A Synthetic Human Insulin Analogue Modified at Position B^{22}. [Lys^{22}-B] Human Insulin[1]

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A human insulin analogue which differs from the parent molecule in that the amino-acid residue arginine at position 22 in the B chain is replaced by lysine has been synthesized. For this purpose a human B chain analogue, in which the arginine residue at position 22 was replaced by a lysine residue, was prepared by the fragment condensation approach and isolated as the *S*-sulphonate. Combination of the latter compound with the thiol form of the human (porcine) insulin A chain yielded the [Lys-^{22}B] human insulin. This analogue was isolated in highly purified form by chromatography on a carboxymethylcellulose column with an exponential sodium chloride gradient. [Lys-^{22}B] insulin shows potencies of 13—14 I.U. mg^{-1} when assayed by the mouse convulsion method and of 8 I.U. mg^{-1} by the radioimmunoassay method (*cf.* 23—25 I.U. mg^{-1} for the natural hormone).

In the structure of insulin from various species, arginine occupies position 22 in the B chain.[2] The only exception is guinea pig insulin[3] which contains aspartic acid at B^{22}. In continuation of our studies aimed at the determination of structure–activity relationships in insulin,[1] we have now modified the hormone molecule at the B^{22} position. In order to gain some understanding of the role of the arginine residue at B^{22} in the expression of the biological profile of this hormone, we have replaced the arginine by a lysine residue in human insulin. The synthetic [Lys^{22}-B] human insulin was found to possess a potency of 13—14 I.U. mg^{-1} by the mouse convulsion assay method, *ca.* 56% of that of the natural hormone (23—25 I.U. mg^{-1}); the radioimmunoassay method gave a value of 8 I.U. mg^{-1}, *ca.* 32% of that of the natural hormone.

X-Ray analysis of the three-dimensional structure of insulin[4] has shown that the B^{20-23} segment forms a U-turn within the protein molecule that results in the placement of the B^{22} residue near the A-chain carboxy-terminus (A^{21}, Asn). Indeed, it appears that a salt bridge is formed between the *C*-terminal carboxy-group of the A^{21} asparagine and the guanidinium group of the B^{22} arginine which contributes to the stabilization of a conformation necessary for biological activity.[4] A stabilizing salt bridge can also be anticipated when the B^{22} arginine (p*K* of the guanidinium group 12·48) is replaced by lysine (p*K* of the ε-amino group 10·53). The high biological activity of [Lys^{22}-B] insulin makes it appear likely that the presence of a positive ion at B^{22} with the concomitant establishment of a salt bridge with the A^{21} carboxylate group contributes greatly to the degree of hormonal activity. The case of guinea pig insulin with aspartic acid at B^{22} (p*K* of the β-carboxylate 3·65) and endowed with substantial biological activity[5] (2·14 I.U. mg^{-1}) does not contradict this speculation. The presence of the B^{22} carboxylate in the vicinity of the A^{21} asparagine resembles the case of maleic acid. The second ionization constant of maleic acid is inordinately small, due to the formation of a hydrogen bond between the undissociated carboxy-group and the carboxylate anion. Thus in essence there exists an interaction between B^{22} and A^{21} positions (although considerably weaker than in the case of the B^{22} arginine or lysine insulins) that contributes to the stabilization of the tertiary structure of the molecule. It is interesting to note that the plot of log (biological activity) *vs.* p*K* of the functional group of the B^{22} amino-acid residue of various insulins is a straight line. We do not exclude the possibility that a contributing factor to the low biological activity of guinea pig insulin resides in the considerable sequential variation that exists between this molecule and the more active insulins.

General Aspects of the Synthesis.—The human insulin analogue was prepared by combination of the *S*-sulphonated human [Lys^{22}] B chain with the thiol form of the human A chain. The latter, which is identical with the respective chain of porcine insulin,[2d] was prepared by reduction with 2-mercaptoethanol of the *S*-sulphonated derivative of that chain prepared by oxidative sulphitolysis of porcine insulin. The sulphitolysis of insulin, the separation of the resulting A and B chain derivatives by continuous flow electrophoresis, and the conversion of the *S*-sulphonated A chain into its thiol form have been described previously.[6] The synthesis of the [Lys^{22}] B chain *S*-sulphonate was patterned after that of the human B chain,[7] since both chains have the same amino-acid sequence, differing only in the B^{22}

