Insulin Peptides. Part XXIV.[1] A Novel Synthesis of the Human Insulin B Chain *S*-Sulphonate

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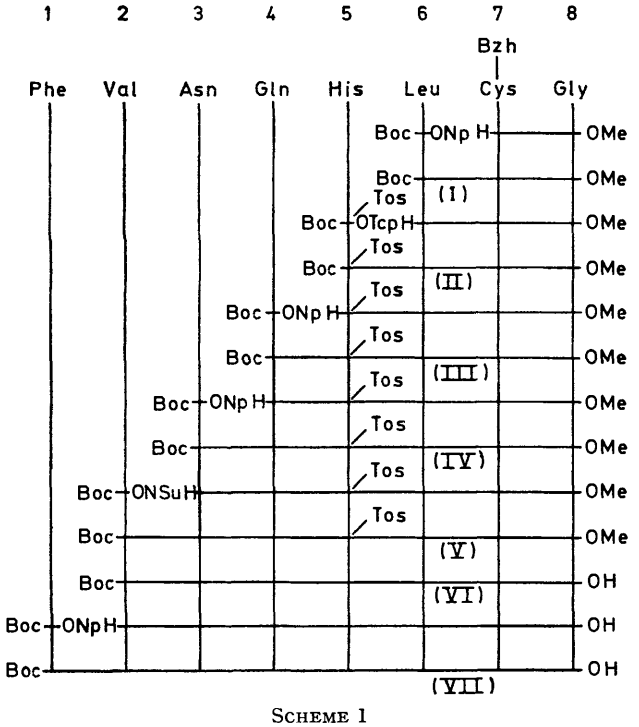
The fragment condensation method was employed to construct the protected triacontapeptide which contains the amino-acid sequence of the human B chain. The four peptide fragments used in this synthesis were prepared stepwise. The protected triacontapeptide was deblocked by treatment with liquid hydrogen fluoride and the resulting reduced peptide chain was converted into the *S*-sulphonated derivative by oxidative sulphitolysis. The *S*-sulphonated chain, purified by chromatography on a CM-cellulose column with a urea–acetate buffer at pH 4·0, was obtained in 33% overall yield, based on the amount of the protected B chain used: a yield of 6—7% was obtained in our original synthesis.

In the preceding paper,[1] we describe the synthesis of a protected hexadecapeptide with the amino-acid sequence found at the *C*-terminal region of the B chain of human insulin (sequence B^{15}—B^{30}). This peptide derivative, which has its functions protected with groups labile to hydrogen fluoride, is the key intermediate in an improved synthesis of the human B chain *S*-sulphonate described in the present paper. The new synthesis was

[1] Part XXIII, G. P. Schwartz and P. G. Katsoyannis, preceding paper.

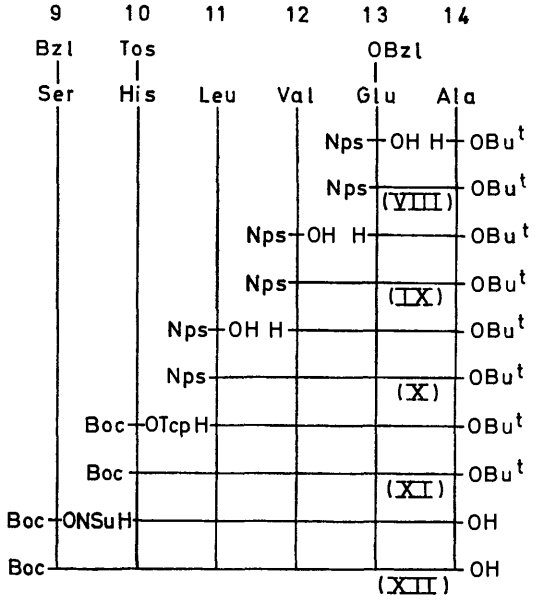
patterned after that of the original synthesis [2] in that it involved the construction of the protected B chain (XIIIb) (Scheme 3), removal of the blocking groups, and conversion of the resulting thiol form of the B chain into the *S*-sulphonated derivative (XIV) by oxidative sulphitolysis.[3] It differs, however, from the original synthesis in the mode of preparation of the intermediates, in the type of blocking groups, and in the deblocking reagent for the final step; instead of sodium in liquid ammonia liquid hydrogen fluoride was used for this purpose. The overall synthesis is given in Schemes 1—3; it involves the coupling of the *C*-terminal hexadecapeptide [1] (sequence B^{15}—B^{30}) with the adjacent hexapeptide (sequence B^{9}—B^{14}) to produce the *C*-terminal docosapeptide (sequence B^{9}—B^{30}), which in turn is condensed with the *N*-terminal octapeptide (sequence B^{1}—B^{8}) to afford the protected B chain.

The *N*-terminal octapeptide fragment (VII) (Scheme 1) was synthesised stepwise with t-butoxycarbonyl as the α-amino-group protector. The imidazole nitrogen atom of histidine was protected with the tosyl group.[4] Deblocking at each synthetic step was carried out by exposure to trifluoroacetic acid. T.l.c. of the penta- (III), hexa- (IV), and hepta-peptide (V) intermediates after deblocking with trifluoroacetic acid each showed a



Scheme 1

major spot and a slower moving minor spot (ninhydrin test), indicative of the presence of a contaminant in all three derivatives. Amino-acid analysis of these three peptide intermediates gave compositions in excellent agreement with the expected values; no attempt, however, was made to secure elemental analysis because of the demonstrated heterogeneity of the deblocked derivatives; we believe that this is due to partial



Scheme 2

detosylation of the histidine residue. Indeed, the totally detosylated heptapeptide (VI) and octapeptide (VII) derivatives were homogeneous on t.l.c. and gave good results on elemental analysis and amino-acid analysis.

The stepwise approach was also used for the synthesis of the hexapeptide fragment (XII) (Scheme 2). The t-butyl group was employed as the α-carboxy-group protector and the t-butoxycarbonyl and *o*-nitrophenylsulphenyl systems as the α-amino-group protectors. The use of this last group in the early stages of the synthesis permitted the selective deblocking of the α-amino-group at each synthetic step with hydrogen chloride in aqueous acetone [5] without any damage to the carboxy-group protector. The dipeptide (VIII) and tripeptide (IX) derivatives were obtained as oils and no attempt was made to convert them into crystalline derivatives for characterization.

