Synthesis of a Biologically Active Insulin Analog Lacking the Intrachain Cyclic System†

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ABSTRACT: The synthesis and isolation in purified form of a biologically active analog of insulin are described. This analog differs from the parent molecule in that the intrachain disulfide system of the A chain (A^6–A^{11}) is missing. For the synthesis of this compound an A-chain analog, in which the cysteine residues at positions A^6 and A^{11} were replaced with alanine residues, was prepared and isolated as the S-sulfonated derivative. Conversion of the latter product to the sulfhydryl form and combination with the S-sulfonated B chain of insulin yielded the [$Ala^{6,11}$-A]insulin. This insulin analog was isolated in purified form by chromatography on a carboxymethylcellulose column with an exponential sodium chloride gradient. [$Ala^{6,11}$-A]insulin possesses a potency ranging from 2 to 2.5 IU per mg when assayed by the mouse convulsion method or by radioimmunoassay. This finding indicates that the intrachain disulfide system does not participate functionally in the mechanism of action of insulin and that it plays an important, but not essential, role in the manifestation of the biological activity of insulin.

Studies are under way in our laboratory directed to the synthesis of analogs of insulin in an attempt to elucidate possible correlations between chemical structure and biological function in this hormone. Our efforts thus far have been directed to the determination of the contribution of amino acid residues located at the amino- and carboxyl-terminal regions of the A and B chains, respectively, to the expression of the biological activity of insulin (Katsoyannis *et al.*, 1971; Katsoyannis and Zalut, 1972a,b). These analogs, and the analogs prepared in other laboratories (for earlier reviews, see Carpenter, 1966, and Lübke and Klostermeyer, 1970), were obtained by the replacement of certain constituent amino acid residues by other natural amino acids, by the replacement or modification of functional groups, or by the deletion of amino acid residues from the primary structure of insulin. The present report describes the synthesis of an analog with drastically altered structural features as compared with the parent insulin molecule. In this analog, the intrachain ring system in the A chain (A^6–A^{11}) has been eliminated by the replacement of the cysteine residues at positions A^6 and A^{11} with alanine residues.

Experimental Procedures and Results

Materials and Techniques. Capillary melting points were determined for all peptide derivatives and are not corrected. Optical rotations were taken with a Zeiss photoelectric precision polarimeter. Thin-layer chromatography (tlc) was performed on 6060 silica gel (Eastman Chromagram Sheet, Eastman Kodak Co., Rochester, N. Y.) except where otherwise indicated in the experimental part. The following solvent systems were used: (A) 1-butanol–acetic acid–water (4:1:5), (B) 1-butanol–acetic acid–pyridine–water (4:1:1:2), (C) 1-butanol–acetic acid–pyridine–water (30:6:20:24), and (D) chloroform–methanol–water (40:15:5) (lower phase). Thin-layer electrophoresis was performed by a method developed in this laboratory (Tometsko and Delihas, 1967) and was carried out with a Wieland-Pfleiderer pherograph (Brinkman Instruments). Amino acid analyses were performed in a Beckman-Spinco amino acid analyzer (Model 120C) equipped with a digital readout system (Model CRS 12AB, Infotronics Corp., Houston, Texas) according to the method of Spackman *et al.* (1958). Acid hydrolysis and calculations of molar ratios were carried out as described previously (Katsoyannis *et al.*, 1967a). 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. Protein determinations were carried out by the method of Lowry *et al.* (1951). Biological assays were carried out by the mouse convulsion method as has been described previously (Katsoyannis and Tometsko, 1966; Katsoyannis *et al.*, 1967c). For radioimmunoassays the method of Hales and Randle (1963) was employed using an "insulin immunoassay kit" (Amersham/Searle Co.). Preswollen microgranular CM-cellulose (Whatman CM 52/1) and Sephadex G-15 (Pharmacia Uppsala) were used in this investigation. Crystalline bovine insulin was generously provided by Eli Lilly and Co. Sodium tetrathionate was prepared as described by Gilman *et al.* (1946).

Synthesis of Sheep [$Ala^{6,11}$-A]Insulin. This analog was prepared by the combination of the sulfhydryl form of the $Ala^{6,11}$-A chain of sheep insulin with the S-sulfonated derivative of the B chain of bovine (sheep, porcine) insulin. The synthesis of $Ala^{6,11}$-A chain in the protected form (XVIIa) was accomplished by a combination of the stepwise elongation and fragment condensation methods of peptide synthesis (for a review, see Hofmann and Katsoyannis, 1963). The key intermediate in this approach was the protected heptadecapeptide (XVI) (sequence 5–21) which contains the C-terminal sequence of the sheep A chain with the cysteine residues of positions 6 and 11 replaced with alanine. Synthesis of this heptadecapeptide derivative was accomplished by the azide coupling of the N-terminal pentapeptide fragment (XV) (sequence 5–9) with the C-terminal dodecapeptide moiety

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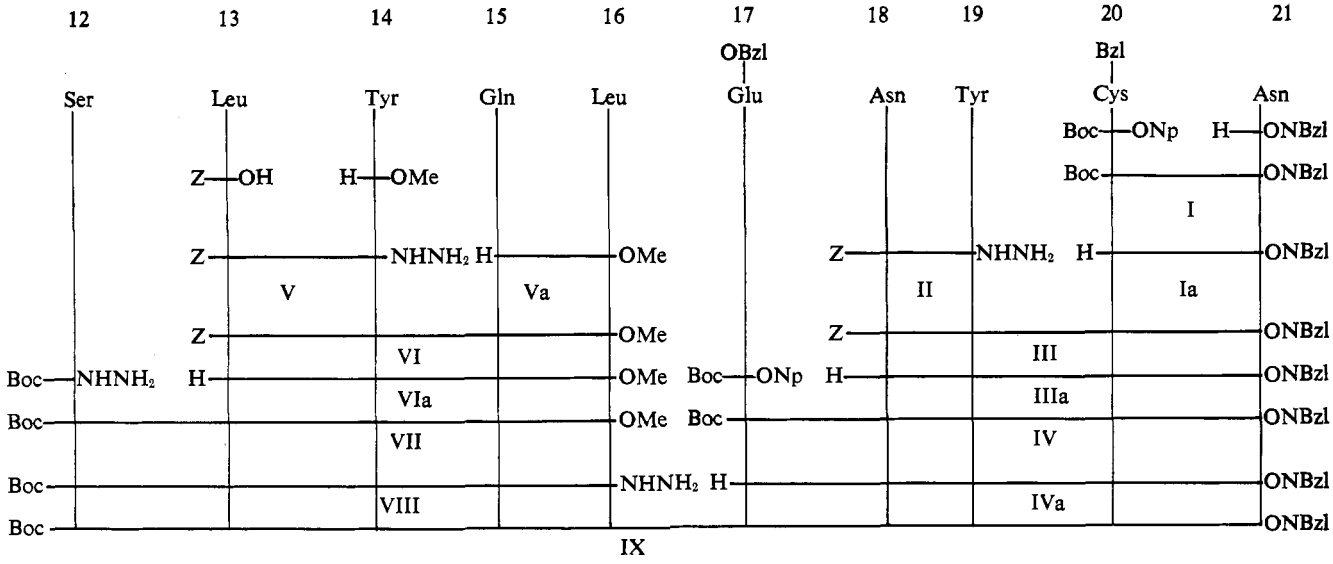
CHART I



(XIIa) (sequence 10–21). The latter compound was constructed by the azide coupling of the C-terminal decapeptide fragment (IXa) (sequence 12–21) with the adjacent dipeptide derivative (XI) (sequence 10–11) followed by selective deblocking at the amino end of the resulting protected peptide (XII). The final synthetic step in the construction of the protected Ala^{6,11}-A chain (XVIIa) involved the removal of the amino- and γ-carboxyl-protecting groups from the blocked heptadecapeptide XVI and the azide coupling of the resulting partially protected derivative (XVIa) with the N-terminal tetrapeptide fragment (XVIb) (sequence 1–4). The blocking of the functional groups of the constituent amino acid residues and the synthesis of the various intermediates were carried out by the standard procedures of peptide chemistry. Upon treatment with trifluoracetic acid the *tert*-butyl ester protecting group was removed from the protected chain XVIIa. Removal of the remaining blocking groups, namely, benzyloxycarbonyl, *S*-benzyl, and *p*-nitrobenzyl ester, from the resulting XVII was accomplished on exposure to sodium in liquid ammonia (Sifferd and du Vigneaud, 1935). The deblocked Ala^{6,11}-A chain on oxidative sulfitolysis (Bailey and Cole, 1959) was converted to the S-sulfonated derivative (XVIII) which, prior to its combination with the S-sulfonated B chain, is transformed to the sulfhydryl form upon exposure to 2-mercaptoethanol. Chart I[1] illustrates the scheme used in the synthesis of the C-terminal-protected decapeptide IX. The overall scheme which was employed for the synthesis of the S-sulfonated Ala^{6,11}-A chain of sheep insulin is summarized in Chart II.

