability of that chain remains to be shown.

In our estimation, the aforementioned comparisons of the physical and chemical properties and recombination abilities of the synthetic material with those of the natural porcine (human) chain justify the conclusion that the synthetic heneicosapeptide is identical with the natural porcine A chain which is considered to be identical with the human A chain.

Experimental Section

The general analytical procedures used were described in paper XVI of this series.[10]

Paper electrophoresis on Whatman No. 3MM paper and thin layer electrophoresis[17] were performed with a Wieland–Pfleiderer pherograph (Brinkmann Instruments). For taking infrared spectra 2 mg of sample 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. Biological assays by the mouse convulsion method were carried out as described previously.[20] Optical rotations were taken with a Zeiss photoelectric precision polarimeter. The following abbreviations are used: Z, carbobenzoxy; Bz, benzyl; NBz, *p*-nitrobenzyl; But, *t*-butyl; N_3, azide; DMF, dimethylformamide; DMSO, dimethyl sulfoxide.

N-Carbobenzoxy-L-glutaminyl-S-benzyl-L-cysteinyl-S-benzyl-L-cysteinyl-L-threonyl-L-serine Hydrazide (IX). A solution of N-carbobenzoxy-L-glutaminyl-S-benzyl-L-cysteinyl-S-benzyl-L-cysteinyl-L-threonyl-L-serine methyl ester[10] (4 g) in DMF (160 ml) containing hydrazine hydrate (8 ml) was stirred at room temperature for 24 hr. The reaction mixture was subsequently poured into water (400 ml) and the pH of the suspension adjusted to 6 with acetic acid. The precipitate was filtered off, washed with water, dried, and reprecipitated from dimethyl sulfoxide–water; 3.0 g (75%); mp 246–248° dec; $[\alpha]^{24}D$ $-16.4°$ (*c* 1, DMSO).

(20) P. G. Katsoyannis and A. Tometsko, *Proc. Natl. Acad. Sci. U. S.*, 55, 1554 (1966).

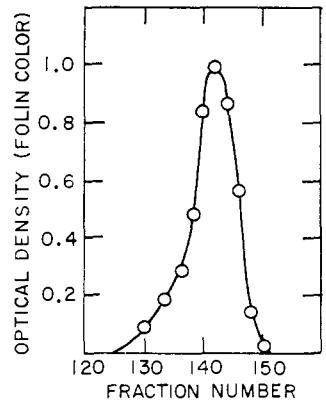


Figure 6. Chromatographic pattern of natural porcine (human) A chain S-sulfonate on a Sephadex G-50 column equilibrated with 1 *M* pyridine.

Anal. Calcd for $C_{40}H_{32}N_8O_{10}S_2$: C, 55.3; H, 5.99; N, 12.90; S, 7.37. Found: C, 54.9; H, 6.57; N, 12.35; S, 6.87.

N-Carbobenzoxy-L-glutaminyl-S-benzyl-L-cysteinyl-S-benzyl-L-cysteinyl-L-threonyl-L-seryl-L-isoleucyl-S-benzyl-L-cysteinyl-L-seryl-L-leucyl-L-tyrosyl-L-glutaminyl-L-leucyl-L-glutamyl-L-asparaginyl-L-tyrosyl-S-benzyl-L-cysteinyl-L-asparagine *p*-Nitrobenzyl Ester (VII). N-Carbobenzoxy-L-isoleucyl-S-benzyl-L-cysteinyl-L-seryl-L-leucyl-L-tyrosyl-L-glutaminyl-L-leucyl-L-glutamyl-L-asparaginyl-L-tyrosyl-S-benzyl-L-cysteinyl-L-asparagine *p*-nitrobenzyl ester[10] (1 g) was dissolved in trifluoroacetic acid (25 ml) and water (0.6 ml), and HBr was passed through this solution for 1 hr at 0°. The decarbobenzoxylated dodecapeptide ester hydrobromide was precipitated by the addition of anhydrous ether, separated by centrifugation, washed several times with ether, and dried over KOH *in vacuo*. This product was dissolved in DMF (25 ml), made slightly basic with triethylamine (0.4 ml), cooled to 0°, and then allowed to react with the pentapeptide azide prepared as follows. Compound IX (0.6 g) was dissolved in DMF (35 ml) and 2 *N* HCl (3 ml) was added. After cooling this solution to −15° (Dry Ice–acetone), $NaNO_2$ (48 mg) dissolved in cold water (0.5 ml) was added. The reaction mixture was stirred at −15° for 5 min and then poured into cold half-saturated sodium chloride solution (150 ml). The precipitated azide was isolated by filtration, washed successively with cold 1 *N* $NaHCO_3$ and water, and dried for 1 hr over P_2O_5 at 0° *in vacuo*. The solid azide was added to the solution of the decarbobenzoxylated dodecapeptide ester prepared as previously described. The reaction mixture was stirred at 0° for 3 days and then poured into methanol (250 ml) containing 1 *N* HCl (0.5 ml). The precipitated product was filtered off, washed successively with dry methanol, 50% aqueous methanol, and water, and reprecipitated from dimethylformamide–water; 0.96 g (70%); mp 274–276° dec; $[\alpha]^{24}D$ $-29.2°$ (*c* 1, DMSO).

