Studies on the Synthesis of Insulin from Natural and Synthetic A and B Chains. III. Synthetic Insulins*

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ABSTRACT: The following insulins have been synthesized by combination of the respective A and B chains and have been isolated in highly purified form: (1) all-synthetic sheep, (2) all-synthetic human, (3) half-synthetic sheep, (4) half-synthetic bovine, (5) half-synthetic porcine, (6) half-synthetic insulin B_AH_B, a hormone not found in nature, which was produced by combination of synthetic human B chain with natural bovine A chain, (7) porcine, and (8) bovine insulins. Based on the amount of the B chain used, the combination yields for the half-synthetic insulins ranged from 17 to 42% and for the all-synthetic insulins ranged from 12 to 22%. Isolation was effected by chromatography on a carboxymethylcellulose column with an exponential sodium chloride gradient. The over-all recovery of insulin by this isolation procedure ranged from 35 to 52%. The synthetic and half-synthetic insulins were compared with their natural counterparts as to specific activity, amino acid composition, and mobility on thin layer electrophoresis and column chromatography. All of these comparisons afforded convincing evidence of the identity of the synthetic insulins with the natural hormones. The all-synthetic sheep and the half-synthetic sheep and bovine insulins were obtained in crystalline form. The synthetic nonnaturally occurring insulin B_AH_B had a biological activity comparable to that of the naturally occurring insulins and was obtained in crystalline form.

Systematic studies in this laboratory during the past several years resulted in the synthesis and isolation in the S-sulfonated form of the A and B chains of sheep (Katsoyannis *et al.*, 1963, 1964, 1966a) and human insulins (Katsoyannis *et al.*, 1966b,c, 1967a). Combination of the chains by the existing procedures (Dixon and Wardlaw, 1960; Du *et al.*, 1961), or more efficiently by procedures developed in this laboratory (Katsoyannis and Tometsko, 1966), led to the generation of sheep and human insulins. Furthermore, combination of these synthetic chains with the corresponding natural chains of bovine and porcine insulins led to the generation of the respective half-synthetic hormones. In our estimation, this work constitutes the first chemical synthesis of insulin (Katsoyannis, 1964a,b, 1966a,b, 1967; Katsoyannis *et al.*, 1966c). Figure 1 illustrates the structure of sheep insulin as proposed by Brown *et al.* (1955) and Table I records the differences found in the aforementioned insulin species.

Isolation of the synthetic insulins by procedures devised for isolation of this protein from natural sources presented complications which are extensively discussed in the preceding publication (Katsoyannis *et al.*, 1967b). Application of acid–alcohol extraction of insulin (Banting *et al.*, 1922) as modified by Du *et al.* (1961) is unsatisfactory. Specifically, in the isolation of all-synthetic bovine insulin (Kung *et al.*, 1966) the over-all recovery of pure material, based on the amounts of the A and B chains used for the combination reaction, was of the order of 0.05–0.1%, and in terms of actual amount of material obtained per experiment, it was of the order of a few hundredths of a milligram. Such a low recovery of material, however, makes imprecise the determination of protein content and biological activity of the synthetic hormone and therefore precludes reliable calculation of its specific activity. Furthermore, other important analytical data such as amino acid composition cannot be obtained. Extensive studies in this laboratory led to the development of efficient methods for the isolation of insulin in highly purified form from combination mixtures of A and B chains (Katsoyannis *et al.*, 1967b). These procedures have been applied in this investigation to the isolation of synthetic and half-synthetic insulins with over-all recoveries (based on the B chain used originally in the combination reaction) at least 100-fold higher than the recoveries reported by Kung *et al.* (1966).

Materials and Methods

S-Sulfonated Derivatives of the A and B Chains of Insulin (A-SSO_3^- and B-SSO_3^-)[1]

Natural A-SSO_3^- and B-SSO_3^- of Bovine and Porcine Insulins. These derivatives were prepared from bovine

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[1] Abbreviations used: A-SSO_3^-, S-sulfonated derivative of the A chain; B-SSO_3^-, S-sulfonated derivative of the B chain.

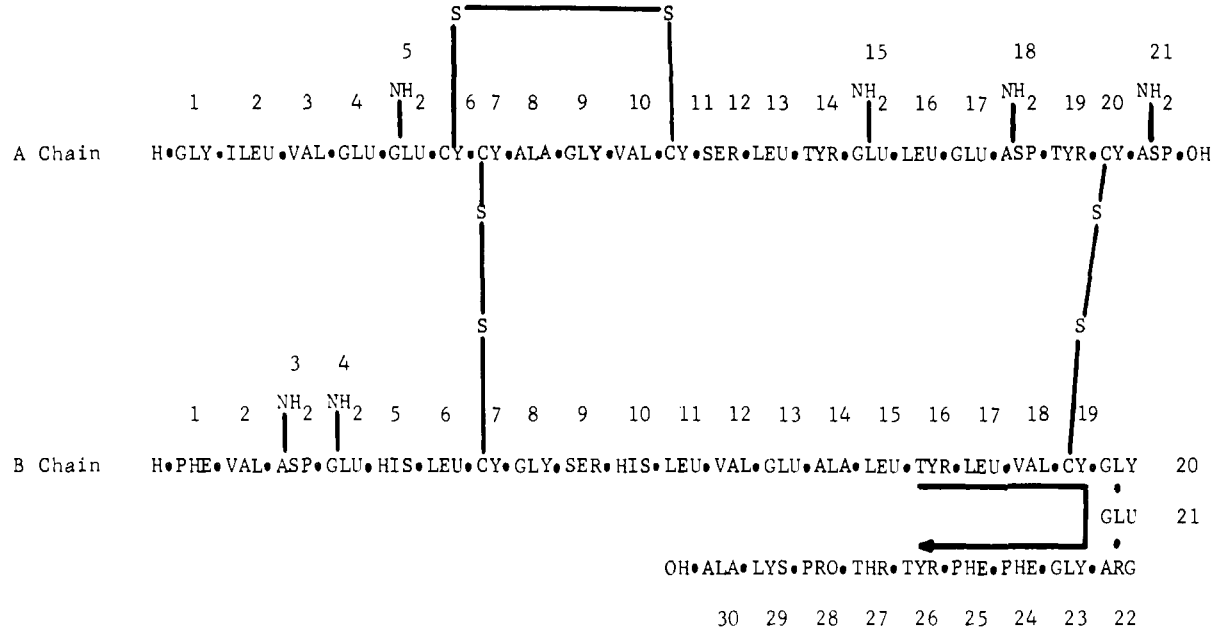

FIGURE 1: Structure of sheep insulin.

TABLE I: Amino Acid Sequences[a] of the Insulins Synthesized.