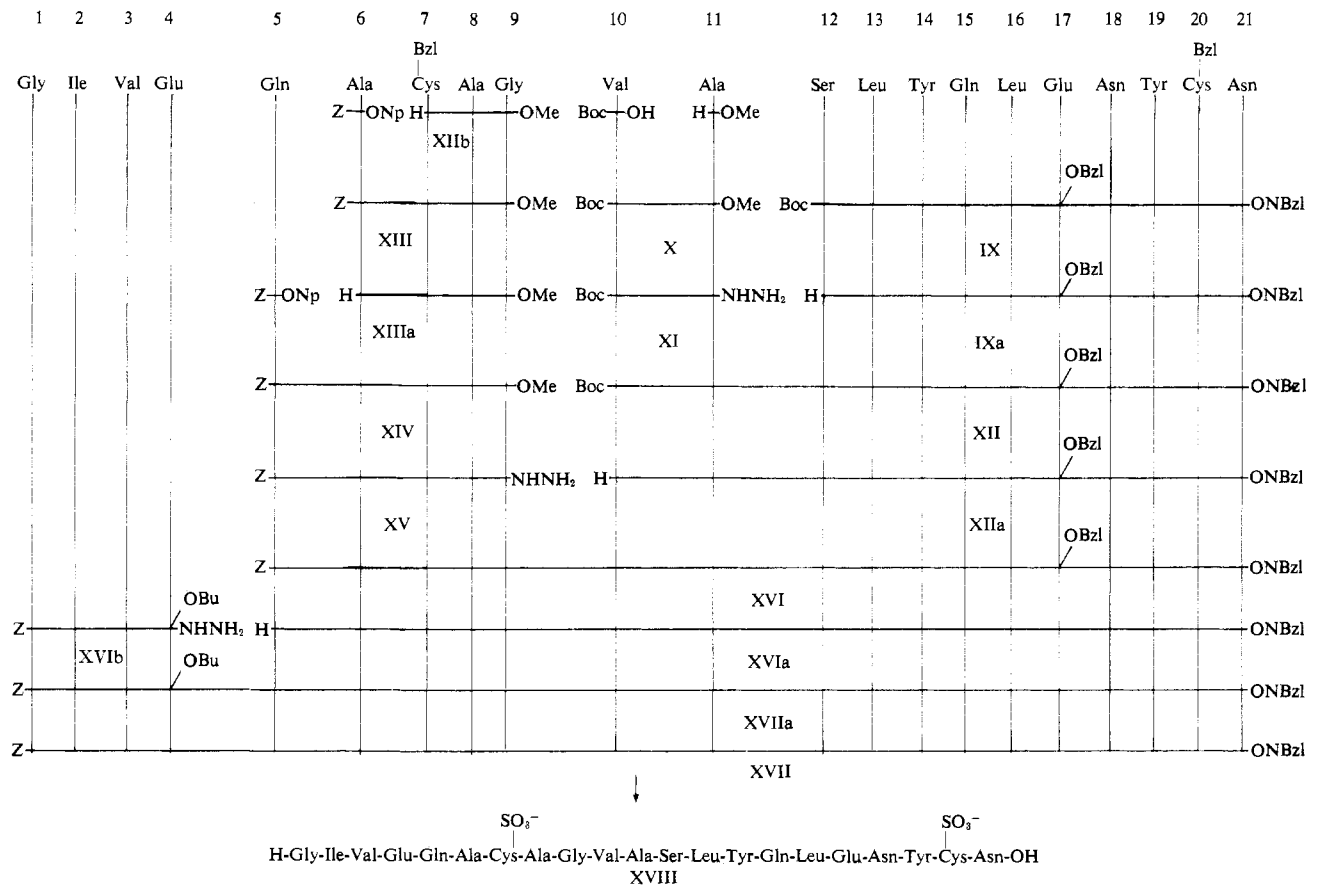
*N-tert-Butyloxycarbonyl-S-benzyl-*L*-cysteinyl-*L*-asparagine p-Nitrobenzyl Ester (I).* A suspension of *N*-benzyloxycarbonyl-L-asparagine *p*-nitrobenzyl ester (24.2 g; Katsoyannis *et al.*, 1966a) in acetic acid (100 ml) was treated with 4 N HBr in acetic acid (120 ml). After 2 hr at room temperature the reaction mixture was poured into ether (800 ml) and the precipitated hydrobromide was filtered off, washed with ether, and dried over KOH *in vacuo*. To a solution of this product in dimethylformamide (100 ml), triethylamine (10 ml) was added, followed by *N-tert*-butyloxycarbonyl-*S*-benzyl-L-cysteine *p*-nitrophenyl ester (26.4 g; Hörnle, 1967). After 24 hr, the reaction mixture was concentrated under reduced pressure to a small volume. Addition of ethyl acetate (100 ml) and water (100 ml) caused the crystallization of the product which was isolated and recrystallized from ethanol; wt 22.6 g (67%), mp 154–155°, $[\alpha]_D^{26}$ −37.8° (*c* 1, dimethylformamide). After deblocking with trifluoroacetic acid, the peptide showed a single spot on thin-layer chromatography (tlc) in systems A and D. *Anal.* Calcd for $C_{26}H_{32}N_4O_8S$: C, 55.6; H, 5.75; N, 10.0. Found: C, 55.8; H, 5.69; N, 10.3.

*N-Benzyloxycarbonyl-*L*-asparaginyl-*L*-tyrosine Hydrazide (II).* A solution of *N*-benzyloxycarbonyl-L-asparaginyl-L-tyrosine ethyl ester (25.4 g; Katsoyannis and Suzuki, 1961) in dimethylformamide (75 ml) was treated with hydrazine hydrate (11 ml). After 24 hr at room temperature, the precipitated crystalline product was collected and washed with methanol; wt 24 g (98%), mp 272–273° dec, $[\alpha]_D^{26}$ +4.6° (*c* 1, Me_2SO). After HBr in acetic acid deblocking, the peptide showed a single spot on tlc in systems A and B. *Anal.* Calcd for $C_{21}H_{25}N_5O_6$: C, 56.9; H, 5.68; N, 15.8. Found: C, 57.2; H, 5.80; N, 16.1.

