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Synthesis of Two Biologically Active Insulin Analogues with Modifications at the N-Terminal and N- and C-Terminal Amino Acid Residues†

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ABSTRACT: The synthesis and isolation in purified form of two analogues of insulin is described. [21-Isoasparagine-A] ([Iasn^{21}-A]) insulin differs from the parent molecule in that the amino acid residue, asparagine, found at the C terminus of the A chain (A^{21}) has been replaced by isoasparagine. [Sar^1,Iasn^{21}-A]insulin differs from insulin in that both the A^1 and A^{21} amino acid residues, glycine and asparagine, have been substituted by sarcosine and isoasparagine, respectively. The synthesis of these analogues followed the pattern employed in this laboratory for the synthesis of insulin and its analogues. The S-sulfonated derivatives of the A chain analogues were chemically synthesized, converted to their sulfhydryl forms, and then combined with the S-sulfonated B chain to produce the respective insulin analogues. Isolation of the insulin analogues from the combination mixtures was effected by chromatography on a carboxymethylcellulose column with an exponential sodium chloride gradient. By the mouse convulsion assay method [Iasn^{21}-A]insulin possessed a potency of 21 IU/mg and [Sar^1,Iasn^{21}-A]insulin 15 IU/mg. The radioimmunoassay method gave values of 16 IU/mg for the former and 7 IU/mg for the latter analogue. The natural hormone has a potency of 23–25 IU/mg by both assay methods. These data indicate that the α- and β-carboxyl groups of the A^{21} amino acid residue are nearly equivalent in terms of their contribution to the expression of the biological activity of insulin. Furthermore, these data strengthen the speculation (Cosmatos, A., Johnson, S., Breier, B., and Katsoyannis, P. G. (1975), *J. Chem. Soc. Perkin Trans. 1,* 2157) that the change in the relative positive charge at the N-terminal amino acid residue of the A chain is responsible for the considerable decrease in the immunoreactivity observed in such modified insulins.

A program has been initiated in this laboratory directed to the synthesis of insulin analogues in an attempt to understand the relationship between chemical structure, biological activity, and immunoreactivity of this hormone. One aspect of these studies has been an attempt to elucidate the role of the amino acid residues found at the amino- and carboxyl-terminal positions of the A chain (A^1 and A^{21}, respectively) in the expression of the biological activity of insulin. We have found that replacement of the α-amino group of the A^1 residue by hydrogen results in a substantial loss (ca. 65%) of the biological activity of the hormone (Katsoyannis and Zalut, 1972). However, substitution of one hydrogen of the α-amino group of the same residue by a methyl group results in a small decrease of the biological activity (ca. 17%) and a pronounced decrease (ca. 63%) of the immunoreactivity of insulin (Okada and Katsoyannis, 1975). On the other hand, substitution of the A^{21} residue, L-asparagine, by its optical isomer (Cosmatos et al., 1975) results in a substantial loss of the biological potency and immunoreactivity of insulin (ca. 67 and 82%, respectively). In the present communication, we describe the synthesis and biological evaluation of two insulin analogues with modifications either at the A^1 position or at both A^1 and A^{21} positions.

Experimental Procedures and Results

Materials and Techniques. Details of the materials and techniques used are given in the preceding paper of this issue (Schwartz and Katsoyannis, 1976). 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 all intermediate peptide derivatives, deblocked at the amino end, was ascertained by thin-layer chromatography (whenever solubility properties allowed it) on 6060 silica gel (Eastman Chromagram Sheet, Eastman Kodak Co.,

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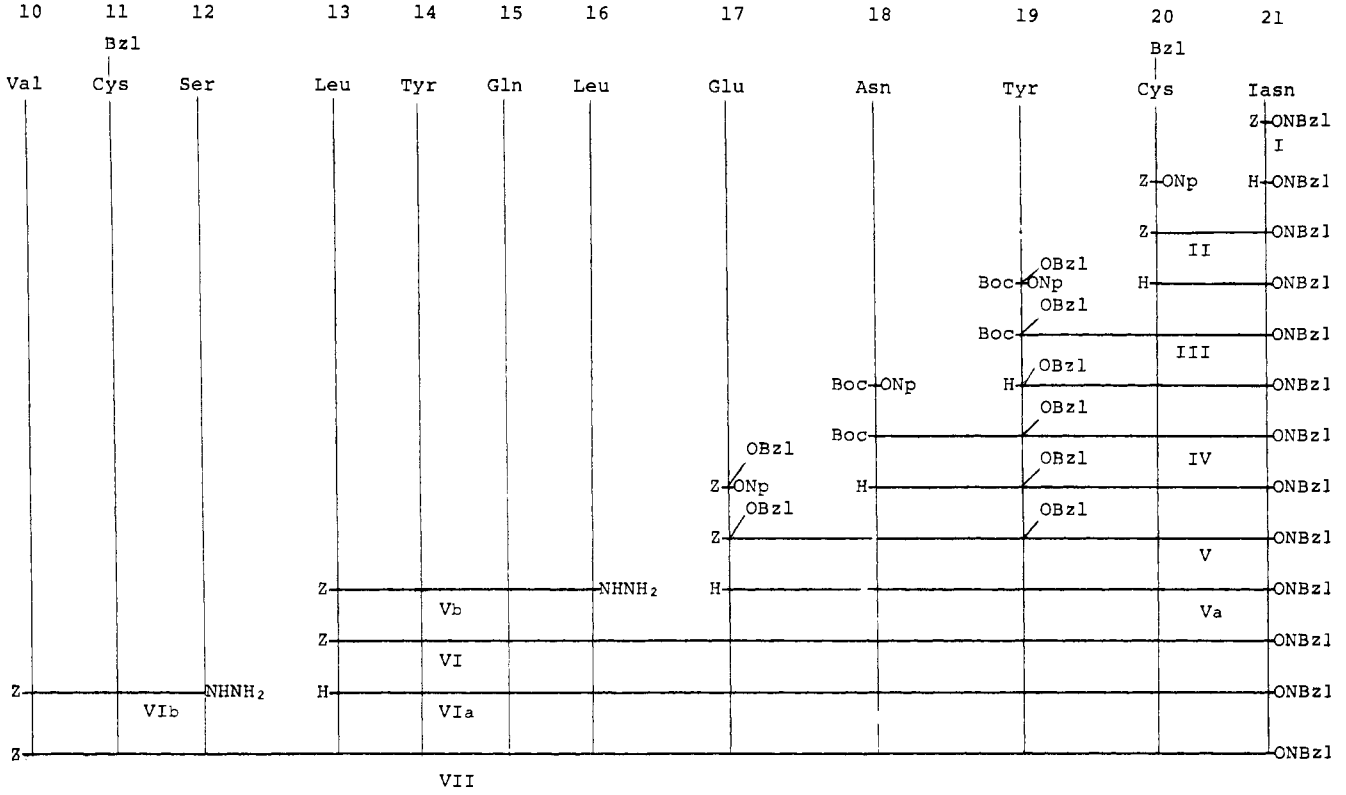
CHART I



