[A-21-D-Asparagine] Sheep Insulin, a Diastereoisomer of the Natural Hormone [1]

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The synthesis and isolation in purified form of [A-D-Asn^{21}] sheep insulin is described. For this purpose [D-Asn^{21}] A chain *S*-sulphonate was synthesized by the fragment condensation approach and isolated in a purified form. Conversion of the latter into its thiol form and combination with the *S*-sulphonated B chain of bovine (sheep) insulin produced the [A-D-Asn^{21}] sheep insulin, isolated by chromatography on a carboxymethylcellulose column with an exponential sodium chloride gradient. The [A-D-Asn^{21}] insulin shows potencies (I.U. mg^{-1}) of 8 when assayed by the mouse convulsion method and of 4 by the radioimmunoassay method (*cf.* 23—25 for the natural hormone).

THE involvement of the carboxy-terminus, 21-asparagine, of the A chain in the expression of high biological activity of insulin has been documented by degradative studies in several laboratories (for a review, see references 2 and 3). It was thus shown that whereas [B-des-Ala^{30}]-insulin retains more than 70% of the biological activity of the native hormone,[3] [B-des-Ala^{30}, A-des-Asn^{21}]-insulin is only slightly active [3] (*ca.* 4% of the activity of insulin). Furthermore, in contrast to [B-des-Ala^{30}]-insulin, [B-des-Ala^{30}, A-des-Asn^{21}]-insulin exhibits o.r.d. and c.d. behaviour considerably different from the natural hormone. These and other data (for a review, see references 2—5) strongly suggest that the A-Asn^{21} is critically involved in the maintenance of a conformation of insulin compatible with high biological activity.

The predicted involvement of A-Asn^{21} in the maintenance of the tertiary structure of insulin is consistent with the findings of the *X*-ray analysis of the three-dimensional structure of this protein.[2,6] The studies of Hodgkin and her co-workers have shown that residues B^{20} to B^{23} form a U-turn within the molecule so that the *C*-terminal region of the B chain (sequence B$^{24-30}$) orients itself in an anti-parallel fashion to the B$^{9-19}$ segment. This folding results in placing A-Asn^{21} and B-Arg^{22} in such juxtaposition as to ensure salt bridge formation between these residues and favourably disposes the –NH– of A-Asn^{21} and the –CO– of B-Gly^{23} for hydrogen-bond formation.[2,6]

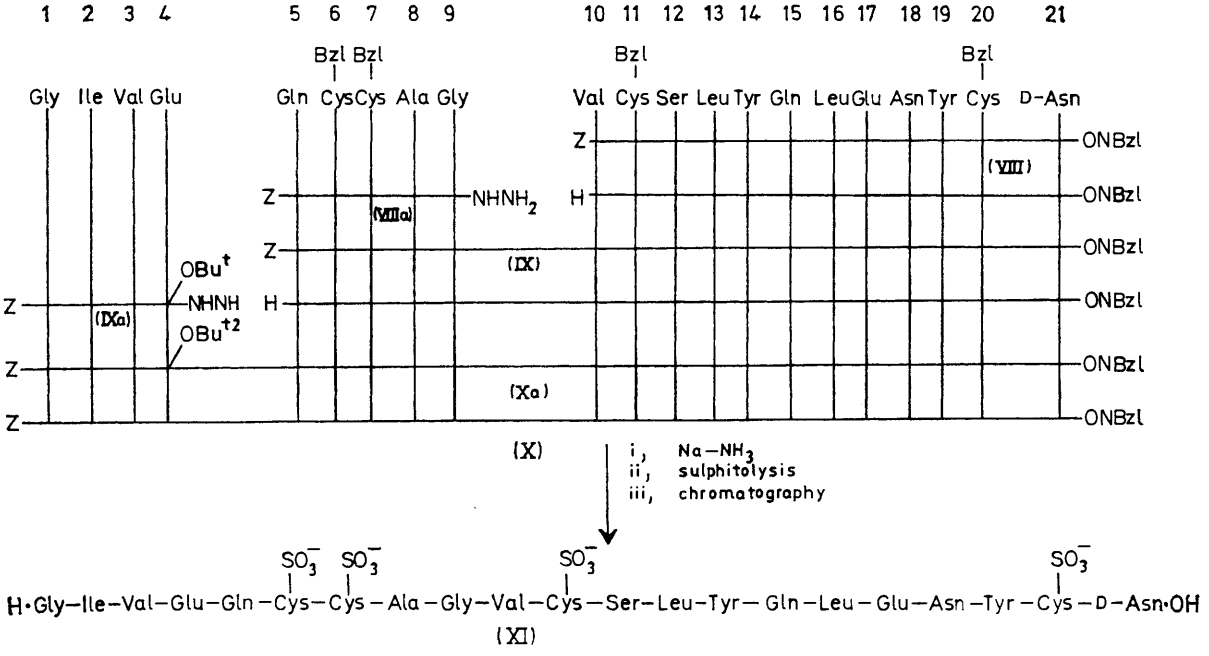
In view of the importance of A-Asn^{21} to the tertiary structure of insulin, we have modified the molecule at the A 21 position. Previous studies have shown that chemical changes in the carboxy-functions of A-Asn^{21} do not affect appreciably the biological activity of insulin. [A-Desamido-Asn^{21}]-insulin [3] and [A-isoasparagine^{21}]-insulin,[7] for example, retain 100 and 80%, respectively, of the biological activity of the natural hormone. However, as reported herein, replacement of the A-L-Asn^{21},

