Synthesis of a Biologically Active Truncated Insulin. Des(pentapeptide B^{26-30}) Human (Porcine) Insulin [1]

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An analogue of human (porcine) insulin which differs from the parent molecule in that the *C*-terminal pentapeptide sequence of the B chain has been eliminated has been synthesized. For this purpose, des(pentapeptide B^{26-30}) B chain *S*-sulphonate was synthesized by the fragment condensation approach and isolated in highly purified form. Interaction of this compound with the thiol form of the A chain of human (porcine) insulin yielded des(p entapeptide B^{26-30}) human (porcine) insulin, which was purified by chromatography on a carboxymethylcellulose column with an exponential sodium chloride gradient. The des(pentapeptide B^{26-30}) insulin shows potencies of 8·5—9 I.U. mg^{-1} when assayed by the mouse convulsion method and of 11 I.U. mg^{-1} by the radioimmunoassay method (*cf.* 23—25 I.U. mg^{-1} for the natural hormone). This indicates that the *C*-terminal pentapeptide sequence of the B chain does not participate functionally in the mechanism of the action of insulin.

CURRENT work in our laboratory is aimed at the elucidation of possible correlations between the chemical structure and the biological activity of insulin. To this end we have synthesized a number of analogues of insulin and determined their biological properties.[1] We have found that elimination of the *C*-terminal tripeptide sequence of the B chain of insulin (B^{28-30}) and conversion of the newly exposed residue at position B^{27} (threonine) into an amino-alcohol or amino-aldehyde derivative does not alter the biological properties of this hormone.[2] We have also shown that elimination of the tetrapeptide sequence B^{27-30} and termination of the B chain with the B^{26} residue, tyrosine, bearing a free carboxy-group results in a molecule possessing *ca.* 50% of the biological potency of insulin.[3] This demonstrates that the *C*-terminal tetrapeptide B^{27-30} does not participate directly in the mechanism of action of the hormone. The drop in biological activity may be attributed [3] to the presence of a carboxylate ion at position B^{26}. Indeed *X*-ray analysis of the three-dimensional structure of insulin has shown that the tyrosine B^{26} residue is part of a non-polar area on the surface of the hormone molecule.[4] This region, which includes several other amino-acid residues that are invariant in most insulin species studied thus far, may be important to the biological activity of insulin.

We have now synthesized des(pentapeptide B^{26-30}) human insulin, in which the B^{25} phenylalanine bearing a free carboxy-group is the new *C*-terminal residue. By the mouse convulsion assay method this analogue was found to possess a specific activity of 8·5—9 I.U. mg^{-1}, *ca.* 35% of that of the natural hormone (23—25 I.U.); the radioimmunoassay method gave a value of 11 I.U. mg^{-1}, *ca.* 45% of that of the natural protein. This indicates that the B^{26-30} sequence is also not involved directly in the manifestation of the biological activity of the hormone. The 15% drop in the biological activity of des(pentapeptide B^{26-30}) insulin as compared to des(tetrapeptide B^{27-30}) insulin might be due to the proximity of a carboxylate ion to the aforementioned non-polar surface of the hormone molecule, with consequent deleterious conformational changes. Alternatively the tyrosine and, to a lesser extent, the threonine at positions B^{26} and B^{27}, respectively, may be necessary for the stabilization of a conformation commensurate with a higher biological activity. The synthesis and biological evaluation of des(pentapeptide B^{26-30}) insulin terminating with phenylalaninamide at B^{25}, now under way, will clarify this possibility.

Preparation of des(pentapeptide B^{26-30}) bovine insulin by enzymic digestion of the natural hormone has been reported by Brandenburg *et al.*;[5] their analogue possesses an activity ranging from 17 to 27% of that of insulin when assayed by the glucose oxidation method.

General Aspects of the Synthesis.—The insulin analogue was prepared by combination of the thiol form of the human (porcine) A chain with the *S*-sulphonated des(pentapeptide B^{26-30}) B chain. The former, which is identical with the corresponding chain of porcine

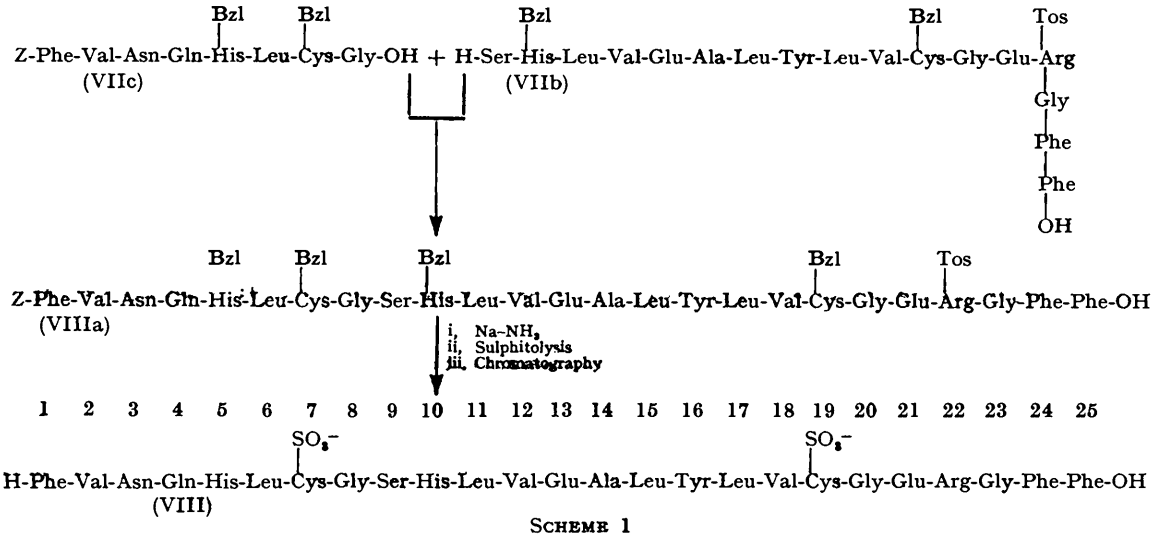
[1] For the previous paper of this series see A. Cosmatos and P. G. Katsoyannis, *J. Biol. Chem.*, 1973, **248**, 7304.

