ion titration curves and will be reported in a subsequent paper.

References

Fox, T. G., and Flory, P. J. (1949), *J. Phys. Colloid Chem. 53*, 197.
Plocke, D. J., Levinthal, C., and Vallee, B. L. (1962), *Biochemistry 1*, 373.
Reithel, F. J. (1963), *Advan. Protein Chem. 18*, 123.
Rothman, F., and Byrne, R. (1963), *J. Mol. Biol. 6*, 330.
Scheraga, H. (1961), Protein Structure, New York, N. Y., Academic.
Schlesinger, M. J. (1965), *J. Biol. Chem. 240*, 4293.
Schlesinger, M. J., and Andersen, L. (1967), *Proc. N. Y. Acad. Sci.* (in press).
Schlesinger, M. J., and Barrett, K. (1965), *J. Biol. Chem. 240*, 4284.
Simmons, N. S., Cohen, C., Szent-Gyorgyi, A. G., Wetlaufer, D. B., and Blout, E. R. (1961), *J. Am. Chem. Soc. 83*, 4766.
Stellwagen, E., and Schachman, H. K. (1962), *Biochemistry 1*, 1056.
Tanford, C. (1962), *Advan. Protein Chem. 17*, 69.
Tanford, C., Kawahara, D., and Lapanje, S. (1967). *J. Am. Chem. Soc. 89*, 729.
Wetlaufer, D. (1962), *Advan. Protein Chem. 14*, 303.

The Synthesis of Triaminoacyl-insulins and the Use of the *t*-Butyloxycarbonyl Group for the Reversible Blocking of the Amino Groups of Insulin*

Daniel Levy† and Frederick H. Carpenter

ABSTRACT: *t*-Butyloxycarbonyl azide reacts with insulin in dimethylformamide to give a derivative in which all three amino groups of insulin are covered and which has a diminished biological activity. Upon treatment of the *t*-butyloxycarbonyl-insulin with anhydrous trifluoroacetic acid, the *t*-butyloxycarbonyl groups are removed to yield a crystalline insulin with high biological activity. Reaction of the *p*-nitrophenyl esters of *t*-butyloxycarbonylamino acids with insulin results in the introduction of three amino acid residues; one on each of the amino terminals of the A and B chain and one on the ε-amino group of lysine 29 of the B chain. Upon treatment of the derivative with anhydrous trifluoroacetic acid, the *t*-butyloxycarbonyl groups are removed to yield triaminoacyl-insulins. Derivatives prepared in this manner included trialanyl-, triasparaginyl-, trilysyl-, trimethionyl-, and by a slight modification, triglutamyl-insulins.

The products were characterized by amino acid composition, DEAE Sephadex chromatography, chemical and enzymatic degradations, and biological activity. Despite the introduction of groups of different size, charge, and hydrophylic–hydrophobic properties, the resulting triaminoacyl-insulins all possessed about the same biological activity amounting to 40–50% of that of bovine insulin in the mouse convulsion assay with slightly higher values in the immunochemical assays.

A continuing study in this laboratory is concerned with the relationship of structure to biological activity of insulin. A recent review (Carpenter, 1966) summarized a number of findings that were obtained by subjecting insulin to various *enzymatic* degradations The present report is concerned with *chemical* modifications involving the free amino groups of insulin.

Reactions at the free amino groups of insulin have been the subject of several previous investigations. Fraenkel-Conrat and Fraenkel-Conrat (1950) covered the amino groups by acetylation with acetic anhydride to yield products which showed little loss of biological activity. On the other hand, Mills (1953) reacted the amino groups with 2,4,5-trinitrotoluene to give products with no biological activity. Since the acetyl and dinitrotolyl residues cannot be removed under mild conditions, they can be considered as irreversible blocking groups. Although several reagents have been proposed for use in the reversible blocking of the amino groups

* From the Department of Biochemistry, University of California, Berkeley, California 94720. *Received July 28, 1967.* This study was supported in part by a grant (AM 00608) of the National Institutes of Health and a grant (GB-5931) of the National Science Foundation. A preliminary account of this work has been presented at the Pacific Slope Biochemical Conference, Davis, Calif., June 15–17, 1967.

† Postdoctoral fellow of the National Institutes of Health, 1965–1967.

