Synthesis of [A-2-αγ-Diaminobutyric Acid, A-19-Glutamic Acid] Sheep Insulin [1]

By **Nicolaos Ferderigos** and **Panayotis G. Katsoyannis,*** Department of Biochemistry, Mount Sinai School of Medicine of the City University of New York, New York 10029, U.S.A.

An analogue of sheep insulin which differs from the parent molecule in that the amino-acid residues isoleucine and tyrosine at positions 2 and 19 in the A chain are replaced by αγ-diaminobutyric acid and glutamic acid, respectively, has been synthesized. For this purpose [A_2bu^2,Glu^{19}] A chain *S*-suphonate was synthesized by the fragment condensation approach and isolated in purified form. Conversion of the latter into the thiol form and interaction with the *S*-sulphonated B chain of bovine (sheep) insulin yielded [A-A_2bu^2.Glu^{19}] sheep insulin, which was purified by chromatography on a carboxymethylcellulose column with an exponential sodium chloride gradient. This analogue, tested by the mouse convulsion assay method and in doses at least 19-fold higher than those normally used for insulin assay, was inactive. It was also inactive when tested by the radioimmunoassay method. Thus the A^2 and/or A^{19} residues must be involved in the expression of the biological activity of insulin.

X-RAY analysis of the three-dimensional structure of insulin [2] has shown that the A chain contains two helical segments, A^{2-8} and A^{13-20}, located in the *N*- and *C*-terminal regions. The *X*-ray model further indicates that within the insulin molecule the A chain is folded upon itself so that the *N*- and *C*-terminal residues are brought together and the aforementioned helical regions orient themselves in an antiparallel fashion.[2c] This arrangement results in the placement of the A^2 residue, isoleucine, close to the A^{19} residue, tyrosine, thus bringing the side chains of these residues into van der Waals contact.[2c]

In the insulin monomer the A-Ile^2 is buried and is part of the hydrophobic interior which contributes to the stabilization of the molecule's structure.[2c] On the other hand, the A-Tyr^{19} residue is on the surface of the monomer and its CO group is involved [2c] in hydrogen-bond formation with the NH of the B-Phe^{25}. Surprisingly, we have found [3] that the simple replacement of A-Ile^2 by norleucine results in an analogue with low biological activity (1 i.u. mg^{-1}; *cf.* 23—25 for the natural hormone). This result suggested that the juxtaposition of A-Ile^2 and A-Tyr^{19} (both invariant residues in all insulin species) and the intra- and/or inter-chain interactions in which they are involved, are of critical importance in the expression of the biological profile of insulin. Strictly speaking, a quantitative delineation of the contributions to the biological activity of insulin ascribable to the intra- and inter-chain interactions of the A^2 and A^{19} residues is not feasible. However, some indications of

[1] Previous paper in this series, A. Cosmatos, Y. Okada, and P. G. Katsoyannis, *Biochemistry*, 1976, **15**, 4076.

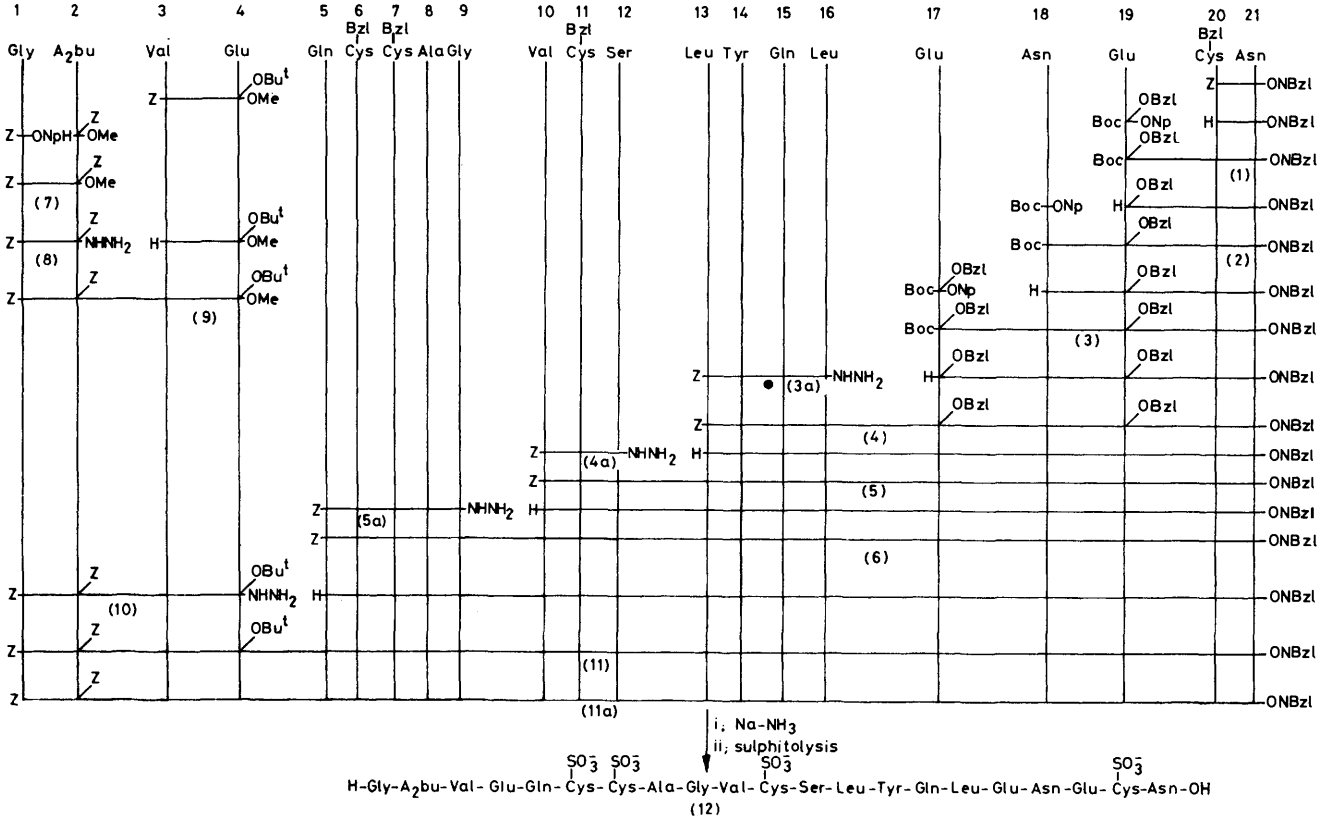
[2] (*a*) M. J. Adams, T. L. Blundell, E. J. Dodson, G. G. Dodson, M. Vijayan, E. N. Baker, M. M. Harding, D. C. Hodgkin, B. Rimmer, and S. Sheat, *Nature*, 1969, **224**, 491; (*b*) T. L. Blundell, G. G. Dodson, E. J. Dodson, D. C. Hodgkin, and M. Vijayan, *Recent Progr. Hormone Res.*, 1971, **27**, 1; (*c*) T. L. Blundell, G. G. Dodson, D. C. Hodgkin, and D. Merola, *Adv. Protein Chem.*, 1972, **26**, 280.

