rhythmias, Dreifus, L. S., and Likoff, W., Ed., New York, N.Y., Grune & Stratton, p 479.
Narayanan, N., and Sulakhe, P. V. (1977), *Mol. Pharmacol. 13,* 1033.
Reddy, N. B., Engel, W. K., and Festoff, B. W. (1976), *Biochim. Biophys. Acta 433,* 365.
Requena, J., DiPolo, R., Brinley, F. J., Jr., and Mullins, F. J. (1977), *J. Gen. Physiol. 70,* 329.
Reuter, H. (1974), *Circ. Res. 34,* 599.
Robinson, N. C., and Tanford, C. (1975), *Biochemistry 14,* 369.
Rosen, O. M., Erlichman, J., and Rubin, G. S. (1975), in Recent Advances in Cyclic Nucleotide Research, Drummond, G. I., Greengard, P., and Robison, G. A., Ed., New York, N.Y., Raven Press, p 253.
Saccomani, G., Spenney, J. G., Urry, D. W., and Sachs, G. (1974), *J. Mol. Cell. Cardiol. 6,* 505.
Schatzman, H. J. (1975), *Curr. Top. Membr. Transp. 6,* 121.
Schwartz, A., Lindenmayer, G. E., and Allen, J. C. (1975), *Pharmacol. Rev. 27,* 3.
Singer, S. J. (1974), *Annu. Rev. Biochem. 43,* 805.
Steckhoven, F. M. A. H. S., Van Heeswikk, M. P. E., De Pont, J. J. H. H. M., and Bonting, S. L. (1976), *Biochim. Biophys. Acta 422,* 210.
St. Louis, P. J. (1975), *Proc. Can. Fed. Biol. Soc. 18,* 153.
St. Louis, P. J., and Sulakhe, P. V. (1976a), *Int. J. Biochem. 7,* 547.
St. Louis, P. J., and Sulakhe, P. V. (1976b), *Can. J. Biochem. 54,* 946.
St. Louis, P. J., and Sulakhe, P. V. (1976c), *Biochem. J. 158,* 535.
Sulakhe, P. V., and St. Louis, P. J. (1978), *Rec. Adv. Stud. Card. Struct. Metab. 11,* 241.
Sulakhe, P. V., Drummond, G. I., and Ng, D. C. (1973a), *J. Biol. Chem. 248,* 4150.
Sulakhe, P. V., Drummond, G. I., and Ng, D. C. (1973b), *J. Biol. Chem. 248,* 4158.
Sulakhe, P. V., Leung, N. L., and St. Louis, P. J. (1976a), *Can. J. Biochem. 54,* 38.
Sulakhe, P. V., Sulakhe, S. J., Leung, N. L., St. Louis, P. J., and Hickie, R. A. (1976b), *Biochem. J. 157,* 705.
Sulakhe, S. J., Leung, N. L., and Sulakhe, P. V. (1976c), *Biochem. J. 157,* 713.
Tada, M., Kirchberger, M. A., and Katz, A. M. (1975), *J. Biol. Chem. 250,* 2640.
Taussky, H. H., and Shorr, E. (1953), *J. Biol. Chem. 202,* 675.
Ueda, T., Rudolph, S. A., and Greengard, P. (1975), *Arch. Biochem. Biophys. 170,* 492.
Warren, G. B., Toon, P. A., Birdsall, N. J. M., Lee, A. G., and Metcalfe, J. C. (1974), *Biochemistry 13,* 5501.
Weber, K., and Osborn, M. (1969), *J. Biol. Chem. 244,* 4406.
Yu, B. P., Masoro, E. J., and Morley, T. F. (1976), *J. Biol. Chem. 51,* 2037.

Synthesis of Des(tetrapeptide B^{1-4}) and Des(pentapeptide B^{1-5}) Human Insulins. Two Biologically Active Analogues†

Gerald Schwartz and Panayotis G. Katsoyannis*

ABSTRACT: Two analogues of human insulin, des(tetrapeptide B^{1-4})- and des(pentapeptide B^{1-5})-insulin, which differ from the parent molecule in that the N-terminal tetrapeptide and pentapeptide sequences, respectively, have been eliminated, have been synthesized. The des(tetrapeptide B^{1-4})-insulin shows potencies of 13 IU/mg by the mouse convulsion assay method and 7.6 IU/mg by the radioimmunoassay method. The des(pentapeptide B^{1-5})-insulin possesses a potency of 1.2 IU/mg when assayed by the glucose-oxidation method in isolated fat cells and 3.7 IU/mg by the radioimmunoassay technique. The natural hormone has a potency of 23–25 IU/mg by both assay methods.

Current studies in our laboratory are directed toward the determination of possible correlations between chemical structure and the biological activity of insulin. One feature of this work is the evaluation of the relative importance of amino acid residues, located at the amino-terminal regions of the A and B chains, for the biological activity of this protein. We have found that removal of the N-terminal tetrapeptide sequence from the A chain leads to the total loss of activity of insulin (Katsoyannis and Zalut, 1972), and even the replacement of the α-amino group of the N-terminal residue of this chain by a hydrogen causes a ca. 65% loss of the biological activity of the hormone. The significance of the N-terminal region of the A chain for the biological activity is also evident from the work carried out in other laboratories [for a review, see Blundell et al., 1972]. However, a different situation exists regarding the role of the amino-terminal region of the B chain to the biological activity of insulin.

Early studies by Brandenburg (1969) have shown that des(B^1)-insulin, produced by selective Edman degradation of the unprotected molecule, is biologically and immunologically identical with the natural hormone. Subsequently, by stepwise degradation of the B-chain moiety of partially protected natural insulin, using the Edman method, Geiger and Langner (1973) found that des(B^1)- and des(dipeptide B^{1-2})-insulin retained almost the full activity of the natural hormone in lowering rabbit blood glucose level. Attempts to prepare the des(tripeptide B^{1-3})-insulin by the same method, however, led

† From the Department of Biochemistry, Mount Sinai School of Medicine, City University of New York, New York, New York 10029. *Received April 14, 1978.* This work was supported by the National Institute for Arthritis, Metabolism, and Digestive Diseases, U.S. Public Health Service (AM-12929). For the previous paper of this series, see Schwartz and Katsoyannis (1977).