Rochester, N.Y.). The solvent systems used were 1-butanol-pyridine-acetic acid-water (4:1:1:2) and 1-butanol-pyridine-acetic acid-water (30:20:6:24). For the enzymatic digestion with leucine aminopeptidase, the method of Hill and Smith (1957) was employed with a chromatographically purified enzyme from Worthington Biochemical Corp., Freehold, N.J. Crystalline bovine insulin was generously provided by Eli Lilly and Co.

General Aspects of the Synthesis of Sheep [Iasn^{21}-A]- and [Sar^{1},Iasn^{21}-A]Insulins.[1] These analogues were prepared by interaction of the S-sulfonated bovine (sheep) B chain with the sulfhydryl form of [Iasn^{21}]A and [Sar^{1},Iasn^{21}]A chains of sheep insulin, respectively. The synthesis of [Iasn^{21}]A and [Sar^{1},Iasn^{21}]A chains in the protected form (IXc and IXd) was achieved by a combination of the stepwise elongation and fragment condensation methods of peptide synthesis (for a review, see Hofmann and Katsoyannis, 1963). The blocking groups from the protected derivatives IXc and IXd were removed upon treatment with sodium in liquid ammonia (Sifferd and du Vigneaud, 1935). The reduced chain analogues on oxidative sulfitolysis (Bailey and Cole, 1959) were converted to the S-sulfonated derivatives (Xa and Xb) that eventually were converted to the sulfhydryl form on exposure to 2-mercaptoethanol. Charts I and II illustrate the overall scheme used for the synthesis of the S-sulfonated [Iasn^{21}]A and [Sar^{1},Iasn^{21}]A chains.

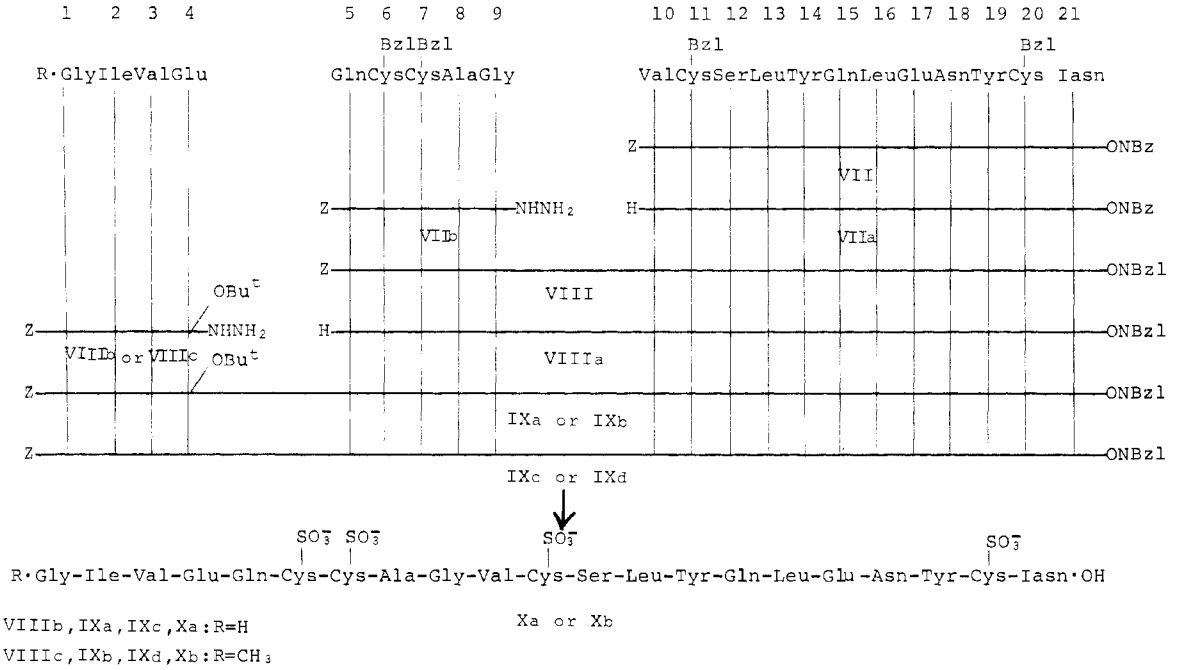
N-Benzyloxycarbonyl-L-isoasparagine p-Nitrobenzyl Ester (I). A solution of *N*-benzyloxycarbonyl-L-isoasparagine (26.7 g; Ressler et al, 1963), *p*-nitrobenzyl chloride (25.7 g), and triethylamine (14 ml) in Me_2Fam (75 ml) was heated at 65 °C for 4 h, cooled to room temperature, and then poured into cold 1 M $KHCO_3$ (750 ml). The precipitate was filtered off, washed with water, triturated with cold methanol (100 ml), and reprecipitated from methanol–water: wt 31.4 g (78%), mp 137–141 °C, $[\alpha]_D^{26}$ −15.4° (c 1, Me_2Fam). Anal. Calcd for $C_{19}H_{19}N_3O_7$: C, 56.9; H, 4.77; N, 10.5. Found: C, 56.9; H, 4.84; N, 10.7.