[1] For the previous paper of this series, see Y. Okada and P. G. Katsoyannis, *J. Amer. Chem. Soc.*, 1975, **97**, 4366.
[2] T. Blundell, G. Dodson, D. Hodgkin, and D. Mercola, *Adv. Protein Chem.*, 1972, **26**, 279.
[3] F. H. Carpenter, *Amer. J. Med.*, 1966, **40**, 750.
[4] T. M. Brugman and E. R. Arquilla, *Biochemistry*, 1973, **12**, 727.
[5] E. R. Arquilla and E. J. Stanford, in 'Insulin Action,' ed. I. Fritz, Academic Press, 1972, p. 29.
[6] (*a*) M. J. Adams, T. Blundell, E. J. Dodson, G. Dodson, M. Vijayan, E. N. Baker, M. M. Harding, D. Hodgkin, B. Rimmer, and S. Sheat, *Nature*, 1969, **224**, 491; (*b*) T. Blundell, G. Dodson, E. J. Dodson, D. Hodgkin, and M. Vijayan, *Rec. Progr. Hormone Res.*, 1971, **27**, 1.
[7] Unpublished data from this laboratory.

present in the natural hormone, by its stereoisomer, D-Asn, results in an analogue with significantly decreased biological activity. By the mouse convulsion assay method this analogue, [A-D-Asn^{21}] sheep insulin, was found to possess a specific activity of 8 I.U. mg^{-1}, *ca.* 32% of that of the natural hormone (23—25 I.U.); the radioimmunoassay method gave a value of 4 I.U. mg^{-1}, *ca.* 16% of that of insulin.

Courtauld atomic models of the natural hormone for the region involved in the salt bridge formation (A^{21}–B^{22}) and hydrogen bond interaction (A^{21} –NH– to B^{23} –CO–) reveal that in the conformation most favourable for salt bridge formation the α- and β-carboxy-groups of A-L-Asn^{21} are nearly equivalent. This may explain why [A-desamido-Asn^{21}]-insulin [3] and [A-isoasparagine^{21}]-insulin [7] have biological activities comparable with that of the natural hormone. On the other hand, Courtauld models of the same region of the [A-D-Asn^{21}]-insulin analogue reveal that the α-carboxy-group (as compared with the natural hormone) is significantly less favourably disposed for salt bridge formation with the guanidinium group of the B-Arg^{22}. We are at present involved in the synthesis of (A-Asn·NH$_2$^{21}]-insulin in order to investigate further the relative significance of the salt bridge between A^{21} and B^{22} amino-acid residues with regard to the biological activity of insulin.

General Aspects of the Synthesis and Isolation of the Insulin Analogue.—[A-D-Asn^{21}] Sheep insulin was synthesised by the procedure used for the synthesis of the natural hormone,[8,9] namely the combination of the *S*-sulphonated form of the B chain of bovine (sheep) insulin with the thiol form of [D-Asn^{21}] A chain. The *S*-sulphonated bovine B chain, which is identical with the corresponding chain of sheep insulin,[10] was prepared by oxidative sulphitolysis of bovine insulin followed by separation of the resulting *S*-sulphonated A and B chains by continuous flow electrophoresis.[11] The thiol form of [D-Asn^{21}] A chain was prepared by reduction with 2-mercaptoethanol of the *S*-sulphonated derivative of [D-Asn^{21}] A chain.[8,9] The synthesis of the latter compound was patterned essentially after that of the natural sheep and human chains reported previously.[12] It involved the synthesis of the protected heneicosapeptide (X) (Scheme 1) containing the entire amino-acid sequence of the [D-Asn^{21}] A chain, removal of the blocking groups, and conversion of the resulting thiol derivative into the *S*-sulphonated form (XI) by oxidative sulphitolysis. The overall synthesis is summarized in Schemes 1 and 2. The *C*-terminal pentapeptide derivative (VI) (sequence 17—21) (Scheme 2) was deblocked with hydrogen bromide in acetic acid and the deblocked product was condensed with the tetrapeptide derivative (VIa) (sequence 13—16) by the azide method to give the protected nonapeptide derivative (VII) (sequence 13—21). Deblocking of the latter compound with hydrogen bromide