Anal. Calcd for $C_{124}H_{160}N_{22}O_{33}S_4$: C, 57.0; H, 6.13; N, 11.8. Found: C, 57.4; H, 6.68; N, 11.9.

Amino acid analysis of an acid hydrolysate of the protected heptadecapeptide showed the following composition expressed in molar ratios: $Asp_{2.0}Ser_{1.7}Thr_{0.9}Glu_{3.0}Ile_{1.0}Leu_{2.0}Tyr_{1.5}$S-benzylcysteine$_{3.3}$. The A chain S-sulfonate prepared from this compound, as will be described later on, was completely digested by LAP.

N-Carbobenzoxyglycyl-L-isoleucyl-L-valyl-γ-*t*-butyl-L-glutamyl-L-glutaminyl-S-benzyl-L-cysteinyl-S-benzyl-L-cysteinyl-L-threonyl-L-seryl-L-isoleucyl-S-benzyl-L-cysteinyl-L-seryl-L-leucyl-L-tyrosyl-L-glutaminyl-L-leucyl-L-glutamyl-L-asparaginyl-L-tyrosyl-S-benzyl-L-cysteinyl-L-asparagine *p*-Nitrobenzyl Ester (III). Protected heptadecapeptide VII (1 g) was dissolved in trifluoroacetic acid (20 ml) containing water (0.4 ml), and HBr was passed through this solution for 1 hr at 0°. Anhydrous ether was then added to the reaction mixture and the precipitated heptadecapeptide ester hydrobromide was isolated by centrifugation, washed with dry ether several times, and dried over KOH *in vacuo*. This material was dissolved in DMF (50 ml) containing triethylamine (0.4 ml), cooled to 0°, and allowed to react with the tetrapeptide azide V which was prepared as follows. A suspension of N-carbobenzoxyglycyl-L-isoleucyl-L-valyl-γ-*t*-butyl-L-glutamic acid hydrazide[16] (0.8 g) in DMF (40 ml) was cooled to −15° and brought into solution by the addition of 2 *N* HCl (3 ml). To this solution was added $NaNO_2$ (82 mg) dissolved in cold water (0.5 ml). After 5 min at −15° the reaction mixture was added to half-saturated sodium chloride solution

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

Amino acid	Acid hydrolysate[a] Theory	Found	Amino acid	LAP digest[a] Theory	Found
Aspartic acid	2.00	1.90	Asparagine	2.00	Emerge on the same position
Threonine	1.00	1.00	Glutamine	2.00	
Serine	2.00	1.80	Threonine	1.00	1.00
Glutamic acid	4.00	4.20	Glutamic acid	2.00	2.00
Glycine	1.00	1.00	Glycine	1.00	1.10
Cysteine	4.00	3.70	S-Sulfocysteine	4.00	4.10[b]
Valine	1.00	0.80	Valine	1.00	1.00
Isoleucine	2.00	1.80	Isoleucine	2.00	2.10
Leucine	2.00	2.00	Leucine	2.00	1.80
Tyrosine	2.00	1.90	Tyrosine	2.00	1.80
Ammonia	4.00	4.20	Serine	2.00	1.80[c]

[a] Number of amino acid residues per molecule. [b] Eluted from the long column of the Beckman–Spinco analyzer after 26 ml of effluent. [c] Separated from glutamine and asparagine in a 30° chromatographic run.

(150 ml) and the precipitated azide was filtered off, washed with cold water, and dried at 0° for 1 hr over P_2O_5 *in vacuo*. This azide was subsequently added to the heptadecapeptide ester which was prepared as described previously. The reaction mixture was stirred at 0°, adding from time to time DMF (total 120 ml) to prevent jelling. After 48 hr the mixture was poured into methanol (500 ml) containing acetic acid (1 ml). The precipitated heneicosapeptide III was isolated by filtration, washed successively with absolute methanol, 50% aqueous methanol, and water, and dried; 0.96 g (82%). For analysis a sample of this material was reprecipitated from dimethyl sulfoxide–water; mp 281–283° dec; $[\alpha]^{24}D$ −27.6° (*c* 1, DMSO).

Anal. Calcd for $C_{146}H_{198}N_{26}O_{39}S_4$: C, 57.1; H, 6.49; N, 11.9. Found: C, 56.9; H, 6.50; N, 11.9.

Amino acid analysis of an acid hydrolysate showed the following composition expressed in molar ratios: $Asp_{2.0}Thr_{1.0}Ser_{1.8}Glu_{4.3}Gly_{1.0}Val_{0.7}Ile_{1.8}Leu_{2.1}Tyr_{1.7}$S-benzylcysteine$_{3.7}$, in good agreement with the values expected from theory.

