Analogs of Insulin. I. Synthesis of Destripeptide B^{28-30} Bovine Insulin and Destripeptide B^{28-30} Porcine (Human) Insulin*

Panayotis G. Katsoyannis,† Clyde Zalut, Alan Harris, and Richard J. Meyer

ABSTRACT: The preparation of an analog of bovine insulin and an analog of porcine (human) insulin is described. These analogs differ from the parent molecules in that the C-terminal tripeptide sequence from their B chain is eliminated and the carboxyl group of the newly exposed amino acid residue is modified. The key intermediate in the synthesis of both analogs is the S-sulfonated derivative of the destripeptide B^{28-30} chain of bovine insulin which was prepared by the sodium-liquid ammonia reduction and sulfitolysis of the latter compound. Combination of the S-sulfonated destripeptide B^{28-30} chain with the sulfhydryl form of the bovine insulin A chain or the porcine (human) insulin A chain afforded the destripeptide B^{28-30} bovine insulin and destripeptide B^{28-30} porcine (human) insulin, respectively. Isolation of these analogs from the combination mixtures was effected by chromatography on a carboxymethylcellulose column with an exponential sodium chloride gradient. By the mouse convulsion assay method the bovine insulin analog possessed a specific activity ranging from 22 to 28 IU per mg, whereas the porcine (human) insulin analog has a potency of 17 IU/mg.

Systematic studies in this laboratory during the past several years resulted in the total synthesis and isolation in highly purified form of sheep and human insulins (Katsoyannis, 1966a; Katsoyannis *et al.*, 1967a). In addition, by combining synthetic or natural chains of one species with the corresponding synthetic or natural chains of another species a number of hybrid insulins have been synthesized and isolated in pure form (Katsoyannis *et al.*, 1967a). The methods of synthesis and combination of the insulin chains and the isolation techniques employed in the purification of the synthetic and hybrid insulins developed in this laboratory have opened unlimited possibilities for the synthesis of insulin analogs and for study of the relationship between chemical structure and biological activity of this protein.

A program has, therefore, been initiated in this laboratory to study what effect changes in the structure of insulin will have on the biological properties of the hormone. The present report describes the synthesis and isolation of two such analogs related to porcine (or human) and bovine insulins. These analogs differ from the parent molecules in that the C-terminal tripeptide sequence from their B chain has been eliminated and the carboxyl group of the new C-terminal amino acid residue has been modified.

Experimental Section

Materials

Crystalline bovine and porcine zinc insulins were generously provided by Eli Lilly and Co. Preswollen microgranular CM-cellulose (Whatman CM 52/1) and Sephadex G-15 (Pharmacia Uppsala) were used in this investigation. Sodium tetrathionate was prepared as described by Gilman *et al.* (1946).

Methods

S-Sulfonated Derivatives of the A and B Chains of Bovine and Porcine Insulins ($A\text{-}SSO_3^-$, $B\text{-}SSO_3^-$).[1] The methods for the preparation of these compounds from natural bovine and procine insulins were described previously (Katsoyannis *et al.*, 1967b).

Sodium in Liquid Ammonia Reduction of Insulin and Sulfitolysis of the Resulting Chains. The classical reduction procedure of Sifferd and du Vigneaud (1935) was employed for the cleavage of insulin to its individual chains following essentially the method used for the deblocking of the protected synthetic insulin A chains (Katsoyannis *et al.*, 1966b, 1967c). In a typical experiment thoroughly dry crystalline bovine zinc insulin (250 mg) was dissolved in anhydrous liquid ammonia (200 ml) in a 500-ml round-bottom flask fitted for magnetic stirring. The reaction was carried out at the boiling point of the solution. Reduction 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 30 sec and was then discharged by the addition of ammonium chloride (*ca.* 2 g). The solution was evaporated at atmospheric pressure to about 10 ml and dried from the frozen state. The reduced product thus obtained was sulfitolyzed as described previously (Katsoyannis, 1966b; Katsoyannis *et al.*, 1967b). Briefly, this material was dissolved in 8 M guanidine hydrochloride (25 ml) containing acetic acid (0.5 ml) and to this solution adjusted to pH 8.9 with dilute ammonium hydroxide was added sodium sulfite (1 g) and freshly prepared sodium tetrathionate (500 mg). The reaction mixture was stirred for 16 hr at room temperature and then dialyzed against five changes of distilled water (4 l. each) at 4° for 20 hr. A Visking $^{18}/_{32}$ dialyzing tubing was used. The dialysate was adjusted to pH 5.0 and the precipitated product was separated by centrifugation. Lyophilization of both the precipitate and the supernatant fluid afforded preparations enriched in $B\text{-}SSO_3^-$ and $A\text{-}SSO_3^-$, respectively.

