Synthesis of Destetrapeptide A^{1-4} Sheep Insulin and Destetrapeptide A^{1-4} Porcine Insulin†

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ABSTRACT: The synthesis and isolation in purified form of an analog of sheep insulin and an analog of porcine insulin are described. These analogs differ from the parent molecules in that the N-terminal tetrapeptide sequence is eliminated from their A chain. For the synthesis of these analogs, the destetrapeptide A^{1-4} chains of sheep and porcine (human) insulins were first chemically synthesized and isolated as the S-sulfonated derivatives. Conversion of the latter materials to their sulfhydryl form and combination with the S-sulfonated B chain of bovine (sheep, porcine) insulin produced the destetrapeptide A^{1-4} sheep insulin and destetrapeptide A^{1-4} porcine insulin, respectively. Isolation of these analogs from the respective combination mixtures was effected by chromatography on a carboxymethylcellulose column with an exponential sodium chloride gradient. By the mouse convulsion assay method, both of these analogs were found to be biologically inactive. This indicates that the amino-terminal region of the A chain in the insulin molecule is critically involved in the expression of the biological properties of this hormone.

Evaluation of the importance of the amino acid residues present at the N- and C-terminal regions of the A and B chains of insulin to the expression of its biological activity has been one feature of our work directed at correlating chemical structure to biological function of this protein. In a previous report (Katsoyannis *et al.*, 1971), we have shown that removal of the C-terminal tripeptide sequence from the B chain of bovine or porcine (human) insulin and modification of the carboxyl group of the newly exposed amino acid residue does not affect the biological activity of the hormone. On the other hand, replacement of the α-amino group of the A chain by hydrogen in the structure of sheep insulin results in a decrease of biological activity of the hormone (Katsoyannis and Zalut, 1972). It was, therefore, of interest to investigate what effect the removal of amino acid residues from the amino terminus of the A-chain moiety of the insulin molecule would have on the biological properties of this protein. The present report describes the synthesis and isolation of two such analogs related to sheep and porcine insulins. These analogs differ from the parent molecules in that the N-terminal tetrapeptide sequence from their A chain has been eliminated.

Experimental Procedures and Results

Materials and Techniques. Sephadex G-50 (Pharmacia Uppsala) and preswollen microgranular CM-cellulose (Whatman CM 52/1) were used. Sodium tetrathionate was prepared as described by Gilman *et al.* (1946). Amino acid analyses were performed according to the method of Spackman *et al.* (1958) in a Beckman-Spinco amino acid analyzer, Model 120C, equipped with a digital readout system (Model CRS-12AB, Infotronics Corp., Houston, Tex.). Acid hydrolysis and calculations of molar ratios were carried out as described previously (Katsoyannis *et al.*, 1967b). 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. Reduction with sodium in liquid ammonia was carried out as described previously (Katsoyannis *et al.*, 1966). Protein determinations were carried out by the method of Lowry *et al.* (1951). Thin-layer electrophoresis by the method of Tometsko and Delihas (1967) was performed with a Wieland-Pfleiderer pherograph (Brinkman Instruments). Biological assays were carried out by the mouse convulsion method as has been described previously (Katsoyannis and Tometsko, 1966; Katsoyannis *et al.*, 1967d).

Synthesis of Destetrapeptide A^{1-4} Sheep and Porcine Insulins. These analogs were prepared by the combination of the S-sulfonated derivative of the B chain of bovine (sheep, porcine) insulin with the sulfhydryl form of the destetrapeptide A^{1-4} chains of sheep and porcine (human; Nicol and Smith, 1960) insulins, respectively. For the synthesis of the desired destetrapeptide A^{1-4} chains, the heptadecapeptide derivatives, containing the amino acid sequence of the respective A chains lacking the N-terminal tetrapeptide segment, were prepared with their functional groups protected. Removal of the blocking groups from the protected derivatives was effected with sodium in liquid ammonia (Sifferd and du Vigneaud, 1935). Oxidative sulfitolysis (Bailey and Cole, 1959) of the resulting reduced products led to the formation of the S-sulfonated destetrapeptide A^{1-4} chains of sheep and porcine (human) insulins, respectively, which, prior to their combination with the S-sulfonated B chain, are transformed to the sulfhydryl form.

