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The Synthesis of a Partial Sequence of Proinsulin Using the A-Chain of Natural Insulin

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Elongation reactions of the *S*-sulfonate of the A-chain of bovine insulin were carried out with acylamino acid-active esters or with acylpeptide azide; the following derivatives of the A-chain were thus synthesized: *S*-sulfonates of N^{G}-nitro-L-arginyl-, L-lysyl-N^{G}-nitro-L-arginyl-, and L-prolyl-L-prolyl-L-glutaminyl-N^{ε}-benzyloxycarbonyl-L-lysyl-N^{G}-nitro-L-arginyl-A-chain. Thus, this was demonstrated to be a useful procedure for the partial synthesis of proinsulin.

Insulin has been successfully synthesized by binding a synthetic A-chain with a natural B-chain[1] or *vice versa*.[2] This is one so-called half-synthesis of complicated polypeptides. The possibility that natural peptide fragments may be used in the synthesis of longer molecules has already been suggested by Goldberger and Anfinsen,[3] and by Offord.[4] However, no examples of successful re- or half-syntheses of natural linear polypeptides from natural or synthetic fragments have yet been reported. Nevertheless, this type of synthesis can be an important preliminary step in the total synthesis of complicated natural polypeptides. The difficulties in this procedure stem from the structure of the peptide fragments involved: when a natural peptide fragment is used as an amino component, all the amino groups except that to be acylated must be protected with suitable groups, and when it is used as a carboxyl component, all functional groups with active hydrogen except that to be activated should be protected.

Recently, proinsulins, the single-chain precursor of insulin, have been isolated from several sources,[5–7]

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[2] C.-i. Niu, Y.-t. Kung, W.-t. Huang, L.-t. Ke, C.-c. Chen, Y.-c. Chen, Y.-c. Du, R.-q. Jiang, C.-l. Tsou, S.-c. Hu, S.-q. Chu and K.-z. Wang, *Scientia Sinica*, **14**, 1386 (1965).

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[6] D. F. Steiner, O. Hallund, A. H. Rubenstein, S. Cho and C. Bayliss, *Diabetes*, **17**, 725 (1968).

[7] J. L. Clark, S. Cho, A. H. Rubenstein and D. F. Steiner, *Biochem. Biophys. Res. Commun.*, **35**, 456 (1969).

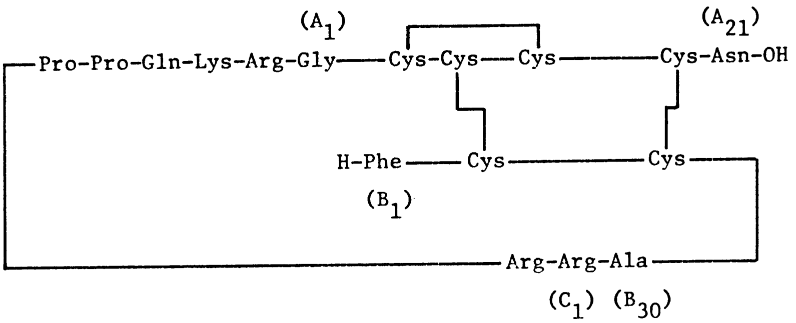

Fig. 1. Structural model of proinsulin from pig$^{5)}$ and ox.$^{8)}$

and the primary structures of those of pig$^{5)}$ and ox$^{8)}$ have been elucidated. As is shown in Fig. 1, proinsulin is a linear polypeptide in which the amino terminus of the A-chain of insulin is linked to the carboxyl terminus of its B-chain by a connecting peptide (C-peptide). The A-chain of insulin has only one active amino group at the amino end of its peptide chain, and is readily obtainable from natural insulin. Thus, the natural A-chain seems to be a suitable starting material for the half-synthesis of proinsulin.

In the present study, the reactivities of the natural A-cain of insulin with acylamino acids and with acylpeptide were examined in the preliminary studies of the synthesis of proinsulin. First, the reactivity of A-chain *S*-sulfonate$^{9)}$ was tested with acylamino acid-active esters. This *S*-sulfonate seemed suitable not only for the protection of the thiol functions, but also for the solubilization of the A-chain in an alkaline medium. Those properties should favor the use of this peptide in the coupling reactions. Next, the author attempted the azide coupling of an acylpeptide with A-chain *S*-sulfonate. The C-terminus of the connecting peptide in proinsulin is arginine, and the coupling of an acylpeptide, having an arginyl residue in the C-terminus, with the natural A-chain by the azide procedure seemed likely to be difficult. Therefore, the author attempted fragment condensation by the azide procedure after a one-step elongation of the A-chain with an N^{G}-nitro-L-arginine residue.