[3] Unpublished data from this laboratory.

the relative importance of each of these interactions may be obtained by synthesis and biological evaluation of the appropriate insulin analogues.

In continuation of our studies of structure–activity relationships in insulin,[1] we have investigated the effect on the biological activity of this hormone of the substitution of the hydrophobic A^2 and A^{19} residues with strongly interacting polar amino-acid residues. To this end, [A-A_2bu^2,Glu^{19}] sheep insulin (A_2bu = αγ-diaminobutyric acid) was synthesized. With this analogue an indication of the significance of the A^2–A^{19} interaction in maintaining a structure compatible with high biological activity of the hormone can be obtained. Biological evaluation by the mouse convulsion assay method has shown that this analogue is inactive in doses up to 19 μg per mouse. The corresponding dosage for insulin is 0.5—1 μg per mouse. By the radioimmunoassay method [A-A_2bu^2,Glu^{19}]insulin was inactive. Undoubtedly the potential interaction between A_2bu and Glu in the analogue is considerably stronger than the van der Waals interaction between Ile and Tyr in the natural hormone. However, our results show that the insertion of A_2bu and Glu at positions 2 and 19, respectively induces structural changes with consequent deleterious effects on biological activity and immunoreactivity.

General Aspects of the Synthesis.—The synthesis of the insulin analogue followed the pattern employed for the total synthesis of insulin [4] and its analogues.[1] It involves the combination of the *S*-sulphonated form of the B chain of bovine (sheep) insulin with the thiol form of [A_2bu^2,Glu^{19}] A chain of sheep insulin. The *S*-sulphonated B chain of bovine insulin, which is identical with the corresponding chain of sheep insulin,[5] was prepared by oxidative sulphitolysis of bovine insulin followed by separation of the resulting *S*-sulphonate derivatives of the A and B chains by chromatography on a carboxymethylcellulose column. The sulphitolysis of insulin and the separation of the A and B chain derivatives have been described previously.[6] The thiol form of [A_2bu^2,Glu^{19}] A chain was prepared by reduction with 2-mercaptoethanol of the *S*-sulphonated derivative of [A_2bu^2,Glu^{19}] A chain as described previously.[4,7] The latter compound was synthesised by classical methods, namely, a combination of the stepwise elongation and fragment condensation approaches, and patterned after that of the natural sheep and human A chains.[8] It involved synthesis of the protected heneicosapeptide (11a) (Scheme)



SCHEME

[4] P. G. Katsoyannis, A. Trakatellis, C. Zalut, S. Johnson, A. Tometsko, G. Schwartz, and J. Ginos, *Biochemistry*, 1967, **6**, 2656.

[5] (*a*) F. Sanger and H. Tuppy, *Biochem. J.*, 1951, **49**, 463; (*b*) F. Sanger and H. Tuppy, *ibid.*, p. 481; (*c*) H. Brown, F. Sanger, and P. Kitai, *ibid.*, 1955, **60**, 556.

[6] P. G. Katsoyannis, A. Tometsko, C. Zalut, S. Johnson, and A. Trakatellis, *Biochemistry*, 1967, **6**, 2635.

[7] P. G. Katsoyannis and A. Tometsko, *Proc. Nat. Acad. Sci. U.S.A.*, 1966, **55**, 1554.

[8] (*a*) P. G. Katsoyannis, A. Tometsko, C. Zalut, and K. Fukuda, *J. Amer. Chem. Soc.*, 1966, **88**, 5625; (*b*) P. G. Katsoyannis, A. Tometsko, and C. Zalut, *ibid.*, 1967, **89**, 4505.

containing the entire amino-acid sequence of the [A_2bu^2,-Glu^{19}] A chain, removal of the blocking groups by sodium in liquid ammonia,[9] and conversion of the resulting thiol derivative into the *S*-sulphonated form by oxidative sulphitolysis. The overall synthesis is summarized in the Scheme. The *C*-terminal protected pentapeptide (3) (sequence 17—21), which was prepared stepwise, was deblocked with trifluoroacetic acid and the product was condensed with the protected tetrapeptide [10] (3a)

TABLE 1

Amino-acid composition * of an acidic hydrolysate and an enzymic digest of the *S*-sulphonated [A_2bu^2,Glu^{19}] A chain of sheep insulin

	Acidic hydrolysis		Enzymic hydrolysis (aminopeptidase M)	
Amino-acid	Theory	Found	Theory	Found
A_2bu	1.0	1.1	1.0	0.8
Asp	2.0	2.0	0	0.1
Gln	0	0	2	Emerge at the same position and not determined
Asn	0	0	2	
Ser	1.0	0.9	1.0	0.9 †
Glu	5.0	4.9	3.0	2.7
Gly	2.0	2.0	2.0	2.0
Ala	1.0	1.1	1.0	1.2
Cys	4.0	3.4 ‡	0	0
Val	2.0	2.0	2.0	2.0
Leu	2.0	2.1	2.0	2.3
Tyr	1.0	0.9	1.0	1.1
S-(SO_3^-)Cys	0	0	4.0	4.0 **

* Number of amino-acid residues per molecule. † Separated from glutamine and asparagine in a 30 °C chromatographic run. ‡ Uncorrected for destruction. ** Eluted from the long column of the Beckman-Spinco analyser after 26 ml of effluent.