Insulin Species	A Chain	B Chain
	8 9 10	30
Bovine	---Ala·Ser·Val--- (Sanger and Thompson, 1953a,b)	---Ala (Sanger and Tuppy, 1951a,b)
Sheep	---Ala·Gly·Val--- (Brown *et al.*, 1955)	---Ala (Brown *et al.*, 1955)
Porcine	---Thr·Ser·Ile--- (Brown *et al.*, 1955)	---Ala (Brown *et al.*, 1955)
Human	---Thr·Ser·Ile--- (Nicol and Smith, 1960)	---Thr (Nicol and Smith, 1960)
Insulin B_AH_B[b]	---Ala·Ser·Val---	---Thr

[a] Sequences not listed are identical. [b] This insulin has not been found in the species examined and it was tentatively designated insulin B_AH_B: B_A for bovine A chain and H_B for human B chain.

and porcine insulins as described previously (Katsoyannis *et al.*, 1967c).

Synthetic A-SSO_3^- and B-SSO_3^- of Sheep Insulin. The synthesis of A-SSO_3^- and B-SSO_3^- of sheep insulin was accomplished as described previously (Katsoyannis *et al.*, 1963, 1964, 1966a; P. G. Katsoyannis, unpublished data).

Synthetic A-SSO_3^- and B-SSO_3^- of Human Insulin. The S-sulfonated derivatives of the A and B chains of human insulin were prepared according to the methods previously described (Katsoyannis *et al.*, 1966b,c, 1967a; P. G. Katsoyannis, unpublished data).

Insulin Synthesis and Isolation Techniques

Insulin synthesis throughout this investigation was performed by interaction of the sulfhydryl form of the A chain with B-SSO_3^- (Katsoyannis and Tometsko, 1966; Katsoyannis *et al.*, 1967b). In a typical experiment, 20 mg of A-SSO_3^- and 5 mg of B-SSO_3^- were used for the combination reaction. After completion of the air-oxidation step a sample (0.8–1.2 ml) of the reaction mixture (approximately 10 ml) was removed for biological assays and the remaining solution was treated as follows. Glacial 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. The entire procedure was carried out at 2°. After 24 hr at 2° the precipitate was collected 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) and the resulting solution was mixed with 40 ml of ice-cold dry acetone containing 4 drops of concentrated hydrochloric acid. A white precipitate was formed immediately. After 2 hr in the cold the precipitate was collected by centrifugation and washed once with dry

TABLE II: Combination Yields and Specific Activities of Isolated Insulins Synthesized by Interaction of A and B Chains.

Type of Chains Used for Combination	Insulin Produced	Combination Yield (%)	Sp Act.[a] (IU/mg)
Natural insulin (bovine, sheep, porcine, and human)			23–25
Synthetic sheep A + natural bovine B	Sheep (half-synthetic)	30–38	25[c]
Natural bovine A + synthetic sheep B	Bovine (half-synthetic)	17–19	22[c]
Synthetic sheep A + synthetic sheep B	Sheep (all-synthetic)	12–16	25[c]
Natural porcine A + natural bovine B	Porcine	35–50	25
Natural bovine A + natural porcine B	Bovine	*b*	23[c]
Synthetic human A + natural bovine B	Porcine (half-synthetic)	30–42	22
Natural bovine A + synthetic human B	Insulin B_AH_B	18–23	22[c]
Synthetic human A + synthetic human B	Human (all-synthetic)	15–22	24

[a] The specific activity was determined on the isolated insulin hydrochloride. [b] Not computed because of a limited number of recombinations. [c] Obtained in crystalline form.

acetone and once with dry ether. A suspension of the precipitate in 3 ml of acetate buffer (pH 3.3; Na^+, 0.04 M) was stirred vigorously for 1 min and the resulting 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 a 0.9 × 23 cm carboxymethylcellulose column and chromatographed as described previously (Katsoyannis *et al.*, 1967b). 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 in order to form the insulin picrate. This picrate was converted to the hydrochloride as described by Randall (1964). Crystallization of insulin, if desirable, was carried out either as described by Randall (1964) or by Epstein and Anfinsen (1963). Protein determinations were performed by the method of Lowry *et al.* (1951) and thin layer electrophoresis was carried out as described by Tometsko and Delihas (1967). Amino acid analyses of acid hydrolysates were performed in a Beckman-Spinco amino acid analyzer (Model 120C) equipped with a digital readout system (Model CRS-10AB, Infotronics Corp., Houston, Texas) according to the method of Spackman *et al.* (1958).

Biological assays were carried out by the mouse convulsion method (Hemmingsen and Krogh, 1926; Marks, 1940; Bangham and Mussett, 1959; for review, see also Stewart, 1960). Each unknown sample was assayed for 3–4 consecutive days. Each day a new insulin standard was used. Two concentrations of the standard and three concentrations of the unknown sample were injected each day into five groups of 12–16 mice each. For the complete assay of a particular sample, a total of 180–200 mice were used. The reported assay values are the average figure of the 3- or 4-day assays. For the preparation of the reference standards, natural bovine crystalline zinc insulin with a potency of 25 IU/mg was used. Specific activities of the synthetic insulins were calculated from measurements of the protein content and the biological activity of individual samples. To test the accuracy of the protein and biological activity determinations of the synthetic materials, a sample of natural crystalline bovine insulin (potency 25 IU/mg) was used as "unknown" and its specific activity was determined at the same time.

TABLE III: Over-All Insulin Recovery from Combination Mixtures of A and B Chains.

Insulin Species Synthesized	Total Act. Processed (IU)	Isolated Insulin Hydrochloride (mg)[b]	Over-All Recov[a] as Insulin-HCl (%)
All-synthetic sheep insulin	86	1.50	43
Half-synthetic sheep insulin	58	0.90	39
Half-synthetic bovine insulin	35	0.53	37
All-synthetic human insulin	78	1.30	41
Half-synthetic porcine insulin	88	1.85	52
Insulin B_AH_B	35	0.50	35
Porcine insulin	130	2.28	42
Bovine insulin	89	1.40	39

[a] The insulin activity present in the combination mixture taken as 100%. [b] Determined by the Folin method.