N-Carbobenzoxyglycyl-L-isoleucyl-L-valyl-L-glutamyl-L-glutaminyl-S-benzyl-L-cysteinyl-S-benzyl-L-cysteinyl-L-threonyl-L-seryl-L-isoleucyl-S-benzyl-L-cysteinyl-L-seryl-L-leucyl-L-tyrosyl-L-glutaminyl-L-leucyl-L-glutamyl-L-asparaginyl-L-tyrosyl-S-benzyl-L-cysteinyl-L-asparagine *p*-Nitrobenzyl Ester (IIIa). Compound III (0.96 g) was dissolved in trifluoroacetic acid (20 ml), and this solution was stored at room temperature for 30 min and then filtered through a sintered glass filter to remove any trace of impurities. Dilution of the filtrate with anhydrous ether caused the precipitation of the partially protected heneicosapeptide IIIa in analytically pure form; 0.9 g (95%); mp 279–282° dec; $[\alpha]^{24}D$ −38.7° (*c* 1, DMSO).

Anal. Calcd for $C_{142}H_{190}N_{26}O_{39}S_4$: C, 56.6; H, 6.31; N, 12.1. Found: C, 56.0; H, 6.14; N, 11.6.

Glycyl-L-isoleucyl-L-valyl-L-glutamyl-L-glutaminyl-S-sulfo-L-cysteinyl-S-sulfo-L-cysteinyl-L-threonyl-L-seryl-L-isoleucyl-S-sulfo-L-cysteinyl-L-seryl-L-leucyl-L-tyrosyl-L-glutaminyl-L-leucyl-L-glutamyl-L-asparaginyl-L-tyrosyl-S-sulfo-L-cysteinyl-L-asparagine (Human Insulin A Chain S-Sulfonate) (IV). A. Azide Coupling of N-Terminal Nonapeptide I with C-Terminal Dodecapeptide II. The partially protected dodecapeptide ester II, prepared from its N-carbobenzoxy derivative (300 mg) as described in the synthesis of VII, was dissolved in DMF (30 ml) containing triethylamine (0.15 ml), cooled to 0°, and allowed to react with the nonapeptide azide I prepared as follows. To a solution of N-carbobenzoxyglycyl-L-isoleucyl-L-valyl-γ-*t*-butyl-L-glutamyl-L-glutaminyl-S-benzyl-L-cysteinyl-S-benzyl-L-cysteinyl-L-threonyl-L-serine hydrazide[10] (300 mg) in DMF (30 ml), cooled to −15° (Dry Ice–acetone), was added 2 *N* HCl (2 ml) followed by a solution of $NaNO_2$ (15.7 mg) in cold water (0.5 ml). After stirring for 5 min at −15° the reaction mixture was poured into half-saturated sodium chloride solution and the precipitated product was immediately filtered, washed with 1 *N* $NaHCO_3$ and water, and dried over P_2O_5 at 0° for 1 hr *in vacuo*. The dried azide was then added to the solution of the dodecapeptide derivative prepared as described previously. After stirring at 0° for 3 days, the reaction mixture was poured into cold water (150 ml) and the precipitated heneicosapeptide was filtered off, washed with water, and dried; 532 mg. This material was dissolved in trifluoroacetic acid (15 ml), and after 30 min the solution was diluted with anhydrous ether (200 ml) and the precipitated product was collected by filtration, washed with ether, and dried over KOH *in vacuo*; 450 mg. The final deblocking of this material was achieved by reduction with sodium–liquid ammonia as follows.[5] The thoroughly dry, partially protected heneicosapeptide (450 mg) was dissolved in anhydrous liquid ammonia (300 ml) in a 500-ml round-bottomed flask fitted for magnetic stirring. The reaction was carried out at the boiling point of the solution. Reduction was accomplished by adding very small pieces of sodium into the solution until a faint blue color appeared throughout. The blue color was allowed to persist for 1 min and was then discharged by the addition of a few crystals of ammonium chloride. The solution was evaporated at atmospheric pressure to about 10 ml and dried from the frozen state. The residue was dissolved in 8 *M* guanidine hydrochloride (25 ml), and to this solution adjusted to pH 8.9 with acetic acid was added sodium sulfite (1.2 g) and sodium tetrathionate[21] (0.6 g). The reaction mixture was stirred at 25° for 24 hr and then dialyzed against four changes of distilled water (4 l. each) at 4° for 20 hr. Visking 18/32 dialyzing tubing was used. Lyophilization of the dialyzate gave the crude A chain S-sulfonate as a white, fluffy powder; 282 mg.