(sequence 13—16) by the azide method to give the protected nonapeptide derivative (4) (sequence 13—21). Deblocking of the derivative (4) with hydrogen bromide in acetic acid and coupling of the product with the protected tripeptide [10] (4a) (sequence 10—12) by the azide method afforded the dodecapeptide derivative (5) (sequence 10—21). Upon exposure to hydrogen bromide in trifluoroacetic acid this derivative was deblocked at the amino-end; it was then condensed by the azide method with the protected pentapeptide [11] (5a) (sequence 5—9) to give the heptadecapeptide fragment (6) (sequence 5—21). *N*-Benzyloxycarbonylglycine *p*-nitrophenyl ester [12] was condensed with N^{γ}-benzyloxycarbonyl-L-$\alpha\gamma$-diaminobutyric acid methyl ester [13] to give the dipeptide derivative (7) (sequence 1—2) which, upon exposure to hydrazine hydrate, afforded the hydrazide (8). Conversion of compound (8) into the azide and coupling with the product obtained by catalytic hydrogenation of *N*-benzyloxycarbonyl-L-valyl-γ-t-butyl L-glutamic acid methyl ester [14] afforded the tetrapeptide derivative (9) (sequence 1—4), which was converted into the corresponding hydrazide (10). In the final synthetic step the protected heptadecapeptide (6) was deblocked with hydrogen bromide in trifluoroacetic acid and the product was condensed by the azide method with the *N*-terminal tetrapeptide (10) to give the protected heneicosapeptide (11). Upon treatment with trifluoroacetic acid compound (11) was converted into the partially protected derivative (11a), which was subsequently deblocked by sodium in liquid ammonia. The product was dissolved in 8M-guanidine hydrochloride and treated with sodium sulphite and sodium tetrathionate at pH 8.9. Dialysis of the mixture followed by lyophilization of the non-diffusible material yielded the *S*-sulphonated [A_2bu^2,-Glu^{19}] A chain (12). Chromatography on Sephadex G-15 yielded a product which was not homogeneous on high voltage thin-layer electrophoresis. This necessitated further purification by chromatography on aminoethylcellulose.

Amino-acid analysis after acidic hydrolysis and enzymic digestion (Table 1), t.l.c. in two solvent systems, and high voltage thin-layer electrophoresis were employed to establish the homogeneity of the purified *S*-sulphonated chain. The conversion of the *S*-sulphonated [A_2bu^2,-Glu^{19}] A chain into its thiol form and its combination with the *S*-sulphonated B chain to produce [A-A_2bu^2,-Glu^{19}] sheep insulin were carried out as described previously.[4,7,15] Isolation and purification of the insulin analogue was effected by chromatography on a carboxymethylcellulose column with an acetate buffer (0.024M; pH 3.3) and an exponential sodium chloride gradient as described previously [4,15] (Figure 1). From the eluate the insulin analogue was isolated *via* the picrate as the hydrochloride. Amino-acid analysis [after acidic hydrolysis (Table 2)] and high voltage thin-layer electrophoresis (Figure 2) were employed to ascertain its homogeneity.

EXPERIMENTAL

In all synthetic steps, coupling of the fragments was followed by detection of the amino-component present with ninhydrin; completion of the reaction was indicated by a negative reaction with ninhydrin. Optical rotations were taken with a Zeiss photoelectric precision polarimeter. M.p.s were taken for samples in capillary tubes. The homogeneity of the intermediate peptide derivatives was ascertained, after deblocking at the amino-end as indicated, by t.l.c. on 6060 silica gel (Eastman Chromagram Sheet) except as otherwise indicated. The following solvent systems were used: (A) acetonitrile–water (3 : 1); (B) chloroform–methanol–water (40 : 15 : 15; lower phase); (C) butan-1-ol–acetic acid–water–pyridine (30 : 6 : 24 : 20); and (D) butan-1-ol–acetic acid–water (4 : 1 : 1). Thin-layer electrophoresis was carried out as described previously [16] with a Wieland-Pfleiderer pherograph (Brinkmann Industries). Amino-acid analyses were performed in a Beckman-Spinco 120C amino-acid analyser according to the

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[14] P. G. Katsoyannis, A. Tometsko, and C. Zalut, *J. Amer. Chem. Soc.*, 1966, **88**, 5622.
[15] P. G. Katsoyannis, A. Trakatellis, S. Johnson, C. Zalut, and G. Schwartz, *Biochemistry*, 1967, **6**, 2642.
[16] A. Tometsko and C. Delihas, *Analyt. Biochem.*, 1967, **18**, 72.

method of Spackman *et al.*[17] Acidic hydrolyses and calculations of molar ratios were carried out as described previously. For the enzymic digestion with aminopeptidase M (Henley and Co., New York) the method of Pfleiderer *et al.*[18] was employed. Preswollen microgranular carboxymethylcellulose (Whatman CM 52/1), Sephadex G-15 (Pharmacia