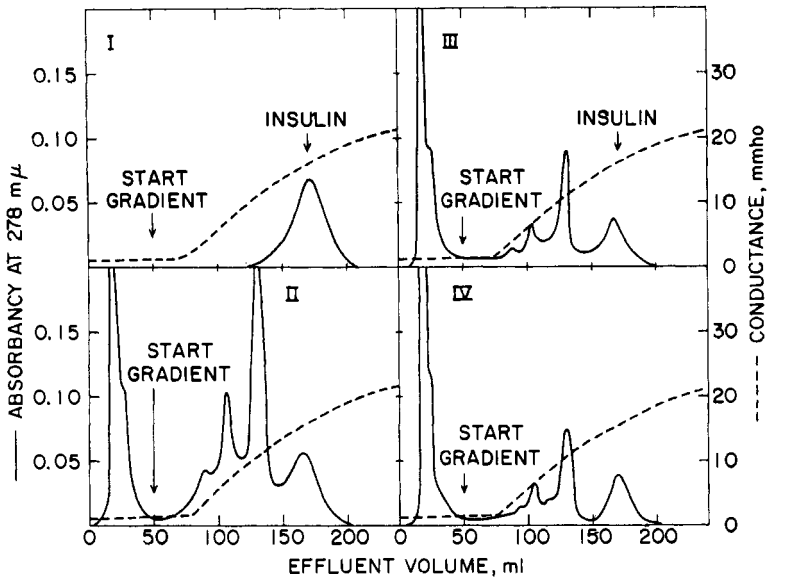

FIGURE 2: Chromatography of natural bovine zinc insulin and of combination mixtures on a 0.9 × 23 cm CM-cellulose column with an exponential NaCl gradient. The column effluent was monitored by a Gilford recording spectrophotometer and by a conductivity meter (Radiometer, Copenhagen). (I) Bovine zinc insulin (75 IU) was dissolved in 2 ml of sodium acetate buffer (pH 3.3; Na^+, 0.04 M) and placed on the column. The insulin was recovered as the hydrochloride (2.4 mg). (II) Combination mixtures containing 86 IU of all-synthetic sheep insulin were processed. The all-synthetic insulin was recovered as the hydrochloride (1.5 mg, sp act. 25 IU/mg). (III) A combination mixture containing 58 IU of half-synthetic sheep insulin was processed. This insulin was recovered as the hydrochloride (0.9 mg, sp act. 25 IU/mg). (IV) A combination mixture containing 35 IU of half-synthetic bovine insulin was processed. The half-synthetic hormone was recovered as the hydrochloride (0.53 mg, sp act. 22 IU/mg).

Natural human insulin was kindly supplied by Dr. I. A. Mirsky. Bovine and porcine insulins were provided by the Eli Lilly Co. Natural sheep insulin was prepared in this laboratory by the method of Randall (1964).

Results

Yields of Insulins Synthesized by Interaction of A and B Chains

The yield of insulin produced by combination of A and B chains is calculated on the basis of the B-SSO_3^- used. In a typical experiment 5 mg of B-SSO_3^- is used and thus the maximum amount of insulin which can be produced is 8 mg or 200 IU. If only 4 mg of insulin or 100 IU is produced, then the combination yield of such a reaction is 50% of the theoretically expected value. Table II records the insulins synthesized in this laboratory, the combination yields obtained, and the specific activities of these synthetic insulins. The combination yields reported are the values obtained from 10 to 20 experiments.

It must be emphasized that the variation of the combination yields of the insulin chains used has no reflection on the specific activity of the insulins produced. As can be seen from Table II the specific activity of all the synthetic insulins isolated are comparable to the potency of the respective natural hormone.

Isolation of Insulins Produced by Combination of Synthetic Sheep A and B Chains

Since sheep and bovine insulins have identical B chains and differ only in the A chain (Brown *et al.*, 1955), combination of sheep B chain with bovine A chain will produce bovine insulin and combination of sheep A chain with bovine B chain will produce sheep insulin.

Insulin Produced by Combination of Synthetic Sheep B Chain with Natural Bovine A Chain (Half-Synthetic Bovine Insulin). Combination mixtures with a total insulin activity of 35 IU were treated as described in Materials and Methods. Isolation of the half-synthetic insulin 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 2-IV. It was previously found (Katsoyannis *et al.*, 1967b) that the components of a recombination mixture are A and B chain components and insulin. In this chromatographic system the A chain components are eluted first without the use of the NaCl gradient. The B chain components and insulin are eluted with application of an exponential NaCl gradient and specifically the insulin is the slowest moving material (Katsoyannis *et al.*, 1967b). The half-synthetic insulin is eluted at the same position where natural bovine insulin emerges in this chromatographic system (Figure 2-I). The effluent containing the active material was con-

TABLE IV: Amino Acid Composition[a] of the All-Synthetic Sheep, the Half-Synthetic Sheep, and the Half-Synthetic Bovine Insulins.

Amino Acid	Sheep Insulin Theory	All-Synthetic Sheep Insulin Found	Half-Synthetic Sheep Insulin Found	Bovine Insulin Theory	Half-Synthetic Bovine Insulin Found
Lysine	1	1.0	1.0	1	1.1
Histidine	2	1.9	2.0	2	2.0
Arginine	1	1.0	1.0	1	0.9
Aspartic acid	3	2.6	3.0	3	3.1
Threonine	1	0.9	1.0	1	0.9
Serine	2	1.8	1.8	3	2.5
Glutamic acid	7	7.2	7.5	7	6.9
Proline	1	1.1	1.0	1	0.9
Glycine	5	5.1	5.0	4	4.0
Alanine	3	3.1	3.0	3	3.2
Valine	5	4.7	4.6	5	4.6
Isoleucine	1	0.8	0.8	1	0.8
Leucine	6	6.2	6.0	6	5.8
Tyrosine	4	*b*	*b*	4	*b*
Phenylalanine	3	3.1	3.0	3	3.1
Half-cystine	6	*b*	*b*	6	*b*

[a] Number of amino acid residues per molecule. [b] Not determined.

centrated in a rotary evaporator to approximately 10 ml, transferred in a centrifuge tube, and mixed with 12 ml of a saturated picric acid solution. After 20 hr at room temperature the precipitated insulin picrate was isolated and converted to insulin hydrochloride (0.53 mg). This recombination and isolation experiment was repeated five times and the results originally obtained were confirmed. Table III summarizes the recovery data of the isolation procedure. Amino acid analysis of the half-synthetic product after acid hydrolysis gave a composition in molar ratios in excellent agreement with the theoretically expected values (Table IV). The synthetic material possessed a specific activity comparable to that of the natural hormone (Table II). On thin layer electrophoresis the synthetic and natural product exhibited an identical behavior (Figure 3). Finally, the half-synthetic insulin was crystallized and the form of the crystals (Figure 4) was similar to that of the natural hormone.

Insulin Produced by Combination of Synthetic Sheep A Chain with Natural Bovine B Chain (Half-Synthetic Sheep Insulin). Combination mixtures with a total insulin activity of 58 IU were processed as described previously. The chromatographic pattern obtained is illustrated in Figure 2-III. The half-synthetic insulin is eluted at the same position where natural bovine insulin emerges in this chromatographic system. The insulin from the effluent was eventually converted to the hydrochloride (0.9 mg) by the same route as described previously and was crystallized (Figure 5). The isolation procedure was repeated three times and the results originally obtained were confirmed. The quantitative data of the isolation process are given in Table III.

The amino acid composition of the synthetic material, expressed in molar ratios, was in excellent agreement with the values expected from theory (Table IV). The specific activity of the synthetic material (25 IU/mg) was identical with the potency of the natural bovine insulin (Table II). Finally, on thin layer electrophoresis, the synthetic insulin moved as a single component and had the same mobility as the natural hormone (Figure 6).