SCHEME 1

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

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

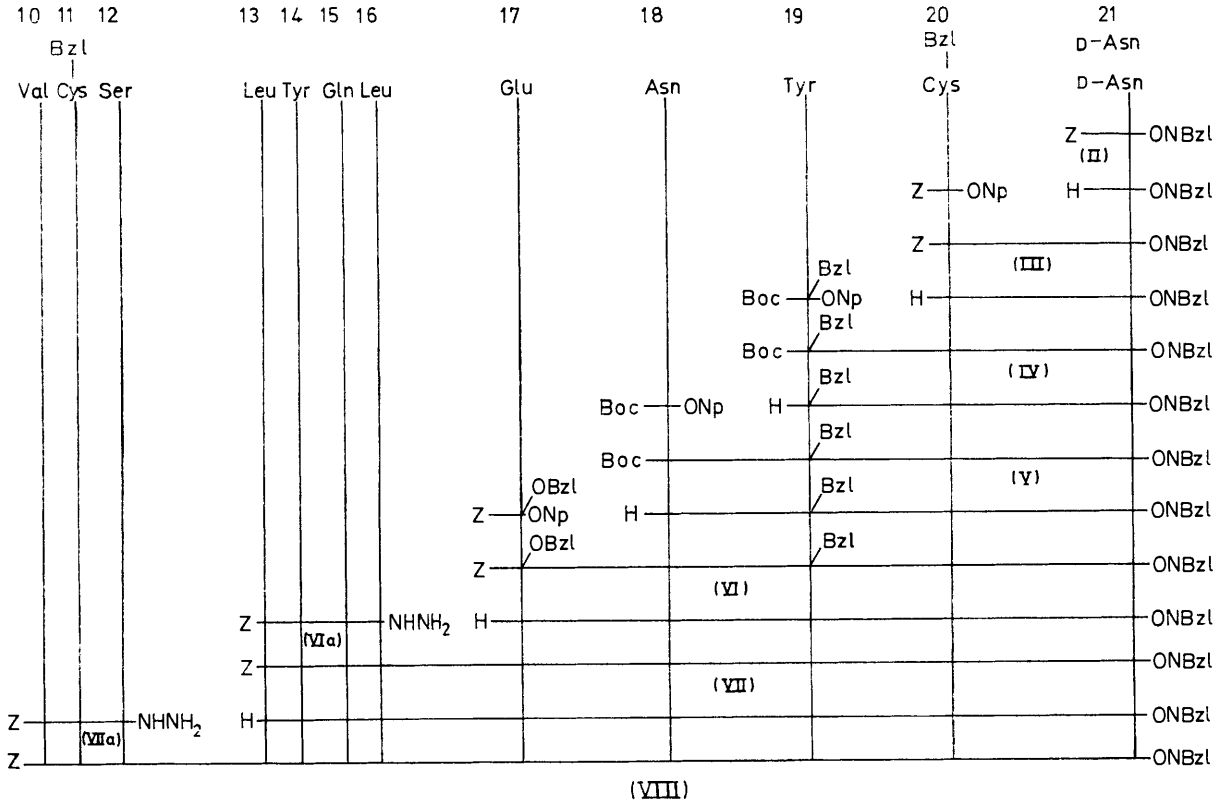
[10] (*a*) F. Sanger and H. Tuppy, *Biochem. J.*, 1951, **49**, 463; (*b*) F. Sanger and H. Tuppy, *ibid.*, p. 481; (*c*) H. Brown, F. Sanger, and P. Kitai, *ibid.*, 1955, **60**, 556.

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

[12] (*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.

in acetic acid and coupling of the resulting product with the tripeptide (VIIa) (sequence 10—12) by the azide method afforded the protected dodecapeptide fragment (VIII) (sequence 10—21). This derivative was deblocked at the amino-end with hydrogen bromide in trifluoroacetic acid and the product was condensed by the azide method with the protected pentapeptide (VIIIa) (sequence 5—9) (Scheme 1) to give the heptadecapeptide fragment (IX) (sequence 5—21). In the final synthetic step the protected heptadecapeptide derivative (IX) was deblocked with hydrogen bromide in trifluoroacetic acid and the resulting product condensed by the azide method with the *N*-terminal tetrapeptide derivative (IXa) to give the protected [D-Asn^{21}] A chain (Xa). On exposure to trifluoroacetic acid the latter was converted into the partially protected derivative (X). The blocking groups, (benzyloxycarbonyl, *S*-benzyl, and *p*-nitrobenzyl) were removed from the protected chain (X) by sodium in liquid ammonia.[13] 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 [D-Asn^{21}] A chain (XI) which was purified by chromatography on Sephadex G-15.

Amino-acid analysis [after acidic hydrolysis and enzymic digestion (Table 1)], t.l.c., and high voltage thin-layer electrophoresis at acidic [Figure 1(A)] and alkaline pH were employed to ascertain the homogeneity of the purified *S*-sulphonated peptide chain. The conversion of [D-Asn^{21}] A chain *S*-sulphonate into its thiol form and its combination with the *S*-sulphonated B chain to produce the [A-D-Asn^{21}] sheep insulin were performed as



SCHEME 2

TABLE 1

Amino-acid composition * of an acidic hydrolysate and an enzymic digest of the *S*-sulphonated [D-Asn^{21}] A chain of sheep insulin

	Acidic hydrolysis		Enzymic hydrolysis (aminopeptidase M)	
Amino-acid	Theory	Found	Theory	Found
Asp	2.0	2.0	0	0
Gln	0	0	2 } †	3.8
Asn	0	0	2 } †	
Ser	1.0	0.9	1.0	1.1 ‡
Glu	4.0	4.0	2.0	1.9
Gly	2.0	1.9	2.0	1.9
Ala	1.0	1.1	1.0	1.0
Cys	4.0	3.6	0	0
Val	2.0	1.5	2.0	2.0
Ile	1.0	0.9	1.0	0.9
Leu	2.0	2.2	2.0	2.2
Tyr	2.0	2.0	2.0	2.0
S-(SO_3^-)Cys	0	0	4.0	3.7

* Number of amino-acid molecules per molecule. † Coincident. ‡ Separated from glutamine and asparagine in a 30 °C chromatographic run.

