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Semisynthesis of Insulin: Specific Activation of the Arginine Carboxyl Group of the B Chain of Desoctapeptide-(B23–30)-insulin (Bovine)†

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ABSTRACT: The semisynthesis of insulin through specific activation, by a combination of enzymatic and chemical means, of the arginine carboxyl of the B chain of desoctapeptide-(B23–30)-insulin (DOI) is described. During this synthesis the amino functions of insulin were protected by the *tert*-butyloxycarbonyl (Boc) group. A1,B1,B29-$(Boc)_3$-insulin (tri-Boc-insulin) was prepared by reaction of di-*tert*-butyl dicarbonate with zinc-free insulin in dimethyl sulfoxide. Digestion of the tri-Boc-insulin with trypsin at pH 8.5 yielded A1,B1-$(Boc)_2$-DOI (di-Boc-DOI) and Gly-Phe-Phe-Tyr-Thr-Pro-(ε-Boc)Lys-Ala (ε-Boc-octapeptide) which were separated by gel chromatography. The trypsin-catalyzed coupling of the arginine carboxyl group of di-Boc-DOI with phenylhydrazine to yield di-Boc-DOI-phenylhydrazide in 87% yield was effected at pH 6.5 in an aqueous organic solvent consisting of dimethyl sulfoxide–2,4-butanediol–water (35:35:30 v/v). A favorable thermodynamic situation for this synthesis of a peptide bond is attributed, in part, to the increase in the pK_a value of the carboxyl group in organic solvents and to the low pK_a value (5.2) of phenylhydrazine. At pH 6.5, this combination of factors allows the un-ionized carboxyl group and the unprotonated amino group to be substantial components of the reaction mixture. Treatment of the di-Boc-DOI-phenylhydrazide with various oxidizing agents yielded the phenyldiimide derivative which was coupled with ε-Boc-octapeptide to resynthesize tri-Boc-insulin. Removal of the Boc groups with trifluoroacetic acid gave a semisynthetic insulin which was indistinguishable from native insulin by several criteria including hormonal activity and susceptibility to trypsin digestion at pH 8.5. This latter point indicates that the chemical coupling through the diimide took place without substantial racemization.

The action of trypsin on insulin has been the subject of several studies from this laboratory (Carpenter & Young, 1959; Young & Carpenter, 1961; Carpenter & Baum, 1962; Wang & Carpenter, 1965, 1967, 1969). When bovine insulin is treated with trypsin at pH values 8–9.5, alanine, a heptapeptide (Gly-Phe-Phe-Tyr-Thr-Pro-Lys), and desoctapeptide-insulin (DOI)[1] are formed. The latter compound, which is missing the C-terminal octapeptide on the B chain, exhibits less than 1% of the hormonal activity of insulin (Young & Carpenter, 1961). Results of the action of carboxypeptidase A on insulin revealed that the C-terminal alanine of the B chain of bovine insulin was nonessential for hormonal activity (Slobin & Carpenter, 1963). Therefore, the presence of some component of the above-mentioned heptapeptide was implicated as a substantial contributor to the hormonal activity. The hepta-

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[1] Abbreviations used: BNPS-skatole, 2-(2-nitrophenylsulfenyl)-3-methyl-3′-bromoindolinine; Boc-, *tert*-butyloxycarbonyl; ε-Boc-octapeptide, Gly-Phe-Phe-Tyr-Thr-Pro-(ε-Boc)Lys-Ala; Boc-ON, 2-[[(*tert*-butyloxycarbonyl)oxy]imino]-2-phenylacetonitrile; OBu^t, *tert*-butyl ester; DEAE-, diethylaminoethyl; di-Boc-DOI, A1,B1-$(Boc)_2$-DOI; DMF, dimethylformamide; Me_2SO, dimethyl sulfoxide; DOI, desoctapeptide-(B23–30)-insulin; $NaDodSO_4$, sodium dodecyl sulfate; tri-Boc-insulin, A1,B1,B29-$(Boc)_3$-insulin; F_3CCOOH, trifluoroacetic acid.