Isolation of $A\text{-}SSO_3^-$ and $B\text{-}SSO_3^-$ by Continuous-Flow Electrophoresis. This method has been described in detail in a

* From the Department of Biochemistry, Mount Sinai School of Medicine, City University of New York, New York, New York 10029. *Received April 15, 1971.* This work was supported by the National Institute for Arthritis and Metabolic Diseases, U. S. Public Health Service (AM 12925). A. H. and R. J. M. thank the New York Diabetes Association for a student Research Fellowship.

† To whom correspondence should be addressed.

[1] Abbreviations used are: $A\text{-}SSO_3^-$, S-sulfonated derivative of the A chain; $B\text{-}SSO_3^-$, S-sulfonated derivative of the B chain.

previous publication (Katsoyannis *et al.*, 1967b). A Model FF electrophoretic separator (Brinkman Instruments) was employed for the separation and purification of the S-sulfonated chains, which was accomplished at 2400 V, 100–130 mA, and 5° using as a supporting buffer a 0.12% NH_4HCO_3 solution adjusted to pH 7.8 with NH_4OH. The electrode rinse of the separator was a solution of 0.315% NH_4HCO_3. The preparation enriched in B-SSO_3^- was suspended in 10 ml of supporting buffer, brought into solution by adjusting the pH to 10 with NH_4OH, and then placed in the continuous-flow separator. The dosing rate was 3.2 ml/hr and the flow rate of the supporting buffer was 150 ml/hr. The collected effluent was monitored with a Beckman DU spectrophotometer at 278 mμ. The tubes containing the B-SSO_3^- derivative were pooled and lyophilized to yield the S-sulfonated chain as a white fluffy material. The A-SSO_3^- was isolated in a similar manner from the preparation enriched in A-SSO_3^-.

Purification of B-SSO_3^- Derivative by Column Chromatography. Purification of B-SSO_3^- derivatives was accomplished with a 4.5 × 50 cm CM-cellulose column using an 8 M urea–0.04 M sodium acetate buffer at pH 4.0. The washing of the resin and the preparation of the column was described previously (Katsoyannis *et al.*, 1967d). During chromatography the effluent was monitored with a Gilford recording spectrophotometer at 278 mμ. For removing the urea, the fractions containing the chain material were titrated with concentrated HCl to pH 3.0 and then placed on a 4 × 60 cm G-15 Sephadex column equilibrated with 5% (v/v) acetic acid. The pooled fractions containing the chain material were concentrated in a rotary evaporator (30°) to about 10 ml and mixed with 12 ml of saturated picric acid solution. After 24 hr at 2° the precipitated picrate of the B-SSO_3^- derivative was isolated by centrifugation and washed with half-saturated picric acid solution. Separation of the picric acid salts from the chain material was accomplished upon chromatography of its picrate on a 1.2 × 50 cm Sephadex G-15 column equilibrated with 0.05 M NH_4HCO_3. The picrate of the B-SSO_3^- derivative was dissolved in 2 ml of 0.05 M NH_4HCO_3 containing a few drops of 1 N NH_4OH and placed on the column. The chromatogram was developed with 0.05 M NH_4HCO_3. Under these conditions the B-SSO_3^- derivative was separated completely from the picric acid salts, and was recovered as a white fluffy material upon lyophilization of the effluent. Both the aforementioned Sephadex columns were monitored by a Gilford recording spectrophotometer.