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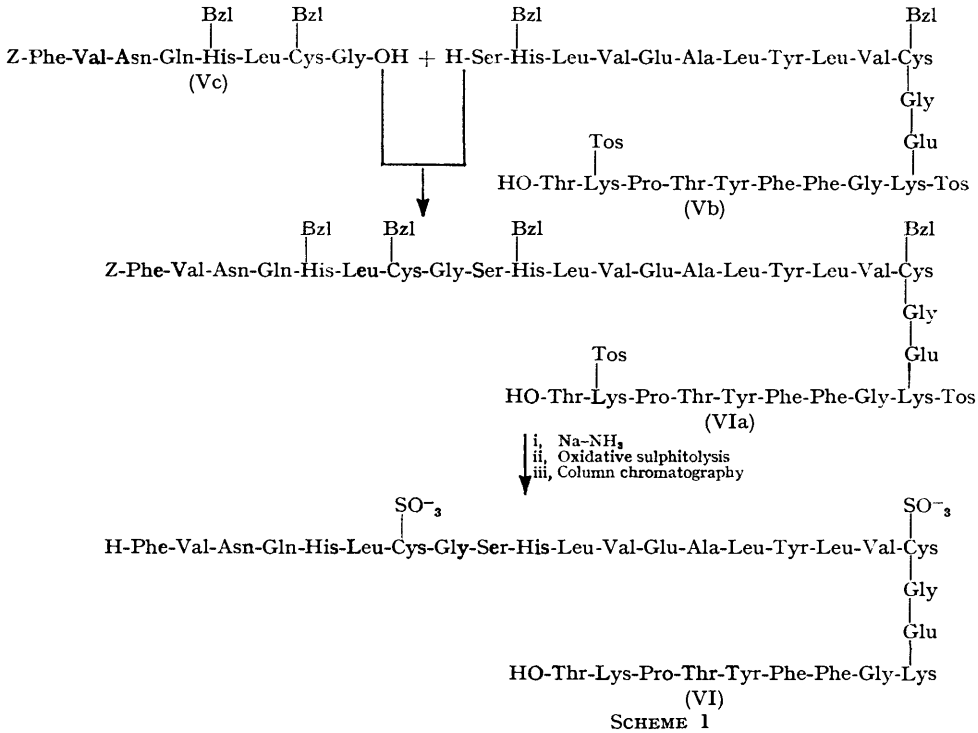
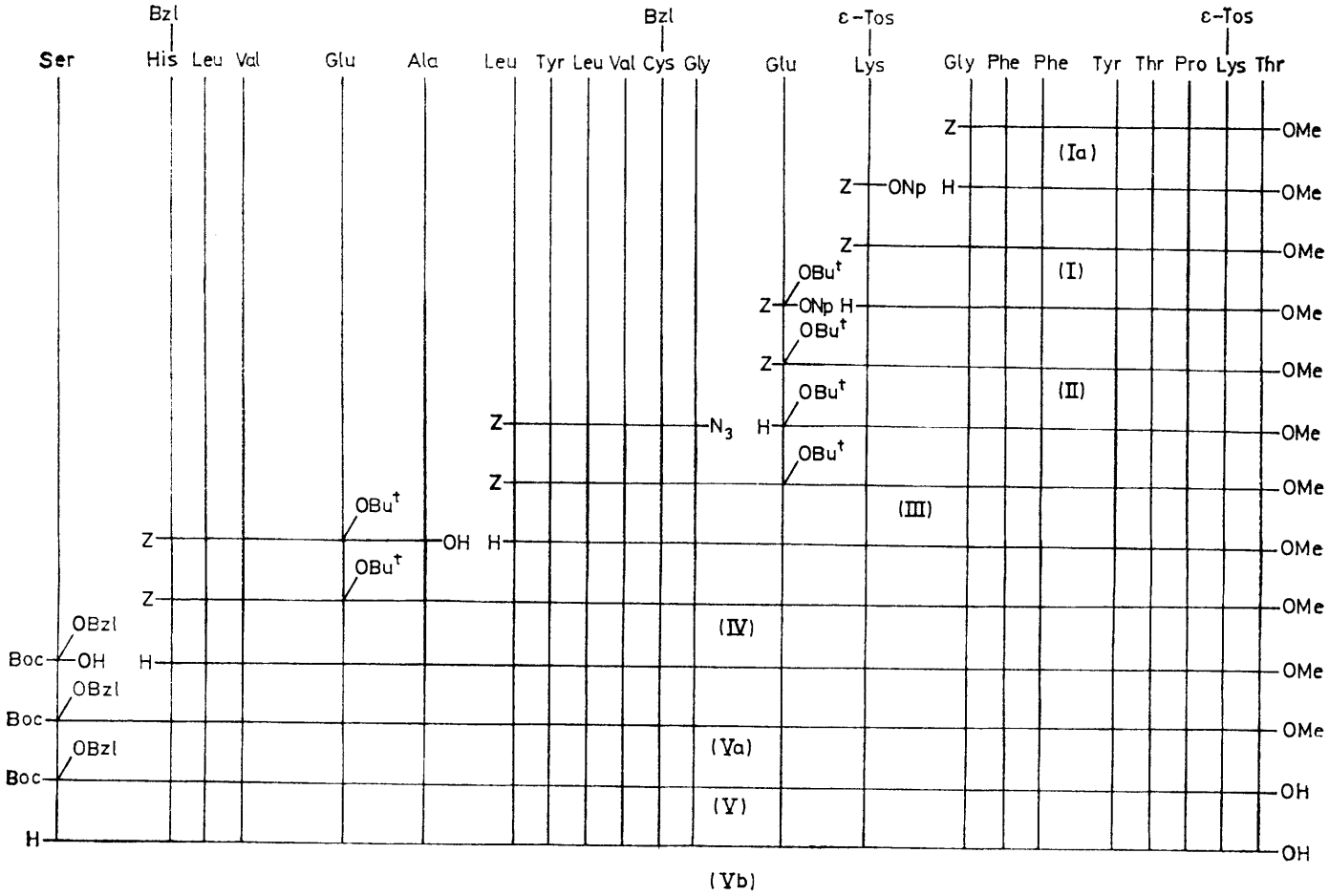
[7] (*a*) P. G. Katsoyannis, A. Tometsko, J. Ginos, and M. Tilak, *J. Amer. Chem. Soc.*, 1966, **88**, 164; (*b*) P. G. Katsoyannis, J. Ginos, C. Zalut, M. Tilak, S. Johnson, and A. Trakatellis, *ibid.*, 1971, **93**, 5877.