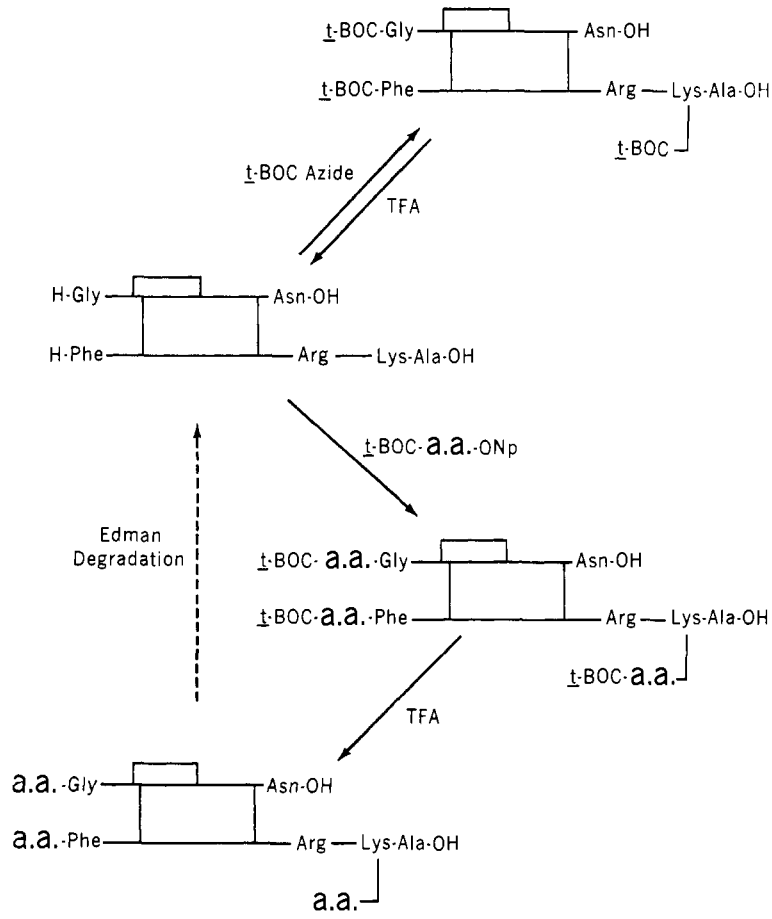

FIGURE 1: Scheme depicting the synthesis and degradation of *t*-BOC-insulin and triaminoacyl-insulins; a.a. refers to an amino acid residue, *t*-BOC refers to *t*-butyloxycarbonyl group, ONp refers to the *p*-nitrophenyl group, and TFA refers to trifluoroacetic anhydride.

of proteins (Goldberger and Anfinsen, 1962; Merigan *et al.*, 1962; Marzotto *et al.*, 1967), they apparently have not been applied to insulin. Ludwig and Byrne (1962) have used ethyl acetamidate to cover the amino groups of the *oxidized* A and B chains of insulin and were subsequently able to remove the acetimidyl blocking groups by treatment with concentrated ammonia. We are reporting on the use of the *t*-butyloxycarbonyl group (*t*-BOC)[1] as a reversible blocking group for the amino residues of insulin.

Another type of modification of the amino groups of insulin involves the addition of amino acid residues by means of the Leuchs' anhydrides. In early work Fraenkel-Conrat (1953) used *N*-carboxy-L-leucine anhydride to react with the amino residues of insulin. More recently, Virupaksha and Tarver (1964) used the *N*-carboxy anhydride of methionine. As one might expect, the products obtained by such procedures consist of a mixture of compounds. Thus, Fraenkel-Conrat found incomplete coverage of the ϵ-amino group of lysine even when he used a large excess of reagent which resulted in the incorporation of over 7.3 leucine residues/mole of insulin. Since there is a total of three amino groups in insulin, such products must consist of a mixture of molecules in which some of the amino groups are unreacted while others are substituted with a single or polyleucyl residue of variable chain length. Understandably, the results of bioassays on such compounds are subject to some ambiguities in interpretation. Nevertheless, it is interesting to note that a methionyl derivative which contained approximately 2.5 residues of methionine/mole of insulin possessed approximately 50% of the biological activity of native insulin (Virupaksha and Tarver, 1964).

This report describes a procedure which allows the addition of various amino acid residues to the three amino groups of insulin. The procedure, which is based on recent developments in peptide chemistry (Carpino *et al.*, 1959; Bodánszky, 1955; Schwyzer and Keppler, 1961), is outlined in Figure 1. The *p*-nitrophenyl esters of *t*-BOC-amino acids are reacted with the amino groups of insulin, followed by removal of the protect-

[1] Abbreviations used: *t*-BOC, the *t*-butyloxycarbonyl group; TPCK-trypsin, trypsin which has be entreated with L-(1-tosylamido-2-phenyl)ethyl chloromethyl ketone; TFA, trifluoroacetic acid.

ing groups in anhydrous trifluoroacetic acid (TFA) to yield the triaminoacyl-insulins. A preliminary report of this work has appeared (Levy and Carpenter, 1966).

Experimental Section

Materials

Bovine zinc insulin was the product of Eli Lilly and Co. (lot no. 836550). TPCK-trypsin was prepared by treating twice-crystallized, salt-free lyophilized trypsin from Worthington Biochemical Corp. (lot no. 6224) with L-(1-tosylamido-2-phenyl)ethyl chloromethyl ketone (Schoellmann and Shaw, 1963) by a modification (Wang and Carpenter, 1965) of the procedure of Kostka and Carpenter (1964). Trypsin treated with TPCK was used in order to minimize the possibility of nontryptic cleavage of peptide bonds by chymotrypsin which is a frequent contaminant of trypsin preparations.

Dimethylformamide (Distillation Products, Inc.) was purified by filtration over a cation-exchange resin (50–100 mesh, Bio-Rad AG 50W-X8) followed by distillation under vacuum (0.2 mm) after refluxing for 2 hr over calcium hydride. Phenyl isothiocyanate (Distillation Products, Inc.) was distilled before using; *t*-BOC-azide was prepared from *t*-butyl carbazate (Aldrich Chemical Co.) according to Carpino *et al.* (1959).

t-BOC-methionine and its *p*-nitrophenyl ester were prepared according to Scoffone *et al.* (1964). α,ϵ-Di-*t*-BOC-lysine *p*-nitrophenyl ester was prepared according to Hofmann *et al.* (1965). *t*-BOC-asparagine *p*-nitrophenyl ester was prepared according to Schröder and Klieger (1964). *t*-BOC-alanine (Calbiochem) was converted to the *p*-nitrophenyl ester according to Tesser and Nivard (1964).