When this study had almost been completed, the author realized that Zahn *et al.*$^{10)}$ had published a paper on the syntheses of three A-chain peptides; L-lysyl-, L-histidyl-, and L-arginyl-A-chain. Especially, Zahn *et al.* used Des-glysine-A-chain, obtained by the Edman-degradation of the A-chain, as a starting peptide for the synthesis of L-arginyl-A-chain. Their procedure may have advantages, since the acylated peptide, which is intended to be coupled with Des-glycine-A-chain, should have a glycine residue in the carboxyl terminus, and it may be activated by various coupling methods. However, this procedure seems unnecessarily complicated if the direct elogation of the A-chain with acylated peptide is possible. The possibility of the direct elongation of the A-chain received special attention in the present investigation.

Experimental

Crystalline bovine insulin was obtained from the Chemical Research Laboratories, Takeda Chemical Industries, Ltd. (Osaka), by the courtesy of Dr. M. Fujino. The *S*-sulfonate of the A-chain was prepared from insulin as has been described in the literature.$^{9),11-12)}$ The dialysis tubing 24/32 was a product of the Visking Co. (Chicago). Sephadex G-25 fine was purchased from the Pharmacia Co. (Uppsala). The chemicals used were of a reagent grade and were used without further purification. Paper electrophoresis was performed at pH 2.1 in a 4M urea-3M formic acid buffer.$^{11)}$ The purity of the A-chain derivatives thus synthesized was confirmed by paper electrophoresis and by the recovery of the constituent amino acids from acid hydrolysates upon amino-acid analysis. All the melting points given are uncorrected.

***S*-Sulfonate of N^{G}-Nitro-L-arginyl-A-chain.** The *S*-sulfonate of the A-chain (413 mg) was dissolved in water (3 m*l*) with sodium bicarbonate (0.16 g), and then a solution of the N^{α}-*p*-methoxybenzyloxycarbonyl-N^{G}-nitro-L-arginine pentachlorophenyl ester$^{13)}$ (1.27 g) in dioxane (6 m*l*) was added. The mixture was mechanically agitated for 2 days at room temperature, washed with ethyl acetate, and then concentrated *in vacuo*. The residue was then again treated with the fresh N^{α}-*p*-methoxybenzyloxycarbonyl-N^{G}-nitro-L-arignine pentachlorophenyl ester (0.63 g) in the same way as has been described above, and the mixture was stirred for one day more at room temperature. Then the reaction mixture was washed with ethyl acetate, concentrated *in vacuo*, and dried over phosphorus pentoxide. The residual powder was dissolved in trifluoroacetic acid

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8) C. Nolan and E. Margoliash, unpublished data (1969).

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10) M. Weinert, D. Brandenburg and H. Zahn, *Hoppe-Seyler's Z. physiol. Chem.*, **350**, 1566 (1969).

11) H. Zahn and E. Drechsel, *ibid.*, **349**, 359 (1968).

12) Y. Shimonishi and H. Zahn, *Biochim. Biophys. Acta*, **154**, 598 (1968).

13) S. Sakakibara, unpublished.

(15 m*l*) together with anisole (0.7 m*l*), and the solution was allowed to stand for 30 min at room temperature. Then it was concentrated *in vacuo*, and the oily residue was dried over phosphorus pentoxide. It was then dissolved in 2% aqueous sodium bicarbonate and dialyzed against distilled water (500 m*l*) for 60 min at room temperature. The dialyzed solution was lyophilized. The lyophilizate was dissolved in a small volume of a 0.1M ammonium bicarbonate solution, charged on a column of Sephadex G-25 (2×50 cm), and eluted using the same buffer solution. The first peak in the eluate, with an absorption at 280 mμ (see Fig. 3), was collected and lyophilized to obtain the *S*-sulfonate of N^G-nitro-L-arginyl-A-chain as a solid; wt. 393 mg.