Insulin Produced by Combination of Synthetic Sheep A and B Chains (All-Synthetic Sheep Insulin). Combination mixtures of all-synthetic sheep A and B chains with a total activity of 86 IU were processed as described previously. The chromatographic pattern obtained and shown in Figure 2-II clearly illustrates that the all-synthetic sheep insulin is eluted at the same position as the natural hormone. The effluent with the active material was processed as described previously and the all-synthetic insulin was isolated as the hydrochloride (1.5 mg). The isolation was repeated four times and the results originally obtained were confirmed. The quantitative data of the isolation procedure are given in Table III. The hydrochloride of the synthetic material was compared with the natural sheep insulin with respect to amino acid composition, biological activity, and electrophoretic mobility on thin layer electrophoresis. Thus, amino acid analysis of the synthetic insulin after acid hydrolysis gave the molar ratios of amino acids shown in Table IV in excellent agreement with the theoretically expected values. On thin layer electrophoresis the synthetic protein exhibited a single Pauly-positive spot and had the same mobility as the natural sheep and bovine

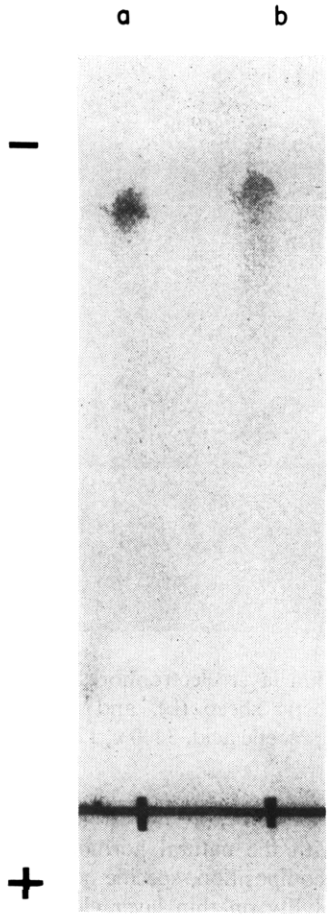

FIGURE 3: Thin layer electrophoresis of half-synthetic bovine (b) and natural bovine (a) insulins; 0.5 N acetic acid, 3400 v, 19 min.

insulins (Figure 7). Finally, the synthetic insulin has a specific activity identical with that of the natural hormone (Table II), *i.e.*, 25 IU/mg.

The synthetic hormone was crystallized and the crystals obtained had the same crystalline form as the natural protein (Figure 8).

Isolation of Insulins Produced by Recombination of Natural Porcine and Bovine Chains

Porcine and human insulins have identical A chains (Brown *et al.*, 1955; Nicol and Smith 1960), and porcine and bovine insulins have identical B chains (Sanger and Tuppy, 1951a,b; Brown *et al.*, 1955). Consequently, recombination of porcine or human A chain with bovine B chain will produce porcine insulin. Since the isolation of insulin produced by combination of synthetic human A chain with natural bovine B chain (half-synthetic porcine insulin) was part of this investigation, it was desirable, for comparison studies, to have available the respective insulin produced by recombination of natural chains. Isolation of insulin produced by recombination of natural bovine A and natural porcine B chains

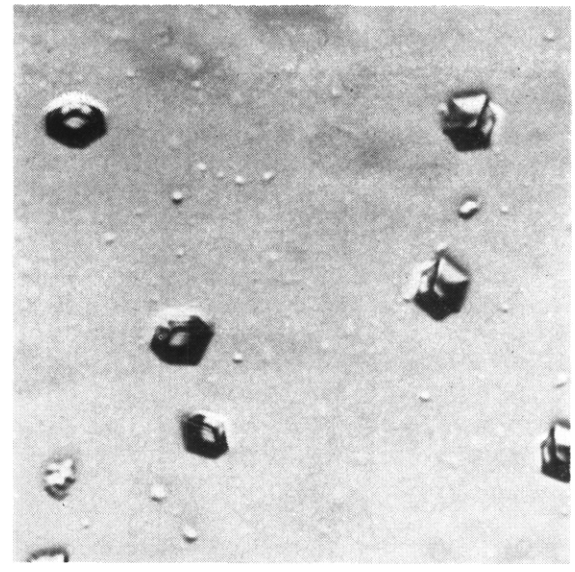

FIGURE 4: Half-synthetic crystalline bovine insulin (natural bovine A chain plus synthetic sheep B chain).

(bovine insulin) was also desirable for reasons which will be considered in the discussion.

Insulin Produced by Recombination of Natural Porcine A and Natural Bovine B Chains (Porcine Insulin). Recombination mixtures with a total activity of 130 IU were processed in the usual manner. The chromatographic pattern obtained is shown in Figure 9-II. As was the case with the other insulins described previously, porcine insulin was eluted at the same position as natural bovine insulin (Figure 9-I). From the effluent containing the active material the insulin was isolated as the hydrochloride (2.28 mg) (for recoveries see Table

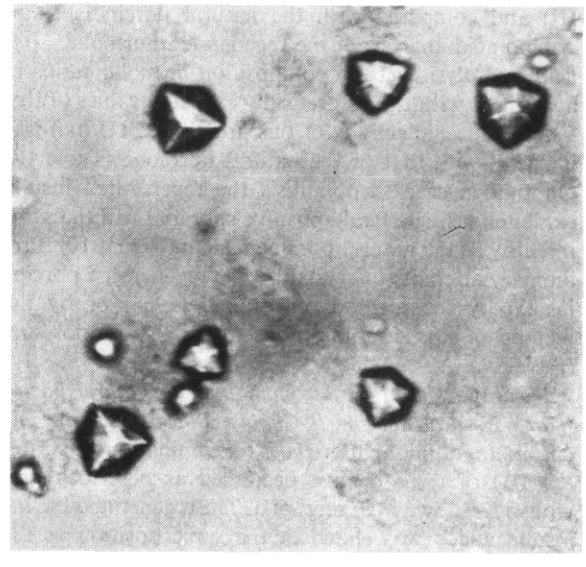

FIGURE 5: Half-synthetic crystalline sheep insulin (synthetic sheep A chain plus natural bovine B chain).

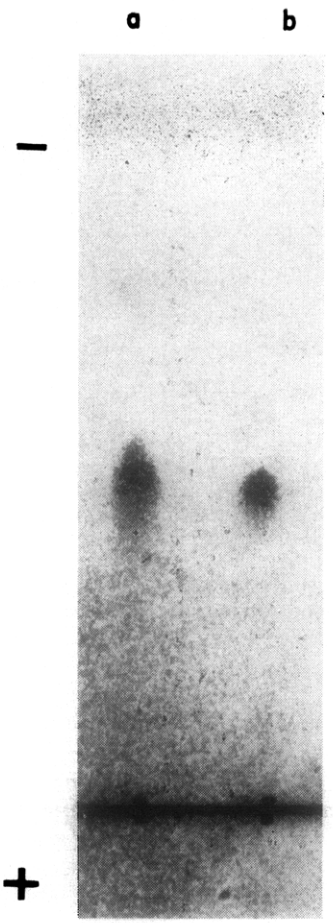

FIGURE 6: Thin layer electrophoresis of half-synthetic sheep (a) and natural sheep (b) insulin; 0.5 N acetic acid, 3400 v, 12 min.