N-Benzyloxycarbonyl-S-benzyl-L-cysteinyl-L-isoasparagine p-Nitrobenzyl Ester (II). A suspension of compound I (10.1 g) in 2 N HBr in acetic acid (150 ml) was stored at room temperature for 2 h. The resulting solution was poured into ether (400 ml) and the precipitate was filtered, washed with ether, and dried over KOH in vacuo. This material was dissolved in Me_2Fam (75 ml) and to this solution, cooled to 5 °C, was added triethylamine (3.5 ml) followed by *N*-benzyloxycarbonyl-*S*-benzyl-L-cysteine *p*-nitrophenyl ester (12.5 g; Bodanzsky and du Vigneaud, 1959). After 18 h at room temperature, the mixture was diluted with 1 N NH_4OH (5 ml), stirred for 30 min, and mixed with ethyl acetate (750 ml). The organic layer was separated, washed with 1 N NH_4OH, water, 1 N HCl, water, and dried over $MgSO_4$. Upon removal of the solvent under reduced pressure and trituration of the residue with ether, 11.7 g (70%) of crystalline product was obtained that was recrystallized from ethyl acetate: mp 131–135 °C, $[\alpha]_D^{26}$ −35.4° (c 1, Me_2Fam). Anal. Calcd for $C_{29}H_{30}N_4O_8S$: C, 58.6; H, 5.08; N, 9.40. Found: C, 58.0; H, 5.26; N, 9.58.

N-tert-Butyloxycarbonyl-O-benzyl-L-tyrosyl-S-benzyl-L-cysteinyl-L-isoasparagine p-Nitrobenzyl Ester (III). Compound II (8.9 g) was treated with 2 N HBr in acetic acid (25 ml) for 1 h. The resulting solution was poured into ether (500 ml) and the precipitated dipeptide hydrobromide was filtered, washed with ether, and dried in vacuo. Into a cooled (0 °C) solution of this material in Me_2Fam (60 ml) triethylamine (2.1 ml) was added followed by *N-tert*-butyloxycar-

[1] Abbreviations used are: Iasn, isoasparagine; NBzl, *p*-nitrobenzyl; Z, benzyloxycarbonyl; Boc, *tert*-butyloxycarbonyl; Bzl, benzyl; Bu^{t}, *tert*-butyl; Np, *p*-nitrophenyl; Me_2Fam, dimethylformamide; Me_2SO, dimethyl sulfoxide; Sar, sarcosyl; CM, carboxymethyl.

CHART II



bonyl-*O*-benzyl-L-tyrosine *p*-nitrophenyl ester (8.1 g; Zahn et al., 1966). After 24 h, the mixture was diluted with 1 N NH_4OH (3 ml), stirred for 1 h, and poured into 1 N NH_4OH (600 ml; 0 °C). The precipitate was filtered, washed with 1 N NH_4OH, water, 10% citric acid and water, dried, and triturated with cold ethyl acetate (30 ml) and warm methanol (50 ml). Upon reprecipitation from dimethylformamide–methanol (30:350 ml) 9.7 g (79%) of product was obtained; mp 191–194 °C (dec); $[\alpha]_D^{26}$ −18.4° (c 1, Me_2Fam). Anal. Calcd for $C_{42}H_{47}N_5O_{10}S$: C, 62.0; H, 5.82; N, 8.60. Found: C, 62.1; H, 5.95; N, 8.78.

N-tert-Butyloxycarbonyl-L-asparaginyl-O-benzyl-L-tyrosyl-S-benzyl-L-cysteinyl-L-isoasparagine p-Nitrobenzyl Ester (IV). Compound III (40.7 g) was treated with trifluoroacetic acid (70 ml) for 1 h at room temperature. The resulting solution was poured into ether (300 ml) and the precipitate was filtered, washed with ether, and dried over KOH in vacuo. To a solution of this product in Me_2Fam (250 ml), cooled to 0 °C, triethylamine (7 ml) was added followed by *N-tert*-butyloxycarbonyl-L-asparagine *p*-nitrophenyl ester (18.5 g; Marshall and Merrifield, 1965). After 48 h, the mixture was diluted with 1 N NH_4OH (10 ml), stirred for 1 h, and poured into 1 N NH_4OH (2 l). The precipitate was collected, washed with 1 N NH_4OH, water, 10% citric acid, and water, dried, and triturated with ethyl acetate and acetone; wt 37.1 g (80%), mp 195–197 °C (dec), $[\alpha]_D^{26}$ −51.4° (c 1, Me_2Fam). Anal. Calcd for $C_{46}H_{53}N_7O_{12}S$: C, 59.5; H, 5.76; N, 10.6. Found: C, 59.7; H, 5.57; N, 10.5.

N-Benzyloxycarbonyl-γ-benzyl-L-glutamyl-L-asparaginyl-O-benzyl-L-tyrosyl-S-benzyl-L-cysteinyl-L-isoasparagine p-Nitrobenzyl Ester (V). A solution of compound IV (18.6) in trifluoroacetic acid (50 ml) was stored at room temperature for 1 h and then poured into cold ether (250 ml). The precipitate was collected, washed with ether, and dried over P_2O_5 in vacuo. To a solution of this material in a mixture of Me_2Fam (75 ml) and hexamethylphosphoramide (100 ml), triethylamine (3 ml) was added, followed by *N*-benzyloxycarbonyl-γ-benzyl-L-glutamic acid *p*-nitrophenyl ester (8.5 g; Goodman and Steuben, 1959). The mixture was stirred for 48 h, diluted with 1 N NH_4OH (10 ml), stirred for 1 h, and poured into cold 1 N NH_4OH (1.5 l). The precipitate was collected, washed with 1 N NH_4OH, water, 1 N HCl, and water, dried, and triturated with methanol (50 ml); wt 21.3 g (90%), mp 210–232 °C (dec), $[\alpha]_D^{26}$ −39.7° (c 1, Me_2Fam). Anal. Calcd for $C_{61}H_{64}N_8O_{15}S$: C, 62.0; H, 5.46; N, 9.48. Found: C, 61.7; H, 5.57; N, 9.18.