SCHEME 1



SCHEME 2

amino-acid residue. The overall synthesis is summarized in Schemes 1 and 2. It involved the construction of the protected triacontapeptide (VIa) containing the entire amino-acid sequence of the [Lys^{22}] B chain, removal of the protecting groups with sodium in liquid

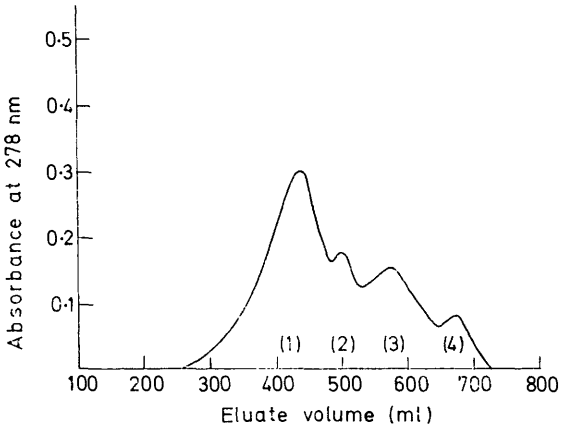


FIGURE 1 Elution pattern from chromatography on a carboxymethylcellulose column (4·3 × 49 cm) with urea–acetate buffer (pH 4·0) of a crude mixture obtained by the sodium–liquid ammonia treatment of synthetic protected human insulin [Lys^{22}] B chain followed by oxidative sulphitolysis, dialysis, and lyophilization

ammonia,[8] and conversion of the resulting thiol derivative into the *S*-sulphonated form (VI) by oxidative sulphitolysis [9] (Scheme 1). The protected triacontapeptide (VIa) was prepared by the coupling of the *N*-terminal octapeptide derivative [10] (Vc) with the *C*-terminal docosapeptide fragment (Vb) by the dicyclohexylcarbodi-imide–*N*-hydroxysuccinimide method.[11] The partially protected docosapeptide derivative was constructed by the fragment condensation approach (Scheme 2).

Removal of the blocking groups from the protected docosapeptide (VIa) was accomplished by sodium in liquid ammonia in a special apparatus.[7b] The reaction was carried out in the presence of sodium amide which effectively inhibited [7b,12] the cleavage of the polypeptide chain between the threonine and proline residues at positions 27 and 28.

The reduced material was dissolved in 8M-guanidine hydrochloride and subjected to oxidative sulphitolysis on exposure to sodium sulphite and sodium tetrathionate at pH 8·9. Dialysis of the mixture followed by lyophilization of the non-diffusible material afforded the crude *S*-sulphonated [Lys^{22}] B chain. Purification was achieved [7b,12] by chromatography of the crude product on a carboxymethylcellulose column equilibrated and eluted with a urea–acetate buffer (pH 4·0) (see Figure 1). The pure [Lys^{22}] B chain *S*-sulphonate was isolated by the procedure applied previously in the synthesis of the sheep insulin B chain.[7b,12] Briefly, the fractions under peak 3 (535—635 ml) were chromatographed on a Sephadex G-15 column (5% acetic acid as eluant), and the peptide material from this eluate was precipitated as the picrate and chromatographed on a Sephadex G-15 column equilibrated and eluted with ammonium hydrogen carbonate. Lyophilization of the eluate yielded the purified [Lys^{22}] B chain *S*-sulphonate. Amino-acid analysis of the purified material after acidic hydrolysis gave a composition in good agreement with the theoretically expected values. The synthetic material was completely digested by aminopeptidase M and on high voltage thin-layer electrophoresis at pH 10, exhibited a single Pauly-positive spot.