Isolation of Synthetic A Chain S-Sulfonate. The crude A chain S-sulfonate was dissolved in 1 *M* pyridine (10 ml) and applied to a Sephadex G-25 column (fine grade, bead form; 2.2 × 90 cm) equilibrated with 1 *M* pyridine. The chromatogram was developed with 1 *M* pyridine at a flow rate of about 34 ml/hr. After 140 fractions of 1.7 ml each were collected, two main peaks, I and II, were detected by the Folin–Lowry method[22] as illustrated in Figure 2. Fractions 65–123 were pooled and lyophilized to give component I (140 mg). Amino acid analysis after acid hydrolysis gave the following composition expressed in micromoles: $Asp_{0.36}Thr_{0.28}Ser_{0.42}Glu_{1.08}Gly_{0.33}Val_{0.24}Ile_{0.44}Leu_{0.38}Tyr_{0.36}$ (cystine was not determined). This composition (high values for glycine, glutamic acid, and isoleucine; low values for leucine and aspartic acid) suggests that component I is the A chain S-sulfonate contaminated with unreacted nonapeptide. Lyophilization of pooled fractions 124–145 yielded component II (112 mg) which, after acid hydrolysis, gave the following amino acid composition in micromoles: $Asp_{0.65}Thr_{0.22}Ser_{0.42}Glu_{1.23}Gly_{0.24}Val_{0.17}Ile_{0.53}Leu_{0.72}Tyr_{0.65}$ (cystine was not determined). This composition, high values for aspartic acid and leucine and low values for glycine and threonine, indicates that II was mainly unreacted dodecapeptide contaminated with A chain S-suifonate. For further purification component I (140 mg) was dissolved in 1% acetic acid (3 ml) and chromatographed on Sephadex G-25 (fine grade, bead form; 2.2 × 90 cm). The flow rate was 34 ml/hr and the fraction volume 1.7 ml. The elution pattern as determined by Folin–Lowry reaction is shown in Figure 3. Fractions 60–76 were pooled and lyophilized to a white powder; 23 mg. Amino acid analysis after acid hydrolysis showed the following composition expressed in micromoles: $Asp_{0.27}Thr_{0.51}Ser_{0.55}Glu_{1.69}Gly_{0.76}Cys_{0.83}$, $Val_{0.56}Ile_{0.75}Leu_{0.30}Tyr_{0.24}$, indicating that this product is unreacted nonapeptide. Fractions 76–94 were pooled and lyophilized to give highly purified A chain S-sulfonate;

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

(22) D. H. Lowry, N. J. Rosebrough, A. L. Farr, and R. J. Randall, *J. Biol. Chem.*, **193**, 265 (1951).

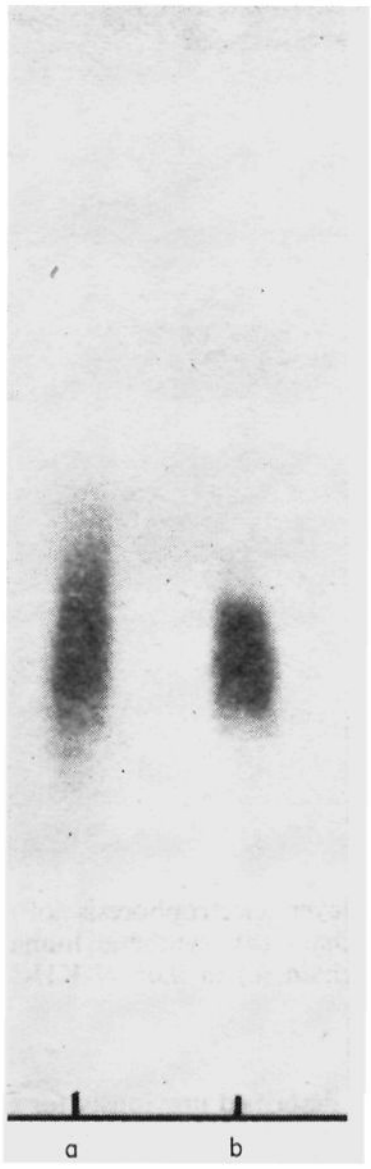


Figure 7. Paper chromatography of the S-sulfonates of synthetic human A chain (a) and natural bovine A chain (b) in the system 1-butanol–pyridine–acetic acid–water (30:20:6:24).

Figure 8. Paper chromatography of the S-sulfonates of natural bovine A chain (a) and synthetic human A chain (b) in the system $CHCl_3$–CH_3OH–H_2O–NH_4OH (4:4:1:1).

