precipitated hydrazide was isolated by filtration, washed with water, dried, and reprecipitated from dimethyl sulfoxide–water; wt, 2.2 g (73%); mp 275° dec; $[\alpha]^{27}D$ −27.5° (*c* 1, DMSO).

Anal. Calcd for $C_{56}H_{78}N_{12}O_{14}S_2$: C, 55.7; H, 6.47; N, 13.9. Found: C, 55.3; H, 6.78; N, 13.7.

This hydrazide was also prepared in 73% yield if instead of the nonapeptide methyl ester the corresponding ethyl ester (compound X) was used.

Acknowledgment. The authors wish to express their appreciation to Miss Karin Scheibe for the amino acid analyses.

Insulin Peptides. XV. The Synthesis of the A Chain of Sheep Insulin and Its Combination with Synthetic or Natural B Chain to Produce Insulin[1,2]

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Received July 8, 1966

Abstract: The A chain of sheep insulin has been synthesized and isolated in a highly purified form as the S-sulfonated derivative. The key intermediate in this synthesis was the partially protected heneicosapeptide that embodies the amino acid sequence of the sheep insulin A chain. This intermediate was prepared by three routes: (a) the carbonyldiimidazole coupling of the N-terminal nonapeptide subunit with the C-terminal dodecapeptide subunit, (b) the azide coupling of a differently blocked derivative of the N-terminal nonapeptide fragment with the C-terminal dodecapeptide fragment, and (c) most efficiently by the azide coupling of the N-terminal tetrapeptide subunit with the C-terminal heptadecapeptide subunit. The partially protected heneicosapeptide obtained by any one of these procedures was converted to the A chain S-sulfonate by removing the protecting groups and sulfitolyzing the ensuing product. Purification of the crude material was accomplished by chromatography on Sephadex. The purified A chain S-sulfonate thus obtained was homogeneous as judged by paper chromatography, paper electrophoresis, and amino acid analysis and optically pure as judged by complete digestion with leucine aminopeptidase. The synthetic product was compared with natural bovine A chain S-sulfonate as to electrophoretic mobility in two pH values, paper chromatography in two solvent systems, chromatographic pattern on Sephadex G-50, and infrared pattern. In all these comparisons the synthetic sheep A chain S-sulfonate and the natural bovine A chain S-sulfonate exhibited an identical behavior. The synthetic A chain was converted to its sulfhydryl form and combined with the natural bovine B chain S-sulfonate. Upon this combination hybrid insulin (one chain synthetic, the other chain natural) was produced in yields ranging from 30 to 38% of theory based on the amount of the B chain S-sulfonate used. Finally, the sulfhydryl form of the synthetic A chain was combined with a synthetic preparation of sheep insulin B chain S-sulfonate. As a result of this combination all-synthetic sheep insulin was produced in yields ranging from 4.7 to 5.7%.

Pioneering studies by Sanger and co-workers, which marked the beginning of a new era in the structural chemistry of proteins, have led to the elucidation of the complete amino acid sequence and subsequently the over-all structure of insulin from various species.[3] Structure I was postulated for sheep insulin.

It may be pointed out that this protein is made up of two polypeptide chains, the A chain containing 21 amino acid residues and the B chain containing 30 amino acid residues, linked together by two disulfide bridges. In addition there is an intra-chain disulfide bridge in the A chain.

Concurrently with the strides in structural protein analysis, there has been a comparable development in the synthetic field.[4] Impressive advances in peptide synthetic methodology, in purification techniques, and in methods of assessing chemical and stereochemical homogeneity have made the synthesis of large polypeptide chains attainable.[5] On this basis, we undertook the synthesis of insulin, making the assumption that air oxidation of a mixture of the sulfhydryl forms of chemically synthesized A and B chains should generate insulin.[5,6] This assumption was verified with natural insulin chains even before the completion of our synthetic work. Dixon and Wardlaw[7] and shortly thereafter Du, *et al.*,[8] reported the cleavage of insulin to its two chains by oxidative sulfitolysis, namely by treatment with sodium sulfite in the pres-

(1) The authors wish to express their appreciation to the U. S. Atomic Energy Commission and to the National Institute of Arthritis and Metabolic Diseases, U. S. Public Health Service, for support of this work.

(2) Preliminary report of part of the results described in this paper has appeared: P. G. Katsoyannis, A. Tometsko, and K. Fukuda, *J. Am. Chem. Soc.*, **85**, 2863 (1963).

(3) F. Sanger and H. Tuppy, *Biochem. J.*, **49**, 463, 481 (1951); F. Sanger and E. O. L. Thompson, *ibid.*, **53**, 353, 366 (1953); H. Brown, F. Sanger, and R. Kitai, *ibid.*, **60**, 556 (1955); J. I. Harris, F. Sanger, and M. A. Naughton, *Arch. Biochem. Biophys.*, **65**, 427 (1956).

(4) For a review see: K. Hofmann and P. G. Katsoyannis, "The Proteins," Vol. 1, H. Neurath Ed., Academic Press Inc., New York, N. Y., 1963, pp 53–188; E. Schröder and K. Lübke, "The Peptides," Vol. II, Academic Press Inc., New York, N. Y., 1966.

(5) P. G. Katsoyannis, *J. Polymer Sci.*, **49**, 51 (1961).

(6) P. G. Katsoyannis, K. Fukuda, and A. Tometsko, *J. Am. Chem. Soc.*, **85**, 1681 (1963).

(7) G. H. Dixon and A. C. Wardlaw, *Nature*, **188**, 721 (1960).

(8) Y.-C. Du, Y.-S. Zhang, Z.-X. Lu, and C.-L. Tsou, *Sci. Sinica*, (Peking), **10**, 84 (1961).

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ence of a mild oxidizing agent such as sodium tetrathionate ($Na_2S_4O_6$). Isolation of the chains in the S-sulfonated form was followed by reconversion to their sulfhydryl form on exposure to mercaptoethanol or thioglycolic acid. Finally insulin was regenerated by air oxidation of a mixture of the sulfhydryl forms of forms of the corresponding synthetic chains according to the scheme in Figure 1 led to the first chemical syntheses of the respective insulins.[12] In the present communication we wish to give detailed experimental procedures for the preparation and isolation in the S-sulfonated form of the A chain of sheep insulin.