N-Benzyloxycarbonyl-L-leucyl-L-tyrosyl-L-glutaminyl-L-leucyl-L-glutamyl-L-asparaginyl-L-tyrosyl-S-benzyl-L-cysteinyl-L-isoasparagine p-Nitrobenzyl Ester (VI). Compound V (11.8 g) was treated with 2 N HBr in acetic acid (100 ml) for 1.5 h at room temperature. The mixture was concentrated to ca. 50 ml under reduced pressure and diluted with ether (500 ml). The precipitate (Va) was filtered off, washed with ether, and dried over KOH and P_2O_5 in vacuo. A cooled (0 °C) solution of this material in Me_2Fam (50 ml) containing triethylamine (3.4 ml) was mixed with a solution of the tetrapeptide azide prepared as follows: *N*-Benzyloxycarbonyl-L-leucyl-L-tyrosyl-L-glutaminyl-L-leucine hydrazide (Vb) (7.5 g; Katsoyannis et al., 1966a) was dissolved in a mixture of Me_2Fam (50 ml) and 1 N HCl in Me_2Fam (22 ml; prepared by adding concentrated hydrochloric acid to Me_2Fam). This solution was cooled to −10 °C and mixed with isoamyl nitrite (1.48 ml). After stirring for 5 min at −10 °C, the mixture was cooled to −40 °C, neutralized with triethylamine (4.7 ml), and diluted with the solution of the deblocked pentapeptide ester prepared as described previously. The mixture was stirred for 36 h and then poured into a mixture (0 °C) of methanol (400 ml), water (500 ml), and 1 N HCl (1.5 ml). The precipitate was isolated, washed with 50% aqueous methanol, and methanol, and dried; wt 12.4 g (82%), mp 232–237 °C (dec), $[\alpha]_D^{26}$ −49.4° (c 1, Me_2Fam). Anal. Calcd for $C_{73}H_{91}N_{13}O_{21}S$: C, 57.7; H, 6.00; N, 12.0; S, 2.1. Found: C, 57.5; H, 6.25; N, 11.6; S, 2.25. Amino acid analysis of an acid hydrolysate showed the following composition expressed in molar ratios: $Asp_{2.0}Glu_{2.0}Leu_{2.0}Tyr_{1.5}$*S*-benzylcysteine$_{0.8}$.

N-Benzyloxycarbonyl-L-valyl-S-benzyl-L-cysteinyl-L-seryl-L-leucyl-L-tyrosyl-L-glutaminyl-L-leucyl-L-gluta-

myl-L-asparaginyl-L-tyrosyl-S-benzyl-L-cysteinyl-L-isoasparagine p-Nitrobenzyl Ester (VII). Compound VI (9.11 g) was treated with 2 N HBr in acetic acid (150 ml) at room temperature for 2 h. To the resulting solution, ether (400 ml) was added and the precipitate (VIa) was filtered, washed with ether, and dried. This product was dissolved in Me_2Fam (50 ml), cooled to 0 °C, and then neutralized with triethylamine (1 ml) just prior to the mixing with a solution of the tripeptide azide prepared as follows: a solution of *N*-benzyloxycarbonyl-L-valyl-*S*-benzyl-L-cysteinyl-L-serine hydrazide (VIb) (3.6 g; Katsoyannis et al., 1966a) in a mixture of Me_2Fam (30 ml) and 1 N HCl in Me_2Fam (13.2 ml) was cooled to −10 °C, mixed with isoamyl nitrite (0.89 ml), and, after 5 min, was cooled to −40 °C, neutralized with triethylamine (2.8 ml), and mixed with the nonapeptide ester prepared as described previously. After 48 h at 4 °C, the mixture was poured into cold 1 N HCl (1 l) and the precipitated material was filtered, washed with water, and triturated with hot methanol and ether; wt 10.2 g (89%), mp 252 °C (dec), $[\alpha]_D^{26}$ −27.1° (Me_2SO). Anal. Calcd for $C_{91}H_{116}N_{16}O_{25}S_2$: C, 57.6; H, 6.12; N, 11.8; S, 3.38. Found: C, 57.0; H, 6.16; N, 11.8; S, 3.25. Amino acid analysis of an acid hydrolysate gave the following ratios: $Asp_{2.0}Ser_{0.8}Glu_{2.0}Val_{0.9}Leu_{2.1}Tyr_{1.5}$*S*-benzylcysteine$_{1.5}$.

N-Benzyloxycarbonyl-L-glutaminyl-S-benzyl-L-cysteinyl-S-benzyl-L-cysteinyl-L-alanylglycyl-L-valyl-S-benzyl-L-cysteinyl-L-seryl-L-leucyl-L-tyrosyl-L-glutaminyl-L-leucyl-L-glutamyl-L-asparaginyl-L-tyrosyl-S-benzyl-L-cysteinyl-L-asparagine p-Nitrobenzyl Ester (VIII). Compound VII (7.6 g) was dissolved in trifluoroacetic acid (70 ml) containing water (1 ml), and hydrogen bromide was passed through the solution for 45 min at 0 °C and then for 30 min at room temperature. Addition of ether (300 ml) caused the precipitation of the hydrobromide of the partially protected dodecapeptide VIIa, which was collected by filtration, washed with ether, and dried. A solution (0 °C) of this material in a mixture of Me_2Fam (40 ml) and Me_2SO (30 ml) containing triethylamine (0.6 ml) was added to a solution of the pentapeptide azide prepared as follows: to a solution of *N*-benzyloxycarbonyl-L-glutaminyl-*S*-benzyl-L-cysteinyl-*S*-benzyl-L-cysteinyl-L-alanylglycine hydrazide (VIIb) (3.62 g; Katsoyannis et al., 1966c) in a mixture of Me_2Fam (50 ml), Me_2SO (10 ml), and 1 N HCl in Me_2Fam (8.8 ml), cooled to −10 °C, isoamyl nitrite (0.59 ml) was added. The mixture was kept at this temperature for 5 min, cooled to −40 °C, neutralized with triethylamine (1.9 ml), and then diluted with the solution of the deblocked dodecapeptide derivative prepared as described previously. After 48 h at 4 °C, the mixture was poured into methanol (700 ml) containing 1 N HCl (2 ml). The precipitate was isolated by centrifugation, washed (warm methanol and ether), and reprecipitated from a solution in a mixture of Me_2Fam (25 ml) and hexamethylphosphoramide (30 ml) by the addition of ether (600 ml); wt 7.2 g (71%); mp 245 °C (dec), $[\alpha]_D^{26}$ −28.7° (c 1, Me_2SO). Anal. Calcd for $C_{121}H_{154}N_{22}O_{31}S_4$: C, 57.2; H, 6.07; N, 12.1; S, 5.05. Found: C, 57.0; H, 6.28; N, 12.3; S, 5.22. Amino acid analysis of an acid hydrolysate gave the following ratios: $Asp_{1.9}Ser_{0.8}Glu_{3.2}Gly_{1.1}Ala_{1.1}Val_{0.8}Leu_{2.0}Tyr_{1.4}$*S*-benzylcysteine$_{3.8}$.