*N-Benzyloxycarbonyl-*L*-asparaginyl-*L*-tyrosyl-S-benzyl-*L*-cysteinyl-*L*-asparagine p-Nitrobenzyl Ester (III).* A solution of I (11.2 g) in trifluoroacetic acid (25 ml) was stored at room temperature for 1 hr and then poured into ether (1000 ml). The precipitated deblocked dipeptide derivative (Ia) was collected, washed with ether and dried over KOH *in vacuo*. A solution of this product in dimethylformamide (30 ml) cooled to 0° was neutralized with triethylamine (5.6 ml) and added to a solution of the dipeptide azide prepared as follows. Compound II (8.87 g) was dissolved in dimethylformamide (35 ml) containing 1 N HCl (42 ml). After cooling this solution to −10°, isoamyl nitrite (2.7 ml) was added. The reaction mixture was stirred for 5 min at −10°, cooled to −30°, and then neutralized with triethylamine (5.6 ml) prior to the addition of the solution of the deblocked dipeptide Ia prepared as described previously. After 48 hr at 4°, the reaction mixture was concentrated under reduced pressure to a small volume. Addition of methanol caused the precipitation of the product, which was collected, washed with methanol, and reprecipitated from dimethylformamide–methanol; wt 17 g (97%), mp 225–227°, $[\alpha]_D^{26}$ −40.0° (*c* 1, dimethylformamide). After HBr in acetic acid treatment, the product showed a

[1] Abbreviations used are: NBzl, *p*-nitrobenzyl; Boc, *tert*-butyloxycarbonyl; Z, benzyloxycarbonyl; Me, methyl; Bzl, benzyl; Np, *p*-nitrophenyl; Bu, *tert*-butyl.

CHART II



single spot on tlc in systems A and D. *Anal.* Calcd for $C_{42}H_{45}N_7O_{12}S$: C, 57.8; H, 5.20; N, 11.3. Found: C, 57.9; H, 5.45; N, 11.1. This tetrapeptide derivative has been prepared previously by a less satisfactory method (Katsoyannis *et al.*, 1966a).

N-tert-Butyloxycarbonyl-γ-benzyl-L-glutamyl-L-asparaginyl-L-tyrosyl-S-benzyl-L-cysteinyl-L-asparagine p-Nitrobenzyl Ester (IV). A suspension of III (5.8 g) in acetic acid (30 ml) was treated with 4 N HBr in acetic acid (30 ml). After 2 hr at room temperature, the resulting solution was poured into ether and the precipitated product (IIa) was collected, washed with ether, and dried over KOH *in vacuo*. To a solution of this material in dimethylformamide (30 ml) containing triethylamine (1.9 ml) was added *N-tert*-butyloxycarbonyl-γ-benzyl-L-glutamic acid *p*-nitrophenyl ester (3.64 g; Li *et al.*, 1963). After 24 hr at room temperature, the reaction mixture was concentrated under reduced pressure to a small volume and mixed with methanol (10 ml). Addition of water (30 ml) to this solution caused the precipitation of the product which was isolated and washed with warm methanol: wt 5.4 g (78%), mp 193–195°, $[\alpha]_D^{26}$ −39.6° (*c* 1, dimethylformamide). After trifluoroacetic acid treatment, the partially protected pentapeptide showed a single spot on tlc in systems A and D. *Anal.* Calcd for $C_{50}H_{60}N_8O_{15}S$: C, 57.5; H, 5.79; N, 10.7. Found: C, 57.3; H, 5.61; N, 10.5. Amino acid analysis after acid hydrolysis showed the following composition expressed in molar ratios: $Asp_{1.9}Glu_{1.0}Tyr_{0.7}$*S*-benzylcysteine$_{0.7}$ (average amino acid recovery 95%).

N-Benzyloxycarbonyl-L-leucyl-L-tyrosine Hydrazide (V). A solution of tyrosine methyl ester hydrochloride (23.1 g) in methanol (30 ml) was treated with triethylamine (14 ml). The precipitated triethylamine hydrochloride was filtered off and the filtrate was concentrated to dryness *in vacuo*. To a solution of this residue in dimethylformamide (150 ml) cooled to −10° *N*-benzyloxycarbonyl-L-leucine (26.5 g) was added followed by *N,N'*-dicyclohexylcarbodiimide (24.8 g). After 24 hr at room temperature, the precipitated by-product was filtered off and the filtrate was concentrated to dryness under reduced pressure. The residue was dissolved in a mixture of ethyl acetate (300 ml) and water (100 ml). The organic phase was washed successively with 1 N HCl, 1 M Na_2CO_3, and water and was dried over $MgSO_4$. The oily residue obtained after evaporation of the solvent under reduced pressure was dissolved in methanol (50 ml) and treated with hydrazine hydrate (15 ml). After 24 hr, the precipitated crystalline dipeptide hydrazide was collected and recrystallized from methanol: wt 23 g (50%), mp 195–197°, $[\alpha]_D^{26}$ −22.4° (*c* 1, dimethylformamide). *Anal.* Calcd for $C_{23}H_{30}N_4O_5$: C, 62.4; H, 6.83; N, 12.7. Found: C, 62.5; H, 6.90; N, 12.9.

N-Benzyloxycarbonyl-L-leucyl-L-tyrosyl-L-glutaminyl-L-leucine Methyl Ester (VI). A solution of *N*-benzyloxycarbonyl-L-glutaminyl-L-leucine methyl ester (8.3 g; Katsoyannis *et al.*, 1963) in acetic acid (30 ml) was mixed with 4 N HBr in acetic acid (30 ml). After 1 hr, the reaction mixture was diluted with ether (600 ml) and the precipitated product was collected, washed with ether, and dried *in vacuo*. A solution of this solid in dimethylformamide (35 ml) cooled to 0° was neutralized with triethylamine (5.6 ml) and added to a solution of the dipeptide azide prepared as follows. Compound V (8.85 g) was dissolved in dimethylformamide (20 ml) containing 1 N

HCl (40 ml) and the solution was cooled to −10° and mixed with isoamyl nitrite (2.7 ml). After stirring for 5 min at −10°, the reaction mixture was cooled to −30°, neutralized with triethylamine (5.6 ml), and diluted with the solution of the deblocked dipeptide ester prepared as described previously. The reaction mixture was stirred at 4° for 48 hr and concentrated under reduced pressure to a small volume. Addition of 1 N HCl to the residue caused the precipitation of the product which was collected, washed with water, and crystallized from aqueous ethanol: wt 11.7 g (85%), mp 238–240°, $[\alpha]_D^{26}$ −28.5° (*c* 1, dimethylformamide). The HBr in acetic acid deblocked peptide showed a single spot on tlc in systems A and D. *Anal.* Calcd for $C_{35}H_{49}N_5O_9$: C, 61.5; H, 7.22; N, 10.2. Found: C, 61.6; H, 7.33; N, 9.9. The same compound has been prepared previously (Katsoyannis *et al.*, 1963) by the stepwise method.

*N-tert-Butyloxycarbonyl-*L*-seryl-*L*-leucyl-*L*-tyrosyl-*L*-glutaminyl-*L*-leucine Methyl Ester (VII).* Compound VI (11 g) was suspended in acetic acid (30 ml) and treated with 4 N HBr in acetic acid (30 ml). After 1 hr at room temperature the reaction mixture was diluted with ether (800 ml) and the precipitated deblocked tetrapeptide VIa was isolated, washed with ether, and dried over KOH *in vacuo*. To a cold solution of this solid in dimethylformamide (25 ml) containing triethylamine (4.5 ml), *N-tert*-butyloxycarbonyl-L-serine azide was added prepared as follows. *N-tert*-Butyloxycarbonyl-L-serine hydrazide (3.94 g; Iselin and Schwyzer, 1961) was dissolved in dimethylformamide (10 ml) and to this solution cooled to −10° 1 N HCl in dimethylformamide (38 ml) was added followed by isoamyl nitrite (2.42 ml). After 5 min at −10°, the reaction mixture was cooled to −30°, neutralized with triethylamine (5.05 ml), and added to the solution of the tetrapeptide derivative VIa prepared as described above. The reaction mixture was stirred at 4° for 48 hr and concentrated under reduced pressure to dryness. The residue was dissolved in ethyl acetate (300 ml) and water (100 ml). The organic phase was washed successively with 10% citric acid solution and water and dried over $MgSO_4$. The solid obtained after evaporation of the solvent under reduced pressure was reprecipitated from aqueous ethanol: wt 9.5 g (79%), mp 215–216° dec, $[\alpha]_D^{26}$ −38.3° (*c* 1, dimethylformamide). After deblocking with trifluoroacetic acid, the peptide showed a single spot on tlc in systems A and D. *Anal.* Calcd for $C_{35}H_{56}N_6O_{11}\cdot H_2O$: C, 55.7; H, 7.74; N, 11.1; O, 25.4. Found: C, 55.5; H, 7.69; N, 11.0; O, 25.6. Amino acid analysis of an acid hydrolysate showed the following composition expressed in molar ratios: $Ser_{0.9}Glu_{1.0}Leu_{2.0}Tyr_{1.0}$ (average amino acid recovery 95%).