S-Sulfonated Derivative of Bovine Insulin B Chain. This compound, which is identical with the corresponding derivative of sheep insulin (Sanger and Tuppy, 1951a,b; Brown *et al.*, 1955) and porcine insulin (Brown *et al.*, 1955), was prepared as described previously (Katsoyannis, *et al.*, 1967b).

L-*Glutaminyl-S-sulfo*-L-*cysteinyl-S-sulfo*-L-*cysteinyl*-L-*alanyl-glycyl*-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-*asparagine* (*Destetrapeptide Sheep A^{1-4} Chain S-Sulfonate*). The heptadecapeptide derivative, *N*-benzyloxycarbonyl-L-glutaminyl-*S*-benzyl-L-cysteinyl-*S*-benzyl-L-cysteinyl-

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TABLE I: Amino Acid Composition[a] of the S-Sulfonated Destetrapeptide A^{1-4} Chain of Sheep Insulin.

	Acid Hydrolysis		Enzymatic Hydrolysis (Leucine Aminopeptidase)	
Amino Acid	Theory	Found	Theory	Found
Aspartic acid	2	1.9	0	0
Glutamine	0	0	2	Emerge at the same position; not determined
Asparagine	0	0	2	
Serine	1	0.9	1	1.0[b]
Glutamic acid	3	3.1	1	1.1
Glycine	1	1.0	1	1.0
Alanine	1	1.0	1	1.0
Half-cystine	4	2.3[c]	0	0
Valine	1	1.0	1	1.0
Leucine	2	2.0	2	2.1
Tyrosine	2	1.9	2	2.0
S-Sulfocysteine	0	0	4	4.0[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.

TABLE II: Amino Acid Composition[a] of the S-Sulfonated Destetrapeptide A^{1-4} Chain of Procine (Human) Insulin.

	Acid Hydrolysis		Enzymatic Hydrolysis (Leucine Aminopeptidase)	
Amino Acid	Theory	Found	Theory	Found
Aspartic acid	2	1.9	0	0
Glutamine	0	0	2	Emerge at the same position; not determined
Asparagine	0	0	2	
Threonine	1	0.9	1	1.0
Serine	2	1.8	2	1.9[b]
Glutamic acid	3	3.0	1	1.0
Half-cystine	4	2.8[c]	0	0
Isoleucine	1	1.0	1	1.0
Leucine	2	2.0	2	2.0
Tyrosine	2	1.9	2	2.0
S-Sulfocysteine	0	0	4	4.2[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.

L-alanylglycyl-L-valyl-*S*-benzyl-L-cysteinyl-L-seryl-L-leucyl-L-tyrosyl-L-glutaminyl-L-leucyl-L-glutamyl-L-asparaginyl-L-tyrosyl-*S*-benzyl-L-cysteinyl-L-asparagine *p*-nitrobenzyl ester, whose synthesis has been previously described (Katsoyannis *et al.*, 1966), was deblocked by the procedure described previously (Katsoyannis *et al.*, 1966). In a typical experiment, the thoroughly dried, protected heptadecapeptide (200 mg) was dissolved in anhydrous liquid ammonia (150 ml) in a 250-ml round-bottom flask fitted for magnetic stirring. The reaction was carried out at the boiling point of the solution. Cleavage of the blocking groups was accomplished by adding small pieces of sodium into the solution until a faint blue color appeared throughout. The blue color was allowed to persist for 1 min and was then discharged by the addition of a few crystals of ammonium chloride. The solution was evaporated at atmospheric pressure to about 10 ml and dried from the frozen state. The reduced product thus obtained was sulfitolyzed for 4 hr using essentially the procedure described previously (Katsoyannis *et al.*, 1966, 1967b). Briefly, this material was dissolved in 8 M guanidine hydrochloride (20 ml) containing acetic acid (0.5 ml), and to this solution, adjusted to pH 8.9 with dilute ammonium hydroxide, was added sodium sulfite (680 mg) and freshly prepared sodium tetrathionate (320 mg). The reaction mixture was stirred for 4 hr at room temperature and dialyzed against four changes of distilled water (4 l. each) at 4° for 20 hr using a Visking 18/32 dialyzing tubing. Upon lyophilization of the dialysate, the destetrapeptide A^{1-4} chain was obtained as a white powder, wt 165 mg. For purification the lyophilized material was chromatographed on a Sephadex G-50 column (fine grade, bead form; 2.4 × 50 cm) equilibrated and eluted with 5% acetic acid. The effluent was monitored with a Gilford recording spectrophotometer at 278 mμ. The chromatographic pattern obtained indicated the presence of a single component which was isolated by lyophilization of the effluent, wt 130 mg (74% based on the protected heptadecapeptide used).