N-t-Butyloxycarbonyl-L-glutamic Acid γ-Methyl Ester α-p-Nitrophenyl Ester. Glutamic acid γ-methyl ester (970 mg, 6.0 mmoles), prepared according to Hanby and Waley (1950), was added to a suspension of magnesium oxide (480 mg, 11.9 mmoles) in 50% aqueous dioxane (30 ml) and stirred for 1 hr. *t*-BOC-azide (2.0 g) was added and the mixture was stirred for 6 hr at 48°. The reaction was poured into cold water (175 ml) and the precipitate was removed by filtration. The aqueous solution was extracted with ethyl acetate to remove the excess *t*-BOC-azide. The ethyl acetate layer was washed with water, the combined aqueous extracts were cooled to 0°, and the pH was adjusted to 3.0 with 10% citric acid (25 ml). The acidified solution was saturated with NaCl, extracted with ethyl acetate, and dried over $MgSO_4$. After filtration, evaporation gave a colorless oil (840 mg, 3.2 mmoles) that was ninhydrin negative and homogeneous on thin layer chromatography; yield 53%. The nuclear magnetic resonance spectrum showed a singlet at τ 8.45 with an integrated value of nine protons for the *t*-butyl group, and a singlet at τ 6.30 for the methyl ester. Although we were unable to obtain this compound in crystalline form, it was satisfactory for use in the preparation of the crystalline *p*-nitrophenyl ester derivative.

The oily *N-t*-BOC-glutamic acid γ-methyl ester (340 mg, 1.3 mmoles) was dissolved in ethyl acetate (5 ml) and cooled to 0°. To this solution was added *p*-nitrophenol (180 mg, 1.3 mmoles) and dicyclohexylcarbodiimide (268 mg, 1.3 mmoles) and the reaction was kept at 0° for 2 hr and then at 4° for 20 hr. The dicyclohexylurea was filtered and the precipitate was washed with fresh ethyl acetate. The organic layer was washed with dilute sodium bicarbonate, dilute citric acid, water, and then dried over $MgSO_4$. Evaporation of the filtered solution afforded a colorless oil that crystallized on standing. The product was recrystallized from ether–petroleum ether (bp 30–60°) to yield crystals (320 mg, 0.84 mmole) of mp 83–86°, yield 65%. This product was homogeneous on thin layer chromatography. The nuclear magnetic resonance spectrum showed peaks at τ 8.47 and 6.30 for the *t*-butyl group and the methyl ester with an integrated intensity of 9:1. The infrared spectrum had peaks at 7.42 and 6.25 μ for the *p*-nitrophenyl ester.

Anal. Calcd for $C_{17}H_{22}N_2O_8$: C, 53.40; H, 5.76; N, 7.33. Found: C, 53.50; H, 5.85; N, 7.20.

Methods

Amino acid analyses were performed on a Beckman–Spinco Model 120B automatic amino acid analyzer (Spackman *et al.*, 1958). All hydrolyses were carried out in 6 N HCl in sealed, evacuated tubes for 6 hr at 120°.

Infrared spectra were run on a Baird-Atomic spectrophotometer utilizing potassium bromide pellets or chloroform solutions. *Nuclear magnetic resonance spectra* were run on a Varian A-60 in chloroform or deuterium oxide. *Ultraviolet* absorption measurements were obtained from a Zeiss spectrophotometer (PM Q II). *Elemental and methoxyl analyses* were performed by the Chemistry Department, University of California, Berkeley, Calif. *Melting points* were taken in open capillary tubes on a Thomas-Hoover melting point apparatus and are uncorrected. *Thin layer chromatography* was performed on plates prepared from silica gel G (E. Merck) with chloroform and ethyl acetate as the eluting solvents. *Biological assays* by the mouse convulsion test and immunoassays were performed at Eli Lilly and Co. From 120 to 150 mice were involved in each convulsion assay.

A *DEAE Sephadex* (A-25) column (2.5 × 30 cm) was used to assess the homogeneity of the various insulin derivatives by the procedure of Bromer and Chance (1967). The samples (5 mg/ml) were dissolved in the chromatographic buffer containing 0.01 M Tris–0.03 M NaCl in 7 M urea and the pH was adjusted to 7.5 with HCl. Elution was performed by a linear gradient obtained by running 0.3 M NaCl (1 l.) into the stirred reservoir containing 1 l. of 0.03 M NaCl (all in 7 M urea–0.01 Tris at pH 7.5). Flow rates were 45–50 ml/hr and fractions of 8–10 ml were collected. Protein was determined by absorbance at 277 mμ.

Modified Insulin Preparations. Insulin hydrochloride (60 mg, 10 μmoles) (Carpenter, 1958) was dissolved in purified dimethylformamide (5.0 ml) and triethyl-

TABLE I: Amino Acid Analyses of Insulin Derivatives.

Amino Acid	Insulin	Trimet-insulin	Triala-insulin	Triasn-insulin	Triglu-insulin	Trilys-insulin
Asp	3	3.08	2.93	**6.00**	3.00	2.85
Thr	1	0.94	0.90	0.95	1.04	0.90
Ser	3	2.92	2.88	2.93	2.91	2.92
Glu	7	6.80	6.85	7.08	**10.05**	6.80
Pro	1	1.02	0.90	0.93	1.06	0.95
Gly	4	4.10	3.96	4.08	4.10	3.94
Ala	3	3.00	**6.04**	3.00	3.00	3.00
Cys	6	5.90	5.93	6.00	6.10	5.82
Val	5	4.87	5.05	4.91	4.93	5.05
Met	0	**2.95**	0.00	0.00	0.00	0.00
Ile	1	0.92	0.92	0.90	0.85	0.97
Leu	6	5.88	5.92	6.02	6.10	5.80
Tyr	4	3.87	3.85	3.85	4.06	3.80
Phe	3	3.02	3.00	3.08	3.03	2.95
Lys	1	1.00	1.00	1.00	1.00	**3.93**
His	2	1.96	2.00	2.15	2.03	2.10
Arg	1	0.93	0.96	0.90	0.91	1.00

