Divergence of the in Vitro Biological Activity and Receptor Binding Affinity of a Synthetic Insulin Analogue, [21-Asparaginamide-A]insulin†

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ABSTRACT: The [21-asparaginamide-A]insulin ([Asn-$(NH_2)^{21}$-A]insulin) was synthesized by the procedures developed in this laboratory to investigate the contribution of the C-terminal residue, asparagine, of the A chain to the biological activity and receptor binding affinity of insulin. Its secondary structure was investigated by circular dichroism studies. The biological behavior of this analogue was compared with that of insulin in in vitro and in vivo tests and in receptor binding assays. In contrast to other naturally occurring insulins and to all insulin analogues synthesized thus far, [Asn-$(NH_2)^{21}$-A]insulin displays a disparity between receptor binding affinity and in vitro biological potency. In stimulating glucose oxidation and lipogenesis the analogue exhibited potencies of 12 and 14.8%, respectively, compared to insulin. In insulin receptor binding assays, however, this analogue was found to possess a relative potency at least fourfold higher than the in vitro biological activities. In rat liver membranes and in isolated fat cells the analogue exhibited affinities of ca. 63.9 and 51.4%, respectively, compared to the natural insulin. Both the synthetic analogue and the natural hormone have the same maximal activity in the in vitro assays and their dose–response curves are parallel. When assayed in vivo by the mouse convulsion test, [Asn$(NH_2)^{21}$-A]insulin displays a potency of ca. 72% that of the native insulin. This might indicate partial amidolysis of the analogue in vivo, resulting in conversion to the natural hormone. The implications of these observations are considered with regard to insulin–receptor interactions and the generation of the physiological response to the hormone.

Degradative studies on insulin [for a review, see Blundell et al. (1974)] and data obtained from synthetic insulin analogues (Cosmatos et al., 1975, 1976; Ferderigos et al., 1979) have established the importance of the A^{21} asparagine in the biological activity of insulin. In continuation of our investigations to delineate the contributions to the biological activity and receptor binding ability to target cells of insulin of the A^{21} residue, we report in the present communication the synthesis, biological evaluation, and receptor binding ability of sheep [Asn$(NH_2)^{21}$-A]insulin. This analogue differs from the parent molecule in that the C-terminal amino acid residue of the A chain, asparagine, has been replaced by asparaginamide.

Experimental Procedures

Capillary melting points were determined for all peptide derivatives and are not corrected. Optical rotations were taken with a Zeiss photoelectric precision polarimeter. Coupling of the intermediate peptide fragments, in all synthetic steps, was followed by detection of the amino component present with ninhydrin; completion of the reaction was indicated by a negative ninhydrin test. Thin-layer chromatography was performed on 6060 silica gel (Eastman Chromagram sheet), with the solvent systems 1-butanol–pyridine–acetic acid–water (30:20:6:24) and 1-butanol–acetic acid–water (4:1:1). The homogeneity of the intermediate peptide derivatives was ascertained, after deblocking at the amino end, by thin-layer chromatography in both of the above-mentioned solvent systems. Thin-layer electrophoresis was performed with a Wieland-Pfleiderer pherograph (Brinkmann Instruments), as described previously (Tometsko & Delihas, 1967). Amino acid analyses were performed in a Beckman 120C amino acid analyzer according to the method of Spackman et al. (1958). Acid hydrolysis and calculations of molar ratios were carried out as previously described (Katsoyannis et al., 1967c). For the enzymatic digestion with leucine aminopeptidase, the method of Hill & Smith (1957) was employed with a chromatographically purified enzyme (Worthington Biochemical Corp.). Preswollen microgranular CM-cellulose[1] (Whatman CM-52) and Ecteola-cellulose (Whatman ET-11) were used in this study. The washing of the resins and the preparation of the columns and of the buffers used were carried out as described by Katsoyannis et al. (1967a,b) and Ferderigos et al. (1979), respectively. Sodium tetrathionate was prepared as described by Gilman et al. (1946). 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). Crystalline bovine insulin was generously provided by Eli Lilly and Co. [1-^{14}C]Glucose (45–55 mCi/mmol), [3-^{3}H]glucose (18 Ci/mmol), and porcine [^{125}I]insulin (107 μCi/μg) were purchased from New England Nuclear. Fatty acid free bovine serum albumin and crude bacterial collagenase were from Sigma Chemical Co., and bovine serum albumin, crystalline and Fraction V, was from Schwarz/Mann. Dinonyl phthalate was purchased from ICN Pharmaceuticals, Inc.

[^{125}*I*]*Insulin Binding: Liver Plasma Membranes.* Plasma membranes were prepared from the livers of fasted rats essentially as previously described (Horvat et al., 1975). Plasma membrane-enriched fractions, separately isolated from crude nuclear (N) fractions and crude microsomal (P) fractions, were pooled for assay. Membranes (40–80 μg of protein) were incubated for 45 min at 24 °C with [^{125}I]insulin [(2–4) × 10^{-10} M and 80–100 μCi/μg], varying concentrations of unlabeled bovine insulin or analogue, and sodium phosphate buffer, 0.1

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[1] Abbreviations used: CM, carboxymethyl; Me_2Fam, dimethylformamide; Me_2SO, dimethyl sulfoxide; Tris, 2-amino-2-(hydroxymethyl)-1,3-propanediol.

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M, pH 7.4, containing 6 mg/mL bovine serum albumin, crystalline or Fraction V, in a total volume of 0.2 mL. After incubation, samples were diluted with 2.0 mL of sodium phosphate buffer, 0.1 M, pH 7.4, containing 1 mg/mL Fraction V bovine serum albumin, and immediately filtered through Millipore EGWP cellulose acetate filters. Filters were rinsed twice with the ice-cold buffer, then dried, and dissolved in Filtron-X (National Diagnostics Co., Somerville, NJ) for counting.

Nonspecific binding of [^{125}I]insulin, defined as radioactivity retained on the filter in the presence of 1×10^{-5} M unlabeled insulin, was less than 10% of the radioactivity retained on the filter in the absence of competitor. Specific binding was obtained by subtracting nonspecific binding from the total radioactivity retained on the filter for each experimental condition. As noted in other laboratories (Cuatrecasas, 1971), filter materials such as cellulose nitrate are unsuitable for this assay because of high and variable binding of [^{125}I]insulin. Relative potency is expressed as

$$\frac{\text{concn of insulin (ng/mL)}}{\text{concn of analogue (ng/mL)}} \times 100$$

required to displace 50% of the specifically bound insulin.

[^{125}I]Insulin Binding: Fat Cells. Fat cells were prepared by the method of Rodbell (1964), and the effect of natural insulin and analogue on the binding of [^{125}I]insulin to receptors was measured essentially as previously described (Cosmatos et al., 1978), with the following modifications. Cells (70–100 mg dry weight) were incubated for 45 min at 37 °C with [^{125}I]insulin (3×10^{-10} M and 80–100 μCi/μg), varying concentrations of insulin or analogue, and Krebs-Ringer bicarbonate buffer containing 30 mg/mL fatty acid free bovine serum albumin in a total volume of 0.6 mL in 1.5-mL plastic microcentrifuge tubes. After incubation, 0.4 mL of dinonyl phthalate was added to the tubes, and the cells were separated from the medium by centrifuging for 30 s in an Eppendorf or Beckman microcentrifuge. Cell cakes were washed by adding 0.3 mL of fresh medium and recentrifuging. Cells were removed with a 200-μL automatic pipet fitted with disposable plastic tips widened by cutting off the ends. The tips, with cells, were placed in scintillation vials, and the cells were dissolved in 1.0 mL of 10% sodium dodecyl sulfate for counting in 10 mL of Aquasol-2 (New England Nuclear). Nonspecific binding, defined as radioactivity remaining with the cells in the presence of 3.3×10^{-6} M unlabeled insulin, was 10–15% of the label associated with the cells in the absence of competitor. Relative potency was expressed as described for receptors in plasma membranes.