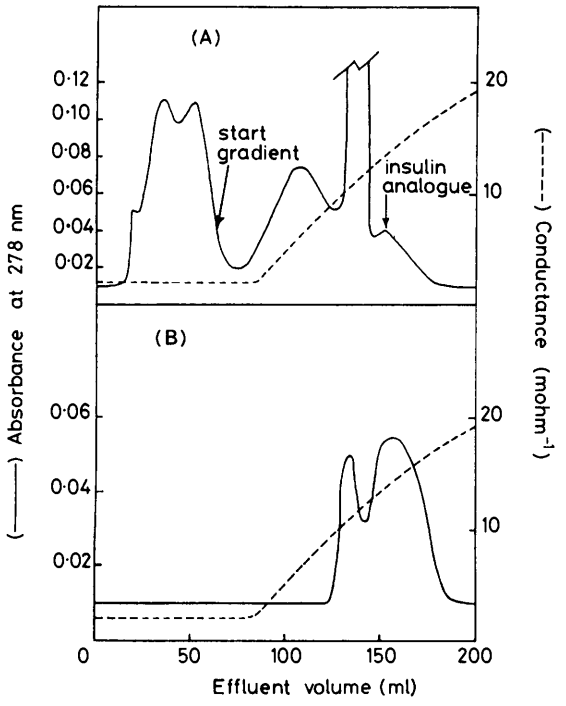

FIGURE 1 (A) Chromatography of two combination mixtures (see Experimental section) of the *S*-sulphonated B chain of bovine (sheep) insulin with the thiol form of sheep [A_2bu^2, Glu^{19}]A chain on a 0.9 × 23 cm carboxymethylcellulose column with acetate buffer (0.024M; pH 3.3) and an exponential sodium chloride gradient. The column eluate was monitored by a Gilford recording spectrophotometer and by a conductivity meter (Radiometer, Copenhagen). The sheep [A-A_2bu^2, Glu^{19}]insulin (145—170 ml of eluate) was recovered as the hydrochloride. (B) The insulin analogue from (A), plus the insulin analogue hydrochloride obtained by processing two more combination mixtures, were combined and rechromatographed on the 0.9 × 23 cm carboxymethylcellulose column in exactly the same way as above. The purified sheep [A-A_2bu^2, Glu^{19}]insulin (145—180 ml of eluate) was recovered as the hydrochloride (1.4 mg)

Uppsala), and aminoethylcellulose (Bio-Rad Cellex-AE) were used. The washing of the resins and the preparation of the columns have been described previously.[4,15] The column eluates were continuously monitored with a Gilford recording spectrophotometer at 278 nm. Protein determinations were carried out by the method of Lowry *et al.*[19] Sodium tetrathionate was prepared as described by Gilman *et al.*[20] Biological assays were carried out by the mouse convulsion method as described previously.[4] Radioimmunoassays were performed by the method of Hales and Randle[21] with an insulin immunoassay kit (Amersham/Searle Co.). Natural bovine insulin was provided by Eli Lilly and Co.

N-t-Butoxycarbonyl-γ-benzyl-L-glutamyl-S-benzyl-L-cysteinyl-L-asparagine p-Nitrobenzyl Ester (1).—A suspension of *N*-benzyloxycarbonyl-*S*-benzyl-L-cysteinyl-L-asparagine *p*-nitrobenzyl ester[10] (14.8 g, 0.025 mol) in acetic acid (50 ml) was treated with 4N-hydrogen bromide in acetic acid (50 ml). After 1 h at room temperature the solution was poured into ether, and the precipitated hydrobromide was filtered off, washed with ether, and dried (KOH and P_2O_5) *in vacuo*. This product was dissolved in dimethylformamide (60 ml) containing triethylamine (3.5 ml) and the precipitated triethylamine hydrobromide was filtered off. To the filtrate *N*-t-butyoxycarbonyl-γ-benzyl-L-glutamic acid *p*-nitrophenyl ester[22] (12.4 g, 0.027 mol) was added. After 24 h at room temperature, the mixture was poured into cold N-ammonia (750 ml) and the *precipitate* was collected, washed (0.5N-NH_4OH, 0.5N-H_2SO_4, and water) and crystallized from dimethylformamide–methanol; yield 14 g (72%); m.p. 184°; $[\alpha]_D^{26}$ −26.2° (*c* 2.0 in Me_2N·CHO). After deblocking with trifluoroacetic acid the peptide

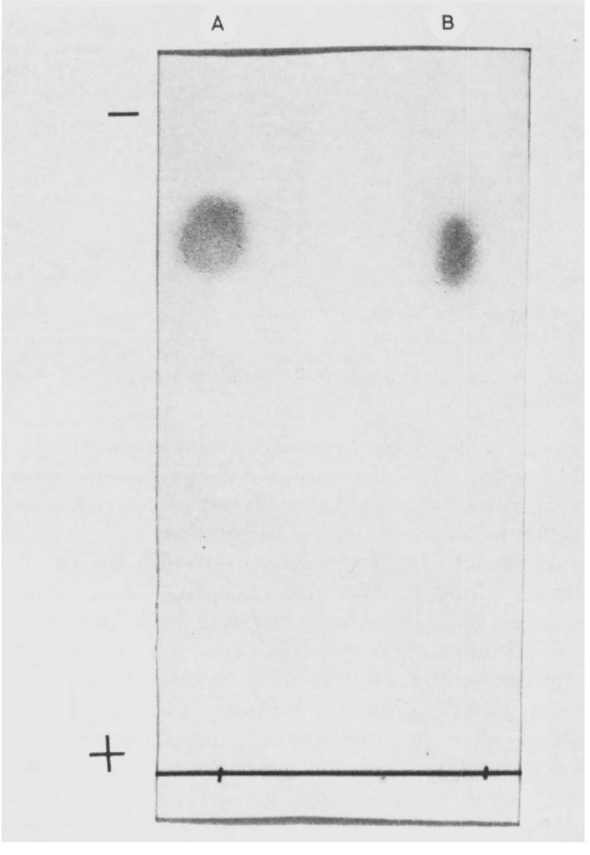

FIGURE 2 High-voltage thin-layer electrophoresis of (A) natural bovine insulin and (B) synthetic [A-A_2bu^2,Glu^{19}] sheep insulin (0.5N-acetic acid; 3 500 V; 15 min)

showed a single spot on t.l.c. in systems (A)—(C) (Found: C, 58.4; H, 5.9; N, 8.9; S, 4.3. $C_{38}H_{45}N_5O_{11}S$ requires C, 58.5; H, 5.8; N, 9.0; S, 4.1%).