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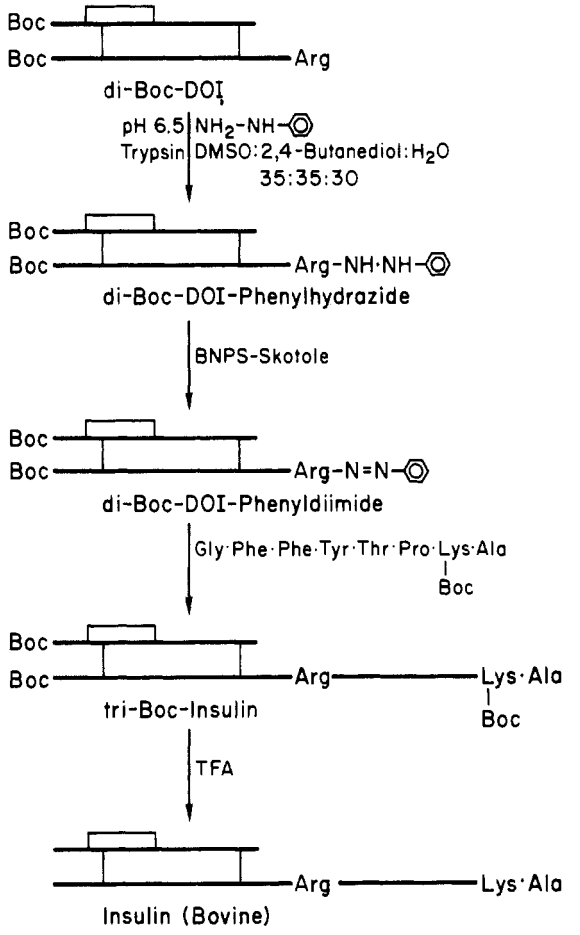
Scheme I: Diagram of the Procedures Used for the Semisynthesis of Insulin from A1,B1-Bis(*tert*-butyloxycarbonyl)desoctapeptide-(B23-30)-insulin (Di-Boc-DOI)



peptide was synthesized (Shields & Carpenter, 1961), and during the ensuing 20 years, a number of chemical schemes for adding back this heptapeptide to the C-terminal of DOI were investigated in this laboratory without substantial success. The problem has received attention from others. A report by Ruttenberg (1972) of a procedure for adding the octapeptide to DOI has been severely criticized (Gattner et al., 1978; Obermeier, 1978). Other methods have encountered side reactions (Shanghai Insulin Research Group, 1973; Obermeier & Geiger, 1976; Obermeier, 1978; Weitzel et al., 1976) which have limited their usefulness as a general procedure. Recently we have investigated the use of enzymatic methods for the attainment of this goal. The subject of this paper concerns the use of phenylhydrazine in the trypsin-catalyzed synthesis of di-Boc-DOI-phenylhydrazide, the conversion of the latter compound to a diimide (Milne & Kilday, 1965; Milne & Most, 1968; Milne & Carpenter, 1968), and the coupling of the di-Boc-DOI-phenyldiimide with ε-Boc-octapeptide to yield, after removal of the Boc groups, insulin (Scheme I).

Experimental Procedures

Materials

Bovine zinc–insulin (Eli Lilly and Co., lot 9SH35AK) was converted to zinc-free insulin by gel chromatography on Sephadex G-50 in 10% acetic acid (Steiner & Oyer, 1967). Urea (Aldrich) was dissolved in distilled water, and a mixed-bed ion exchanger [Bio-Rad Laboratories, AG 501-X8(D)] was added to remove ammonium cyanate (Stark et al., 1960). The mixture was stirred overnight and then filtered. The Sephadex gel chromatography resins were purchased from Pharmacia Fine Chemicals, as was the ion-exchange resin, DEAE-Sephacel. Bio-Gel P-2, 200–400 mesh, was obtained from Bio-Rad Laboratories, as were the reagents for polyacrylamide gel electrophoresis. Urea for gel electrophoresis was the ultrapure grade from Schwarz/Mann. Trypsin treated with L-1-(tosylamido)-2-phenylethyl chloromethyl ketone to inhibit contaminant chymotryptic activity (Kostka & Carpenter, 1964) was purchased from Worthington Biochemical Corp. Dimethyl sulfoxide (Pierce Chemical Corporation) and dimethylformamide (Eastman) were distilled under vacuum (15 mm) after refluxing for 2 h over calcium hydride. The distilled solvents were stored at −10 °C. Triethylamine (Sequanal grade), *N*-ethylmorpholine, and BNPS-skatole were from Pierce Chemical Corp. Di-*tert*-butyl dicarbonate (99%) and Boc-ON were obtained from Aldrich.

Methods

Amino acid analyses were performed on a Beckman-Spinco Model 120B automatic amino acid analyzer (Spackman et al., 1958) equipped with a system AA automatic integrator. Acid hydrolyses were performed in 6 N HCl and 0.1% phenol in sealed, evacuated tubes for 6–12 h at 120 °C (Carpenter & Chrambach, 1962).