The coupling of the octa-, hexa-, and hexadecapeptide fragments to produce eventually the *S*-sulphonated human B chain is summarized in Scheme 3. The hexapeptide (XII) and hexadecapeptide (XIIa) fragments were condensed *via* the dicyclohexylcarbodiimide–*N*-hydroxysuccinimide method.[6] The resulting protected docosapeptide (XIII) had the correct elemental analytical figures and amino-acid composition;

[2] (*a*) P. G. Katsoyannis, K. Fukuda, A. Tometsko, K. Suzuki, and M. Tilak, *J. Amer. Chem. Soc.*, 1964, **86**, 930; (*b*) P. G. Katsoyannis, A. Tometsko, J. Ginos, and M. Tilak, *ibid.*, 1966, **88**, 164; (*c*) P. G. Katsoyannis, J. Ginos, C. Zalut, M. Tilak, S. Johnson, and A. Trakatellis, *ibid.*, 1971, **93**, 5877.

[3] J. L. Bailey and R. D. Cole, *J. Biol. Chem.*, 1959, **234**, 1733.

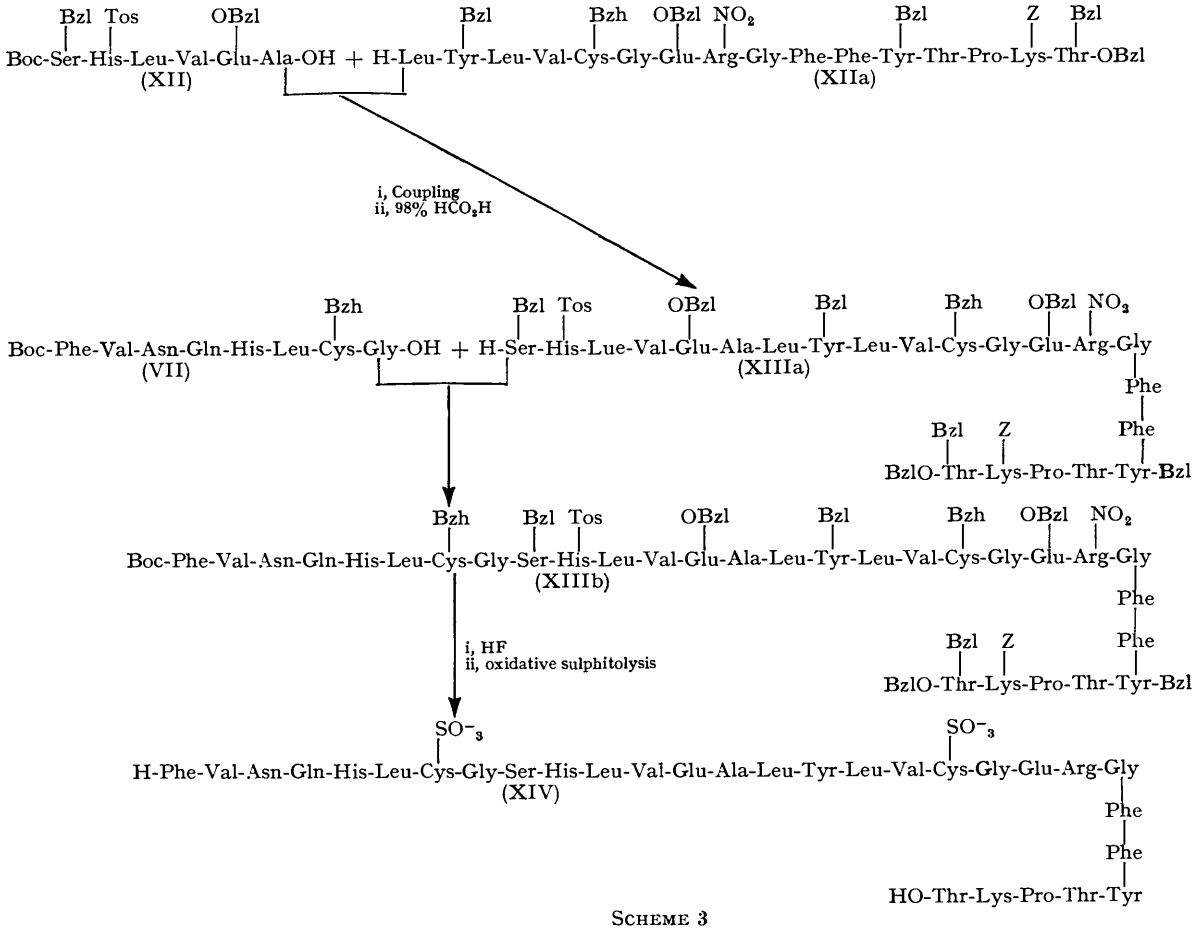
[4] T. Fugii and S. Sakakibara, *Bull. Chem. Soc. Japan*, 1961, **42**, 1466.

[5] G. C. Stelakatos, A. Paganou, and L. Zervas, *J. Chem. Soc.* (*C*), 1966, 1191.

[6] F. Weygand, D. Hoffman, and E. Wünsch, *Z. Naturforsch.*, 1966, **21b**, 426.

owing to its insolubility, t.l.c. data, however, could not be obtained. Deblocking of the protected docosapeptide with formic acid and coupling of the ensuing

For purification,[2c,9] the crude *S*-sulphonated B chain was chromatographed on a CM-cellulose column equilibrated and eluted with a urea–acetate buffer (pH 4·0).



SCHEME 3

partially protected derivative (XIIIa) with the *N*-terminal octapeptide fragment (VII) *via* the hydroxybenzotriazole–dicyclohexylcarbodi-imide method[7] afforded the protected B chain (XIIIb). In the fragment condensations mentioned above complete couplings were attained by using the activated carboxylic components at concentrations in excess, such that their levels did not fall below 0·05M at completion. The protected B chain was exposed to liquid hydrogen fluoride, whereupon the t-butoxycarbonyl, diphenylmethyl, tosyl, nitro-, benzyloxycarbonyl, and benzyl groups were removed.[8] The product was dissolved in 8M-guanidine hydrochloride and subjected to oxidative sulphitolysis by treatment with sodium sulphite and sodium tetrathionate at pH 8·9.[2c] Dialysis of the mixture followed by lyophilization of the non-diffusible material afforded the *S*-sulphonated B chain (XIV).