N-Benzyloxycarbonylglycyl-L-isoleucyl-L-valyl-L-glutamyl-L-glutaminyl-S-benzyl-L-cysteinyl-S-benzyl-L-cysteinyl-L-alanylglycyl-L-valyl-S-benzyl-L-cysteinyl-L-seryl-L-leucyl-L-tyrosyl-L-glutaminyl-L-leucyl-L-glutamyl-L-asparaginyl-L-tyrosyl-S-benzyl-L-cysteinyl-L-isoasparagine p-Nitrobenzyl Ester (IXc). The protected heptadecapeptide (VIII) (2.54 g) was deblocked with HBr in trifluoroacetic acid (30 ml) and water (1 ml) as described in the synthesis of (VIII). The hydrobromide of the deblocked heptadecapeptide derivative (VIIIa) was precipitated by addition of ether (200 ml), filtered, washed with ether, and dried. The dried material was dissolved in a mixture of Me_2Fam (25 ml) and Me_2SO (25 ml) containing triethylamine (0.15 ml), cooled to 0 °C, and added to the solution of the tetrapeptide azide prepared as follows: *N*-benzyloxycarbonylglycyl-L-isoleucyl-L-valyl-γ-*tert*-butyl-L-glutamic hydrazide (VIIIb) (0.68 g; Katsoyannis et al., 1966b) was dissolved in a mixture of Me_2Fam (15 ml) and Me_2SO (3 ml), and to this solution, cooled to −10 °C, 1 N HCl in Me_2Fam (2.2 ml) was added, followed by isoamyl nitrite (0.15 ml). After 5 min, the mixture was cooled to −40 °C, neutralized with triethylamine (0.45 ml), and mixed with the solution of the deblocked heptadecapeptide prepared as described previously. After 48 h at 4 °C, the mixture was poured into methanol (700 ml) containing acetic acid (2 ml). The precipitate was collected by centrifugation, washed with warm methanol and ether, and dried. A solution of this material in trifluoroacetic acid (25 ml) was stored at room temperature for 30 min and then diluted with ether (200 ml). The precipitated partially protected heneicosapeptide IXc was collected by centrifugation, washed with ether, and dried; wt 2.1 g (70%), mp 282 °C (dec), $[\alpha]_D^{26}$ −39.8° (c 0.5, hexamethylphosphoramide). Anal. Calcd for $C_{139}H_{184}N_{26}O_{37}S_4$: C, 56.8; H, 6.26; N, 12.4; S, 4.36. Found: C, 56.2; H, 6.15; N, 11.9; S, 4.66. Amino acid analysis of an acid hydrolysate gave the following ratios: $Asp_{1.9}Ser_{0.8}Glu_{4.2}Gly_{2.1}Ala_{1.2}Val_{1.5}Ile_{0.5}Leu_{2.0}Tyr_{1.4}$*S*-benzylcysteine$_{3.4}$.

Glycyl-L-isoleucyl-L-valyl-L-glutamyl-L-glutaminyl-S-sulfo-L-cysteinyl-S-sulfo-L-cysteinyl-L-alanylglycyl-L-valyl-S-sulfo-L-cysteinyl-L-seryl-L-leucyl-L-tyrosyl-L-glutaminyl-L-leucyl-L-glutamyl-L-asparaginyl-L-tyrosyl-S-sulfo-L-cysteinyl-L-isoasparagine (Sheep Insulin [Iasn^{21}]A Chain S-Sulfonate) (Xa). Compound IXc (300 mg) was reduced with sodium in liquid ammonia (300 ml) and the reduced product was subsequently sulfitolized (Katsoyannis et al., 1966c). After evaporation of the ammonia, the residue was dissolved in 8 M guanidine hydrochloride (30 ml) and to this solution, adjusted to pH 8.9 with acetic acid or dilute NH_4OH (depending on the pH of the solution), sodium sulfite (1 g) and sodium tetrathionate (0.5 g) were added. The reaction mixture was stirred at 25 °C for 4 h and then dialyzed in a Visking 18/32 dialyzing tubing at 4 °C for 24 h against four changes of water (4 l each). The dialysate was lyophilized and the crude [Iasn^{21}]A chain *S*-sulfonate (Xa) was obtained as a white powder. For purification, this material was chromatographed on a Sephadex G-15 column (2.4 × 50 cm) equilibrated, and eluted with 0.015 M ammonium hydrogen carbonate. The effluent corresponding to the main peak, as monitored by a Gilford recording spectrophotometer, was lyophilized and the purified A chain analogue was obtained as a white fluffy material; wt 239 mg (89% based on IXc used).

Amino acid analysis after acid hydrolysis gave the molar ratios $Asp_{1.9}Ser_{0.8}Glu_{4.2}Gly_{2.0}Ala_{1.2}Cys_{3.2}$(uncorrected), $Val_{1.6}Ile_{0.6}Leu_{2.0}Tyr_{1.6}$,[2] in good agreement with the theoretically expected values. Digestion of the synthetic polypeptide with leucine aminopeptidase and amino acid analysis of the digest gave the following ratios: $Asp_{0.2}Ser_{0.8}$ (separated from Gln and Asn in a 30 °C chromatographic run), $Glu_{2.1}Gly_{2.0}Ala_{1.1}Val_{1.9}Leu_{2.2}Tyr_{1.8}$ *S*-sulfocysteine$_{4.1}$. Gln and Asn

[2] A sample of the hydrolysate placed on the short column of the Beckman-Spinco analyzer indicated the total absence of 3-benzyltyrosine.

emerge on the same position and were not determined. Similarly, Iasn and Ile emerge very close to each other and were not determined. It is apparent that the synthetic analogue was completely digested by the enzyme.