Glucose Oxidation. Conversion of [1-^{14}C]glucose into $^{14}CO_2$ by isolated fat cells was performed as previously described (Cosmatos et al., 1978) with the following modifications. Triplicate samples containing cells (20–30 mg dry weight) were preincubated for 45 min before addition of [1-^{14}C]glucose; after addition of label, incubation was continued for 1 h. The buffer contained 30 mg/mL fatty acid free bovine serum albumin. Stimulation of 0 and 100% represents $^{14}CO_2$ production in the absence and presence, respectively, of 1.89×10^{-8} M bovine insulin. Relative potency is calculated as

$$\frac{\text{concn of insulin (ng/mL)}}{\text{concn of analogue (ng/mL)}} \times 100$$

required to achieve 50% of the maximum stimulation of $^{14}CO_2$ production.

Lipogenesis. Isolated fat cells were incubated with [3-^{3}H]glucose, and the conversion of labeled glucose into lipids was measured by extracting the incubation mixtures with a toluene-based scintillation fluid as described by Moody et al. (1974). Triplicate incubations in plastic scintillation vials contained cells (20–30 mg dry weight), [3-^{3}H]glucose (5×10^{-4} M and 0.5 μCi/mL), varying concentrations of bovine insulin or insulin analogue, and Krebs-Ringer bicarbonate buffer containing 30 mg/mL fatty acid free bovine serum albumin in a total volume of 1.0 mL. Mixtures were preincubated at 37 °C for 45 min before addition of [3-^{3}H]glucose and then further incubated for 1 h before extraction. The aqueous phase was not removed before counting. In the absence of cells, unchanged glucose is essentially uncounted (<0.01% of added [3-^{3}H]glucose). Stimulation of 0 and 100% and relative potency are expressed as described for glucose oxidation.

Receptor Occupancy. Receptor occupancy was calculated as the ratio of the concentration of insulin or analogue required to elicit 50% of the maximal response in lipogenesis or glucose oxidation to the concentration required to displace 50% of the [^{125}I]insulin from the receptor preparation.

Circular Dichroism (CD). The CD measurements of the [Asn$(NH_2)^{21}$-A] analogue and zinc-free bovine insulin were obtained with a JASCO J-500A automatic recording spectropolarimeter equipped with a data processor. Spectra were taken at 26 °C, under nitrogen, in 0.01 M phosphate buffer (pH 7.63) and 0.01 M HCl (pH 2.2) with protein concentrations of ca. 0.1–1.0 mg/mL. Cylindrical quartz cells with optical path lengths of 0.10 and 1.00 cm were used. Each sample was scanned 4 or 8 times, and an average spectrum was obtained by using the data processor and correcting for the base line. Mean residue ellipticity ($[\theta]^{mrw}$) was calculated from

$$[\theta]^{mrw} = \frac{\theta \bar{M}}{cl}$$

where θ is the observed ellipticity, $\bar{M}$ is the mean residual molecular weight (mrw) calculated to be 112 for insulin and the analogue, c is the protein concentration in milligrams per milliliter, and l is the optical path length in centimeters.

General Aspects of Synthesis of Sheep [Asn$(NH_2)^{21}$-A]-insulin. This analogue was synthesized by combination of the S-sulfonated form of the B chain of bovine (sheep) insulin with the sulfhydryl form of the [Asn$(NH_2)^{21}$]A chain of sheep insulin. The S-sulfonated B chain was prepared by oxidative sulfitolysis of bovine insulin and separation of the resulting S-sulfonated A and B chains by column chromatography (Katsoyannis et al., 1967a). The sulfhydryl form of the [Asn$(NH_2)^{21}$]A chain was obtained by reduction of the S-sulfonated derivative of that chain. The synthesis of the latter compound was accomplished by classical methods of peptide synthesis. It involved the synthesis of the protected heneicosapeptide IX, containing the entire amino acid sequence of the [Asn$(NH_2)^{21}$]A chain, removal of the protecting group by sodium in liquid ammonia, and conversion of the resulting thiol derivative into the S-sulfonated form X by oxidative sulfitolysis.

N-(Benzyloxycarbonyl)-L-asparaginamide (I). N-(Benzyloxycarbonyl)-L-asparagine *p*-nitrophenyl ester (Bodanszky & du Vigneaud, 1959) (19.3 g) was dissolved in a mixture of Me_2Fam (100 mL) and methylene chloride (150 mL), and ammonia gas was passed through the solution (5 °C) for 30 min. The mixture was then diluted with methylene chloride (500 mL), and the precipitated product was collected by filtration, washed with 0.5 N NH_4OH and water, and dried. This material was dissolved in 95% aqueous acetic acid (170 mL) and precipitated by the addition of water: wt 12 g (90%); mp 230 °C dec; $[\alpha]^{26}_D$ +16.8° (*c* 1, 95% acetic acid). Anal.

Calcd for $C_{12}H_{15}N_3O_4$: C, 54.3; H, 5.70; N, 15.8. Found: C, 54.4; H, 5.68; N, 15.6.

N-(Benzyloxycarbonyl)-S-benzyl-L-cysteinyl-L-asparaginamide (II). A solution of compound I (13.3 g) in acetic acid (50 mL) was treated with 4 N HBr in acetic acid (50 mL). After 1 h the mixture was diluted with anhydrous ether (350 mL) and the precipitated product was collected by filtration, washed thoroughly with ether, and dried over KOH in vacuo. To a solution of this product in Me_2Fam (60 mL) was added triethylamine (7 mL) followed by *N*-(benzyloxycarbonyl)-*S*-benzyl-L-cysteine *p*-nitrophenyl ester (Bodanszky & du Vigneaud, 1959) (25 g). After 48 h the mixture was poured into cold 5% aqueous $NaHCO_3$ (1 L) and the precipitated product was collected by filtration, washed with 5% $NaHCO_3$ and water, and dried: wt 21.5 g (95%); mp 231–232 °C; $[\alpha]^{26}_D$ −21.7° (*c* 1, Me_2Fam). A sample for analysis was precipitated from a solution in Me_2Fam by the addition of water, melting point unchanged. Anal. Calcd for $C_{22}H_{26}N_4O_5S$: C, 57.6; H, 5.72; N, 12.2; S, 7.0. Found: C, 57.8; H, 5.87; N, 12.3; S, 7.3.

N-(tert-Butoxycarbonyl)-O-benzyl-L-tyrosyl-S-benzyl-L-cysteinyl-L-asparaginamide (III). A solution of compound II (13.8 g) in acetic acid (30 mL) was treated with 4 N HBr in acetic acid (30 mL). After 1 h the mixture was diluted with anhydrous ether and the resulting precipitate was collected by filtration and reprecipitated from a solution in methanol by the addition of ether. To a solution of this product in Me_2Fam (150 mL) was added triethylamine (4.2 mL), followed by *N*-(*tert*-butoxycarbonyl)-*O*-benzyl-L-tyrosine *p*-nitrophenyl ester (Zahn et al., 1966) (14.5 g). After 48 h at room temperature, the solution was diluted with 1 N NH_4OH (50 mL), stirred for 1 h, and poured into cold 0.5 N NH_4OH (1.5 L). The precipitated product was filtered off, washed (0.5 N NH_4OH, water, 10% citric acid, and water), dried, and reprecipitated from Me_2Fam–water: wt 19.9 g (98%); mp 199–200 °C; $[\alpha]^{26}_D$ −4.1° (*c* 1, Me_2Fam). Anal. Calcd for $C_{35}H_{43}N_5O_7S$: C, 62.0; H, 6.39; N, 10.3. Found: C, 61.9; H, 6.51; N, 10.4.