0006-2960/78/0417-4550$01.00/0

to the conversion of the newly formed N terminus, glutamine (B^4), to pyroglutamic acid. This product was found to possess ca. 70% of the activity of insulin. Using a modified procedure of the original method, Geiger and Langner (1975) reported recently, without experimental details, the preparation of des(tetrapeptide B^{1-4})-insulin by stepwise degradation of natural insulin. This analogue was reported to possess ca. 70% of the activity of the natural hormone. Stepwise Edman degradation of the B-chain moiety of protected natural insulin was also reported recently by Saunders and Offord (1977). These investigators, using different protecting groups than those employed by Geiger and Langner (1975), reported that they have prepared the des(B^1)-, des(dipeptide B^{1-2})-, des(tripeptide B^{1-3})-, des(tetrapeptide B^{1-4})-, and des(pentapeptide B^{1-5})-insulin derivatives. All these derivatives have the α- and ϵ-amino groups of the A^1 Gly and B^{29} Lys protected with the ethoxycarbonylmethionyl function. However, neither the preparation nor the biological activities of the unprotected analogues were reported.

The present report describes the chemical synthesis, isolation in pure form, and biological evaluation of des(tetrapeptide B^{1-4}) and des(pentapeptide B^{1-5}) human insulins. These analogues differ from the parent molecule in that the N-terminal tetrapeptide and pentapeptide sequences, respectively, have been eliminated from their B chain moiety.

Experimental Procedures and Results

Materials and Techniques. Capillary melting points were determined for all derivatives and are not corrected. Optical rotations were taken with a Zeiss photoelectric precision polarimeter. 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. The homogeneity of the intermediate peptide derivatives was ascertained, after deblocking at the amino end, by thin-layer chromatography on 6060 silica gel (Eastman Chromagram sheet). The solvent systems used were: (A) 1-butanol–pyridine–acetic acid–water (30:20:6:24) and (B) 1-butanol–acetic acid–water (4:1:1). Thin-layer electrophoresis was performed by a method developed in this laboratory (Tometsko and Delihas, 1967) and was carried out with a Wieland–Pfleiderer pherograph (Brinkmann Instruments, Westbury, N.Y.) Amino acid analyses were performed in a Beckman–Spinco amino acid analyzer according to the method of Spackman et al. (1958). Acid hydrolysis and calculations of molar ratios were carried out as described previously (Katsoyannis et al., 1967a). Enzymatic digestion with aminopeptidase M (Sigma Chemical Co.) was performed by the method of Pfleiderer et al. (1964). Preswollen microgranular CM[1]-cellulose (Whatman CM 52) was used in this study. The washing of the resin and the preparation of the columns and of the buffers used were described previously (Katsoyannis et al., 1967a,b). The column eluates were continuously monitored with a Gilford recording spectrophotometer at 287 nm. Sodium tetrathionate was prepared as described by Gilman et al. (1946). Desalting of the B chain analogues after column chromatography was performed by the hollow-fiber technique in a Bio-Fiber 50 beaker, as described in Bulletin No. 1004 of the Bio-Rad Laboratories (Richmond, Calif.) Protein determinations were carried out by the method of Lowry et al. (1951). Biological assays by the mouse convulsion assay method were carried out as described previously (Katsoyannis et al., 1967c). Biological evaluation by the glucose oxidation method in isolated fat cells was performed as described by Rodbell (1964). For radioimmunoassays, the double-antibody technique of Hales and Randle (1963) was employed using an "insulin immunoassay kit" (Amersham/Searle Co.). Crystalline porcine insulin was generously provided by Eli Lilly and Co.

General Aspects of the Synthesis of Human Des(tetrapeptide B^{1-4})- and Des(pentapeptide B^{1-5})-insulin. These analogues were prepared by the interaction of the thiol form of the human A chain with the S-sulfonated des(tetrapeptide B^{1-4}) and des(pentapeptide B^{1-5}) B chains of human insulin, respectively. The thiol form of the human A chain, which is identical with the respective chain of porcine insulin (Nicol and Smith, 1960), was obtained by reduction with 2-mercaptoethanol of the S-sulfonated derivative of that chain, prepared by oxidative sulfitolysis of porcine insulin (Katsoyannis et al., 1967a). The synthesis of the S-sulfonated des(tetrapeptide B^{1-4}) and des(pentapeptide B^{1-5}) human B chains was patterned after that of our novel synthesis of human B chain (Schwartz and Katsoyannis, 1973b). It involved the construction of the protected hexacosapeptide and pentacosapeptide derivatives containing the entire amino acid sequence of des(tetrapeptide B^{1-4}) and des(pentapeptide B^{1-5}) B chains, respectively, removal of the protecting groups with liquid hydrogen fluoride, and conversion of the resulting thiol derivatives into the corresponding S-sulfonated forms III and IV, by oxidative sulfitolysis.

N-tert-Butoxycarbonyl-L-leucyl-S-diphenylmethyl-L-cysteinylglycine (I). To a suspension of *S*-diphenylmethyl-L-cysteinylglycine (Hiskey et al., 1971) (3.3 g) in Me_2Fam (70 mL), *N-tert*-butoxycarbonyl-L-leucine *N*-hydroxysuccinimide ester (Anderson et al., 1964) (3.3 g) was added, followed by triethylamine (0.1 mL). After stirring at room temperature for 24 h, the resulting clear solution was poured into a mixture of ether (1 L) and water (300 mL). The organic layer was washed successively with water, 0.2 N sulfuric acid, and water, dried with $MgSO_4$, and concentrated to a small volume. Upon cooling, the product was crystallized out, collected by filtration, and recrystallized from ethyl acetate–ether: wt 4.4 g (80%); mp 110–111 °C; $[\alpha]_D^{26}$ −39.1° (*c* 1, Me_2Fam). Anal. Calcd for $C_{29}H_{40}N_3O_6S$: C, 62.3; H, 7.2; N, 7.5. Found: C, 61.8; H, 7.2; N, 7.9. After treatment with trifluoroacetic acid: homogeneous on TLC in solvents A and B.