[13] R. H. Sifferd and V. du Vigneaud, *J. Biol. Chem.*, 1935, **108**, 753.

described previously.[8,9,14] The insulin analogue was isolated by chromatography on a carboxymethylcellulose column with an acetate buffer (0.024M; pH 3.3) and an exponential sodium chloride gradient, according

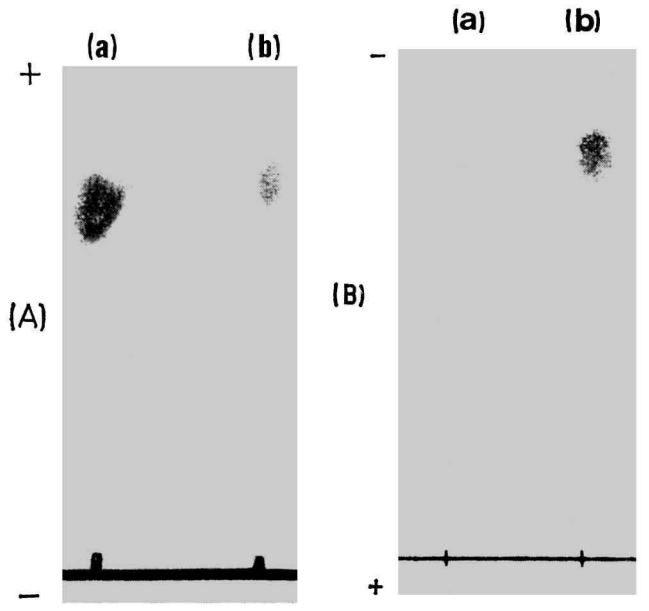


FIGURE 1 (A) High-voltage thin-layer electrophoresis of (a) natural bovine A chain *S*-sulphonate and (b) synthetic [D-Asn[21]] A chain *S*-sulphonate; 0.5N acetic acid, 3 500 V, 25 min; (B) high-voltage thin-layer electrophoresis of (a) synthetic [A-D-Asn[21]] sheep insulin and (b) natural bovine insulin; 0.5N-acetic acid, 3 500 V, 15 min

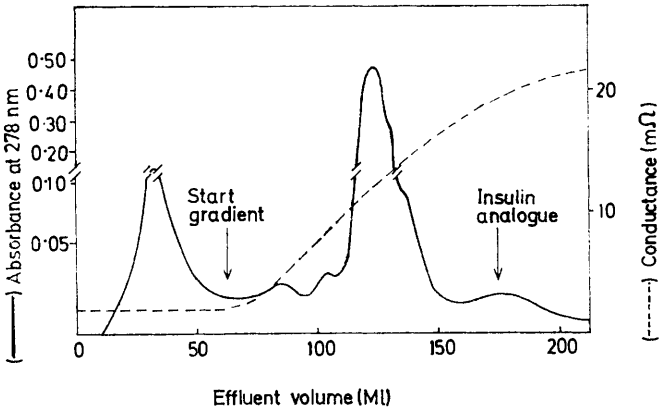


FIGURE 2 Chromatography of two combination mixtures (see Experimental section) of the thiol form of sheep [D-Asn[21]] A chain with the *S*-sulphonated bovine (sheep) B chain on a carboxymethylcellulose column (0.9 × 23 cm) with acetate buffer (0.024M; pH 3.3) and an exponential sodium chloride gradient. The column eluate was monitored by a Gilford recording spectrophotometer and by a conductivity meter (Radiometer, Copenhagen). The [A-D-Asn[21]] sheep insulin (160—200 ml of eluate) was recovered *via* the picrate as the hydrochloride

to the procedure reported previously[9,14] (Figure 2). The insulin analogue was isolated from the eluate *via* the picrate as the hydrochloride.[9,14] Amino-acid analysis [after acidic hydrolysis (Table 2)] and high voltage thin-layer electrophoresis [Figure 1(B)] were employed to ascertain its homogeneity.

TABLE 2

Amino-acid composition * of an acidic hydrolysate of [A-D-Asn[21]] sheep insulin

Amino-acid	Theory	Found	Amino-acid	Theory	Found
Lys	1.0	1.0	Gly	5.0	5.0
His	2.0	2.0	Ala	3.0	3.2
Arg	1.0	1.0	Cys	6.0	4.7 †
Asp	3.0	2.9	Val	5.0	4.7
Thr	1.0	0.9	Ile	1.0	0.6
Ser	2.0	2.0	Leu	6.0	6.0
Pro	1.0	0.9	Tyr	4.0	2.8 ‡
Glu	7.0	7.1	Phe	3.0	3.0

* Number of amino-acid residues per molecule. † Not corrected for destruction. ‡ Under the conditions used in our laboratory tyrosine recoveries after acidic hydrolysis of natural or synthetic insulins and analogues range from 65 to 80% of theory.

EXPERIMENTAL

Details of the techniques employed are given in reference 15. Elemental analysis, amino-acid analysis, and t.l.c. (whenever the solubility properties were favourable) were used to confirm the purity of the intermediates.