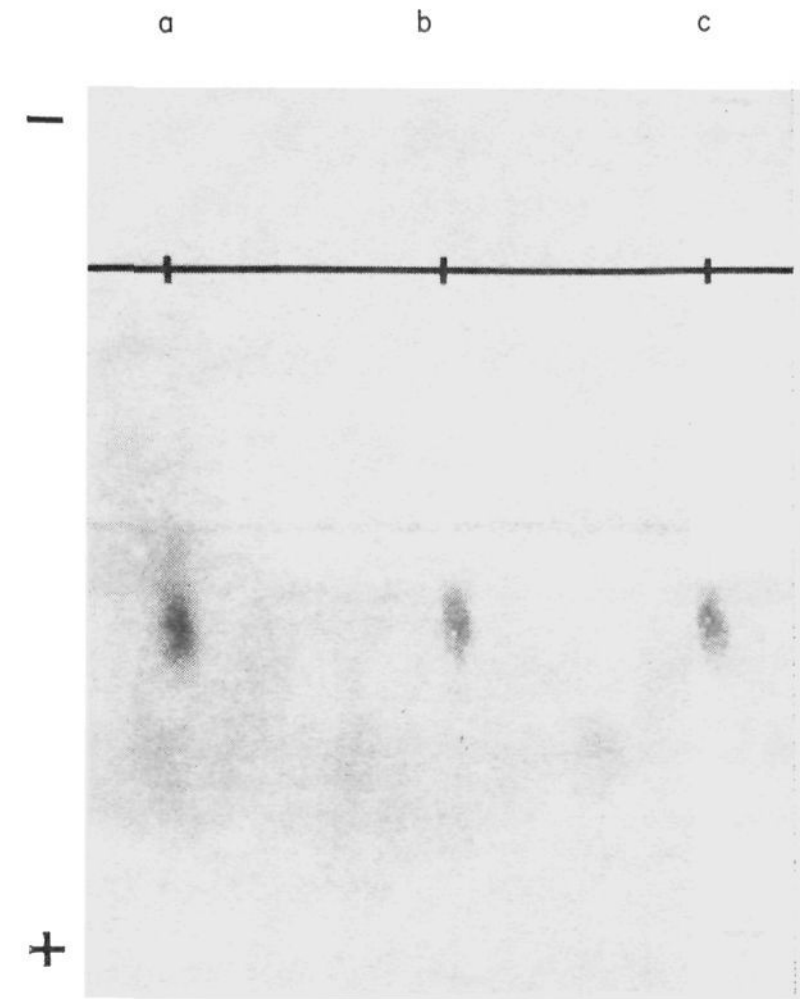


Figure 9. Paper electrophoresis of the S-sulfonates of natural bovine A chain (a), synthetic human A chain (b), and natural porcine A chain (c) in 0.5 *N* acetic acid, pH 2.9 (2000 v, 1 hr).

100 mg (25%, based on the amount of C-terminal dodecapeptide used for the original coupling).

B. Deblocking of Partially Protected Heneicosapeptide IIIa. The deblocking of IIIa (329 mg) with sodium–liquid ammonia (250 ml) was carried out by the procedure described previously. After evaporation of the ammonia the residue was dissolved in 8 *M* guanidine hydrochloride (25 ml) and to this solution, adjusted to pH 8.9 with acetic acid, was added sodium sulfite (1.5 g) and sodium tetrathionate (0.75 g). The reaction mixture was stirred at 25° for 24 hr and then dialyzed in Visking 18/32 dialyzing tubing at 4° for 24 hr against four changes (4 l. each) of distilled water. Upon lyophilization of the dialyzate, the A chain S-sulfonate was obtained as a white powder; 290 mg.

Isolation of Synthetic A Chain S-Sulfonate. The lyophilized material (290 mg) was dissolved in 1 *M* pyridine (10 ml) and chromatographed on a Sephadex G-50 column (fine grade, bead form) in 1 *M* pyridine under the following conditions: column size, 2.2 × 90 cm; flow rate, 34 ml/hr; fraction volume, 1.7 ml. The elution pattern of this column as determined by Folin–Lowry analysis of the effluent indicated the presence of a single peak as shown in Figure 5. The fractions under the peak were pooled and lyophilized to give highly purified A chain S-sulfonate; 250 mg (86%; based on IIIa used).

Criteria of Chemical and Stereochemical Homogeneity of Synthetic Human A Chain S-Sulfonate and Comparison with Natural Porcine (Human) and Bovine A Chain S-Sulfonate. Identical material was obtained by any one of the two synthetic procedures as judged by amino acid analysis, paper chromatography, paper electrophoresis, thin layer electrophoresis, LAP digestibility, and infrared spectroscopic analysis. Consequently, for evaluating chemical and stereochemical purity and for comparison purposes, synthetic material prepared by both procedures was used indiscriminately.

The synthetic material possessed the specific rotation $[\alpha]^{24}D$ $-91 \pm 1°$ (*c* 0.1, water), compared to $[\alpha]^{24}D$ $-93 \pm 1°$ (*c* 0.1, water) which we found for natural porcine (human) A chain S-sulfonate. 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 and ammonia shown in Table I, in excellent agreement with the theoretically expected values. Digestion of the synthetic material with LAP and amino acid