Amino acid analysis of the purified product after acid hydrolysis gave the composition shown in Table I in good agreement with the theoretically expected values. Digestion of the synthetic material with leucine aminopeptidase and amino acid analysis of the digest gave the amino acid molar ratios shown in Table I which are practically identical with the theoretically expected values. This proves that the synthetic chain was completely digested with leucine aminopeptidase and, consequently, that the optical configuration of the constituent amino acids was preserved during the synthetic processes. On thin-layer electrophoresis in 0.5 N acetic acid (3400 V) and in 0.01 N NH_4HCO_3 (pH 10.0, 2900 V) the synthetic product moved as a single component (Pauly reaction).

L-*Glutaminyl*-*S*-*sulfo*-L-*cysteinyl*-*S*-*sulfo*-L-*cysteinyl*-L-*threonyl*-L-*seryl*-L-*isoleucyl*-*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-*asparagine* (*Destetrapeptide Porcine* (*Human*) A^{1-4} *Chain S-Sulfonate*). The deblocking of the heptadecapeptide derivative, *N*-benzyloxycarbonyl-L-glutaminyl-*S*-benzyl-L-cysteinyl-*S*-benzyl-L-cysteinyl-L-threonyl-L-seryl-L-isoleucyl-*S*-benzyl-L-cysteinyl-L-seryl-L-leucyl-L-tyrosyl-L-glutaminyl-L-leucyl-L-glutamyl-L-asparaginyl-L-tyrosyl-*S*-benzyl-L-cysteinyl-L-asparagine *p*-nitrobenzyl ester, whose synthesis was described in a previous paper (Katsoyannis *et al.*, 1967a), its conversion to the S-sulfonated derivative, and the purification of the final product were accomplished by exactly the same procedures described above in the synthesis of the respective sheep chain derivative. From 200 mg of protected heptadecapeptide 125 mg (71% based on the protected heptadecapeptide used) of purified destetrapeptide porcine (human) A^{1-4} chain *S*-sulfonate was obtained.

Amino acid analyses after acid and enzymatic (leucine aminopeptidase) hydrolysis gave the compositions shown in Table II in a very good agreement with the theoretically ex-

TABLE III: Amino Acid Composition[a] of the Destetrapeptide A^{1-4} Sheep and Destetrapeptide A^{1-4} Porcine Insulins.

Amino Acid	Sheep Insulin Analog		Porcine Insulin Analog	
	Theory	Found	Theory	Found
Lysine	1	1.0	1	1.1
Histidine	2	1.7	2	2.0
Arginine	1	1.1	1	1.0
Aspartic acid	3	2.8	3	3.1
Threonine	1	0.9	2	1.8
Serine	2	1.9	3	2.8
Glutamic acid	6	6.1	6	6.1
Proline	1	1.1	1	0.9
Glycine	4	4.0	3	3.1
Alanine	3	2.9	2	2.0
Half-cystine	6	4.5[b]	6	3.9[b]
Valine	4	3.8	3	3.0
Isoleucine	0	0	1	1.0
Leucine	6	5.9	6	5.9
Tyrosine	4	2.4[b]	4	2.8[b]
Phenylalanine	3	2.7	3	2.7

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

pected values. On thin-layer electrophoresis in 0.5 N acetic acid (3400 V) and in 0.01 N NH_4HCO_3 (pH 10.0, 2900 V) the synthetic material moved as a single component (Pauly reaction).