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[21] C. N. Hales and P. J. Randle, *Biochem. J.*, 1963, **88**, 137.
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N-*t*-*Butoxycarbonyl*-L-*asparaginyl*-γ-*benzyl*-L-*glutamyl*-S-*benzyl*-L-*cysteinyl*-L-*asparagine* p-*Nitrobenzyl Ester* (2).—A solution of compound (1) (12.5 g, 0.016 mol) in trifluoroacetic acid (25 ml) was stored at room temperature for 45 min and then poured into ether (200 ml). The precipitate was filtered off, washed with ether, and dried (KOH and P_2O_5) *in vacuo*. To a solution of this material in dimethylformamide (80 ml; 0 °C), triethylamine (2.2 ml) and *N*-t-butoxycarbonyl-L-asparagine *p*-nitrophenyl ester [23] (6.1 g, 0.018 mol) were added. After 48 h at room temperature, the mixture was poured into cold N-ammonia (750 ml) and the *precipitate* was filtered off, washed (0.5N-NH_4OH, 0.5N-H_2SO_4, and water) and triturated with methanol; yield 10.4 g (80%); m.p. 194°; $[\alpha]_D^{26}$ −47.2° (*c* 2.0 in Me_2N·CHO); on t.l.c. it was homogeneous in solvents (A) and (C) after deblocking with trifluoroacetic acid (Found: C, 56.4; H, 5.85; N, 11.1; S, 3.8. $C_{42}H_{51}N_7O_{13}S$ requires C, 56.4; H, 5.75; N, 11.0; S, 3.6%). Amino-acid analysis after acidic hydrolysis gave the following ratios: $Asp_{2.0}Glu_{1.0}Cys(Bzl)_{0.6}$.

N-*t*-*Butoxycarbonyl*-γ-*benzyl*-L-*glutamyl*-L-*asparaginyl*-γ-*benzyl*-L-*glutamyl*-S-*benzyl*-L-*cysteinyl*-L-*asparagine* p-*Nitrobenzyl Ester* (3).—A solution of compound (2) (4.5 g, 0.005 mol) in trifluoroacetic acid (10 ml) was stored at room temperature for 45 min and then poured into ether. The *precipitate* was filtered off, washed with ether, and dried (P_2O_5 and KOH) *in vacuo*. To a solution of this material in dimethylformamide (20 ml), triethylamine (0.7 ml) and *N*-t-butoxycarbonyl-γ-benzyl-L-glutamic acid *p*-nitrophenyl ester (2.5 g, 0.005 5 mol) were added. After 24 h the mixture was poured into cold N-ammonia (500 ml) and the *precipitate* was filtered off, washed (0.5N-NH_4OH and water), dried, and reprecipitated from dimethylformamide–ethyl acetate; yield 4.1 g (74.5%); m.p. 185—186°, $[\alpha]_D^{26}$ −35.2° (*c* 2 in Me_2N·CHO); homogeneous in solvents (A) and (C) after deblocking with trifluoroacetic acid (Found: C, 58.2; H, 5.6; N, 10.2; S, 2.9. $C_{54}H_{64}N_8O_{16}S$ requires C, 58.3; H, 5.8; N, 10.1; S, 2.9%). Amino-acid analysis after acidic hydrolysis gave the following ratios: $Asp_{2.0}Glu_{2.0}Cys(Bzl)_{0.5}$.

N-*Benzyloxycarbonyl*-L-*leucyl*-L-*tyrosyl*-L-*glutaminyl*-L-*leucyl*-γ-*benzyl*-L-*glutamyl*-L-*asparaginyl*-γ-*benzyl*-L-*glutamyl*-S-*benzyl*-L-*cysteinyl*-L-*asparagine* p-*Nitrobenzyl Ester* (4).—A solution of compound (3) (1.1 g, 0.001 mol) in trifluoroacetic acid (8 ml) was stored at room temperature for 1 h and then poured into ether (150 ml). The *precipitate* was filtered off, washed with ether, and dried (P_2O_5 and KOH) *in vacuo*. This product was dissolved in dimethylformamide (4 ml) containing triethylamine (0.14 ml), cooled to 0 °C, and condensed with the tetrapeptide azide prepared as follows. To a solution of *N*-benzyloxycarbonyl-L-leucyl-L-tyrosyl-L-glutaminyl-L-leucine hydrazine [10] (0.75 g, 0.001 1 mol) in a mixture of dimethylformamide (3.5 ml), dimethyl sulphoxide (1.5 ml), and 5.6N-hydrochloric acid in dioxan (0.7 ml), cooled to −15 °C, t-butyl nitrite (0.13 ml) was added. After 5 min the mixture was neutralized with triethylamine (0.55 ml) and mixed with the solution of the pentapeptide ester prepared as described previously. After 48 h (4 °C) the mixture was poured into cold water (500 ml) and the *precipitate* was filtered off, washed (0.5N-HCl, water, and methanol), dried, and reprecipitated from dimethylformamide–methanol; yield 1.3 g (80%); m.p. 234—236°, $[\alpha]_D^{26}$ −34.0° (*c* 1.0 in Me_2SO). After deblocking with hydrogen bromide in acetic acid, the peptide did not migrate on t.l.c. in systems (A)—(D) (Found: C, 60.0; H, 6.0; N, 11.0. $C_{83}H_{101}N_{13}O_{22}S$ requires C, 59.9; H, 6.15; N, 10.9%). Amino-acid analysis after acidic hydrolysis gave the following ratios: $Asp_{1.9}Glu_{2.9}Leu_{2.1}Tyr_{0.6}Cys(Bzl)_{0.7}$.