N-tert-Butoxycarbonyl-N^{im}-tosyl-L-histidyl-L-leucyl-S-diphenylmethyl-L-cysteinylglycine (II). A solution of compound I (4.4 g) in trifluoroacetic acid (25 mL) was stored at room temperature for 30 min. The solvent was removed under reduced pressure, and to the residue was added a saturated solution of sodium acetate to give a pH 5.5. After cooling the mixture for 2 h, the precipitated peptide derivative was isolated by filtration and washed with water and ether. To a suspension of this product in Me_2Fam (60 mL) *N-tert*-butoxycarbonyl-N^{im}-tosyl-L-histidine 2,4,5-trichlorophenyl ester (Schwartz and Katsoyannis, 1973a) (5.8 g) was added, followed by triethylamine (0.5 mL). After stirring for 24 h at room temperature, the resulting clear solution was diluted with a mixture of ether (200 mL) and petroleum ether (400 mL) and cooled at −20 °C for 5 h. The precipitated product was collected and crystallized from methylene chloride–ether: wt 5 g (74%); mp 134 °C (dec); $[\alpha]_D^{26}$ −32° (*c* 1, Me_2Fam). Anal. Calcd for $C_{41}H_{51}N_6O_9S_2$: C, 58.9; H, 6.1; N, 10.0. Found: C, 58.4; H, 6.1; N, 9.6. After treatment with trifluoroacetic acid: homogeneous on TLC in solvents A and B.

L-Histidyl-L-leucyl-S-sulfo-L-cysteinylglycyl-L-seryl-L-histidyl-L-leucyl-L-valyl-L-glutamyl-L-alanyl-L-leucyl-

[1] Abbreviations used are: CM, carboxymethyl; TLC, thin-layer chromatography; Me_2Fam, dimethylformamide; Tris-HCl, 2-amino-2-hydroxymethyl-1,3-propanediol.

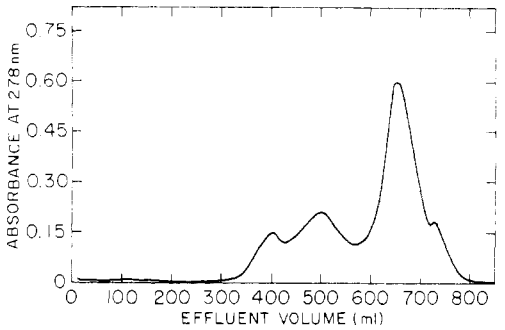

FIGURE 1: Elution pattern from chromatography on a CM-cellulose column (4 × 60 cm) with urea–acetate buffer (pH 4.0) of a crude synthetic S-sulfonated human des(tetrapeptide B^{1-4}) B chain obtained by liquid hydrogen fluoride deblocking of the protected hexacosapeptide followed by oxidative sulfitolysis, desalting, and lyophilization. From the effluent (590–720 mL), 45 mg of purified des(tetrapeptide B^{1-4}) B chain *S*-sulfonate was obtained.

L-tyrosyl-L-leucyl-L-valyl-S-sulfo-L-cysteinylglycyl-L-glutamyl-L-arginylglycyl-L-phenylalanyl-L-phenylalanyl-L-tyrosyl-L-threonyl-L-prolyl-L-lysyl-L-threonine [Des(tetrapeptide B^{1-4}) B Chain S-Sulfonate] (III). A solution of the protected docosapeptide *N-tert*-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^{ω}-nitro-L-arginylglycyl-L-phenylalanyl-L-phenylalanyl-*O*-benzyl-L-tyrosyl-L-threonyl-L-prolyl-N^{ϵ}-benzyloxycarbonyl-L-lysyl-*O*-benzyl-L-threonine benzyl ester (Schwartz and Katsoyannis, 1973b) (0.4 g) 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 Me_2Fam (25 mL). This solution, cooled to 0 °C, was diluted with 1 N NH_4OH (5 mL) and immediately poured into ice-cold saturated aqueous sodium chloride (300 mL). The pH of the mixture was adjusted to 9.5 with 1 N NH_4OH, and the precipitated free base of the docosapeptide was filtered off, washed successively with water, 2-propanol, and petroleum ether, and dried. This product was dissolved in hexamethylphosphoramide (6 mL) and to this solution, cooled to 4°C, the tetrapeptide (II) was added which was activated as follows. To a solution of the tetrapeptide derivative (II) (1.1 g) in Me_2Fam (6 mL), cooled to 4 °C, 1-hydroxybenzotriazole (0.2 g) and *N,N'*-dicyclohexylcarbodiimide (0.26 g) were added. The mixture was stirred for 1 h at 4 °C and for 1 h at room temperature and then added to the solution of the free base of the docosapeptide prepared as described above. After 48 h at room temperature, the reaction mixture was poured into cold water (500 mL) containing 1 N NH_4OH (10 mL). The precipitated crude hexacosapeptide derivative (protected B chain analogue) was isolated by centrifugation, washed successively with water, 50% aqueous methanol, absolute methanol, and ether, and reprecipitated from a solution in hexamethylphosphoramide–dimethylformamide (20 mL; 1:1) by the addition of methanol, wt 0.33 g. An acid hydrolysate of this material showed the following composition in molar ratios: $Lys_{1.2}His_{2.0}Arg_{0.8}Thr_{2.0}Ser_{1.1}Glu_{2.0}Pro_{1.2}Gly_{3.4}Ala_{1.0}Cys_{1.3}Val_{1.8}Leu_{3.9}Tyr_{0.8}Phe_{2.1}$.