On thin-layer chromatography or high-voltage thin-layer electrophoresis in 0.5 N acetic acid (pH 2.9 and 3500 V) and in 0.05 M potassium bicarbonate (pH 8.4 and 1800 V) the synthetic material moved as a single component (Pauly reaction).

Sarcosyl-L-isoleucyl-L-valyl-L-glutamyl-L-glutaminyl-S-sulfo-L-cysteinyl-S-sulfo-L-cysteinyl-L-alanylglycyl-L-valyl-S-sulfo-L-cysteinyl-L-seryl-L-leucyl-L-tyrosyl-L-glutaminyl-L-leucyl-L-glutamyl-L-asparaginyl-L-tyrosyl-S-sulfo-L-cysteinyl-L-isoasparagine (Sheep Insulin [Sar^1,Iasn^{21}]A Chain S-Sulfonate) (Xb). The protected heptadecapeptide VIII (1 g) was dissolved in trifluoroacetic acid (20 ml) containing water (0.4 ml) and HBr passed through the solution for 1.6 h at 0 °C. Addition of ether caused the precipitation of the hydrobromide of the partially deblocked heptadecapeptide (VIIIa), which was isolated by filtration, washed with ether, and dried over KOH in vacuo. To a solution of this material in a mixture of Me_2Fam (5 ml) and Me_2SO (5 ml) containing triethylamine (0.11 ml) and cooled to 0 °C was added the tetrapeptide azide prepared as follows. *N*-Benzyloxycarbonylsarcosyl-L-isoleucyl-L-valyl-γ-*tert*-butyl-L-glutamic acid hydrazide (VIIIc) (0.48 g; Okada and Katsoyannis, 1975) was dissolved in a mixture of Me_2Fam (5 ml) and Me_2SO (5 ml). To this solution, cooled to −10 °C, 1 N HCl in Me_2Fam (1.6 ml) was added, followed by isoamyl nitrite (0.11 ml). After 5 min, the mixture was neutralized with triethylamine (0.22 ml) and then added to the solution of the partially protected heptadecapeptide prepared as described previously. The mixture was stirred for 48 h at 4 °C and then poured into methanol (600 ml). The precipitated protected heneicosapeptide IXb was isolated by centrifugation, washed with methanol and ether, and dried. A solution of this product in trifluoroacetic acid (25 ml) was stored at room temperature for 30 min and then poured into ether (700 ml). The precipitated partially protected heneicosapeptide IXd was isolated by centrifugation, washed with ether, and dried over KOH in vacuo; wt 0.64 g (55% based on VIII used), mp 288–290 °C (dec).

The reduction of IXd (200 mg) with sodium in liquid ammonia (200 ml) and the oxidative sulfitolysis of the reduced product were accomplished by the procedure we have described previously (Katsoyannis et al., 1966c). After evaporation of the ammonia, the residue was dissolved in 8 M guanidine hydrochloride (18 ml) and to this solution, adjusted to pH 8.9, was added sodium sulfite (1.36 g) and sodium tetrathionate (0.64 g). The mixture was stirred at room temperature for 4 h and dialyzed in a Visking 18/32 dialysis tubing at 4 °C for 24 h against four changes of distilled water (4 l each). Lyophilization of the dialysate afforded the crude [Sar^1,Iasn^{21}]A chain *S*-sulfonate (Xb) as a white powder; wt 180 mg. For purification the lyophilized material was chromatographed on a Sephadex G-25 column (2.4 × 90 cm) equilibrated and eluted with 0.015 M ammonium bicarbonate. The elution pattern of this column, as determined by monitoring the effluent by a Gilford recording spectrophotometer, indicated the presence of a single component. Upon lyophilization of the effluent, the purified [Sar^1,Iasn^{21}]A chain *S*-sulfonate (Xb) was obtained as a white fluffy material; wt 110 mg (62% based on the amount of IXd used).

Amino acid analysis of the synthetic material after acid hydrolysis gave the following molar ratios in good agreement with the theoretically expected values: $Asp_{1.8}Sar_{1.0}Ser_{0.8}Glu_{4.1}Gly_{1.2}Ala_{1.2}Cys_{2.4}$(uncorrected),$Val_{1.8}Ile_{0.8}Leu_{2.0}Tyr_{1.7}$.[2] Digestion of the synthetic analogue with leucine aminopeptidase and amino acid analysis of the digest gave the following molar ratios: $Asp_{0.2}Ser_{0.8}Glu_{2.2}Gly_{1.0}Ala_{1.1}Val_{1.9}Leu_{2.1}Tyr_{2.1}$*S*-sulfocysteine$_{4.1}$. Gln, Asn, and Sar emerge on the same position and were not determined. Similarly, Iasn and Ile emerge very close to each other and were not determined. As can be seen, the synthetic chain was completely digested by the enzyme, indicating, within the limits of error of the enzymatic technique, that the stereochemical homogeneity of the constituent amino acids was preserved during the synthetic processes. On thin-layer chromatography or high voltage thin-layer electrophoresis in 0.5 N acetic acid (pH 2.9 and 3400 V) and in 0.01 N ammonium bicarbonate (adjusted to pH 10 with NH_4OH; 2900 V) the synthetic [Sar^1,Iasn^{21}]A chain *S*-sulfonate moved as a single component (Pauly reaction).

S-Sulfonated Derivatives of the A and B Chains of Bovine Insulin. The B chain of bovine insulin is identical with the corresponding chain of sheep insulin (Sanger and Tuppy, 1951a,b; Brown et al., 1955). The S-sulfonated bovine A and B chains were prepared by the procedure we have described previously (Katsoyannis et al., 1967a).