Three systems were employed for polyacrylamide slab gel electrophoresis. System A: Electrophoresis was performed in the presence of 8 M urea and 0.9 M acetic acid at pH 3.5 in 15% acrylamide (Poole et al., 1974). The gel was stained with amido black (Racusen, 1973). System B: $NaDodSO_4$–polyacrylamide slab gel electrophoresis was performed by the method of Ferro-Luzzi Ames (1974) at pH 8.8 in 15% acrylamide with the modification that the proteins were not treated with β-mercaptoethanol. System C: Electrophoresis at pH 8.8 without $NaDodSO_4$ was performed as described by Laemmli (1970) and the gel stained with Coomassie Brilliant Blue R-250. Gel filtration and ion-exchange chromatography separations were monitored with a 2138 Uvicord S detector (LKB). Protein concentration was determined by absorbance at 277 nm (Carpenter & Hess, 1956). Free amino groups were measured by using 2,4,6-trinitrobenzenesulfonic acid (Fields, 1971). The stimulation of lipogenesis in rat epididymal fat cells was performed by the method of Moody et al. (1974).

Preparation of Tri-Boc-insulin. Zinc-free insulin (360 mg, 60 μmol) was dissolved in 8 mL of Me_2SO containing 200 μmol of triethylamine. Di-*tert*-butyl dicarbonate (200 μmol) was added dropwise. The reaction was allowed to proceed with stirring for 3 h at room temperature. The solution was cooled in ice, diluted with 20 mL of 0.1 M NH_4HCO_3, pH 8.0, and subjected to gel filtration on Sephadex G-25 (fine) (4.5 × 50 cm) in 0.1 M NH_4HCO_3, pH 8.0. The appropriate fractions were pooled and lyophilized (yield 96%). The protein was then dissolved in 90 mL of 1 M NH_4OH and reacted at 5 °C for 30 h. The solution was diluted to 500 mL and lyophilized. The modified hormone was then subjected to ion-exchange chromatography at 5 °C on DEAE-Sephacel equilibrated in 0.09 M NaCl, 0.01 M Tris, pH 7.2, and 7 M urea. The materials in the various peaks were dialyzed against distilled water and lyophilized. The material in the main peak was desalted on a Sephadex G-25 (fine) column (4.5 × 50 cm) in 0.1 M NH_4HCO_3, pH 8.0, to yield 255 mg (74%) of tri-Boc-insulin.

Preparation of Di-Boc-DOI. Tri-Boc-insulin (150 mg) was dissolved in 150 mL of 0.1 M *N*-ethylmorpholine and 1 mM $CaCl_2$, adjusted to pH 8.6 with 10% acetic acid. Trypsin (7.5 mg) was added, and the incubation was conducted at room temperature for 24 h. The mixture was then lyophilized, and the residue was subjected to chromatography on a Sephadex

G-50 (fine) column (3 × 80 cm) equilibrated with 0.1 M NH_4HCO_3, pH 8.0. The di-Boc-DOI and ϵ-Boc-octapeptide fractions were separately pooled and lyophilized (yield di-Boc-DOI 92%). The ϵ-Boc-octapeptide fraction was desalted on a Bio-Rad P-2 (200–400 mesh) column (2.5 × 45 cm) equilibated with 0.1 M NH_4HCO_3, pH 8.0.

Preparation of Di-Boc-DOI-phenylhydrazide. Di-Boc-DOI (80 mg) was dissolved in 28 mL of Me_2SO. An equal volume of 1,4-butanediol was added, and the solution was cooled in an ice bath. Ice-cold 5 mM $CaCl_2$ (4 mL) was added. An aqueous phenylhydrazine solution (20 mL), containing a 1000-fold molar excess adjusted to pH 6.5 with 6 N HCl, was added slowly. The solution was incubated with 8 mg of trypsin added in four equal portions at 8–16 h intervals for a total of 48 h at room temperature. The mixture was dialyzed against 10% acetic acid at 5 °C and then lyophilized. The phenylhydrazide was purified by ion-exchange chromatography at 5 °C on a DEAE-Sephacel column (2.5 × 45 cm) equilibrated in 0.08 M NaCl, 0.01 M Tris, pH 7.2, and 7 M urea. The material in the main peak was pooled, dialyzed against distilled water at 5 °C, and lyophilized (yield 87%).