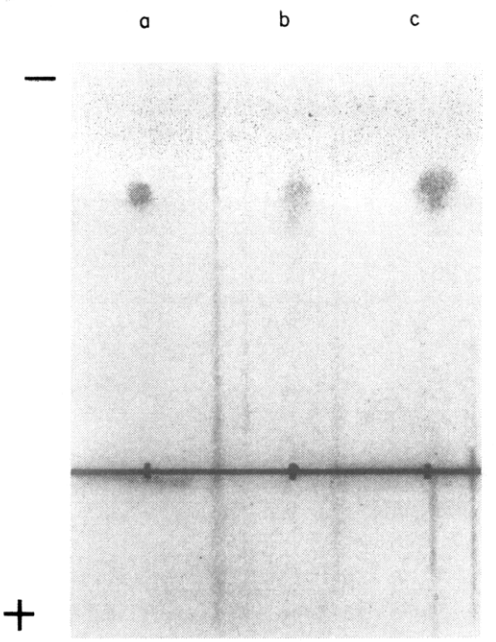

FIGURE 7: Thin layer electrophoresis of natural sheep (a), all-synthetic sheep (b), and natural bovine (c) insulins; 0.5 N acetic acid, 3400 v, 12 min.

III) and compared with the natural porcine insulin. Amino acid analysis of the regenerated hormone, after acid hydrolysis, gave a composition in molar ratios in agreement with the theoretically expected values (Table V). The specific activity of this protein (25 IU/mg) was identical with that of the natural hormone (Table II). On thin layer electrophoresis the regenerated insulin exhibited a single Pauly-positive spot and had the same mobility as the natural porcine insulin (Figure 10). Preliminary attempts to crystallize the regenerated porcine insulin by the method of Randall (1964) or by the method of Epstein and Anfinsen (1963) were not successful.

Insulin Produced by Recombination of Natural Bovine A and Natural Porcine B Chains (Bovine Insulin). The chromatographic pattern of recombination mixtures (89 IU) of these chains, processed as described previously, is shown in Figure 9-III. The regenerated bovine insulin which was eluted at the same position as the natural hormone was converted to the hydrochloride (1.4 mg). Quantitative data of the isolation method are listed in Table III. The regenerated bovine insulin was compared with the natural hormone with respect to amino acid composition, specific activity, and electrophoretic mobility on thin layer electrophoresis. Thus amino acid analysis after acid hydrolysis gave a composition in molar ratios in excellent agreement with the theoretically expected values (Table V). The specific activity of the regenerated hormone (23 IU/mg) was

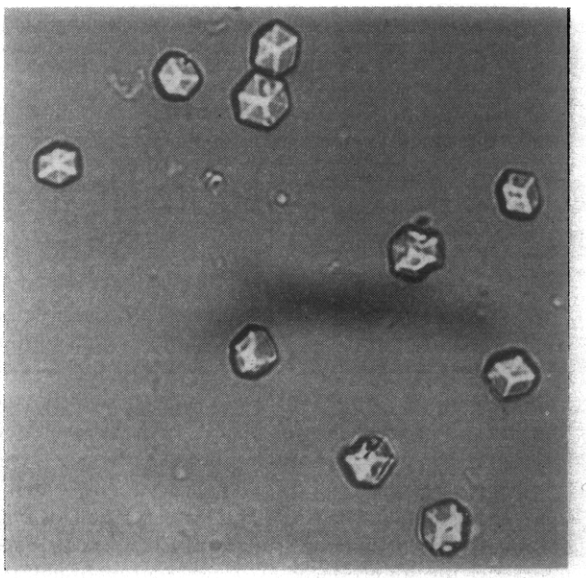

FIGURE 8: All-synthetic crystalline sheep insulin (synthetic sheep A chain plus synthetic sheep B chain).

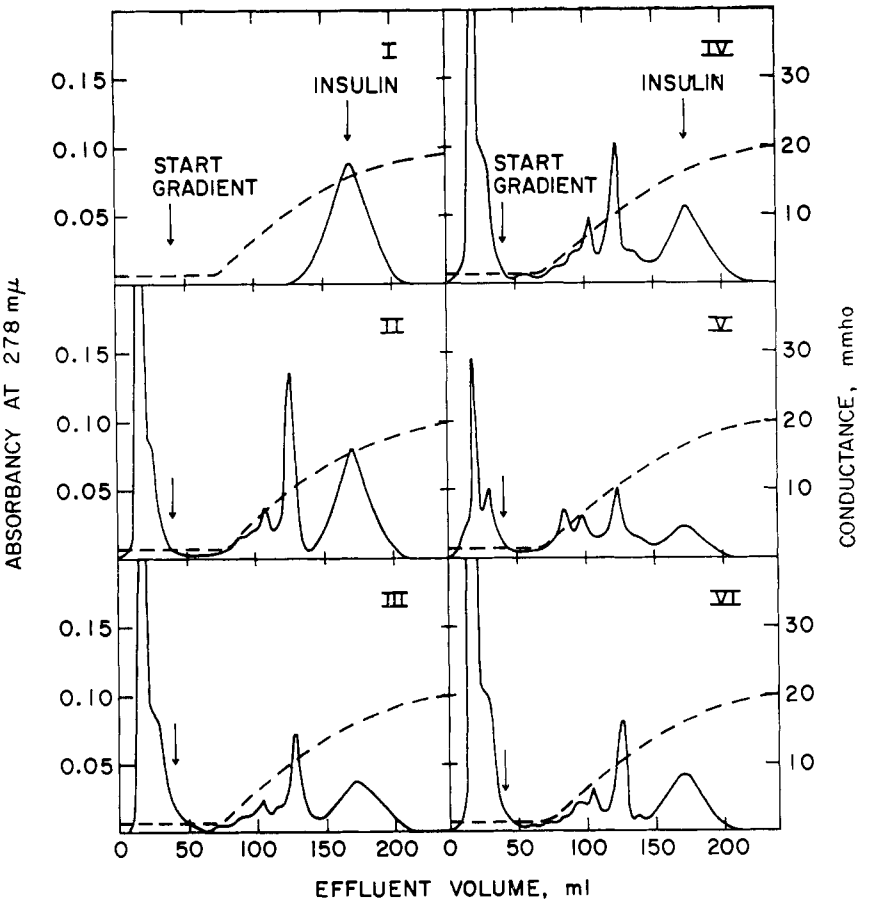

FIGURE 9: Chromatography of natural bovine zinc insulin and of combination mixtures on a 0.9 × 23 cm CM-cellulose column with an exponential NaCl gradient. The column effluent was monitored by a Gilford spectrophotometer and by a conductivity meter. (I) Natural bovine zinc insulin (87.5 IU) was dissolved in 3 ml of sodium acetate buffer and placed on the column. The insulin was recovered as the hydrochloride (3.0 mg). (II) Combination mixtures containing 130 IU of porcine insulin (porcine A plus bovine B) were processed. The hormone was recovered as hydrochloride (2.28 mg, sp act. 25 IU/mg). (III) A combination mixture containing 89 IU of bovine insulin (bovine A plus porcine B) was processed. This insulin was recovered as the hydrochloride (1.4 mg, sp act. 23 IU/mg). (IV) Combination mixtures containing 88 IU of half-synthetic porcine insulin (synthetic human A plus bovine B) were processed. The half-synthetic hormone was recovered as the hydrochloride (1.85 mg, sp act. 22 IU/mg). (V) A combination mixture containing 35 IU of insulin B_AH_B was processed. The insulin B_AH_B was recovered as the hydrochloride (0.5 mg, sp act. 22 IU/mg). (VI) Combination mixtures containing 78 IU of all-synthetic human insulin were processed. This insulin was recovered as the hydrochloride (1.3 mg, sp act. 24 IU).

identical with the potency of the natural product (Table II). On thin layer electrophoresis the regenerated insulin migrated as a single component and had the same mobility as the natural hormone (Figure 11).