N^{α},N^{ε}-Di-*t*-butyloxycarbonyl-L-lysine *N*-Hydroxysuccinimide Ester. N^{α},N^{ε}-Di-*t*-butyloxycarbonyl-L-lysine[14,15] (1.38 g, 4.0 mmol) and *N*-hydroxysuccinimide (0.46 g, 4.0 mmol) were dissolved in absolute tetrahydrofuran (20 m*l*), after which the solution was cooled to −10°C. A solution of *N*,*N'*-dicyclohexylcarbodiimide (0.82 g, 4.0 mmol) in absolute tetrahydrofuran (10 m*l*) was added to the chilled solution. The solution was stirred for an hour at the same temperature and then for 24 hr at room temperature; the urea thus formed was filtered off and washed with ethyl acetate. The filtrate and washings were combined and concentrated to a syrup *in vacuo*, which was crystallized by trituration with petroleum ether; wt. 1.70 g. The crude material was recrystallized from ethyl acetate and petroleum ether; wt. 1.54 g (87.0%); mp 92—96°C, $[\alpha]_D^{18}$ −30.6° (*c* 2, ethanol).

Found: C, 54.18; H, 7.96; N, 9.19%. Calcd for $C_{20}H_{33}O_8N_3$: C, 54.16; H, 7.50; N, 9.48%.

***S*-Sulfonate of L-Lysyl-N^G-nitro-L-arginyl-A-chain.** The *S*-sulfonate of N^G-nitro-L-arginyl-A-chain (104 mg) was dissolved in water (1.5 m*l*) containing sodium bicarbonate (0.04 g) and allowed to react with the H^{α},N^{ε}-di-*t*-butyloxycarbonyl-L-lysine *N*-hydroxysuccinimide ester (0.22 g) dissolved in dioxane (2 m*l*). The reaction mixture was then treated as has been described for the synthesis of the *S*-sulfonate of N^G- nitro-L-arginyl -A-chain. The yield of the *S*-sulfonate of L-lysyl-N^G -nitro-L-arginyl-A-chain was 93.8 mg. This material had a slower mobility on paper by electrophoresis at pH 2.1 than the *S*-sulfonate of N^G-nitro-L-arginyl-A-chain.

***t*-Amyloxycarbonyl-L-prolyl-L-proline *N*-Hydroxysuccinimide Ester.** A solution of *t*-amyloxycarbonyl-L-prolyl-L-proline[16] (3.26 g, 10 mmol) and *N*-hydroxysuccinimide (1.15 g, 10 mmol) in absolute tetrahydrofuran (30 m*l*) was cooled to 0—5°C, and then *N*,*N'*-dicyclohexylcarbodiimide (2.06 g, 10 mmol) was added to the chilled solution. The mixture was stirred for an hour at the same temperature and then for 20 hr at room temperature, and the precipitate thus formed was removed by filtration. The filtrate was concentrated to a syrup *in vacuo*; crystals formed on storage in a refrigerator. The crude product was collected by filtration with petroleum ether; wt. 4.29 g. It was recrystallized from ethyl acetate and petroleum ether; wt. 4.04 g (95.5%), mp 111.5—112.5°C, $[\alpha]_D^{29}$ −103.8° (*c* 2, ethanol).

Found: C, 56.52; H, 7.91; N, 9.72%. Calcd for $C_{20}H_{29}O_7N_3$: C, 56.72; H, 6.90; N, 9.92%.

***o*-Nitrophenylsulfenyl-L-glutaminyl-N^{ε}-benzyloxycarbonyl-L-lysine Methyl Ester.** N^{ε}-Benzyloxycarbonyl-L-lysine methyl ester hydrochloride[17] (2.68 g, 8.09 mmol) and triethylamine (1.13 m*l*) were dissolved in dimethylformamide (25 m*l*), and then the *o*-nitrophenylsulfenyl-L-glutamine *N*-hydroxysuccinimide ester[18] (2.67 g, 6.74 mmol) was added to the solution. After having been stirred for 2 days at room temperature, the reaction mixture was diluted with ethyl acetate (*ca.* 200 m*l*). The solution was washed successively with 0.1N hydrochloric acid, 5% sodium bicarbonate, and water, and dried over anhydrous sodium sulfate. The dried solution was concentrated to obtain green crystals, which were then collected with ethyl acetate and petroleum ether; wt. 3.11 g. The crude product was recrystallized from methanol and water; wt. 3.00 g (77.3%), mp 155—157°C.