This material was converted to the S-sulfonated des(tetrapeptide B^{1-4}) B chain by deblocking with liquid hydrogen fluoride followed by oxidative sulfitolysis as described previously (Schwartz and Katsoyannis, 1973b). Briefly, the thoroughly dried protected crude B chain analogue (200 mg) was placed in a Diaflow reaction vessel of a hydrogen fluoride apparatus (Toho Co., Japan) and treated with dry hydrogen fluoride (9 mL) and anisole (1 mL) at 10 °C for 1 h. After evaporation of the hydrogen fluoride, the residue was dried in high vacuum (over KOH) for 24 h and triturated with ethyl acetate. The reduced product was dissolved in 8 M guanidine hydrochloride (20 mL), and to this solution, adjusted to pH 8.9 (dilute NH_4OH), were added sodium sulfite (1.2 g) and freshly prepared sodium tetrathionate (0.7 g). After stirring at room temperature for 3 h, the mixture was placed in an 18/32 Visking dialysis tubing and dialyzed against four changes of distilled water (4-L each) at 4 °C for 24 h. Lyophilization of the dialysate gave the crude des(tetrapeptide B^{1-4}) B chain S-sulfonate as a white powder. For purification, this material was dissolved in urea–acetate buffer (6 mL) [0.04 M sodium acetate, 8 M urea (pH 4.0)] and placed on a CM-cellulose column (4 × 60 cm) equilibrated and eluted with the same buffer (Katsoyannis et al., 1967a,b). The chromatographic pattern obtained by monitoring the effluent with a Gilford recording spectrophotometer (Figure 1) indicated the presence of one major component, the des(tetrapeptide B^{1-4}) B chain *S*-sulfonate, and only small amounts of other components. The effluent under the major peak (590–720 mL) was collected and desalted using the hollow-fiber technique as described previously (Schwartz and Katsoyannis, 1976). Lyophilization of the desalted solution afforded the S-sulfonated des(tetrapeptide B^{1-4}) B chain as a white fluffy material, wt 45 mg.

TABLE I: Amino Acid Composition[a] of an Acid Hydrolysate and an Enzymatic Digest (Aminopeptidase M) of the S-Sulfonated Des(tetrapeptide B^{1-4}) B Chain of Human Insulin.

amino acid	acid hydrolysis		enzymatic hydrolysis	
	theory	found	theory	found
Lys	1	1.0	1	1.2
His	2	2.0	2	1.9
Arg	1	1.0	1	1.0
Thr	2	2.0	2	2.1
Ser	1	1.0	1	1.2
Glu	2	1.9	2	1.8
Pro	1	0.9	1	0.9
Gly	3	3.0	3	2.9
Ala	1	1.1	1	1.0
½-Cys	2	1.2[b]	0	0
Val	2	1.9	2	2.0
Leu	4	4.0	4	4.1
Tyr	2	1.6[b]	2	1.9
Phe	2	2.0	2	2.2
S-sulfoCys	0	0	2	2.1[c]

[a] Number of amino acid residues per molecule. [b] Uncorrected for destruction. [c] Eluted from the long column of the Beckman–Spinco analyzer after 26 mL of effluent.

Amino acid analysis after acid hydrolysis gave the molar ratios of amino acids shown in Table I in good agreement with the theoretically expected values. Digestion of the synthetic material with aminopeptidase M and amino acid analysis of the digest gave the amino acid ratios shown in Table I in agreement with the theoretically expected values. On high-voltage thin-layer electrophoresis in 0.01 M ammonium hydrogen carbonate (adjusted to pH 10 with NH_4OH) at 2500 V, the synthetic chain moved as a single component (Pauly reaction) (Figure 2).

L-Leucyl-S-sulfo-L-cysteinylglycyl-L-seryl-L-histidyl-L-leucyl-L-valyl-L-glutamyl-L-alanyl-L-leucyl-L-tyrosyl-L-leucyl-L-valyl-S-sulfo-L-cysteinylglycyl-L-glutamyl-L-arginylglycyl-L-phenylalanyl-L-phenylalanyl-L-tyrosyl-L-threonyl-L-prolyl-L-lysyl-L-threonine [Des(pentapeptide B^{1-5}) B Chain S-Sulfonate] (IV). The protected docosapep-

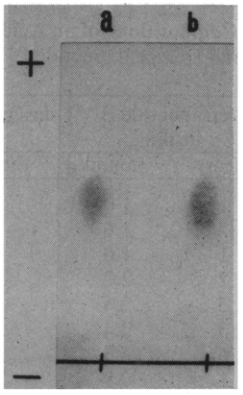

FIGURE 2: High-voltage thin-layer electrophoresis of the *S*-sulfonated derivatives of synthetic human des(tetrapeptide B^{1-4}) B chain (a) and natural porcine B chain (b); 0.01 M NH_4HCO_3 adjusted to pH 10.0 with NH_4OH (2500 V, 25 min).

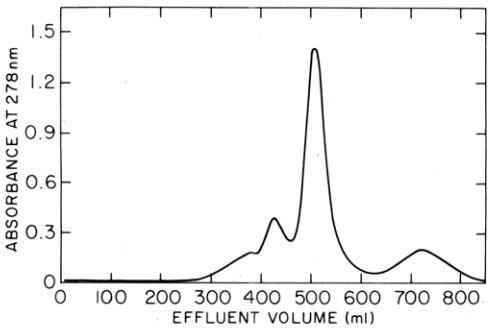

FIGURE 3: Elution pattern from chromatography on a CM-cellulose column (4 × 60 cm) with urea–acetate buffer (pH 4.0) of a crude synthetic S-sulfonated human des(pentapeptide B^{1-5}) B chain obtained by liquid hydrogen fluoride deblocking of the protected pentacosapeptide followed by oxidative sulfitolysis, desalting, and lyophilization. From the effluent (460–600 mL), 55 mg of purified des(pentapeptide B^{1-5}) B chain *S*-sulfonate was obtained.

tide (used also for the synthesis of compound III) (0.4 g) was deblocked and converted to the free base as described above. To a solution of this product in hexamethylphosphoramide (6 mL), cooled to 4 °C, the tripeptide (I) was added, which was activated as follows. The tripeptide derivative (I) (1.1 g) was dissolved in Me_2Fam (6 mL) and to this solution, cooled to 4 °C, 1-hydroxybenzotriazole (0.3 g) and *N,N'*-dicyclohexylcarbodiimide (0.4 g) were added. After stirring for 1 h at 4 °C and at room temperature 1 h, the solution of the activated tripeptide was added to the solution of the docosapeptide prepared as described above. The reaction mixture was stirred for 48 h at room temperature and then diluted with cold water (500 mL) containing 1 N NH_4OH (10 mL). The precipitated crude pentacosapeptide derivative (protected B-chain analogue) was collected by centrifugation, washed successively with water, 50% aqueous methanol, absolute methanol, and ether, and reprecipitated from a solution in Me_2Fam (20 mL) by the addition of methanol, wt 0.3 g. Amino acid analysis of this product after acid hydrolysis gave the following ratios: $Lys_{1.1}His_{0.9}Arg_{0.7}Thr_{2.0}Ser_{1.0}Glu_{2.0}Pro_{1.1}Gly_{3.3}Ala_{1.0}$-$Cys_{1.5}Val_{1.8}Leu_{3.7}Tyr_{0.6}Phe_{2.0}$.