*N-tert-Butyloxycarbonyl-*L*-seryl-*L*-leucyl-*L*-tyrosyl-*L*-glutaminyl-*L*-leucine Hydrazide (VIII).* A solution of VII (9.5 g) in methanol (70 ml) was treated with hydrazine hydrate (2.9 ml). After 48 hr at room temperature, the precipitated product was collected and reprecipitated from dimethylformamide–methanol–ether: wt 8.2 g (86%), mp 232–233° dec, $[\alpha]_D^{26}$ −21.8° (*c* 1, dimethylformamide). *Anal.* Calcd for $C_{34}H_{56}N_8O_{10}$: C, 55.4; H, 7.66; N, 15.2. Found: C, 55.3; H, 7.94; N, 15.3.

*N-tert-Butyloxycarbonyl-*L*-seryl-*L*-leucyl-*L*-tyrosyl-*L*-glutaminyl-*L*-leucyl-γ-benzyl-*L*-glutamyl-*L*-asparaginyl-*L*-tyrosyl-S-benzyl-*L*-cysteinyl-*L*-asparagine p-Nitrobenzyl Ester (IX).* A solution of IV (3.14 g) in trifluoroacetic acid (11 ml) was stored at room temperature for 1 hr and then diluted with ether (500 ml). The precipitated deblocked pentapeptide (IVa) was isolated, washed with ether, and dried over KOH *in vacuo*. A solution of this material in dimethylformamide (10 ml) cooled to 0° was neutralized with triethylamine (0.64 ml) and added to a solution of the pentapeptide azide prepared as follows. The protected pentapeptide hydrazide VIII (2.2 g) was dissolved in dimethylformamide (10 ml) and to this solution cooled to −10° 1 N HCl in dimethylformamide (6.6 ml) was added followed by isoamyl nitrite (0.4 ml). After 5 min at −10°, the mixture was cooled to −30°, neutralized with triethylamine (0.84 ml), and added to the solution of the pentapeptide ester IVa prepared as described previously. After 48 hr at 4°, the reaction solution was concentrated under reduced pressure to dryness and the residue mixed with water. The solid product was isolated and reprecipitated from dimethylformamide–methanol: wt 7.4 g (74%), mp 223–225° dec, $[\alpha]_D^{26}$ −38.1° (*c* 1, dimethylformamide). After deblocking with trifluoroacetic acid, the decapeptide ester showed single spot on tlc in systems A and D. *Anal.* Calcd for $C_{80}H_{104}N_{14}O_{23}S$: C, 57.8; H, 6.31; N, 11.8. Found: C, 57.6; H, 6.54; N, 11.5. Amino acid analysis of an acid hydrolysate showed the following composition expressed in molar ratios: $Asp_{2.0}Ser_{0.8}Glu_{2.0}Leu_{2.0}Tyr_{1.8}$*S*-benzylcysteine$_{0.7}$ (average amino acid recovery 88%).

*N-tert-Butyloxycarbonyl-*L*-valyl-*L*-alanine Methyl Ester (X).* A solution of *N-tert*-butyloxycarbonyl-L-valine (20.7 g; Anderson and McGregor, 1957) in freshly distilled tetrahydrofuran (100 ml) containing triethylamine (14 ml) was cooled to −10° and isobutyl chlorocarbonate (13.4 ml) was added. After 15 min at −10°, a cold solution of L-alanine methyl ester in dimethylformamide (50 ml) (prepared from 13.9 g of alanine methyl ester hydrochloride and 14 ml of triethylamine as described for tyrosine methyl ester in the synthesis of V) was added. The reaction mixture was stirred at 0° for 30 min and at room temperature for 3 hr and evaporated to dryness *in vacuo*. The residue was dissolved in ethyl acetate (300 ml) and water (100 ml). The organic layer was separated, washed successively with water, 1 M Na_2CO_3, and water, dried over $MgSO_4$, and concentrated to a small volume *in vacuo*. Upon addition of petroleum ether (bp 30–60°) the product crystallized out: wt 14 g (47%), mp 141–142°, $[\alpha]_D^{26}$ −52.9° (*c* 1, methanol). *Anal.* Calcd for $C_{14}H_{26}N_2O_5$: C, 55.6; H, 8.68; N, 9.3. Found: C, 55.8; H, 8.68; N, 8.8.

*N-tert-Butyloxycarbonyl-*L*-valyl-*L*-alanine Hydrazide (XI).* A solution of X (13 g) in methanol (50 ml) was treated with hydrazine hydrate (6 ml). Evaporation of the solvent, to a small volume, and addition of ether caused the crystallization of the product which was collected and recrystallized from methanol–ether: wt 8 g (62%), mp 122–123°, $[\alpha]_D^{26}$ −54.4° (*c* 1, methanol). *Anal.* Calcd for $C_{13}H_{26}N_4O_4$: C, 51.6; H, 8.67; N, 18.5. Found: C, 51.4; H, 8.72; N, 18.3.

*N-tert-Butyloxycarbonyl-*L*-valyl-*L*-alanyl-*L*-seryl-*L*-leucyl-*L*-tyrosyl-*L*-glutaminyl-*L*-leucyl-γ-benzyl-*L*-glutamyl-*L*-asparaginyl-*L*-tyrosyl-S-benzyl-*L*-cysteinyl-*L*-asparagine p-Nitrobenzyl Ester (XII).* A solution of the protected decapeptide IX (2.22 g) in trifluoroacetic acid (7 ml) was stored at room temperature for 1 hr. Upon addition of ether (400 ml) the partially deblocked decapeptide IXa was precipitated, collected, washed with ether, and dried over KOH *in vacuo*. This product was dissolved in dimethylformamide (15 ml), cooled to 0° and then neutralized with triethylamine (0.38 ml) just prior to the addition of the dipeptide azide prepared as follows. To a solution of the dipeptide hydrazide XI (0.45 g) in dimethylformamide (7 ml) cooled to −15° was added 1 N HCl in dimethylformamide (3.2 ml) followed by isoamyl nitrite (0.21 ml). After 5 min triethylamine (0.42 ml) was

added and the solution was mixed with the decapeptide ester prepared as described above. The reaction mixture was stirred for 48 hr at 4°, concentrated under reduced pressure to a small volume and mixed with 50% (v/v) aqueous methanol (200 ml). The precipitated protected dodecapeptide was collected, washed with water, and triturated with warm methanol: wt 1.97 g (81%), mp 233–235° dec, $[\alpha]_D^{26}$ −40.1° (*c* 1, dimethylformamide). After deblocking with trifluoroacetic acid, the peptide showed a single spot on tlc in systems A and D. *Anal.* Calcd for $C_{88}H_{118}N_{16}O_{25}S$: C, 57.7; H, 6.49; N, 12.2. Found: C, 57.4; H, 6.72; N, 12.1. Amino acid analysis of an acid hydrolysate showed the following composition expressed in molar ratios: $Asp_{2.0}Ser_{0.8}Glu_{2.1}Ala_{1.0}Val_{1.0}Leu_{2.0}Tyr_{1.5}$*S*-benzylcysteine$_{0.7}$ (average amino acid recovery 91%).