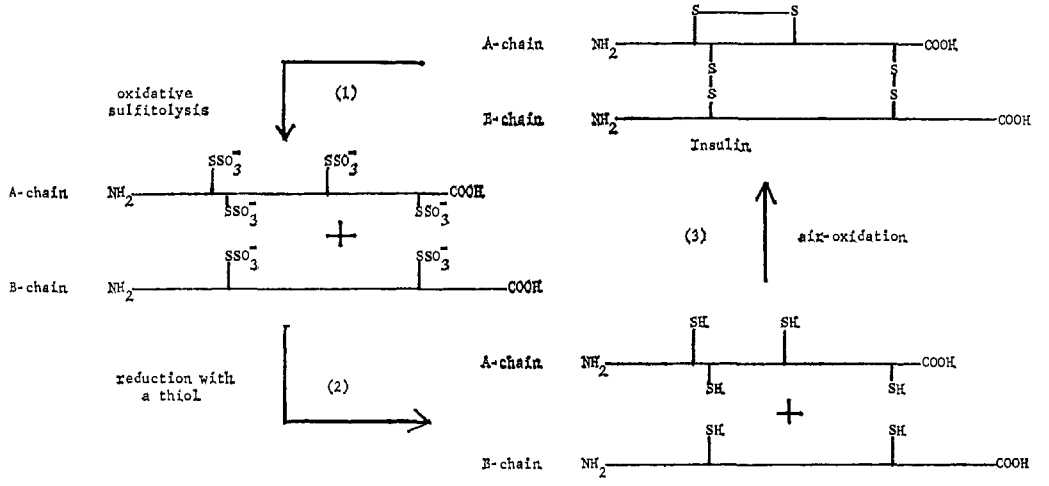


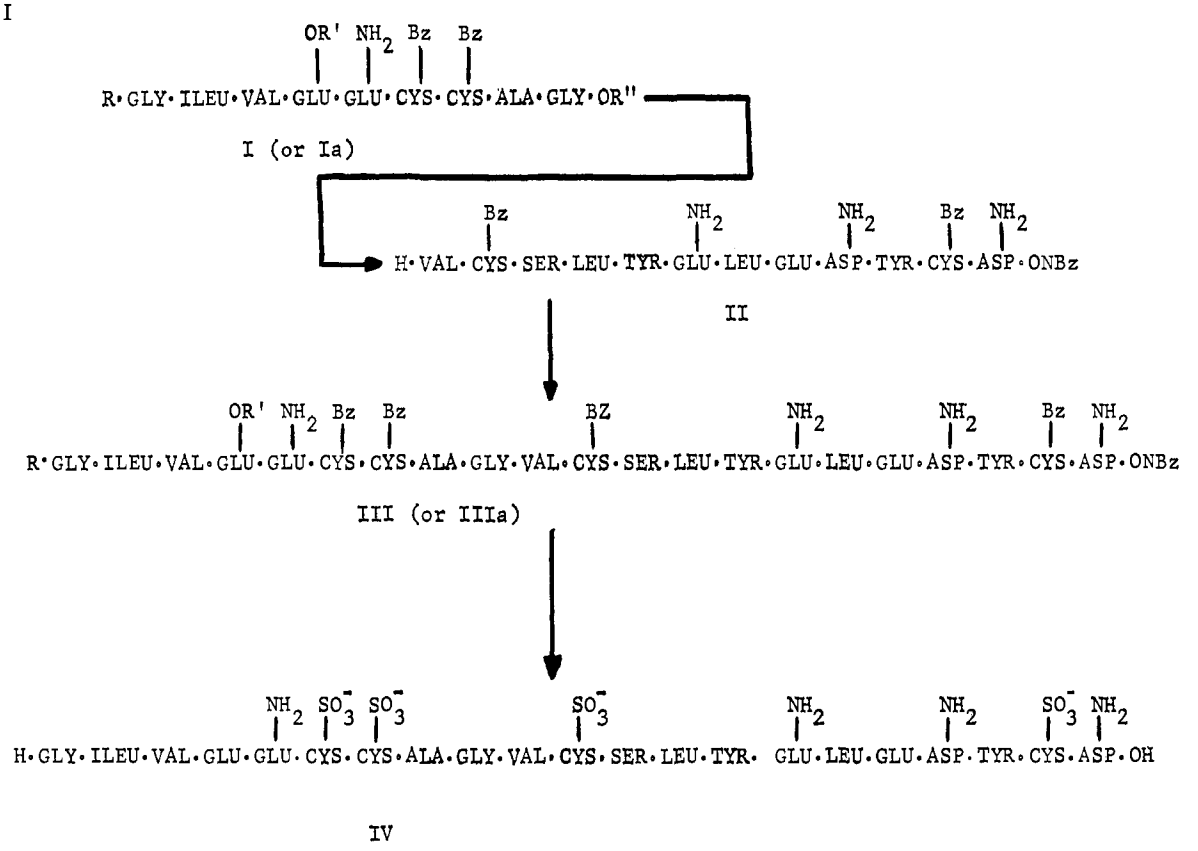
Figure 1. Cleavage and resynthesis of insulin: (1) oxidative sulfitolysis with $Na_2SO_3 + Na_2S_4O_6$ to give the S-sulfonated derivatives of the A and B chains, (2) conversion of the S-sulfonated chains to the respective sulfhydryl form on exposure to a thiol, (3) air oxidation of a mixture of the sulfhydryl chains to form insulin.

the two chains. The cleavage and regeneration of insulin is illustrated in Figure 1. Following these reports we have completed and reported in preliminary communications the synthesis and isolation in the S-sulfonated form of the A and B chains of sheep[2,9] and human insulin.[10,11] Combination of the sulfhydryl

In view of the fact[4] that the protecting groups N-carbobenzoxy, N-*p*-nitrocarbobenzoxy, O-benzyl, O-*p*-nitrobenzyl, and S-benzyl could be cleaved by sodium in liquid ammonia and that the protecting group O-*t*-butyl could be cleaved on exposure to trifluoroacetic acid,[13] we thought that if we could prepare the protected A chain III (Chart I), the removal of the

(9) P. G. Katsoyannis, K. Fukuda, A. Tometsko, K. Suzuki, and M. Tilak, *J. Am. Chem. Soc.*, 86, 930 (1964).
(10) P. G. Katsoyannis, A. Tometsko, J. Ginos, and M. Tilak, *ibid.*, 88, 164 (1966).
(11) P. G. Katsoyannis, A. Tometsko, and C. Zalut, *ibid.*, 88, 166 (1966).
(12) P. G. Katsoyannis, *Am. J. Med.*, 40, 652 (1966).
(13) (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).

Chart I



I, R=NZ, R'=t-But, R''=H

Ia, R=Z, R'=H, R''=N_3

III, R=NZ, R'=t-But

IIIa, R=Z, R'=H

blocking groups with trifluoroacetic acid and sodium in liquid ammonia[14] followed by sulfite–tetrathionate treatment should give the S-sulfonate of the A chain.

In the early synthetic studies[2] the desired protected heneicosapeptide III was prepared (procedure A) by coupling the N-terminal nonapeptide subunit N-*p*-nitrocarbobenzoxyglycyl-L-isoleucyl-L-valyl-γ-*t*-butyl-L-glutamyl-L-glutaminyl-S-benzyl-L-cysteinyl-S-benzyl-L-cysteinyl-L-alanylglycine[6] (I) with the C-terminal dodecapeptide subunit L-valyl-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) which in turn was prepared from its N-carbobenzoxy derivative[15] by treatment with HBr in trifluoroacetic acid.

Condensation of I with II was effected by the use of N,N^1-carbonyldiimidazole according to the method of Anderson.[16] Without any further purification the protected heneicosapeptide III was exposed successively to HBr in trifluoroacetic acid and to sodium in liquid ammonia, whereupon the *p*-nitrocarbobenzoxy, *t*-butyl, benzyl, and *p*-nitrobenzyl protecting groups were removed. In the early stages of this work, it appeared that during the sodium in liquid ammonia treatment of the protected A chain some degree of desulfuration had taken place.[17] The cysteine content of the isolated A chain S-sulfonate, as judged by amino acid analysis of an acid hydrolysate, was less than theoretically expected (molar ratio 3.5 instead of 4.0) whereas the alanine content was high[2] (molar ratio 1.4 instead of 1.0). In subsequent studies, however, we were able to avoid this side reaction by adding the sodium to the liquid ammonia solution of the partially protected heneicosapeptide in very small pieces and allowing the faint blue color, indicative of the completion of the reaction, to persist for only 1 min. The reduced material obtained after evaporation of the ammonia was subjected to oxidative sulfitolysis by treatment with sodium sulfite and sodium tetrathionate at pH 8.9, upon which the sulfhydryl functions were converted to the S-sulfonates.

For purification, the S-sulfonated form of the sheep insulin A chain (IV) thus obtained was passed at first through a Sephadex G-25 column equilibrated with 1 *M* pyridine and then through a Sephadex G-25 column equilibrated with 1% acetic acid. The purified material was obtained as a white fluffy powder after lyophilization of the effluent from the last Sephadex column. Based on the amount of the dodecapeptide subunit used, the over-all yield in pure A chain S-

(14) R. H. Sifferd and V. du Vigneaud, *J. Biol. Chem.*, 108, 753 (1935).

(15) P. G. Katsoyannis, A. Tometsko, and C. Zalut, *J. Am. Chem. Soc.*, 88, 5618 (1966).

(16) G. W. Anderson and R. Paul, *ibid.*, 80, 4423 (1958); R. Paul and G. W. Anderson, *ibid.*, 82, 4596 (1960).

(17) P. G. Katsoyannis, Excerpta Medica International Congress Series, No. 83, Excerpta Medical Foundation, New York, N. Y., 1964, pp 1216–1230.

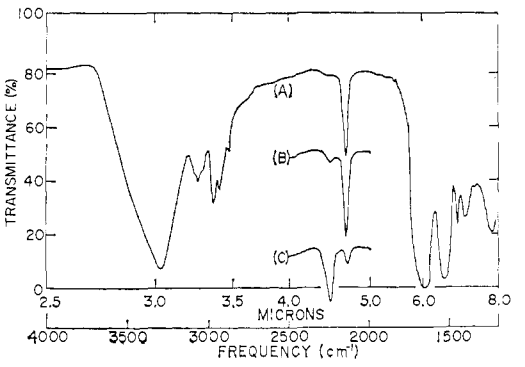


Figure 2. Infrared spectrum of the N-terminal nonapeptide azide (Ia): azide absorption 4.75 μ, isocyanate absorption 4.5 μ; (A) 3 hr at 0°; (B) 74 hr at 0°; (C) 124 hr at 25°.