In contrast to the regenerated porcine insulin, the regenerated bovine hormone was readily crystallized.

Isolation of Insulins Produced by Combination of Synthetic Human A and B Chains

The combination of synthetic human A chain with natural bovine B chain will produce half-synthetic porcine insulin as was mentioned previously. On the other hand, the combination of natural bovine A chain with synthetic human B chain will produce a half-synthetic insulin which has not been found in any of the species examined. This insulin is tentatively designated as insulin B_AH_B: B_A for bovine A chain and H_B for human B chain.

Insulin Produced by Combination of Synthetic Human A Chain and Natural Bovine B Chain (Half-Synthetic Porcine Insulin). Combination reactions between synthetic human A chain and natural bovine B chain were carried out as described in Materials and Methods and the resulting reaction mixtures with a total activity of 88 IU were processed as indicated previously. The chromatographic pattern obtained from the CM-cellulose column is illustrated in Figure 9-IV and indicates that the half-synthetic porcine insulin is eluted at the same position as the porcine insulin regenerated from natural chains (Figure 9-II) and the natural bovine insulin

TABLE V: Amino Acid Composition[a] of Regenerated Porcine and Bovine Insulins.

Amino Acid	Porcine Insulin Theory	Porcine Insulin Regenerated Found	Bovine Insulin Theory	Bovine Insulin Regenerated Found
Lysine	1	1.0	1	1.0
Histidine	2	2.0	2	1.9
Arginine	1	1.0	1	1.0
Aspartic acid	3	3.0	3	3.0
Threonine	2	1.8	1	1.0
Serine	3	2.4	3	2.8
Glutamic acid	7	7.0	7	7.2
Proline	1	1.1	1	1.1
Glycine	4	4.0	4	4.2
Alanine	2	2.0	3	3.2
Valine	4	3.8	5	4.8
Isoleucine	2	1.7	1	0.7
Leucine	6	6.1	6	6.0
Tyrosine	4	*b*	4	*b*
Phenylalanine	3	3.1	3	3.0
Half-cystine	6	*b*	6	*b*

[a] Number of amino acid residues per molecule. [b] Not determined.

TABLE VI: Amino Acid Composition[a] of the All-Synthetic Human Insulin and the Half-Synthetic Porcine and B_AH_B Insulins.

Amino Acid	All-Synthetic Human Insulin Theory	All-Synthetic Human Insulin Found	Half-Synthetic Porcine Insulin Theory	Half-Synthetic Porcine Insulin Found	Insulin B_AH_B Theory	Insulin B_AH_B Found
Lysine	1	0.8	1	0.9	1	0.9
Histidine	2	1.9	2	1.9	2	1.9
Arginine	1	1.0	1	1.0	1	0.9
Aspartic acid	3	2.8	3	2.9	3	2.9
Threonine	3	2.5	2	1.9	2	1.8
Serine	3	2.6	3	2.5	3	2.8
Glutamic acid	7	7.1	7	7.2	7	6.9
Proline	1	0.9	1	1.1	1	0.8
Glycine	4	4.1	4	4.0	4	4.1
Alanine	1	1.1	2	2.0	2	2.0
Valine	4	3.9	4	3.9	5	4.9
Isoleucine	2	1.9	2	2.1	1	0.8
Leucine	6	6.1	6	6.2	6	6.0
Tyrosine	4	*b*	4	*b*	4	*b*
Phenylalanine	3	3.0	3	3.1	3	3.1
Half-cystine	6	*b*	6	*b*	6	*b*

[a] Number of amino acid residues per molecule. [b] Not determined.

(Figure 9-I). From the effluent, the active material was isolated, *via* picrate, as the hydrochloride (1.85 mg). The isolation procedure was repeated twice with comparable results. Recovery data are given in Table III.

Amino acid analysis of the half-synthetic insulin gave data (Table VI) in agreement with the theoretically expected values. The specific activity of this insulin (Table II) was comparable to that of the natural hor-

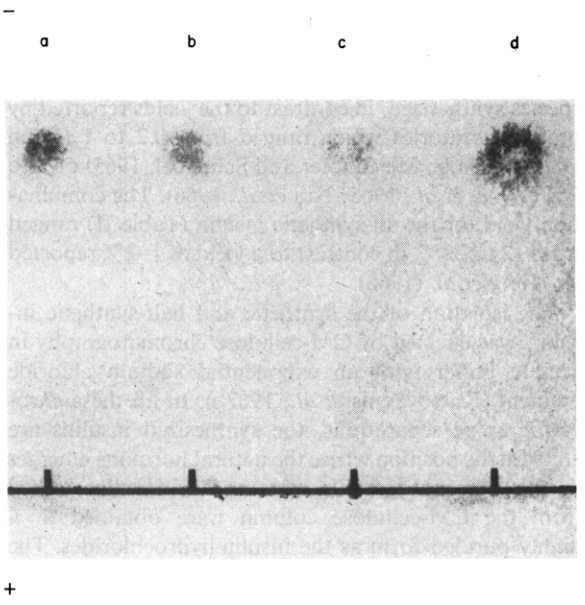

FIGURE 10: Thin layer electrophoresis of half-synthetic insulin B_AH_B (a), half-synthetic porcine (b), regenerated porcine (c), and natural porcine (d) insulins; 0.5 N acetic acid, 3200 v, and 12 min.

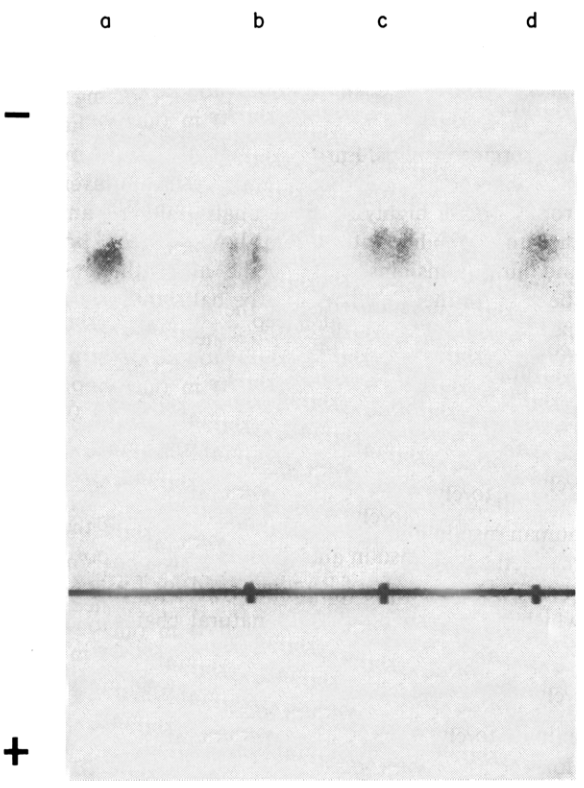

FIGURE 11: Thin layer electrophoresis of natural bovine (a), regenerated bovine (b), all-synthetic human (c), and natural human (d) insulins; 0.5 N acetic acid, 3400 v, and 15 min.