Deblocking of this material (200 mg) by liquid hydrogen fluoride and oxidative sulfitolysis of the reduced product was carried out in exactly the same way as described in the synthesis of compound III. Purification of the crude des(pentapeptide B^{1-5}) B chain *S*-sulfonate was performed on a CM-cellulose column (4 × 60 cm) as described in the purification of III. The peptide material in the column eluate was monitored continuously with a Gilford recording spectrophotometer

TABLE II: Amino Acid Composition[a] of an Acid Hydrolysate and an Enzymatic Digest (Aminopeptidase M) of the S-Sulfonated Des(pentapeptide B^{1-5}) B Chain of Human Insulin.

	acid hydrolysis		enzymatic hydrolysis	
amino acid	theory	found	theory	found
Lys	1	1.0	1	1.2
His	1	1.0	1	0.9
Arg	1	1.0	1	1.0
Thr	2	1.9	2	2.1
Ser	1	1.1	1	1.0
Glu	2	2.0	2	1.8
Pro	1	1.0	1	0.9
Gly	3	3.2	3	3.0
Ala	1	1.1	1	1.0
½-Cys	2	1.4[b]	0	0
Val	2	2.0	2	2.0
Leu	4	3.7	4	4.3
Tyr	2	1.7[b]	2	1.8
Phe	2	2.0	2	2.0
S-sulfoCys	0	0	2	2.1[c]

[a] Number of amino acid residues per molecule. [b] Uncorrected for destruction. [c] Eluted from the long column of the Beckman–Spinco analyzer after 26 mL of effluent.

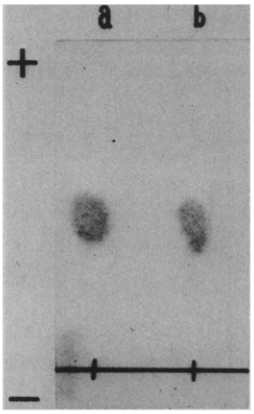

FIGURE 4: High-voltage thin-layer electrophoresis of the S-sulfonated derivatives of synthetic human des(pentapeptide B^{1-5}) B chain (a) and natural porcine B chain (b); 0.01 M NH_4HCO_3 adjusted to pH 10.0 with NH_4OH (2500 V, 25 min).

at 278 nm. The chromatographic pattern obtained is shown in Figure 3. The eluate under the major peak (460–600 mL) was collected and desalted using the hollow-fiber technique. Lyophilization of the desalted solution yielded the S-sulfonated des(pentapeptide B^{1-5}) B chain as white fluffy material, wt 55 mg.

Amino acid analysis of the purified material after acid hydrolysis gave a composition expressed in molar ratios in good agreement with the theoretically expected values (Table II). The synthetic material was completely digested by aminopeptidase M (Table II). On high-voltage thin-layer electrophoresis in 0.01 M ammonium hydrogen carbonate (adjusted to pH 10.0 with NH_4OH) and 2500 V, the synthetic-chain analogue moved as a single component (Pauly reaction) (Figure 4).

S-Sulfonated Derivatives of A and B Chains. These compounds were prepared by oxidative sulfitolysis of porcine insulin followed by separation of the resulting S-sulfonated chains by CM-cellulose chromatography as described previously (Katsoyannis et al., 1967a).

Synthesis and Isolation of Des(tetrapeptide B^{1-4}) Human Insulin. This analogue was synthesized by the interaction of the thiol form of the A chain of human (porcine) insulin with

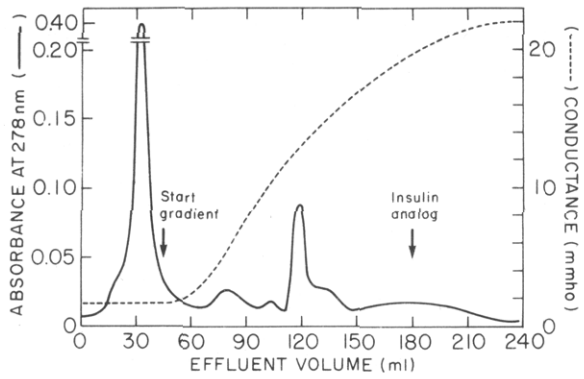

FIGURE 5: Chromatography of a combination mixture (see Experimental Procedures and Results) of synthetic human des(tetrapeptide B^{1-4}) B chain *S*-sulfonate with the thiol form of human (porcine) A chain on a CM-cellulose column (0.9 × 23 cm) with acetate buffer (pH 3.3; $[Na^+]$ 0.024 M) and an exponential sodium chloride gradient. The column eluate was monitored by a Gilford recording spectrophotometer and by a conductivity meter (Radiometer, Copenhagen). The purified human des(tetrapeptide B^{1-4})-insulin (150–220 mL of eluate) was recovered via picrate as the hydrochloride (1.4 mg).