N-*Benzyloxycarbonyl*-L-*valyl*-S-*benzyl*-L-*cysteinyl*-L-*seryl*-L-*leucyl*-L-*tyrosyl*-L-*glutaminyl*-L-*leucyl*-L-*glutamyl*-L-*asparaginyl*-L-*glutamyl*-S-*benzyl*-L-*cysteinyl*-L-*asparagine* p-*Nitrobenzyl Ester* (5).—Compound (4) (1.7 g, 0.001 mol) was suspended in acetic acid (10 ml) and treated with 4N-hydrogen bromide in acetic acid (10 ml). After 1 h the solution was poured into ether and the precipitated hydrobromide of the nonapeptide ester was filtered off, washed with ether, and dried (P_2O_5 and KOH) *in vacuo*. This product was dissolved in dimethylformamide (4 ml) and dimethyl sulphoxide (1 ml) containing triethylamine (0.5 ml), cooled to 0 °C, and condensed with the tripeptide azide prepared as follows. To a solution of *N*-benzyloxycarbonyl-L-valyl-*S*-benzyl-L-cysteinyl-L-serine hydrazide [10] (1.1 g, 0.002 mol) in dimethylformamide (6 ml), dimethyl sulphoxide (2 ml), and 5.6N-hydrochloric acid in dioxan (0.9 ml), cooled to −15 °C, t-butyl nitrite (0.24 ml) was added. After 5 min, the mixture was neutralized with triethylamine (0.7 ml) and added to the nonapeptide ester prepared as described previously. After 48 h at 4 °C, the mixture was poured into cold water (200 ml) and the *precipitate* was filtered off, washed (0.5N-HCl, water, and methanol) and triturated with warm methanol; yield 1.5 g (83%); m.p. 239—246°, $[\alpha]_D^{26}$ −31.8° (*c* 1.0 in Me_2SO). After deblocking with HBr in acetic acid, the peptide did not migrate on t.l.c. in systems (A)—(D) (Found: C, 56.4; H, 6.35; N, 11.8. $C_{87}H_{114}N_{16}O_{26}S_2$ requires C, 56.1; H, 6.15; N, 12.0%). Amino-acid analysis after acidic hydrolysis gave the following ratios: $Asp_{1.9}Glu_{2.9}Val_{1.0}Leu_{2.1}Ser_{0.9}Tyr_{0.8}Cys(Bzl)_{1.5}$.

N-*Benzyloxycarbonyl*-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-*glutamyl*-S-*benzyl*-L-*cysteinyl*-L-*asparagine* p-*Nitrobenzyl Ester* (6).—Compound (5) (1.8 g, 0.001 mol) was dissolved in trifluoroacetic acid (30 ml) containing water (0.9 ml) and hydrogen bromide was passed through the solution for 1 h at 0 °C. Addition of ether (150 ml) caused precipitation of the deblocked dodecapeptide hydrobromide, which was filtered off, washed with ether, and dried (P_2O_5 and KOH) *in vacuo*. To a solution of this material in dimethylformamide (10 ml) cooled at 0 °C, triethylamine (0.42 ml) was added followed by the protected pentapeptide azide prepared as follows. *N*-Benzyloxycarbonyl-L-glutaminyl-*S*-benzyl-L-cysteinyl-*S*-benzyl-L-cysteinyl-L-alanyl-L-glycine hydrazide [11] (0.9 g, 0.001 1 mol) was dissolved in dimethylformamide (10 ml), dimethyl sulphoxide (8 ml), and 5.6N-hydrochloric acid in dioxan (0.6 ml), and to this solution cooled to −15 °C, t-butyl nitrite (0.12 ml) was added. After 5 min, the mixture was neutralized with triethylamine (0.5 ml) and added to the dodecapeptide ester prepared as described above. After 72 h at 4 °C, the mixture was poured into methanol (200 ml) and N-hydrochloric acid (50 ml). The *precipitate* was filtered off, washed with methanol, triturated with warm methanol, and reprecipitated from dimethyl sulphoxide–methanol; yield 1.5 g (62%); m.p. 244—249°; $[\alpha]_D^{26}$ −31.5° (*c* 1.0 in Me_2SO). The heptadecapeptide ester hydrobromide did not move on t.l.c. in systems (A)—(D) (Found: C, 55.9; H, 6.1; N, 12.4. $C_{117}H_{152}N_{22}O_{32}S_4$ requires C, 56.0; H, 6.1; N, 12.3%). Amino-acid analysis after acidic hydrolysis gave the follow-

[23] G. R. Marshall and R. B. Merrifield, *Biochemistry*, 1965, **4**, 2394.

ing ratios: $Asp_{1.9}Ser_{0.8}Glu_{4.0}Gly_{0.9}Ala_{1.0}Val_{1.0}Leu_{2.1}Tyr_{0.4}Cys(Bzl)_{3.3}$.

N-Benzyloxycarbonylglycyl-Nγ-benzyloxycarbonyl-L-αγ-diaminobutyric Acid Methyl Ester (7). *Nγ*-Benzyloxycarbonyl-L-αγ-diaminobutyric acid methyl ester hydrochloride [13] (4.5 g, 0.015 mol) was partitioned between ethyl acetate and 0.5M-sodium carbonate, and the organic layer was separated, washed with water, dried ($MgSO_4$), and concentrated to *ca.* 30 ml. To this solution *N*-benzyloxycarbonylglycine *p*-nitrophenyl ester [12] (5.4 g, 0.017 mol) was added. After 24 h the mixture was poured into ethyl acetate (250 ml) and the organic phase was washed (0.5N-HCl, 0.5N-NH_4OH, and water), dried ($MgSO_4$), and concentrated to dryness to give an oil (4.8 g, 70%).