N-Benzyloxycarbonyl-D-asparagine (I).—To a cold (0 °C) mixture of D-asparagine hydrate (150 g) and magnesium oxide (100 g) in water (2 l), benzyloxycarbonyl chloride (175 ml) was added during 15 min with stirring. Stirring, was continued at 0 °C for 4 h during which time water (1 l) was added to permit effective mixing. The pH of the mixture was then adjusted to 2—3 with hydrochloric acid and the precipitate was filtered off and washed with dilute hydrochloric acid and water. The moist material was dissolved in boiling methanol (*ca.* 1.5 l), the solution was filtered, and the filtrate cooled at 0 °C. The *precipitate* was filtered off, washed with cold methanol and ether, and dried; yield 217 g (81%); m.p. 162—164°; $[\alpha]_D^{26}$ +6.5° (*c* 1.0 in Me_2N·CHO) (Found: C, 54.4; H, 5.3; N, 10.7. $C_{12}H_{14}N_2O_5$ requires C, 54.1; H, 5.3; N, 10.5%).

N-Benzyloxycarbonyl-D-asparagine p-Nitrobenzyl Ester (II).—A solution of *N*-benzyloxycarbonyl-D-asparagine (53.4 g), *p*-nitrobenzyl chloride (51.4 g), and triethylamine (28 ml) in Me_2N·CHO (150 ml) was heated at 65 °C for 4 h, cooled to room temperature, and poured into cold M-potassium hydrogen carbonate (1.5 l) with stirring. The *precipitate* was filtered off and washed with water and methanol (1.5 l); yield 62 g (77%); m.p. 167—169°; $[\alpha]_D^{26}$ +14.1° (*c* 1.0 in Me_2N·CHO) {the specific rotation for the corresponding L-isomer is −15° (*c* 1.0 in Me_2N·CHO) and not −63.3° (*c* 0.98 in Me_2N·CHO) as erroneously recorded previously;[16] lit.,[17] $[\alpha]_D^{20}$ −12.5 ± 1° (*c* 2.0 in Me_2N·CHO)} (Found: C, 56.7; H, 4.9; N, 10.3. $C_{19}H_{19}N_3O_7$ requires C, 56.9; H, 4.75; N, 10.5%).

N-Benzyloxycarbonyl-S-benzyl-L-cysteinyl-D-asparagine p-Nitrobenzyl Ester (III).—*N*-Benzyloxycarbonyl-D-asparagine *p*-nitrobenzyl ester (20.2 g) was treated with a solution (300 ml) of 2N-hydrogen bromide in acetic acid. After 2 h

[14] P. G. Katsoyannis, A. Trakatellis, S. Johnson, C. Zalut, and G. Schwartz, *Biochemistry*, 1967, **6**, 2642.

[15] P. G. Katsoyannis, J. Ginos, G. Schwartz, and A. Cosmatos, *J.C.S. Perkin I*, 1974, 1311.

[16] P. G. Katsoyannis and K. Suzuki, *J. Amer. Chem. Soc.*, 1961, **83**, 4057.

[17] D. Theodoropoulos and I. Souchleris, *J. Org. Chem.*, 1966, **31**, 4009.

at room temperature. the mixture was poured into anhydrous ether (700 ml) and the precipitated hydrobromide was filtered off, washed with ether, and dried (KOH) *in vacuo*. To a cold (5 °C) solution of this product in Me_2N·CHO (150 ml), triethylamine (7 ml) was added, followed by *N*-benzyloxycarbonyl-*S*-benzyl-L-cysteine *p*-nitrophenyl ester [18] (25 g). The mixture was kept overnight at room temperature, then N-ammonia (10 ml) was added and the mixture was stirred for 30 min and then diluted with ethyl acetate (1.5 l). The organic layer was washed (N-NH_4OH, water, N-HCl, and water) and concentrated to dryness. The *residue* was triturated with ether and crystallized from ethyl acetate; yield 22.4 g (75%); m.p. 182—184°; $[\alpha]_D^{26}$ −20.2° (*c* 1.0 in Me_2N·CHO) (Found: C, 58.9; H, 5.1; N, 9.7. $C_{29}H_{304}N_4O_8S$ requires C, 58.6; H, 5.1; N, 9.4%). The following amino-acid ratios were found after deblocking (2N-HBr in acetic acid) and digestion with aminopeptidase M: $Asn_{1.0}$benzylcysteine $_{1.0}$ (average amino-acid recovery 75%).

N-*t*-*Butoxycarbonyl*-O-*benzyl*-L-*tyrosyl*-S-*benzyl*-L-*cysteinyl*-D-*asparagine* p-*Nitrobenzyl Ester* (IV).—Compound(III) (29.7 g) was treated with 2N-hydrogen bromide in acetic acid (100 ml) for 1 h. The mixture was then poured into ether (1 l) and the precipitated hydrobromide was filtered off, washed with ether, and dried (P_2O_5 and KOH) *in vacuo*. Into a cooled (0 °C) solution of this hydrobromide in Me_2N·CHO (150 ml), triethylamine (7 ml) was added, followed by *N*-t-butoxycarbonyl-*O*-benzyl-L-tyrosine *p*-nitrophenyl ester [19] (25.5 g). After 24 h at room temperature, the mixture was diluted with N-ammonia (10 ml), stirred for 1 h, and poured into N-ammonia (1.5 l; 0 °C). The *precipitate* was filtered off, washed (N-NH_4OH, water, 10% citric acid, and water), dried *in vacuo*, and triturated with cold t-butyl acetate (100 ml) and warm methanol (300 ml); yield 29.3 g (72%); m.p. 169—173° (decomp.); $[\alpha]_D^{26}$ −5.4° (*c* 1.0 in Me_2N·CHO) (Found: C, 62.2; H, 6.0; N, 8.7. $C_{42}H_{47}N_5O_{10}S$ requires C, 62.0; H, 5.8; N, 8.6%).