Insulin Synthesis by Combination of the A and B Chains and Isolation Techniques. Insulin synthesis was carried out by interaction of the sulfhydryl form of the A chain with the S-sulfonated form of the B chain (Katosyannis and Tometsko, 1966; Katsoyannis *et al.*, 1967d). In a typical experiment, an aqueous solution of 20 mg of A-SSO_3^- was treated at pH 5.0 with 2-mercaptoethanol for 6 min at 100°. The reaction mixture was cooled to 5° and then extracted with ethyl acetate to remove the 2-mercaptoethanol and the ensuing reduced A chain was allowed to react with 5 mg of B-SSO_3^- at pH 9.6. After 18–22 hr at 2°, a sample (1–2 ml) of the reaction mixture (approximately 10 ml) was removed for biological assays and the remaining solution was treated as follows. Acetic acid was added to the reaction mixture to give a final concentration of 10% (v/v) and the resulting solution was mixed with an equal volume of saturated picric acid solution. After 24 hr at 2° the precipitated picrate was isolated by centrifugation and washed twice with 5-ml portions of a half-saturated picric acid solution. This solid was dissolved in a few drops of acetone–water (4:1, v/v) and the resulting solution was mixed with 40 ml of cold dry acetone containing 4 drops of concentrated HCl. The precipitated white product was allowed to settle for 2 hr at 2°, collected by centrifugation, and washed successively with dry acetone and dry ether. Separation of insulin from this precipitate was accomplished as follows. A suspension of the precipitate (which consists of the hydrochlorides of the various products formed by the combination of the A and B chains) in 1 ml of acetate buffer (pH 3.3; Na^+, 0.04 M) was stirred for 1 min and any insoluble material was removed by centrifugation and washed with 2 ml of the same buffer. If more than one recombination mixture was needed, each mixture was treated separately up to this point. The combined supernatants were placed on the 0.9 × 23 cm CM-cellulose column and chromatographed using a sodium chloride gradient as described previously (Katsoyannis *et al.*, 1967a,d). The effluent containing the insulin was concentrated in a rotary evaporator (20–25°) to approximately 10 ml and mixed with an equal volume of saturated picric acid solution. The precipitated insulin (or insulin analog) picrate was collected by centrifugation and converted to the hydrochloride (Randall, 1964).

Additional Techniques. Amino acid analyses were performed according to the method of Spackman *et al.* (1958) in a Beckman-Spinco amino acid analyzer, Model 120B, equipped with a digital readout system. Acid hydrolysis was performed in constant-boiling HCl under nitrogen at 108° for 24 hr. To calculate the molar ratios the average micromoles of glutamic acid, glycine and leucine found were assumed to be equal to the theoretical number of residues in accordance with the known number of each of these residues in the compound to be analyzed. 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). Protein determinations were performed by the method of Lowry *et al.* (1951). Determination of insulin activity was carried out by the mouse convulsion method as has been described previously (Katsoyannis and Tometsko, 1966; Katsoyannis *et al.*, 1967a). Specific activities of the synthetic analogs were calculated from measurements of the protein content and the biological activity of individual samples.

Results

Preparation of S-Sulfonated B Chain Lacking the C-Terminal Tripeptide Sequence. The sodium in liquid ammonia treatment of insulin results in the cleavage of all three disulfide bridges of that molecule and the formation of the sulfhydryl forms of the A and B chains. Oxidative sulfitolysis of the mixture of the reduced chains results in the formation of the S-sulfonated derivatives (SH → S·SO_3^-). The sodium in liquid ammonia reduction, however, besides splitting the disulfide bridges of insulin causes also cleavage of the B chain between the threonine and proline residues at positions 27–28. This results in the elimination of the Pro-Lys-Ala segment from the C-terminal sequence of that chain and the formation of multiple forms of the new C-terminal residue, threonine (Katsoyannis *et al.*, 1971b; Benisek and Cole, 1965; Benisek *et al.*, 1967). Separation of the A-SSO_3^- and destripeptide B-SSO_3^- was accomplished by precipitation at pH 5.0 and then continuous-flow electrophoresis. From 250 mg of bovine insulin 80 mg of destripeptide B-SSO_3^- was obtained. Further purification of the B-SSO_3^- derivative was achieved by chromatography on the 4.5 × 50 cm CM-cellulose column using urea–acetate buffer (pH 4.0). Figure 1-I shows the

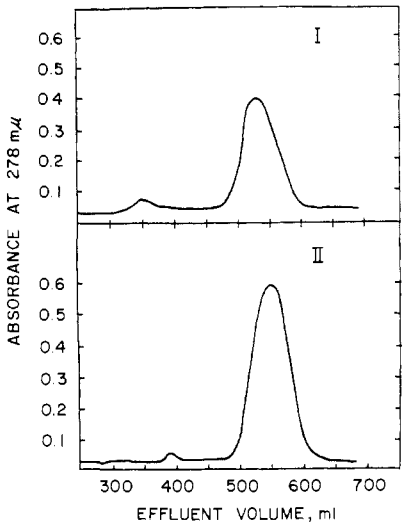

FIGURE 1: Chromatography of B chain derivatives on 4.5 × 50 cm CM-cellulose column with 8 M urea–0.04 M acetate buffer (pH 4.0). (I) Destripeptide B^{28-30} chain *S*-sulfonate (30 mg) which has been separated from A-SSO_3^- by continuous-flow electrophoresis was placed on the column. The amount of purified product was about 18 mg. (II) B-SSO_3^- (50 mg), which was obtained by oxidative sulfitolysis of insulin followed by continuous-flow electrophoresis, was placed on the column and about 33 mg of material was recovered.