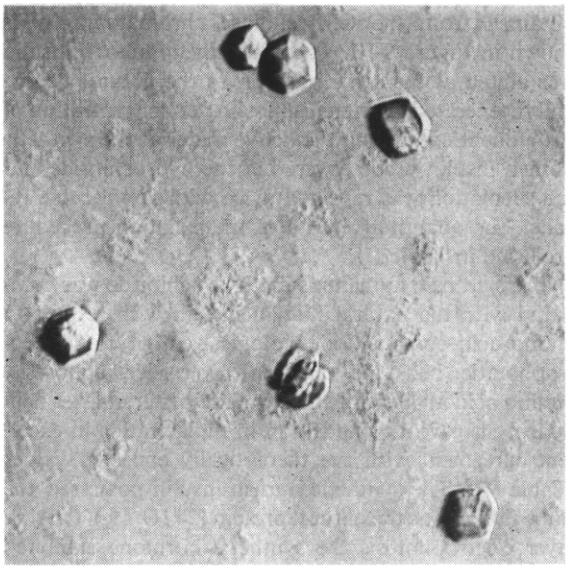

FIGURE 12: Half-synthetic crystalline insulin B_AH_B (natural bovine A plus synthetic human B).

mone. On thin layer electrophoresis the half-synthetic porcine insulin migrated as a single component (Pauly positive) and exhibited the same mobility as the natural and the regenerated porcine insulins (Figure 10). As was the case with the porcine insulin regenerated by recombination of natural chains, the half-synthetic porcine insulin, in preliminary experiments, did not crystallize by the method of Randall (1964) or the method of Epstein and Anfinsen (1963).

Insulin Produced by Combination of Natural Bovine A Chain and Synthetic Human B Chain (Half-Synthetic Insulin B_AH_B). The chromatographic pattern of combination mixtures (35 IU) processed as described previously is illustrated in Figure 9-V. This nonnaturally occurring insulin is also eluted at the same position as the other insulins described in this work. From the effluent, the active material was recovered, *via* picrate, as the hydrochloride (0.5 mg). This isolation was repeated twice with comparable results. Quantitative data on the isolation process are given in Table III. Amino acid analysis of this hormone after acid hydrolysis gave a composition in molar ratios in excellent agreement with the theoretically expected values (Table VI). The synthetic material has a potency (22 IU/mg) comparable to that of the naturally occurring hormones. On thin layer electrophoresis this product exhibited a single Pauly-positive spot and had the same mobility as the natural or half-synthetic porcine insulins (Figure 10). Finally the synthetic hormone was readily crystallized (Figure 12).

Insulin Produced by Combination of Human Synthetic A and B Chains (All-Synthetic Human Insulin). Combination of the synthetic human A and B chains was carried out as discussed in the Materials and Methods and combination mixtures with a total activity of 78 IU were processed in the usual manner. The chromatogram

obtained from the CM-cellulose chromatography is shown in Figure 9-VI. The all-synthetic human insulin was eluted at the same position as the natural bovine and regenerated porcine insulins. After concentration of the effluent containing the active material the synthetic human insulin was recovered as the hydrochloride (1.3 mg). Isolation was repeated twice with comparable results. The quantitative data of the isolation procedure are listed in Table III.

The synthetic human insulin hydrochloride was compared with the natural human insulin with respect to amino acid composition, biological activity, and electrophoretic behavior on thin layer electrophoresis. Amino acid analysis of the synthetic material after acid hydrolysis gave a composition in molar ratios in excellent agreement with the theoretically expected values (Table VI). The synthetic human insulin possessed the same potency as the natural protein (24 IU/mg). On thin layer electrophoresis the synthetic hormone exhibited a single Pauly-positive spot and had the same mobility as the natural human and bovine insulins (Figure 11). Preliminary attempts to crystallize the synthetic protein by the methods of Randall (1964) or the method of Epstein and Anfinsen (1963) were not successful. It might be recalled that a similar situation was encountered with the porcine insulin produced by recombination of natural chains and the half-synthetic porcine insulin. All three insulins have as a common denominator identical A chains.

Discussion

Insulin is essentially the exclusive product formed, among the many theoretically possible isomers, upon interaction of the A and B chains (Katsoyannis *et al.*, 1967b). The implication, therefore, arises that the necessary information for folding and orientation of the chains in a manner that permits their spontaneous combination to form insulin is contained within the primary structure of the chains. Consequently, the problem of insulin synthesis is in essence the problem of synthesis of its individual chains. The A and B chains of sheep and human insulin were synthesized, according to the proposed structures, in this laboratory, and their equivalence with their natural counterparts was established by many criteria (Katsoyannis *et al.*, 1963, 1964, 1966a–c, 1967a; P. G. Katsoyannis, unpublished data).

Consequently the availability of methods of preparing purified A and B chains and the development of highly efficient techniques, both for their combination (Katsoyannis and Tometsko, 1966; Katsoyannis *et al.*, 1967b) and the isolation of the insulin so formed (Katsoyannis *et al.*, 1967b), has now opened the way for the synthesis of various insulins and the preparation of analogs of this hormone.

This investigation has dealt with the isolation, from combination mixtures, of all-synthetic sheep and human insulins, half-synthetic bovine, sheep, and porcine insulins, and of an insulin which has not been encountered in any species examined thus far. This insulin, designated insulin B_AH_B, was formed by the interaction of synthetic human B chain with natural bovine A chain. The combination yields of the half-synthetic insulins (Table II) ranged from 17 to 42%, depending on the insulin species synthesized, in contrast to the yields reported by other laboratories which ranged from 0.2 to 1 (Zahn *et al.*, 1965a,b; Meienhofer and Schnabel, 1965) or 5 to 7% (Wang *et al.*, 1965; Niu *et al.*, 1966). The combination yield for the all-synthetic insulin (Table II) ranged from 12 to 22%, in contrast to a yield of 1–2% reported by Kung *et al.* (1966).

The isolation of the synthetic and half-synthetic insulins was effected by CM-cellulose chromatography in acetate buffer with an exponential sodium chloride gradient (Katsoyannis *et al.*, 1967b). In all these chromatographic separations, the synthesized insulins are eluted at the position where the natural hormone emerges in this chromatographic system. The insulins eluted from the CM-cellulose column were obtained in a highly purified form as the insulin hydrochlorides. The over-all recovery of insulin hydrochloride ranged from 35 to 52% based on the original insulin activity present in the combination mixture (Table III).

