

[CONTRIBUTION FROM THE BIOCHEMISTRY DEPARTMENT, UNIVERSITY OF PITTSBURGH SCHOOL OF MEDICINE, PITTSBURGH, PENNA.]

**Insulin Peptides. V. The Synthesis of a Protected Nonapeptide Amide Corresponding to Positions 11 to 19 in the B-Chain of the Insulin Sequence**BY PANAYOTIS G. KATSOYANNIS<sup>1</sup> AND KENJI SUZUKI

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The protected nonapeptide N-carbobenzoxy-L-leucyl-L-valyl- $\gamma$ -benzyl-L-glutamyl-L-alanyl-L-leucyl-L-tyrosyl-L-leucyl-L-valyl-S-benzyl-L-cysteinamide was synthesized. This peptide corresponds to positions 11 to 19 in the B-chain of the insulin sequence. Its preparation was accomplished by the condensation of the *p*-nitrophenyl ester of N-carbobenzoxy-L-leucyl-L-valine with the partially protected heptapeptide  $\gamma$ -benzyl-L-glutamyl-L-alanyl-L-leucyl-L-tyrosyl-L-leucyl-L-valyl-S-benzyl-L-cysteinamide. The latter compound was prepared by the stepwise elongation approach starting with S-benzyl-L-cysteinamide.

In the structure<sup>2</sup> of insulins isolated from different species, two polypeptide chains are present: the A-chain containing twenty-one amino acid residues and the B-chain containing thirty amino acid residues. In the insulin molecule these two chains are linked together by two disulfide bridges formed between the half-cystine residues in positions A7 and B7 and A20 and B19 of the A- and B-chains, respectively, thus forming an interchain ring system containing twenty-seven amino acid residues (Fig. 1).

Studies have been undertaken in this Laboratory directed to the construction of this ring system as part of a broader program aimed at the synthesis of the insulin molecule. In previous communications we have reported the preparation of peptides containing amino acid sequences found in the A-chain moiety of the interchain ring system of sheep insulin.<sup>3,4</sup> We have now prepared the protected nonapeptide N-carbobenzoxy-L-leucyl-L-valyl- $\gamma$ -benzyl-L-glutamyl-L-alanyl-L-leucyl-L-tyrosyl-L-leucyl-L-valyl-S-benzyl-L-cysteinamide (IX). The sequence corresponding to this peptide occupies positions 11 to 19 in the B-chain and it is a part of the B-chain moiety of the interchain ring system.

The protected nonapeptide amide IX was prepared by coupling a partially protected heptapeptide amide with the *p*-nitrophenyl ester of an acyl dipeptide. Thus the condensation of N-carbobenzoxy-L-leucyl-L-valine *p*-nitrophenyl ester (VIII) with  $\gamma$ -benzyl-L-glutamyl-L-alanyl-L-leucyl-L-tyrosyl-L-leucyl-S-benzyl-L-cysteinamide (VII) in dimethylformamide afforded the desired protected nonapeptide amide IX in 91% yield.

The partially blocked heptapeptide amide (VII) was obtained from the fully protected heptapeptide amide VI by selective removal of its amino protecting group. The synthesis of the fully protected heptapeptide amide VI was accomplished by the stepwise elongation approach. Starting from the C-terminal residue, S-benzyl-L-cysteinamide, the peptide chain was built up by attaching one amino acid residue at a time to the amino terminal end of the chain. The *p*-nitrophenyl esters of the appropriate carbobenzoxy amino acids served as the activated "carboxyl component" in each synthetic step. The yields of the individual synthetic steps range from 90 to 97% of theory with the exception of one step where an 82% yield was obtained. The chemical purity of the protected heptapeptide amide VI and of the intermediate peptides was established by elemental analysis and by paper chromatography of

the decarbobenzoxylated derivatives. In all cases, sharp single spots were obtained, indicating the presence of single components.