amine (10 mg) was added along with the *p*-nitrophenyl ester of a *t*-BOC-amino acid (300 μmoles). The reaction was allowed to proceed for 18 hr at room temperature after which time the insulin derivative was precipitated by the addition of ether and washed with fresh ether. The resulting product, which was ninhydrin negative, was dried thoroughly over phosphorous pentoxide under high vacuum and then dissolved in 2.0 ml of anhydrous TFA. The solution was kept for 1 hr at room temperature by which time the ninhydrin color had reached a maximum value. The insulin derivative was precipitated with ether, washed with fresh ether, and dried. The residue (~70 mg) was subjected to two isoelectric precipitations. Trimethionyl-, trialanyl-, and triasparaginyl-insulins were dissolved in 7.0 ml of 0.5 M acetic acid (containing 1.0 mg of zinc acetate) and precipitated at pH 5.9 with 5 M ammonium hydroxide, while trilysyl-insulin was precipitated at pH 7.5. In each case the final precipitate was collected by centrifugation, washed with water, acetone, ether, and dried under vacuum; yield ~65 mg, 90–95%. A control sample of insulin was put through all of the manipulations involved in this synthesis with the exception of the deletion of the acylating agent.

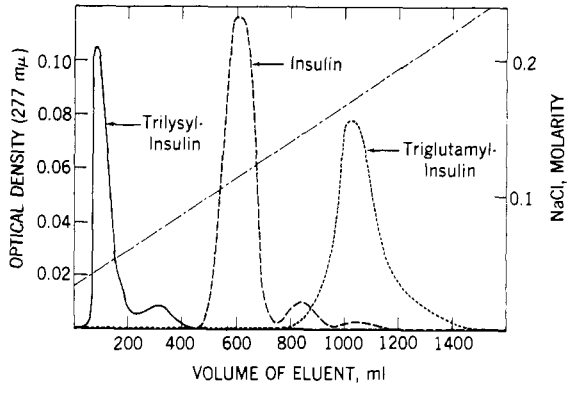

FIGURE 2: DEAE Sephadex chromatography with 0.01 M Tris buffer in 7 M urea with a 0.03–0.30 M NaCl gradient (pH 7.5) of trilysyl-insulin (———), triglutamyl-insulin (.....), and an insulin control (– – – –); sodium chloride gradient (— · —).

Triglutamyl-γ-methyl ester-insulin was prepared in the same manner as described above. To effect hydrolysis of the methyl ester, the following procedure was performed. Triglutamyl-γ-methyl ester-insulin (100 mg, 16.6 μmoles) was dissolved in 8 ml of 0.034 M carbonate buffer (pH 11.0). The solution was maintained at room temperature for 3.0 hr. The pH was then adjusted to 3.6 with 1 N HCl and the precipitate was collected by centrifugation. The precipitate was washed with water and then lyophilized. After drying over P_2O_5 under high vacuum, the product (85 mg) was treated with TFA as described above and then subjected to an isoelectric precipitation at pH 4.2 to afford the triglutamyl-insulin (Figure 2) derivative (80 mg, 13.3 μmoles, 80% yield). The methoxyl analysis value for triglutamyl-γ-methyl ester-insulin was 1.65% for a calculated value of 1.50%. After base hydrolysis, the per cent methoxyl was observed to be less than 0.1%. The amino acid analyses of the aminoacyl-insulin derivatives are shown in Table I.

The Action of Trypsin on Trimethionyl-insulin. Trimethionyl-insulin (5.3 mg, 0.88 μmole) was dissolved in 2.0 ml of pH 8.0 carbonate buffer. To the solution was added 0.22 ml of a TPCK-trypsin solution (pH 3.0, 0.001 M $CaCl_2$, 1.2 mg/ml) and the reaction was kept at 35° for 90 min. To stop the reaction the pH

TABLE II: Amino Acid Analyses of Insulin and Insulin Derivatives.

Amino Acid	Insulin	Dimethionyl-desoctapeptide-insulin	Nonapeptide	Regenerated Insulin[a]	Regenerated Insulin[b]
Asp	3	3.06		3.06	3.03
Thr	1		0.97	1.05	0.98
Ser	3	3.02		2.85	2.98
Glu	7	6.68		7.15	6.96
Pro	1		1.00	0.88	1.06
Gly	4	2.94	1.06	3.95	3.97
Ala	3	2.08	1.03	3.12	2.90
Cys	6	5.68		5.95	5.62
Val	5	4.66		4.85	4.75
Met	0	1.81	1.08		
Ile	1	0.78		0.97	0.82
Leu	6	5.74		6.04	6.00
Tyr	4	2.79	1.01	3.79	3.64
Phe	3	1.00	2.00	3.00	3.00
Lys	1		0.93	1.00	1.00
His	2	1.97		2.13	2.12
Arg	1	0.91		0.90	1.03

[a] Insulin regenerated from trialanyl-insulin. [b] Insulin regenerated from trimethionyl-insulin.

was adjusted with 4 N HCl to pH 2.0. A portion of the reaction mixture was analyzed for free amino acids by ion-exchange resin chromatography according to Spackman *et al.* (1958). Acetone was added to the remainder of the reaction mixture and the precipitated protein was purified by ion-exchange chromatography according to Carpenter and Baum (1962). The protein peak was collected and subjected to amino acid analysis. The aqueous phase after the acetone precipitation which contained the nonapeptide was taken to dryness, the residue was hydrolyzed, and subjected to amino acid analysis. The results are shown in Table II.