Isolation of the Human Insulin Analogue.—The conversion of the *S*-sulphonated A chain of human (porcine) insulin into its thiol form was carried out by interaction with 2-mercaptoethanol at pH 5·0 and 100 °C. Combination of the reduced chain with the *S*-sulphonated [Lys^{22}] B chain to produce the human insulin analogue was accomplished by the procedure reported previously.[6b,13] Isolation of the insulin analogue in a highly purified form from the combination mixture of its two chains was achieved by chromatography on a 0·9 × 23 cm carboxymethylcellulose column with an acetate buffer (pH 3·3) and an exponential sodium chloride gradient (Figure 2). The insulin analogue was isolated from the eluate *via* the picrate as the hydrochloride, as described previously.[6b,13b]

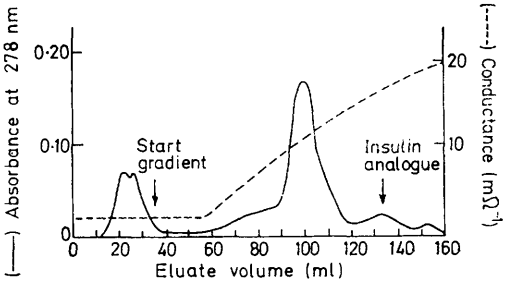


FIGURE 2 Chromatography of four combination mixtures (see Experimental section) of human [Lys^{22}] B chain *S*-sulphonate with the thiol form of human (porcine) A chain on a carboxymethylcellulose column (0·9 × 23 cm) with acetate buffer (0·024M; pH 3·3) and an exponential sodium chloride gradient. The column eluate was continuously monitored by a Gilford recording spectrophotometer and by a conductivity meter (Radiometer, Copenhagen). The purified [Lys^{22}-B] human insulin (120—145 ml of eluate) was recovered *via* the *picrate* as the hydrochloride

Amino-acid analysis of an acidic hydrolysate of this analogue gave a composition expressed in molar ratios in good agreement with the theoretically expected values

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(Table). The synthetic analogue was homogeneous on thin-layer electrophoresis in 0·5N-acetic acid at 3400 V. The activity of the human [Lys^{22}-B] insulin was 13—14 I.U. mg^{-1} by the mouse convulsion assay method (natural insulin 23—25 I.U. mg^{-1}) and 8 I.U. mg^{-1} by the radioimmunoassay method.

Amino-acid composition [a] of an acidic hydrolysate of the [Lys^{22}-B] human insulin

Amino-acid	Theory	Found
Lys	2	2·0
His	2	2·2
Asp	3	2·8
Thr	3	2·4 [b]
Ser	3	2·8 [b]
Glu	7	6·6
Pro	1	0·9
Gly	4	4·2
Ala	1	1·2
Half-cystine	6	3·6 [b]
Val	4	3·6 [c]
Ile	2	1·3 [c]
Leu	6	5·7
Tyr	4	2·8 [b,d]
Phe	3	3·2

[a] Number of amino-acid residues per molecule. [b] Uncorrected for destruction. [c] Low values are due to the well-known resistance of Ile-Val to acidic hydrolysis. [d] 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 used are given in ref. 1. 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 ninhydrin test. Elemental analysis, amino-acid analysis, and paper chromatography (ninhydrin detection) (whenever the solubility properties were favourable) were used to confirm the purity of the intermediates.

N$^{\alpha}$-*Benzyloxycarbonyl*-N$^{\epsilon}$-*tosyl*-L-*lysylglycyl*-L-*phenylalanyl*-L-*phenylalanyl*-L-*tyrosyl*-L-*threonyl*-L-*prolyl*-N$^{\epsilon}$-*tosyl*-L-*lysyl*-L-*threonine Methyl Ester* (I).—*N*-Benzyloxycarbonylglycyl-L-phenylalanyl-L-phenylalanyl-L-tyrosyl-L-threonyl-L-prolyl-*N*$^{\epsilon}$-tosyl-L-lysyl-L-threonine methyl ester [14] (Ia) (3 g) was hydrogenated for 3·5 h over 10% palladium–charcoal (0·6 g) in methanol (50 ml) containing 2N-hydrogen chloride (1·3 ml). The catalyst was filtered off and the filtrate concentrated under reduced pressure. To a solution of the residue in dimethylformamide (DMF) (20 ml) cooled to 2°, triethylamine (0·33 ml) was added followed by *N*$^{\alpha}$-benzyloxycarbonyl-*N*$^{\epsilon}$-tosyl-L-lysine *p*-nitrophenyl ester [15] (1·3 g). After 48 h at room temperature the mixture was poured into cold N-ammonia (300 ml) saturated with sodium chloride. The *precipitate* was collected, washed (N-NH_4OH, 0·5N-HCl, and water), dried, and reprecipitated twice from a solution in ethanol by the addition of ether; yield 2·9 g (90%); m.p. indefinite; after hydrogenolysis (in methanol–HCl) R_F^1 0·94, R_F^2 3·73 × His, single spot (Found: C, 59·6; H, 6·4; N, 10·0. $C_{76}H_{96}N_{11}O_{19}S_2$ requires C, 59·6; H, 6·3; N, 10·1%); amino-acid analysis after acidic hydrolysis: $Lys_{1\cdot8}Thr_{1\cdot8}Pro_{1\cdot0}Gly_{1\cdot0}Phe_{2\cdot0}Tyr_{0\cdot7}$.