Regeneration of Insulin. Di-Boc-DOI-phenylhydrazide (50 mg, 10 μmol) was dissolved in 5 mL of DMF containing 11 μmol of pyridine. A 100-fold molar excess of phenol was added to act as scavenger, and then a 50-fold molar excess of BNPS-skatole was added to convert the phenylhydrazide to the phenyldiimide. This mixture was reacted at room temperature for 30 min in the dark. The pH was adjusted to approximately 9.2 with triethylamine, and a 10-fold molar excess of ϵ-Boc-octapeptide, generated from the tryptic digestion of tri-Boc-insulin, was added. The reaction was allowed to proceed at room temperature in the dark for 20–24 h. The solution was cooled in ice, and the product was precipitated with 5 volumes of diethyl ether and collected by centrifugation. The pellet was dissolved in 0.1 M NH_4HCO_3, pH 8.0, and chromatographed on a Sephadex G-50 (fine) column (3 × 80 cm). The materials in the various peaks were pooled and lyophilized. The yield of crude tri-Boc-insulin was 76%. This material was dissolved in trifluoroacetic acid (2 mL), and the resulting solution was stirred at room temperature for 2 h. The solution was cooled in ice, and the protein was precipitated with 5 volumes of diethyl ether, collected by centrifugation, and dried. The regenerated insulin was isolated by ion-exchange chromatography on DEAE-Sephacel equilibrated in 0.035 M NaCl, 0.01 M Tris, pH 7.9, and 7 M urea. The materials in the various peaks were dialyzed against distilled water and lyophilized. The material from the main peak was further purified by gel filtration on a Sephadex G-50 (fine) column (2.5 × 45 cm), using 0.1 M NH_4HCO_3 as eluant, to yield 9 mg (18% overall recovery by weight).

Results

Since the presence of free amino groups could lead to undesirable side reactions in the trypsin-catalyzed synthesis of the phenylhydrazide, in the oxidation of the hydrazide to the diimide, or in the coupling reaction with the octapeptide (Scheme I), the amino groups were protected with a blocking group (Boc) which was known to be removable from insulin (Levy & Carpenter, 1967). The desired intermediates, di-Boc-DOI and ϵ-Boc-octapeptide, were prepared by the action of trypsin at pH 8.5 on tri-Boc-insulin. The latter compound was made by reaction of Boc-ON or di-*tert*-butyl dicarbonate with insulin. The tri-Boc-insulin was separated from mono- and disubstituted derivatives present in these reaction mixtures by ion-exchange chromatography on DEAE-Sephacel. Under conditions which yielded tri-Boc-insulin as the major component, the elution profile for the tri-BOC-insulin was unsymmetrical (Figure 1). Since materials isolated from the leading and trailing edges of this unsymmetrical peak exhibited identical behavior and the expected mobility for a trisubstituted derivative upon electrophoresis under basic or acidic conditions, the dissymmetry was attributed to the presence of byproducts formed by the reaction of the phenolic or hydroxyl groups of insulin with the acylating reagents. Various modifications of the reaction conditions were explored in unsuccessful attempts to avoid the side reactions: the solvent, Me_2SO, was replaced by DMF, by 50% aqueous dioxane, by methanol-5% aqueous triethylamine (4:1), or by 0.1 M aqueous *N*-ethylmorpholine; the base, triethylamine, was replaced by the more hindered diisopropylethylamine; the acylating reagent was added in limiting amounts. Addition of 2,4-dinitrophenol as a scavenger (Martinez et al., 1979) reduced the amount of byproducts but did not completely eliminate them. However, when the crude reaction products were exposed to 1 M NH_4OH at 5 °C for 30 h and the resulting products subjected to chromatography on DEAE-Sephacel, symmetrical peaks were observed in the elution profile (Figure 1). The material isolated from the various peaks was subjected to polyacrylamide gel electrophoresis in system A (acidic urea) with the results shown in the inset to Figure 1. Fractions I, II, and III exhibited relative mobilities corresponding to the mono–, di–, and trisubstituted derivatives, respectively. This assignment was confirmed by the color yield upon reaction of the various fractions with trinitrobenzenesulfonic acid (Fields, 1971). The tri-Boc-insulin exhibited 6% of the activity of the native hormone in the stimulation of lipogenesis in the rat epididymal fat cell assay (Figure 2). However, after the tri-Boc-insulin had been treated for 2 h at room temperature with anhydrous F_3CCO-OH, the resulting deblocked product exhibited full hormonal activity in the fat cell assay. The material in fraction IV (Figure 1) constituted a significant proportion of the products formed in the Boc-ylation of insulin. High salt concentrations