Edman Degradation of Trimethionyl-insulin. Trimethionyl-insulin (2.00 mg, 0.33 μmole) was dissolved in 1.2 ml of a buffer prepared by mixing 15.0 ml of pyridine, 10.0 ml of water, and 1.0 ml of dimethylaminoethanol. To this solution was added 50 μl of freshly distilled phenyl isothiocyanate and the pH of the solution was adjusted to pH 9.0 with 12% aqueous TFA. The reaction was flushed with nitrogen and kept in a glass-stoppered vessel for 1 hr at 40°. The reaction was washed several times with benzene to remove the excess phenyl isothiocyanate and then 0.5 ml of water was added to the aqueous phase and the solution was lyophilized in the presence of P_2O_5 and NaOH. The residue was washed with a small amount of ethyl acetate and the sample was dried under high vacuum for 30 min. The residue was then dissolved in 100 μl of TFA and the tube was flushed with nitrogen and the glass-stoppered tube was placed in an oil bath at 40° for 1 hr. The TFA was evaporated and the residue was extracted with ethylene chloride to obtain the thiazolinone. After evaporation of the ethylene chloride the thiazolinone was hydrolyzed in 2 ml of 0.1 N NaOH in an evacuated vessel for 12 hr at 120° (Africa and Carpenter, 1966). The hydrolysate was neutralized with 0.2 N HCl and evaporated to dryness before subjecting it to amino acid analysis. The protein residue remaining after extraction with ethylene chloride was hydrolyzed in acid and subjected to amino acid analysis. The results of the amino acid analysis on the protein hydrolysate are shown in Table II. The analysis of the hydrolyzed thiazolinone afforded one peak corresponding to methionine.

Modified Edman Degradation of Trialanyl-insulin. The trifluoroacetyl salt of trialanyl-insulin (100 mg, 16.6 μmoles) was dissolved in 40 ml of water. The pH was adjusted to 8.0 with 1 N NaOH and maintained at 40° and at pH 8.0 in a pH-Stat. Distilled phenyl isothiocyanate (100 μl, 0.84 mmole) was added to the above solution; after 3 hr, a further 100 μl of the reagent was added. The mixture was stirred under nitrogen for the duration of the reaction. After 6 hr the reaction was extracted five times with 20-ml portions of benzene and then once with 15 ml of ether. The aqueous phase was frozen and lyophilized. The resultant product was dried for 20 hr over P_2O_5 in high vacuum. The white solid was then dissolved in 10 ml of TFA, and the vessel was flushed with dry nitrogen, stoppered, and kept at 40° for 1 hr. The solution was evaporated to a small volume (~1 ml) and then extracted with ethyl acetate (three 30-ml portions). The residue, after evaporation of the TFA, was dried to yield 75 mg of an amorphous white solid. This

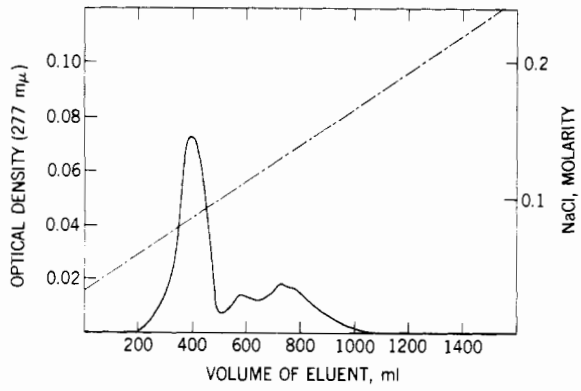

FIGURE 3: DEAE Sephadex chromatography of insulin regenerated from trialanyl-insulin (———); sodium chloride gradient (— - —). Conditions are the same as for Figure 2.

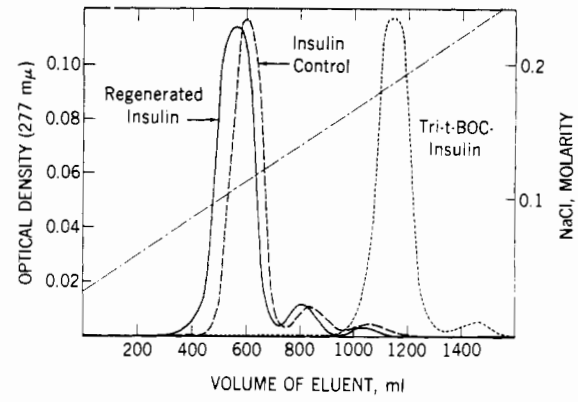

FIGURE 4: DEAE Sephadex chromatography of tri-*t*-BOC-insulin (.....), insulin control (— — —), and regenerated insulin (———); sodium chloride gradient (— - —). Conditions are the same as Figure 2.

material was dissolved in 9 ml of 0.5 M acetic acid, containing 1 mg of zinc acetate. The pH was adjusted to 5.8 with 5 M NH_4OH and the resultant precipitate was permitted to sit at 0° for 20 hr. The white precipitate was washed with water, acetone, ether, and dried to afford 68 mg of a white solid. A sample was subjected to acid hydrolysis and amino acid analysis (Table II). Biological assays are shown in Table III and chromatographic analysis in Figure 3.

Preparation of t-BOC-insulin. Insulin hydrochloride (90 mg, 15.0 μmoles) was dissolved in 12 ml of purified dimethylformamide to which was added triethylamine (20 mg, 200 μmoles) and *t*-BOC-azide (780 mg, 5.45 mmoles). The reaction was allowed to proceed for 5 hr at 40° after which time it was cooled to room temperature. The protein was precipitated with ether and after isolation by centrifugation the precipitate was washed with fresh ether and dried. The white solid was subjected to an isoelectric precipitation at pH 4.5 by dissolving the solid (90 mg) in 10 ml of 0.5 M ammonium acetate (pH 6.4) and adjusting the pH with 5.0 M acetic acid in the presence of zinc acetate (1.0 mg). The resultant white precipitate was isolated by centrifugation, washed with water, and lyophilized (78 mg, 12.8 μmoles, 85%). The ninhydrin test was negative. The biological assay result is shown in Table III and the chromatographic analysis is given in Figure 4.