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

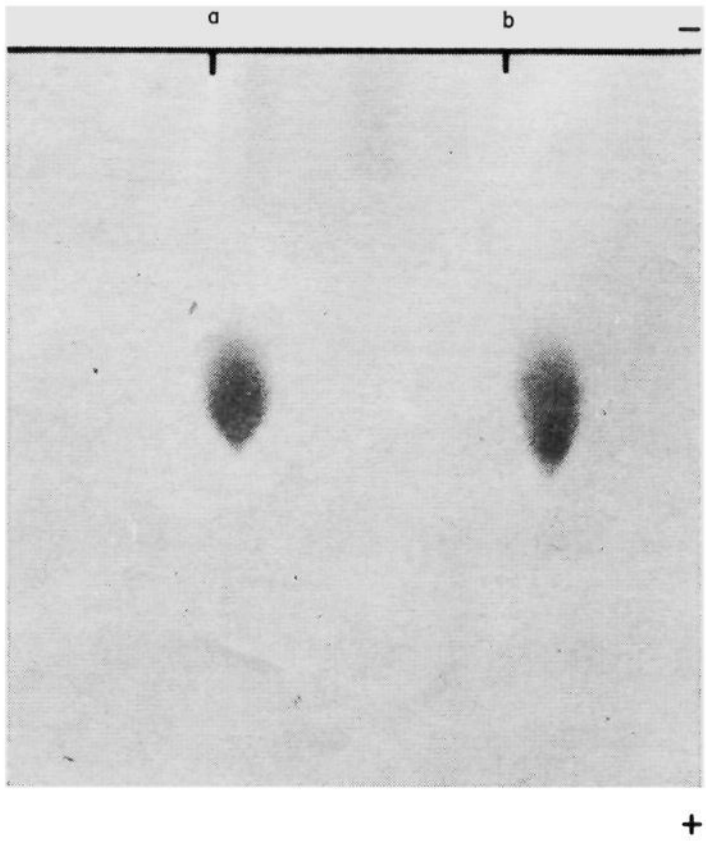


Figure 10. Paper electrophoresis of the S-sulfonates of natural bovine A chain (a) and synthetic human A chain (b) in 0.05 *N* $KHCO_3$, pH 8.5 (1400 v, 1 hr).

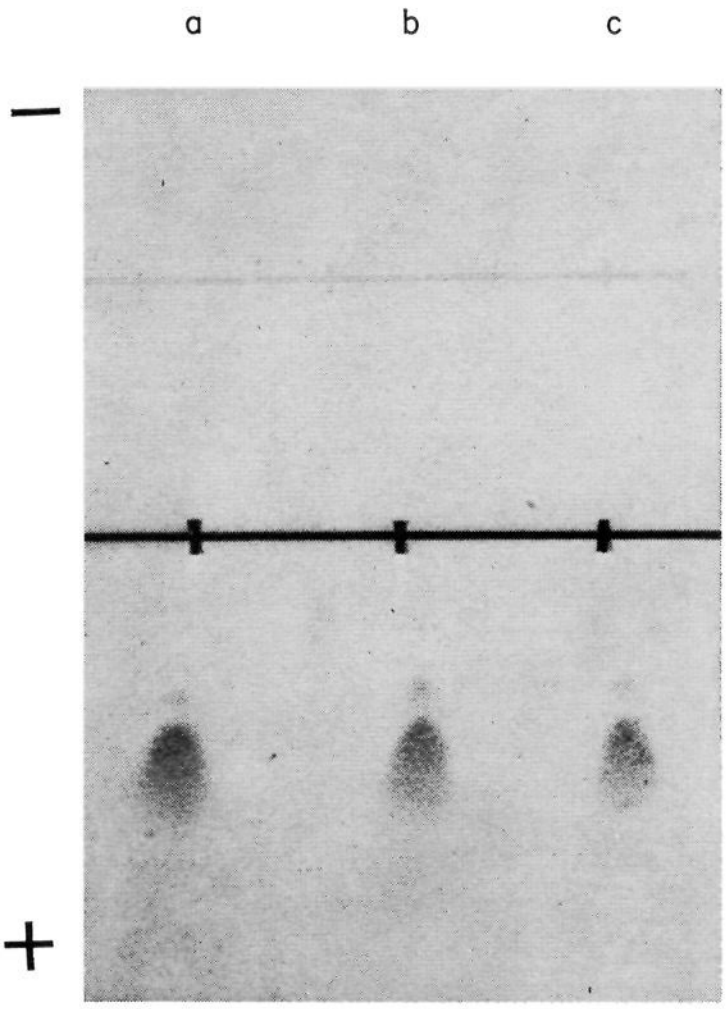


Figure 11. Thin layer electrophoresis of the S-sulfonates of natural bovine A chain (a), synthetic human A chain (b), and natural porcine A chain (c) in 0.5 *N* acetic acid, pH 2.9 (3400 v, 20 min).

analysis of the digest with an 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 chain was completely digested by LAP and, consequently, that the optical configuration of the constituent amino acids was preserved during the synthetic processes. Chromatography of the natural porcine (human) A chain S-sulfonate on a Sephadex G-50 column equilibrated with 1 *M* pyridine under