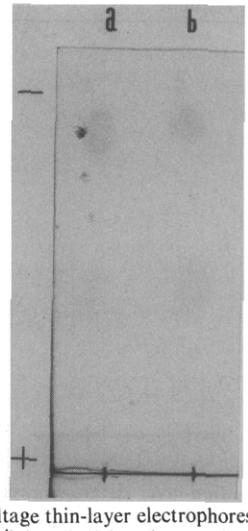

FIGURE 6: High-voltage thin-layer electrophoresis of a synthetic human des(tetrapeptide B^{1-4})-insulin (a) and natural porcine insulin (b); 0.5 N acetic acid, 3400 V, and 15 min.

the S-sulfonated form of the des(tetrapeptide B^{1-4}) B chain using the procedure that we have reported recently (Schwartz and Katsoyannis, 1976). Briefly, a solution of 20 mg of human (porcine) A chain S-sulfonate in 0.1 M Tris-HCl buffer (pH 8.3) was treated with 2-mercaptoethanol at 37 °C for 6 min under nitrogen. After cooling to 4 °C, the reaction mixture was diluted with acetic acid–2-propanol–ethyl acetate (1:30:50), and the precipitated thiol form of the A chain was isolated by centrifugation, washed successively with ethyl acetate and petroleum ether, and dried. This product was then allowed to react with 10 mg of des(tetrapeptide B^{1-4}) B chain *S*-sulfonate for 16 h at pH 10.6 and 4 °C. The combination mixture was subsequently treated as described previously (Katsoyannis et al., 1967b,c). Isolation of the insulin analogue from the combination mixture and purification were carried out by chromatography on a 0.9 × 23 cm CM-cellulose column with an acetate buffer (Na^+ 0.024 M, pH 3.3) and an exponential NaCl gradient, as was described previously (Katsoyannis et al., 1967b,c). The chromatographic pattern obtained is shown in Figure 5. The insulin analogue was eluted with application of the gradient and was the slowest moving component. The effluent containing the insulin analogue (150–200 mL) was processed as we have described previously (Katsoyannis et al., 1967c), and the human des(tetrapeptide B^{1-4})-insulin was isolated via picrate as the hydrochloride (1.4 mg).

TABLE III: Amino Acid Composition[a] of an Acid Hydrolysate of Des(tetrapeptide B^{1-4}) and Des(pentapeptide B^{1-5}) Human Insulins.

amino acid	des(tetrapeptide B^{1-4}) insulin		des(pentapeptide B^{1-5}) insulin	
	theory	found	theory	found
Lys	1	1.0	1	1.1
His	2	1.6	1	0.8
Arg	1	1.0	1	0.9
Asp	2	2.2	2	2.0
Thr	3	2.8	3	2.4
Ser	3	2.4	3	2.8
Glu	6	6.3	6	6.4
Pro	1	0.9	1	0.9
Gly	4	4.1	4	3.9
Ala	1	1.2	1	1.0
½-Cys	6	3.1[b]	6	3.2[b]
Val	3	2.7	3	2.6
Ile	2	1.2	2	1.2
Leu	6	5.5	6	5.7
Tyr	4	2.9[b]	4	2.8[b]
Phe	2	2.1	2	1.9

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

Amino acid analysis of this analogue after acid hydrolysis gave a composition expressed in molar ratios, shown in Table III, in agreement with theoretically expected values. On thin-layer electrophoresis in 0.5 N acetic acid and 3400 V, the synthetic analogue moved as a single component (Pauly reaction) (Figure 6). The des(tetrapeptide B^{1-4}) human insulin, by the mouse convulsion assay method, was found to possess a potency of 13 IU/mg and, by radioimmunoassay, had a potency of 7.6 IU/mg (ca. 54 and 32%, respectively, of that of the natural hormone).

Synthesis and Isolation of Des(pentapeptide B^{1-5}) Human Insulin. The synthesis of this analogue by the interaction of the thiol form of human (porcine) A chain with the S-sulfonated form of human des(pentapeptide B^{1-4}) B chain and its purification were accomplished by exactly the same procedure outlined above in the synthesis of des(tetrapeptide B^{1-4}) human insulin. Chromatography of two combination mixtures, each corresponding to 20 mg of human (porcine) A chain *S*-sulfonate and 10 mg of des(pentapeptide B^{1-5}) human B chain S-sulfonate, gave the pattern shown in Figure 7. The human des(pentapeptide B^{1-5})-insulin was eluted with application of the gradient and was isolated from the effluent (116–150 mL) via picrate as the hydrochloride (0.650 mg).

Amino acid analysis of this analogue after acid hydrolysis gave a composition expressed in molar ratios, shown in Table III, in agreement with the theoretically expected values. The synthetic analogue was homogeneous on thin-layer electrophoresis (0.5 N acetic acid, 3400 V) (Figure 8). The human des(pentapeptide B^{1-5})-insulin showed a potency of 1.2 IU/mg (ca. 5% of that of insulin) when assayed by the glucose-oxidation method in isolated fat cells; the radioimmunoassay method gave a value of 3.7 IU/mg, ca. 15% of that of the natural hormone.

Discussion

In the amino-terminal region of the B chain of insulin from several mammalian species, the sequence (B^1–B^7) phenylalanyl-valyl-asparaginyl-glutaminyl-histidyl-leucyl-cysteinyl is present (Smith, 1966). A considerable sequence variation was found, however, in the B^1–B^4 portion of this segment of

TABLE IV: Comparison of Amino Acid Sequences in the Amino-Terminal Region of the B Chain of Insulins.[a]

insulin species	B chain 0	1	2	3	4	5	6	7	references
human		Phe	Val	Asn	Gln	His	Leu	Cys-	(Nicol and Smith, 1960)
bovine		Phe	Val	Asn	Gln	His	Leu	Cys-	(Sanger and Tuppy, 1951a,b)
elephant		Phe	Val	Asn	Gln	His	Leu	Cys-	(Smith, 1966)
guinea pig		Phe	Val	Ser	Arg	His	Leu	Cys-	(Smith, 1966)
chicken		Ala	Ala	Asn	Gln	His	Leu	Cys-	(Smith, 1966)
cod	Met	Ala	Pro	Pro	Gln	His	Leu	Cys-	(Reid et al., 1968)
angler fish	Val	Ala	Pro	Ala	Gln	His	Leu	Cys-	(Smith, 1966)
rat, mouse (I,II)[b]		Phe	Val	Lys	Gln	His	Leu	Cys-	(Smith, 1966; Markussen, 1971)
hagfish		Arg	Thr	Thr	Gly	His	Leu	Cys-	(Peterson et al., 1975)

[a] In several of the insulins, variations also occur in positions beyond position 7. [b] Two different insulins, I and II, have been found in the rat and mouse.