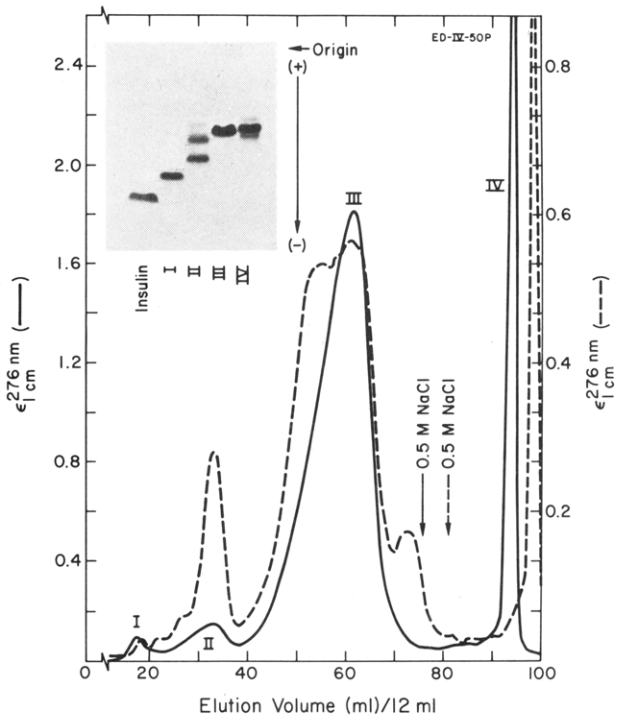


FIGURE 1: Chromatography of tri-Boc-insulin on DEAE-Sephacel (2.5 × 45 cm) with 0.09 M NaCl and 0.01 M Tris (pH 7.2) in 7 M urea at 5 °C before (- - -) and after (—) treatment with 1 M NH_4OH for 30 h at 5 °C. (Inset) Gel electrophoresis in acidic urea (system A) of the various fractions eluted from the column.

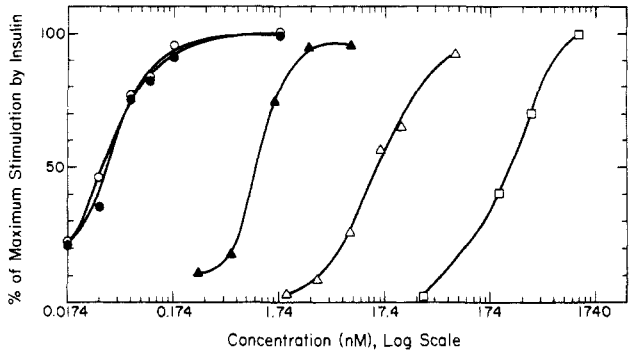


FIGURE 2: Dose response of the various insulin derivatives in the stimulation of lipogenesis in rate epididymal fat cells: bovine insulin standard (●), resynthesized insulin (○), tri-Boc-insulin (▲), di-Boc-desoctapeptide-insulin (△), di-Boc-desoctapeptide-insulin phenylhydrazide (□).

Table I: Amino Acid Ratios[a]

amino acid	bovine insulin theory	bovine insulin found	regenerated insulin found	di-Boc-DOI theory	di-Boc-DOI found
Lys	1	1.03	0.94	0	0.02
His	2	2.04	1.88	2	1.92
Arg	1	1.01	0.98	1	0.98
Asp	3	2.98	2.94	3	2.96
Thr	1	1.06	0.93	0	<0.05
Ser	3	2.65	2.71	3	2.89
Glu	7	7.02	7.06	7	7.04
Pro	1	1.13	0.88	0	0.08
Gly	4	4.02	4.00	3	3.01
Ala	3	3.03	2.98	2	2.00
$^1/_2$-Cys	6	4.40	4.73	6	4.88
Val	5	4.53	4.57	5	4.22
Ile	1	0.61	0.65	1	0.47
Leu	6	5.93	5.94	6	5.81
Tyr	4	3.86	3.72	3	2.96
Phe	3	2.94	2.82	1	1.02

[a] Hydrolyzed for 6 h at 120 °C in 6 N HCl. Ratios are calculated on the basis of Asp + Glu = 10.

(0.5 M NaCl) were needed to elute the material, which indicated that it was strongly bound to the resin. This tight binding and the tendency for the material to smear in gel electrophoresis (inset to Figure 1) were indicative of polymeric forms. Gel electrophoresis in the presence of $NaDodSO_4$ (system B) confirmed the presence of dimer and higher aggregates of insulin (data not shown).