The stereochemical homogeneity of the decarbobenzoxylated heptapeptide amide VI and certain of the intermediates was established by digestion with leucine aminopeptidase (LAP) followed by paper chromatography of the digests. The chromatograms exhibited ninhydrin-positive spots with *R<sub>f</sub>* values corresponding to the expected amino acids only. Since no other ninhydrin-reactive components were present, it is concluded that the digestion was complete. This suggests<sup>5</sup> that no racemization of the constituent amino acids had occurred during the synthetic processes leading to the preparation of the protected heptapeptide VI. The chemical purity of the protected nonapeptide IX was established by elemental analysis, paper chromatography of the deblocked derivative and amino acid analysis of an acid hydrolysate. In the latter case the constituent amino acids were obtained in the ratios predicted by theory (see Experimental section) with an average recovery of 96%. Low recovery of tyrosine from acid hydrolysates of carbobenzoxy peptides has been noticed previously by Hofmann, *et al.*<sup>6</sup> Although S-benzylcysteine was present in a paper chromatogram of the hydrolysate, it was not eluted after twenty-hour chromatography on a long column (150 cm.) using the automatic Stein-Moore technique.<sup>7</sup> Attempts to ascertain the stereochemical homogeneity of the final product by digestion with LAP were unsuccessful because the decarbobenzoxylated nonapeptide amide was practically insoluble in the incubation medium.

The over-all scheme which was employed for the synthesis of the protected nonapeptide (IX) is summarized in Chart I.

N-Carbobenzoxy-L-valyl-S-benzyl-L-cysteinamide (I) was prepared in 95% yield and in crystalline form by the interaction of N-carbobenzoxy-L-valine *p*-nitrophenyl ester<sup>8</sup> with S-benzyl-L-cysteinamide.<sup>9</sup> Hydrogen bromide in acetic acid was employed for removal of the carbobenzoxy group. The decarbobenzoxylated derivative which was fully digestible with LAP was treated with N-carbobenzoxy-L-leucine *p*-nitrophenyl ester<sup>10</sup> to give the crystalline N-carbobenzoxy-L-leucyl-L-valyl-S-benzyl-L-cysteinamide (II) in 93% yield.

On exposure to hydrogen bromide in acetic acid the protected tripeptide was decarbobenzoxylated and the resulting product was condensed with N-carbobenzoxy-O-benzyl-L-tyrosine *p*-nitrophenyl ester<sup>10</sup> to yield N-

(1) This work was supported by a Research Career Development Award (GM-K3-15151) from the Public Health Service and a grant (A-3067) from the National Institute of Arthritis and Metabolic Diseases, Public Health Service, for which we wish to express our appreciation.

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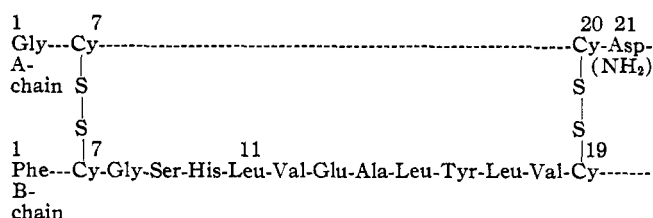
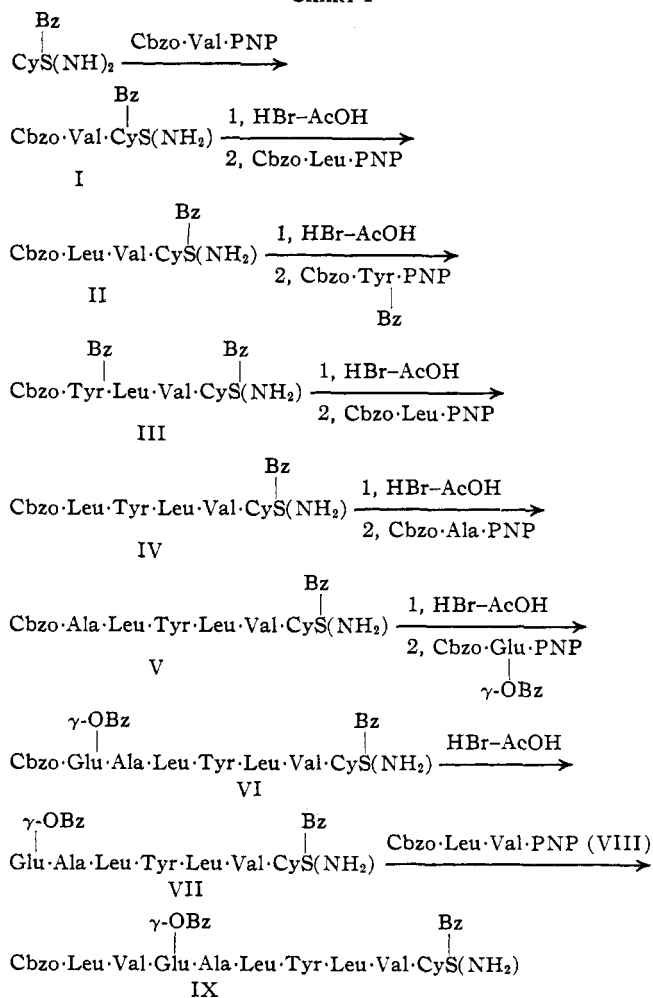