N-Benzyloxycarbonylglycyl-Nγ-benzyloxycarbonyl-L-αγ-diaminobutyrohydrazide (8).—The oily compound (7) (4.8 g) was dissolved in methanol (25 ml) containing hydrazine hydrate (4 ml). After 24 h at room temperature, the precipitated peptide *hydrazide* was filtered off and washed with methanol; yield 4.3 g (90%); m.p. 167°; $[\alpha]_D^{26}$ −11.3° (*c* 2.0 in Me_2N·CHO) (Found: C, 57.6; H, 5.9; N, 15.5. $C_{22}H_{27}N_5O_6$ requires C, 57.8; H, 5.95; N, 15.3%).

N-Benzyloxycarbonylglycyl-Nγ-benzyloxycarbonyl-L-αγ-diaminobutyryl-L-valyl-γ-t-butyl-L-glutamic Acid Methyl Ester (9).—A solution of *N*-benzyloxycarbonyl-L-valyl-γ-t-butyl-L-glutamic acid methyl ester [14] (2.3 g, 0.005 mol) in methanol (50 ml) was hydrogenated over 10% palladium–charcoal (0.7 g). After 2 h the catalyst was filtered off and the filtrate was concentrated to dryness *in vacuo*. The residue was dissolved in ethyl acetate (5 ml), cooled to 0 °C, and then mixed with a solution of the dipeptide azide prepared as follows. To a solution of compound (8) (2.5 g, 0.005 5 mol) in dimethylformamide (10 ml) and 2N-hydrochloric acid (5.7 ml), cooled to −10 °C, sodium nitrite (386 mg), dissolved in cold water (2 ml), was added. The mixture was stirred at −10 °C for 5 min and diluted with ethyl acetate (35 ml). The organic phase was then washed (M-$KHCO_3$ and water), dried (Na_2SO_4), and added to the solution of the dipeptide ester prepared as described previously. After 48 h at 4 °C the ethyl acetate was evaporated off *in vacuo*; the residue was dissolved in dimethylformamide (15 ml) and mixed with water (200 ml). The *precipitate* was filtered off, washed (0.5N-HCl, M-$KHCO_3$, and water), and reprecipitated from methanol; yield 2.7 g (73%); m.p. 176°; $[\alpha]_D^{26}$ −18.4° (*c* 1.0 in Me_2N·CHO); homogeneous in solvents (C) and (D) (Found: C, 60.0; H, 6.75; N, 9.3. $C_{37}H_{51}N_5O_{11}$ requires C, 59.9; H, 6.9; N, 9.4). Amino-acid analysis after acidic hydrolysis gave the following ratios: $A_2bu^3_{1.1}Gly_{1.0}Val_{1.0}Glu_{1.0}$.

N-Benzyloxycarbonylglycyl-Nγ-benzyloxycarbonyl-L-αγ-diaminobutyryl-L-valyl-γ-t-butyl-L-glutamic Acid Hydrazide (10).—To a solution of compound (9) (2.1 g) in methanol (100 ml), hydrazine hydrate (5 ml) was added. After 24 h the precipitated *hydrazide* was filtered off and washed with methanol; yield 1.6 g (76%); m.p. 217°; $[\alpha]_D^{26}$ −17.6° (*c* 1.0 in Me_2N·CHO) (Found: C, 58.3; H, 6.85; N, 13.3. $C_{36}H_{51}N_7O_{10}$ requires C, 58.3; H, 6.9; N, 13.2%).

N-Benzyloxycarbonylglycyl-Nγ-benzyloxycarbonyl-L-αγ-diaminobutyryl-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-glutamyl-S-benzyl-L-cysteinyl-L-asparagine p-Nitrobenzyl Ester (11a).—The protected heptadecapeptide (6) (1.5 g 0.000 6 mol) was dissolved in trifluoroacetic acid (30 ml) containing water (1 ml), and hydrogen bromide was passed through this solution for 1 h at 0 °C. Addition of ether (200 ml) caused precipitation of the deblocked heptadecapeptide hydrobromide, which was filtered off, washed with ether, and dried (KOH and P_2O_5) *in vacuo*. This material was dissolved in dimethylformamide (5 ml) and dimethyl sulphoxide (5 ml) containing triethylamine (0.5 ml), cooled to 0 °C, and mixed with the tetrapeptide azide prepared as follows. To a solution of *N*-benzyloxycarbonylglycyl-*Nγ*-benzyloxycarbonyl-L-αγ-diaminobutyryl-L-valyl-γ-t-butyl-L-glutamic acid hydrazide (10) (1.1 g, 0.001 5 mol) in dimethylformamide (7 ml) containing 5.6N-hydrochloric acid in dioxan (0.7 ml), cooled to −15 °C, t-butyl nitrite (0.2 ml) was added. After 5 min the mixture was neutralized with triethylamine (0.55 ml) and then added to the partially protected heptadecapeptide prepared as described above. After 48 h at 4 °C, the mixture was poured into methanol (200 ml) and N-hydrochloric acid (25 ml). The precipitated protected heneicosapeptide (11) was isolated by centrifugation, washed (warm methanol), and dried. A solution of this material in trifluoroacetic acid (30 ml) was stored at room temperature for 30 min and then diluted with ether (300 ml). The precipitated partially protected heneicosapeptide (11a) was isolated by centrifugation, washed with ether, and dried (P_2O_5 and KOH) *in vacuo*; yield 1.1 g (61%); m.p. 242—245°; $[\alpha]_D^{26}$ −43.2° (*c* 1.0 in Me_2SO). After deblocking (HBr–AcOH) the peptide did not move on t.l.c. in solvents (A)—(D). Amino-acid analysis after acidic hydrolysis gave the following ratios: $A_2bu_{1.0}Asp_{1.9}Ser_{0.8}Glu_{4.9}Gly_{2.0}Ala_{1.0}Val_{2.1}Leu_{2.1}Tyr_{0.6}Cys(Bzl)_{3.5}$.