[2] P. G. Katsoyannis, C. Zalut, A. Harris, and R. J. Meyer, *Biochemistry*, 1971, **10**, 3884.

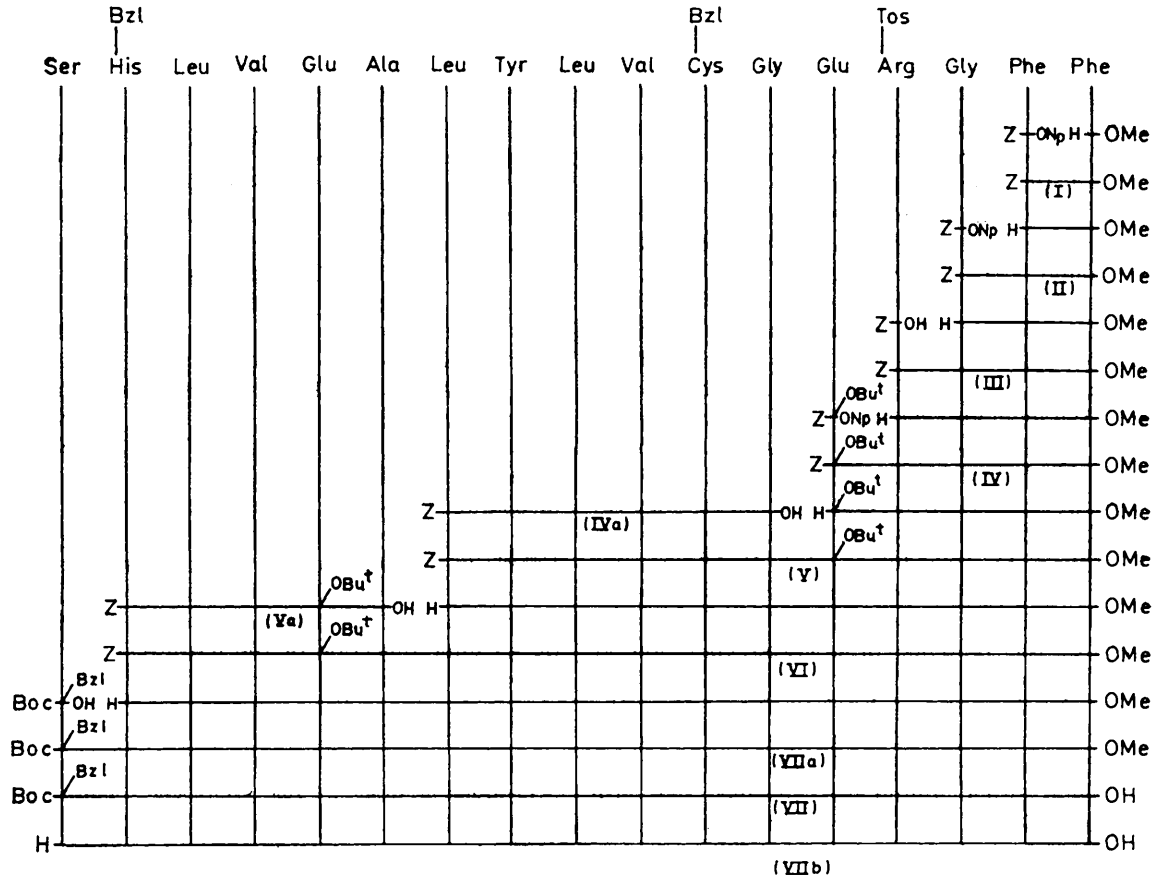
[3] P. G. Katsoyannis, J. Ginos, A. Cosmatos, and G. P. Schwartz, *J. Amer. Chem. Soc.*, 1973, **95**, 6427.

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

[5] D. Brandenburg, W.-D. Busse, H.-G. Gattner, H. Zahn, A. Wollmer, J. Glieman, and W. Puls, in 'Peptides 1972,' eds. H. Hanson and H.-D. Jakubke, North-Holland Publishing Company, Amsterdam, 1973, p. 270.



SCHEME 1



SCHEME 2

insulin,[6] was obtained by reduction with 2-mercaptoethanol of the *S*-sulphonated derivative of that chain, prepared by oxidative sulphitolysis of porcine insulin.[7] The synthesis of the *S*-sulphonated des(pentapeptide B^{26-30}) human B chain was patterned after that of the natural sheep and human B chains reported previously.[8] It involved the construction of a protected pentacosapeptide (VIIIa) containing the entire amino-acid sequence of des(pentapeptide B^{26-30}) B chain

[6] D. S. H. W. Nicol and L. F. Smith, *Nature*, 1960, **187**, 483.

[7] (*a*) P. G. Katsoyannis, A. Tometsko, C. Zalut, S. Johnson, and A. Trakatellis, *Biochemistry*, 1967, **6**, 2635; (*b*) P. G. Katsoyannis, A. Trakatellis, S. Johnson, C. Zalut, and G. Schwartz, *ibid.*, 1967, **6**, 2642.

[8] (*a*) P. G. Katsoyannis, K. Fukuda, A. Tometsko, K. Suzuki, and M. Tilak, *J. Amer. Chem. Soc.*, 1964, **86**, 930; (*b*) P. G. Katsoyannis, C. Zalut, A. Tometsko, M. Tilak, S. Johnson, and A. Trataktellis, *ibid.*, 1971, **93**, 5871; (*c*) P. G. Katsoyannis, J. Ginos, C. Zalut, M. Tilak, S. Johnson, and A. Trakatellis, *ibid.*, 1971, **93**, 5877.