N-(tert-Butoxycarbonyl)-L-asparaginyl-O-benzyl-L-tyrosyl-S-benzyl-L-cysteinyl-L-asparaginamide (IV). A solution of compound III (17 g) in 98% formic acid (100 mL) was stored at room temperature for 4 h and subsequently concentrated under reduced pressure. The residue was triturated with cold 0.5 N NH_4OH, washed with water, and dried. To a solution of this product in Me_2Fam (75 mL) were added *N*-(*tert*-butoxycarbonyl)-L-asparagine *p*-nitrophenyl ester (Marshall & Merrifield, 1965) (9 g) and a few drops of triethylamine. After 48 h at room temperature, the solution was diluted with 1 N NH_4OH (10 mL), stirred for 30 min, and poured into cold 0.5 N NH_4OH (500 mL). The precipitate was filtered off, washed (1 N NH_4OH, water, 5% citric acid, and water), and dried: wt 19 g (95%); mp 233–235 °C dec; $[\alpha]^{26}_D$ −31.9° (*c* 1, Me_2Fam). Anal. Calcd for $C_{39}H_{49}N_7O_9S$: C, 59.1; H, 6.24; N, 12.4. Found: C, 59.2; H, 6.40; N, 12.6. An acid hydrolysate of this material showed the following composition in molar ratios: $Asp_{2.1}Tyr_{0.8}$*S*-BzlCys$_{0.9}$.

N-(tert-Butoxycarbonyl)-γ-benzyl-L-glutamyl-L-asparaginyl-O-benzyl-L-tyrosyl-S-benzyl-L-cysteinyl-L-asparaginamide (V). A solution of compound IV (15.8 g) in 98% formic acid (75 mL) was stored at room temperature for 4 h and subsequently concentrated under reduced pressure. The residue was triturated with ether and dissolved in Me_2Fam (40 mL). This solution, cooled to 0 °C, was poured into cold 0.5 N NH_4OH (250 mL) saturated with NaCl. The precipitated free base of the tetrapeptide was filtered off, washed with water, and dried over P_2O_5 in vacuo. To a solution of this product in Me_2Fam (50 mL) were added *N*-(*tert*-butoxycarbonyl)-γ-benzyl-L-glutamic acid *p*-nitrophenyl ester (Sandrin & Boissonnas, 1963) (10 g) and a few drops of triethylamine. After 48 h at room temperature, the solution was diluted with 1 N NH_4OH (10 mL), stirred for 30 min, and poured into cold 0.5 N NH_4OH (500 mL). The precipitated protected pentapeptide was filtered off, washed (1 N NH_4OH, water, 5% citric acid, and water), and dried: wt 19.8 g (98%); mp 228 °C dec; $[\alpha]^{26}_D$ −25.8° (*c* 1, Me_2Fam). Anal. Calcd for $C_{51}H_{62}N_8O_{12}S$: C, 60.6; H, 6.18; N, 11.1. Found: C, 60,8; H, 6.28; N, 11.2. Amino acid analysis of an acid hydrolysate gave the following composition expressed in molar ratios: $Asp_{1.9}Glu_{1.0}$*S*-BzlCys$_{0.9}$Tyr$_{0.8}$.

N-(tert-Butoxycarbonyl)-L-seryl-L-leucyl-L-tyrosyl-L-glutaminyl-L-leucyl-γ-benzyl-L-glutamyl-L-asparaginyl-O-benzyl-L-tyrosyl-S-benzyl-L-cysteinyl-L-asparaginamide (VI). A solution of compound V (2.3 g) in trifluoroacetic acid (7 mL) containing anisole (0.5 mL) was stored at 0 °C for 1 h and diluted with ether (300 mL). The precipitated deblocked pentapeptide derivative was isolated by filtration, washed with ether, and dried over KOH in vacuo. A solution of this material in Me_2Fam (20 mL), cooled to 0 °C, was neutralized with triethylamine (0.64 mL) and mixed with a solution of the pentapeptide azide prepared as follows. *N*-(*tert*-Butoxycarbonyl)-L-seryl-L-leucyl-L-tyrosyl-L-glutaminyl-L-leucine hydrazide (Katsoyannis et al., 1973) (1.67 g) was dissolved in Me_2Fam (15 mL), and to this solution, cooled to −10 °C, was added 5.4 N HCl in dioxane (0.85 mL) followed by isoamyl nitrite (0.31 mL). After 5 min at −10 °C, the mixture was cooled to −30 °C, neutralized with triethylamine (0.64 mL), and added to the solution of the deblocked pentapeptide amide prepared as described previously. After 48 h at 4 °C, the reaction mixture was poured into methanol (300 mL) and the precipitated protected decapeptide was collected by centrifugation, washed with methanol, and dried: wt 2.7 g (73.6%); mp 260–264 °C dec; $[\alpha]^{26}_D$ −18.6° (*c* 1, Me_2SO). Anal. Calcd for $C_{80}H_{106}N_{14}O_{20}S$: C, 59.5; H, 6.62; N, 12.1. Found: C, 59.5; H, 6.92; N, 12.0. Amino acid analysis of an acid hydrolysate showed the following composition expressed in molar ratios: $Asp_{2.1}Ser_{0.8}Glu_{2.1}Leu_{1.9}Tyr_{1.6}$*S*-BzlCys$_{0.9}$ (average amino acid recovery 96%).

N-(tert-Butoxycarbonyl)glycyl-L-valyl-S-benzyl-L-cysteinyl-L-seryl-L-leucyl-L-tyrosyl-L-glutaminyl-L-leucyl-γ-benzyl-L-glutamyl-L-asparaginyl-O-benzyl-L-tyrosyl-S-benzyl-L-cysteinyl-L-asparaginamide (VII). A solution of compound VI (1.62 g) in trifluoroacetic acid (6 mL) containing anisole (0.5 mL) was stored at 0 °C for 1 h. Addition of ether (400 mL) to the reaction mixture caused precipitation of the trifluoroacetate of the decapeptide derivative, which was collected by filtration, washed with ether, and dried over KOH in vacuo. To a cooled (0 °C) solution of this product in a mixture of Me_2Fam (10 mL) and Me_2SO (15 mL) was added triethylamine (0.28 mL), followed by the tripeptide azide prepared as follows. *N*-(*tert*-Butoxycarbonyl)glycyl-L-valyl-*S*-benzyl-L-cysteine hydrazide (Okada & Katsoyannis, 1975) (0.72 g) was dissolved in Me_2Fam (10 mL), and to this solution, cooled to −10 °C, was added 5.4 N HCl in dioxane (0.55 mL), followed by isoamyl nitrite (0.20 mL). After 5 min at −10 °C, the mixture was cooled to −30 °C, neutralized with triethylamine (0.42 mL), and added to the solution of the decapeptide amide prepared as described previously. After 72 h at 4 °C, the reaction mixture was poured into methanol (700 mL) and the precipitated product was collected by centrifugation, washed with methanol, and dried: wt 1.9 g

(96.7%); mp 272–274 °C dec; $[\alpha]^{26}_D$ −21.5° (*c* 1, Me_2SO). Anal. Calcd for $C_{97}H_{129}N_{17}O_{23}S_2$: C, 59.3; H, 6.63; N, 12.1. Found: C, 59.5; H, 6.88; N, 11.9. Amino acid analysis of an acid hydrolysate showed the following composition expressed in molar ratios: $Asp_{2.1}Ser_{0.8}Glu_{2.1}Gly_{1.0}Val_{1.0}Leu_{2.0}Tyr_{1.6}$*S*-$BzlCys_{1.5}$ (average amino acid recovery 94%).