*N-Benzyloxycarbonyl-*L*-alanyl-S-benzyl-*L*-cysteinyl-*L*-alanylglycine Methyl Ester (XIII).* A suspension of *N*-benzyloxycarbonyl-*S*-benzyl-L-cysteinyl-L-alanylglycine methyl ester (15 g; Katsoyannnis *et al.*, 1966b) in acetic acid (100 ml) was treated with 4 N HBr in acetic acid (100 ml). After 1 hr, the resulting solution was poured into ether (1000 ml) and the precipitated hydrobromide of the partially protected tripeptide XIIb was isolated, washed with ether, and dried over KOH *in vacuo*. To a cooled (0°) solution of this product in dimethylformamide (120 ml), triethylamine (6.9 ml) was added followed by *N*-benzyloxycarbonyl-L-alanine *p*-nitrophenyl ester (9.9 g; Goodman and Stueben, 1959). After 24 hr at room temperature, the reaction mixture was poured into cold 1 N NH_4OH (800 ml). The precipitated product was collected by filtration, washed successively with 1 N NH_4OH, water, 1 N HCl, and water, dried, and reprecipitated from dimethylformamide–water: wt 12.9 g (88%), mp 215–217°, $[\alpha]_D^{26}$ −25.2° (*c* 1, dimethylformamide). After HBr in acetic acid treatment, the deblocked peptide showed a single spot on tlc in systems A and B. *Anal.* Calcd for $C_{27}H_{34}N_4O_7S$: C, 58.1; H, 6.09; N, 10.0. Found: C, 58.0; H, 6.08; N, 9.9.

*N-Benzyloxycarbonyl-*L*-glutaminyl-*L*-alanyl-S-benzyl-*L*-cysteinyl-*L*-alanylglycine Methyl Ester (XIV).* Compound XIII (12.9 g) was suspended in acetic acid (75 ml) and treated with 4 N HBr in acetic acid (75 ml). After 1 hr, the resulting solution was poured into ether (1000 ml) and the precipitated hydrobromide of the tetrapeptide ester (XIIIa) was isolated, washed with ether, and dried over KOH *in vacuo*. This material was dissolved in dimethylformamide (170 ml) and to this solution was added triethylamine (7 ml), followed by *N*-benzyloxycarbonyl-L-glutamine *p*-nitrophenyl ester (9.5 g; Bodanszky and du Vigneaud, 1959). After 24 hr, the reaction mixture was poured into cold 1 N NH_4OH (1000 ml), and the precipitated product was washed successively with 1 N NH_4OH, water, 1 N HCl, and water, dried, and reprecipitated from dimethylformamide–water: wt 14.4 g (91%), mp 275–278°, $[\alpha]_D^{26}$ −19.9° (*c* 1, Me_2SO). *Anal.* Calcd for $C_{32}H_{42}N_6O_9S$: C, 56.0; H, 6.12; N, 12.2. Found: C, 55.4; H, 6.15; N, 11.7.

*N-Benzyloxycarbonyl-*L*-glutaminyl-*L*-alanyl-S-benzyl-*L*-cysteinyl-*L*-alanylglycine Hydrazide (XV).* A solution of the pentapeptide ester XIV (3.6 g) in a mixture of dimethylformamide (270 ml) and methanol (70 ml) was treated with hydrazine hydrate (8 ml). After 24 hr, the reaction mixture was poured into methanol (300 ml) and the precipitated pentapeptide hydrazide was collected and washed with methanol: wt 2.9 g (81%), mp 259–261°, $[\alpha]_D^{26}$ −18.5° (*c* 1, Me_2SO). After HBr in acetic acid treatment, the deblocked peptide showed a single spot on tlc in systems A and B. *Anal.* Calcd for $C_{31}H_{42}N_8O_8S$: C, 54.2; H, 6.12; N, 16.3. Found: C, 54.3; H, 6.33; N, 16.1. Amino acid analysis of an acid hydrolysate showed the following composition expressed in molar ratios: $Glu_{1.0}Gly_{1.0}Ala_{2.0}$*S*-benzylcysteine$_{0.8}$ (average amino acid recovery 95%).

*N-Benzyloxycarbonyl-*L*-glutaminyl-*L*-alanyl-S-benzyl-*L*-cysteinyl-*L*-alanylglycyl-*L*-valyl-*L*-alanyl-*L*-seryl-*L*-leucyl-*L*-tyrosyl-*L*-glutaminyl-*L*-leucyl-γ-benzyl-*L*-glutamyl-*L*-asparaginyl-*L*-tyrosyl-S-benzyl-*L*-cysteinyl-*L*-asparagine p-Nitrobenzyl Ester (XVI).* A solution of the protected dodecapeptide XII (2 g) in trifluoroacetic acid (25 ml) was stored for 2 hr at room temperature and then poured into ether (400 ml). The precipitated deblocked dodecapeptide XIIa was collected, washed with ether, and dried over KOH *in vacuo*. To a cold solution of this product in dimethylformamide (10 ml) containing triethylamine (0.3 ml) was added the pentapeptide azide prepared as follows. The protected pentapeptide hydrazide XV (0.7 g) was dissolved in a mixture of dimethylformamide (10 ml) and Me_2SO (10 ml) containing 1 N HCl in dimethylformamide (2.2 ml). This solution was cooled to −15°, mixed with isoamyl nitrite (0.16 ml), and after 5 min was neutralized with triethylamine (0.28 ml) and added to the dodecapeptide ester prepared as described previously. After 72 hr at 4°, the reaction mixture was poured into methanol (1000 ml) and the precipitated product was collected and washed with methanol: wt 1.4 g (54%), mp 270–273° dec, $[\alpha]_D^{26}$ −24.4° (*c* 1 Me_2SO). The HBr in acetic acid deblocked heptadecapeptide showed a single spot on tlc in systems B and C. *Anal.* Calcd for $C_{114}H_{148}N_{22}O_{31}S_2$: C, 57.4; H, 6.25; N, 12.9. Found: C, 57.5; H, 6.50; N, 13.0. Amino acid analysis of an acid hydrolysate showed the following composition expressed in molar ratios: $Asp_{2.0}Ser_{0.8}Glu_{3.0}Gly_{0.9}Ala_{2.9}Val_{1.0}Leu_{2.1}Tyr_{1.5}$*S*-benzylcysteine$_{1.7}$ (average amino acid recovery 85%).

*N-Benzyloxycarbonylglycyl-*L*-isoleucyl-*L*-valyl-*L*-glutamyl-*L*-glutaminyl-*L*-alanyl-S-benzyl-*L*-cysteinyl-*L*-alanylglycyl-*L*-valyl-*L*-alanyl-*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 (XVII).* The protected heptadecapeptide XVI (1.2 g) was dissolved in trifluoroacetic acid (20 ml) containing water (0.6 ml) and hydrogen bromide was passed through the solution for 75 min at 0°. Addition of ether to this solution caused the precipitation of the hydrobromide of the partially protected heptadecapeptide XVIa which was collected, washed with ether, and dried over KOH *in vacuo*. To a solution of this solid in a mixture of dimethylformamide (5 ml) and Me_2SO (5 ml) cooled to 0°, triethylamine (0.14 ml) was added followed by the protected tetrapeptide azide prepared as follows. *N*-Benzyloxycarbonylglycyl-L-isoleucyl-L-valyl-γ-*tert*-butyl-L-glutamic acid hydrazide (XVIb, 0.75 g) whose synthesis was described previously (Katsoyannis *et al.*, 1966b) was dissolved in a mixture of dimethylformamide (5 ml) and Me_2SO (5 ml). To this solution cooled to −10° 1 N HCl in dimethylformamide (2.4 ml) was added followed by isoamyl nitrite (0.16 ml). After 5 min, the reaction mixture was neutralized with triethylamine (0.34 ml) and added to the partially protected heptadecapeptide (XVIa) prepared as described previously. The reaction mixture was stirred for 48 hr at 4° and then poured into methanol (500 ml). The precipitated heneicosapeptide derivative XVIIa was isolated by centrifugation, washed with methanol, and dried: wt 1.1 g, mp 275–277° dec. A solution of this material in trifluoroacetic acid (30 ml) was stored at room temperature for 30 min and then poured into ether (300 ml). The precipitated partially protected heneicosapeptide XVII was collected and washed with ether: wt 1 g (72% based on XVI

used), mp 278–280° dec, $[\alpha]_D^{26}$ −32.7° (*c* 1, Me_2SO). After deblocking with HBr in trifluoroacetic acid, the heneicosapeptide derivative showed a single spot on tlc in systems B and C. *Anal.* Calcd for $C_{125}H_{172}N_{26}O_{37}S_2 \cdot 2H_2O$: C, 55.0; H, 6.49; N, 13.3; O, 22.9. Found: C, 54.6; H, 6.73; N, 13.1; O, 22.6. Amino acid analysis of an acid hydrolysate showed the following composition expressed in molar ratios: $Asp_{1.9}Ser_{0.8}Glu_{4.1}Gly_{2.2}Ala_{3.2}Val_{2.0}Ile_{0.8}Leu_{1.9}Tyr_{1.2}$*S*-benzylcysteine$_{1.5}$ (average amino acid recovery 85%).