(Scheme 1), removal of the protecting groups with sodium in liquid ammonia,[9] and conversion of the resulting thiol derivative into the *S*-sulphonated form (VIII) by oxidative sulphitolysis.[10] The protected pentacosapeptide was synthesised by classical methods of peptide synthesis, namely, a combination of the 'stepwise elongation' and 'fragment condensation' approaches. The overall synthesis is summarised in Schemes 1 and 2; it involves the coupling of the *N*-terminal octapeptide fragment[11] (VIIc) (Scheme 1) with the *C*-terminal heptadecaptide derivative (VIIb) by the dicyclohexylcarbodi-imide–*N*-hydroxysuccinimide method.[12] The partially protected heptadecapeptide derivative (VIIb) (Scheme 2) was constructed by the fragment condensation approach. Benzyloxycarbonyl and, in one instance, t-butoxycarbonyl were used as the α-amino-group protectors. The acylated amino-acids used at each step in the stepwise synthesis of the various fragments were activated in all but two instances by conversion into the corresponding *p*-nitrophenyl esters. Activation of N^{α}-benzyloxycarbonyl-N^{ω}-tosyl-L-arginine[13] in the synthesis of the tetrapeptide derivative (III) and of *N*-t-butoxycarbonyl-*O*-benzyl-L-serine[14] in the synthesis of the heptadecapeptide derivative (VIIa) was performed by use of 3-(2-ethylisoxazol-5-ylio)benzenesulphonate[15] and dicyclohexylcarbodi-imide–*N*-hydroxysuccinimide, respectively. The various fragments were condensed by the dicyclohexylcarbodi-imide–*N*-hydroxysuccinimide method.[12] The benzyloxycarbonyl group was removed by catalytic hydrogenation or by exposure to hydrogen bromide in trifluoroacetic acid, and the t-butoxycarbonyl group by treatment with trifluoroacetic acid.

The benzyloxycarbonyl, benzyl, and tosyl groups were removed from the protected pentacosapeptide (VIIIa) by sodium in liquid ammonia in a special apparatus.[8c] 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 des(pentapeptide B^{26-30}) B chain (VIII).

The crude *S*-sulphonated chain was purified[8b,c] by chromatography on a carboxymethylcellulose column equilibrated and eluted with a urea–acetate buffer (pH 4·0) [see Figure 1(b)]. The pure material was isolated as described previously.[8b,c] Briefly, the fractions under the main peak were chromatographed on a Sephadex G-15 column (5% acetic acid as eluant), and the peptide material from this eluate was precipitated as the picric acid salt and was subsequently chromatographed on a Sephadex G-15 column with ammonium hydrogen carbonate as eluant. Lyophilization of the eluate from the latter column afforded the purified chain analogue. Amino-acid analysis, after acidic hydrolysis and enzymic digestion (Table 1), chromatography on a carboxymethylcellulose column [Figure 1(c)], and high voltage thin-layer electrophoresis [Figure 2(A)] were employed to ascertain the homogeneity of the purified *S*-sulphonated peptide chain.

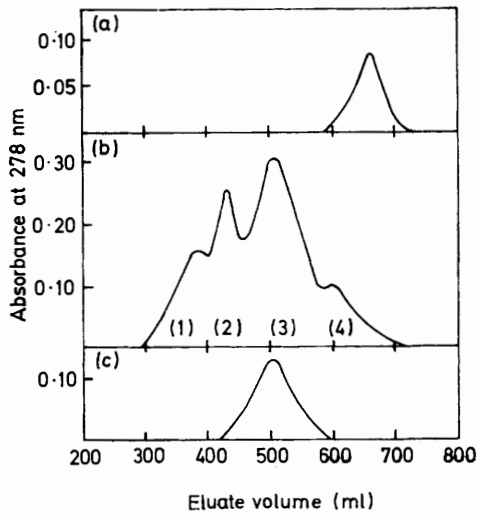


FIGURE 1 Elution patterns from chromatography on a carboxymethylcellulose column (4·3 × 49 cm) with urea–acetate buffer (pH 4·0): (a) natural insulin B chain *S*-sulphonate; (b) crude mixture obtained by the sodium–liquid ammonia treatment of synthetic protected des(pentapeptide B^{26-30}) B chain followed by oxidative sulphitolysis, dialysis, and lyophilization; (c) purified synthetic des(pentapeptide B^{26-30}) B chain *S*-sulphonate obtained from crude material upon chromatography in the same chromatographic system

TABLE 1

Amino-acid composition * of the *S*-sulphonated des(pentapeptide B^{26-30}) B chain of insulin

	Acidic hydrolysis		Enzymic hydrolysis (aminopeptidase M)	
Amino-acid	Theory	Found	Theory	Found
His	2·0	2·0	2·0	1·9
Arg	1·0	0·9	1·0	0·8
Asp	1·0	1·1	0	0
Gln	0	0	1·0 †	2·2
Asn	0	0	1·0 †	
Ser	1·0	0·9	1·0	1·0 ‡
Glu	3·0	2·9	2·0	1·6
Gly	3·0	3·0	3·0	3·0
Ala	1·0	1·1	1·0	1·2
Cys	2·0	1·6	0	0
Val	3·0	2·9	3·0	3·0
Leu	4·0	4·3	4·0	4·3
Tyr	1·0	0·9	1·0	1·0
Phe	3·0	2·7	3·0	2·9
S-(SO_3^-)Cys	0	0	2·0	1·9 §

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

The *S*-sulphonated A chain of human (porcine)

[9] R. H. Sifferd and V. du Vigneaud, *J. Biol. Chem.*, 1935, **108**, 753.
[10] J. L. Bailey and R. D. Cole, *J. Biol. Chem.*, 1959, **234**, 1733.
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[12] F. Weygand, D. Hoffman, and E. Wünsch, *Z. Naturforsch.*, 1966, **21b**, 426.
[13] J. Ramachandran and C. H. Li, *J. Org. Chem.*, 1962, **27**, 4006.
[14] E. Wünsch and A. Zwick, *Chem. Ber.*, 1964, **97**, 2497.
[15] R. B. Woodward, R. A. Olofson, and H. Mayer, *J. Amer. Chem. Soc.*, 1961, **83**, 1010.

insulin was converted into its thiol form by interaction with 2-mercaptoethanol at pH 5·0 and 100 °C. The reduced chain was then combined with the *S*-sulphonated des(pentapeptide B^{26-30}) human B chain to produce