N-(*tert-Butoxycarbonyl*)-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*-γ-*benzyl*-L-*glutamyl*-L-*asparaginyl*-*O*-*benzyl*-L-*tyrosyl*-*S*-*benzyl*-L-*cysteinyl*-L-*asparaginamide* (*VIII*). A solution of the protected tridecapeptide VII (1.5 g) in trifluoroacetic acid (12 mL) containing anisole (0.5 mL) was stored at 0 °C for 1 h and then poured into ether (300 mL). The precipitated partially protected tridecapeptide derivative was isolated, washed with ether, and dried over KOH in vacuo. To a cold solution of this product, in a mixture of Me_2Fam (8 mL) and Me_2SO (10 mL) containing triethylamine (0.21 mL), was added the tetrapeptide azide prepared as follows. The protected tetrapeptide hydrazide *N*-(*tert*-butoxycarbonyl)-L-glutaminyl-*S*-benzyl-L-cysteinyl-*S*-benzyl-L-cysteinyl-L-alanine hydrazide (Okada & Katsoyannis, 1975) (0.71 g) was dissolved in a mixture of Me_2Fam (8 mL) and Me_2SO (5 mL), and to this solution, cooled to −10 °C, was added 5.4 N HCl in dioxane (0.37 mL) followed by isoamyl nitrite (0.13 mL). After 5 min at −10 °C, the reaction mixture was neutralized with triethylamine (0.28 mL) and added to the solution of the tridecapeptide derivative prepared as described previously. After 72 h at 4 °C, the reaction mixture was poured into methanol (700 mL) and the precipitated protected heptadecapeptide was isolated by centrifugation, washed with methanol, and dried: wt 1.5 g (77.1%); mp 279–280 °C dec; $[\alpha]^{26}_D$ −25.03° (*c* 1, Me_2SO). Anal. Calcd for $C_{125}H_{164}N_{22}O_{28}S_4$: C, 58.8; H, 6.49; N, 12.1. Found: C, 58.2; H, 6.59; N, 12.1. Amino acid analysis of an acid hydrolysate showed the following composition expressed in molar ratios: $Asp_{2.0}Ser_{0.8}Glu_{3.2}Gly_{1.0}Ala_{1.0}Val_{1.0}Leu_{1.9}Tyr_{1.6}$*S*-$BzlCys_{3.5}$ (average amino acid recovery 80%).

N-(*Benzyloxycarbonyl*)*glycyl*-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*-γ-*benzyl*-L-*glutamyl*-L-*asparaginyl*-*O*-*benzyl*-L-*tyrosyl*-*S*-*benzyl*-L-*cysteinyl*-L-*asparaginamide* (*IX*). A solution of the protected heptadecapeptide amide VIII (0.8 g) in trifluoroacetic acid (10 mL) containing anisole (0.5 mL) was stored at room temperature for 30 min and then poured into ether (300 mL). The precipitated product was collected, washed with ether, and dried over KOH in vacuo. To a solution of this solid in a mixture of Me_2Fam (5 mL) and Me_2SO (5 mL), cooled to 0 °C, was added triethylamine (0.09 mL), followed by the protected tetrapeptide azide prepared as follows. *N*-(Benzyloxycarbonyl)glycyl-L-isoleucyl-L-valyl-γ-*tert*-butyl-L-glutamic acid hydrazide (Katsoyannis et al., 1966a) (0.38 mg) was dissolved in a mixture of Me_2Fam (5 mL) and Me_2SO (5 mL). To this solution, cooled to −10 °C, was added 5.4 N HCl in dioxane (0.23 mL), followed by isoamyl nitrite (0.08 mL). After 5 min at −10 °C, the reaction mixture was neutralized with triethylamine (0.19 mL) and subsequently added to the partially protected heptadecapeptide prepared as described previously. The reaction mixture was stirred for 48 h at 4 °C and then poured into methanol (600 mL). The protected heneicosapeptide IXa was isolated by centrifugation, washed with methanol, and dried: wt 0.75 g; mp 297–299 °C dec. A solution of this solid in trifluoroacetic acid (15 mL) containing anisole (1 mL) was stored at room temperature for 30 min and then poured into ether (400 mL). The precipitated partially protected heneicosapeptide was isolated, washed with ether, and dried: wt 0.7 g (75.7%); mp 286–290 °C dec; $[\alpha]^{26}_D$ −28.2° (*c* 1, Me_2SO). An acid hydrolysate gave the following composition expressed in molar ratios: $Asp_{1.9}Ser_{0.7}Glu_{4.2}Gly_{2.0}Ala_{1.1}Val_{1.5}Ile_{0.6}Leu_{1.8}Tyr_{1.5}$*S*-$BzlCys_{3.5}$ (average amino acid recovery 78%).

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-*asparaginamide* (*Sheep Insulin* [*Asn*$(NH_2)^{21}$]*A Chain S-Sulfonate*) (*X*). The reduction of the partially protected heneicosapeptide IX and the oxidative sulfitolysis of the reduced product were carried out as described previously (Katsoyannis et al., 1966b). Briefly, the thoroughly dry compound (200 mg) was dissolved in anhydrous liquid ammonia (200 mL) in a 500-mL round-bottomed flask fitted for magnetic stirring. Reduction was accomplished at the boiling point of the solution by adding small pieces of sodium into the solution until a faint blue color appeared throughout. The blue color was allowed to persist for 10 s and was then discharged by the addition of a few crystals of ammonium chloride. The solution was concentrated at atmospheric pressure to dryness, and the residue was dried in vacuo and dissolved in 8 M guanidine hydrochloride (18 mL). To this solution, adjusted to pH 8.9 with acetic acid or dilute NH_4OH (depending on the pH of the solution), were added sodium sulfite (1.36 g) and freshly prepared sodium tetrathionate (0.64 g). The reaction mixture was stirred at room temperature for 4 h and then placed in Spectrapor membrane tubing No. 3 and dialyzed against four changes of distilled water (4 L each) at 4 °C for 20 h. Upon lyophilization of the dialysate, the crude [$Asn(NH_2)^{21}$]A chain *S*-sulfonate was obtained as a white powder. For purification, the lyophilized material was dissolved in 0.015 M ammonium bicarbonate (5 mL) and chromatographed on a Sephadex G-15 column (4.5 × 45 cm), equilibrated and eluted with 0.015 M ammonium bicarbonate at a flow rate of 40 mL/h. The effluent corresponding to the main peak, as monitored by a Gilford recording spectrophotometer, was lyophilized, and the [$Asn(NH_2)^{21}$]A chain *S*-sulfonate was obtained as a white powder, wt 166 mg. For further purification this material (50 mg) was dissolved in 0.1 M Tris-HCl buffer (pH 7.0, 5 mL) and placed on an Ecteola-cellulose (Whatman ET-11) column (1.2 × 25 cm) which was equilibrated with the same buffer. Subsequently the column was eluted with 0.1 M Tris-HCl buffer (pH 7.0) and a linear NaCl gradient, as described previously (Ferderigos et al., 1979). The column effluent was monitored with a Gilford recording spectrophotometer and a conductivity meter (Radiometer, Copenhagen). The elution pattern is shown in Figure 1. The eluate under the major peak (195–235 mL) was collected and dialyzed in Spectrapor membrane tubing No. 3 at 4 °C for 24 h against four changes of distilled water (4 L each). Lyophilization of the dialysate gave the purified [$Asn(NH_2)^{21}$]A chain *S*-sulfonate as a white powder (35 mg). On high-voltage thin-layer electrophoresis in 0.5 N acetic acid (pH 2.9 and 3500 V) the synthetic chain moved as a single component (Pauly-positive spot). Amino acid analysis of the purified product after acid hydrolysis gave the molar ratios shown in Table I, in good agreement with the expected values. Digestion of the synthetic material with leucine aminopeptidase and amino acid analysis of the digest gave the molar ratios shown in Table I, in good agreement with the expected values.