The isolated insulin hydrochlorides were compared with their natural counterparts with respect to amino acid composition, specific activity, and electrophoretic mobility on thin layer electrophoresis. As is shown in Table IV–VI, amino acid analyses of the isolated insulin hydrochlorides, after acid hydrolysis, gave amino acid compositions expressed in molar ratios in excellent agreement with the theoretically expected values. Similarly, the specific activities of the insulin hydrochlorides ranged from 22 to 25 IU/mg (Table II), as compared to the specific activity of 23–25 IU/mg found in our laboratory for the natural, bovine, sheep, human, and porcine insulins. Furthermore, the synthetic insulins exhibited single Pauly-positive spots on thin layer electrophoresis, a highly sensitive analytical tool, and had the same mobility with natural bovine, sheep, porcine, and human insulins. Finally, the all-synthetic sheep, the half-synthetic sheep, and the half-synthetic bovine insulins were crystallized and the crystalline form of these synthetic insulins was similar to that of the natural hormones. All these comparisons, in our estimation, afforded convincing evidence of the identity of these synthetic insulins with the natural sheep and bovine insulins, respectively.

Preliminary attempts to crystallize the all-synthetic human insulin and the half-synthetic porcine insulin by the methods of Randall (1964) or Epstein and Anfinsen (1963) were unsuccessful. Similarly, the porcine insulin, which was regenerated from natural chains, failed to crystallize by the aforementioned methods. On the other hand, the insulin (insulin B_AH_B) produced by combination of the synthetic human B chain with the natural bovine A chain and the insulin produced by combination of the natural porcine B chain with the natural bovine A chain were crystallized.

The pattern thus emerged that insulins produced by combination of natural or synthetic human (porcine) A chain with B chain are not crystallizable by the methods mentioned before, whereas replacement of the A chain

in these combinations with natural bovine A or sheep synthetic A chains resulted in regeneration of crystallizable insulins. Possible explanations of this phenomenon could be that either the human (porcine) A chain has undergone during its preparation subtle chemical changes not detected by the routine laboratory procedures (Katsoyannis *et al.*, 1967a) or that the aforementioned chain, on combination with B chain, produced an insulin molecule with a conformation different from that of the natural hormone. In this respect the work of Berson and Yallow (1961) is of considerable importance. These investigators have indeed found that sperm whale and porcine insulins, which have the same amino acid sequences, exhibit rather marked immunological differences, and they attribute that difference to "certain conformational aspects related to unknown differences in their secondary or tertiary structures" (Berson and Yallow, 1966). However, it should be pointed out that whatever the nature of this phenomenon might be, the specific activity and the electrophoretic and chromatographic mobility of the regenerated insulins is not affected.

The synthesis of insulin B_AH_B, which has not been found in nature and which is endowed with the full potency of the natural hormones, constitutes the first synthesis of an insulin analog which embodies within its structure naturally occurring A and B chains (hybrid insulin). The preparation of this protein indicates that it is possible to synthesize insulins which do not occur in nature by combining A and B chains of naturally occurring hormones. Whether combinations of all-natural chains can always be accomplished, and whether the insulins thus produced would have the full biological activity of the natural hormone, remains to be shown. Finally, the methods of synthesis and combination of the insulin chains developed in this laboratory and the isolation techniques described in the present investigation open unlimited possibilities for the synthesis of insulin analogs and for study of the relationship between chemical structure and biological activity of this protein.

Acknowledgments

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β-Aspartyl Peptide Formation from an Amino Acid Sequence in Ribonuclease*

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ABSTRACT: Exhaustive enzymic hydrolysates of bovine ribonuclease have a much higher β-aspartyl peptide content than digests of other proteins of known structure previously studied. β-Aspartyl di-, tri-, and tetrapeptides are shown to derive from 22 to 34% of a certain amino acid sequence beginning with asparagine at residue 67 of ribonuclease. Evidence is presented that the β-aspartyl linkage does not preexist in the protein but forms spontaneously and nonenzymatically in fragments liberated during the digestion.

Previous experiments (Haley *et al.*, 1966; Pisano *et al.*, 1966), in which β-aspartyl peptides were found in enzymic digests of several proteins, failed to demonstrate conclusively that these linkages originated from the natural amino acid sequence of the molecules. In proteins of known sequence molar yields of Asp(Gly),[1] the most abundant peptide, were always below 4% of the theoretical, a value too low to exclude with certainty the occasional presence of a side-chain glycine attached *in vivo* by transamidation (Loewy *et al.*, 1966). Our results indicated but did not prove that β-aspartyl bonds formed during the digestions and did not preexist.

Because the nonenzymic conversion of Asn-Gly to Asp(Gly) was found to proceed at approximately ten times the rate of the conversion of Asp-Gly to the β isomer it was felt that bovine ribonuclease, which has one -Asn-Gly- and no -Asp-Gly- sequences (Hirs *et al.*, 1960; Smyth *et al.*, 1962, 1963), might yield more Asp(Gly) than hemoglobin or lysozyme, which have -Asp-Gly- but no -Asn-Gly- sequences (Konigsberg *et al.*, 1962, 1963; Canfield, 1963). Digestions of native as well as performic acid oxidized ribonuclease by methods we previously used with other proteins resulted in molar yields of Asp(Gly) not much different from those obtained with hemoglobin and lysozyme, but in total yields of β-aspartyl di-, tri-, and tetrapeptides as high as 34% of theory and matching a known sequence in the protein. Isolation and study of the 19 residue peptide, *O*-Trp 2,[2] and the heptapeptide (*O*-Trp 2)-Chy 3[3] (Hirs *et al.*, 1956), which contain the sequence, give convincing evidence that the β-aspartyl peptides arise from the sequence. The results point strongly to the nonenzymic conversion of an N-terminal asparaginyl to a β-aspartyl linkage during the digestions.

Experimental Section

Materials. Bovine ribonuclease and chymotrypsin, both three times crystallized, were purchased from Worthington; ribonuclease A, 100 Kunitz units/mg, from Sigma; and trypsin, free of chymotrypsin, from Calbiochem. Asp(Gly-Gln) was prepared as described elsewhere (Dorer *et al.*, 1967). Other hydrolytic enzymes and aspartyl peptides were described previously (Haley *et al.*, 1966).

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[1] Abbreviations used: Asp-Gly, α-L-aspartylglycine; Asp(Gly), β-L-aspartylglycine; Asp(Gly-Gln), β-L-aspartylglycyl-L-glutamine, etc.; VK, Viokase; PRN, Pronase; LAP, leucine aminopeptidase; CA, carboxypeptidase A; CB, carboxypeptidase B; TR, trypsin; Chy, chymotrypsin; PRL, prolidase.

[2] *O*-Trp 2 is Asn-Gly-Gln-Thr-Asn-$CySO_3H$-Tyr-Gln-Ser-Tyr-Ser-Thr-$MetSO_2$-Ser-Ile-Thr-Asp-$CySO_3H$-Arg.

[3] (*O*-Trp 2)Chy 3 is Asn-Gly-Gln-Thr-Asn-$CySO_3H$-Tyr.