N-*t*-*Butoxycarbonyl*-L-*asparaginyl*-O-*benzyl*-L-*tyrosyl*-S-*benzyl*-L-*cysteinyl*-D-*asparagine* p-*Nitrobenzyl Ester* (V).—A solution of compound (IV) (20.4 g) in trifluoroacetic acid (35 ml) was stored at room temperature for 1 h, and then poured into cold ether (200 ml). The precipitate was filtered off, washed with ether and dried (P_2O_5 and KOH) *in vacuo*. To a solution of this material in Me_2N·CHO (150 ml; 0 °C), triethylamine (3.5 ml) and *N*-t-butoxycarbonyl-L-asparagine *p*-nitrophenyl ester [20] (9.3 g) were added. After 48 h the mixture was diluted with N-ammonia (10 ml), stirred for 1 h, and poured into N-ammonia (1 l). The *precipitate* was collected by filtration, washed (N-NH_4OH, water, 10% citric acid, and water), dried, and triturated with ethyl acetate and acetone; yield 16.3 g (70%); m.p. 197—198° (decomp.); $[\alpha]_D^{26}$ −31.4° (*c* 1.0 in Me_2N·CHO) (Found: C, 59.3; H, 5.95; N, 10.4. $C_{46}H_{53}N_7O_{12}S$ requires C, 59.5; H, 5.75; N, 10.6%).

N-*Benzyloxycarbonyl*-γ-*benzyl*-L-*glutamyl*-L-*asparaginyl*-O-*benzyl*-L-*tyrosyl*-S-*benzyl*-L-*cysteinyl*-D-*asparagine* p-*Nitrobenzyl Ester* (VI).—A solution of compound (V) (9.3 g) in trifluoroacetic acid (30 ml) was stored at room temperature for 1 h and then poured into ether (150 ml). The precipitate was collected by filtration, washed with ether, and dried. To a solution of this material in a mixture of Me_2N·CHO (40 ml) and hexamethylphosphoramide (50 ml), triethylamine (1.5 ml) was added, followed by *N*-benzyloxycarbonyl-γ-benzyl-L-glutamic acid *p*-nitrophenyl ester [21] (4.3 g). The mixture was stirred at room temperature for 2 days, diluted with N-ammonia (10 ml) and after stirring for an additional 1 h was poured into N-ammonia (1 l; 0 °C). The *precipitate* was filtered off, washed (N-NH_4OH, water, N-HCl, and water), dried, and triturated with hot methanol; yield 10.2 g (86%); m.p. 214—216° (decomp.); $[\alpha]_D^{26}$ −26.2° (*c* 1.0 in Me_2N·CHO) (Found: C, 61.9; H, 5.4; N, 9.8; S, 2.6. $C_{61}H_{64}N_8O_{15}S$ requires C, 62.0; H, 5.45; N, 9.5; S, 2.7%).

N-*Benzyloxycarbonyl*-L-*leucyl*-L-*tyrosyl*-L-*glutaminyl*-L-*leucyl*-L-*glutamyl*-L-*asparaginyl*-L-*tyrosyl*-S-*benzyl*-L-*cysteinyl*-D-*asparagine* p-*Nitrobenzyl Ester* (VII).—Compound (VI) (5.9 g) was treated with 2N-hydrogen bromide in acetic acid (50 ml) for 1 h at room temperature. The mixture was concentrated to *ca.* 25 ml under reduced pressure and diluted with ether (300 ml). The precipitate was filtered off, washed with ether, and dried (KOH and P_2O_5) *in vacuo*. This product was dissolved in Me_2N·CHO (30 ml) containing triethylamine (2 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 hydrazide [22] (VIa) (4.1 g) in a mixture of acetic acid (35 ml), Me_2N·CHO (50 ml), and 2N-hydrochloric acid (7 ml) cooled to −10 °C, sodium nitrite (460 mg) in cold water (2 ml) was added. The mixture was stirred at −10 °C for 10 min and then poured into cold saturated sodium chloride solution (300 ml). The precipitated tetrapeptide azide was filtered off, washed (ice-cold water, N-$NaHCO_3$, and water) and dried (P_2O_5) at 0 °C for 2 h *in vacuo*. This azide was then added to the solution of the deblocked pentapeptide. The mixture was stirred for 24 h at 0 °C and then poured into a mixture of methanol (200 ml), water (300 ml), and N-hydrochloric acid (1 ml). The *precipitate* was filtered off, washed (50% aqueous methanol), and dried; yield 5.8 g (75%); m.p. 245° (decomp.); $[\alpha]_D^{26}$ −40.7° (*c* 1.0 in Me_2N·CHO) (Found: C, 57.8; H, 6.3; N, 11.7; S, 2.1. $C_{73}H_{91}N_{13}O_{21}S$ requires C, 57.7; H, 6.0; N, 12.0; S, 2.1%). Amino-acid analysis after acidic hydrolysis gave the following ratios: $Asp_{1.9}$-$Glu_{2.0}Leu_{2.1}Tyr_{1.4}$S-Benzylcysteine$_{0.8}$.