Found: C, 54.33; H, 5.60; N, 12.00; S, 5.47%. Calcd for $C_{26}H_{33}O_8N_5S$: C, 54.25; H, 5.78; N, 12.17; S, 5.57%.

***t*-Amyloxycarbonyl-L-prolyl-L-prolyl-L-glutaminyl-N^{ε}-benzyloxycarbonyl-L-lysine Methyl Ester.** The *o*-nitrophenylsulfenyl-L-glutaminyl-N^{ε}-benzyloxycarbonyl-L-lysine methyl ester (6.34 g, 11 mmol) was suspended in distilled dioxane (40 m*l*), and then treated with 4N HCl in dioxane (15 m*l*) for 15 min. The clear solution thus formed was concentrated to a syrup and dried over sodium hydroxide *in vacuo*. Then it was thoroughly washed with a mixture of acetone and ether. The residue was dissolved in dimethylformamide (40 m*l*), and triethylamine (1.6 m*l*) and the *t*-amyloxycarbonyl-L-prolyl-L-proline *N*-hydroxysuccinimide ester (3.95 g, 9.35 mmol) were added to the solution. After having been stirred for 2 days at room temperature, the reaction mixture was diluted with ethyl acetate (*ca.* 300 m*l*). The diluted solution was washed successively with 0.1N hydrochloric acid, 5% sodium bicarbonate, and water, and dried over anhydrous sodium sulfate. The dried solution was concentrated *in vacuo* to a syrup; wt. *ca.* 5.6 g. This material has not yet been crystallized.

***t*-Amyloxycarbonyl-L-prolyl-L-prolyl-L-glutaminyl-N^{ε}-benzyloxycarbonyl-L-lysine Hydrazide.** The syrupy material of the *t*-amyloxycarbonyl-L-prolyl-L-prolyl-L-glutaminyl-N^{ε}-benzyloxycarbonyl-L-lysine methyl ester (*ca.* 5.6 g) was dissolved in ethanol (80 m*l*), and then 100% hydrazine hydrate (8 m*l*) was added to the solution. After standing for 24 hr at room temperature, the mixture was concentrated to a syrup; crystals were obtained by rubbing the syrup with petroleum ether; wt. 5.40 g. The crude product was recrystallized from ethanol and ether; wt. 4.82 g (70.5% of the theoretical yield based on the content of the *t*-amyloxycarbonyl-L-prolyl-L-proline *N*-hydroxysuccinimide ester); mp 104°C, $[\alpha]_D^{18}$ −68.5° (*c* 2, ethanol).

Found: C, 57.47; H, 7.55; N, 15.04%. Calcd for

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17) R. A. Boissonnas, S. Guttmann, R. L. Huguenin, P. A. Jaquenoud and E. Sandrin, *Helv. Chim. Acta*, **41**, 1867 (1958).

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$C_{35}H_{54}O_9N_8$: C, 57.52; H, 7.54; N, 15.33%.

S-Sulfonate of L-Prolyl-L-prolyl-L-glutaminyl-N^{ε}-benzyloxycarbonyl-L-lysyl-N^{G}-nitro-L-arginyl-A-chain. *t*-Amyloxycarbonyl-L-prolyl-L-prolyl-L-glutaminyl-N^{ε}-benzyloxycarbonyl-L-lysine hydrazide (366 mg) was dissolved in dimethylformamide (3 m*l*) and cooled to −40°C. The solution was mixed with 1N HCl in tetrahydrofuran (1.0 m*l*) and then with isoamylnitrite (70 mg) dissolved in tetrahydrofuran (1.0 m*l*) at the same temperature.[19] After 30 min, triethylamine (0.17 m*l*) and a solution of the *S*-sulfonate of N^{G}-nitro-L-arginyl-A-chain (280 mg) and sodium bicarbonate (0.12 g) dissolved in water (2 m*l*) were added to the solution. The mixture was stirred mechanically at −20°C for 2 days, and then dialyzed against distilled water (500 m*l*) for 60 min at room temperature and lyophilized. The residue was dried over phosphorus pentoxide *in vacuo* and then treated with trifluoroacetic acid (15 m*l*) at 5°C for 30 min. The solution was concentrated *in vacuo*, and the residual oil was dried over phosphorus pentoxide. This was dissolved in 2% aqueous sodium bicarbonate at room temperature. After 30 min, the solution was dialyzed against distilled water and lyophilized. The residue was dissolved in a small volume of a 0.1M ammonium bicarbonate solution and fractionated on a column of Sephadex G-25 (2×50 cm) using the same buffer solution. The first peak in the eluate with an absorption at 280 m*μ*, as has been described in the synthesis of the N^{G}-nitro-L-arginyl derivative, was collected and lyophilized. Thus L-prolyl-L-prolyl-L-glutaminyl-N^{ε}-benzyloxycarbonyl-L-lysyl-N^{G}-nitro-L-arginyl-A-chain *S*-sulfonate was obtained as a powder; wt. 163 mg.