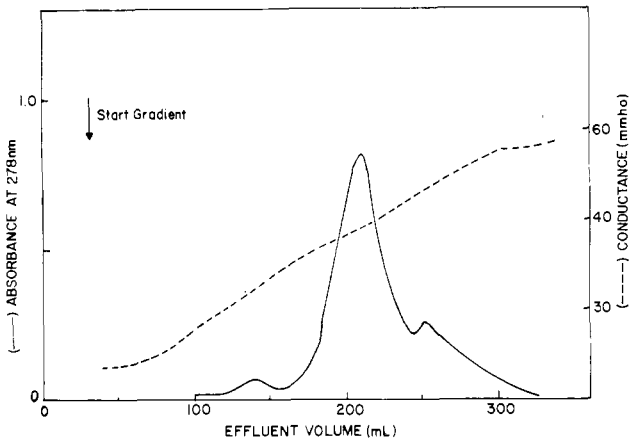

FIGURE 1: Chromatography of crude [Asn$(NH_2)^{21}$]A chain *S*-sulfonate on a 1.2 × 25 cm Ecteola-cellulose column with 0.1 M Tris-HCl buffer (pH 7.0) and a linear NaCl gradient. The column effluent was monitored by a Gilford recording spectrophotometer and by a conductivity meter (Radiometer, Copenhagen).

Table I: Amino Acid Composition[a] of an Acid Hydrolysate and an Enzymatic Digest (Leucine Aminopeptidase) of the S-Sulfonated [Asn$(NH_2)^{21}$]A Chain of Sheep Insulin

	acid hydrolysis		enzymatic hydrolysis	
amino acid	theory	found	theory	found
Asn(NH_2)	0	0	1	emerges in the same position with ammonia and not determined[b]
Asp	2	1.8	0	0
Gln	0	0	2	emerges in the same position and not determined
Asn	0	0	1	
Ser	1	0.8	1	0.8[c]
Glu	4	4.1	2	1.9
Gly	2	2.0	2	2.1
Ala	1	1.1	1	1.0
$^1/_2$-Cys	4	3.1[d]	0	0
Val	2	1.7	2	2.2
Ile	1	0.7	1	1.1
Leu	2	1.9	2	2.1
Tyr	2	1.5[d]	2	1.8
S-sulfo-Cys	0	0	4	3.9[e]

[a] Number of amino acid residues per molecule. [b] In model experiments it was found that incubation of asparaginamide with leucine aminopeptidase yields only a trace of asparagine. [c] Separated from glutamine and asparagine in a 30 °C chromatographic run. [d] Uncorrected for destruction. [e] Eluted from the long column of the Beckman analyzer after 26 mL of effluent.

It is apparent that the synthetic polypeptide was completely digested by the enzyme, indicating, within the limits of error of the enzymatic techique, that the stereochemical homogeneity of the constituent amino acids was preserved during the synthetic processes.

S-Sulfonated Derivative of the B chain. This compound was prepared by oxidative sulfitolysis of bovine insulin followed by separation of the resulting S-sulfonated A and B chains by CM-cellulose chromatography, as described previously (Katsoyannis et al., 1967a). The B chains of bovine and sheep insulin are identical (Sanger & Tuppy, 1951; Brown et al., 1955).

Synthesis and Isolation of Sheep [Asn$(NH_2)^{21}$-A]insulin. The synthesis of this analogue was accomplished by the interaction of the sulfhydryl form of the [Asn$(NH_2)^{21}$]A chain with the S-sulfonated form of the bovine (sheep) B chain by the procedure we have described previously (Katsoyannis & Tometsko, 1966; Katsoyannis et al., 1967b,c). In a typical experiment, an aqueous solution of 20 mg of [Asn$(NH_2)^{21}$]A chain *S*-sulfonate was reacted at pH 5.0 with 2-mercaptoethanol for 6 min at 100 °C under nitrogen. After being cooled to 5 °C, the mixture was extracted 4 times with ethyl acetate annd then allowed to react with 5 mg of the S-sulfonated B chain for 20 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 2. As was the case with synthetic insulin (Katsoyannis et al., 1967c), the [Asn$(NH_2)^{21}$-A]insulin was eluted with application of the gradient and is the slowest moving component. The effluent containing the insulin analogue (239–289 mL of effluent) was concentrated to approximately 5 mL in a rotary evaporator (20–25 °C), and the protein material was isolated via picrate as the hydrochloride (1.02 mg). Amino acid analysis of an acid hydrolysate of this analogue gave a com-

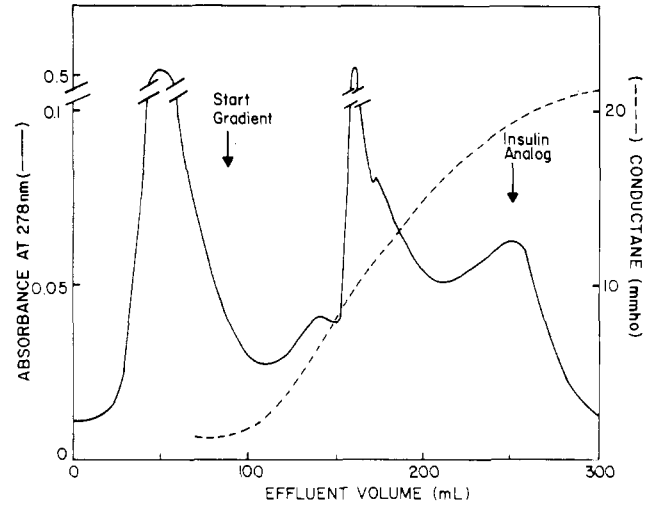

FIGURE 2: Chromatography of two combination mixtures (see Experimental Procedures) of the sulfhydryl form of sheep [Asn$(NH_2)^{21}$]A chain with the S-sulfonated sheep (bovine) B chain on a 0.9 × 23 cm CM-cellulose column with acetate buffer (0.024 M, pH 3.3) and an exponential NaCl gradient. The column effluent was monitored by a Gilford recording spectrophotometer and by a conductivity meter (Radiometer, Copenhagen). The sheep [Asn$(NH_2)^{21}$-A]insulin (239–289 mL of effluent) was recovered as the hydrochloride.

Table II: Amino Acid Composition[a] of an Acid Hydrolysate of the Sheep [Asn$(NH_2)^{21}$-A]insulin

amino acid	theory	found
Lys	1	1.1
His	2	1.8
Arg	1	1.0
Asp	3	3.1
Thr	1	1.1
Ser	2	2.0
Pro	1	0.9
Glu	7	7.0
Gly	5	5.0
Ala	3	3.0
$^1/_2$-Cys	6	4.0[b]
Val	5	4.5
Ile	1	0.8
Leu	6	6.3
Tyr	4	2.9[b]
Phe	3	2.7

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

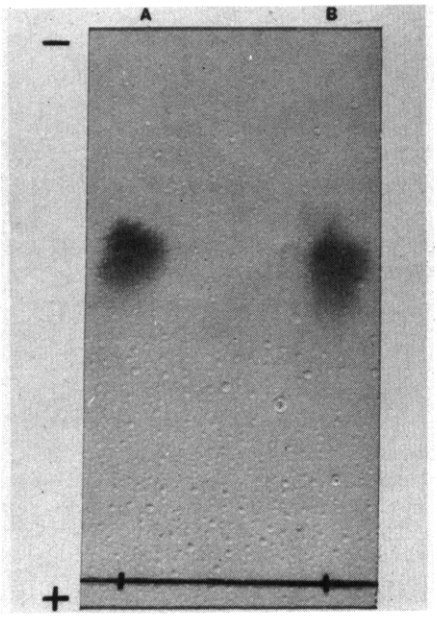

FIGURE 3: Thin-layer electrophoresis of natural bovine insulin (A) and synthetic sheep [Asn(NH_2)^{21}-A]insulin (B); 0.5 N acetic acid, 3400 V, and 15 min.