N-*Benzyloxycarbonyl*-γ-*t*-*butyl*-L-*glutamyl*-N$^{\epsilon}$-*tosyl*-L-*lysylglycyl*-L-*phenylalanyl*-L-*phenylalanyl*-L-*tyrosyl*-L-*threonyl*-L-*prolyl*-N$^{\epsilon}$-*tosyl*-L-*lysyl*-L-*threonine Methyl Ester* (II).—The protected nonapeptide (I) (2·9 g) was hydrogenated for 3·5 h over 10% palladium–charcoal (0·5 g) in methanol (35 ml) containing 2N-hydrogen chloride (0·8 ml). After removal of the catalyst and evaporation of the solvent the residue was dissolved in DMF (20 ml). To this solution cooled to 2°, triethylamine (0·22 ml) and *N*-benzyloxycarbonyl-γ-t-butyl-L-glutamic acid p-nitrophenyl ester [16] (0·71 g) were added. After 48 h the mixture was poured into cold 0·5N-ammonia (300 ml) saturated with sodium chloride. The *precipitate* was collected, washed (0·5N-NH_4OH, 0·5N-acetic acid, and water), dried, and reprecipitated from methanol (60 ml); yield 2 g (74%), m.p. 205—206°; $[\alpha]_D^{26}$ −24° (*c* 1·0 in DMF); after hydrogenolysis (methanol–acetic acid) R_F^1 0·96, R_F^2 4·22 × His (Found: C, 59·4; H, 6·6; N, 9·8. $C_{84}H_{111}N_{12}O_{22}S_2$ requires C, 59·4; H, 6·5; N, 9·8%); amino-acid analysis after acidic hydrolysis: $Lys_{1\cdot8}Thr_{1\cdot8}Glu_{1\cdot0}Pro_{0\cdot9}Gly_{1\cdot0}Phe_{2\cdot0}Tyr_{0\cdot7}$.

N-*Benzyloxycarbonyl*-L-*leucyl*-L-*tyrosyl*-L-*leucyl*-L-*valyl*-S-*benzyl*-L-*cysteinylglycyl*-γ-*t*-*butyl*-L-*glutamyl*-N$^{\epsilon}$-*tosyl*-L-*lysylglycyl*-L-*phenylalanyl*-L-*phenylalanyl*-L-*tyrosyl*-L-*threonyl*-L-*prolyl*-N$^{\epsilon}$-*tosyl*-L-*lysyl*-L-*threonine Methyl Ester* (III).—A solution of the protected decapeptide (II) (1·9 g) in a mixture of methanol (110 ml) and DMF (30 ml) containing acetic acid (0·7 ml) was hydrogenated for 5 h over 10% palladium–charcoal (1 g). The catalyst was filtered off and washed with methanol (30 ml) and DMF (100 ml). The combined filtrates and washings were concentrated to a small volume *in vacuo* and then mixed with ether. The precipitated decapeptide acetate was filtered off, washed with ether, and dried. This product was dissolved in DMF (20 ml) containing triethylamine (0·11 ml), cooled to −5°, and allowed to react with the hexapeptide azide prepared as follows. *N*-Benzyloxycarbonyl-L-leucyl-L-tyrosyl-L-leucyl-L-valyl-*S*-benzyl-L-cysteinylglycine hydrazide [17] (1·05 g) was dissolved in DMF (20 ml) containing 2N-hydrogen chloride (3 ml). After cooling the solution to −15°, sodium nitrite (81 mg) dissolved in cold water (2 ml) was added. The mixture was stirred at −15° for 6 min and then poured into a cold mixture of saturated sodium chloride (80 ml) and sodium hydrogen carbonate (10 ml). The precipitated hexapeptide azide was filtered off, washed with water (0°), and dried for 2 h (P_2O_5) *in vacuo*. The i.r. spectrum of the dry product exhibited the characteristic azide band at 4·75 μm without any trace of the isocyanate band. This azide was then added to the solution of the deblocked decapeptide prepared as described previously, along with cold DMF (15 ml). The mixture was stirred for 48 h at 0° and then poured into a mixture of methanol (300 ml), water (130 ml), and acetic acid (1 ml). The *precipitate* was isolated by centrifugation, washed (methanol, ether), and dried; yield 1·8 g (97%), m.p. 267—269°; $[\alpha]_D^{26}$ −31·9° (*c* 1 in DMF) (Found: C, 60·4; H, 6·6; N, 10·2. $C_{123}H_{165}N_{18}O_{29}S_3$ requires C, 60·2; H, 6·75; N, 10·3%); amino-acid analysis after acidic hydrolysis: $Lys_{2\cdot0}Thr_{1\cdot8}Glu_{1\cdot0}Pro_{0\cdot9}Gly_{2\cdot0}Val_{1\cdot0}Leu_{2\cdot0}Phe_{1\cdot9}Tyr_{1\cdot7}$-*S*-Benzylcysteine$_{0\cdot9}$.