Isolation of Insulin Analogs Produced by Combination of Destetrapeptide A^{1-4} Chains with B Chain. The synthesis of these analogs was carried out by interaction of the sulfhydryl form of the corresponding destetrapeptide A^{1-4} chain with the S-sulfonated form of the B chain of bovine insulin according to the procedures developed in this laboratory (Katsoyannis and Tometsko, 1966; Katsoyannis *et al.*, 1967c). In a typical combination experiment, 20 mg of destetrapeptide A^{1-4} chain was converted to the sulfhydryl form upon exposure to 2-mercaptoethanol and then was allowed to react with 5 mg of S-sulfonated B chain. Isolation of the insulin analog thus produced was carried out by chromatography on a 0.9 × 23 cm CM-cellulose column with an exponential NaCl gradient, as was described in detail in previous reports (Katsoyannis *et al.*, 1967c,d).

Destetrapeptide A^{1-4} Sheep Insulin. Two combination mixtures, each corresponding to the amounts of materials indicated above, were processed. The chromatographic pattern obtained is shown in Figure 1. The insulin analog is eluted with application of the NaCl gradient and is the slowest moving component. A similar pattern is obtained when regenerated, all-synthetic, and half-synthetic insulins are chromatographed in the same system (Katsoyannis *et al.*, 1967d). 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.*, 1967d).

Amino acid analysis of this analog after acid hydrolysis gave a composition in molar ratios in very good agreement with the theoretically expected values (Table III). On thin-layer electrophoresis in 0.5 N acetic acid (3500 V) the synthetic analog moved as a single component and had a mobility

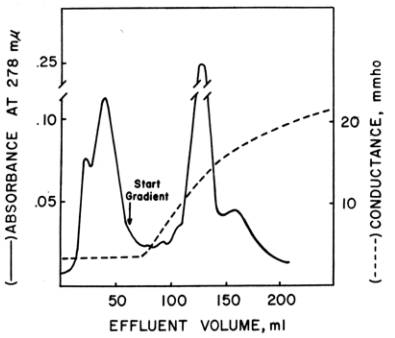

FIGURE 1: Chromatography of a combination mixture of destetrapeptide A^{1-4} chain of sheep insulin and B chain of bovine (sheep) insulin on a 0.9 × 23 cm CM-cellulose column with acetate buffer (0.024 M, pH 3.3) and an exponential NaCl gradient. The column effluent was monitored by a Gilford recording spectrophotometer and by a conductivity meter (Radiometer, Copenhagen). Two combination mixtures (see Experimental Procedures and Results) were processed. The destetrapeptide A^{1-4} sheep insulin (slowest moving component; 145–195 ml of effluent) was recovered as the hydrochloride (0.8 mg).

slightly different from that of bovine insulin (Figure 2). The destetrapeptide A^{1-4} sheep insulin, in doses up to 20 μg/mouse, was found to be biologically inactive by the mouse convulsion assay method. Crystallization of this analog was not attempted.

Destetrapeptide A^{1-4} Porcine Insulin. Two combination mixtures, each corresponding to the amounts of material in-

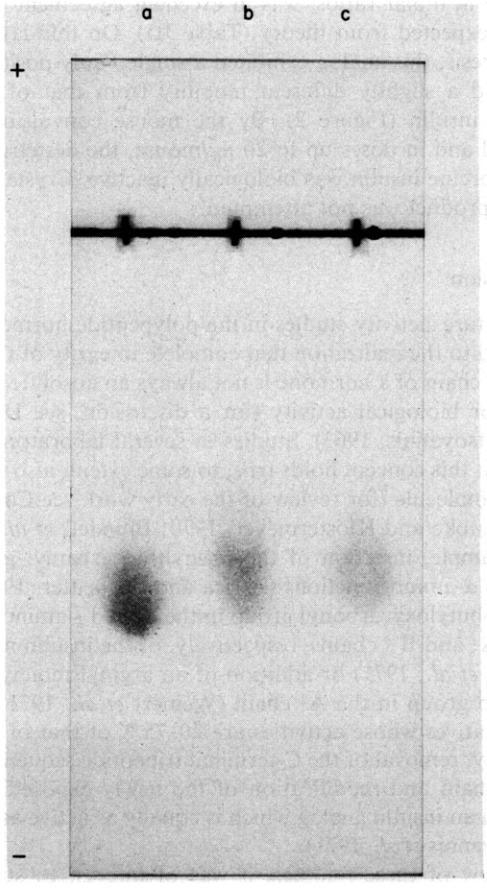

FIGURE 2: Thin-layer electrophoresis of natural bovine insulin (a), destetrapeptide A^{1-4} porcine insulin (b), and destetrapeptide A^{1-4} sheep insulin (c): 0.5 N acetic acid (3500 V, 13 min).