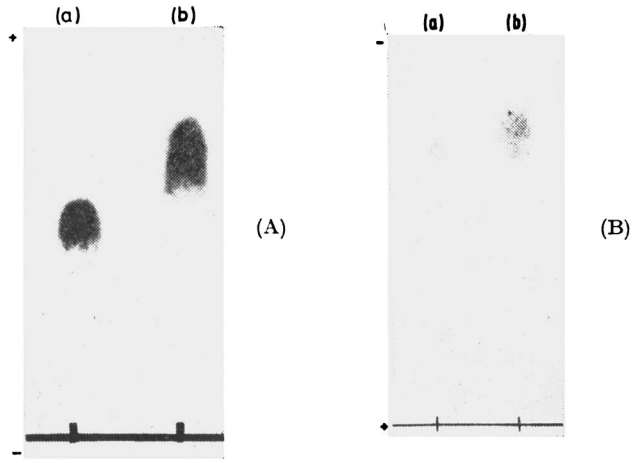

FIGURE 2 (A) High-voltage thin-layer electrophoresis of (a) natural porcine B chain *S*-sulphonate and (b) synthetic des(pentapeptide B^{26-30}) B chain *S*-sulphonate (0·01M-NH_4HCO_3 adjusted to pH 10·0 with NH_4OH; 2700 V; 25 min); (B) High-voltage thin-layer electrophoresis of (a) des(pentapeptide B^{26-30}) human (porcine) insulin and (b) natural bovine insulin (0·5N-acetic acid; 3400 V; 15 min)

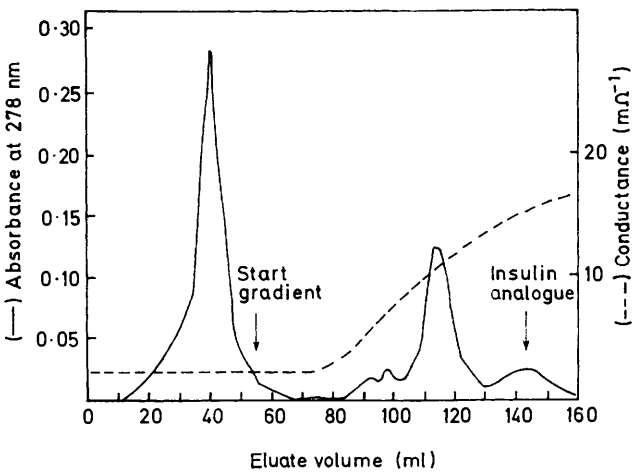

FIGURE 3 Chromatography of four combination mixtures (see Experimental section) of des(pentapeptide B^{26-30}) B chain *S*-sulphonate with the thiol form of human (porcine) A chain on a cellulose column (0·9 × 23 cm) with acetate buffer (0·024M; pH 3·3) and an exponentional sodium chloride gradient. The column eluate was monitored by a Gilford recording spectrophotometer and by a conductivity meter (Radiometer, Copenhagen). The des(pentapeptide B^{26-30}) human (porcine) insulin (130—160 ml of eluate) was recovered as the hydrochloride

the insulin analogue according to the procedure reported previously.[7b,16] The product was isolated by the procedure we have employed [7b,16b] in the synthesis of sheep and human insulin; namely, chromatography on a carboxymethylcellulose column with an acetate buffer (pH 3·3) and an exponential sodium chloride gradient (Figure 3). From the eluate the insulin analogue was isolated *via* the picrate as the hydrochloride.[7b,16b] Amino-acid anslysis, after acidic hydrolysis (Table 2), and high voltage thin-layer electrophoresis [Figure 2(B)] were employed to ascertain its homogeneity. No attempt was made to obtain it in crystalline form.

TABLE 2

Amino-acid composition * of an acidic hydrolysate of des(pentapeptide B^{26-30}) human (porcine) insulin

Amino-acid	Theory	Found
His	2	2·2
Arg	1	1·1
Asp	3	2·8
Thr	1	0·8
Ser	3	2·6 †
Glu	7	6·6
Gly	4	4·2
Ala	1	1·2
Cys	6	4·0 †
Val	4	3·7
Ile	2	1·3
Leu	6	5·9
Tyr	3	2·0 ‡
Phe	3	2·9

* 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

M.p.s were taken for samples in capillary tubes. Optical rotations were taken with a Zeiss photoelectric precision polarimeter. Amino-acid analyses were performed with a Beckman-Spinco 120C amino-acid analyser equipped with a digital readout system (model CRS 12 AB, Infotronics Corp., Houston, Texas) according to the method of Spackman *et al.*[17] Acidic hydrolyses and calculations of molar ratios were carried out as described previously.[7a] For the enzymic digestion with aminopeptidase M the method of Pfleiderer *et al.*[18] was employed; the enzyme was purchased from Henley and Co., New York. For paper chromatography, the protected peptides were deblocked as indicated and the resulting unprotected compounds were chromatographed on Whatman no. 1 paper at room temperature. R_F^1 values refer to the Partridge system; [19] R_F^2 values refer to the system [20] butan-1-ol–pyridine–acetic acid–water (30 : 20 : 6 : 24 v/v) and are expressed as a multiple of the distance travelled by a histidine marker. Thin-layer electrophoresis was carried out according to a method developed in this laboratory [21] and was performed with a Wieland–Pfleiderer pherograph (Brinkmann Instruments, Westbury, New York). Elemental analyses were carried out by the Schwarzkopf Microanalytical Laboratory, Woodside, New York. Preswollen microgranular carboxymethylcellulose (Whatman CM 52/1) and Sephadex G-15 (Pharmacia Uppsala) were used. The washing of the resins and the preparation of

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[18] G. Pfleiderer, P. G. Celliers, M. Stanulovic, E. M. Wachsmuth, H. Determann, and G. Braunitzer, *Biochem. Z.*, 1964, **340**, 552.

[19] S. M. Partridge, *Biochem. J.*, 1948, **42**, 238.

[20] S. G. Waley and G. Watson, *Biochem. J.*, 1953, **55**, 328.