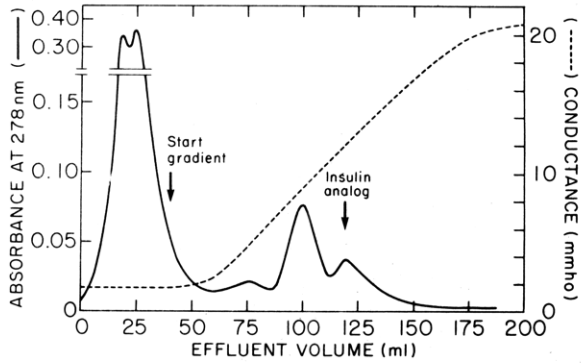

FIGURE 7: Chromatography of two combination mixtures (see Experimental Procedures and Results) of synthetic human des(pentapeptide B^{1-5}) B chain *S*-sulfonate with the thiol form of human (porcine) A chain on a CM-cellulose column (0.9 × 23 cm) with acetate buffer (pH 3.3; [Na^+] 0.024 M) and an exponential sodium chloride gradient. The eluate was monitored by a Gilford recording spectrophotometer and by a conductivity meter. The purified human des(pentapeptide B^{1-5})-insulin (116–150 mL of eluate) was recovered via picrate as the hydrochloride (0.65 mg).

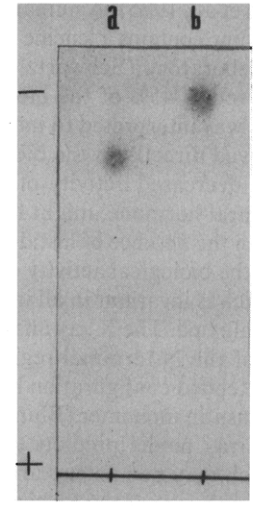

FIGURE 8: High-voltage thin-layer electrophoresis of synthetic human des(pentapeptide B^{1-5})-insulin (a) and natural porcine insulin (b); 0.5 N acetic acid, 3400 V, and 15 min.

the B chain of insulin in several other species (Table IV). This sequential variation implies that the B^1–B^4 segment of the B chain may not be essential in the expression of the biological activity of insulin. The predicted noninvolvement of these N-terminal residues of the B chain in the expression of the biological activity of insulin is consistent with the findings of the X-ray analysis of the three-dimensional structure of this protein (Blundell, et al., 1972a). The elegant studies of Hodgkin and her co-workers have indeed shown that the N-terminal segment of the B chain is on the surface of the monomeric and dimeric forms of insulin. Furthermore, this segment, from position B^7 (which is the position of the interchain disulfide bridge A^7–B^7), is folded across and forms an extended chain lying in an antiparallel fashion to the central portion of the A chain moiety of the molecule. In this configuration, the first three residues (sequence B^1–B^3) do not appear to be involved in stabilizing interactions that might be consequential to the tertiary structure of the protein and hence to its biological activity (Blundell et al., 1971, 1972a). However, hydrogen-bond contacts appear to be established between the B^4 (Gln) and A^{11} (Cys) residues and between the imidazole nitrogen of the B^5 histidine and the carbonyl oxygen of the A^7 cysteine.

An unequivocal evaluation of the contribution of the individual amino acid residues at the amino terminus of the B chain to the biological activity of insulin can be provided by the synthesis of analogues with modifications of these residues. Des(B^1) (Brandenburg, 1969; Geiger and Langner, 1973) and des(dipeptide B^{1-2}) (Geiger and Langner, 1973) insulins have been prepared in pure form by a modification of the natural hormone and found to possess the full activity of insulin. This proves that the B^1 and B^2 amino acid residues are not necessary for the biological activity of the hormone. The des(tripeptide B^{1-3})-insulin which was prepared by a modification of the natural hormone (Geiger and Langner, 1973) has the newly formed N terminus of the B chain, glutamine (B^4) cyclized to pyroglutamic acid. The high biological activity, however, of this modified analogue, ca. 70% of that of insulin, suggests that the B^3 residue also is not important for the biological activity of the hormone.

In the present report, we describe the synthesis, isolation in pure form, and biological activity of des(tetrapeptide B^{1-4}) human insulin. This permitted us to evaluate the significance of the N-terminal tetrapeptide sequence of the B chain of insulin in the expression of the biological activity of the hormone. Furthermore, our data, in conjunction with the biological evaluation of the des(B^1)-, des(dipeptide B^{1-2})-, and des(tripeptide B^{1-3})-[pyroglutamic acid-B^4]-insulin, allowed us to assess the role of the B^4 residue, glutamine, in the manifestation of the biological profile of the hormone. By the mouse convulsion assay method, the des(tetrapeptide B^{1-4})-insulin was found to possess a specific activity of 13 IU/mg, ca. 54% of that of the natural hormone (23–25 IU/mg); the radioimmunoassay method gave a value of 7.6 IU/mg, ca. 32% of that of the

natural protein. This indicates that the B^4 (Gln), as is the case with the B^1 (Phe), B^2 (Val), and B^3 (Asn), is not involved directly in the expression of the biological activity of the hormone. The decrease in the biological activity of des(tetrapeptide B^{1-4})-insulin as compared to the des(B^1)- and des(dipeptide B^{1-2})-insulin might be due to the elimination of the hydrogen bond between the B^4 and A^{11} residues (Blundell et al., 1972) with consequent small changes in the tertiary structure and hence reduction in the biological activity of the hormone.