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[16] E. Schnabel, *Annalen*, 1963, **667**, 171.
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N$^{\alpha}$-*Benzyloxycarbonyl*-N^{im}-*benzyl*-L-*histidyl*-L-*leucyl*-L-*valyl*-γ-t-*butyl*-L-*glutamyl*-L-*alanyl*-L-*leucyl*-L-*tyrosyl*-L-*leucyl*-L-*valyl*-S-*benzyl*-L-*cysteinylglycyl*-L-*glutamyl*-N$^{\epsilon}$-*tosyl*-L-*lysylglycyl*-L-*phenylalanyl*-L-*phenylalanyl*-L-*tyrosyl*-L-*threonyl*-L-*prolyl*-N$^{\epsilon}$-*tosyl*-L-*lysyl*-L-*threonine Methyl Ester* (IV).—The protected hexadecapeptide (III) (1·72 g) was dissolved in trifluoroacetic acid (20 ml) containing water (0·2 ml), and hydrogen bromide was passed through the solution for 1 h. Concentration under reduced pressure and addition of ether precipitated the deblocked hexadecapeptide ester hydrobromide. This product was filtered off, washed with ether, and dried (KOH). To a solution of this material in DMF (15 ml) cooled to 5°, triethylamine (0·2 ml) was added followed by the partially protected pentapeptide N^{α}-benzyloxycarbonyl-N^{im}-benzyl-L-histidyl-L-leucyl-L-valyl-γ-t-butyl-L-glutamyl-L-alanine [18] which was activated as follows. A solution of the partially protected pentapeptide (1·19 g) and *N*-hydroxysuccinimide (0·16 g) in a mixture of DMF (20 ml) and hexamethylphosphoramide (5 ml) was cooled to −10° and dicyclohexylcarbodiimide (0·29 g) was added. After 1 h at −10° and 5 h at 4° the mixture was filtered through a sintered-glass funnel and the filtrate was added to the solution of the free base of the hexadecapeptide ester prepared as described above. After 48 h at room temperature the mixture was poured into methanol (300 ml) containing acetic acid (3 ml). The precipitated *heneicosapeptide derivative* (IV) was isolated by centrifugation, washed (hot methanol and ether), and dried; yield, 2 g (91%), m.p. 268—269°; $[\alpha]_D^{26}$ −22·4° (*c* 1 in Me_2SO) (Found: C, 60·0; H, 6·9; N, 11·2. $C_{155}H_{212}N_{25}O_{36}S_3$ requires C, 60·1; H, 6·9; N, 11·3%); amino-acid analysis after acidic hydrolysis: $Lys_{1.6}Thr_{1.8}Glu_{2.0}Pro_{1.0}Gly_{2.2}Ala_{1.1}Val_{1.9}Leu_{3.0}Tyr_{1.6}Phe_{2.0}$-*S*-Benzylcysteine$_{0.5}$Benzylhistidine$_{1.0}$.

N-*t*-*Butoxycarbonyl*-O-*benzyl*-L-*seryl*-N^{im}-*benzyl*-L-*histidyl*-L-*leucyl*-L-*valyl*-L-*glutamyl*-L-*alanyl*-L-*leucyl*-L-*tyrosyl*-L-*leucyl*-L-*valyl*-S-*benzyl*-L-*cysteinylglycyl*-L-*glutamyl*-N$^{\epsilon}$-*tosyl*-L-*lysylglycyl*-L-*phenylalanyl*-L-*phenylalanyl*-L-*tyrosyl*-L-*threonyl*-L-*prolyl*-N$^{\epsilon}$-*tosyl*-L-*lysyl*-L-*threonine* (V).—The heneicosapeptide derivative (IV) (1 g) was dissolved in trifluoroacetic acid (15 ml) containing water (0·15 ml), and hydrogen bromide was passed through the solution for 1 h. After most of the solvent was removed under reduced pressure the residue was mixed with ether. The precipitated heneicosapeptide ester hydrobromide was collected, triturated with warm propan-2-ol, and washed with ether. The dried product was dissolved in hexamethylphosphoramide (20 ml) containing triethylamine (0·13 ml) and this solution was mixed with *N*-t-butoxycarbonyl-*O*-benzyl-L-serine which was activated as follows. To a solution of *N*-t-butoxycarbonyl-*O*-benzyl-L-serine (1·47 g) and *N*-hydroxysuccinimide (0·57 g) in DMF (15 ml) cooled to −10°, dicyclohexylcarbodi-imide (1·03 g) was added. After 1 h at −5° and 4 h at 4° the *NN'*-dicyclohexylurea was filtered off, and to the filtrate the solution of the free base of the heneicosapeptide ester, prepared as described above, was added. The mixture was stirred for 24 h at room temperature and then poured into ether (500 ml). The precipitated protected docosapeptide (Va) was isolated by centrifugation, washed (dilute acetic acid), and dried. To a solution of this product (0·84 g) in hexamethylphosphoramide (10 ml) cooled to 10°, 1N-sodium hydroxide (2·1 ml) and water (3 ml) were added over 30 min. After being stirred for another 30 min (10—15°) this solution was cooled (5°) and diluted with 2N-hydrogen chloride (1·05 ml) and water (200 ml). The precipitated *partially protected docosapeptide* (V) was isolated, washed with water, and dried; yield 0·83 g, m.p. 278—281°, $[\alpha]_D^{25}$ −21·6° (*c* 1 in Me_2SO) (Found: C, 60·0; H, 6·7; N, 11·4. $C_{157}H_{215}N_{26}O_{38}S_2$ requires C, 59·5; H, 6·8; N, 11·5%); amino-acid analysis after acidic hydrolysis: $Lys_{1.6}Thr_{1.8}Ser_{0.8}Glu_{2.0}Pro_{1.0}Gly_{2.3}Ala_{0.8}Val_{1.9}$-$Leu_{3.0}Tyr_{1.6}Phe_{2.1}$*S*-Benzylcysteine$_{1.0}$Benzylhistidine$_{0.7}$.