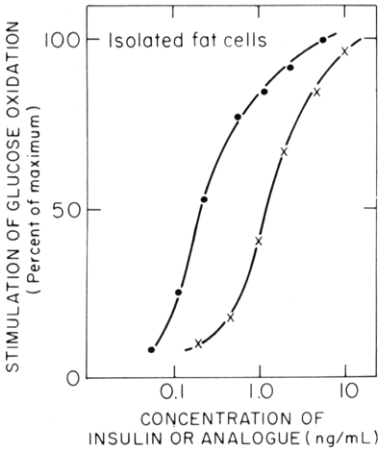

FIGURE 4: Effect of bovine insulin (●) and [Asn(NH_2)^{21}-A]insulin (×) on glucose oxidation in isolated fat cells (see Experimental Procedures). The stimulation of glucose oxidation, expressed as percent of maximum, is plotted as a function of the concentration of insulin and analogue. Data are from a typical experiment which was repeated 4 times.

position in agreement with the expected values (Table II). On thin-layer electrophoresis in 0.5 N acetic acid (pH 2.9 and 3500 V) the synthetic analogue moved as a single component (Pauly reaction) (Figure 3).

Biological Evaluation of Sheep [Asn(NH_2)^{21}-A]insulin. Biological evaluation of this analogue was performed in vivo by the mouse convulsion assay method and in vitro by glucose oxidation, lipogenesis, and receptor binding assays in rat liver membranes and in isolated fat cells. By the mouse convulsion assay method, [Asn(NH_2)^{21}-A]insulin was found to possess 17.3 ± 2.0 IU/mg (ca. 72% of the activity of the natural hormone).

The synthetic analogue and the natural hormone have the same maximal activity in stimulating glucose oxidation, as measured by the production of $^{14}CO_2$ from [1-^{14}C]glucose, and lipogenesis, as measured by the incorporation of [3-^{3}H]glucose into organic solvent extractable material, and their dose–response curves in these assays appear parallel, as shown in Figures 4 and 5. The relative potency of [Asn(NH_2)^{21}-A]-

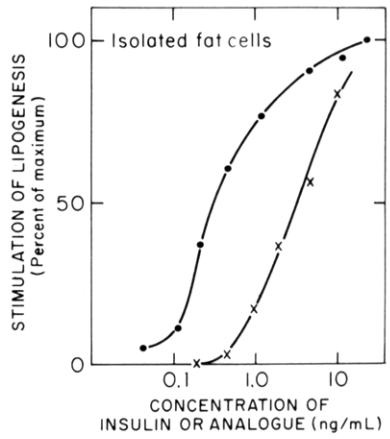

FIGURE 5: Effect of bovine insulin (●) and [Asn(NH_2)^{21}-A]insulin (×) on lipogenesis in isolated fat cells (see Experimental Procedures). The stimulation of lipogenesis, expressed as percent of maximum, is plotted as a function of the concentration of insulin and analogue. Data are from a typical experiment which was repeated 3 times.

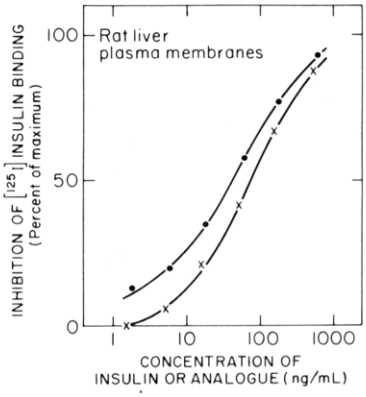

FIGURE 6: Effect of bovine insulin (●) and [Asn(NH_2)^{21}-A]insulin (×) on porcine [^{125}I]insulin binding to rat liver plasma membranes (see Experimental Procedures). The inhibition of [^{125}I]insulin binding, expressed as percent of maximum, is plotted as a function of the concentration of unlabeled insulin and analogue. Data are from a typical experiment which was repeated 3 times.

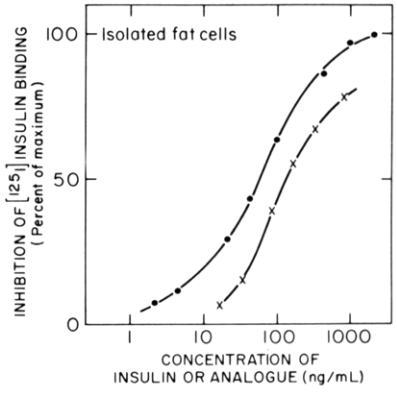

FIGURE 7: Effect of bovine insulin (●) and [Asn(NH_2)^{21}-A]insulin (×) on porcine [^{125}I]insulin binding to isolated fat cells (see Experimental Procedures). The inhibition of [^{125}I]insulin binding, expressed as percent of maximum, is plotted as a function of the concentration of unlabeled insulin and analogue. Data are from a typical experiment which was repeated 3 times.

insulin is ca. 12% in glucose oxidation and ca. 14.8% in lipogenesis, as compared to insulin. In insulin receptor binding assays this analogue was found to possess a relative potency of 63.9% in rat liver membranes and 51.4% in isolated fat cells as compared to the natural hormone. Representative dose–response curves are shown in Figures 6 and 7. Table III summarizes the results obtained in three or four separate

Table III: Relative Binding Affinity and in Vitro Biological Activity of Sheep [Asn(NH_2)^{21}-A]insulin

% of bovine insulin (mean ± SEM)			
receptor binding			
liver membranes	fat cells	glucose oxidation	lipogenesis
63.9 ± 3.7	51.4 ± 6.4	14.8 ± 4.0	12.0 ± 3.4

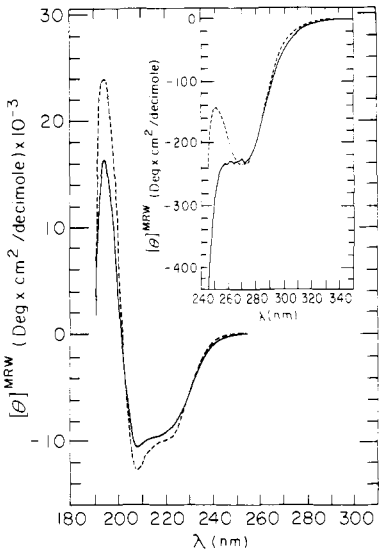

FIGURE 8: The near- and far-UV CD spectra of [Asn(NH_2)^{21}-A]-insulin (—) and zinc-free insulin (---) in 0.01 M phosphate buffer at pH 7.63 and 1.75 × 10^{-5} M concentrations.

experiments for each type of in vitro assay.

Circular Dichroism Studies. Figure 8 illustrates the near- and far-ultraviolet circular dichroic spectra of the analogue and zinc-free insulin. At pH 7.63, 1.74 × 10^{-5} M, the [Asn(NH_2)^{21}-A] analogue showed negative minima at 208 and 222 nm and a complex series of minima at 260–280 nm. Similar minima at 208 and 222 nm were observed for insulin under the same conditions. A distinct minimum at 275 nm and a shoulder at 282 and 268 nm are noted with insulin. The natural hormone displays a positive maximum at 194–195 nm; the analogue exhibits a slightly shifted maximum at 193–194 nm of lesser intensity. Figure 9 illustrates the far-ultraviolet spectrum of insulin and the analogue in 0.01 M HCl (pH 2.2) at the above concentration. Both compounds exhibit minima at 208 nm, and maxima are observed at 195 nm for insulin and 193–194 nm for the [Asn(NH_2)^{21}-A] analogue. Insulin at pH 2.2 displays a shallow minimum at 222 nm whereas the analogue exhibits a shoulder at this wavelength.