In the structure of insulin from various species, histidine residues occupy positions 5 and 10 in the B chain. X-ray analysis suggests (Blundell et al., 1972a) that the B^{10} histidine is important for the formation of stable zinc–insulin hexamers. Guinea pig (Smith, 1966), coypu (Smith, 1972), and hagfish (Peterson et al., 1975) insulins which do not contain a histidine at B^{10} do not form stable zinc–insulin hexamers (Blundell et al., 1972a,b; Peterson et al., 1975). A human insulin analogue, which instead of histidine contains a leucine residue at B^{10}, was synthesized in our laboratory (Schwartz and Katsoyannis, 1977) and found to retain 45% of the biological activity of natural insulin. This was interpreted to indicate that the B^{10} histidine is not involved directly in the biological activity of insulin and that the decreased activity of this analogue, as compared to the natural hormone, might be due to a change in polarity rather than the absence of histidine at position B^{10}. The significance for the biological activity of insulin of the B^5 histidine residue, which is invariant in all insulin species, had, however, not been evaluated. The X-ray analysis indicates that this residue is part of the N-terminal region of the B chain which assumes an extended configuration lying against the A chain moiety of the insulin monomer (Blundell et al., 1972a). Furthermore, the X-ray model predicts the formation of a hydrogen bond, which may be of structural importance, between the imidazole nitrogen of the B^5 histidine and the carbonyl group of the A^7 cysteine. The present investigation has permitted us to establish the crucial role of the B^5 histidine residue and supports the X-ray model prediction (Blundell et al., 1972a) that this residue is involved in intramolecular interactions of structural importance. This was achieved by the synthesis, isolation in pure form, and biological evaluation of an insulin analogue lacking the N-terminal pentapeptide sequence from the B chain moiety of the parent molecule. The synthetic des(pentapeptide B^{1-5}) human insulin was found to possess a potency of 1.2 IU/mg by the glucose-oxidation method in isolated fat cells, ca. 5% of that of the natural hormone (23–25 IU/mg); the radioimmunoassay method gave a value of 3.7 IU/mg, ca. 15% of that of the natural hormone. These findings indicate that the B^5 histidine residue, in contrast to the B^{10} residue, is critically involved in interactions which confer on the insulin molecule topochemical features commensurate with high biological activity. Interestingly, a comparison of the yields of the synthetic des(tetrapeptide B^{1-4})- and des(pentapeptide B^{1-5})-insulin produced by combination of the respective chains (see Experimental Section) shows that the yield of the former analogue is about four times higher than that of the latter. It is tempting to speculate that the histidine residue at position B^5 is so involved in interchain interactions as to dispose the A and B chains for a more efficient combination.

Acknowledgments

The authors express their appreciation to Dr. Kang Cheng and Mrs. Mei-Ying Liu for the biological assays and to Mrs. Edith Li for the radioimmunoassays and the amino acid and enzymatic analyses.

References

Anderson, G. W., Zimmerman, J. E., and Callahan, F. M. (1964), *J. Am. Chem. Soc. 86,* 1839.

Blundell, T. L., Dodson, G. G., Dodson, E. J., Hodgkin, D. C., and Vijayan, M. (1971), *Recent Prog. Horm. Res. 27,* 1.

Blundell, T. L., Dodson, G. G., Hodgkin, D. C., and Mercola, D. (1972a), *Adv. Protein Chem. 26,* 279.

Blundell, T. L., Cutfield, J. F., Cutfield, S. M., Dodson, E. J., Dodson, G. G., Hodgkin, D. C., and Mercola, D. (1972b), *Diabetes Suppl. 2,* 492.

Brandenburg, D. (1969), *Hoppe-Seyler's Z. Physiol. Chem. 350,* 741.

Geiger, R., and Langner, D. (1973), *Hoppe-Seyler's Z. Physiol. Chem. 354,* 1285.

Geiger, R., and Langner, D. (1975), in *Pept. Proc. Eur. Pept. Symp., 14th, 1974,* 173.

Gilman, A., Philips, F. S., and Koelle, E. S. (1946), *Am. J. Physiol. 146,* 348.

Hales, C. N., and Randle, P. J. (1963), *Biochem. J. 88,* 137.

Hiskey, R. G., Beacham, L. M., III, Matl, V. G., Smith, J. N., Williams, E. B., Jr., Thomas, A. M., and Wolters, E. G. (1971), *J. Org. Chem. 36,* 488.

Katsoyannis, P. G., and Zalut, C. (1972), *Biochemistry 11,* 3065.

Katsoyannis, P. G., Tometsko, A., Zalut, C., Johnson, S., and Trakatellis, A. C. (1967a), *Biochemistry 6,* 2635.

Katsoyannis, P. G., Trakatellis, A. C., Johnson, S., Zalut, C., and Schwartz, G. (1967b), *Biochemistry 6,* 2642.

Katsoyannis, P. G., Trakatellis, A. C., Zalut, C., Johnson, S., Tometsko, A., Schwartz, G., and Ginos, J. (1967c), *Biochemistry 6,* 2656.

Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951), *J. Biol. Chem. 193,* 265.

Markussen, J. (1971), *Int. J. Protein Res. 3,* 149.

Nicol, D. S. H. W., and Smith, L. F. (1960), *Nature (London) 187,* 483.

Peterson, J. D., Steiner, D. F., Emdin, S. D., and Falkmer, S. (1975), *J. Biol. Chem. 250,* 5183.

Pfleiderer, G., Celliers, P. G., Stanulovic, M., Wachsmuth, E. D., Determann, H., and Braunitzer, G. (1964), *Biochem. Z. 340,* 552.

Reid, K. B. M., Grant, P. T., and Youngson, A. (1968), *Biochem. J. 110,* 289.

Rodbell, M. (1964), *J. Biol. Chem. 239,* 375.

Sanger, F., and Tuppy, H. (1951a), *Biochem. J. 49,* 463.

Sanger, F., and Tuppy, H. (1951b), *Biochem. J. 49,* 481.

Saunders, D. J., and Offord, R. (1977), *Biochem. J. 165,* 479.

Schwartz, G., and Katsoyannis, P. G. (1973a), *J. Chem. Soc., Perkin Trans. 1,* 2890.

Schwartz, G., and Katsoyannis, P. G. (1973b), *J. Chem. Soc., Perkin Trans. 1,* 2894.

Schwartz, G., and Katsoyannis, P. G. (1976), *Biochemistry 15,* 4071.

Schwartz, G., and Katsoyannis, P. G. (1977), *J. Chem. Res. (S),* 220.

Schwartz, G., and Katsoyannis, P. G. (1977), *J. Chem. Res. (M),* 2453.

Smith, L. F. (1966), *Am. J. Med. 40,* 662.

Smith, L. F. (1972), *Diabetes 21, Suppl. 2,* 457–460.

Spackman, D. H., Stein, W. H., and Moore, S. (1958), *Anal. Chem. 30,* 1190.

Tometsko, A., and Delihas, N. (1967), *Anal. Biochem. 18,* 272.