The chromatographic pattern obtained (Figure 1) indicates the presence of one major component, which is

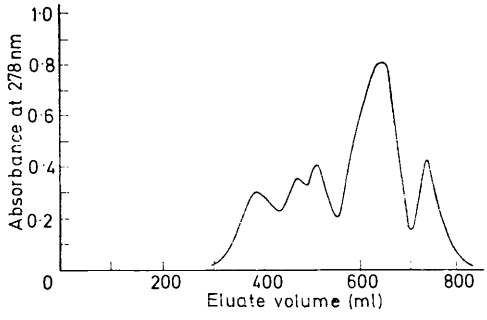


FIGURE 1 Elution pattern from a CM-cellulose chromatography on a 40 × 60 cm column with a urea—acetate buffer (pH 4·0) of crude synthetic *S*-sulphonated human B chain obtained by hydrogen fluoride deblocking of the protected triacontapeptide, followed by oxidative sulphitolysis, dialysis, and lyophilization. From the eluate (550—680 ml) 50 mg of purified B chain *S*-sulphonate were obtained

eluted at the position where natural B chain *S*-sulphonate emerges in this chromatographic system, and small amounts of other components.[2c,9] The latter were not

[7] W. Konig and R. Geiger, *Chem. Ber.*, 1970, **103**, 788.
[8] S. Sakakibara, 'Chemistry and Biochemistry of Amino Acids, Peptides and Proteins,' ed. B. Weinstein, Marcel Dekker, New York, 1971, p. 51.
[9] P. G. Katsoyannis, C. Zalut, A. Tometsko, M. Tilak, S. Johnson, and A. Trakatellis, *J. Amer. Chem. Soc.*, 1971, **93**, 5871.

characterized. The main component was isolated by the procedure applied previously in the synthesis of the sheep insulin B chain.[9] This procedure consists of chromatography of the fractions under the main peak on a Sephadex G-15 column (5% acetic acid as eluant), precipitation of the peptide material from this eluate as the picric acid salt, and chromatography of the picrate on a Sephadex G-15 column equilibrated and eluted with ammonium hydrogen carbonate. Lyophilization of the eluate from the latter column afforded the purified B chain *S*-sulphonate as a white fluffy material. The overall yield, based on the amount of crude protected B chain used, was 33%; a yield of 6—7% was obtained by the original method.[2c,9]

Amino-acid analysis of the purified material after acidic hydrolysis gave a composition in good agreement with the theoretically expected values for human B chain (Table). Digestion of this material with amino-

Amino-acid composition [a] of the synthetic human insulin B chain *S*-sulphonate

	Acidic hydrolysis		Enzymic hydrolysis (aminopeptidase M)	
Amino-acid	Theory	Found	Theory	Found
Lysine	1·0	1·0	1·0	1·1
Histidine	2·0	2·0	2·0	2·0
Arginine	1·0	1·0	1·0	1·0
Aspartic acid	1·0	1·0	0	0
Glutamine	0	0	1·0	Emerge at the same position; not determined
Asparagine	0	0	1·0	
Serine	1·0	0·9 [b]	1·0	
Threonine	2·0	1·9	2·0	1·6
Glutamic acid	3·0	3·0	2·0	1·9
Proline	1·0	1·0	1·0	0·8
Glycine	3·0	3·1	3·0	2·7
Alanine	1·0	1·0	1·0	1·1
Half-cystine	2·0	1·5 [b]	0	0
Valine	3·0	3·0	3·0	3·2
Leucine	4·0	4·0	4·0	4·3
Tyrosine	2·0	1·6 [b]	2·0	2·1
Phenylalanine	3·0	2·9	3·0	3·1
S-sulphocysteine	0	0	2·0	2·1

[a] Number of amino-acid residues per molecule. [b] Uncorrected for destruction.

peptidase M and amino-acid analysis of the digest showed that the constituent amino acids were present in the theoretically expected ratios and thus established the stereochemical homogeneity of the synthetic chain within the limits of error of the enzymic technique (Table). On high voltage thin-layer electrophoresis at pH 10·0 (Figure 2) and at pH 2·9, the synthetic material exhibited a single Pauly-positive spot and had the same mobility as the natural bovine insulin B chain *S*-sulphonate. We compared our synthetic product with the latter material since natural human insulin is not available in sufficient quantity to permit isolation of its individual chains for comparison. The synthetic human B chain was combined with natural porcine A chain, which is identical with the corresponding human chain;[10,11] the human insulin isolated was identical with the natural hormone.

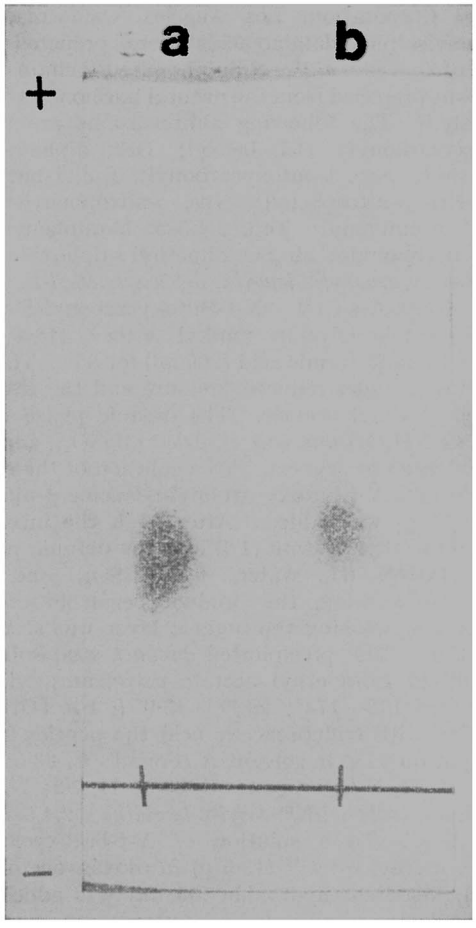


FIGURE 2 High-voltage thin-layer electrophoresis of synthetic *S*-sulphonated human B chain (a) and natural bovine B chain *S*-sulphonate (b) (0·01M-NH_4HCO_3 adjusted to pH 10·0 with NH_4OH; 2800 V; 25 min)

EXPERIMENTAL

The general analytical procedures used are described in the preceding paper.[1] For t.l.c. the protected peptides were deblocked by exposure to trifluoroacetic acid or hydrogen chloride in acetone and chromatographed on 6060 silica gel. The enzymatic digestion with aminopeptidase M was carried out according to the method of Pfleiderer *et al.*[12] with an enzyme purchased from Henley and Co., New York. Thin-layer electrophoresis, performed according to a method developed in this laboratory,[13] was carried out with a Wieland–Pfleiderer pherograph (Brinkman Instruments, New York). Pre-swollen microgranular CM-cellulose (Whatman CM52/1) and Sephadex G-15 (Pharmacia Uppsala) were used in this investigation. The washing of the resins and the preparation of the columns and of the buffers used have been described previously.[14,15]

[10] D. S. H. W. Nicol and L. F. Smith, *Nature*, 1960, **187**, 483.
[11] H. Brown, F. Sanger, and R. Kitai, *Biochem. J.*, 1955, **60**, 566.
[12] G. Pfleiderer, P. G. Cellicrs, M. Stanulovic, E. D. Wachsmuth, H. Determann, and G. Braunitzer, *Biochem. Z.*, 1964, **340**, 552.
[13] A. Tometsko and N. Delihas, *Analyt. Biochem.*, 1967, **18**, 72.
[14] P. G. Katsoyannis, A. Tometsko, C. Zalut, S. Johnson, and A. Trakatellis, *Biochemistry*, 1967, **6**, 2635.
[15] P. G. Katsoyannis, A. Trakatellis, S. Johnson, C. Zalut, and G. Schwartz, *Biochemistry*, 1967, **6**, 2642.