Glycyl-L-*isoleucyl*-L-*valyl*-L-*glutamyl*-L-*glutaminyl*-L-*alanyl*-*S*-*sulfo*-L-*cysteinyl*-L-*alanylglycyl*-L-*valyl*-L-*alanyl*-L-*seryl*-L-*leucyl*-L-*tyrosyl*-L-*glutaminyl*-L-*leucyl*-L-*glutamyl*-L-*asparaginyl*-L-*tyrosyl*-*S*-*sulfo*-L-*cysteinyl*-L-*asparagine* (*Ala*^{6,11} *Sheep A Chain S-Sulfonate*) (*XVIII*). The reduction of the partially protected heneicosapeptide XVII (200 mg) with sodium in liquid ammonia (200 ml) and the oxidative sulfitolysis of the reduced product were accomplished by the procedure described in a previous publication (Katsoyannis *et al.*, 1966c). After evaporation of the ammonia, the residue was dissolved in 8 M guanidine hydrochloride (20 ml) and 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 (680 mg) and freshly prepared sodium tetrathionate (320 mg). The reaction mixture was stirred at room temperature for 18 hr and then placed in a Visking $^{18}/_{32}$ dialysis tubing and dialyzed against four changes of distilled water (4 l. each) at 4° for 20 hr. Upon lyophilization of the dialysate, the crude Ala^{6,11}-A chain *S*-sulfonate (XVIII) 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 (2.2 × 45 cm) equilibrated and eluted with 0.015 M ammonium bicarbonate at a flow rate of about 40 ml/hr. The elution pattern of this column, as determined by monitoring the effluent by a Gilford recording spectrophotometer, is shown in Figure 1. Lyophilization of the effluent (fractions 50–110) afforded the purified final product XVIII as a white fluffy powder: wt 160 mg (90% based on XVII used). The synthetic material possessed the specific rotation $[\alpha]_D^{26}$ −88.9° (*c* 0.1, water). Amino acid analysis after acid hydrolysis gave the molar ratios shown in Table I in a very good agreement with the theoretically expected values. Digestion of the synthetic product with leucine aminopeptidase and amino acid analysis of the digest gave the molar ratios shown in Table I. As can be seen, the synthetic chain was completely digested by the enzyme indicating that the optical configuration of the constituent amino acids was preserved during the synthetic processes. On tlc in the systems B (Figure 2) and C (Figure 3), the synthetic chain exhibited a single Pauly- and ninhydrin-positive spot and had a mobility very similar to that of the natural bovine A-chain *S*-sulfonate. Finally on thin-layer electrophoresis in 0.5 N acetic acid pH 2.9 and 3500 V (Figure 4) and in 0.1 M NH_4HCO_3 (pH 10.0) and 2900 V (Figure 5), the synthetic chain moved as a single component (Pauly reaction) and, as was expected, had a different mobility from that of the natural bovine A chain *S*-sulfonate.

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

Synthesis and Isolation of Ala^{6,11}*-A Sheep Insulin.* This insulin analog was synthesized by the interaction of the sulfhydryl form of the Ala^{6,11}-A chain with the S-sulfonated form of the B chain (Katsoyannis and Tometsko, 1966; Katsoyannis *et al.*, 1967b). In a typical combination experiment 20 mg of the S-sulfonated Ala^{6,11}-A chain was converted

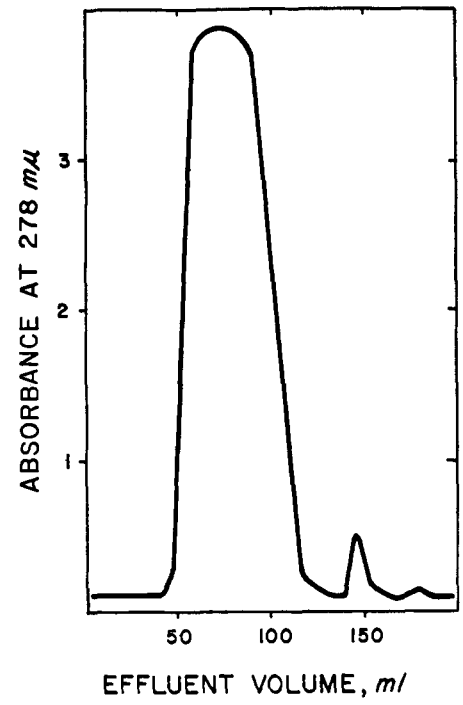


FIGURE 1: Chromatographic pattern of synthetic Ala^{6,11} sheep A chain *S*-sulfonate on a 2.2 × 45 cm Sephadex G-15 column equilibrated and eluted with 0.015 M NH_4HCO_3. From the column effluent which was monitored by a Gilford recording spectrophotometer 160 mg of synthetic chain was recovered by lyophilization of the effluent (50–110 ml).

TABLE I: Amino Acid Composition[a] of the S-Sulfonated Ala^{6,11}-A Chain of Sheep Insulin.

	Acid Hydrolysis		Enzymatic Hydrolysis (Leucine Aminopeptidase)	
Amino Acid	Theory	Found	Theory	Found
Aspartic acid	2.0	2.1	0	0
Glutamine	0	0	2	Emerge on the same position and not determined
Asparagine	0	0	2	
Serine	1.0	1.0	1.0	0.9[b]
Glutamic acid	4.0	4.0	2.0	2.2
Glycine	2.0	2.0	2.0	2.1
Alanine	3.0	2.9	3.0	2.9
Half-cystine	2.0	1.6[c]	0	0
Valine	2.0	1.6	2.0	2.0
Isoleucine	1.0	0.8	1.0	1.1
Leucine	2.0	2.0	2.0	2.1
Tyrosine	2.0	1.5	2.0	2.1
S-Sulfocysteine	0	0	2.0	2.1[d]

[a] Number of amino acid residues per molecule. [b] Separated from glutamine and asparagine in a 30° chromatographic run. [c] Uncorrected for destruction. [d] Eluted from the long column of the Beckman-Spinco analyzer after 26 ml of effluent.