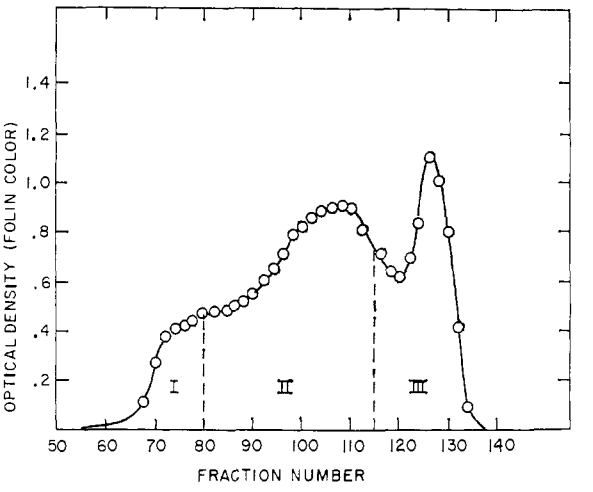


Figure 3. Elution pattern of crude synthetic sheep A chain S-sulfonate from a G-25 Sephadex column equilibrated with 1 *M* pyridine.

sulfonate (IV) is approximately 25%. This over-all yield includes the coupling step between the nonapeptide and dodecapeptide subunits, the deblocking steps, and the purification steps.

In later synthetic studies the preparation of the protected heneicosapeptide was approached (procedure B) by the azide coupling of the N-terminal nonapeptide fragment with the C-terminal dodecapeptide derivative. Thus, interaction of an excess of N-carbobenzoxyglycyl-L-isoleucyl-L-valyl-L-glutamyl-L-glutaminyl-S-benzyl-L-cysteinyl-S-benzyl-L-cysteinyl-L-alanylglycine azide (Ia, Chart I), which was prepared from the respective hydrazide[18] in the usual way, with L-valyl-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) afforded the partially protected heneicosapeptide IIIa. In preliminary experiments this azide coupling was effected by adding an excess of the solid azide Ia to a solution of II in DMF and allowing the reaction to proceed for 24 hr at 0°. Under these conditions, however, the yield of the final product was very low. This prompted us to investigate the stability of the azide Ia by infrared spectroscopy as suggested by Schwyzer and Kappeler,[19] taking advantage of the fact that while an azide has a characteristic band at 4.75 μ, its principal decomposition product, namely the isocyanate, shows a sharp band at 4.5 μ. The results of this study are illustrated in Figure 2. As can be seen, the nonapeptide azide Ia is an extremely stable compound. After 72 hr at 0° a trace of isocyanate has been formed and only after 124 hr at 25° the azide has been converted largely to isocyanate. In view of this observation we allowed the azide Ia to react with the dodecapeptide II for 72 hr at 5° and we obtained the desired product IIIa in satisfactory yields.

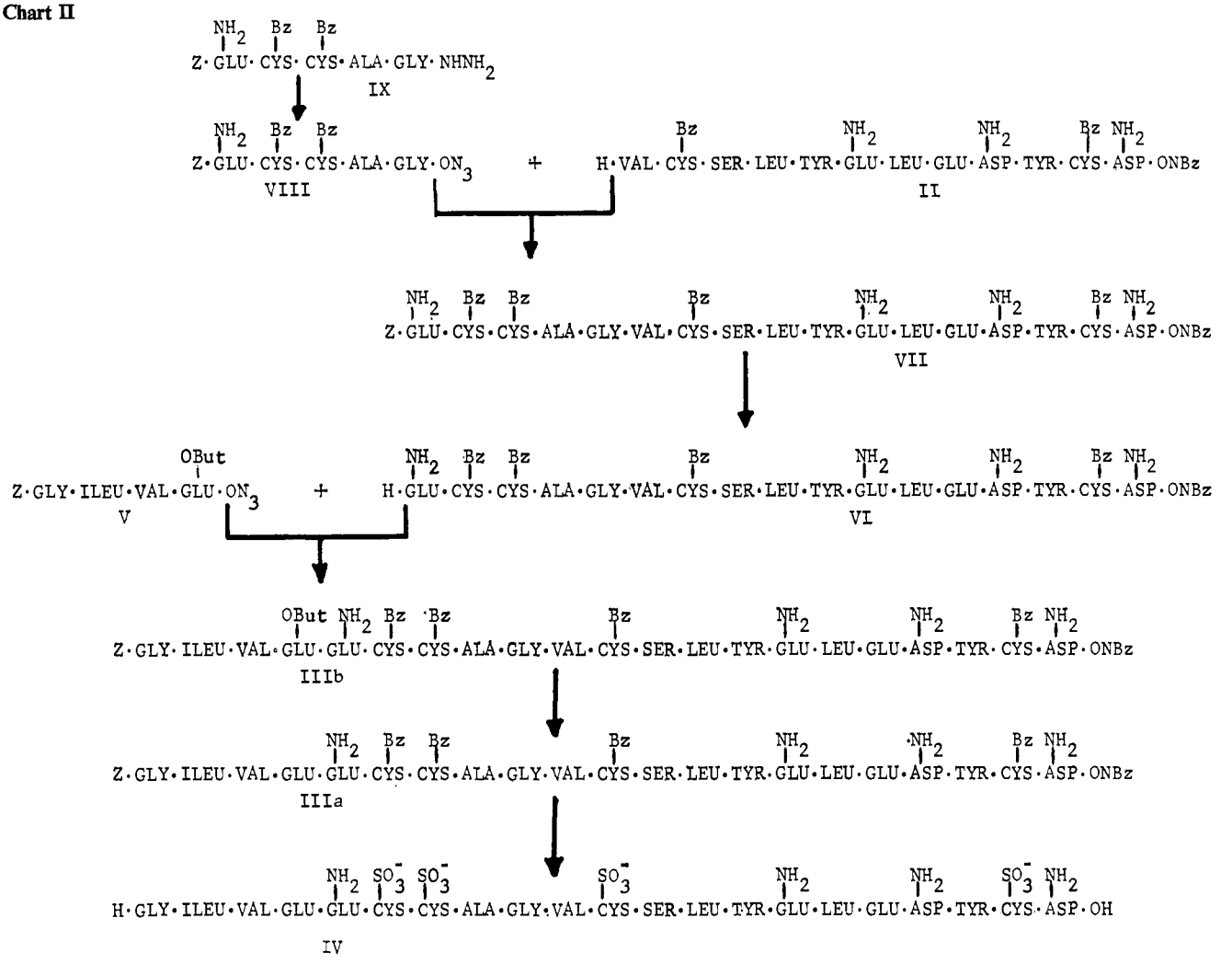
On exposure to sodium in liquid ammonia all the protecting groups from IIIa were cleaved and the reduced product obtained was dissolved in 8 *M* guanidine hydrochloride and sulfitolyzed at pH 8.9 for 24 hr. Dialysis of the reaction mixture against water followed by lyophilization gave the crude A chain S-sulfonate as a white fluffy powder. Sulfitolysis of the reduced A chain in 8 *M* guanidine hydrochloride followed by dialysis and lyophilization has many advantages over the procedure discussed earlier. This method eliminates completely the formation of insoluble products which accompany sulfitolysis in water and which must be filtered off before proceeding to the chromatographic step. All inorganic salts and low molecular weight by-products formed during the liquid ammonia cleavage are removed by dialysis, and thus over-loading the Sephadex column with nonpeptide material is avoided. This last aspect becomes of great importance when large-scale preparations are to be carried out.

For purification the lyophilized material was dissolved in 1 *M* pyridine, applied to a Sephadex G-25 column equilibrated with 1 *M* pyridine, and eluted with the same buffer. From the elution pattern of this column, shown in Figure 3, it is apparent that three components (I, II, and III) are present and partly separated. Amino acid analysis of acid hydrolysates of each of these components indicated that I is mostly unreacted nonapeptide, II is mostly A chain S-sulfonate, and III is mostly unreacted dodecapeptide. Apparently the A chain, because it contains aromatic amino acids, is retarded on the column[20] and is eluted after the nonapeptide, although the latter has a lower molecular weight. Fractions under peak II were pooled and lyophilized, and the solid residue obtained was dissolved in 1% acetic acid and passed through a second Sephadex G-25 column equilibrated with 1% acetic acid. The elution pattern is shown in Figure 4. The fractions under peak II, upon lyophilization, yielded highly purified A chain S-sulfonate. The over-all yield in purified material, based on the amount of the dodecapeptide used, is approximately 37% of theory. The synthesis of the A chain S-sulfonate by this procedure was repeated 10 times and the results originally obtained were confirmed.