Regeneration of Insulin from t-BOC-insulin. Anhydrous *t*-BOC-insulin (60 mg, 9.83 μmoles) was dissolved in anhydrous TFA (2 ml) and stirred in a glass-stoppered vessel for 2 hr at room temperature. After the reaction was cooled in an ice bath, ether was added to precipitate the protein. The precipitate was centrifuged and washed twice with fresh ether and dried. The solid product was dissolved in 7.0 ml of 0.5 M acetic acid and in the presence of zinc acetate (1.0 mg), precipitated at pH 5.8 with 5 M ammonium hydroxide. This crystalline material (50 mg, 8.62 μmoles, 88%) possessed a ninhydrin color value of the same intensity as unreacted insulin. On DEAE Sephadex chromatography it behaved in nearly the same fashion as the starting insulin (Figure 4). The biological assay result is shown in Table III.

TABLE III: Biological Assays.

Derivative	Biol Act. (units/mg)	
	Mouse Convulsion Assay	Immuno-assay
Insulin control	20.4 ± 4.5	23.1
Trimethionyl-insulin	12.3 ± 1.7	15.3
Triglutamyl-insulin	8.3 ± 1.1	8.3
Trilysyl-insulin	9.9 ± 2.6	11.5
Triasparaginyl-insulin	9.7 ± 2.4	14.4
Trialanyl-insulin	10.0 ± 0.8	14.1
Edman-degraded trialanyl-insulin	9.0 ± 1.5	16.3
Tri-*t*-BOC-insulin	5.7 ± 1.3	13.6
Regenerated insulin	15.3 ± 3.0	22.8
Insulin control	18.9 ± 3.2	24.2

Results

Synthesis of the Triaminoacyl-insulins. With the exception of the triglutamyl derivative all of the aminoacyl-insulins were synthesized by essentially the same procedure: (1) coupling of the *t*-BOC-amino acid *via* the active *p*-nitrophenyl esters with the free amino groups of insulin in dimethylformamide in the presence of triethylamine, (2) removal of the *t*-BOC group in anhydrous TFA, and (3) isolation of the product by isoelectric precipitation. In the first step a large excess of the *t*-BOC-amino acid *p*-nitrophenyl ester was used

in order to ensure complete reaction of the amino groups. The coupled product was readily separated from the excess reagent owing to their differential solubility in ether. In the second step, involving the removal of the *t*-BOC group in TFA, it was found quite essential to rigorously dry the *t*-BOC derivative before dissolving in TFA in order to obtain complete removal of the *t*-BOC groups in a reasonable amount of time. In the third step, the pH of the isoelectric precipitation was varied according to the nature of the amino acid residues which had been added to the insulin. For the alanine, asparagine, and methionine derivatives, the precipitation was performed at pH 5.9 near the normal isoelectric point of insulin. While for the lysine and glutamic acid derivatives, isoelectric precipitation was performed at pH values of 7.5 and 4.2, respectively. It should be noted that a control sample of insulin was carried through the complete procedure with the omission of the acylating agent and was recovered in crystalline form with essentially full biological activity.

An extra step was involved in the synthesis of the triglutamyl-insulin. In this synthesis, the use of *t*-BOC-glutamic acid γ-methyl ester α-*p*-nitrophenyl ester resulted in a derivative in which the γ-carboxyl groups of the added glutamic acids were present as methyl esters. The γ-methyl groups were saponified at pH 11 before removal of the *t*-BOC groups in order to avoid a possible cyclization reaction (Beecham, 1954). One should mention, however, that there is some possibility of transamidation (transpeptidation) occurring during the saponification forming a γ-glutamyl peptide (Battersby and Robinson, 1955).

Chemical Characterization. The ninhydrin reaction was used to follow the addition of the *t*-BOC-amino acids to the insulin. The fact that a negative ninhydrin reaction was given by the *t*-BOC-aminoacyl derivatives indicated that all of the three amino groups of insulin had been covered. Amino acid analysis on the aminoacyl-insulins (as well as on the *t*-BOC-aminoacyl-insulins) indicated, in each instance, the addition of three amino acid residues. This fact along with the ninhydrin results is strong evidence that the aminoacyl residues have been added to each of the three amino groups of insulin and to no other groups (imidazole, hydroxyl, or phenolic) of insulin. Two other lines of evidence are in agreement with this conclusion. When the Edman degradation was performed on trimethionyl- and trialanyl-insulins, only the expected amino acids, methionine and alanine, respectively, were released in the reaction. This indicates complete substitution of the N-terminal glycine and phenylalanine of insulin. Furthermore, the product of the Edman degradation of trimethionyl- and trialanyl-insulins had the amino acid composition of starting insulin which indicates that all three of the added residues are subject to removal by the Edman degradation. Finally, evidence for the presence of one of the added residues on the ϵ-amino of lysine 29 of B chain was obtained by trypsin degradation of the molecule. Trypsin catalyzes the hydrolysis of the arginyl bond at position 22 and the lysyl bond at position 29 of the B chain to yield free alanine, a heptapeptide (Gly-Phe-Phe-Tyr-Thr-Pro-Lys) and a large fragment (desoctapeptide-insulin) (Young and Carpenter, 1961). When trimethionyl-insulin was treated with trypsin, no free alanine was released, a nonapeptide with the composition Gly,-Phe,Phe,Tyr,Thr,Pro,Lys,Met,Ala and a large fragment with the composition of desoctapeptide-dimethionyl-insulin were produced (Table II). The failure to liberate alanine indicates that the ϵ-amino group of the lysine is covered (Anfinsen *et al.*, 1956) and this is confirmed by the isolation of the nonapeptide containing the added methionine. Thus all of the evidence indicates that the triaminoacyl-insulins contain an additional amino acid residue at each of the N-terminal positions of the A and B chains and a further residue on the ϵ-amino group of the lysine residue at position 29 of the B chain.