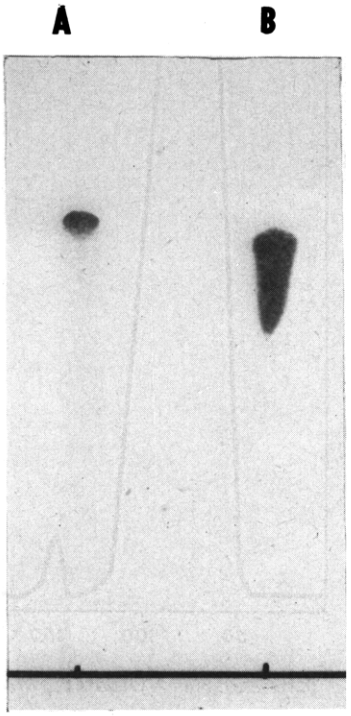


FIGURE 2: Thin-layer chromatography of the *S*-sulfonates of synthetic Ala^{6,11} sheep A chain (A) and natural bovine A chain (B) in the system 1-butanol–acetic acid–pyridine–H_2O (4:1:1:2).

to the sulfhydryl form upon exposure to 2-mercaptoethanol and allowed to react with 5 mg of S-sulfonated B chain. The combination mixture was then treated as described previously (Katsoyannis *et al.*, 1967b,c). Isolation of the insulin analog was carried out by chromatography on a 0.9 × 23

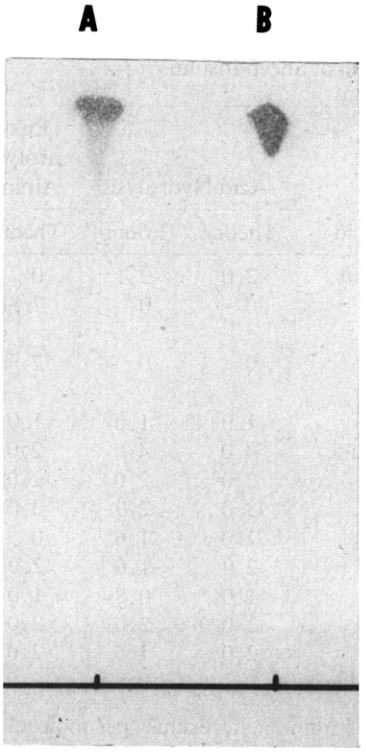


FIGURE 3: Thin-layer chromatography of the *S*-sulfonates of synthetic Ala^{6,11} sheep A chain (A) and natural bovine A chain (B) in the system 1-butanol–pyridine–acetic acid–H_2O (30:20:6:24).

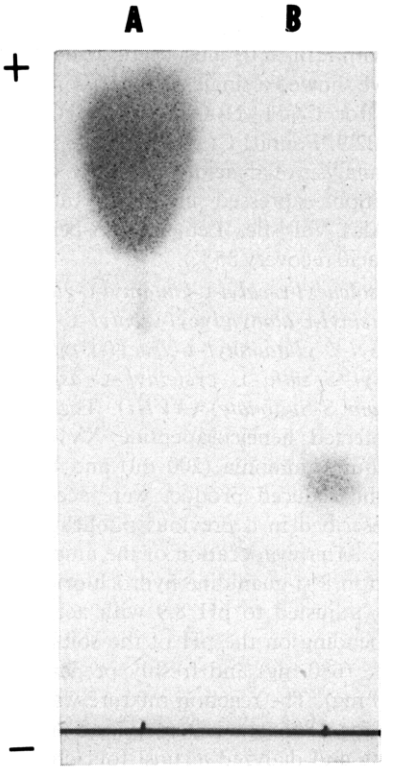


FIGURE 4: Thin-layer electrophoresis of the *S*-sulfonates of natural bovine A chain (A) and synthetic Ala^{6,11} sheep A chain (B); 0.5 N acetic acid (3500 V, 20 min).

cm CM-cellulose column with an exponential NaCl gradient (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 6-I. As was the case with natural, all-synthetic and half-synthetic insulins (Katsoyannis *et al.*, 1967c), and with other insulin analogs (Katsoyannis *et al.*, 1971; Katsoyannis and Zalut, 1972a,b), the [Ala^{6,11}-A]insulin is eluted with applica-

TABLE II: Amino Acid Composition[a] of the Sheep[Ala^{6,11}-A]-Insulin.

Amino Acid	Theory	Found
Lysine	1	1.0
Histidine	2	1.8
Arginine	1	1.1
Aspartic acid	3	3.3
Threonine	1	1.0
Serine	2	2.1
Glutamic acid	7	6.6
Proline	1	1.1
Glycine	5	5.2
Alanine	5	4.8
Half-cystine	4	2.0[b]
Valine	5	4.9
Isoleucine	1	0.5
Leucine	6	5.8
Tyrosine	4	3.4
Phenylalanine	3	2.8

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

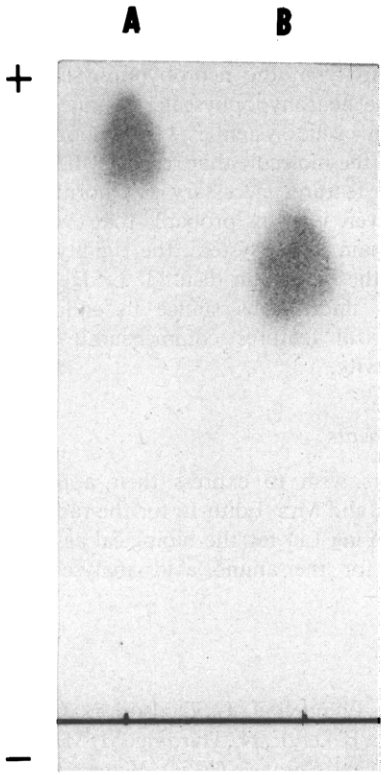


FIGURE 5: Thin-layer electrophoresis of the *S*-sulfonates of natural bovine A chain (A) and synthetic Ala^{6,11} sheep A chain (B); 0.01 M NH_4HCO_3 adjusted to pH 10.0 with NH_4OH (2900 V, 18 min).

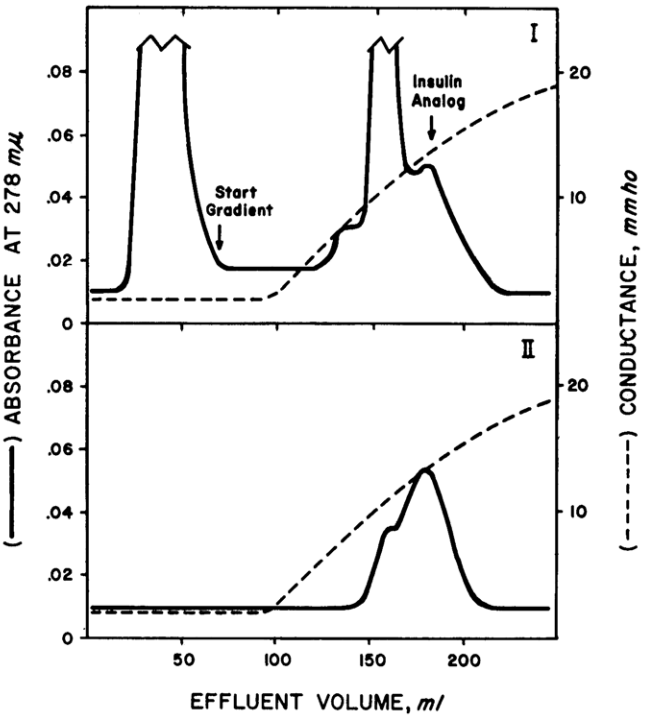


FIGURE 6: (I) Chromatography of two combination mixtures (see Materials and Techniques) of synthetic Ala^{6,11} sheep A chain and natural bovine (sheep) B chain on a 0.9 × 23 cm CM-cellulose column with acetate buffer (0.024 M, pH 3.3) and an exponential NaCl gradient. The column effluent was monitored by a Gilford recording spectrophotometer and by a conductivity meter (Radiometer, Copenhagen). The sheep [Ala^{6,11}-A]insulin (175–215 ml of effluent) was recovered as the hydrochloride (0.8 mg). (II) The insulin analog (0.8 mg) from (I), plus the hydrochloride obtained by processing a single combination mixture, were combined and rechromatographed on the 0.9 × 23 cm CM-cellulose column in exactly the same way as above. The purified sheep [Ala^{6,11}-A]insulin (168–210 ml of effluent) was recovered as the hydrochloride (1 mg).

tion of the NaCl gradient and is the slowest moving component. The insulin analog from the effluent was isolated *via* picrate as the hydrochloride (0.8 mg) according to the procedure described previously (Katsoyannis *et al.*, 1967c). This material was combined with the hydrochloride obtained by processing, in exactly the same way as above, another combination mixture, and rechromatographed on the 0.9 × 23 cm CM-cellulose column. Figure 6-II shows the chromatographic pattern obtained. From the effluent (168–210 ml), 1 mg of sheep [Ala^{6,11}-A]insulin was recovered as the hydrochloride.