Discussion

In the last several years many insulin analogues have been prepared, either by modification of the natural hormone [for an early review, see Blundell et al. (1974)] or by chemical synthesis (Katsoyannis, 1979). By in vivo and/or in vitro assays, these analogues were found to exhibit potencies ranging from zero to values equal to that of the natural hormone. In general, with the analogues, the potencies obtained by an in vitro assay differed from those obtained by in vivo tests (Freychet et al., 1974; Cosmatos et al., 1978; Katsoyannis, 1979). This difference may be attributed to the multiplicity of processes (e.g., absorption, distribution, and degradation of the analogue as compared to insulin) involved in the in vivo assays. It was found, however, that for all these insulin analogues, their receptor binding affinities and in vitro bio-

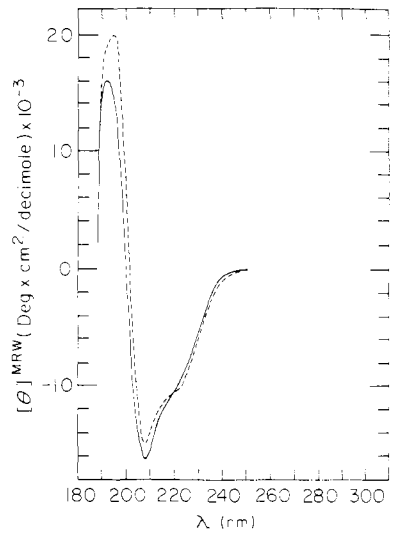

FIGURE 9: The far-UV CD spectra of [Asn(NH_2)^{21}-A]insulin (—) and zinc-free insulin (---) in 0.01 M HCl (pH 2.2) and 1.75 × 10^{-5} M concentrations.

logical potencies, relative to those of insulin, were the same. In addition, all biologically active analogues exhibited the same maximal response in the in vitro biological assays and receptor binding tests, and their dose–response curves were parallel to those of insulin. These data were generally interpreted to indicate that the biological activities of the analogues were wholly a consequence of their binding affinity to the insulin membrane receptors; thus insulin's "binding site" and the region associated with the initiation of cellular processes were indistinguishable (Freychet et al., 1974; Gliemann & Gammeltoft, 1974; Cosmatos et al., 1978).

The present investigation demonstrates that [Asn-(NH_2)^{21}-A]insulin behaves differently from all other modified insulins and suggests that our ideas regarding biological activity and receptor binding ability may need reevaluation. [Asn-(NH_2)^{21}-A]insulin, in contrast to all other modified insulins, displays a disparity between receptor binding activity and biological potency relative to native insulin. As shown in Figures 4 and 5, the synthetic analogue and the natural hormone have the same maximal activity in the in vitro assays and their dose–response curves are parallel. By the in vitro assays (stimulation of glucose oxidation and lipogenesis) the analogue exhibited activities of ca. 12 and 14.8%, respectively, compared to insulin. However, in insulin receptor binding assays, this analogue was found to possess a relative potency at least fourfold higher than the in vitro biological potency. In rat liver membranes (Figure 6) and in isolated fat cells (Figure 7) the analogue exhibited affinities of ca. 63.9 and 51.4%, respectively, compared to the natural hormone (Table III). It should be noted that [Asn(NH_2)^{21}-A]insulin, when assayed in vivo by the mouse convulsion test, displays a potency of ca. 72% that of native insulin. This might indicate partial amidolysis of the analogue in vivo, resulting in conversion to the natural hormone.

Emdin et al. (1977) have reported that hagfish insulin, like the [Asn(NH_2)^{21}-A] analogue, exhibits a disparity between in vitro biological activity and receptor binding affinity. They further report that when hagfish and porcine insulins are present at submaximally stimulating levels, they act in an additive manner with no evidence of antagonism. In several experiments (data not shown) we observed similar behavior for our analogue in the presence of bovine insulin. It should be noted that recently Muggeo et al. (1979) reported no divergence between biological activity and receptor binding

potency of hagfish insulin. These investigators suggest that further experiments are required to clarify this issue.

As mentioned above, the binding of the [Asn$(NH_2)^{21}$-A] analogue to the receptor is impaired. Evidently, the simple conversion of the A^{21} carboxyl group of insulin to a carboxamide alters the region of the hormone molecule which is involved in receptor binding. This is not surprising since a salt bridge between A^{21} asparagine and B^{22} arginine, indicated by the X-ray model of insulin (Blundell et al., 1974) and believed to contribute to the conformational integrity of the hormone, cannot be formed with the analogue. It has been suggested (Pullen et al., 1976; Blundell et al., 1974) that the B^{24}–B^{29} stretch of insulin is intimately involved in the binding of the hormone to its receptor in a manner similar to its involvement in dimerization. A comparison of the circular dichroic (CD) spectra of the [Asn$(NH_2)^{21}$-A] analogue and insulin suggests that it is in fact the B^{24}–B^{29} stretch which has undergone some conformational modification.

Figure 8 shows the near- and far-ultraviolet CD spectra of the [Asn$(NH_2)^{21}$-A] analogue and zinc-free insulin, at comparable concentrations (ca. 0.1 mg/mL) and pH 7.63. Figure 9 shows the far-ultraviolet spectra of this analogue and insulin in 0.01 N HCl (pH 2.2). The essential similarity of their CD spectra clearly indicates that there is no large overall conformational difference between the synthetic compound and the natural hormone. It is also apparent that their CD spectra exhibit small differences. CD studies of insulin and various analogues (Morris et al., 1968; Goldman & Carpenter, 1974; Strickland & Mercola, 1976) have demonstrated that in the 260–280-nm region, the negative ellipticity may be ascribed primarily to the aromatic tyrosyl and phenylalanyl residues, with the principal circular dichroic band for insulin at 274 nm attributed to the tyrosyl residues (Morris et al., 1968; Strickland & Mercola, 1976). The intensity and discreteness of this band have been shown to be directly related to the degree of association of monomeric insulin and analogues to dimers and hexamers (Strickland & Mercola, 1976; Wood et al., 1975). This association is, in turn, dependent on the presence and maintenance of the conformational integrity as a β strand of the B^{24}–B^{29} segment of the monomer (Goldman & Carpenter, 1974; Strickland & Mercola, 1976; Brugman & Arquilla, 1973; Brandenburg et al., 1972). It is apparent from Figure 8 that the most significant difference between the CD spectra of insulin and the [Asn$(NH_2)^{21}$-A] analogue is noted in the 274-nm region, indicative of a conformational modification in the B^{24}–B^{29} segment. The intensity of the dichroic band at 222 nm for insulin may, in large measure, be attributed to the β strands B^{2}–B^{7} and B^{24}–B^{29} (Quadrifoglio & Ury, 1968); the dichroic band at 208 nm primarily reflects the α-helical segments, A^{2-8}, A^{13-19}, and B^{9-19} (Blundell et al., 1974; Chen et al., 1974; Chou & Fasman, 1974). The ratio of ellipticities $[\Theta]_{208}/[\Theta]_{222}$ has been taken as a measure of the β-strand character of these segments in insulin analogues. The values reported (Wood et al., 1975; Yeung et al., 1979) for bovine insulin, 1.15–1.33 (pH ca. 7.8), may be compared with the much larger values, $\geq$1.50, found for either insulin analogues lacking the B^{24}–B^{29} segment or those analogues which do not dimerize or do so with difficulty. At pH 7.63, ratios of 1.18 and 1.31, and in 0.01 N HCl (pH 2.2), 1.59 and 1.46, were recorded for the [Asn$(NH_2)^{21}$-A]insulin and the natural hormone, respectively. These results are also consistent with the view that the B^{24}–B^{29} stretch of this analogue has undergone some conformational modification. The moderate but significant reduction in binding affinity of the analogue to the receptor probably results from this distortion of the β conformation of the B^{24}–B^{29} stretch.

It is now well established that insulin target tissues are maximally stimulated by concentrations of insulin and physiologically active analogues far lower than those required to displace tracer quantities of [^{125}I]insulin from insulin–receptor preparations derived from these tissues. In the present study, 50% maximal biological response is achieved with insulin at a calculated receptor occupancy of 0.2–0.5%; for the [Asn-$(NH_2)^{21}$-A] analogue, this figure is 2–3% (see Experimental Procedures).