Synthesis and Isolation of Sheep [Iasn^{21}-A]Insulin. The synthesis of this analogue was accomplished by the interaction of the sulfhydryl form of the [Iasn^{21}]A chain with the S-sulfonated form of the bovine (sheep) B chain by the procedure we have described previously (Katsoyannis and Tometsko, 1966; Katsoyannis et al., 1967b,c). In a typical experiment an aqueous solution of 20 mg of [Iasn^{21}]A chain *S*-sulfonate was reacted at pH 5.0 with 2-mercaptoethanol for 6 min at 100 °C under nitrogen. After cooling to 5 °C, the mixture was extracted four times with ethyl acetate and then allowed to react with 5 mg of the S-sulfonated B chain for 16 h at pH 10–10.5 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). Chromatography of two combination mixtures, each corresponding to the amounts of materials indicated above, gave the pattern shown in Figure 1. As was the case with synthetic insulin (Katsoyannis et al., 1967b) and other synthetic insulin analogues (Katsoyannis et al., 1975), the [Iasn^{21}-A] insulin was eluted with application of the gradient and is the slowest moving component. The effluent containing the insulin analogue (190–220 ml of effluent) was concentrated to approximately 10 ml in a rotary evaporator (20–25 °C) and the protein material was isolated via picrate as the hydrochloride (0.6 mg).

Amino acid analysis of an acid hydrolysate of this analogue gave the following ratios in good agreement with the theoretically expected values: $Lys_{1.1}His_{1.8}Arg_{1.0}Asp_{2.9}Thr_{1.0}Ser_{2.0}Glu_{7.1}Pro_{1.0}Gly_{5.1}Ala_{3.3}Cys_{3.2}$(uncorrected for destruction),$Val_{4.5}Ile_{0.8}Leu_{5.7}Tyr_{3.1}$(uncorrected),$Phe_{2.7}$. On thin-layer electrophoresis in 0.5 N acetic acid and 3400 V the synthetic analogue moved as a single component and had a mobility similar to that of natural insulin (Pauly reaction). The sheep [Iasn^{21}-A]insulin by the mouse convulsion assay method was found to possess a potency of 21 IU/mg and by radioimmunoassay had a potency of 16 IU/mg.

Synthesis and Isolation of Sheep [Sar^1,Iasn^{21}-A]Insulin.

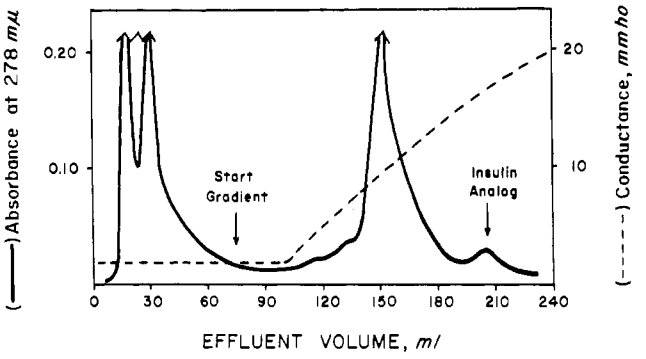


FIGURE 1: Chromatography of two combination mixtures (see Experimental Procedures and Results) of synthetic [Iasn^{21}] sheep A chain and natural bovine (sheep) B chain on a 0.9 × 23 cm CM-cellulose column with acetate buffer (0.024 M, pH 3.3) and an exponential NaCl gradient. The column effluent was monitored by a Gilford recording spectrophotometer and by a conductivity meter (Radiometer, Copenhagen). The [Iasn^{21}-A]insulin (190–220 ml of effluent) was recovered as the hydrochloride (0.6 mg).

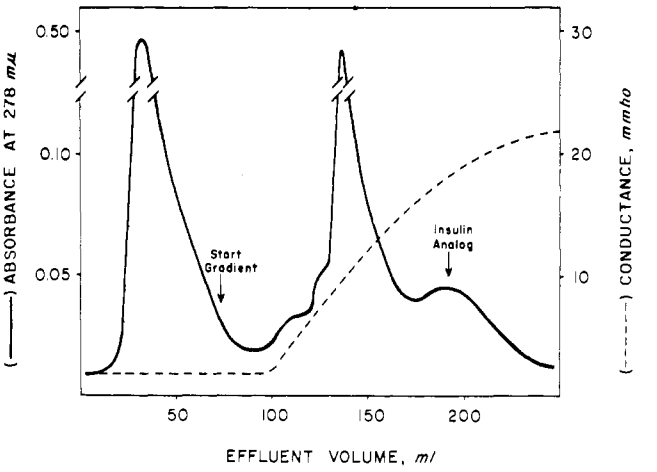


FIGURE 2: Chromatography of two combination mixtures (see Experimental Procedures and Results) of synthetic [Sar^{1},Iasn^{21}] sheep A chain and natural bovine (sheep) B chain on a 0.9 × 23 cm CM-cellulose column with acetate buffer (0.024 M, pH 3.3) and an exponential NaCl gradient. The column effluent was monitored by a Gilford recording spectrophotometer and by a conductivity meter (Radiometer, Copenhagen). The [Sar^{1},Iasn^{21}-A]insulin (180–220 ml of effluent) was recovered as the hydrochloride (1.03 mg).

The synthesis of this analogue by the interaction of the sulfhydryl form of [Sar^{1},Iasn^{21}] sheep A chain with the *S*-sulfonate form of sheep (bovine) B chain and its purification were accomplished by exactly the same procedure as described above in the synthesis of sheep [Iasn^{21}-A]insulin. Chromatography of two combination mixtures each corresponding to 20 mg of [Sar^{1},Iasn^{21}]A chain *S*-sulfonate and 5 mg of bovine (sheep) B chain *S*-sulfonate gave the pattern shown in Figure 2. The sheep [Sar^{1}-,Iasn^{21}-A]insulin is eluted with application of the gradient and was isolated from the effluent (180–220 ml) via picrate as the hydrochloride (1.03 mg).