[21] A. Tometsko and N. Delihas, *Analyt. Biochem.*, 1967, **18**, 72.

the columns and of the buffers have been described previously.[7a,b] Sodium tetrathionate was prepared as described by Gilman *et al.*[22] Natural bovine and porcine insulins were provided by Eli Lilly and Co. Biological assays were carried out by the mouse convulsion method.[16a,b] Radioimmunoassays were performed by the method of Hales and Randle [23] with an insulin immunoassay kit (Amersham/Searle Co.). Protein determinations were carried out by the method of Lowry *et al.*[24]

N-*Benzyloxycarbonyl*-L-*phenylalanyl*-L-*phenylalanine Methyl Ester* (I).—To a solution of L-phenylalanine methyl ester hydrochloride (21·5 g) and N-benzyloxycarbonyl-L-phenylalanine *p*-nitrophenyl ester [25] (42 g) in dimethylformamide (DMF) (150 ml) cooled to 0 °C, triethylamine (14 ml) was added in three portions during 3 h. The mixture was further stirred at room temperature for 20 h and poured into ethyl acetate (600 ml) and water (150 ml). The organic phase was washed (0·5N-NH_4OH, 0·5N-HCl, and water), dried and evaporated and the solid product was crystallized from 95% ethanol; yield 34·5 g (75%); m.p. 146—148°, $[\alpha]_D^{26}$ −20·1° (*c* 1 in DMF) {lit.,[26] m.p. 148—149°, $[\alpha]_D^{26}$ −17·5° (*c* 2 in DMF)}; paper chromatography after hydrogenolysis in the presence of hydrogen chloride: R_F^1 0·84, R_F^2 3·12 × His.

N-*Benzyloxycarbonylglycyl*-L-*phenylalanyl*-L-*phenylalanine Methyl Ester* (II).—Compound (I) (15 g) was dissolved in methanol (300 ml) containing concentrated hydrogen chloride (2·5 ml) and hydrogenated for 3·5 h over 10% palladium–charcoal (2 g). The catalyst was filtered off and the filtrate was concentrated under reduced pressure. The residue was dried by addition of methanol followed by evaporation under reduced pressure and then dissolved in DMF (100 ml). To this solution, cooled to 0 °C, *N*-benzyloxycarbonylglycine *p*-nitrophenyl ester [27] (9·9 g) was added, followed by triethylamine (3·9 ml). After 24 h the mixture was poured into ethyl acetate (600 ml) and water (150 ml) and the organic phase was washed (0·5N-NH_4OH, 0·5N-HCl, and water), dried, and evaporated. The solid *residue* (15·7 g, 92%) was dissolved in methanol (130 ml) and precipitated with water (50 ml); yield 13·5 g (79%); m.p. 127—128°, $[\alpha]_D^{26}$ −15·2° (*c* 1 in DMF); paper chromatography after hydrogenolysis (in methanol–HCl–HCl): R_F^1 0·85; R_F^2 3·57 × His (Found: C, 67·0; H, 5·95; N, 8·4. $C_{29}H_{31}N_3O_6$ requires C, 67·3; H, 6·05; N, 8·1%).

N^{α}-*Benzyloxycarbonyl*-N^{ω}-*tosyl*-L-*arginylglycyl*-L-*phenylalanyl*-L-*phenylalanine Methyl Ester* (III).—*N*-Benzyloxycarbonylglycyl-L-phenylalanyl-L-phenylalanine methyl ester (5·18 g) was hydrogenated for 3 h over 10% palladium–charcoal (1 g) in methanol (150 ml) containing 2N-hydrochloric acid (5·5 ml). Filtration and evaporation left the solid tripeptide ester hydrochloride, which was dried by addition of methanol followed by evaporation and was then used for condensation with N^{α}-benzyloxycarbonyl-N^{ω}-tosyl-L-arginine. A solution of the latter (5·1 g) in acetonitrile (140 ml) and DMF (15 ml) was cooled to 0 °C, and triethylamine (1·54 ml) was added followed by 3-(2-ethylisoxazol-5-ylio)benzenesulphonate (2·79 g). After 1 h at 0 °C the mixture was diluted with a solution of the foregoing tripeptide ester hydrochloride in DMF (40 ml) and acetonitrile (40 ml) containing triethylamine (1·5 ml). The mixture was stirred at room temperature for 48 h then poured into ice-cold 0·5N-sodium hydrogen carbonate saturated with sodium chloride. The separated semi-solid product was extracted into ethyl acetate (500 ml) and the organic layer was washed successively with 0·5N-sodium hydrogen carbonate saturated with sodium chloride, 0·5N-hydrochloric acid saturated with sodium chloride, and finally saturated aqueous sodium chloride. The residue obtained after removal of the ethyl acetate was dissolved in methanol (60 ml) and precipitated with saturated aqueous sodium chloride (300 ml). The *solid* was isolated, washed with cold water, and dried; yield 7·1 g (86%); m.p. undetermined (the peptide sinters at 90 °C and it is converted into a liquid at 112 °C), $[\alpha]_D^{26}$ −11·8° (*c* 1 in DMF); paper chromatography after hydrogenolysis (in methanol–HCl): R_F^1 0·90, R_F^2 4·08 × His. Amino-acid analysis after acidic hydrolysis showed the expected composition: $Arg_{1\cdot0}Gly_{1\cdot0}$ $Phe_{2\cdot0}$ (Found: C, 60·4; H, 6·2; N, 11·4. $C_{42}H_{49}N_7O_9S$ requires C, 60·9; H, 5·95; N, 11·8%).