chromatogram obtained with the destripeptide B-SSO_3^-, whereas Figure 1-II illustrates the chromatographic pattern of purified B-SSO_3^- obtained from oxidative sulfitolysis of bovine insulin (Katsoyannis *et al.*, 1967b). On high-voltage thin-layer electrophoresis in 0.01 M NH_4HCO_3 adjusted to pH 10 with NH_4OH and 3400 V the purified destripeptide B-SSO_3^- exhibited a single Pauly-positive spot and had the same mobility as natural bovine B-SSO_3^-. Amino acid analysis of the purified material after acid hydrolysis gave a composition in molar ratios in excellent agreement with the theoretically expected values (Table I). The purified product possessed the specific rotation of $[\alpha]_D^{25}$ −95.4° (*c* 0.1, 0.5 N acetic acid) compared to $[\alpha]_D^{25}$ −95.8 ± 4.3° (*c* 0.1, 0.5 N acetic acid) which was found for natural bovine B-SSO_3^-.

TABLE I: Amino Acid Composition[a] of the S-Sulfonated Destripeptide B^{28-30} Chain of Bovine Insulin.

Amino Acid	Theory	Found
Lysine	0	0.09
Histidine	2	2.0
Arginine	1	1.0
Aspartic acid	1	1.0
Serine	1	0.9
Threonine	0	0.1
Glutamic acid	3	3.0
Proline	0	0
Glycine	3	3.0
Alanine	1	1.0
Half-cystine	2	1.4[b]
Valine	3	2.9
Leucine	4	3.8
Tyrosine	2	1.9
Phenylalanine	3	3.0

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

TABLE II: Amino Acid Composition[a] of the Destripeptide B^{28-30} Bovine and Destripeptide B^{28-30} Porcine (Human) Insulins.

	Bovine Insulin Analog		Porcine (Human) Insulin Analog	
Amino Acid	Theory	Found	Theory	Found
Lysine	0	0	0	0.1
Histidine	2	2.1	2	1.9
Arginine	1	1.0	1	1.0
Aspartic acid	3	3.0	3	3.0
Threonine	0	0	1	1.0
Serine	3	2.7	3	2.7
Glutamic acid	7	7.1	7	7.0
Proline	0	0	0	0
Glycine	4	4.1	4	4.0
Alanine	2	2.1	1	1.3
Half-cystine	6	4.6[b]	6	4.8[b]
Valine	5	4.6	4	3.7
Isoleucine	1	0.7	2	1.5
Leucine	6	5.9	6	6.0
Tyrosine	4	3.1[b]	4	2.8[b]
Phenylalanine	3	2.9	3	2.9

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

Isolation of Insulins Produced by Combination of Destripeptide B Chain with Natural A Chains. Bovine, sheep, and porcine insulins have identical B chains (Sanger and Tuppy, 1951a,b; Brown *et al.*, 1955) which differ from the human insulin B chain in the C-terminal amino acid residue; instead of alanine the human B chain contains threonine (Nicol and Smith, 1960). It is, therefore, obvious that the destripeptide B-SSO_3^- is the same for all the aforementioned insulins. The structure of sheep insulin with appropriate numbering of the constituent amino acid residues is shown in Figure 2. Porcine and human insulins have identical A chains (Brown *et al.*, 1955; Nicol and Smith, 1960). Consequently, combination of porcine A chain with destripeptide B chain will result in the formation of an insulin which can be considered as human (or porcine) insulin lacking the C-terminal tripeptide sequence. Natural A and B chains produced by oxidative sulfitolysis of insulin upon recombination according to the procedure described in Materials and Methods produce insulin in a yield of approximately 50% of the theoretically expected value based on the B-SSO_3^- used (Katsoyannis *et al.*, 1967a). The ability of B-SSO_3^- to recombine with the A chain and produce insulin was not impaired when the C-terminal tripeptide sequence of that chain was split by sodium in liquid ammonia. Thus, the yield of the insulin analog produced by combination of destripeptide B-SSO_3^- with natural A chain was also approximately 50% of the value expected by theory.