The tri-Boc-insulin was treated with trypsin at pH 8.6 in 0.1 M *N*-ethylmorpholine buffer at room temperature until a complete digestion was indicated by the measurement of released amino groups by reaction with trinitrobenzenesulfonate. The liberated di-Boc-DOI and ε-Boc-octapeptide were separated by chromatography on Sephadex G-50. The isolated di-Boc-DOI was virtually free of any contaminating tri-Boc-insulin as judged by gel electrophoresis and amino acid composition (Table I). It exhibited a hormonal activity of 0.4% that of insulin (Figure 2).

The trypsin-catalyzed addition of phenylhydrazine to the C-terminal arginine of di-Boc-DOI was performed at pH 6.5 in a mixed solvent system consisting of Me_2SO–1,4-butanediol–water (35:35:30). The reaction mixture was dialyzed against 10% HOAc, lyophilized, and subjected to ion-exchange chromatography on DEAE-Sephacel, with the results shown in Figure 3. In the solvent system used for this chromatography (0.08 M NaCl, 0.01 M Tris, pH 7.2, and 7 M urea) the di-Boc-DOI-phenylhydrazide (Figure 3, solid line) is well separated from any unreacted di-Boc-DOI (Figure 3, dashed line). Fractions containing the main peak (Figure 3, solid line)

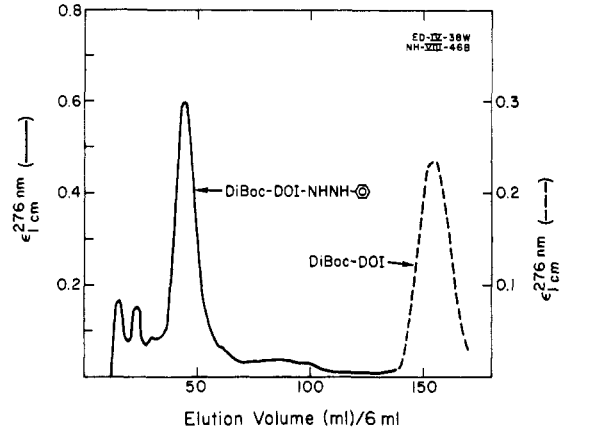


FIGURE 3: Chromatography on DEAE-Sephacel (2.5 × 45 cm) with 0.08 M NaCl and 0.01 M Tris (pH 7.2) in 7 M urea at 5 °C of di-Boc-desoctapeptide-insulin phenylhydrazide (—) and di-Boc-desoctapeptide-insulin (- - -).

were pooled, dialyzed against distilled water, and lyophilized to yield 87% of di-Boc-DOI-phenylhydrazide. The phenylhydrazide derivative exhibited 0.01% of the activity of insulin in the stimulation of lipogenesis in the epididymal fat cell assay (Figure 2).

For determination of the best conditions for the oxidation of phenylhydrazide to the diimide and its subsequent coupling to the amine component, pilot experiments were performed in which the di-Boc-DOI-phenylhydrazide was subjected to various oxidizing agents, followed by coupling with a large excess of Met-OBu^t. After gel chromatography to remove excess reagents, the products were subjected to amino acid analysis to determine the degree of coupling of Met-OBu^t and of possible destruction of other amino acid residues. Also, the rate and degree of formation of the phenyldiimide by the various oxidants were followed by the change in absorption at 280 nm (Milne & Most, 1968). *N*-Bromosuccinimide and the milder oxidizing agent *N*-chlorosuccinimide (Milne & Kilday, 1965) yielded the phenyldiimide and good coupling with Met-OBu^t but resulted in partial destruction of histidine and tyrosine. *o*-Iodosobenzoic acid (Mahoney & Hermodson, 1979) produced the diimide which coupled completely with Met-OBu^t without appreciable damage to histidine or tyrosine. However, the insolubility of the reagent in water or ether made its separation from the protein inconvenient. BNPS-skatole (Omenn et al., 1970) oxidized the phenylhydrazide to the diimide which gave complete coupling with the Met-OBu^t without destruction of histidine and with only small effects (<10%) on the tyrosine. So that the possible oxidation of tyrosine could be avoided, phenol was added as a scavenger in subsequent reactions using BNPS-skatole.