Figure 1.

carbobenzoxy-O-benzyl-L-tyrosyl-L-leucyl-L-valyl-S-benzyl-L-cysteinamide (III) in crystalline form and in 82% yield. The deblocked tetrapeptide amide was completely digested by LAP. Removal of the carbobenzoxy and benzyl groups from III by treatment with hydrogen bromide in acetic acid and coupling of the ensuing product with N-carbobenzoxy-L-leucine *p*-nitrophenyl ester<sup>10</sup> afforded the protected pentapeptide

CHART I



Bz: benzyl  
PNP: *p*-nitrophenyl ester  
Cbz: carbobenzoxy

N-carbobenzoxy-L-leucyl-L-tyrosyl-L-leucyl-L-valyl-S-benzyl-L-cysteinamide (IV) in 96% yield. N-Carbobenzoxy-L-alanyl-L-leucyl-L-tyrosyl-L-leucyl-L-valyl-S-benzyl-L-cysteinamide (V) was obtained in 90% yield by decarbobenzoxylating the peptide derivative IV on exposure to hydrogen bromide in acetic acid and coupling the resulting product with N-carbobenzoxy-L-alanine *p*-nitrophenyl ester.<sup>11</sup>

The protected heptapeptide amide VI was readily obtained in 97% yield by the condensation of N-carbobenzoxy- $\gamma$ -benzyl-L-glutamic acid *p*-nitrophenyl es-

ter<sup>11,12</sup> with the product obtained by decarbobenzoxylation of the protected hexapeptide V with hydrogen bromide in acetic acid. The chemical and stereochemical homogeneity of the protected heptapeptide was discussed previously.

Coupling of N-carbobenzoxy-L-leucyl-L-valine<sup>13</sup> with *p*-nitrophenol by the N,N'-dicyclohexylcarbodiimide method<sup>14</sup> yielded the respective dipeptide *p*-nitrophenyl ester VIII in crystalline form. Condensation of this ester with  $\gamma$ -benzyl-L-glutamyl-L-alanyl-L-leucyl-L-tyrosyl-L-leucyl-L-valyl-S-benzyl-L-cysteinamide (VII), which in turn was obtained by decarbobenzoxylation of its protected derivative VI, afforded the desired protected nonapeptide amide IX in 91% yield. The chemical purity of this compound was discussed previously.

The chemical purity of all the intermediates was established by elemental analysis of the carbobenzoxyated derivatives and by paper chromatography of the deblocked compounds. In all cases, decarbobenzoxylation was effected by treatment of the protected peptide with hydrogen bromide in acetic acid.

### Experimental

Capillary melting points were determined for all compounds and are corrected. For paper chromatography, the protected peptides were deblocked on exposure to 2 *N* hydrogen bromide in acetic acid. The ensuing hydrobromides were chromatographed on paper, Whatman No. 1, using the Partridge system.<sup>15</sup> The reported yields were calculated on the basis of the carboxyl component used.

**N-Carbobenzoxy-L-valyl-S-benzyl-L-cysteinamide (I).**—To a solution of S-benzyl-L-cysteinamide (1.05 g.) in dimethylformamide (10 ml.), N-carbobenzoxy-L-valine *p*-nitrophenyl ester (1.95 g.) was added. After 24 hours the solid reaction mixture was mixed with ether (40 ml.) and the crystalline product was collected by filtration and washed thoroughly with ether; wt. 2.1 g. (91%), m.p. 215°. A sample for analysis was recrystallized from aqueous acetic acid; m.p. 217°,  $[\alpha]_D^{25} -33.4^\circ$  (*c* 0.97, dimethylformamide).