INSULIN PRODUCED BY COMBINATION OF NATURAL BOVINE A CHAIN WITH DESTRIPEPTIDE B CHAIN (DESTRIPEPTIDE-B^{28-30} BOVINE INSULIN). Combination mixtures with a total insulin activity of 75 IU were treated as described in Materials and

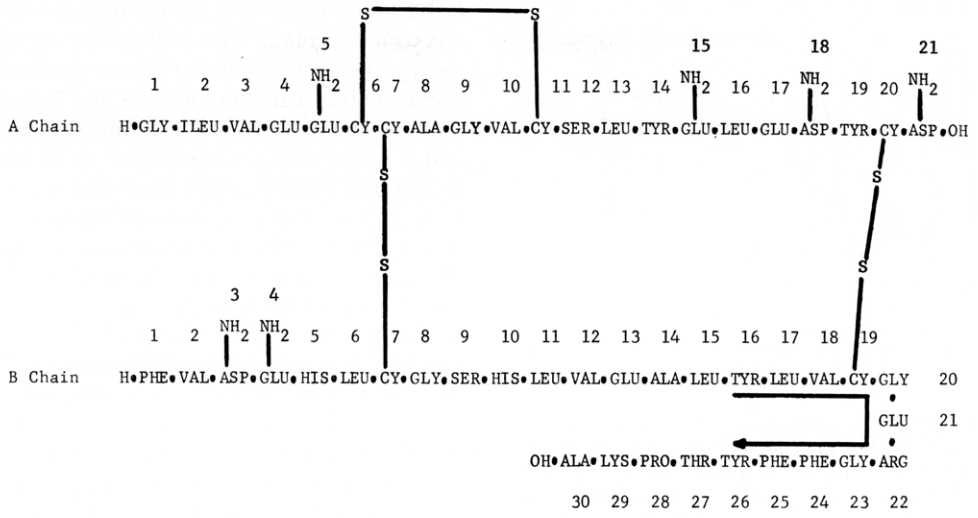

FIGURE 2: Structure of sheep insulin.

Methods. Isolation of the insulin analog was accomplished by chromatography on the 0.9 × 23 cm CM-cellulose column with an exponential NaCl gradient. Figure 3 illustrates the chromatographic pattern obtained. The insulin analog is eluted with application of the NaCl gradient and specifically the insulin analog is the slowest moving material. A similar situation exists with the chromatographic pattern of natural insulin, all synthetic, half-synthetic and hybrid insulins as was reported previously (Katsoyannis *et al.*, 1967a). The effluent containing the active materials was concentrated in a rotary evaporator to approximately 10 ml and the destripeptide insulin was isolated as the hydrochloride (1.1 mg) following the procedure discussed in Materials and Methods. 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 II). The synthetic analog possessed a specific activity (22–28 IU/mg) comparable to that of the natural bovine insulin. Finally on thin-layer electrophoresis (0.5 N acetic acid, 3400 V) the destripeptide insulin moved as a single component (Pauly reaction) and had a mobility slightly different from that of the natural bovine hormone (Figure 4). All attempts to crystallize this analog by existing techniques (Epstein and Anfinsen, 1963; Randall, 1964) were unsuccessful.

INSULIN PRODUCED BY COMBINATION OF NATURAL PORCINE (HUMAN) A CHAIN WITH DESTRIPEPTIDE B CHAIN (DESTRIPEP-

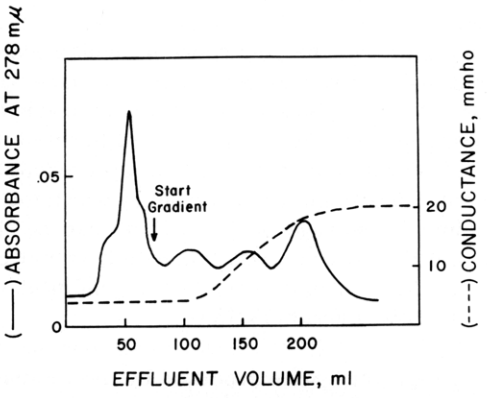

FIGURE 3: Chromatography of a combination mixture o destripeptide B^{28-30} and A chains of bovine insulin on a 0.9 × 23 cm CM-cellulose column with acetate buffer (pH 3.3; Na^+ 0.04 M) and an exponential NaCl gradient. The column effluent was monitored by a Gilford recording spectrophotometer and by a conductivity meter (Radiometer, Copenhagen). Combination mixtures containing 75 IU of destripeptide B^{28-30} bovine insulin were processed. The insulin analog (slowest moving component) was recovered as the hydrochloride (1.1 mg, specific activity 22–28 IU).