N-*Benzyloxycarbonyl*-γ-*t*-*butyl*-L-*glutamyl*-N^{ω}-*tosyl*-L-*arginylglycyl*-L-*phenylalanyl*-L-*phenylalanine Methyl Ester* (IV).—Compound (III) (6·04 g) was hydrogenated for 3 h over 10% palladium–charcoal (1·5 g) in methanol (150 ml) containing 2N-hydrochloric acid (3·8 ml). Filtration and evaporation left a residue which was dried by repeated addition and evaporation of methanol, dissolved in DMF (60 ml), and cooled to 0 °C. Triethylamine (1·0 ml) was added, followed by *N*-benzyloxycarbonyl-L-glutamic acid γ-t-butyl α-*p*-nitrophenyl diester [28] (3·4 g). After being stirred at room temperature for 24 h, the mixture was poured into ethyl acetate (400 ml) and water (100 ml). The organic layer was washed (0·5N-NH_4OH, cold 0·2N-HCl, and water), dried, and evaporated. The *product* was precipitated from a solution in ethyl acetate (60 ml) with petroleum (150 ml) and crystallized from methanol (100 ml); yield 6·2 g (90%); m.p. 154—155°, $[\alpha]_D^{26}$ −11·3° (*c* 1 in DMF); paper chromatography after hydrogenolysis in the presence of acetic acid: R_F^1 0·92, R_F^2 5·57 × His; amino-acid analysis after acidic hydrolysis: $Arg_{1\cdot0}Glu_{1\cdot0}$ $Gly_{1\cdot0}Phe_{2\cdot0}$ (Found: C, 60·6; H, 6·6; N, 11·2. $C_{51}H_{64}N_8O_{12}S$ requires C, 60·5; H, 6·35; N, 11·1%).

N-*Benzyloxycarbonyl*-L-*leucyl*-L-*tyrosyl*-L-*leucyl*-L-*valyl*-S-*benzyl*-L-*cysteinylglycyl*-γ-*t*-*butyl*-L-*glutamyl*-N^{ω}-*tosyl*-L-*arginylglycyl*-L-*phenylalanyl*-L-*phenylalanine Methyl Ester* (V).—The pentapeptide derivative (IV) (2·34 g) was hydrogenated for 4 h over 10% palladium–charcoal (0·7 g) in a solution of methanol (80 ml) and DMF (6 ml) containing glacial acetic acid (0·8 ml). Filtration and evaporation left a residue which was dried by repeated addition and evaporation of methanol under reduced pressure. The dried acetate of the pentapeptide ester (1·88 g) was used for condensation with *N*-benzyloxycarbonyl-L-leucyl-L-tyrosyl-L-leucyl-L-valyl-*S*-benzyl-L-cysteinylglycine [3] (IVa). To a solution of the latter (2·57 g) in DMF (15 ml) cooled to −10 °C, *N*-hydroxysuccinimide (0·47 g) was added, followed by dicyclohexylcarbodi-imide (0·6 g). After 12 h at 5 °C the mixture was filtered through a sintered-glass funnel and to the clear filtrate was added the acetate

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of the pentapeptide ester (1·88 g), followed by triethylamine (0·28 ml). The reaction was allowed to proceed at room temperature with stirring for 48 h, during which time it became necessary to add more DMF (10 ml) to control the increasing viscosity of the solution. The mixture was then poured into methanol (300 ml) and the precipitated *product* was isolated by centrifugation and washed with methanol and ether; yield 3·37 g (96%); m.p. 256—257°, $[\alpha]_D^{26}$ −26·6° (*c* 1 in DMF) (Found: C, 60·6; H, 6·8; N, 11·1. $C_{89}H_{118}N_{14}O_{19}S_2$ requires C, 61·0; H, 6·8; N, 11·2%); amino-acid analysis after acidic hydrolysis: $Arg_{1.0}Glu_{1.0}Gly_{2.0}Val_{1.0}Leu_{2.0}Tyr_{0.9}Phe_{1.9}$S-Benzylcysteine$_{0.9}$.

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$^{\omega}$-*tosyl*-L-*arginylglycyl*-L-*phenylalanyl*-L-*phenylalanine Methyl Ester* (VI).—The protected undecapeptide (V) (1·6 g) was dissolved in cold trifluoroacetic acid (15 ml) containing water (0·15 ml) and hydrogen bromide was passed through the solution for 1 h at room temperature. The solvent was evaporated off under reduced pressure and the product was triturated with ether and reprecipitated from methanol (20 ml) with ether. The white precipitate was isolated by centrifugation, washed with ether, dried (KOH) *in vacuo*, dissolved in DMF (15 ml), and cooled to 0 °C. It was then neutralized with triethylamine (0·26 ml) just prior to addition of the pentapeptide N$^{\alpha}$-benzyloxycarbonyl-N^{im}-benzyl-L-histidyl-L-leucyl-L-valyl-γ-t-butyl-L-glutamyl-L-alanine [3] (Va), which was activated as follows. To a solution of compound (Va) (1·52 g) and *N*-hydroxysuccinimide (0·21 g) in DMF (30 ml) cooled to −10 °C, dicyclohexylcarbodi-imide (0·37 g) was added. The mixture was stirred for 12 h at 5 °C and then filtered through a sintered-glass funnel. This filtrate was mixed with the solution of the deblocked undecapeptide ester. After 48 h at room temperature the mixture was poured into methanol (500 ml) containing acetic acid (3 ml). The *product* was isolated by centrifugation, washed (methanol and water), and dried; yield 1·9 g (88%); m.p. 265—266°, $[\alpha]_D^{26}$ −19·1° (*c* 1 in Me_2SO) (Found: C, 59·8; H, 6·85; N, 12·3; O, 18·2. $C_{121}H_{163}N_{21}O_{26}S_2,H_2O$ requires C, 60·3; H, 6·9; N, 12·2; O, 17·9%), amino-acid analysis after acidic hydrolysis: $Arg_{1.0}Glu_{2.0}Gly_{2.1}Ala_{0.9}Val_{1.9}Leu_{2.9}Tyr_{0.7}Phe_{1.8}$Benzylhistidine$_{1.0}$S-Benzylcysteine$_{0.7}$.