Although the preparation of the A chain S-sulfonate by coupling the N-terminal nonapeptide subunit with the C-terminal dodecapeptide fragment either by the carbonyldiimidazole method or preferably by the azide method was satisfactory, we felt that confirmation of the synthesis by a third route was desirable.

(18) P. G. Katsoyannis, A. Tometsko, and C. Zalut, *J. Am. Chem. Soc.*, 88, 5622 (1966).
(19) R. Schwyzer and H. Kappeler, *Helv. Chim. Acta*, 44, 1991 (1961).
(20) J. Porath, *Biochim. Biophys. Acta*, 39, 193 (1960).

Chart II



Consequently, we have worked out a third approach which, because of the high yields and the facile purification of the final product, is in our opinion the best method for the synthesis of the A chain S-sulfonate. In this new approach, which is illustrated in Chart II, the protected heneicosapeptide is constructed by the coupling of a tetrapeptide fragment with a heptadecapeptide subunit. The pentapeptide N-carbobenzoxy-L-glutaminyl-S-benzyl-L-cysteinyl-S-benzyl-L-cysteinyl-L-alanylglycine methyl ester, whose synthesis was described in the preceding communication,[18] on exposure to hydrazine afforded the hydrazide IX which in turn was converted to the corresponding azide VIII and then condensed with the dodecapeptide derivative II to give the heptadecapeptide N-carbobenzoxy-L-glutaminyl-S-benzyl-L-cysteinyl-S-benzyl-L-cysteinyl-L-alanylglycyl-L-valyl-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 75% yield. This heptadecapeptide derivative contains the amino acid sequence found at the carboxyl terminus of the A chain of sheep insulin. Elemental analysis and amino acid analysis of an acid hydrolysate established the chemical purity of this compound. The decarbobenzoxylated derivative did not move in any of the solvent systems routinely used in our work for paper chromatography and hence no paper chromatographic data could be obtained. Decarbobenzoxylation of VII on exposure to HBr in trifluoroacetic acid and coupling of the ensuing product VI with an excess of the azide of the tetrapeptide N-carbobenzoxyglycyl-L-isoleucyl-L-valyl-γ-*t*-butyl-L-glutamic acid[18] (V),

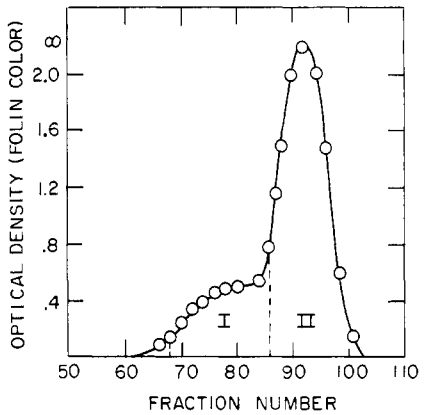


Figure 4. Elution pattern of partially purified (G-25 Sephadex, 1 *M* pyridine) synthetic sheep A chain S-sulfonate from a G-25 Sephadex column equilibrated with 1% acetic acid.

which contains the amino acid sequence found at the amino terminus of the A chain, afforded the protected heneicosapeptide IIIb in 85% yield. On treatment with trifluoroacetic acid the *t*-butyl group was removed from IIIb and the partially protected derivative, N-

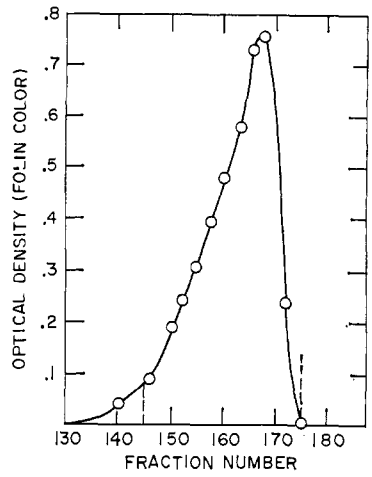


Figure 5. Elution pattern of synthetic sheep A chain S-sulfonate from a G-50 Sephadex column equilibrated with 1 *M* pyridine.

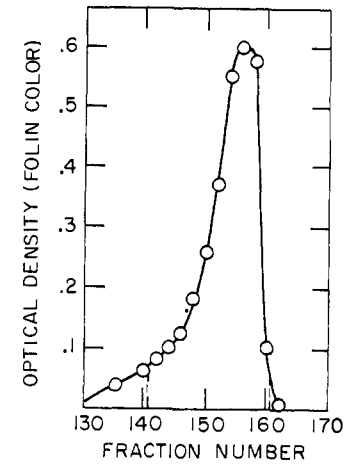


Figure 6. Elution pattern of natural bovine A chain S-sulfonate from a G-50 Sephadex column equilibrated with 1 *M* pyridine.

carbobenzoxyglycyl-L-isoleucyl-L-valyl-L-glutamyl-L-glutaminyl-S-benzyl-L-cysteinyl-S-benzyl-L-cysteinyl-L-alanylglycyl-L-valyl-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) was obtained in 93% yield. Elemental analysis and amino acid analysis of an acid hydrolysate demonstrated that the latter compound was obtained in analytically pure form. The sodium in liquid ammonia method was employed to cleave all the blocking groups from the heneicosapeptide derivative IIIa. The reduced product obtained after evaporation of the ammonia was sulfitolyzed in 8 *M* guanidine hydrochloride at pH 8.9, dialyzed against water, and lyophilized. Chromatography of the lyophilized product on Sephadex G-50 using 1 *M* pyridine as the eluting buffer afforded the A chain S-sulfonate in a highly purified form and in 86% yield. The elution pattern of this chromatographic step is shown in Figure 5. An identical elution pattern was obtained when natural bovine A chain S-sulfonate[21] was chromatographed on a similar column as shown in Figure 6. The synthesis of the A chain S-sulfonate by this procedure has been repeated three times and the results originally obtained were confirmed.

Amino acid analysis of an acid hydrolysate of the synthetic material, prepared by any of the three procedures discussed earlier, gave a composition in molar ratios in excellent agreement with the theoretically expected. Digestion of the synthetic product with leucine aminopeptidase (LAP) and amino analysis of the digest showed that the constituent amino acids were present in the ratios expected by theory and thus established the stereochemical homogeneity of the synthetic chain. On paper chromatography in two solvent systems and on high voltage paper electrophoresis at two pH values the synthetic material exhibited a single Pauly- and ninhydrin-positive spot indicative of the presence of a single component.

Since natural sheep A chain S-sulfonate was not available to us, we compared the synthetic material with natural bovine A chain S-sulfonate[21] which differs from the sheep A chain in only one amino acid residue. Instead of a glycine residue in position 9 the bovine A chain contains a serine residue. On paper chromatography in two solvent systems, on high voltage paper electrophoresis in two pH values, and on Sephadex G-50 chromatography the synthetic A chain S-sulfonate and the natural bovine A chain S-sulfonate exhibit the same behavior. No differences were detected in the infrared patterns of these compounds. The synthetic material possessed the specific activity $[\alpha]^{27}D$ −73.9° (*c* 1, water) compared to $[\alpha]^{22}D$ −70° (*c* 1, water) for natural bovine A chain S-sulfonate.

The synthetic sheep A chain S-sulfonate was converted to its sulfhydryl form and combined with the natural bovine B chain S-sulfonate. The combination was effected by the method developed recently in our laboratory[22] with a slight modification. Thus, instead of air oxidizing the mixture of A and B chains at pH 10.6, we carried out this step at pH 9.6. No difference in the yield of insulin formed could be detected by the change in pH. Upon this combination, hybrid insulin was produced in yields ranging from 30 to 38% of theory (based on the amount of the B chain S-sulfonate used) and crystallized.[12] The experimental details of the isolation of crystalline hybrid insulin will be reported in a later paper.[23] The synthetic A chain was further combined by the same procedure with a synthetic preparation of sheep insulin B chain S-sulfonate.[9] Upon this combination, all-synthetic insulin was formed in yields ranging from 4.7 to 5.7% of theory (based on the amount of the B chain S-sulfonate used) and isolated in crystalline form.[23]

After publication of a preliminary report of this work, Zahn, *et al.*,[24,25] reported the synthesis of a protected derivative of the A chain of sheep insulin by coupling the N-terminal nonapeptide with the C-terminal dodecapeptide fragments. Without conversion to its S-sulfonate form, this synthetic material

(21) Prepared from bovine insulin by a new procedure (see ref 12); P. G. Katsoyannis and A. Tometsko, unpublished data.