The di-Boc-DOI-phenylhydrazide was oxidized with BNPS-skatole in DMF, and the resulting diimide was reacted, without isolation, with a 10-fold molar excess of ε-Boc-octapeptide. The products were precipitated with ether and subjected to gel chromatography on Sephadex G-50, with the results shown in Figure 4. Peak I of Figure 4 contained material of high molecular weight and high absorption at 280 nm. Peak III contained the excess ε-Boc-octapeptide. Gel electrophoresis in system C of the material in peak II (inset, Figure 4) revealed that it was composed primarily of tri-Boc-insulin with small amounts of di-Boc-DOI-phenylhydrazide and di-Boc-DOI. Without further purification, the material in peak II was deblocked in anhydrous F_3CCOOH, and the resulting mixture was subjected to ion-exchange chromatography, with the results shown in Figure 5. Peak I (Figure 5) has the expected mobility for insulin in this solvent

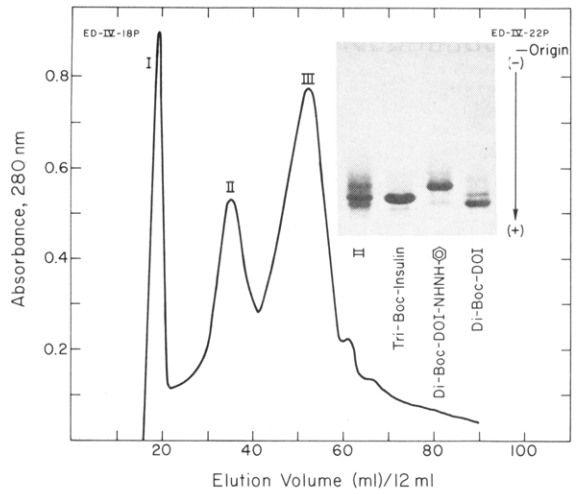


FIGURE 4: Chromatography on Sephadex G-50 (fine) (3 × 80 cm) with 0.1 M NH_4HCO_3 (pH 8.0) of the crude mixture obtained from reaction of di-Boc-desoctapeptide-insulin phenyldiimide with excess ϵ-Boc-octapeptide. (Inset) Polyacrylamide gel electrophoresis (system C) of fraction II, tri-Boc-insulin, di-Boc-desoctapeptide-insulin phenylhydrazide (di-Boc-DOI-$NHNHC_6H_5$), and di-Boc-desoctapeptide-insulin (di-Boc-DOI).

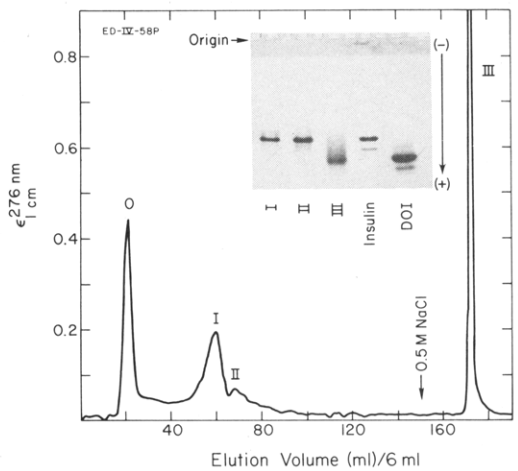


FIGURE 5: Chromatography on DEAE-Sephacel (2 × 35 cm) with 0.035 M NaCl and 0.01 M Tris (pH 7.9) in 7 M urea at 5 °C of crude resynthesized insulin. (Inset) Polyacrylamide gel electrophoresis (system C) of fractions I–III isolated from the chromatography and of standard insulin and desoctapeptide-insulin (DOI).

system. The various fractions were pooled, dialyzed against water, and lyophilized. Gel electrophoresis (system C) of the various lyophilized fractions revealed that peaks I and II contained material moving like insulin (inset, Figure 5). Both were free of desamido-insulin which is a contaminant in the insulin used as a standard. DOI (and/or polymers of it) was eluted in peak III. Peak 0 contained nonproteinaceous material absorbing at 280 nm. The material in peak I was subjected to further purification by gel chromatography on Sephadex G-50, followed by lyophilization of the insulin-containing fraction to yield 9 mg (18% yield from di-Boc-DOI-phenylhydrazide) of pure regenerated insulin. This last gel chromatography step is necessitated by the fact that the solutions recovered from the DEAE-Sephacel chromatography invariably contain some high molecular weight, nonproteinaceous components which are solubilized by the urea-containing solutions from either the DEAE-Sephacel or the dialysis tubing. The regenerated insulin had the amino acid compositons (Table I) expected for insulin when it is hydrolyzed under these conditions (Carpenter & Chrambach, 1962; Africa & Carpenter, 1970). It possessed full hormonal activity in the lipogenesis assay (Figure 2) and was hydrolyzed by trypsin at pH 8.5 to give DOI (determined by gel electrophoresis) and 2 equiv of amino groups (by reaction with trinitrobenzenesulfonate). This latter observation indicates that the coupling reaction through the diimide took place without substantial racemization of the arginine residue, which confirms previous experience in the diimide coupling of model peptides (Milne & Carpenter, 1968).