The column eluates were continuously monitored with a Gilford recording spectrophotometer at 278 nm. Sodium tetrathionate was prepared as described by Gilman *et al.*[16] t-Butoxycarbonylamino-acids were purchased from Cyclo Chemical Corporation, Los Angeles, California, and *o*-nitrophenylsulphenylamino-acids were prepared by the method of Zervas *et al.*[17] *S*-Sulphonated B chain of bovine insulin was prepared from the natural hormone as described previously.[14] The following abbreviations are used: Z, benzyloxycarbonyl; Bzl, benzyl; Bzh, diphenylmethyl; Me, methyl; Boc, t-butoxycarbonyl; Bu^t, t-butyl; Tos, tosyl; Np, *p*-nitrophenyl; Nps, *o*-nitrophenylsulphenyl; NSu, *N*-succinimidyl; Tcp, 2,4,5-trichlorophenyl; DMF, dimethylformamide; Me_2SO, dimethyl sulphoxide.

N-*t-Butoxycarbonyl*-L-*leucyl*-S-*diphenylmethyl*-L-*cysteinyl*-*glycine Methyl Ester* (I).—*N*-t-Butoxycarbonyl-*S*-diphenylmethyl-L-cysteinylglycine methyl ester[1] (18·4 g) was treated with 98% formic acid (200 ml) for 3 h. The solvent was removed under reduced pressure and the residue was dissolved in ethyl acetate. The organic phase was then washed (N-NH_4OH and water), dried ($MgSO_4$), and concentrated *in vacuo* to dryness. To a solution of the residue in DMF (60 ml), *N*-t-butoxycarbonyl-L-leucine *p*-nitrophenyl ester[18] (14 g) was added. After 24 h the mixture was poured into ethyl acetate (1 l) and the organic phase was washed (N-NH_4OH, water, 0·2N-H_2SO_4, and water). During the washing, the product began to crystallize; after the last washing the organic layer was stored overnight at 0°. The precipitated *product* was isolated and recrystallized from ethyl acetate–petroleum; yield 21 g (91%); m.p. 173—174°; $[\alpha]_D^{26}$ −43·9° (*c* 1 in DMF); after deblocking with trifluoroacetic acid the peptide showed a single spot on t.l.c. in solvent A (Found: C, 63·0; H, 7·1; N, 7·3. $C_{30}H_{41}N_3O_6S$ requires C, 63·0; H, 7·2; N, 7·3%).

N-*t-Butoxycarbonyl*-N^{im}-*tosyl*-L-*histidine* 2,4,5-*Trichlorophenyl Ester*.—To a solution of *N*-t-butoxycarbonyl-L-histidine methyl ester[19] (13·5 g) in dioxan–acetone (1 : 1; 100 ml), N-sodium hydroxide (50 ml) was added. After 1 h the solution was diluted with 2M-potassium carbonate (30 ml) and cooled to 0°. To this solution was then added during 30 min with vigorous stirring a solution of tosyl chloride (11·5 g) in acetone (30 ml). After stirring for an additional hour the mixture was diluted with cold water (100 ml) and extracted four times with ether. The aqueous layer was then acidified to pH 3·5 with solid citric acid and the oil formed was extracted into ethyl acetate. The organic layer was washed with cold water, dried ($MgSO_4$), and concentrated under reduced pressure. The residue solidified upon trituration with petroleum to give the crude *N*-t-butoxycarbonyl-*N^{im}*-tosyl-L-histidine (16·5 g, 80%). To a solution of this crude product in dichloromethane (150 ml), 2,4,5-trichlorophenol (7·5 g) was added, followed after cooling to −20° by dicyclohexylcarbodiimide (7·6 g). After 24 h at −20° the precipitated dicyclohexylurea was filtered off and the filtrate concentrated to dryness *in vacuo*. The *residue* crystallized from propan-2-ol; yield 18 g (76%); m.p. 140—141°; $[\alpha]_D^{26}$ −11·9° (*c* 1 in DMF) (Found: C, 48·9; H, 4·3; Cl, 17·7; N, 7·4. $C_{24}H_{24}Cl_3N_3O_6S$ requires C, 48·9; H, 4·1; Cl, 18·1; N, 7·1%).

N-*t-Butoxycarbonyl*-N^{im}-*tosyl*-L-*histidyl*-L-*leucyl*-S-*diphenylmethyl*-L-*cysteinylglycine Methyl Ester* (II).—A solution of compound (I) (5·7 g) in trifluoroacetic acid (20 ml) was stored at room temperature for 25 min. The solvent was removed under reduced pressure, the remaining oil was dissolved in ethyl acetate (600 ml), and the organic phase was washed (cold N-NH_4OH and cold water). The free base of the peptide, which was obtained after evaporation of the ethyl acetate (dried over $MgSO_4$) was dissolved in DMF (60 ml), and *N*-t-butoxycarbonyl-*N^{im}*-tosyl-L-histidine 2,4,5-trichlorophenyl ester (5·8 g) was added. After 24 h the mixture was poured into ethyl acetate (250 ml) and the organic phase was washed (0·2N-NH_4OH, water, 0·2N-H_2SO_4, and water). Concentration of the ethyl acetate solution (dried over $MgSO_4$) to a small volume and addition of ether caused crystallization. Recrystallization from ethyl acetate–ether afforded the *product* (6·5 g, 76%), m.p. 170—171°, $[\alpha]_D^{26}$ −30·0° (*c* 1 in DMF); after deblocking with trifluoroacetic acid the peptide showed a single spot on t.l.c. in solvent A (Found: C, 59·7; H, 6·4; N, 9·5. $C_{43}H_{54}N_6O_9S_2$ requires C, 59·8; H, 6·3; N, 9·7%).