*Anal.* Calcd. for  $C_{23}H_{29}N_3O_4S$ : C, 62.3; H, 6.58; N, 9.5. Found: C, 62.9; H, 6.62; N, 9.6.

The peptide amide hydrobromide which was obtained from the protected derivative I was fully digestible with LAP.

**N-Carbobenzoxy-L-leucyl-L-valyl-S-benzyl-L-cysteinamide (II).**—N-Carbobenzoxy-L-valyl-S-benzyl-L-cysteinamide (14 g.) was dissolved in 2 *N* HBr in acetic acid (100 ml.). After 1 hour at room temperature anhydrous ether (800 ml.) was added. The heavy oil which separated was washed with ether by decantation and dried over KOH *in vacuo*. To a cold solution of this product in dimethylformamide (100 ml.), triethylamine (7 ml.) was added followed by N-carbobenzoxy-L-leucine *p*-nitrophenyl ester (11.2 g.). After standing overnight at room temperature the solution was diluted with 1 *N*  $NH_4OH$  (10 ml.), stirred for 1 hour and mixed with ethyl acetate (500 ml.). The resulting solution was washed successively with 1 *N*  $NH_4OH$ , water, 1 *N* HCl and water and dried with  $MgSO_4$ . Evaporation to dryness left a crystalline residue which was recrystallized from aqueous acetic acid; wt. 14.8 g. (93%), m.p. 230–231°,  $[\alpha]_D^{25} -29.9^\circ$  (*c* 0.9, dimethylformamide); *R*<sub>f</sub> (hydrobromide) 0.77, single sharp ninhydrin-positive spot.

*Anal.* Calcd. for  $C_{28}H_{40}N_4O_5S$ : C, 62.6; H, 7.26; N, 10.1. Found: C, 62.9; H, 7.26; N, 10.2.

The decarbobenzoxylated derivative was fully digestible with LAP.

**N-Carbobenzoxy-O-benzyl-L-tyrosyl-L-leucyl-L-valyl-S-benzyl-L-cysteinamide (III).**—Compound II (14.7 g.) was dissolved in acetic acid (60 ml.) and 4 *N* HBr in acetic acid (60 ml.) was added to the solution. After 45 minutes at room temperature, anhydrous ether (500 ml.) was added and the precipitated product was washed with ether and dried over KOH *in vacuo*. This solid was dissolved in dimethylformamide (100 ml.) and triethylamine (3.7 ml.) was added, followed by N-carbobenzoxy-O-benzyl-L-tyrosine *p*-nitrophenyl ester (12.6 g.). After standing at room temperature the yellow solution was diluted with 1 *N*  $NH_4OH$  (10 ml.), stirred for 1 hour and mixed with ethyl acetate (500 ml.). This solution was subsequently washed with 1 *N*  $NH_4OH$ , water, 1 *N* HCl and again with water. On re-

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removal of the ethyl acetate, a crystalline residue remained which was recrystallized from aqueous acetic acid; wt. 16 g. (82%), m.p. 263–264°,  $[\alpha]_D^{25} -27^\circ$  (*c* 1, dimethylformamide);  $R_f$  (hydrobromide) 0.85, single ninhydrin- and tyrosine-positive spot.

*Anal.* Calcd. for  $C_{45}H_{65}N_7O_7S$ : C, 66.7; H, 6.86; N, 8.6. Found: C, 66.5; H, 6.61; N, 8.4.

The peptide hydrobromide which was obtained from the protected derivative was completely digested by LAP.

**N-Carbobenzoxyl-L-leucyl-L-tyrosyl-L-leucyl-L-valyl-S-benzyl-L-cysteinamide (IV).**—N-Carbobenzoxyl-O-benzyl-L-tyrosyl-L-leucyl-L-valyl-S-benzyl-L-cysteinamide (13 g.) was suspended in acetic acid (60 ml.) and treated with 4 *N* HBr in acetic acid (60 ml.). After 1 hour at room temperature, dry ether (500 ml.) was added, the precipitate which formed was filtered, washed with ether, dried and dissolved in dimethylformamide (100 ml.). To this solution triethylamine (2.3 ml.) was added followed by N-carbobenzoxyl-L-leucine *p*-nitrophenyl ester (6.1 g.). After 24 hours at room temperature, the yellow solution was diluted with 1 *N*  $NH_4OH$  (10 ml.), stirred for 1 hour and poured into ice-cold 1 *N*  $NH_4OH$  (300 ml.). The precipitated product was collected by filtration, washed with water, 1 *N* HCl and water and reprecipitated from aqueous acetic acid; wt. 12.6 g. (96%), m.p. 248–250°,  $[\alpha]_D^{25} -38.9^\circ$  (*c* 1.03, dimethylformamide);  $R_f$  (hydrobromide) 0.84, single ninhydrin- and tyrosine-positive spot.