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

(23) P. G. Katsoyannis, A. Trakatellis, A. Tometsko, S. Johnson, C. Zalut, and G. Schwartz, to be published.

(24) H. Zahn, H. Bremer, and R. Zabel, *Z. Naturforsch.*, **20b**, 653 (1965).

(25) J. Meienhofer, E. Schnabel, H. Bremer, O. Brinkhoff, R. Zabel, W. Sroka, H. Klostermeyer, D. Brandenburg, T. Okuda, and H. Zahn, *ibid.*, **18b**, 1120 (1963).

was mixed with the natural bovine B chain S-sulfonate or with a synthetic preparation of protected B chain, and the mixture was treated with sodium in liquid ammonia. Air oxidation of the reaction mixture in both instances produced insulin in yields ranging from 0.2 to 1% of theory.[26]

Very recently, Wang, *et al.*, reported in a preliminary note without any experimental details the synthesis of bovine A chain S-sulfonate also by coupling the N-terminal nonapeptide with the C-terminal dodecapeptide subunits.[27] On combination with the natural B chain after conversion to its sulfhydryl form, these authors reported that the synthetic material produced insulin in 7% average yield. In a later paper, they further reported that the synthetic bovine A chain, on combination with a synthetic preparation of bovine B chain, afforded all-synthetic insulin in yields ranging from 1 to 2% of theory.[28] Although the quantitative aspects of the work reported by the Chinese investigators are quite unclear, it appears that the yield[8,28] of insulin produced is not calculated on the basis of the amounts of the chains used for the combination but on the basis of the specific activity of the final oxidation product. Since the final oxidation product has undergone a certain degree of purification, namely precipitation at an acidic pH, its specific activity is not a measure of the over-all combination yield.

Experimental Section

General analytical procedures used were those described in paper XIII of this series.[15]

For taking infrared spectra the following procedure was followed: 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. Pressures up to 20,000 lb were also used without any change in the final results. The infrared spectrum was taken with a Perkin–Elmer Infracord, Model 337. All operations were performed at 25°.

Paper electrophoresis was performed with a Wieland-Pfleiderer pherograph (Brinkmann Instruments) on Whatman No. 3MM paper. Biological assays by the mouse convulsion method were carried out as described in a previous report.[22]

The following abbreviations are used: $A_S\text{-}SSO_3^-$ = synthetic A chain S-sulfonate; $A_N\text{-}SSO_3^-$ = natural A chain S-sulfonate; Z = carbobenzoxy; NZ = *p*-nitrocarbobenzoxy; Bz = benzyl; NBz = *p*-nitrobenzyl; But = *t*-butyl; DMF = dimethylformamide; DMSO = dimethyl sulfoxide.

N-Carbobenzoxy-L-glutaminyl-S-benzyl-L-cysteinyl-S-benzyl-L-cysteinyl-L-alanylglycine Hydrazide (IX). A solution of N-carbobenzoxy-L-glutaminyl-S-benzyl-L-cysteinyl-S-benzyl-L-cysteinyl-L-alanylglycine methyl ester[18] (4 g) in DMF (250 ml) and methanol (20 ml) containing hydrazine hydrate (8 ml) was stirred at 40° for 24 hr and at room temperature for 48 hr. The reaction mixture was subsequently poured into water (500 ml) and the pH of the suspension adjusted to 6 with acetic acid. The precipitated product was filtered off, washed with water, dried, and reprecipitated from dimethyl sulfoxide–water; wt, 3.3 g (82%); mp 244–245°; $[\alpha]^{27}D$ −24° (*c* 1, DMSO).

Anal. Calcd for $C_{38}H_{48}N_8O_8S_2$: C, 56.4; H, 5.94; N, 13.9. Found: C, 56.3; H, 5.99; N, 13.6.

The same compound was obtained in 82% yield when the pentapeptide ethyl ester[6] was used instead of the methyl ester.

N-Carbobenzoxy-L-glutaminyl-S-benzyl-L-cysteinyl-S-benzyl-L-cysteinyl-L-alanylglycyl-L-valyl-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-valyl-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[15] (1 g) was dissolved in trifluoroacetic acid (20 ml) and water (0.4 ml) and HBr was passed through this solution for 1 hr at 0°. Addition of anhydrous ether to the reaction mixture caused the decarbobenzoxylated dodecapeptide ester to precipitate as the hydrobromide which was filtered off, washed with ether, and dried over KOH *in vacuo*. This product was dissolved in DMF (25 ml) containing triethylamine (0.4 ml), cooled to 0°, and caused to react with the pentapeptide azide VIII prepared as follows. N-Carbobenzoxy-L-glutaminyl-S-benzyl-L-cysteinyl-S-benzyl-L-cysteinyl-L-alanylglycine hydrazide (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), $NaNO_2$ (52 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 water (150 ml). The precipitated pentapeptide azide was isolated immediately 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*. Infrared spectrum of the product taken after drying for 3 hr exhibited the characteristic azide band at 4.75 μ without any trace of the isocyanate band. The solid azide was added to the solution of the decarbobenzoxylated dodecapeptide ester prepared as described previously. 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 protected heptadecapeptide was filtered off, washed successively with anhydrous methanol, 50% aqueous methanol, and water, and reprecipitated from dimethyl sulfoxide–water; wt, 1 g (75%); mp 270–271° dec; $[\alpha]^{27}D$ −31.2° (*c* 1, DMSO).

Anal. Calcd for $C_{121}H_{154}N_{22}O_{31}S_4$: C, 57.2; H, 6.07; N, 12.1. Found: C, 56.3; H, 5.92; N, 11.8.

Amino acid analysis of an acid hydrolysate of the protected heptadecapeptide showed the following composition expressed in molar ratios: $Asp_{2.0}Ser_{0.8}Glu_{3.0}Gly_{1.0}Ala_{1.0}Val_{1.0}Leu_{2.0}Tyr_{1.6}$S-benzylcysteine$_{3.7}$. The A chain S-sulfonate prepared from this compound, as it will be described later on, was completely digested by LAP.

Glycyl-L-isoleucyl-L-valyl-L-glutamyl-L-glutaminyl-S-sulfo-L-cysteinyl-S-sulfo-L-cysteinyl-L-alanylglycyl-L-valyl-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 (Sheep Insulin A Chain S-Sulfonate) (IV). A. Carbonyldiimidazole Coupling of N-Terminal Nonapeptide I with C-Terminal Dodecapeptide II. N-*p*-Nitrocarbobenzoxyglycyl-L-isoleucyl-L-valyl-γ-*t*-butyl-L-glutamyl-L-glutaminyl-S-benzyl-L-cysteinyl-S-benzyl-L-cysteinyl-L-alanylglycine[6] (I, 182 mg) was dissolved in DMF (20 ml) and N,N′-carbonyldiimidazole[29] (33 mg) was added and stirred for 1 hr at 0° and for 30 min at 25°. To this mixture was added a solution of the partially deblocked dodecapeptide II, prepared from its carbobenzoxy derivative (300 mg) as described in the synthesis of VII, in DMF (5 ml) containing triethylamine (0.2 ml). After stirring for 12 hr at 0° and for 24 hr at 25° the reaction mixture was poured into 0.5 *N* HCl (60 ml). The precipitated product (crude III) was filtered off, washed with water, and dried over P_2O_5; wt, 400 mg. This material was dissolved in trifluoroacetic acid (10 ml) and water (0.2 ml) and HBr was passed through the solution for 1 hr. Addition of ether to the reaction mixture caused precipitation of the partially protected heneicosapeptide which was then isolated by filtration, washed with ether, and dried over KOH *in vacuo*. This material was converted to the A chain S-sulfonate by reduction with sodium in liquid ammonia followed by oxidative sulfitolysis with sodium sulfite and sodium tetrathionate.

The thoroughly dry, partially protected heneicosapeptide was dissolved in anhydrous liquid ammonia (100 ml) in a 200-ml, round-bottomed flask fitted for magnetic stirring. The reaction was carried out at the boiling point of the solution. Cleavage 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 water (25 ml) and the pH of the solution was adjusted to 8.9 with 1 *N* acetic acid. To this solution sodium sulfite (500 mg) and freshly

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(29) We wish to express our appreciation to Dr. G. W. Anderson for a generous sample of N,N′-carbonyldiimidazole.