N-*t-Butoxycarbonyl*-L-*glutaminyl*-N^{im}-*tosyl*-L-*histidyl*-L-*leucyl*-S-*diphenylmethyl*-L-*cysteinylglycine Methyl Ester* (III).—The tetrapeptide (II) (6·5 g) was treated with trifluoacetic acid (30 ml). After 30 min, the solvent was removed under reduced pressure and the residue was dissolved in ethyl acetate (250 ml). The organic phase was washed (M-Na_2CO_3 and water), dried ($MgSO_4$), and concentrated *in vacuo*. To a solution of the residue in DMF (50 ml), *N*-t-butoxycarbonyl-L-glutamine *p*-nitrophenyl ester[20] (3 g) was added. After 24 h the mixture was poured into ethyl acetate (250 ml) and the organic phase was washed (M-$NaHCO_3$, water, 0·2N-H_2SO_4, and water), dried ($MgSO_4$), and concentrated to dryness *in vacuo*. The residue was precipitated from a solution in dichloromethane by addition of ether and was reprecipitated from acetone–ether; yield 5·8 g (77%), m.p. 162—164°; after deblocking with trifluoroacetic acid the peptide showed two spots on t.l.c. in solvent A. Amino-acid analysis after acidic hydrolysis gave the following ratios: $His_{1·0}Glu_{1·0}Gly_{1·0}Cys_{0·6}Leu_{1·0}$.

N-*t-Butoxycarbonyl*-L-*asparaginyl*-L-*glutaminyl*-N^{im}-*tosyl*-L-*histidyl*-L-*leucyl*-S-*diphenylmethyl*-L-*cysteinylglycine Methyl Ester* (IV).—A solution of the pentapeptide derivative (III) (5·8 g) in trifluoroacetic acid (20 ml) was stored at room temperature for 30 min and then poured into ether (600 ml). The precipitated pentapeptide trifluoroacetate was isolated, washed with ether, and dissolved in DMF (50 ml), and *N*-t-butoxycarbonyl-L-asparagine *p*-nitrophenyl ester[21] (2 g) was added, followed by triethylamine (0·84 ml). After 24 h the mixture was poured into ether and the precipitated product was isolated by filtration and washed with ethyl acetate–ether (1 : 1). This material was then dissolved in DMF (50 ml), precipitated by the addition of water (500 ml) and saturated aqueous sodium carbonate (20 ml), isolated by filtration, and washed [water, 2% acetic acid, water, and acetone–ether (1 : 1)]; yield 5·8 g (89%), m.p. indefinite; after deblocking with trifluoroacetic acid the peptide showed two spots on t.l.c. in solvent A. Amino-

[16] A. Gilman, F. S. Philips, and E. S. Koelle, *Amer. J. Physiol.*, 1946, **146**, 348.
[17] L. Zervas, D. Borovas, and E. Gazis, *J. Amer. Chem. Soc.*, 1963, **85**, 3660.
[18] K. Vogler, R. O. Studer, P. Lanz, W. Lergier, and W. Böhne, *Helv. Chim. Acta*, 1965, **48**, 1161.
[19] B. O. Handford, T. A. Hylton, K. T. Wang, and B. Weinstein, *J. Org. Chem.*, 1968, **33**, 4251.
[20] H. Zahn, W. Danho, and B. Gutte, *Z. Naturforsch.*, 1966, **21b**, 763.
[21] G. R. Marshall and R. B. Merrifield, *Biochemistry*, 1965, **4**, 2394.

acid analysis of an acidic hydrolysate gave the following amino-acid ratios: $His_{1.0}Asp_{1.0}Glu_{1.0}Gly_{0.9}Cys_{0.7}Leu_{1.0}$.

N-*t-Butoxycarbonyl*-L-*valyl*-L-*asparaginyl*-L-*glutaminyl*-N^{im}-*tosyl*-L-*histidyl*-L-*leucyl*-S-*diphenylmethyl*-L-*cysteinyl*-*glycine Methyl Ester* (V).—The protected hexapeptide (IV) (5·8 g) was deblocked with trifluoroacetic acid and the peptide trifluoroacetate was isolated as described in the synthesis of (IV). To a solution of the hexapeptide trifluoroacetate in DMF (40 ml) containing triethylamine (0·9 ml), *N*-t-butoxycarbonyl-L-valine *N*-hydroxysuccinimide ester [22] (3 g) was added. After 48 h the mixture was poured into cold ether (700 ml) and the precipitated product was collected and washed with acetone–ether (1 : 1); yield 6 g (94%), m.p. 217—219°; after deblocking with trifluoroacetic acid the peptide showed two spots on t.l.c. in solvent A. Amino-acid analysis of an acidic hydrolysate gave the following ratios: $His_{1.0}Asp_{1.0}Glu_{1.0}Gly_{1.0}Cys_{0.6}Val_{1.0}Leu_{1.0}$.

N-*t-Butoxycarbonyl*-L-*valyl*-L-*asparaginyl*-L-*glutaminyl*-L-*histidyl*-L-*leucyl*-S-*diphenylmethyl*-L-*cysteinylglycine* (VI).—To a suspension of the fully protected heptapeptide ester (V) (6 g) in a mixture of DMF (20 ml) and methanol (30 ml), N-sodium hydroxide (10 ml) was added during 15 min. After stirring for an additional 45 min, N-sodium hydroxide (1 ml) was added to the resulting solution, which was further stirred for 20 min. The mixture was then poured into cold water (200 ml) containing N-hydrochloric acid (11 ml). The *precipitate* was isolated by centrifugation, washed with water, and triturated with propan-2-ol; yield 3·2 g (61%); m.p. 237—238°; $[\alpha]_D^{26}$ −43·7° (*c* 1 in Me_2SO); after deblocking with trifluoroacetic acid the peptide showed a single spot on t.l.c. in solvent A (Found: C, 57·2; H, 7·15; N, 14·6. $C_{49}H_{69}N_{11}O_{12}S$ requires C, 56·7; H, 6·7; N, 14·8%). Amino-acid analysis after acidic hydrolysis gave the following ratios: $His_{1.0}Asp_{1.0}Glu_{1.0}Gly_{1.0}Cys_{0.6}Val_{1.0}Leu_{1.0}$ and $ammonia_{2.0}$.