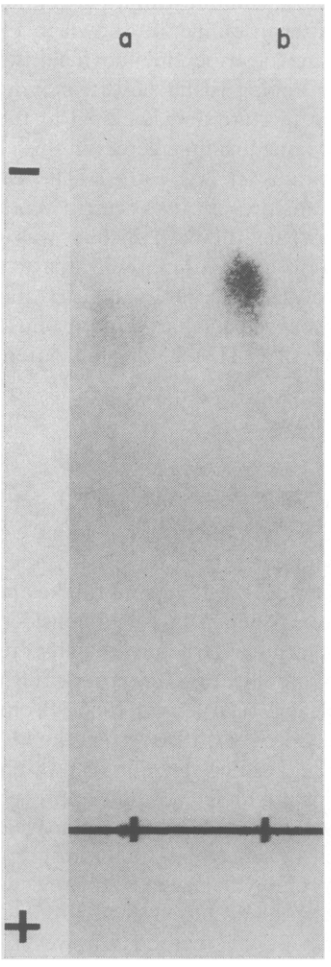

FIGURE 4: Thin-layer electrophoresis of destripeptide B^{28-30} bovine insulin (a) and natural bovine insulin (b): 0.5 N acetic acid, 3400 V, and 12 min.

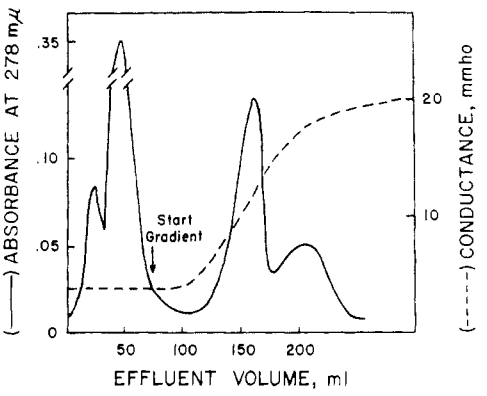

FIGURE 5: Chromatography of a combination mixture of destripeptide B^{28-30} chain (from bovine insulin) and A chain of porcine (human) insulin on a 0.9 × 23 cm CM-cellulose column with acetate buffer (pH 3.3; Na^+ 0.04 M) and an exponential NaCl gradient. The column effluent was monitored by a Gilford recording spectrophotometer and by a conductivity meter. Combination mixtures containing about 90 IU of destripeptide B^{28-30} porcine (human) insulin were processed. The insulin analog (slowest moving component) was recovered as the hydrochloride (1.8 mg, specific activity 17 IU).

TIDE B^{28-30} PORCINE (HUMAN) INSULIN). Combination mixtures with an insulin activity of about 90 IU were treated as described previously. Isolation of the porcine (human) insulin analog was accomplished by chromatography on the 0.9 × 23 cm CM-cellulose column with a NaCl gradient. The chromatographic pattern obtained is shown in Figure 5. As was the case with the destripeptide bovine insulin the porcine (human) insulin analog is the slowest moving component of the combination mixture and is eluted at the same position where natural bovine insulin emerges in this chromatographic system (Katsoyannis *et al.*, 1967a). The effluent with the active material was processed as described previously and the destripeptide porcine (human) insulin was isolated as the hydrochloride (1.8 mg). Amino acid analysis of the analog after acid hydrolysis gave data (Table II) in agreement with the theoretically expected values. This insulin analog possessed a specific activity of 17 IU/mg. Crystallization of this product was not attempted.

Discussion

Investigations of the relationship of the chemical structure to biological activity in the insulin field have been carried out in several laboratories (for reviews, see Carpenter, 1966, Trakatellis and Schwartz, 1968, and Lübke and Klostermeyer, 1970). An approach which provided considerable amount of information regarding structure–activity relationships involved chemical modifications of various functional groups of the insulin molecule (Carpenter, 1966; Bromer *et al.*, 1967; Levy and Carpenter, 1967, 1970; Africa and Carpenter, 1970). Equally interesting results were obtained by enzymatic modifications of the insulin molecule. This approach consists in the selective removal of one or more amino acid residues from the amino or carboxyl termini of the insulin chains with the aid of various proteolytic enzymes (for review, see Carpenter, 1966, and Lübke and Klostermeyer, 1970).