Glycyl-L-αγ-diaminobutyryl-L-valyl-L-glutamyl-L-glutaminyl-S-sulpho-L-cysteinyl-S-sulpho-L-cysteinyl-L-alanylglycyl-L-valyl-S-sulpho-L-cysteinyl-L-seryl-L-leucyl-L-tyrosyl-L-glutaminyl-L-leucyl-L-glutamyl-L-asparaginyl-L-glutamyl-S-sulpho-L-cysteinyl-L-asparagine (*Sheep Insulin [A_2bu^2,Glu^{19}]A chain S-Sulphonate*) (12).—Reduction of the partially protected heneicosapeptide (11a) (300 mg) with sodium in liquid ammonia (300 ml) and oxidative sulphitolysis of the product were accomplished as described previously.[8a] After evaporation of the ammonia, the residue was dissolved in 8M-guanidine hydrochloride (30 ml), and to this solution, adjusted to pH 8.9 with acetic acid or dilute ammonia (depending on the pH of the solution), were added sodium sulphite (900 mg) and freshly prepared sodium tetrathionate (420 mg). The mixture was stirred at room temperature for 4 h and then placed in an 18/32 Visking dialysing tube and dialysed against four changes of distilled water (4 l each) at 4 °C for 24 h. Upon lyophilization of the non-diffusible material the crude [A_2bu^2,Glu^{19}]A chain *S*-sulphonate (12) was obtained as a white powder. For purification the lyophilized material was chromatographed on a Sephadex G-15 column (2.4 × 50 cm), which was equilibrated and eluted with 0.015M-ammonium hydrogen carbonate. The effluent corresponding to the main peak, as monitored by a Gilford recording spectrophotometer, was lyophilized and the peptide material was obtained as a white powder (200 mg, 80%). On high voltage thin-layer electrophoresis in 0.5N-acetic acid (pH 2.9 and 3 500 V) this material was not homogeneous. For further purification the product (50 mg) was dissolved in 0.02M-ammonium hydrogen carbonate (pH 9; 10 ml) and placed on an aminoethylcellulose column (2.4 × 45 cm) which was equilibrated with the same buffer. Subsequently the column was washed with 0.02M-ammonium hydrogen carbonate (500 ml) and eluted with 0.04M-ammonium hydrogen carbonate (flow rate *ca.* 120 ml h^{-1}).

The elution pattern, as determined by monitoring the eluate with a Gilford recording spectrophotometer, indicated the presence of a single component. Lyophilization of the eluate under the peak gave the purified [A_2bu^2,Glu^{19}]A chain *S*-sulphonate as a white powder (30 mg). On t.l.c. (Cellulose 6065, Eastman Chromagram Sheet) in systems (C) and (D) and on high voltage thin-layer electrophoresis in 0.5N-acetic acid (pH 2.9 and 3 500 V) the synthetic chain moved as a single component (Pauly-positive spot). Amino-acid analysis of the purified product after acidic hydrolysis gave the molar ratios shown in Table 1, in good agreement with the expected values. Digestion of the synthetic material with aminopeptidase M and amino-acid analysis of the digest gave the molar ratios shown in Table 1, in agreement with the expected values.

S-*Sulphonated Derivatives of A and B Chains of Bovine Insulin*.—These compounds were prepared by oxidative sulphitolysis of bovine insulin by the method described previously.[6]

Synthesis and Isolation of [*A-*A_2bu^2,Glu^{19}] *Sheep Insulin*.—The synthesis was accomplished by combination of the thiol form of sheep [A_2bu^2,Glu^{19}]A chain with the *S*-sulphonated derivative of the B chain of bovine (sheep) insulin according to the procedure reported previously.[7,15] In a typical experiment *S*-sulphonated [A_2bu^2,Glu^{19}]A chain (20 mg) was converted into the thiol form by exposure to 2-mercaptoethanol (in water, pH 5.0; 6 min; 100 °C) and treated with *S*-sulphonated B chain (5 mg). The mixture was then treated as described previously.[4,15] The insulin analogue was isolated by chromatography on a carboxymethylcellulose column (0.9 × 23 cm) with an acetate buffer (0.024M; pH 3.3) and an exponential sodium chloride gradient according to the procedure reported previously.[4,15] Two mixtures, each corresponding to the amounts of material mentioned above, were chromatographed and gave the pattern shown in Figure (A). The insulin analogue from the eluate (145—170 ml) was isolated *via* the picrate as the hydrochloride according to the procedure described previously.[4] This material was combined with the hydrochloride obtained by processing, in exactly the same way as above, two more mixtures, and rechromatographed on the 0.9 × 23 cm carboxymethylcellulose column. Figure (B) shows the chromatographic pattern obtained. From the eluate (145—180 ml) the [A-A_2bu^2,Glu^{19}]insulin was isolated as the hydrochloride (1.4 mg).

Amino-acid analysis of this analogue after acidic hydrolysis gave a composition in agreement with the expected values (Table 2). On thin-layer electrophoresis in 0.5N-acetic acid (pH 2.9 and 3 500 V) the synthetic analogue moved as a single component (Pauly reaction) (Figure 2). By the mouse convulsion assay method, and in doses up to 19 μg per mouse, this analogue was biologically inactive. It was also inactive by the radioimmunoassay method.

TABLE 2

Amino-acid composition * of an acidic hydrolysate of [A-A_2bu^2,Glu^{19}] sheep insulin

Amino-acid	Theory	Found
A_2bu	1.0	1.1
Lys	1.0	1.0
His	2.0	2.0
Arg	1.0	1.0
Asp	3.0	3.0
Thr	1.0	0.9
Ser	2.0	1.8
Glu	8.0	7.7
Pro	1.0	1.0
Gly	5.0	5.0
Ala	3.0	3.0
Cys	6.0	4.1 †
Val	5.0	5.2
Leu	6.0	5.7
Tyr	3.0	2.4 †
Phe	3.0	2.9

* Number of amino-acid residues per molecule. † Uncorrected for destruction.

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