N-*Benzyloxycarbonyl*-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*-D-*asparagine* p-*Nitrobenzyl Ester* (VIII).—Compound (VII) (4.6 g) was treated with 2N-hydrogen bromide in acetic acid (60 ml) at room temperature for 1 h. Dilution of the mixture with ether (300 ml) caused the precipitation of the deblocked nonapeptide hydrobromide. This was filtered off, washed with ether, and dried (KOH and P_2O_5) *in vacuo*. To a solution of this derivative in Me_2N·CHO (50 ml) cooled to 0 °C, triethylamine (0.6 ml) was added, followed by the tripeptide azide prepared as follows. To a solution of *N*-benzyloxycarbonyl-L-valyl-*S*-benzyl-L-cysteinyl-L-serine hydrazide [22] (VIIa) (2.5 g) in a mixture of acetic acid (70 ml), Me_2N·CHO (30 ml), and 2N-hydrochloric acid (6 ml) cooled to −10 °C, sodium nitrite (320 mg) in cold water (2 ml) was added. The mixture was stirred at −10 °C for

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10 min and then poured into a cold saturated sodium chloride solution (350 ml). The precipitated tripeptide azide was filtered off, washed (ice-cold water, N-$NaHCO_3$, and water) and dried (P_2O_5) at 0 °C for 1 h *in vacuo*. This material was then added to the solution of the deblocked nonapeptide derivative. After 24 h at 0 °C the mixture was poured into N-hydrochloric acid (400 ml). The *precipitate* was filtered off, washed with water, triturated with hot methanol, and reprecipitated from Me_2N·CHO–H_2O; yield 4.9 g (85%); m.p. 260° (decomp.); $[\alpha]_D^{26}$ −24.6° (*c* 1.0 in Me_2N·CHO) (Found: C, 56.9; H, 6.25; N, 11.8; S, 3.4. $C_{91}H_{116}N_{16}O_{25}S_2$ requires C, 57.6; H, 6.1; N, 11.8; S, 3.4%). Amino-acid analysis of an acidic hydrolysate gave the following ratios: $Asp_{1.9}Ser_{0.9}Glu_{2.0}$-$Val_{1.0}Leu_{2.0}Tyr_{1.5}$S-benzylcysteine$_{1.6}$.

*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*-tyrosyl-*S*-benzyl-*L*-cysteinyl-*D*-asparagine* p-*Nitrobenzyl Ester* (IX).—Compound (VIII) (3.8 g) was dissolved in trifluoroacetic acid (35 ml) containing water (0.5 ml) and hydrogen bromide was passed through this solution for 45 min at 0 °C and then for 30 min at room temperature. Addition of ether (150 ml) caused precipitation of the deblocked dodecapeptide hydrobromide, which was filtered off, washed with ether, and dried (KOH and P_2O_5) *in vacuo*. This material was dissolved in a mixture of Me_2N·CHO (20 ml) and Me_2SO (15 ml) containing triethylamine (0.3 ml), cooled to 0 °C, and added to a solution of the pentapeptide azide prepared as follows. To a solution of *N*-benzyloxycarbonyl-L-glutaminyl-*S*-benzyl-L-cysteinyl-*S*-benzyl-L-cysteinyl-L-alanylglycine hydrazide [12a] (VIIIa) (1.8 g) in a mixture of Me_2N·CHO (25 ml) and Me_2SO (5 ml) containing N-hydrochloric acid in Me_2N·CHO (4.4 ml), cooled to −10°, isopentyl nitrite (0.3 ml) was added. The mixture was kept at this temperature for 5 min, cooled to −40 °C, neutralized with triethylamine (0.9 ml), and then diluted with the solution of the deblocked dodecapeptide prepared as described above. After 48 h at 4 °C the mixture was poured into methanol (350 ml) containing N-hydrochloric acid (1 ml). The *precipitate* was collected by centrifugation, washed (warm methanol and ether), and reprecipitated from a solution in a mixture of Me_2N·CHO and hexamethylphosphoramide (15 ml) by addition of ether (300 ml); yield 4 g (80%); m.p. 270° (decomp.); $[\alpha]_D^{26}$ −24.0° (*c* 1.0 in Me_2SO) (Found: C, 56.8; H, 6.15; N, 11.8; S, 5.05. $C_{121}H_{154}N_{22}O_{31}S_4$ requires C, 57.2; H, 6.05; N, 12.1; S, 5.05%). Amino-acid analysis of an acidic hydrolysate gave the following ratios: $Asp_{1.9}$-$Ser_{0.8}Glu_{3.0}Gly_{1.0}Ala_{1.0}Val_{1.0}Leu_{2.0}Tyr_{1.4}$S-benzylcysteine$_{3.3}$.