The development of methods, however, for preparing purified A and B chains and the development of highly efficient techniques, both for their combination (Katsoyannis and Tometsko, 1966; Katsoyannis *et al.*, 1967d) and the isolation of the insulin thus produced (Katsoyannis *et al.*, 1967a,d) has opened unlimited possibilities for the synthesis of insulin analogs and for study of structure–activity relationships. To this end a number of analogs of the A and B chains have been synthesized, submitted to combination experiments with natural B and A chains, respectively (Weber *et al.*, 1967a,b, 1968, 1969; Weber and Weitzel, 1968; Hörnle *et al.*, 1968; Weitzel *et al.*, 1969, 1970), and the combination mixtures were tested for biological activity. Biological evaluation of combination mixtures of A and B chains, however, is insufficient for drawing meaningful conclusions regarding structure–activity relationships. Generation of a certain amount of biological activity by combination of modified A and B chains might indicate that either the chains have combined very efficiently and the product, "insulin analog," is not very active or that the chains do not combine efficiently but the analog is highly active. Obviously, then, isolation of an analog in purified form is a prerequisite for meaningful conclusions regarding structure–activity studies.

This investigation has dealt with the isolation in purified form of an analog of porcine (human) insulin and an analog of bovine insulin. Both analogs differ from the parent molecules in that their B chain lacks the C-terminal tripeptide sequence and the carboxyl group of the newly exposed amino acid residue is modified. The key intermediate in the synthesis of both of these analogs is a B chain which lacks the C-terminal tripeptide sequence. The destripeptide B chain is prepared by the sodium in liquid ammonia reduction of bovine insulin. This treatment results in the splitting of the disulfide bridges of insulin and, in addition, causes the cleavage of the B chain between the threonine and proline residues at positions 27–28. For all practical purposes the cleavage of the threonyl–proline bond is almost quantitative as shown from the amino acid analysis of an acid hydrolysate of the isolated destripeptide B-SSO_3^- (Table I). The molar ratios of Pro, Lys, and Ala which occupy the C-terminal tripeptide sequence in the B chain indicate that only traces of these amino acid residues are present in the acid hydrolysate of that chain. The elimination of the C-terminal tripeptide sequence, Pro-Lys-Ala, results in the formation of a new C-terminal amino acid residue, threonine. It has been shown, however (Benisek *et al.*, 1967), that the sodium–liquid ammonia cleavage of acyl–proline bonds leads to the formation of multiple forms of the new C-terminal amino acid residue which in this case is threonine. It appears that the predominant forms of the new C terminus are the corresponding amino alcohol and amino aldehyde derivatives. Since either of these derivatives will be decomposed upon acid hydrolysis, the acid hydrolysate of the destripeptide B-SSO_3^- contains only traces of threonine (Table I). It might be pointed out that a procedure has been worked out (Katsoyannis *et al.*, 1971b) by which the sodium–liquid ammonia cleavage of the B chain can be effectively prevented. This procedure was successfully employed in the synthesis of the B chain of sheep and human insulin (Katsoyannis *et al.*, 1964, 1966a,b, 1971a,b).

The destripeptide B^{28-30} bovine and porcine (human) insulin analogs which were produced by the combination of the destripeptide B^{28-30} chain with the corresponding A chains were isolated by CM-cellulose chromatography in acetate buffer with an exponential sodium chloride gradient (Katsoyannis *et al.*, 1967a,d). In these chromatographic separations, both analogs are eluted at the position where the natural hormone emerges in this chromatographic system (Katsoyannis *et al.*, 1967a). The purified analogs eluted from the CM-cellulose column were obtained as the hydrochlorides and

their homogeneity was ascertained by amino acid analysis after acid hydrolysis and high-voltage thin-layer electrophoresis. The specific activity of the destripeptide B^{28-30} bovine insulin ranged from 22 to 28 IU per mg as compared to the specific activity of 23–25 IU/mg found in our laboratory for the natural bovine, sheep, human, and porcine insulins (Katsoyannis *et al.*, 1967a). The specific activity of the destripeptide B^{28-30} porcine (human) insulin, however, was 70% of that of the natural hormone, namely, 17 IU/mg. We are unable as yet to ascertain whether the lower specific activity of the porcine (human) insulin analog is inherent to that molecule or is due to subtle chemical changes of the porcine (human) A chain (Katsoyannis *et al.*, 1967a) which cannot be detected by routine laboratory procedures.

The high biological activity of the destripeptide B^{28-30} insulin demonstrates that the three amino acid residues Pro, Lys, and Ala, which are found at the C terminus of the parent molecule, are not necessary for biological activity of insulin. It also further implies that an intact threonine residue at position B^{27} in the parent molecule is not necessary for biological activity of the hormone, since the new C terminus of both active analogs contains the amino alcohol or amino aldehyde derivative of that amino acid.