N-*t-Butoxycarbonyl*-L-*phenylalanyl*-L-*valyl*-L-*asparaginyl*-L-*glutaminyl*-L-*histidyl*-L-*leucyl*-S-*diphenylmethyl*-L-*cysteinylglycine* (VII).—The partially protected heptapeptide (VI) (3·2 g) was deblocked as described in the synthesis of (IV). To a solution of the ensuing peptide trifluoroacetate in DMF (30 ml), *N*-t-butoxycarbonyl-L-phenylalanine *p*-nitrophenyl ester [23] (3·8 g) was added, followed by triethylamine (0·8 ml). After 48 h the mixture was poured into cold ethyl acetate and the precipitate was isolated, washed with ether, and dried. This product was suspended in DMF (60 ml) and after stirring for a few minutes the mixture was poured into cold 0·05N-ammonia (200 ml). The pH of the mixture was then adjusted to 6·0 with acetic acid and the precipitated partially protected *octapeptide* was isolated, washed with water, triturated with warm propan-2-ol, and reprecipitated from Me_2SO–H_2O; yield 2 g (54%), m.p. 240° (decomp.); $[\alpha]_D^{26}$ −35·9° (*c* 1 in Me_2SO); after deblocking with trifluoroacetic acid the peptide showed a single spot on t.l.c. in solvents A and B (Found: C, 57·6; H, 6·8; N, 14·4; O, 17·5. $C_{58}H_{78}N_{12}O_{13}S,0{\cdot}5H_2O$ requires C, 58·4; H, 6·85; N, 14·1; O, 18·1%). Amino-acid analysis of an acidic hydrolysate gave the following amino acid ratios: $His_{1.0}Asp_{1.0}Glu_{1.0}Gly_{1.0}Cys_{0.7}Val_{1.0}Leu_{1.0}Phe_{1.0}$.

N-O-*Nitrophenylsulphenyl*-γ-*benzyl*-L-*glutamyl*-L-*alanine t-Butyl Ester* (VIII).—*N*-Benzyloxycarbonyl-L-alanine t-butyl ester (14 g) (prepared by the procedure described for the synthesis of the corresponding DL-ester [24]) was hydrogenated for 3 h over 10% palladium–charcoal (3 g) in methanol (150 ml). The catalyst was filtered off and the filtrate was concentrated to dryness under reduced pressure. To a solution of the residue in DMF (100 ml) containing *N*-*o*-nitrophenylsulphenyl-γ-benzyl-L-glutamic acid [prepared from *N*-*o*-nitrophenylsulphenyl-γ-benzyl-L-glutamic acid dicyclohexylamine salt (28·5 g) in the usual way [17]], cooled to 0°, *N*-hydroxysuccinimide (2·9 g) and dicyclohexylcarbodi-imide (10·2 g) were added. After 48 h at room temperature, the mixture was cooled to 0°, the precipitated dicyclohexylurea was filtered off, and the filtrate was poured into ethyl acetate (500 ml). The organic layer was washed (N-NH_4OH, water, 0·2N-H_2SO_4, and water), dried ($MgSO_4$), and concentrated to dryness *in vacuo*. The remaining oil (17 g, 71%) was used directly in the next step. The free base of the peptide prepared as described in the following preparation showed a single spot on t.l.c. in solvent A.

N-O-*Nitrophenylsulphenyl*-L-*valyl*-γ-*benzyl*-L-*glutamyl*-L-*alanine t-Butyl Ester* (IX).—The peptide (VIII) in the oil form (17 g) was dissolved in acetone (300 ml) and 6N-hydrochloric acid (20 ml) was added. After 5 min the solvent was removed under reduced pressure and the residue was mixed with cold water (300 ml) and ether (300 ml). The insoluble bis-*o*-nitrophenyl disulphide was filtered off and the aqueous layer was separated and extracted several times with ether to remove the residual *o*-nitrophenylsulphenyl chloride. The pH of the aqueous phase was adjusted to 9·5 (N-NH_4OH) and the free base of the dipeptide ester was extracted into ethyl acetate. The organic layer was washed with water, dried ($MgSO_4$), and concentrated to dryness *in vacuo*. To a solution of the remaining oil in DMF (100 ml) containing *N*-*o*-nitrophenylsulphenyl-L-valine [prepared from its dicyclohexylamine salt (18 g)], cooled to 0°, *N*-hydroxysuccinimide (2·3 g) and dicyclohexylcarbodi-imide (6·9 g) were added. The protected tripeptide was isolated by the procedure described in the synthesis of (VIII); this product (oil) (14 g, 69%) was used directly in the next step. The deblocked peptide (HCl–acetone) showed a single spot on t.l.c. in solvent A.

N-O-*Nitrophenylsulphenyl*-L-*leucyl*-L-*valyl*-γ-*benzyl*-L-*glutamyl*-L-*alanine t-Butyl Ester* (X).—The tripeptide (IX) in the oil form (14 g) was dissolved in acetone (250 ml) and 6N-hydrochloric acid (15 ml) and processed as described in the synthesis of (IX). The resulting free base of the tripeptide was dissolved in DMF (100 ml) containing *N*-*o*-nitrophenylsulphenyl-L-leucine [prepared from its dicyclohexylamine salt (14·3 g)]. To this solution, cooled to 0°, *N*-hydroxysuccinimide (2·2 g) and dicyclohexylcarbodiimide (5·3 g) were added. After 48 h at room temperature the mixture was cooled to 0°, the precipitated dicyclohexylurea was filtered off, and the filtrate was poured into ethyl acetate (500 ml). The organic layer was washed (water, N-NH_4OH, water, 0·2N-H_2SO_4, and water) and dried ($MgSO_4$). Concentration to a small volume, under reduced pressure, and cooling for several hours caused the crystallization of the *product* (6·6 g, 40%), m.p. 183—184°; $[\alpha]_D^{26}$ −10·9° (*c* 1 in DMF). After deblocking with acetone–hydrochloric acid [as described in the synthesis of (XI)] the peptide showed a single spot on t.l.c. in solvent A (Found:

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[23] E. Sandrin and R. A. Boissonnas, *Helv. Chim. Acta*, 1963, **46**, 1637.

[24] G. W. Anderson and F. M. Callahan, *J. Amer. Chem. Soc.*, 1960, **82**, 3358.

C, 59·6; H, 7·1; N, 9·2. $C_{36}H_{51}N_5O_9S$ requires C, 59·2; H, 7·05; N, 9·6%).