Amino acid analysis of an acid hydrolysate of this analogue gave the following ratios in good agreement with the theoretically expected values: $Lys_{1.1}His_{1.9}Arg_{0.9}Asp_{2.8}Thr_{1.0}$-$Sar_{1.2}Ser_{2.0}Glu_{7.0}Pro_{0.9}Gly_{4.2}Ala_{3.2}Cys_{3.2}$(uncorrected for destruction),$Val_{4.6}Ile_{0.7}Leu_{5.9}Tyr_{2.9}$(uncorrected),$Phe_{2.9}$. On thin-layer electrophoresis in 0.5 N acetic acid and 3500 V, the synthetic analogue moved as a single component (Pauly reaction). The sheep [Sar^{1},Iasn^{21}-A]insulin was found to possess 15 IU/mg by the mouse convulsion assay method and 7 IU/mg by the radioimmunoassay method.

Discussion

Studies in several laboratories with modified insulins, prepared by use of proteolytic enzymes, strongly suggest that the C terminus of the A chain, A^{21} asparagine, is critically involved in the manifestation of the biological profile of insulin (for an excellent review of this subject, see Blundell et al., 1972). Furthermore, these studies indicate that the involvement of the A^{21} asparagine in the biological behavior of insulin is related to its contribution in the maintenance of the tertiary structure of the hormone (see Blundell et al., 1972; Arquilla and Stanford, 1972; Carpenter, 1966). The subsequent elucidation of the three-dimensional structure of insulin (Adams et al., 1969; Blundell et al., 1971; 1972) has made apparent the role of the A^{21} residue in the maintenance of the tertiary structure of this protein. The elegant studies of Hodgkin and co-workers (Blundell et al., 1972) have indeed shown that in the x-ray model of insulin the A^{21} asparagine is near the B^{22} arginine. This arrangement not only results in the formation of a salt bridge between these two residues, but also favorably disposes the NH of the A^{21} asparagine and the CO of the B^{23} glycine to form a hydrogen bond (Blundell et al., 1972). Both these interactions contribute to the stabilization of the conformation of the insulin molecule necessary for high biological activity. It can be anticipated that substitutions of the A^{21} and B^{22} amino acid residues, which permit analogous interactions between the A^{21} and B^{22} positions, would result in biologically active modified insulins. Thus, substitutions of B^{22} arginine with lysine (Katsoyannis et al., 1975) or aspartic acid (guinea pig insulin; Zimmerman et al., 1972) and of A^{21} L-asparagine with aspartic acid (Carpenter, 1966) or D-asparagine (Cosmatos et al., 1975), in all of which interactions between A^{21} and B^{22} positions may be retained (Katsoyannis et al., 1975), yield biologically active compounds.

Courtauld atomic models for the region of the hormone molecule that involves the residues participating in the A^{21}-B^{22} salt bridge and the A^{21}-B^{23} interaction indicate (Cosmatos et al., 1975) that in the conformation most favorable for salt bridge formation the α- and β-carboxyl groups of A^{21} L-asparagine are nearly equivalent. This suggests that substitution of A^{21} asparagine with isoasparagine would not hinder salt bridge formation with the B^{22} arginine and hence not appreciably affect the biological activity of the molecule. The present investigation has allowed us to substantiate this suggestion. [Iasn^{21}-A]insulin was synthesized and isolated in purified form and its biological activity and immunoreactivity was evaluated. This analogue, by the mouse convulsion assay method, was found to possess a potency of 21 IU/mg, ca. 87% of that of the natural hormone; the radioimmunoassay method gave a value of 16 IU/mg, ca. 66% of that of insulin.

We have reported recently the synthesis of an insulin analogue in which the basic character of the A^{1} residue of the A chain was increased in comparison to that of the natural hormone (Okada and Katsoyannis, 1975). This analogue, [Sar^{1}-A]insulin, differs from the parent molecule in that the N terminus of the A chain, glycine (pK_2 = 9.6), has been replaced with sarcosine (pK_2 = 10.0). This modification results in a small decrease in the biological activity but a pronounced decrease in the immunoreactivity of the molecule (20 and 9 IU/mg, respectively, vs. 23–25 IU/mg for the natural hormone) (Table I). This finding suggested (Okada and Katsoyannis, 1975) that the change in the relative positive charge at the A chain N terminus was responsible for the considerable decrease in the immunoreactivity of this analogue. The present com-

TABLE I: Comparison of the Biological Activity and Immunoreactivity of Insulin and the Insulin Analogues.

Insulin Species	Biological Act.[a] IU/mg	Biological Act.[a] % of Natural	Immunoreactivity[b] IU/mg	Immunoreactivity[b] % of Natural	Ratio of Biological Act. to Immunoreactivity
Natural	23–25	100	23–25	100	1.0
Sar^1-A	20	83	9	37	2.2
$Iasn^{21}$-A	21	87	16	66	1.3
Sar^1,$Iasn^{21}$-A	15	62	7	29	2.1

[a] Mouse convulsion method. [b] Radioimmunoassay, double-antibody technique.

munication gives further support to this speculation. This was accomplished by the synthesis and isolation in purified form of [Sar^1,$Iasn^{21}$-A]insulin, an analogue which differs from the parent molecule [$Iasn^{21}$-A]insulin in that the glycine at the A^1 position has been replaced with sarcosine. As was the case with [Sar^1-A]insulin, this analogue possesses a modestly lower biological activity but a considerably lower immunoreactivity than the parent molecule (15 and 7 IU/mg, respectively, vs. 21 and 16 IU/mg for [$Iasn^{21}$-A]insulin). Of particular interest is the observation that the substitution of glycine with sarcosine in the A^1 position of insulin and [$Iasn^{21}$-A]insulin results, for each pair (parent molecule vs. analogue), in nearly equivalent percental decrease in both the biological activity and immunoreactivity of the parent molecules (Table I). These relationships are simply illustrated by comparing the ratio of the biological activity and immunoreactivity of the parent molecules (insulin and [$Iasn^{21}$-A]insulin) and their "analogues". As shown in Table I, the ratio of these activities for natural insulin and [$Iasn^{21}$-A]insulin is in the range of 1–1.3 and for [Sar^1-A]insulin and [Sar^1-,$Iasn^{21}$-A]insulin 2.1–2.2. We must emphasize, however, that several more analogues must be prepared before the observed differential effect on biological activity and immunoreactivity of insulin is ascribed primarily to changes in the basicity of the N terminus of the A chain.

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