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

Number of amino acid residues per molecule					
	Acid hydrolysate			LAP digest	
Amino acid	Theory	Found	Amino acid	Theory	Found
Aspartic acid	2.00	2.00	Asparagine	2.00	Emerge on the same position, and not determined
Serine	1.00	0.90	Glutamine	2.00	
Glutamic acid	4.00	4.20	Serine	1.00	0.90[a]
Glycine	2.00	2.00	Glutamic acid	2.00	2.00
Alanine	1.00	1.10	S-Sulfocysteine	4.00	4.30[b]
Cysteine	4.00	4.00[c]	Glycine	2.00	2.00
Valine	2.00	1.80	Alanine	1.00	1.00
Isoleucine	1.00	0.70	Valine	2.00	2.00
Leucine	2.00	2.00	Isoleucine	1.00	1.00
Tyrosine	2.00	2.00	Leucine	2.00	2.00
Ammonia	4.00	4.00	Tyrosine	2.00	2.00

[a] Separated from glutamine and asparagine in a 30° chromatographic run. [b] Eluted from the long column of the Beckman-Spinco analyzer after 26 ml of effluent. [c] Determined as cysteic acid: S. Moore, *J. Biol. Chem.*, **238**, 235 (1963).

prepared sodium tetrathionate[30] (250 mg) was added. After stirring for 24 hr at room temperature, the reaction mixture was filtered to remove insoluble material, and the filtrate was lyophilized.

Isolation of Synthetic A Chain S-Sulfonate. The white fluffy material obtained upon lyophilization was dissolved in 1 *M* pyridine (7 ml) and applied to a Sephadex G-25 column (medium grade block polymerizate; 3 × 80 cm) which had been equilibrated with 1 *M* pyridine. The chromatogram was developed with 1 *M* pyridine at a flow rate of about 18 ml/hr. Fractions of 3 ml each were collected and the location of the eluted material was determined by the Folin–Lowry method.[31] Pooling of fractions 80–130, containing the fastest moving component, followed by lyophilization, afforded a fluffy white material (160 mg). This product was dissolved in 1% acetic acid (3 ml) and chromatographed on a Sephadex G-25 column (medium grade block polymerizate; 3 × 50 cm) which had been equilibrated with 1% acetic acid. Elution was carried out with 1% acetid acid at a flow rate of about 18 ml/hr, and fractions of 3 ml each were collected. Folin–Lowry color reaction indicated the presence of a fast-moving single peak with only traces of a slower moving component. Fractions under main peak (tubes 40–70) were pooled and lyophilized to yield the A chain S-sulfonate in a highly purified form; wt, 102 mg (25%, based on the amount of II used). Criteria of homogeneity will be discussed later on in this report.

B. Azide Coupling of N-Terminal Nonapeptide Ia with C-Terminal Dodecapeptide II. Partially protected dodecapeptide ester II, prepared from its carbobenzoxy derivative (300 mg) as described in the synthesis of VII, was dissolved in DMF (25 ml) containing triethylamine (0.2 ml), cooled to 0°, and allowed to react with the nonapeptide azide Ia prepared as follows. N-Carbobenzoxyglycyl-L-isoleucyl-L-valyl-L-glutamyl-L-glutaminyl-S-benzyl-L-cysteinyl-S-benzyl-L-cysteinyl-L-alanylglycine hydrazide[18] (300 mg) was dissolved in DMF (25 ml) containing 2 *N* HCl (2 ml) and cooled to −15° (Dry Ice–acetone). To this solution $NaNO_2$ (17.2 mg) dissolved in cold water (0.5 ml) was added. After stirring for 5 min at −15° the reaction mixture was poured into cold water (120 ml) and the precipitated azide was immediately filtered, washed with 1 *N* $NaHCO_3$ and water, and dried over P_2O_5 at 0° for 1 hr. The infrared spectroscopic analysis of the azide is shown in Figure 2. The dry nonapeptide 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 partially protected heneicosapeptide was filtered off, washed with water, and dried; wt, 500 mg. The final deblocking of this material with sodium in liquid ammonia (250 ml) was carried out as in procedure A. After removal 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 (1.2 g) and sodium tetrathionate (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. A Visking 18/32 dialyzing tubing was used. Lyophilization of the dialyzate gave the crude A chain S-sulfonate as a white fluffy powder; wt, 360 mg.

Isolation of Synthetic A Chain S-Sulfonate. The lyophilized material (220 mg) 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, three main peaks, I, II, and III, were detected by the Folin–Lowry method as illustrated in Figure 3.

Fractions 65–80 were pooled and lyophilized to give component I (20 mg). Amino acid analysis after acid hydrolysis gave the following composition expressed in micromoles: $Asp_{0.25}Ser_{0.12}Glu_{1.20}Gly_{0.83}Ala_{0.44}Val_{0.44}Ile_{0.28}Leu_{0.28}Tyr_{0.25}$ (cystine was not determined). This composition (high values for glycine and alanine, low values for leucine and aspartic acid) suggests that component I is mainly unreacted nonapeptide which is contaminated with A chain S-sulfonate.

Fractions 81–115 were pooled and lyophilized to yield component II (130 mg) which, after acid hydrolysis, gave an amino acid composition in micromoles, $Asp_{0.36}Ser_{0.20}Glu_{1.07}Gly_{0.50}Ala_{0.29}Val_{0.46}Ile_{0.18}Leu_{0.44}Tyr_{0.43}$ (cystine not determined), fairly close to the theoretically expected values for A chain S-sulfonate.

Finally, lyophilization of pooled fractions 116–130 yielded component III (60 mg) which, after acid hydrolysis, gave the following amino acid composition in micromoles: $Asp_{0.52}Ser_{0.24}Glu_{0.75}Gly_{0.15}Ala_{0.10}Val_{0.29}Ile_{0.06}Leu_{0.63}Tyr_{0.55}$ (cystine not determined). This composition (high values for aspartic acid and leucine, low values for isoleucine, glycine, and alanine) indicates that III is mainly unreacted dodecapeptide contaminated with A chain S-sulfonate.

For further purification, component II (100 mg) was combined with a similar product (160 mg) from another run on a larger scale. The material (260 mg) was dissolved in 1% acetic acid (10 ml) and chromatographed on Sephadex G-25 (fine grade, bead form) in 1% acetic acid 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 reaction is shown in Figure 4. Fractions 87–98 under the main peak were pooled and lyophilized to give highly purified A chain S-sulfonate; wt, 185 mg (37%, based on the amount of the C-terminal dodecapeptide used for the original coupling).

C. Azide Coupling of N-Terminal Tetrapeptide V with C-Terminal Heptadecapeptide VI. 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 filtration, washed with ether, 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[18] (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-

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

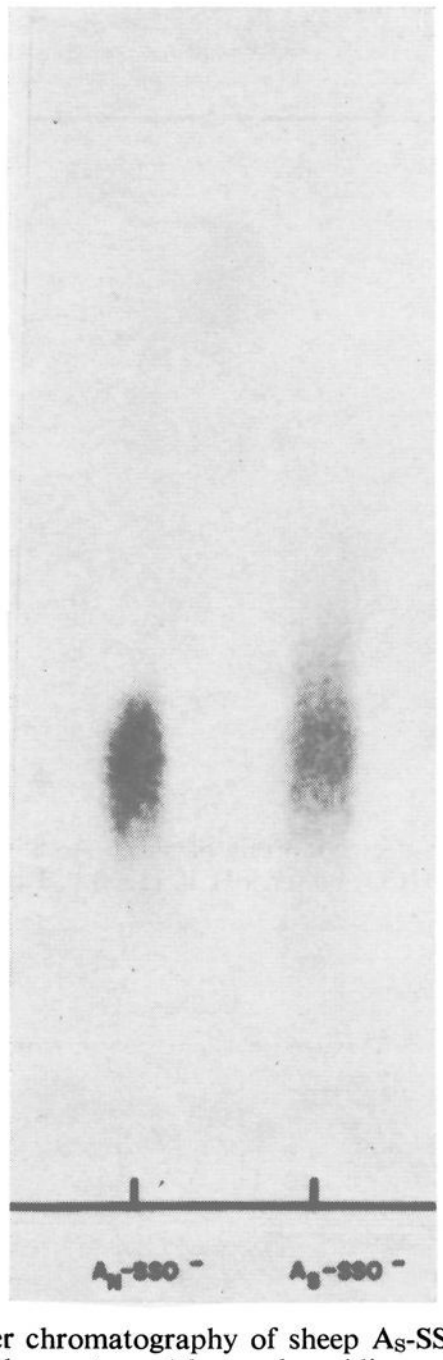

Figure 7. Paper chromatography of sheep A_S-SSO_3^- and bovine A_N-SSO_3^- in the system 1-butanol–pyridine–acetic acid–water (30:20:6:24).