N-*Benzyloxycarbonylglycyl-*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-*D*-asparagine* p-*Nitrobenzyl Ester* (X).—The protected heptadecapeptide (IX) was deblocked (HBr in trifluoroacetic acid) as described in the synthesis of (IX). Addition of ether (200 ml) to the mixture 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 a mixture of Me_2N·CHO (25 ml) and Me_2SO (25 ml) containing triethylamine (0.15 ml), cooled to 0 °C, and added to a solution of the tetrapeptide azide prepared as follows. To a solution of *N*-benzyloxycarbonylglycyl-L-isoleucyl-L-valyl-γ-t-butyl-L-glutamic acid hydrazide [23] (IXa) (0.68 g) in a mixture of Me_2N·CHO (15 ml) and Me_2SO (3 ml) containing N-hydrochloric acid in Me_2N·CHO (2.2 ml) cooled to −10 °C, isopentyl nitrite (0.15 ml) was added. After 5 min at this temperature, the mixture was cooled to −40 °C, neutralized with triethylamine (0.45 ml) and then added to the solution of the deblocked heptadecapeptide prepared as described above. After 48 h at 4 °C the mixture was poured into methanol (750 ml) containing acetic acid (2 ml). The precipitated protected heneicosapeptide (Xa) was collected by centrifugation, washed (warm methanol and ether), and dried. A solution of this material in trifluoroacetic acid (25 ml) was stored at room temperature for 30 min and then diluted with ether (200 ml). The precipitated *partially protected heneicosapeptide* (X) was collected by centrifugation, washed with ether, and dried (KOH and P_2O_5) *in vacuo*; yield 2.3 g (78%); m.p. 279° (decomp.); $[\alpha]_D^{26}$ −31.8° [*c* 0.5 in $PO(NMe_2)_3$] (Found: C, 56.3; H, 6.2; N, 12.1; S, 4.3. $C_{139}H_{184}N_{26}O_{37}S_4$ requires C, 56.8; H, 6.25; N, 12.4; S, 4.5%). Amino-acid analysis after acidic hydrolysis gave the following ratios: $Asp_{1.9}Ser_{0.8}Glu_{3.8}Gly_{1.7}Ala_{1.0}Val_{1.5}Ile_{0.5}Leu_{2.0}Tyr_{1.4}$S-benzylcysteine$_{3.6}$.

*Glycyl-*L*-isoleucyl-*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*-tyrosyl-*S*-sulpho-*L*-cysteinyl-*D*-asparagine* (*Sheep Insulin* [D-*Asn*[21]] *A Chain* S-*Sulphonate*) (XI).—The reduction of the partially protected heneicosapeptide (X) (300 mg) with sodium in liquid ammonia (250 ml) was carried out as described previously.[12a] After evaporation of the ammonia, the residue was dissolved in 8M-guanidine hydrochloride (20 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 (680 mg) and freshly prepared sodium tetrathionate (340 mg). The mixture was stirred at room temperature for 18 h and then placed in an 18/32 Visking dialysis tube and dialysed against four changes of distilled water (4 l each) at 4 °C for 20 h. Upon lyophilization of the non-diffusible material the crude [D-Asn[21]] A chain *S*-sulphonate was obtained as a white powder. For purification the lyophilized material was dissolved in 0.015M-ammonium hydrogen carbonate (5 ml) and chromatographed on a Sephadex G-15 column (2.2 × 45 cm) equilibrated and eluted with 0.015M-ammonium hydrogen carbonate (flow rate *ca.* 40 ml h^{-1}). The elution pattern, as determined by monitoring the eluate with a Gilford recording spectrophotometer, indicated the presence of a major component and only small amounts of other components. Lyophilization of the eluate under the major peak gave the purified [D-Asn[21]] A chain *S*-sulphonate as a white powder (230 mg, 86%). Amino-acid analysis of the purified chain 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 amino-acid ratios shown in Table 1, in good agreement with the expected values. On t.l.c. [Cellulose 6 065, Eastman Chromagram Sheet; butan-1-ol–pyridine–acetic acid–water (30 : 20 : 6 : 24)], or high voltage thin-layer electrophoresis in 0.5N-acetic acid (pH 2.9 and 3500 V)

[23] P. G. Katsoyannis, A. Tometsko, and C. Zalut, *J. Amer. Chem. Soc.*, 1966, **88**, 5622.

(Figure 1) and in 0.5N-potassium hydrogen carbonate (pH 8.4 and 1800 V) the synthetic chain moved as single component (Pauly-positive spot).

S-*Sulphonated Derivatives of the A and B Chains of Bovine Insulin.*—These derivatives were prepared by oxidative sulphitolysis of bovine insulin followed by separation of the resulting *S*-sulphonated chains by continuous flow electrophoresis as described previously.[11]

Synthesis and Isolation of [A-D-Asn^{21}*] Sheep Insulin.*—The synthesis of this analogue by the interaction of the thiol form of sheep [D-Asn^{21}] A chain and the *S*-sulphonated form of the B chain of bovine (sheep) insulin was carried out as described previously.[8,14] In a typical experiment, [D-Asn^{21}] A chain *S*-sulphonate (20 mg) was converted into the thiol form upon treatment with 2-mercaptoethanol (in water, pH 5.0; 6—8 min and 100 °C) and treated with B chain *S*-sulphonate (5 mg). The mixture was treated as described previously.[9,14] 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 by the procedure reported previously.[9,14] Chromatography of two reaction mixtures, each corresponding to the amounts of materials indicated above, gave the pattern shown in Figure 2. The [A-D-Asn^{21}]-insulin was eluted with application of the gradient and was isolated from the eluate (160—200 ml) *via* the picrate as the hydrochloride [9] (0.6 mg).

Amino-acid analysis of this analogue after acidic hydrolysis gave a composition in good agreement with the expected values (Table 2). On thin-layer electrophoresis in 0.5N-acetic acid (pH 2.9; 3500 V) the synthetic analogue moved as a single component (Pauly reaction) and had a mobility similar to that of natural bovine insulin (Figure 3). It showed potencies of 8 I.U. mg^{-1} by the mouse convulsion assay method and 4 I.U. mg^{-1} by the radioimmunoassay method (33 and 16%, respectively, of the activity of the natural hormone).

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