Results and Discussion

The coupling reaction was, first, carried out by mixing an aqueous solution of A-chain *S*-sulfonate with a solution of the N^{α}-*p*-methoxybenzyloxycarbonyl-N^{α}-nitro-L-arginine pentachlorophenyl ester in dioxane under the standard conditions used in peptide synthesis.[20] A large molar excess of the N^{α}-*p*-methoxybenzyloxycarbonyl-N^{G}-nitro-L-arginine pentachlorophenyl ester was employed, since it was thought that the quantitative acylation of the A-chain *S*-sulfonate would facilitate the purification procedure. When a 10—15 fold molar excess of this reagent was allowed to react with A-chain *S*-sulfonate for 2 days at room temperature, a little unreacted A-chain was detected in the reaction mixture by paper electrophoresis using a formic acid buffer solution at pH 2.1. However, the repeated addition of 5—7 fold molar excesses of the pentachlorophenyl ester to the reaction mixture resulted in the quantitative acylation of the A-chain *S*-sulfonate, as is shown in Fig. 2. After removing the protective group with trifluoroacetic acid, the reaction product was purified by gel-filtration on Sephadex G-25, using ammonium bicarbonate buffer as a solvent, as is shown on Fig. 3. The first fraction of the eluate, N^{G}-nitro-L-arginyl-A-chain *S*-sulfonate, was collected and lyophilized to yield a cotton-like residue. This compound was identified by paper electrophoresis and by an amino-acid analysis of its acid hydrolysate, as is shown in Fig. 2 and Table 1 respectively.

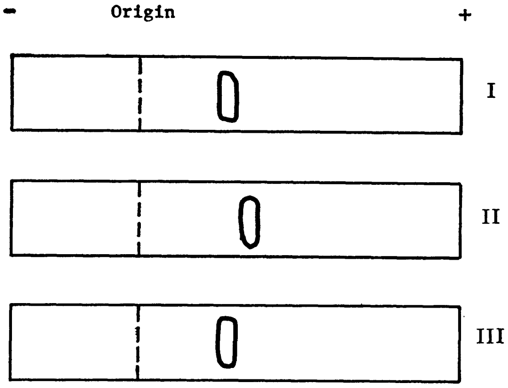

Fig. 2. Diagram of paper electropherogram stained with Pauly reagent (urea-formic acid buffer, pH 2.1, 7.5 V/cm). Bands I, II and III correspond to the *S*-sulfonate of A-chain, reaction mixture after reaction of the *S*-sulfonate of A-chain with N^{α}-*p*-methoxybenzyloxycarbonyl-N^{G}-nitro-L-arginine pentachloropyenyl ester, and the *S*-sulfonate of N^{G}-nitro-L-arginyl-A-chain, respectively.

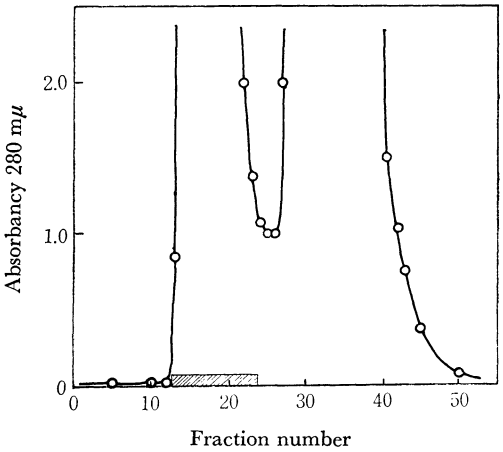

Fig. 3. Gel-filtration of mixture after reaction of the *S*-sulfonate of A-chain and N^{α}-*p*-methoxybenzyloxycarbonyl-N^{G}-nitro-L-arginine pentachlorophenyl ester and removing the *N*-terminal protective group by treatment with trifluoroacetic acid on Sephadex G-25 (2×50 cm).