Discussion

Our first attempts to synthesize a peptide bond involved the observation that the minimum negative free-energy change in the hydrolysis of an internal peptide bond in aqueous solution occurs at pH 6.5. It was further noted in 1960 (Carpenter, 1960) that the major contribution to the free-energy change involved ionization of carboxyl and amino groups. If it were possible to devise conditions where the predominant species were un-ionized carboxyl groups and unprotonated amino groups, the free-energy change would favor synthesis of a peptide bond. When di-Boc-DOI was treated with 2 M hydrazine in the presence of trypsin at pH 6.5, about 15% of di-Boc-DOI-hydrazide was formed (Canova-Davis & Carpenter, 1978). Shortly thereafter, Inouye et al. (1979) reported the use of trypsin in the synthesis of peptide bonds at pH 6.5 in the presence of varying proportions of organic solvents. Their application of this scheme to di-Boc-DOI (porcine) and a derivative of the human octapeptide, Gly-Phe-Phe-Tyr-Thr-Pro-Lys-Thr, resulted, after removal of protecting groups, in the semisynthesis of human insulin. Subsequently this procedure has been used in the synthesis of insulins in which the phenylalanines of the octapeptide were replaced by leucine (Tager et al., 1980; Gattner et al., 1980). When organic solvents were incorporated into the solvent system used for trypsin-catalyzed synthesis of di-Boc-DOI-hydrazide, the yields were improved to about 30%. The resulting di-Boc-DOI-hydrazide was purified and treated with nitrous acid to yield the azide which in turn was coupled with methionine *tert*-butyl ester. The *tert*-butyl groups were removed by F_3CCOOH (Levy & Carpenter, 1966) to yield DOI-Met^{B23} (Canova-Davis & Carpenter, 1980).

The improved yields of peptide bonds observed in the presence of organic solvents as compared to water are attributed not only to a decrease in the activity of water but also more importantly to an increase in the apparent pK_a value of the carboxyl group in the organic solvent mixture (Homandberg et al., 1978). The latter results in a substantial increase in the concentration of the un-ionized carboxyl group at pH 6.5. On the other hand, the apparent pK_a values of primary amines are affected little by the presence of substantial amounts of organic solvents (Duggan & Schmidt, 1943). The better yields (~60%) in the coupling reaction observed by Inoyue et al. (1979) with amino acid esters (pK_a 7.7) as compared with our results with hydrazine (pK_a 8.5) can be attributed to the lower pK_a values of the amino acid esters (or amides) which afford a relatively higher concentration of the unprotonated amino component. So that we could take further advantage of this pK_a effect, an amino component was sought which would have a pK_a value below that of amino acid esters but which could also be activated for subsequent peptide bond formation by chemical means. An amino component that satisfied these conditions proved to be phenylhydrazine (pK_a 5.2).

There are some limits to the direct procedure (Inouye et al., 1979; Tager et al., 1980) which are surmounted by the intermediate formation of the phenylhydrazide. In our hands,

the direct addition of glycine methyl or ethyl ester was unsuccessful. It appears that the conditions for direct addition of various peptides may vary with the nature of the peptide (Gattner et al., 1980). The procedure developed here involves the specific activation of the C-terminal arginine of DOI and should be applicable to the addition of any variety of peptide derivatives regardless of their configurations or compositions. The success of the method derives in part from the low pK_a value of phenylhydrazine which results in high yields (>87%) of the synthesized phenylhydrazide. Another factor is the absence in insulin of tryptophan and methionine which allows the use of a variety of oxidizing agents to convert the phenylhydrazide to the phenyldiimide. The method has the disadvantages of involving an extra step and also the potential that the reactive phenyldiimide or the reactions involved in forming the phenyldiimide may lead to undesirable side products. Nevertheless, the resynthesis of insulin from di-Boc-DOI-phenylhydrazide and the natural ε-Boc-octapeptide proceeded in sufficient overall yield (18%) to make this procedure a feasible method for investigating the relationship of structure to activity in the C-terminal octapeptide of the B chain of insulin.

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