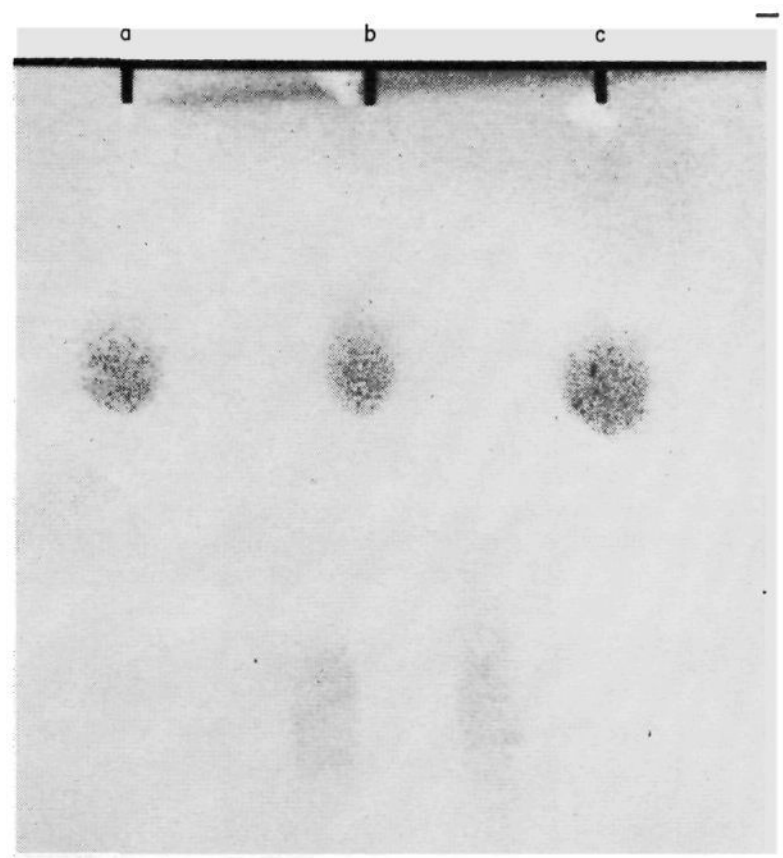


Figure 12. Thin layer electrophoresis of the S-sulfonates of natural bovine A chain (a), synthetic human A chain (b), and natural porcine A chain (c) in 0.05 *N* $KHCO_3$, pH 8.4 (1900 v, 20 min).

the same conditions described previously for the synthetic material (Figure 5) showed the pattern illustrated in Figure 6. On paper chromatography in the system 1-butanol–pyridine–acetic acid–water[24] (30:20:6:24) the synthetic chain exhibited a single Pauly- and ninhydrin-positive spot and has the same mobility (using histidine as a marker: R_f 1.4 × His) as the natural bovine A chain S-sulfonate (Figure 7). Similarly, on paper chromatography in the system chloroform–methanol–water–ammonium hydroxide (4:4:1:1) the synthetic human and natural bovine chains exhibited an identical behavior (R_f 1.07 × His) as shown in Figure 8.

On high-voltage paper electrophoresis in 0.5 *N* acetic acid, pH 2.9, and 2000 v, the synthetic chain moved as a single component (Pauly and ninhydrin reaction) and had the same mobility as the natural porcine and bovine chains (Figure 9). Paper electrophoresis in 0.05 *N* $KHCO_3$, pH 8.5, and 1400 v showed (Figure 10) that the synthetic human and natural bovine chains moved as single components (Pauly and ninhydrin reaction) and had the same mobility.

On thin layer electrophoresis in 0.5 *N* acetic acid, pH 2.9, and 3400 v, and in 0.05 *N* $KHCO_3$, pH 8.4, and 1900 v, the synthetic human and the natural porcine and bovine chains moved as single components (Pauly reaction) and had the same mobility, as shown in Figures 11 and 12, respectively.

Finally, no differences were detected in the infrared spectra of the synthetic and natural porcine (human) chains, as shown in Figure 13.

Combination of Synthetic Human A Chain with Natural Bovine B Chain to Generate Insulin. A solution of synthetic human A chain S-sulfonate (20 mg) in water (5 ml), which had been deaerated by boiling under nitrogen, was adjusted to pH 5 with 1 *N* acetic acid, mixed with 1 *M* 2-mercaptoethanol (0.8 ml), and heated in a boiling water bath for 6 min. The entire process was carried out in a nitrogen atmosphere. The reaction mixture was cooled to 10–15° and extracted four times with 40-ml portions of ethyl acetate. After the last extraction, the traces of ethyl acetate were removed by flushing the reaction vessel with a stream of nitrogen. The resulting jelly-like product was mixed with natural bovine insulin B chain S-sulfonate (5 mg) and water (7 ml). After adjusting the pH of the resulting suspension to 9.6 with 1 *N* NaOH a clear solution was obtained. This solution was diluted with 0.1 *M* glycine buffer (0.8 ml), pH 9.6, and stirred for 20 hr at 2° in contact with CO_2-free air. In four experiments the solution of the combination mixture (volume about 10 ml) was shown to possess, by the mouse convul-

(24) S. G. Waley and G. Watson, *Biochem. J.*, **55**, 328 (1953).

sion method, 6–8.4 insulin units/ml. Since the theoretical amount of insulin formed, based on the amount of B chain S-sulfonate used, is 20 units/ml (0.8 mg of insulin/ml), the combination yield in these experiments ranges from 30 to 42%. This half-synthetic human insulin and, furthermore, the all-synthetic human insulin were isolated in highly purified form as the hydrochloride salts and found to possess by the mouse convulsion method 22–24 IU/mg. The experimental details of this part of the work will be reported in a later communication.[25]

Combination of Natural Porcine (Human) A Chain and Natural Bovine B Chain. Porcine insulin was sulfitolyzed and its A and B chains were isolated in the S-sulfonated form using the conditions employed for the preparations of the bovine insulin chains.[19] Combination of the A chain S-sulfonate (20 mg) thus obtained with natural bovine B chain S-sulfonate (5 mg) was carried out as described previously. The solution of the recombination mixture (total volume approximately 10 ml) upon assay by the mouse convulsion method was shown to possess 7–10 insulin units/ml. On the basis of the amount of the B chain S-sulfonate used, the recombination yield for the natural chains is 35–50% of the theoretically expected value.