Second, the N^{G}-nitro-L-arginyl-A-chain *S*-sulfonate obtained above was allowed to react with the N^{α},N^{ε}-di-*t*-butyloxycarbonyl-L-lysine *N*-hydroxysuccinimide ester. The reaction was car-

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[19] J. Honzl and J. Rudinger, *Collect. Czech. Chem. Commun.*, **26**, 2333 (1961).

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ried out in the same way in dioxane-water under weakly alkaline conditions. The extent of the coupling reaction was also monitored by paper electrophoresis at pH 2.1, and L-lysyl-N^G-nitro-L-arginyl-A-chain *S*-sulfonate was isolated similarly. The amino-acid analysis of its acid hydrolysate also gave satisfactory results, as is shown in Table 1.

TABLE 1. AMINO ACID COMPOSITIONS OF VARIOUS DERIVATIVES OF THE A-CHAIN[a]

Amino acid	*S*-Sulfonate of A-chain[b]	I[c]	II[c]	III[c]
Asp	1.88 (2)	1.88	1.80	1.94
Ser	1.67 (2)	1.62	1.57	1.69
Glu	4.15 (4)	4.06	3.68	4.88
Pro				2.19
Gly	1.09 (1)	1.00	1.00	1.00
Ala	1.00 (1)	1.00	1.00	1.00
Cys	1.69 (2)	1.35	1.53	1.50
Val	1.67 (2)	1.56	1.63	1.69
Ile	0.55 (1)	0.53	0.61	0.66
Leu	2.00 (2)	1.91	1.98	2.03
Tyr	1.85 (2)	1.41	1.49	1.28
Arg+Lys[d]		1.06	1.78	1.81

a) Samples were hydrolyzed in 6N hydrochloric acid without added phenol in sealed tubes, for 24 hr, at 110°C, and analyzed in a Hitachi KLA-3B analyzer. All values shown are uncorrected.

b) This compound was analyzed for comparison with the synthesized compounds.

c) Compounds I, II and III mean *S*-sulfonates of N^G-nitro-L-arginyl-, L-lysyl-N^G-nitro-L-arginyl-, and L-prolyl-L-prolyl-L-glutaminyl-N^ε-benzyloxycarbonyl-L-lysyl-N^G-nitro-L-arginyl-A-chain, respectively.

d) Total values of lysine and arginine are showed, because lysine was not separated from ornithine derived from nitroarginine in this analysis.

Third, N^G-nitro-L-arginyl-A-chain *S*-sulfonate was coupled with *t*-amyloxycarbonyl-L-prolyl-L-prolyl-L-glutaminyl-N^ε-benzyloxycarbonyl-L-lysine by the azide procedure described in the introduction. In this case, the reaction product could not be detected by paper electrophoresis at pH 2.1, because it did not separate distinctly from unreacted *S*-sulfonate. The reaction was allowed to proceed for 2 days at −20°C, and then the product was isolated after removing the *N*-terminal protective group by treatment with trifluoroacetic acid. The product was found to migrate slower than A-chain *S*-sulfonate at pH 2.1, and seemed to be almost pure L-prolyl-L-prolyl-L-glutaminyl-N^ε-benzyloxycarbonyl-L-lysyl-N^G-nitro-L-arginyl-A-chain *S*-sulfonate. The amino-acid analysis of the hydrolysate also gave good results, as is shown in Table 1. This compound will be used as material for further elongation reactions, since the ε-amino group of the lysyl residue and the thiol group of cysteinyl residues are still suitably protected.

The above results clearly show that the natural A-chain of insulin can be used as a starting material in the partial synthesis of proinsulin, in which acylamino acid-active esters are used for step-by step elongation reactions and fragment condensation by means of the azide procedure can be applied the to N^G-nitro-L-arginyl-A-chain. The success of azide coupling with N^G-nitro-L-arginyl-A-chain is especially noteworthy. Thus, the use of Des-glycine-A-chain for the elongation reactions does not seem always to be necessary. Attempts at direct condensation with the A-chain by the azide procedure remain unsuccessful.

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