Chromatographic Characterization. The various insulin derivatives were subjected to ion-exchange chromatography on DEAE Sephadex in buffers containing urea according to the procedures of Bromer and Chance (1967). This system is particularly sensitive for the detection of charge differences among various insulin preparations. The dashed line in Figure 2 shows the results obtained on the insulin used as starting material in these syntheses. In addition to the major peak with an elution value of 610 ml, two additional small peaks are eluted at 850 and 1050 ml. These are undoubtedly desamido forms of insulin which bear additional negative charges and require higher salt concentrations to be eluted from the resin. The presence of such desamido forms in crystalline insulin preparations has long been recognized (Harfenist and Craig, 1952; Harfenist, 1953; Carpenter and Chrambach, 1962). As would be expected, the trilysyl-insulin which has three additional positive charges over that of native insulin is eluted at a much lower salt concentration than insulin (solid line of Figure 2) while the triglutamyl-insulin which bears three additional negative charges is eluted at a much higher salt concentration than insulin (dotted line of Figure 2). In each of these derivatives, the major portion of the material eluted as a well-defined peak, but there were also small amounts of materials eluting at higher salt concentrations, which can be attributed to the desamido form present in the original preparation. It is particularly pertinent to note that neither the trilysyl- nor triglutamyl-insulin could have been contaminated with any appreciable quantity of unreacted insulin.

On the Regeneration of Insulin from Trialanyl-insulin. A problem of current investigation in this laboratory involves the chemical degradation of the insulin molecule from the N-terminal ends. Degradation of insulin by the Edman procedure as it is used in peptide sequence work (Doolittle, 1965) has led to inactive materials.[2] This loss of activity may be due to the

[2] Unpublished results of H. O. Van Orden and F. H. Carpenter.

removal of the N-terminal residues or may be due to some unknown reaction(s) taking place at some point in the degradation procedure. It occurred to us that the triaminoacyl-insulin should serve as ideal model compounds to study this reaction. Removal of the three amino acid residues should regenerate the original insulin structure. If a triaminoacyl-insulin could be subjected to a degradation which resulted in the formation of a crystalline insulin of full biological activity, not only would the degradation procedure be validated, but also it would give additional information on the nature of the changes involved in the original conversion of insulin to the triaminoacyl derivatives. With this goal in mind, trialanyl-insulin was subjected to a modified Edman degradation devised by Mr. Bruce Africa of this laboratory. The chief modification in this degradation is to perform the reaction with phenyl isothiocyanate in aqueous solution in a pH-Stat instead of in pyridine buffers. The product obtained by the application of this procedure to trialanyl-insulin had the correct amino acid composition of insulin (Table II, column 5) but it was not obtained in crystalline form, it possessed only about 50% of the activity of native insulin and its chromatographic behavior in DEAE Sephadex (Figure 3) showed it to be quite heterogeneous with the principal component moving somewhat faster than the starting insulin. The interpretation of these results is somewhat equivocal: either approximately 50% of the molecules underwent some irreversible change in the synthesis of the trialanyl-insulin or in its degradation. The latter appears more probable but the former cannot be completely eliminated.

t-BOC-insulin. The reaction of *t*-BOC-azide with insulin in dimethylformamide was followed by the ninhydrin reaction. The fact that the *t*-BOC-insulin was ninhydrin negative indicates that the three primary amino groups of insulin were covered in the reaction. In analogy with the formation of the tri-*t*-BOC-aminoacyl derivatives, it is presumed that only three *t*-BOC groups were introduced. Although we have no direct evidence to indicate that substitution did not take place on other, albeit neutral, groups in the molecule, experience with *t*-BOC-azide in peptide synthesis would appear to make such reactions unlikely (Schröder and Lübke, 1965). The *t*-BOC-insulin formed in this reaction behaved as expected on DEAE Sephadex chromatography (Figure 4, dotted line) in which the majority of the material was eluted at a salt concentration equivalent to that for the emergence of triglutamyl-insulin. This is in agreement with blocking of three amino groups to yield a compound possessing a higher negative charge at pH 7.5 than insulin. The *t*-BOC-insulin was unique among the derivatives reported here in that there was quite poor agreement between the biological assays performed by the mouse convulsion test (5.7 ± 1.3 units/mg) and the immunoassay (13.6 units/mg) (Table III). Upon treating the *t*-BOC-insulin with anhydrous TFA, a product was regenerated which possessed three free amino groups (as judged by ninhydrin assay), the correct amino acid composition of insulin, the chromatographic behavior of insulin (Figure 4, solid line), the ability to crystallize, and a biological activity equivalent to that of an insulin control and nearly as high as native insulin (Table III). These results indicate that the *t*-BOC group can be used as a reversible blocking group for the amino groups of insulin.

Biological Activity. The results of the bioassays (Table III) show that all of the triaminoacyl-insulins possessed about the same activity. In the case of the mouse convulsion assay, the activities were about 40–50% of the activity usually associated with crystalline zinc-insulin (25 units/mg). The immunoassay gave slightly higher results. On DEAE chromatography, each of these compounds appeared to be as homogeneous as the starting insulin. Because of the marked difference in chromatographic behavior between the trilysyl- and triglutamyl-insulins and insulin, one can report with some assurance that at least in the case of these derivatives, their biological activity cannot be attributed to contamination with unreacted insulin. It should be noted that the biological assays were performed on material before it was subjected to chromatography in the 7 M urea system. Therefore, the decreased activity of these derivatives cannot be attributed to irreversible denaturation by urea. The chemical and chromatographic tests largely eliminate the possibility that some unusual rearrangement is taking place which inactivates about half of the molecules and it therefore appears reasonable to conclude that the triaminoacyl-insulins represent essentially homogeneous products possessing approximately half of the biological activity of native insulin.