N-*t-Butoxycarbonyl*-N^{im}-*tosyl*-L-*histidyl*-L-*leucyl*-L-*valyl*-*γ*-*benzyl*-L-*glutamyl*-L-*alanine t-Butyl Ester* (XI).—A solution of the crystalline tetrapeptide (X) (5·4 g) in warm acetone (25 ml) was cooled to room temperature and diluted with 6N-hydrochloric acid (2·8 ml). After 5 min the solvent was removed *in vacuo* and the residue triturated with ether and cooled to 0°. The precipitated tetrapeptide ester hydrochloride was isolated by filtration and purified by suspension in propan-2-ol (30 ml) and precipitation with ether (300 ml). The dried product was dissolved in DMF (50 ml) containing triethylamine (1 ml) and treated with *N*-t-butoxycarbonyl-*N*^{im}-tosyl-L-histidine 2,4,5-trichlorophenyl ester (4 g). After 24 h the mixture was poured into ethyl acetate (500 ml) and the organic layer was washed (0·2N-NH_4OH, water, 0·2N-H_2SO_4, and water), dried ($MgSO_4$), and concentrated to dryness *in vacuo*. The protected *pentapeptide* was isolated from the residue by precipitation with petroleum from a solution in ether; yield 6·2 g (87%), m.p. indefinite (180—192°); $[\alpha]_D^{26}$ −15·7° (*c* 1 in DMF); single spot on t.l.c. in solvent A after deblocking with trifluoroacetic acid (Found: C, 59·6; H, 7·1; N, 10·0. $C_{48}H_{69}N_7O_{12}S$ requires C, 59·5; H, 7·2; N, 10·1%).

N-*t-Butoxycarbonyl*-O-*benzyl*-L-*seryl*-N^{im}-*tosyl*-L-*histidyl*-L-*leucyl*-L-*valyl*-*γ*-*benzyl*-L-*glutamyl*-L-*alanine* (XII).—A solution of the protected pentapeptide (XI) (3 g) in trifluoroacetic acid was stored at room temperature for 1 h and then poured into cold ether (500 ml). The precipitated deblocked pentapeptide trifluoroacetate was collected, washed with ether, and reprecipitated from a suspension in propan-2-ol (30 ml) by addition of ether (300 ml). The dried peptide salt was dissolved in DMF (20 ml) containing triethylamine (0·5 ml) and this solution was added to *N*-t-butoxycarbonyl-*O*-benzyl-L-serine which was activated as follows. To a solution of *N*-t-butoxycarbonyl-*O*-benzyl-L-serine (950 mg) and *N*-hydroxysuccinimide (348 mg) in acetonitrile (30 ml), cooled to 0°, dicyclohexylcarbodi-imide (600 mg) was added. After 3 h at 0° the dicyclohexylurea was filtered off and the filtrate concentrated to dryness *in vacuo*. To this residue was then added the solution of the deblocked pentapeptide prepared as just described. After 24 h the mixture was poured into cold ether (700 ml) and the precipitated partially protected *hexapeptide* was collected and reprecipitated from acetone–ether and dimethylformamide–water; yield 2·1 g (61%), m.p. indefinite (130—150°); $[\alpha]_D^{26}$ −13·6° (*c* 1 in DMF). After deblocking with trifluoroacetic acid the peptide showed a single spot on t.l.c. in solvents A and B (Found: C, 58·3; H, 6·8; N, 10·1; O, 21·9. $C_{54}H_{72}N_8O_{14}S,H_2O$ requires C, 58·6; H, 6·75; N, 10·1; O, 21·7%). Amino-acid analysis of an acidic hydrolysate gave the following ratios: $His_{1.0}Ser_{0.8}Glu_{1.0}Ala_{1.0}Val_{1.0}Leu_{1.0}$.

N-*t-Butoxycarbonyl*-O-*benzyl*-L-*seryl*-N^{im}-*tosyl*-L-*histidyl*-L-*leucyl*-L-*valyl*-*γ*-*benzyl*-L-*glutamyl*-L-*alanyl*-L-*leucyl*-O-*benzyl*-L-*tyrosyl*-L-*leucyl*-L-*valyl*-S-*diphenylmethyl*-L-*cysteinylglycyl*-*γ*-*benzyl*-L-*glutamyl*-N$^{\omega}$-*nitro*-L-*arginylglycyl*-L-*phenylalanyl*-L-*phenylalanyl*-O-*benzyl*-L-*tyrosyl*-L-*threonyl*-L-*prolyl*-N$^{\varepsilon}$-*benzyloxycarbonyl*-L-*lysyl*-O-*benzyl*-L-*threonine Benzyl Ester* (XIII).—A solution of the protected hexadecapeptide *N*-t-butoxycarbonyl-L-leucyl-*O*-benzyl-L-tyrosyl-L-leucyl-L-valyl-*S*-diphenylmethyl-L-cysteinylglycyl-*γ*-benzyl-L-glutamyl-N$^{\omega}$-nitro-L-arginylglycyl-L-phenylalanyl-L-phenylalanyl-*O*-benzyl-L-tyrosyl-L-threonyl-L-prolyl-N$^{\varepsilon}$-benzyloxycarbonyl-L-lysyl-*O*-benzyl-L-threonine benzyl ester [1] (800 mg) in 98% formic acid (30 ml) was stored at room temperature for 3 h and subsequently concentrated under reduced pressure. The residue was triturated with ether and dissolved in DMF (25 ml). This solution, cooled to 0°, was diluted with N-ammonia (5 ml) and immediately poured into cold saturated aqueous sodium chloride (300 ml). The pH of the mixture was adjusted to 9·5 (N-NH_4OH) and the precipitated free base of the hexadecapeptide (XIIa) was collected, washed successively with water, propan-2-ol, and petroleum, and dried. This product was dissolved in hexamethylphosphoramide (5 ml) and DMF (5 ml) and to this solution, cooled to 4°, the hexapeptide derivative (XII) (1 g) was added. The mixture was stirred for a few minutes and to the resulting solution *N*-hydroxysuccinimide (116 mg) and dicyclohexylcarbodi-imide (200 mg) were added. After 24 h at 4° the mixture was diluted with hexamethylphosphoramide (20 ml), stirred for an additional 5 h, and poured into cold saturated aqueous sodium chloride (400 ml) containing 2M-sodium carbonate (20 ml). The precipitated protected *docosapeptide* was filtered off, washed (water, acetone, and ether), and reprecipitated from dimethylformamide–propan-2-ol; yield 1 g (93%), m.p. >260°, $[\alpha]_D^{26}$ −19·9° (*c* 1 in Me_2SO) (Found: C, 63·4; H, 6·6; N, 10·4. $C_{200}H_{247}N_{29}O_{40}S_2$ requires C, 63·8; H, 6·65; N, 10·8%). Amino-acid analysis of an acidic hydrolysate gave the following ratios: $Lys_{1.0}His_{1.0}Arg_{0.7}Thr_{1.7}Ser_{1.0}Glu_{2.0}Pro_{0.9}Gly_{2.0}Ala_{1.0}Cys_{0.4}Val_{2.1}Leu_{3.0}Tyr_{0.8}Phe_{1.8}$.