N-*t*-*Butyloxycarbonyl*-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$^{\omega}$-*tosyl*-L-*arginylglycyl*-L-*phenylalanyl*-L-*phenylalanine* (VII).—The hexadecapeptide derivative (VI) (0·9 g) was dissolved in cold trifluoroacetic acid (17 ml) containing water (0·3 ml) and hydrogen bromide was passed through the solution for 70 min at room temperature. Most of the solvent was removed under reduced pressure and the remaining product was mixed with ether. The precipitated peptide ester hydrobromide was washed with ether, reprecipitated from a suspension in propan-2-ol with ether, and dried (KOH) *in vacuo*. To a solution of this product in hexamethylphosphoramide (15 ml) cooled to 0 °C, triethylamine (0·16 ml) was added followed by a solution in DMF of *N*-t-butyloxycarbonyl-*O*-benzyl-L-serine which was activated as follows. A solution of *N*-t-butyloxycarbonyl-*O*-benzyl-L-serine (1·33 g) and *N*-hydroxysuccinimide (0·52 g) in DMF (15 ml) was cooled to −10 °C, and dicyclohexylcarbodi-imide (0·93 g) was added. After 1 h at −10 °C, and 3 h at 5 °C, the mixture was filtered through a sintered-glass funnel and the filtrate added to the solution of the hexadecapeptide ester, followed by hexamethylphosphoramide (5 ml). After 24 h at room temperature the mixture was poured into methanol (500 ml) and the precipitated product (VIIa) was isolated by centrifugation, washed with methanol and dried; yield 0·66 g (71%); m.p. 268—272°; amino-acid analysis after acidic hydrolysis: $Arg_{1.1}Ser_{0.8}Glu_{1.9}Gly_{2.0}Ala_{0.8}Val_{1.7}Leu_{2.7}Tyr_{0.7}Phe_{2.0}$Benzylhistidine$_{0.7}$-*S*-Benzylcysteine$_{0.7}$.

A suspension of the protected heptadecapeptide (VIIa) (0·82 g) in hexamethylphosphoramide (14 ml) was stirred at 40 °C until a clear solution was obtained. To this solution, cooled to 5 °C, were added (in portions during 30 min) N-sodium hydroxide (2 ml) and water (1 ml). The mixture was stirred for an additional 30 min at room temperature, diluted with cold water (20 ml), and acidified with 2N-hydrochloric acid (1 ml). Addition of water (120 ml) completed the precipitation of the partially protected *heptadecapeptide* (VII), which was isolated by centrifugation, washed (water, methanol, and ether) and dried; yield 0·7 g (86%); m.p. 282°, $[\alpha]_D^{26}$ −22·6° (*c* 1 in Me_2SO) (Found: C, 59·1; H, 6·75; N, 12·2; O, 19·0. $C_{123}H_{166}N_{22}O_{28}S_2,H_2O$ requires C, 59·5; H, 6·8; N, 12·4; O, 18·7%); Amino-acid analysis of an acidic hydrolysate: $Arg_{0.9}Ser_{0.8}Glu_{2.0}Gly_{2.2}Ala_{1.0}Val_{2.0}Leu_{3.0}Tyr_{0.8}Phe_{1.8}$Benzylhistidine$_{1.0}$S-Benzylcysteine$_{0.7}$.

L-*Phenylalanyl*-L-*valyl*-L-*asparaginyl*-L-*glutaminyl*-L-*histidyl*-L-*leucyl*-S-*sulphonato*-L-*cysteinylglycyl*-L-*seryl*-L-*histidyl*-L-*leucyl*-L-*valyl*-L-*glutamyl*-L-*alanyl*-L-*leucyl*-L-*tyrosyl*-L-*leucyl*-L-*valyl*-S-*sulphonato*-L-*cysteinylglycyl*-L-*glutamyl*-L-*arginylglycyl*-L-*phenylalanyl*-L-*phenylalanine* [*Des*(*pentapeptide B*$^{26-30}$) *B Chain* S-*Sulphonate*] (VIII).—A solution of the partially protected heptadecapeptide (VII) (0·70 g) in trifluoroacetic acid (10 ml) was stored at room temperature for 1 h. Addition of ether caused precipitation of the trifluoroacetate of *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$^{\omega}$-tosyl-L-arginylglycyl-L-phenylalanyl-L-phenylalanine (VIIb), which was isolated by centrifugation, washed with ether, and dried (KOH) *in vacuo*. This heptadecapeptide derivative was dissolved in hexamethylphosphoramide (15 ml) by stirring and warming to 40 °C and to the resulting solution, cooled to 0 °C, was added triethylamine (0·17 ml) just before the addition of the *N*-terminal octapeptide *N*-benzyloxycarbonyl-L-phenylalanyl-L-valyl-L-asparaginyl-L-glutaminyl-N^{im}-benzyl-L-histidyl-L-leucyl-*S*-benzyl-L-cysteinylglycine (VIIc), which was activated in the following manner. The derivative (VIIc) (1·0 g) and *N*-hydroxysuccinimide (90 mg) were dissolved in a mixture of DMF (10 ml) and hexamethylphosphoramide (7 ml) and to this solution cooled to −5 °C was added dicyclohexylcarbodi-imide (167 mg). The mixture was stored at 5 °C for 12 h and then added to the solution of the heptadecapeptide derivative (VIIb). After 40 h at room temperature the mixture was acidified with acetic acid (0·5 ml) and poured into methanol (400 ml). The precipitated partially protected pentacosapeptide (VIIIa) was collected by centrifugation, washed with warm methanol and ether, and dried; yield 0·48 g; amino-acid analysis of an acidic hydrolysate: $Arg_{0.9}Asp_{1.0}Ser_{0.7}Glu_{3.1}Gly_{3.1}Ala_{0.9}Val_{2.7}Leu_{3.5}Tyr_{0.6}Phe_{2.7}$Benzylhistidine$_{2.0}$S-Benzylcysteine$_{1.6}$. This material was converted into des(pentapeptide B$^{26-30}$) B chain *S*-sulphonate (VIII) by reduction with sodium in