Amino acid analysis of an acid hydrolysate of this analog gave a composition expressed in molar ratios in good agreement with the theoretically expected values (Table II). On thin-layer electrophoresis in 0.5 N acetic acid and 3500 V, the synthetic analog moved as a single component (Pauly reaction) and had a mobility slightly different from that of bovine insulin (Figure 7). The sheep [Ala^{6,11}-A]insulin was found to possess 2–2.5 IU/mg by the mouse convulsion assay method and 2.1 IU/mg by the radioimmunoassay method. Crystallization of this analog was not attempted.

Discussion

In the structure of insulin, as determined by Sanger (Ryle *et al.*, 1955; Brown *et al.*, 1955), the A and B chains are linked together by two disulfide bridges at positions A^7 to B^7 and A^{20} to B^{19}. In addition, there is an intrachain disulfide linkage in the A chain between cysteine residues located at positions A^6 and A^{11}. The latter structural feature results in the formation of a 20-membered cyclic system in the A chain. It may be recalled that a cyclic disulfide of the same size was originally found in the posterior pituitary hormones (du Vigneaud *et al.*, 1953a–c).

Intensive studies by many investigators have demonstrated that in the posterior pituitary hormones the disulfide system is not essential for hormonal activity, although a 20-membered cyclic structure in these hormones is indeed a requirement for biological activity (for a recent review on the subject, see Rudinger, 1971). Thus, acyclic analogs (Beránková and Šorm, 1961; Jošt *et al.*, 1964; Huguenin and Guttmann, 1965; Nesvadba *et al.*, 1968) and analogs with an enlarged ring structure (Jarvis and du Vigneaud, 1964) possess a very low hormonal activity. The low biological activity exhibited by the acyclic analogs has been interpreted (Jošt and Rudinger, 1969) to indicate that portions of their molecules are capable of assuming a "pseudocyclic" conformation that results in the establishment of topochemical features resembling those of the biologically active cyclic compounds.

In view of these findings, it was of great interest to investigate the role of the intrachain disulfide system in the expression of the biological activity of insulin. To this end, an analog of the sheep insulin A chain in which the cystine residue in positions A^6–A^{11} was replaced by cystathionine has been synthesized (Jošt *et al.*, 1968) and combined with the B chain. The presence of biological activity in the combination mixture implied that, as in the case of the neurohypophyseal hormones, the intrachain disulfide system does not participate functionally in the mechanism of action of insulin. Elimination of the intrachain disulfide system from insulin has been claimed by Weber *et al.* (1968) to result essentially in biological

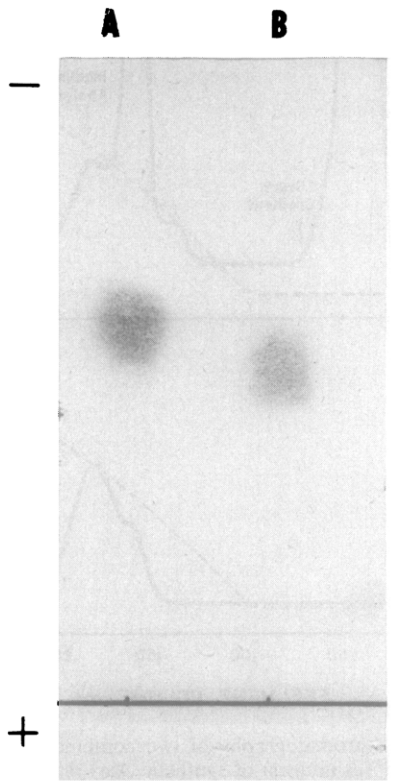


FIGURE 7: Thin-layer electrophoresis of natural bovine insulin (A) and synthetic sheep [Ala^{6,11}-A]insulin (B); 0.5 N acetic acid (3500 V, 15 min).

inactivation. These authors report with no experimental details that the combination mixture of what presumably is Ala^{6,11}-A chain with natural B chain exhibited insulin-like activity ranging from 0.04 to 0.2% of that of insulin.

The present investigation has permitted us to establish that elimination of the intrachain disulfide system does not lead to biological inactivation and further to evaluate the relative importance of this system in the manifestation of the biological activity of the hormone. This was achieved by the synthesis, isolation in purified form, and biological evaluation of an insulin analog lacking the intrachain cyclic system in the A chain moiety of the parent molecule. For the preparation of this analog the Ala^{6,11}-A chain, which differs from the natural chain in that the cysteine residue at positions 6 and 11 have been replaced by alanine, has been synthesized and isolated in a highly purified form. From the combination mixture of this chain analog with B chain, the [Ala^{6,11}-A] insulin was isolated. It was found by the mouse convulsion method and by radioimmunoassay that this analog possesses a potency of 2–2.5 IU/mg, which is approximately 10% of the biological activity of insulin (25 IU/mg).

The X-ray analysis of the three-dimensional structure of this hormone (Adams *et al.*, 1969; Blundell *et al.*, 1971) has shown that the A^{6}–A^{11} disulfide bridge is completely buried and is part of the hydrophobic core of the insulin molecule. Hence, its inaccessibility to the environment outside the insulin molecule makes it a most unlikely candidate to be involved directly in any chemical interplay between the hormone and the responsive cell. The findings presented in this paper are in complete accord with this interpretation. However, elimination of the cyclic system resulting in a 90% loss of biological activity probably indicates that the intrachain ring structure confers on the insulin molecule topochemical features essential for its full activity.

It might be interesting at this point to compare the role of the intrachain disulfide bridge in the expression of the biological activity of the neurohypophyseal hormones and insulin. In the neurohypophyseal hormones, it appears that the intrachain cyclic system is very important in imposing constrains on the molecule that result in the formation of the topochemical features necessary for hormonal activity. In insulin, however, it seems probable that even in the absence of the intrachain cyclic system, the rigidity imposed on the molecule by the interchain disulfide bridge and other intra- or interchain interactions suffice to endow the hormone with geometrical features commensurate with a reduced biological activity.

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Application of the Phase Partition Method to a Hydrophobic Membrane Protein, Phospholipase A1 from *Escherichia coli*†

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ABSTRACT: Phase partition has been applied to the purification of hydrophobic membrane proteins. Phase systems of up to four liquid phases contained various polymers and detergents in aqueous solution. The polymers dextran, Ficoll, poly(ethylene glycol), and poly(propylene glycol) produced phases with distinctive hydrophobic and hydrophilic characteristics. Partitions of the membrane-bound phospholipase A1 of *Escherichia coli* brought about a several-hundredfold purification of the enzyme and a separation from phospholipid and added detergents. The added polymers are also removed from the enzyme.

Isolation of pure membrane proteins is essential for a study of their properties and an understanding of their role in the intact membrane. The hydrophobic nature of membrane proteins presents special problems for conventional protein separation methods. Hydrophobic proteins form aggregates which can be broken only by drastic treatment with detergents, organic solvents, or extremes of pH. Membrane proteins also show a strong affinity for lipids.

We undertook to see whether phase partition could be used to separate hydrophobic proteins. With this technique, a substance may partition between two or several phases composed of different polymers dissolved in water. Previously, this technique has been applied only to hydrophilic substances such as the water-soluble proteins, nucleic acids, viruses, and cell organelles. The method should also be applicable to hydrophobic proteins, provided these can be solubilized and polymers with varying hydrophobicity are used as phase formers.

In order to make the phase partition technique generally applicable to hydrophobic proteins we sought first to apply it to phospholipase A1 of *Escherichia coli* (Scandella and Kornberg, 1971), an enzyme firmly bound to the outer membrane of *E. coli*. It is a stable enzyme with well-defined properties and is easily assayed.

We have found that polymer phase systems together with nonionic detergents were effective in the purification of phospholipase A1 several-hundredfold. It is hoped that the procedure will be applicable to other hydrophobic enzymes.

Experimental Procedures and Results

Materials and Methods. Dextran (10% moisture assumed) and Ficoll were obtained from Pharmacia Fine Chemicals,

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