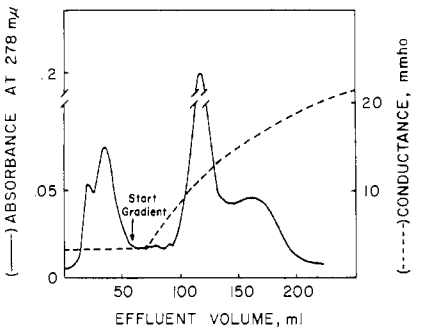

FIGURE 3: Chromatography of a combination mixture of destetra peptide A^{1-4} chain of porcine (human) insulin and B chain of bovine (porcine) insulin on a 0.9 × 23 cm CM-cellulose column with acetate buffer (0.024 M, pH 3.3) and an exponential NaCl gradient. The column effluent was monitored by a Gilford recording spectrophotometer and by a conductivity meter. Two combination mixtures, each corresponding to the amounts of materials indicated in the Experimental Section, were processed. The destetrapeptide A^{1-4} porcine insulin (slowest moving component; 145–200 ml of effluent) was recovered as the hydrochloride (1.1 mg).

dicated previously, were processed. Figure 3 illustrates the chromatographic pattern obtained. As was the case with the sheep insulin analog, the destetrapeptide A^{1-4} porcine insulin is the slowest moving component of the combination mixture. The insulin analog from the effluent was isolated and eventually converted to the hydrochloride (1.1 mg) as described previously (Katsoyannis *et al.*, 1967d).

The amino acid composition of the synthetic analog, expressed in molar ratios, was in excellent agreement with the values expected from theory (Table III). On thin-layer electrophoresis, this analog exhibited a single Pauly-positive spot and had a slightly different mobility from that of natural bovine insulin (Figure 2). By the mouse convulsion assay method and in doses up to 20 μg/mouse, the destetrapeptide A^{1-4} porcine insulin was biologically inactive. Crystallization of this product was not attempted.

Discussion

Structure–activity studies in the polypeptide hormone field have led to the realization that complete integrity of the polypeptide chain of a hormone is not always an absolute requirement for biological activity (for a discussion, see Hofmann and Katsoyannis, 1963). Studies in several laboratories indicate that this concept holds true, to some extent, also with the insulin molecule (for review of the early work see Carpenter, 1966; Lübke and Klostermeyer, 1970; Blundell *et al.*, 1971). For example, insertion of the phenylthiocarbamyl group in the two α-amino functions (Africa and Carpenter, 1970) and the *tert*-butyloxycarbonyl group in the α- and ε-amino groups in the A^1 and B^{29} chains, respectively, of the insulin molecule (Geiger *et al.*, 1971) or addition of an arginyl moiety on the α-amino group in the A^1 chain (Weinert *et al.*, 1971) results in derivatives whose activities are 20–75% of that of insulin. Similarly, removal of the C-terminal tripeptide sequence from the B chain and modification of the newly exposed residue result in an insulin analog which is equally as active as insulin (Katsoyannis *et al.*, 1971).

In view of these findings, it was of interest to study the effect of the removal of amino acid residues at the amino end of the insulin chains on the biological activity of this hormone. Preliminary data obtained from the action of leucine aminopeptidase on insulin (Smith *et al.*, 1958) suggest that the first six amino acid residues of the B chain do not appear to be essential for biological activity. They further imply that the loss of activity observed may be the result either of the removal of residues from the amino-terminal region of the A chain or of hydrolysis past the neighboring interchain disulfide bridge in the B chain.

The present investigation has permitted us to determine unequivocally the effect of the removal of amino acid residues from the amino end of the A chain of insulin on the biological activity of the hormone. This was accomplished by the synthesis, isolation in purified form, and biological evaluation of destetrapeptide A^{1-4} sheep and porcine insulins. These analogs differ from the parent molecules in that their A chain lacks the N-terminal tetrapeptide sequence. It was found that these analogs are biologically inactive when assayed by the mouse convulsion method.