Two models are considered to explain the discrepancy between binding and in vitro biological activity noted with the [Asn$(NH_2)^{21}$-A]insulin analogue. Both models accommodate the fact that relatively few of the available receptor sites need be occupied to achieve high activity. We recognize that other models may be postulated. We fell that the merit in presenting these models resides in illustrating that reasonable and substantially different interpretations of the relationship between the binding behavior and physiological activity of insulin may be accommodated. The first model assumes that (a) an analogue–receptor complex is not necessarily as effective as the insulin–receptor complex in generating the biological response and (b) occupancy of relatively few of the available receptor sites effects near-maximal biological activity and further filling of receptors results in little further increase in biological activity. This latter assumption, commonly known as the "spare-receptor" hypothesis, has often been invoked to account fot the observed disparity between receptor occupancy and hormone physiological activity [e.g., Kahn (1976)]. This model presumes that the conversion of the A^{21} carboxyl to a carboxamide group has had a dual effect: (1) it has reduced the binding affinity of the analogue for the receptor by distorting the B^{24}–B^{29} segment, and (2) it has impaired the efficiency of the analogue–receptor complex in initiating the physiological response. Therefore, to achieve a given biological response requires a greater concentration of analogue–receptor complex than is the case with insulin. A necessarily larger concentration of analogue is required than would be calculated on the basis of the reduced affinity of the analogue for the receptor to achieve this level of analogue–receptor complex. The A^{21} carboxyl group in this model plays a functional role in the expression of the biological activity of insulin, and its conversion to a carboxamide diminishes its effectiveness. With those analogues where no disparity is found between relative receptor binding affinity and biological potency, this model presumes that the analogue–receptor complex is equally effective in generating the biological response as is the insulin–receptor complex and thus the binding affinity becomes a direct measure of biological potency.

Implicit in this model is the assumption that interaction of receptor and hormone is followed by some chemical or physical change which initiates an easily saturable chain of events leading to the biological response. Ginsberg et al. (1976) and Krupp & Livingston (1979) have reported that interaction of insulin with its receptor results in the conversion of a high molecular weight species to a smaller form exhibiting altered insulin binding properties. If this dissociation occurs in vivo as part of the chain of events leading to insulin action, the [Asn$(NH_2)^{21}$-A] analogue might cause it to occur at a different rate or extent than is the case for insulin. This presumption allows for some degree of dissociation between relative biological potency and relative binding affinity of insulin vs. the analogue.

Larner et al. (1979) have described a material generated from muscle by insulin which they propose as a mediator for

the action of insulin. If these findings prove general, they represent another possible mechanism for the dissociation of binding affinity and biological activity displayed by [Asn-$(NH_2)^{21}$-A]insulin.

The second model accepts the commonly held view that binding alone dictates activity. It assumes that the insulin-responsive cell membrane contains two classes of receptors, A and B, to which the hormone may bind (Hollenberg & Cuatrecasas, 1976). One class of receptors, say A, when occupied by hormone is physiologically much more active than class B. The affinity of the hormone (insulin or analogue) to the A receptors is much greater than that to the B receptors, and the number of A receptors is substantially smaller than the number of B receptors. It then follows that binding studies of receptor and hormone yield only the "affinity (or dissociation) constants" of the class B receptors. Physiological responses observed at substantially lower concentrations of hormone than those employed in binding studies are taken as a measure of binding affinity to class A receptors. When the experimentally determined ratio of "dissociation constants", $K_{insulin}/K_{analogue}$, to the class B sites is the same as that which, if determined directly by binding studies, would be found for class A sites, then and only then would the experimentally observed ratio of concentrations, [insulin]/[analogue], which effect equivalent physiological response, be equal to the $K_{insulin}/K_{analogue}$ ratio determined by binding studies. This appears to be the case for most analogues investigated. It is not so with [Asn$(NH_2)^{21}$-A]insulin. On the basis of this model, the binding of the [Asn$(NH_2)^{21}$-A] analogue to class A sites is considerably weaker than that which would be predicted on the basis of its binding affinity to class B receptors, and consequently a given physiological response requires a higher concentration of analogue than binding affinity predicts. Our data do not permit us to make a definite choice between these models.

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Evidence for a Physiological Role of Corticosteroid Binder IB†

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ABSTRACT: Glucocorticoid binding proteins in liver and kidney of adrenalectomized rats have been analyzed by rapid ion-exchange chromatography, gel filtration, and specific interactions with antibodies against purified liver cytosol receptor and purified transcortin. In both tissues, the unactivated form of the [^{3}H]triamcinolone acetonide–receptor complex is eluted by 0.4–0.5 M KCl from DEAE-Sephadex minicolumns. After heat activation, the major form in liver cytosol, corticosteroid binder II, is eluted by 0.2 M KCl from the DEAE minicolumn; a minor component in the buffer wash is identified as corticosteroid binder IB. Binders II and IB are shown to be DNA-binding proteins whereas the unactivated forms do not bind to DNA. In contrast, the major activated form in kidney cytosol has the properties of corticosteroid binder IB (e.g., it is eluted in the buffer wash of the DEAE minicolumn, is a DNA-binding protein, and has a Stokes radius of 20–26 Å). Evidence is presented which suggests that IB is not a proteolytic product of II. Antibody to highly purified rat liver glucocorticoid receptor was immobilized on Sepharose 4B-CL. The major unactivated and activated [^{3}H]triamcinolone acetonide–receptor complexes from rat liver are adsorbed by the immunoaffinity column. Liver binder IB, however, is not adsorbed. The major unactivated and activated forms in kidney are not adsorbed by the antibody column; thus, both rapid ion-exchange and immunoaffinity chromatography suggest that the kidney form is similar to liver IB. Kidney cortex was separated from the medulla. The medulla contained corticosteroid binder II as defined by ion-exchange and immunoreactivity. This small quantity of medulla binder II is masked when cytosols are prepared from the whole kidney. In cytosols from heart, thymus, brain, and muscle, the receptor is similar to the liver and kidney medulla receptors. Further analysis of corticosteroid-binding proteins in kidney was carried out by using an antibody to purified serum transcortin. The kidney contains a large quantity of transcortin-like protein that binds [^{3}H]corticosterone. After removal of this protein by immunoprecipitation, the remaining receptor in kidney has the properties of corticosteroid binder IB. Taken together, these results show that binder IB may be a second glucocorticoid receptor in liver and the only glucocorticoid receptor in kidney cortex.

Glucocorticoids regulate gene expression in eukaryotic cells via a two-step mechanism common for all steroid hormones (Litwack et al., 1973; Kalimi et al., 1973; Rousseau et al., 1973). A complex of steroid with a specific receptor protein forms in the cytoplasm of the target cell (Litwack et al., 1965; Baxter & Tomkins, 1971; Beato et al., 1971). The hormone–receptor complex must undergo activation to enable translocation to the nucleus (Litwack et al., 1973; Kalimi et al., 1973; Baxter et al., 1972; Higgins et al., 1973; Beato et al., 1973; Wira & Munck, 1974; Atger & Milgrom, 1976) and binding to chromatin (Simons et al., 1976) or in vitro to purified DNA (Baxter et al., 1972; Wira & Munck, 1974; Milgrom et al., 1973; Cake et al., 1978).

Although numerous reports exist on specific glucocorticoid receptors in various target tissues, the few comparative studies which have been done (Ballard et al., 1974; Acs et al., 1975; Lippmann & Thompson, 1974; Feldman et al., 1978) suggest that receptors in different target organs are similar. Deciding whether receptors from different targets are identical is important to the understanding of the general mechanism of steroid hormone action. Kidney was selected in addition to the liver, which is a well characterized target tissue for glucocorticoids (Litwack et al., 1965). Besides its role as a target tissue for mineralocorticoids, the kidney displays gluconeogenic activity (Krebs et al., 1963) which is responsive to administered glucocorticoids (Londau, 1960). Specific glucocorticoid receptors have been identified in kidney cytosol (Rousseau et al., 1972; Funder et al., 1973). The results here indicate the existence of a distinctive and identical glucocorticoid receptor type in all the tissues studied except for kidney cortex, whose

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