*Anal.* Calcd. for  $C_{44}H_{60}N_8O_8S$ : C, 63.4; H, 7.25; N, 10.0. Found: C, 63.2; H, 7.14; N, 9.7.

**N-Carbobenzoxyl-L-alanyl-L-leucyl-L-tyrosyl-L-leucyl-L-valyl-S-benzyl-L-cysteinamide Hydrate (V).**—Compound IV (6 g.) was dissolved in 2 *N* HBr in acetic acid (60 ml.). After 1 hour at room temperature, dry ether was added. The precipitate was washed with ether, dried briefly over KOH and dissolved in dimethylformamide (50 ml.). Triethylamine (1 ml.) was added to this solution followed by N-carbobenzoxyl-L-alanine *p*-nitrophenyl ester (2.4 g.). After 24 hours at room temperature, the reaction mixture was diluted with 1 *N*  $NH_4OH$  (200 ml.). The precipitated product was isolated by filtration, washed with water, 1 *N* HCl and water and reprecipitated from aqueous acetic acid; wt. 6 g. (90%), m.p. 259°,  $[\alpha]_D^{25} -41.7^\circ$  (*c* 1, dimethylformamide);  $R_f$  (hydrobromide) 0.91, single ninhydrin- and tyrosine-positive spot.

*Anal.* Calcd. for  $C_{47}H_{65}N_9O_8S \cdot H_2O$ : C, 61.2; H, 7.34; N, 10.7. Found: C, 61.2; H, 7.06; N, 10.8.

**N-Carbobenzoxyl- $\gamma$ -benzyl-L-glutamyl-L-alanyl-L-leucyl-L-tyrosyl-L-leucyl-L-valyl-S-benzyl-L-cysteinamide (VI).**—Compound V (3.7 g.) was suspended in acetic acid (10 ml.) and treated with 4 *N* HBr in acetic acid (10 ml.). After 1 hour, dry ether (200 ml.) was added, the precipitate which was formed was collected by filtration, washed with ether and dried over KOH *in vacuo*. This solid was dissolved in dimethylformamide (20 ml.); triethylamine (0.6 ml.) was added followed by N-carbobenzoxyl- $\gamma$ -benzyl-L-glutamic acid *p*-nitrophenyl ester (1.8 g.). After 24 hours the yellow solution was diluted with 1 *N*  $NH_4OH$  (2 ml.),

stirred for 1 hour and poured into ice-cold 1 *N*  $NH_4OH$  (50 ml.). The precipitate was collected by filtration, washed with water, 1 *N* HCl and water and purified on reprecipitation from aqueous acetic acid; wt. 4 g. (97%), m.p. 254–260°,  $[\alpha]_D^{25} -27.9^\circ$  (*c* 0.48, dimethylformamide);  $R_f$  (hydrobromide) 0.72, single ninhydrin- and tyrosine-positive spot.

*Anal.* Calcd. for  $C_{59}H_{78}N_{10}O_{10}S$ : C, 63.1; H, 7.00; N, 10.0. Found: C, 62.9; H, 7.11; N, 10.0.

The heptapeptide amide hydrobromide which was formed from the carbobenzoxyl derivative on exposure to HBr in acetic acid was fully digested with LAP.

**N-Carbobenzoxyl-L-leucyl-L-valine *p*-Nitrophenyl Ester (VIII).**—To a precooled solution of N-carbobenzoxyl-L-leucyl-L-valine (1.0 g.) in ethyl acetate (15 ml.) and tetrahydrofuran (5 ml.) was added *p*-nitrophenol (0.45 g.) followed by *N,N'*-dicyclohexylcarbodiimide (0.57 g.). After 30 minutes at 0° and 2 hours at room temperature the *N,N'*-dicyclohexylurea which was separated was filtered off and the filtrate concentrated to dryness *in vacuo*. On reprecipitation from ether-petroleum ether, 0.62 g. (47%) of crystalline product was obtained, m.p. 126°,  $[\alpha]_D^{25} +16.8^\circ$  (*c* 0.53, dimethylformamide).