L-*Phenylalanyl*-L-*valyl*-L-*asparaginyl*-L-*glutaminyl*-L-*histidyl*-L-*leucyl*-S-*sulpho*-L-*cysteinylglycyl*-L-*seryl*-L-*histidyl*-L-*leucyl*-L-*valyl*-L-*glutamyl*-L-*alanyl*-L-*leucyl*-L-*tyrosyl*-L-*leucyl*-L-*valyl*-S-*sulpho*-L-*cysteinylglycyl*-L-*glutamyl*-L-*lysyl*-*glycyl*-L-*phenylalanyl*-L-*phenylalanyl*-L-*tyrosyl*-L-*threonyl*-L-*prolyl*-L-*lysyl*-L-*threonine* (*Human Insulin* [Lys^{22}] *B Chain* S-*Sulphonate*) (VI).—A solution of the docosapeptide derivative (V) (820 mg) in trifluoroacetic acid (10 ml) was stored at room temperature for 1 h and then diluted with ether. The precipitated deblocked docosapeptide salt (Vb) was collected, washed with ether, and dried. A solution of this product in hexamethylphosphoramide (15 ml) was cooled to 0° and neutralized with triethylamine (0·17 ml) just prior to the addition of the *N*-terminal partially protected octapeptide (Vc) which was activated as follows. *N*-Benzyloxycarbonyl-L-phenylalanyl-L-valyl-L-asparaginyl-L-glutaminyl-N^{im}-benzyl-L-histidyl-L-leucyl-*S*-benzyl-L-cysteinylglycine (Vc) (1 g) and *N*-hydroxysuccinimide (93 mg) were dissolved in a mixture of DMF (10 ml) and hexamethylphosphoramide (7 ml), and to this solution cooled to −5° was added dicyclohexylcarbodiimide (167 mg). After 12 h at 5° the mixture was filtered and the filtrate added to the solution of the free base of the docosapeptide derivative prepared as described above. After 40 h at room temperature the mixture was poured into methanol (300 ml) containing acetic acid (0·4 ml). The precipitated crude protected B chain analogue (VIa) was isolated by centrifugation, washed (methanol and ether), and dried: yield 768 mg (68%); amino-acid analysis after acidic hydrolysis: $Lys_{1.9}Asp_{1.1}Thr_{1.4}Ser_{0.7}$-$Glu_{3.0}Pro_{0.8}Gly_{3.4}Ala_{0.7}Val_{3.0}Leu_{4.0}Tyr_{1.1}Phe_{2.9}$*S*-Benzylcysteine$_{1.8}$Benzylhistidine$_{2.3}$. This material was converted into the *S*-sulphonated [Lys^{22}] B chain (VI) by deblocking with sodium in liquid ammonia in the presence of sodium amide, followed by oxidative sulphitolysis. The deblocking procedure was essentially that described in the synthesis of the human B chain *S*-sulphonate and was carried out in the glass apparatus devised for sodium–liquid ammonia reactions.[7b] The dried protected B chain analogue (VIa) (200 mg) was dissolved in anhydrous liquid ammonia (200 ml) containing sodium amide (60 mg). The reduction was carried out at the b.p. of the solution by the dropwise addition of a liquid ammonia solution of sodium. The faint blue colour indicating the end-point of the reaction (excess of sodium) was allowed to persist for 20 s and then discharged by the addition of a few crystals of ammonium chloride. The resulting clear solution was concentrated at atmospheric pressure to *ca.* 10 ml and dried from the frozen state. The residue was dissolved in 8M-guanidine hydrochloride (30 ml) and to this solution, adjusted to pH 8·9 (dilute acetic acid or ammonia depending on the pH of the solution), was added freshly prepared sodium tetrathionate (250 mg) and 30 min later sodium sulphite (500 mg). The mixture was stirred for 3 h and then placed in an 18/32 Visking dialysis tube and dialysed against four changes of distilled water

[18] P. G. Katsoyannis, J. Ginos, A. Cosmatos, and G. Schwartz, *J. Amer. Chem. Soc.*, 1973, **95**, 6427.