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-*arginylglycyl*-L-*phenylalanyl*-L-*phenylalanyl*-L-*tyrosyl*-L-*threonyl*-L-*propyl*-L-*lysyl*-L-*threonine* (*Human Insulin B Chain* S-*Sulphonate*) (XIV).—The protected docosapeptide (XIII) (0·8 g) was deblocked with 98% formic acid as described in the synthesis of (XIII). The resulting free base of the docosapeptide (XIIIa) was dissolved in hexamethylphosphoramide (10 ml) and DMF (10 ml) and to this solution the octapeptide derivative (XII) (2 g) was added. The mixture was stirred for a few minutes and to the resulting solution 1-hydroxybenzotriazole (270 mg) and dicyclohexylcarbodi-imide (400 mg) were added. After 24 h at room temperature the mixture was poured into cold water (500 ml) containing N-ammonia (10 ml). The precipitated protected crude B chain (XIIIb) was isolated by centrifugation, washed (water, 50% aqueous methanol, absolute methanol, and ether), and reprecipitated from a solution in hexamethylphosphoramide–dimethylformamide (20 ml; 1 : 1) by addition of methanol; yield 800 mg (78%). Amino-acid analysis after acidic hydrolysis gave the following amino-acid ratios: $Lys_{1.1}His_{1.9}Arg_{0.9}Asp_{1.0}Thr_{1.8}Ser_{0.9}Glu_{3.0}Pro_{0.9}Gly_{3.1}Ala_{1.0}Cys_{1.3}Val_{3.0}Leu_{4.0}Tyr_{0.8}Phe_{2.9}$.

This material was converted into the *S*-sulphonated B chain by deblocking with liquid hydrogen fluoride followed by oxidative sulphitolysis as follows. The thoroughly dried protected crude B chain (200 mg) and anisole (1 ml) were placed in a Daiflon reaction vessel of a hydrogen fluoride apparatus (Toho Company, Japan) and treated with hydrogen fluoride according to the procedure of Sakakibara.[8] The hydrogen fluoride used (10 ml) was redistilled over cobalt trifluoride and the deblocking reaction was performed at 10° for 1 h. The hydrogen fluoride was removed by use of an aspirator protected with a solid carbon dioxide trap. The residue was dried in high

vacuum (KOH) for 24 h and triturated thoroughly with ethyl acetate. The reduced crude B chain was then dissolved in 8M-guanidine hydrochloride (20 ml) and to this solution, adjusted to pH 8·9 (dilute NH_4OH) were added sodium sulphite (1·2 g) and freshly prepared sodium tetrathionate (0·7 g). The mixture was stirred for 3 h at room temperature and then placed in an 18/32 Visking dialysis tube and dialysed against four changes of distilled water (3 l each) at 4° for 24 h. Upon lyophilization of the non-diffusible material the crude human B chain *S*-sulphonate was obtained as a white powder; yield 120 mg (81% based on the crude protected B chain used). Amino-acid analysis of this material after acidic hydrolysis gave the following ratios: $Lys_{1.1}His_{1.8}Arg_{1.0}Asp_{0.8}Thr_{1.6}Ser_{0.7}Glu_{2.4}Pro_{0.8}$-$Gly_{3.1}Ala_{1.1}Cys_{1.6}Val_{3.1}Leu_{4.4}Tyr_{1.7}Phe_{2.5}$.

Purification of Synthetic Human B Chain S-*Sulphonate*.—The lyophilized material (120 mg) was dissolved in urea–acetate buffer (6 ml) (0·04M-sodium acetate, 8M-urea; pH 4·0) and placed on a CM-cellulose column (4 × 60 cm) equilibrated and eluted with the same buffer. The preparation of the column has been described in detail.[14,15] The peptide material in the column eluate was monitored continuously with a Gilford recording spectrophotometer at 278 nm. The chromatographic pattern obtained (Figure 1) indicates the presence of one major component, the human B chain *S*-sulphonate, and only small amounts of other components. The eluate under the major peak (550—680 ml) was collected, the pH was adjusted to 3·0 (concentrated HCl), and the solution was placed on a Sephadex G-15 column (4 × 60 cm) [5% (v/v) acetic acid as eluant]. By this procedure the urea of the buffer was completely removed. Peptide material was detected by continuous monitoring of the eluate with a Gilford recording spectrophotometer at 278 nm. The eluate containing the peptide material was collected, concentrated (at 25—30°) to a small volume (*ca.* 15 ml), and mixed with an equal volume of saturated picric acid solution. After 24 h at 4° the precipitated picric acid salt of the peptide chain was isolated by centrifugation and dissolved in 0·05N-ammonium hydrogen carbonate (2 ml) with the aid of a few drops of dilute ammonia. This solution was then placed on a Sephadex G-15 column (1·2 × 50 cm) equilibrated and eluted with 0·05N-ammonium hydrogen carbonate. By this procedure the peptide material was separated completely from the picric acid. Continuous monitoring of the eluate with a Gilford recording spectrophotometer at 278 nm was used to locate the peptide material, which was recovered by lyophilization as a white fluffy powder; yield 50 mg (33% based on the protected crude B chain used).

Criteria of Homogeneity of Synthetic Human B Chain S-*Sulphonate*.—Amino-acid analysis of the synthetic peptide chain after acidic hydrolysis gave the molar ratios of amino acids shown in the Table, in good agreement with the expected values. Digestion of the synthetic chain with aminopeptidase M and amino-acid analysis of the digest gave the amino-acid ratios shown in the Table, in good agreement with the expected values. This established the stereochemical homogeneity of the synthetic chain within the limits of error of the enzymic technique. The synthetic human insulin B chain *S*-sulphonate on CM-cellulose chromatography is eluted at the same position as the natural bovine insulin B chain *S*-sulphonate. On high voltage thin-layer electrophoresis in 0·01M-ammonium hydrogen carbonate (pH 10 and 2800 V) (Figure 2) and in 0·5N-acetic acid (pH 2·9 and 3000 V) the synthetic chain moved as a single component (Pauly-positive spot) and had the same mobility as the natural bovine chain. The synthetic human B chain was combined with natural A chain by the procedure described previously;[25] the human insulin isolated was identical with the natural protein.

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