*Anal.* Calcd. for  $C_{25}H_{31}N_3O_7$ : C, 61.8; H, 6.42; N, 8.6. Found: C, 62.0; H, 6.42; N, 8.4.

**N-Carbobenzoxyl-L-leucyl-L-valyl- $\gamma$ -benzyl-L-glutamyl-L-alanyl-L-leucyl-L-tyrosyl-L-leucyl-L-valyl-S-benzyl-L-cysteinamide One and One-half Hydrate (IX).**—N-Carbobenzoxyl- $\gamma$ -benzyl-L-glutamyl-L-alanyl-L-leucyl-L-tyrosyl-L-leucyl-L-valyl-S-benzyl-L-cysteinamide (0.3 g.) was dissolved in 2 *N* HBr in acetic acid (4 ml.). After 50 minutes at room temperature dry ether (50 ml.) was added and the precipitated material was isolated by filtration and washed thoroughly with dry ether and dried over KOH *in vacuo*. To a solution of this product in dimethylformamide (8 ml.), triethylamine (0.1 ml.) was added followed by N-carbobenzoxyl-L-leucyl-L-valine *p*-nitrophenyl ester (0.12 g.). After 24 hours the reaction mixture was diluted with 1 *N*  $NH_4OH$  (0.5 ml.), stirred 30 minutes and poured into ice-cold 1 *N*  $NH_4OH$  (50 ml.). The precipitated product was collected by filtration, washed with 1 *N*  $NH_4OH$ , water, 1 *N* HCl and water again. On precipitation from dimethylformamide-water, 0.28 g. (91%) of product was obtained, m.p. 262–266°,  $[\alpha]_D^{25} -32.8^\circ$  (*c* 0.18, dimethylformamide);  $R_f$  (hydrobromide) 0.93, single sharp ninhydrin- and tyrosine-positive spot.

*Anal.* Calcd. for  $C_{70}H_{98}N_{10}O_{14}S \cdot 1.5 H_2O$ : C, 61.7; H, 7.39; N, 10.3. Found: C, 61.4; H, 7.22; N, 10.7.

Amino acid analysis by a Beckman-Spinco analyzer after acid hydrolysis showed the expected composition expressed in molar ratios: glu<sub>0.98</sub>ala<sub>1.0</sub>val<sub>2.0</sub>leu<sub>3.0</sub>tyr<sub>0.78</sub> (S-benzylcysteine present on paper chromatogram but not determined). The amino acid recovery was 96% of the theoretical value.

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[CONTRIBUTION FROM THE BIOCHEMISTRY DEPARTMENT, UNIVERSITY OF PITTSBURGH SCHOOL OF MEDICINE, PITTSBURGH, PENNA.]

## Insulin Peptides. VI. The Synthesis of a Partially Protected Nonapeptide Corresponding to the First Nine Amino Acid Residues of the A-Chain of Insulin<sup>1</sup>

BY PANAYOTIS G. KATSOYANNIS, KOUHEI FUKUDA AND ANDREW TOMETSKO

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The partially protected nonapeptide N-*p*-nitrocarbobenzoxylglycyl-L-isoleucyl-L-valyl- $\gamma$ -*tert*-butyl-L-glutamyl-L-glutamyl-L-S-benzyl-L-cysteinyl-L-S-benzyl-L-cysteinyl-L-alanylglycine, containing the N-terminal sequence of the A-chain of sheep insulin, has been synthesized. The key step for the synthesis of this compound involved the condensation of an acyltetrapeptide subunit with a pentapeptide ester subunit. Both these subunits were synthesized by stepwise elongation of the peptide chain from the amino end.

Regeneration of insulin activity from the separated A- and B-chains or their benzyl derivatives has been accomplished independently by two groups of investigators.<sup>2–5</sup> This provides sufficient assurance that

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the eventual synthesis of insulin can be achieved if chemically synthesized A- and B-chains are available.

Peptide chemistry probably has not developed to the level of sophistication necessary to cope with the synthesis of large protein molecules.<sup>6</sup> However, the present synthetic methodology and the development of purification techniques has undoubtedly paved the

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