(3 l each) at 4° for 24 h. Upon lyophilization of the non-diffusible material the crude [Lys^{22}] B chain *S*-sulphonate was obtained as a white powder (yield 116 mg). This material was dissolved in urea–acetate buffer (6 ml) (0·04M-sodium acetate, 8M-urea; pH 4·0) and placed on a carboxymethylcellulose column (4·3 × 49 cm) equilibrated and eluted with the same buffer. The eluant was monitored continuously with a Gilford recording spectrophotometer at 278 nm. The chromatographic pattern obtained (Figure 1) indicated the presence of four components 1—4. Components 1, 2, and 4 were not characterized. The eluate under peak 3 (535—635 ml) was collected, acidified (pH 3; concentrated HCl), and placed on a Sephadex G-15 column (4 × 60 cm) equilibrated and eluted with 5% (v/v) acetic acid. The urea-free eluate from this column containing the peptide material (detected with a Gilford recording spectrophotometer at 278 nm) was collected, concentrated (25—30°) to *ca.* 10 ml, and mixed with a saturated picric acid solution (12 ml). After 24 h at 5° the precipitated picrate of the [Lys^{22}] B chain *S*-sulphonate was isolated by centrifugation and dissolved in 0·05N-ammonium hydrogen carbonate (2 ml) with the aid of a few drops of 1N-ammonia. This solution was placed on a Sephadex G-15 column (1·2 × 50 cm) equilibrated and eluted with 0·05N-ammonium hydrogen carbonate. The peptide material, located by continuous monitoring of the eluate with a Gilford recording spectrophotometer at 278 nm, was recovered as a white powder by lyophilization of the eluate; yield 16 mg; amino-acid analysis of the purified [Lys^{22}] B chain *S*-sulphonate after acidic hydrolysis in good agreement with the expected values: $Lys_{1.9}His_{1.8}Asp_{1.0}Thr_{1.4}Ser_{0.9}Glu_{3.0}Pro_{1.0}Gly_{3.2}Ala_{1.0}Cys_{2.1}Val_{3.0}Leu_{4.2}Tyr_{2.0}Phe_{3.0}$. The synthetic material was completely digested by aminopeptidase M. On high voltage thin-layer electrophoresis in 0·01M-ammonium hydrogen carbonate (pH 10; 2800 V) the synthetic chain moved as single component.

S-Sulphonate of the A Chain of Human (Porcine) Insulin.—The A chain of human insulin is identical with the corresponding chain of porcine insulin. The *S*-sulphonated A chain of human (porcine) insulin was prepared by oxidative sulphitolysis of porcine insulin followed by continuous flow electrophoretic separation of the resulting mixture of the A and B chains as we have reported previously.[6a]

Synthesis and Isolation of [Lys^{22}-B] *Human Insulin.*—The synthesis of this insulin analogue was accomplished by the procedures we have described in detail previously; namely, the interaction of the thiol form of the human (porcine) A chain with the *S*-sulphonated [Lys^{22}] B chain. In a typical combination experiment a solution of human (porcine) A chain *S*-sulphonate (20 mg) in water (5 ml) was treated with 1M-mercaptoethanol (0·9 ml) at pH 5 for 6 min at 100°. The resulting thiol form of the A chain was allowed to react with the *S*-sulphonated [Lys^{22}] B chain (5 mg) at pH 9·6 and the combination mixture was treated as described previously. Isolation of the insulin analogue from the combination mixture was carried out by chromatography on a 0·9 × 23 cm carboxymethylcellulose column with an acetate buffer (0·024 M; pH 3·3) and an exponential sodium chloride gradient following the procedure we have reported previously. Chromatography of four combination mixtures, each corresponding to the amounts of materials indicated above, gave the pattern shown in Figure 2. The [Lys^{22}-B] insulin is eluted with the application of the sodium chloride gradient and was isolated from the eluate (120—145 ml) *via* the picrate as the hydrochloride (0·3 mg) according to the procedure described previously.

Amino-acid analysis of an acidic hydrolysate of this analogue gave a composition expressed in molar ratios in good agreement with the expected values (Table). On thin-layer high-voltage electrophoresis in 0·5N-acetic acid at 3400 V, the synthetic analogue moved as a single component (Pauly reaction) and had a mobility slightly different from that of bovine insulin. The human [Lys^{22}-B] insulin was found to possess a potency of 13—14 I.U. mg^{-1} by the mouse convulsion assay method (*cf.* 23—25 I.U. mg^{-1} for the natural hormone) and 8 I.U. mg^{-1} by the radioimmunoassay method.

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