In previous work, it has been shown that the A^1 residue, glycine, is important for the biological activity of insulin. Removal of this residue (Brandenburg and Ooms, 1969; Africa and Carpenter, 1970) or even a modification of it (Katsoyannis and Zalut, 1972) results in a substantial decrease in biological activity. These findings, in conjunction with the data presented in this report, clearly demonstrate that the amino-terminal region of the A chain in the molecule of insulin is decisively involved in the manifestation of the biological properties of this protein. It is not known, however, whether the lack of biological activity of the analogs described in this report is inherent to their covalent structure or is caused by covalent-induced changes in the conformation of their molecules.

It might be pointed out that the above conclusions are based on the assumption that in the structure of the A^{1-4} destetrapeptide analogs, the recombined chains have the same disulfide bonds as insulin. Although no attempt was made to prove this point by degradative studies, all the existing evidence strongly supports this assumption.

(1) Recombination of chains to produce insulin or analogs involves an intramolecular ring closure to form the 20-membered disulfide system of the A chain and an intermolecular disulfide bond formation between A and B chains. From the original work on the synthesis of the neurohypopheseal hormones (du Vigneaud *et al.*, 1954, 1958) and on the oxidation of L-cysteinyltetraglycyl-L-cysteine (Rydon, 1958), it is evident that when the intermediate sulfhydryl peptides are air oxidized, intramolecular disulfide ring closure to form the 20-membered cyclic monomer

is favored over the linear polymerization or the intermolecular disulfide bond formation. Consequently, it is reasonable to expect that when the sulfhydryl form of the A chain or its analogs and the S-sulfonated form of the B chain are subjected to air-oxidation, intramolecular ring closure to form the intrachain 20-membered cyclic system of the A chain will precede the interchain disulfide bond formation between A (or analogs) and B chains. Consequently, the possibility of wrong disulfide pairing during the formation of the interchain bonds is substantially reduced (Katsoyannis and Tometsko, 1966). The successful synthesis of insulin from various species (for a review, see Katsoyannis, 1969) has indeed proven that, upon recombination of the insulin chains, the proper disulfide

pairing does occur in the formation of the intrachain cyclic system and also in the establishment of the interchain disulfide bridges. The proper disulfide pairing takes place not only during recombination with natural or synthetic chains but also during formation of hybrid insulins (Katsoyannis *et al.* 1967d) and during formation of other truncated insulins (Katsoyannis *et al.*, 1971). In all these cases where biologically active molecules were produced, as well as in the present investigation where a biologically inactive molecule was obtained, identical conditions were used for the combination of the chains and isolation and purification of the final product.

(2) Previous studies have shown that a recombination mixture of insulin chains consists exclusively of unreacted A and B chain components and of insulin (Katsoyannis *et al.*, 1967c,d). Furthermore, the chromatographic patterns obtained during the isolation of insulin or its biologically active analogs (Katsoyannis *et al.*, 1967d, 1971; Katsoyannis and Zalut, 1972) are identical with the chromatographic profiles obtained in the isolation of the biologically inactive A^{1-4} destetrapeptide insulins. It is thus reasonable to assume that in all these cases a comparable situation exists.

(3) One could argue that the four amino acid residues at the N terminus of the A chain, particularly, the polar glutamic acid residue at A^4, might exert an important influence on the process of recombination of the A and B chains, and their absence might lead to improper disulfide pairing. The existing data indicate that the N-terminal region of the A chain is in proximity to the C terminus of the B chain (Blundell *et al.*, 1971; Zahn and Meienhofer, 1958), and amino acid residues contained in these regions, such as Glu at A^4 and Lys at B^{29}, appear to be involved in interactions with each other (Blundell *et al.*, 1971). It was found, however (Katsoyannis *et al.*, 1971), that the destripeptide B^{28-30} chain readily recombines with the A chain to produce a fully biologically active insulin analog. This indicates that interactions of the N-terminal and C-terminal regions of the A and B chains, respectively, do not appear to influence the proper recombination of these chains. Similarly, it was found that an analog of the A chain, which has attached an arginyl residue at the N terminus, readily combines with B chain to produce a biologically active molecule, in spite of the fact that the polarity of the N-terminal region of the A chain has undergone such a radical change (Weinert *et al.*, 1971).

Acknowledgments

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