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

(25) P. G. Katsoyannis, A. Trakatellis, C. Zalut, S. Johnson, A. Tometsko, G. Schwartz, and J. Ginos, *Biochemistry*, in press.

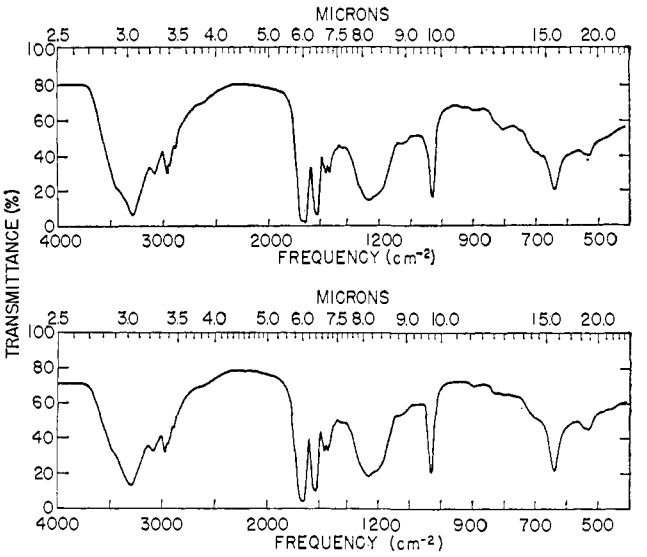


Figure 13. Infrared spectrum of the S-sulfonates of synthetic human A chain (top) and natural porcine A chain (bottom).

Studies on the Mechanism of Biotin Catalysis. II[1,2]

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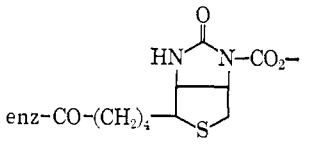
Contribution from the Department of Biochemistry, Yale University, New Haven, Connecticut 06510. *Received March 22, 1967*

Abstract: A kinetic study of the decarboxylation of a carboxybiotin analog, N-carboxy-2-imidazolidone, has been carried out. The rate law for decarboxylation is: rate = $k_1(S) + k_2(H_3O^+)(S) + k_3(BH)(S)$, where (S) and (BH) are the concentrations of the carboxyimidazolidone anion and acidic form of the buffer. At 25°, k_1 is 2.5×10^{-3} min^{-1}, k_2 is 3.5×10^5 M^{-1} min^{-1}, and k_3 is proportional to the acid dissociation constant of the buffer. At 6° the Brønsted α value, determined from a study with nine acidic catalysts, is 0.9. Metal ions prevent decarboxylation of carboxyimidazolidone; the dissociation constants for the metal complexes with Cu(II) and Mn(II) are 5.5×10^{-5} M and 3.5×10^{-3} M, respectively. The refractoriness of the carboxyimidazolidone anion is attributed to the poor leaving ability of the imidazolidone anion, and the insensitivity of this compound to general and specific acid catalysis is ascribed to the low basicity of the ureido system of the carbamic acid.

Carbon dioxide transfer reactions mediated by biotin–enzymes have been demonstrated to proceed by a mechanism in which an N-carboxybiotin intermediate I is formed in an initial reaction with ATP and bicarbonate.[3] Although this pathway is well documented, relatively little is known about the kinetic properties of N-carboxybiotin or the susceptibility of this compound to different types of catalytic interactions. We report here a detailed study of the kinetics of the decarboxylation of a similar compound, N-carboxy-2-imidazolidone, and the reaction of this compound with metal ions, hydronium ion, and general acids.



I

Experimental Section

Materials. Phenyl chloroformate containing ^{14}C in the carbonyl group was synthesized by the dropwise addition of 4.1 ml of a freshly prepared 2.44 M aqueous sodium phenolate solution to an ice-cold solution containing 1 mcurie (0.6 mcurie/mmole) of phosgene-^{14}C dissolved in 10 ml of a 12.5% solution of phosgene in benzene. After the addition of phenolate was complete, 4 ml of redistilled nonradioactive phenyl chloroformate was added.

(1) Supported by a grant from the National Institutes of Health (GM 11820).

(2) For the first paper in this series see M. Caplow, *J. Am. Chem. Soc.*, **87**, 5774 (1965).

(3) (a) S. Ochoa and Y. Kaziro, *Comp. Biochem.*, **16**, 210 (1966); (b) F. Lynen, J. Knappe, and E. Lorch, *Proc. Intern. Congr. Biochem.*, *5th, Moscow, 1961*, **4**, 225 (1963); (c) H. G. Wood and M. F. Utter, *Essays Biochem.*, **1**, 1 (1965).