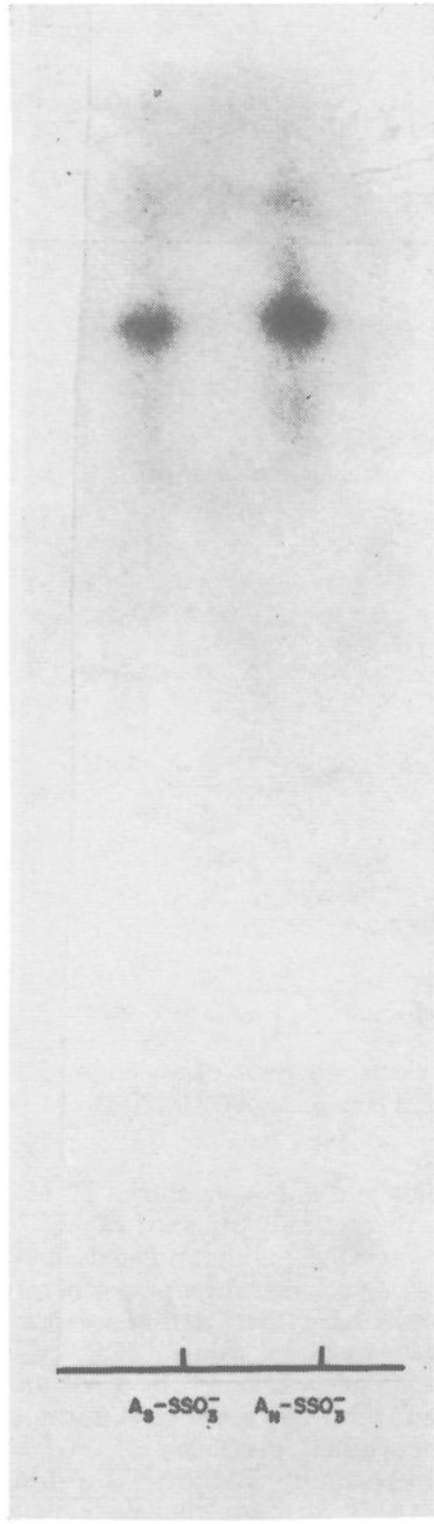

Figure 8. Paper chromatography of sheep A_S-SSO_3^- and bovine A_N-SSO_3^- in the system $CHCl_3$–CH_3OH–H_2O–NH_4OH (4:4:1:1).

saturated NaCl (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 100 ml) to prevent it from becoming solid. After 48 hr the mixture was poured into methanol (500 ml) containing acetic acid (1 ml). The precipitated protected heneicosapeptide IIIb was isolated by filtration, washed successively with absolute methanol, 50% aqueous methanol, and water, and dried; wt, 0.98 g (85%). A solution of this material in trifluoroacetic acid (20 ml) was stored at room temperature for 30 min and then filtered through a sintered glass filter to remove any traces of impurities. Dilution of the filtrate with anhydrous ether caused the precipitation of the partially protected heneicosapeptide, N-carbobenzoxyglycyl-L-isoleucyl-L-valyl-L-glutamyl-L-glutaminyl-S-benzyl-L-cysteinyl-S-benzyl-L-cysteinyl-L-alanylglycyl-L-valyl-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), in analytically pure form; wt, 0.9 g (93%); mp 288–290° dec; $[\alpha]^{27}D$ −19.1° (*c* 0.5, hexamethylphosphoramide).

Anal. Calcd for $C_{139}H_{184}N_{26}O_{37}S_4$: C, 56.8; H, 6.26; N, 12.4. Found: C, 56.2; H, 6.24; N, 12.4.

Amino acid analysis of an acid hydrolysate showed the following composition expressed in molar ratios: $Asp_{2.1}Ser_{0.9}Glu_{4.3}Gly_{2.0}$-$Ala_{1.0}Val_{1.7}Ile_{0.7}Leu_{2.1}Tyr_{1.7}$S-benzylcysteine$_{4.0}$, in good agreement with the values expected from theory.

The reduction of IIIa (327 mg) with sodium in liquid ammonia (250 ml) was accomplished 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 a 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; wt, 288 mg.

Sephadex G-50 Chromatography of Synthetic A Chain Sulfonate. The lyophilized material (288 mg) was dissolved in 1 *M* pyridine (10 ml) and applied to a Sephadex G-50 column (fine grade, bead form; 2.2 × 90 cm) which had been equilibrated with 1 *M* pyridine. The chromatogram was developed with 1 *M* pyridine at a flow rate of about 34 ml/hr. Fractions of 1.7 ml each were collected. Folin–Lowry analysis of the effluent gave the pattern shown in Figure 5. Fractions 145–175 were pooled and lyophilized to give highly purified A chain S-sulfonate; wt, 250 mg (86%, based on IIIa used.)

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

The synthetic material possessed the specific rotation $[\alpha]^{27}D$ −73.9° (*c* 1, water) compared to $[\alpha]^{22}D$ −70° (*c* 1, water) for natural bovine A chain S-sulfonate found by Wang, *et al.*[27]

Amino acid analysis by the method of Spackman, Stein, and Moore[32] of the synthetic chain after acid hydrolysis gave the molar

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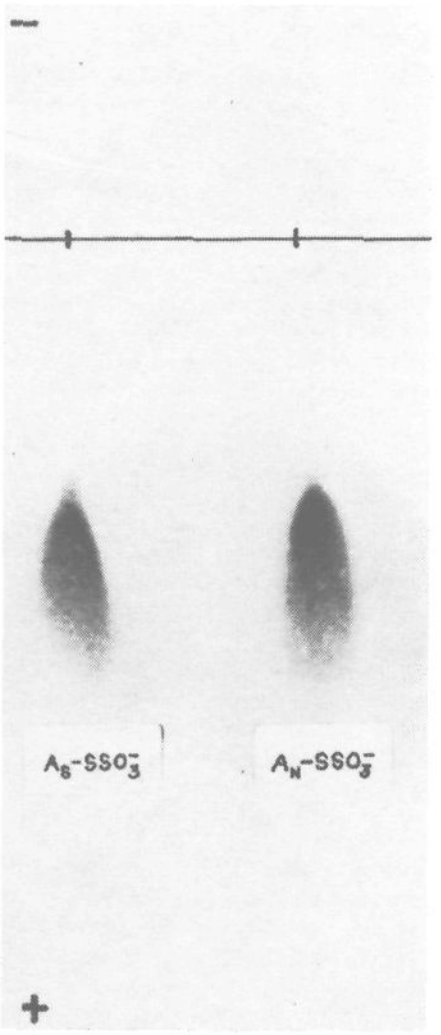

Figure 9. Paper electrophoresis of sheep A_S-SSO_3^- and bovine A_N-SSO_3^- in 0.5 *N* acetic acid, pH 2.9 (2000 v, 1 hr).

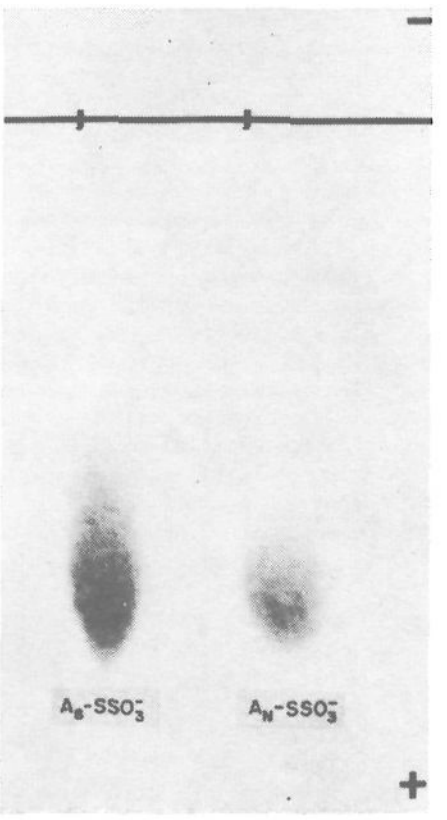

Figure 10. Paper electrophoresis of sheep A_S-SSO_3^- and bovine A_N-SSO_3^- in NH_4HCO_3 buffer, pH 10 (1900 v, 1 hr).