liquid ammonia followed by oxidative sulphitolysis with sodium sulphite and sodium tetrathionate; the procedure was essentially that employed previously in the synthesis of the insulin B chain.[8b,c] The sodium in liquid ammonia reduction was carried out in a special glass apparatus.[8c] To a solution of the thoroughly dried crude pentacosapeptide derivative (200 mg) in anhydrous liquid ammonia (200 ml) was added dropwise a dilute solution of sodium in liquid ammonia. The faint blue colour which was obtained at the end point of the reaction was allowed to persist for 20 s and subsequently discharged by addition of a few crystals of ammonium chloride. The clear solution was concentrated at atmospheric pressure to about 10 ml and lyophilized. The residue was dissolved in 8M-guanidine hydrochloride (30 ml) and to this solution, adjusted to pH 8·9 (with acetic acid or NH_4OH depending on the pH of the solution), were added freshly prepared sodium tetrathionate (250 mg) and, after 30 min, sodium sulphite (500 mg). After being stirred for 3 h at room temperature the mixture was placed in an 18/32 Visking dialysis tube and dialysed against four changes of distilled water (4 l each) at 4 °C for 24 h. Upon lyophilization of the non-diffusible material the crude des(pentapeptide B^{26-30}) B chain *S*-sulphonate was obtained as a white powder; yield 140 mg; amino-acid analysis of an acidic hydrolysate: $His_{1.9}Arg_{0.9}Asp_{1.0}Ser_{0.7}Glu_{3.0}Gly_{3.1}Ala_{1.1}Cys_{1.5}Val_{3.0}Leu_{4.4}$-$Tyr_{0.8}Phe_{2.5}$.

Isolation of Des(pentapeptide B^{26-30}) B Chain S-*Sulphonate*.—A carboxymethylcellulose column (pre-swollen microgranular, Whatman CM/52/1; 4·3 × 49 cm) equilibrated and eluted with a urea–acetate buffer (0·04M-sodium acetate, 8M-urea; pH 4·0) was used for the isolation. The washing of the resin and the preparation of the column have been described previously.[7a,b] A solution of the lyophilized crude material (130 mg) in *ca.* 5 ml of buffer was applied to the column and the chromatogram was developed with the same buffer at 120 ml h^{-1}. The eluted material was monitored continuously with a Gilford recording spectrophotometer at 278 nm. Figure 1(b) illustrates the chromatographic pattern obtained and indicates the presence of the components (1)—(4). A similar chromatographic profile was obtained when crude synthetic B chain *S*-sulphonate of bovine or human insulin was chromatographed in the same system.[8b,c] Figure 1(a) illustrates the chromatographic pattern of natural bovine B chain *S*-sulphonate [7a] in the same system. No attempt was made to identify components (1), (2), and (4). The eluate containing component (3) (460—560 ml) was adjusted to pH 3·0 (concentrated HCl) and placed on a Sephadex G-15 column (4 × 60 cm); 5% (v/v) acetic acid was used as the eluting buffer. Peptide material was detected with a Gilford recording spectrophotometer at 278 nm and collected. The eluate containing the peptide material was concentrated in a rotary evaporator (30 °C) to 10—12 ml and mixed with an equal volume of saturated picric acid solution. The precipitated picrate of des(pentapeptide B^{26-30}) B chain *S*-sulphonate was allowed to settle overnight at 4°, isolated by centrifugation, and washed twice with half-saturated picric acid solution. The picrate salt was then dissolved in 0·05N-ammonium hydrogen carbonate (2 ml) with the aid of a few drops of dilute ammonia and placed on a Sephadex G-15 column (1·2 × 50 cm) equilibrated and eluted with 0·05N-ammonium hydrogen carbonate. The des(pentapeptide B^{26-30}) B chain was separated completely from the picric acid and was recovered by lyophilization of the eluate (which was continuously monitored with a Gilford spectrophotometer) as a white fluffy powder; yield 27 mg. Rechromatography of this material on the carboxymethylcellulose column under the conditions described previously gave a single peak [Figure 1(c)]. Amino-acid analysis of the purified material after acidic hydrolysis gave a composition in excellent agreement with the theoretically expected values (Table 1). 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. This proves that the digestion was complete and that the optical configuration of the constituent amino-acids was preserved during the synthesis of the B chain analogue. On high-voltage thin-layer electrophoresis in 0·01M-ammonium hydrogen carbonate (adjusted to pH 10·0 with NH_4OH) at 2700 V the synthetic material moved as a single component (Pauly reaction) and had a greater mobility towards the anode than the natural *S*-sulphonated porcine B chain (Figure 2A).

S-*Sulphonated Derivatives of the A and B Chains of Porcine Insulin*.—These compounds were prepared by oxidative sulphitolysis of porcine insulin followed by separation of the resulting *S*-sulphonated chains by continuous flow electrophoresis as described in a previous report.[7a]

Synthesis and Isolation of Des(pentapeptide B^{26-30}) Human (Porcine) Insulin.—The synthesis was accomplished by the interaction of the thiol form of the A chain of human (porcine) insulin and the *S*-sulphonated form of the des-(pentapeptide B^{26-30}) B chain following the procedures we have reported previously.[7b,16,a,b] In a typical experiment, an aqueous solution of the *S*-sulphonated human A chain (20 mg) was converted into the thiol form by exposure to 2-mercaptoethanol at pH 5 and at 100 °C. The reduced chain was then treated with *S*-sulphonated des(pentapeptide B^{26-30}) B chain (5 mg) and the combination mixture was treated as described previously.[7b,16a,b] The insulin analogue was isolated by chromatography on a carboxymethylcellulose column (0·9 × 23 cm) with an acetate buffer (pH 3·3) and an exponential sodium chloride gradient according to the procedure reported previously.[7b,16b] Four combination mixtures, each corresponding to the amounts of materials indicated above, were chromatographed and gave the pattern shown in Figure 3. The insulin analogue was eluted with application of the sodium chloride gradient as the slowest moving component, and was isolated [7b,16b] from the eluate *via* the picrate as the hydrochloride (0·35 mg).

Amino-acid analysis of this analogue after acidic hydrolysis gave a composition in agreement with the expected values (Table 2). On thin-layer electrophoresis in 0·5N-acetic acid (pH 2·9 and 3400 V) the synthetic analogue moved as a single component (Figure 2B). It showed potencies of 8·5—9 I.U. mg^{-1} by the mouse convulsion assay method and 11 I.U. mg^{-1} by the radioimmunoassay method (35 and 45%, respectively, of the activity of the natural hormone).

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