Acknowledgments

The authors express their appreciation to Mrs. Mei-Ying Liu for the biological assays and to Miss Karin Scheibe for amino acid analyses. They also thank the Eli Lilly and Co. for generous supplies of bovine and porcine insulins.

References

Africa, B., and Carpenter, F. H. (1970), *Biochemistry 9*, 1962.

Benisek, W. F., and Cole, R. D. (1965), *Biochem. Biophys. Res. Commun. 20*, 655.

Benisek, W. F., Raftery, M. A., and Cole, R. D. (1967), *Biochemistry 6*, 3780.

Bromer, W. W., Sheehan, K. S., Berns, A. W., and Arquilla, E. R. (1967), *Biochemistry 6*, 2378.

Brown, H., Sanger, F., and Kitai, R. (1955), *Biochem. J. 60*, 556.

Carpenter, F. H. (1966), *Amer. J. Med. 40*, 750.

Epstein, C. J., and Anfinsen, C. B. (1963), *Biochemistry 2*, 461.

Gilman, A., Philips, F. S., and Koelle, E. S. (1946), *Amer. J. Physiol. 164*, 348.

Hörnle, S., Weber, U., and Weitzel, G. (1968), *Hoppe-Seyler's Z. Physiol. Chem. 349*, 1428.

Katsoyannis, P. G. (1966a), *Science 154*, 1509.

Katsoyannis, P. G. (1966b), *Amer. J. Med. 40*, 652.

Katsoyannis, P. G., Fukuda, K., Tometsko, A., Suzuki, K., and Tilak, M. (1964), *J. Amer. Chem. Soc. 86*, 930.

Katsoyannis, P. G., Ginos, J., Zalut, C., Tilak, M., Johnson, S., and Trakatellis, A. C. (1971a), *J. Amer. Chem. Soc.* (in press).

Katsoyannis, P. G., and Tometsko, A. (1966), *Proc. Nat. Acad. Sci. U. S. 55*, 1554.

Katsoyannis, P. G., Tometsko, A., Ginos, J., and Tilak, M. (1966a), *J. Amer. Chem. Soc. 88*, 164.

Katsoyannis, P. G., Tometsko, A., and Zalut, C. (1967c), *J. Amer. Chem. Soc. 89*, 4505.

Katsoyannis, P. G., Tometsko, A., Zalut, C., and Fukuda, K. (1966b), *J. Amer. Chem. Soc. 88*, 5625.

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

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

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

Katsoyannis, P. G., Zalut, C., Tometsko, A., Tilak, M., Johnson, S., and Trakatellis, A. (1971b), *J. Amer. Chem. Soc.* (in press).

Levy, D., and Carpenter, F. H. (1967), *Biochemistry 6*, 3559.

Levy, D., and Carpenter, F. H. (1970), *Biochemistry 9*, 3215.

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

Lübke, K., and Klostermeyer, H. (1970), *Advan. Enzymol. 33*, 445.

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

Randall, S. S. (1964), *Biochim. Biophys. Acta 90*, 472.

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

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

Sifferd, R. H., and du Vigneaud, V. (1935), *J. Biol. Chem. 108*, 753.

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

Tometsko, A., and Delihas, N. (1967), *Anal. Biochem. 18*, 72.

Trakatellis, A. C., and Schwartz, G. P. (1968), *Progr. Chem. Org. Natural Products 26*, 120.

Weber, U., Hörnle, S., Grieser, G., Herzog, K.-H., and Weitzel, G. (1967b), *Hoppe-Seyler's Z. Physiol. Chem. 348*, 1715.

Weber, U., Hörnle, S., Köhler, P., Nagelschneider, G., Eisele, K., and Weitzel, G. (1968), *Hoppe-Seyler's Z. Physiol. Chem. 349*, 512.

Weber, U., Schneider, F., Köhler, P., and Weitzel, G. (1967a), *Hoppe-Seyler's Z. Physiol. Chem. 348*, 947.

Weber, U., Herzog, K.-H., Grossmann, H., Hornle, S., and Weitzel, G. (1969), *Hoppe-Seyler's Z. Physiol. Chem. 350*, 1425.

Weber, U., and Weitzel, G. (1968), *Hoppe-Seyler's Z. Physiol. Chem. 349*, 1431.

Weitzel, G., Eisele, K., Zollner, H., and Weber, U. (1969), *Hoppe-Seyler's Z. Physiol. Chem. 350*, 1480.

Weitzel, G., Weber, U., Eisele, K., Zollner, H., and Martin, J. (1970), *Hoppe-Seyler's Z. Physiol. Chem. 351*, 263.