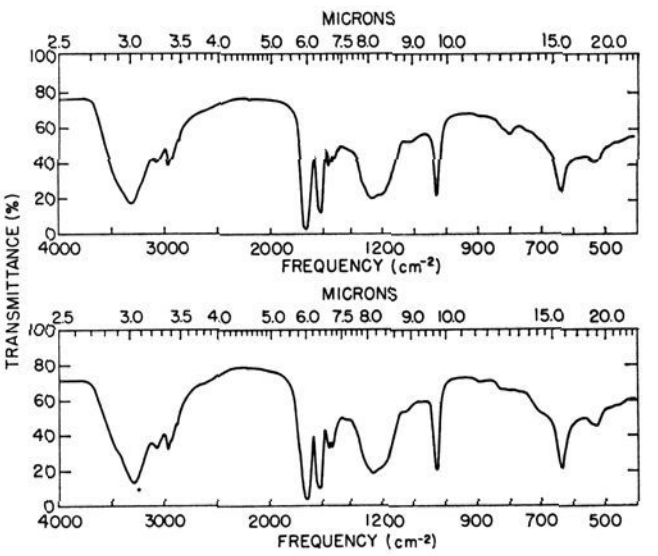

Figure 11. Infrared spectrum of sheep A_S-SSO_3^- (top) and bovine A_N-SSO_3^- (bottom).

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 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 theroretically 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.

On paper chromatography in the system 1-butanol–pyridine–acetic acid–water[33] (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 $CHCl_3$–CH_3OH–H_2O–NH_4OH (4:4:1:1) the synthetic and natural chains exhibited an identical behavior (R_f 1.07 × His) as shown in Figure 8. When 288 mg of the synthetic chain S-sulfonate was applied on Sephadex G-50 in the pyridine system, the pattern shown in Figure 5 was obtained. A similar chromatographic pattern, shown in Figure 6, was obtained when 50 mg of natural bovine A chain S-sulfonate was applied on the same column.

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 chain (Figure 9). Paper electrophoresis in NH_4HCO_3 buffer, pH 10 (1.4 g of NH_4HCO_3 was dissolved in 2 l. of H_2O and brought to pH 10.0 with concentrated NH_4OH), and 1900 v showed (Figure 10) that the synthetic and natural chains moved as single components (Pauly and ninhydrin reaction) and had the same mobility.

Finally, no differences were detected in the infrared spectra of the synthetic and natural chains as shown in Figure 11.

Combination of Synthetic A Chain with Natural B Chain to Generate Insulin. Synthetic sheep A chain S-sulfonate (20 mg) was dissolved in water (5 ml) which had been deaerated by extensive boiling under nitrogen. The solution was adjusted to pH 5 with 1 *N* acetic acid (glass electrode), cooled to 0°, and after adding 1 *M* mercaptoethanol (0.8 ml) and deaerating with an aspirator, heated in a boiling water bath for 6 min under nitrogen. The reaction mixture was then cooled to 10–15° and extracted four times with 40-ml portions of ethyl acetate. The resulting sulfhydryl form of the A chain was obtained as a jelly-like product after the traces of the ethyl acetate were removed from the mixture with a stream of nitrogen. This product was mixed with natural bovine B chain S-sulfonate (5 mg) and water (7 ml) and the ensuing suspension was solubilized by adjusting the pH to 9.6 with 1 *N* NaOH. The 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 five experiments the solution of the combination mixture (total volume about 10 ml) upon assay by the mouse convulsion method was shown to possess 6.1–7.8 insulin units per milliliter. Since the theoretical yield of insulin formed, based on the amount of B chain S-sulfonate used, is 20 units per milliliter, the combination yield in these experiments ranged from 30 to 38%. The insulin formed has been isolated in crystalline form[12,23] and the experimental details of this part of the work will be reported in a later communication.

Combination of Synthetic A Chain with Synthetic B Chain to Generate Insulin. Synthetic sheep A chain S-sulfonate (20 mg) was converted to the sulfhydryl form and allowed to react with a synthetic preparation of sheep B chain S-sulfonate[9] (5 mg) according to the procedure described previously. The solution of the recombination mixture (total volume 9.6 ml) upon assay by the mouse convulsion method was shown to possess 0.98–1.19 insulin units per milliliter. Based on the amount of the B chain S-sulfonate used, the combination yield for the all-synthetic sheep insulin is 4.7 to 5.7% of theory.

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

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 the Eli Lilly and Co. for a generous gift of crystalline bovine insulin.

Molecular Interactions in β-Lactoglobulin. X. The Stoichiometry of the β-Lactoglobulin Mixed Tetramerization[1]

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Abstract: The aggregation of β-lactoglobulins A, B, and mixtures of A and B at pH 4.65 has been measured by light scattering and sedimentation. The measurements have been subjected to detailed analysis. The previously postulated stoichiometry, that 30% of the β-lactoglobulin B can form mixed tetramers with 90% of the β-lactoglobulin A, has been discarded. A new, simpler interpretation is based on the assumption that all molecules can aggregate with three types of bonds of different strengths (A–A, B–B, A–B). These three bond types are sufficient to describe quantitatively the progressive tetramerization of mixed lactoglobulins, which can involve fifteen different aggregated species.

In previous communications[3-5] it has been reported that at low temperatures β-lactoglobulin A (β-A) undergoes a reversible tetramerization (from a 36,000 Dalton isoelectric unit to one of 144,000[6]) between pH 3.7 and 5.2 with 90% of the total protein capable of aggregation.[10] The tetramer formed was found to be a closed structure with 422 symmetry.[11,12] The values of the thermodynamic parameters are all negative.[3] The entropy and enthalpy differences become less negative as the pH is increased while the change in free energy passes through a maximum at pH 4.4–4.7.[3,4]

Light scattering and sedimentation data on mixtures of β-A and β-B were interpretable in terms of a stoichiometry in which 30% of the β-lactoglobulin B (β-B) could form mixed tetramers with 90% of the β-A[3,4] while, by itself, β-B could form aggregates no larger than dimers. This interpretation implied that β-A and β-B have the same specific bonding in the aggregation reaction. The aforementioned conclusion was based upon the observed unimodal sedimentation pattern with the B variant under aggregating conditions,[13] while in the case of β-A a bimodal pattern is observed. Gilbert,[14] in his earlier publications, had concluded that in aggregating systems bimodality could be observed in moving boundary experiments only if the degree of aggregation was to species greater than a dimer, while monomer–dimer equilibrium resulted in a single peak. It was assumed, therefore, that in the case of β-B, the formation of aggregates greater than a dimer was excluded; in β-A–β-B mixtures, however, the formation of mixed tetramers was necessary to account both for the light scattering and sedimentation results.[3,10] While this mechanism described in a quantitatively acceptable manner the light scattering data for a variety of β-A–β-B mixtures, the stoichiometry was rather cumbersome.

Recently, Gilbert[15] has shown that when aggregation is weak, the hydrodynamic concentration-dependent retardation of moving boundaries assumes major proportions and may overwhelm the bimodality of a reaction boundary, vitiating our previous reasons for limiting the aggregation of β-B to the dimer state. Also, it was found that a hypothetical 30% dimerization of β-B, in the absence of β-A, using the same change in free energy as an (A–A) bond, could not account for recent light scattering (Figure 2) and sedimentation velocity (Figure 7) results. It was then decided that the problem should be reexamined in terms of an alternate stoichiometry, not restricted to a dimerization model. The most reasonable mechanism was found to be one which permitted both genetic variants (A and B) to undergo the tetramerization with different bond strengths, and which abandoned the concept of heterogeneity of the genetic variants with respect to this

(1) This work was presented in part at the 150th National Meeting of the American Chemical Society, Atlantic City, N. J., Sept 1965.

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(6) In actuality the 36,000-Dalton isoelectric unit is a dimer of two identical chains held together by strong noncovalent bonds.[7] Under the conditions treated here, this dimer undergoes a tetramerization reaction to the 144,000-Dalton octamer; this reaction will be referred to as tetramerization. For purposes of treating the association reaction discussed in this paper, the pertinent kinetic units will be referred to as monomer, dimer, trimer, and tetramer, keeping in mind that they are composed of two, four, six, and eight chains,[7] respectively, with molecular weights of 36,000, 72,000, 108,000, and 144,000. Even though the true chemical subunit is a single chain of 18,000 molecular weight,[8,9] the present usage seems justified, since, under the experimental conditions of this paper, the 36,000-Dalton unit does not dissociate into its two identical subunits.

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