Discussion

In contrast to earlier procedures for the addition of amino acid residues to insulin which made use of the Leuchs' anhydrides and which gave products with variable substitution of the amino groups (Fraenkel-Conrat, 1953; Virupaksha and Tarver, 1964), the procedure presented in this paper yields a product in which one amino acid residue has been added to each of the amino groups of insulin. The effect of such additions on the properties of the molecule is much more amenable to interpretation in the case of the present synthesis than in the synthesis involving the Leuchs' anhydrides.

The derivatives reported here involve amino acid residues bearing side chains of varying charge, size, and hydrophilic–hydrophobic properties. In three of the derivatives, neutral amino acid residues were added so that the total charge on the resulting molecule was the same as that of native insulin. However, among the three there was considerable difference in the size of the side chain (alanine *vs.* methionine) and in their hydrophilic properties (asparagine *vs.* methionine). In two derivatives, triglutamyl-insulin and *t*-BOC-insulin, the product contained three additional negative charges over that of insulin. In another derivative, trilysyl-insulin, just the opposite effect was obtained

in that three additional positive charges were added to the molecule. In view of this wide diversity of properties of the substituent groups, it is rather surprising to find that all of the derivatives had approximately the same biological activity, which was about 50% that of native insulin.

It seems clear that the biological activity of the triaminoacyl-insulin must be a property of the preparation and not due to a 50% contamination with unreacted insulin. It is possible that the reduced activity of these compounds is due to some undetected rearrangement of the molecules not specifically associated with the substitution of the amino groups. The fact that a control sample, which was subjected to all of the procedures with the exception of the acylation reagent, was recovered unchanged and with full biological activity appears to rule out a solvent-mediated rearrangement. If the modified Edman degradation of trialanyl-insulin had yielded material with the complete properties of native insulin, the decreased biological activity of the triaminoacyl-insulin could have been attributed unambiguously to the substitution of the amino groups. The fact that this degradation yielded a rather heterogeneous material with about 50% of the activity of native insulin prevents a rigorous interpretation. However, the failure to regain fully active material is probably attributable to an unknown reaction in the Edman degradation rather than in the original synthesis.

In the case of the *t*-BOC-insulin which had a biological activity of 5.7 ± 1.3 units/mg in the mouse convulsion assay, it was possible to regenerate a crystalline compound with almost full activity upon removal of the *t*-BOC groups. So in this case the decreased activity of *t*-BOC-insulin must be associated with the addition of the *t*-BOC groups. Fraenkel-Conrat and Fraenkel-Conrat (1950) have reported that acetylation of the amino groups in insulin results in only a 25% decrease in biological activity, whereas Mills (1953) found that addition of three dinitrotoluene groups resulted in complete loss of activity. The bioassays indicate that the *t*-BOC group lies in between these two extremes. It should be noted that the acetyl group might be removed in the body, whereas this would be unlikely for the *t*-BOC or dinitrotoluene groups.

The decreased activity observed in these derivatives may be attributed to any one of a number of causes. A free amino group may be needed for proper binding of the hormone to its receptor site. When the amino groups are blocked or their relative position changed by the addition of new amino acids, the effectiveness in the biosystem may be reduced. On the other hand, the loss of activity may be caused by steric hindrance either by direct interaction with the site of action in the biosystem or by effecting a conformational change in the molecule which results in a change in the efficiency by which the insulin is transported or by which it interacts with the receptor sites. Current investigations are involved with examining the chemical and physical properties of these derivatives to see how they compare with native insulin.

In the compounds reported here the two peptide chains of insulin have been extended by the addition of one amino acid residue on each amino-terminal end. In addition, the compounds also contain an amino acid residue attached to the ϵ-amino group of lysine at position 29 of the B chain. It would be desirable to determine whether the decreased activity of these derivatives was a result of extension of the peptide chains or due to substitution of the ϵ-amino group of lysine. In view of the fact that several naturally occurring species of insulin have an additional amino acid residue at the amino terminus of the B chain (Smith, 1966), it is tempting to attribute the decrease in activity observed in these derivatives to substitution of the ϵ-amino group of lysine. Future investigation will be directed to this point.

It should be noted that in contrast to most proteins, insulin possesses remarkable stability in acid, in mild alkali, and in most organic solvents. It is these properties which make it possible to expose it to the conditions encountered in the chemical synthesis of peptides as exemplified in the syntheses of the triaminoacyl derivatives. The formation of *t*-BOC-insulin by the reaction of *t*-BOC-azide with insulin in dimethylformamide is another example of the application of a reagent in frequent use in peptide synthesis. In this instance the amino groups of insulin are blocked with a group which subsequently can be removed without undue damage to the molecule. The availability of this reversible blocking agent for the amino groups of insulin will make it possible to perform specific reactions on other parts of the molecule, such as addition of amino acid residues to the carboxyl end of the molecule, without fear of reacting with the amino groups of insulin.

In recent years at least four different groups have reported on the total synthesis of insulin (Meienhofer *et al.*, 1963; Katsoyannis *et al.*, 1964; Kung *et al.*, 1965; Marglin and Merrifield, 1966). The availability of these synthetic procedures should greatly abet the determination of the relationship of structure to biological activity of insulin. However, in all of these syntheses, the final step involves the condensation of the two chains through the formation of disulfide bonds. This final step is not unambiguous in that correct pairing of the disulfide bonds is left to chance. When the two natural chains are brought together under the proper conditions, a portion of the molecules, which is greater than would be predicted on statistical basis, condenses to give the correct pairing of disulfide bonds. Evidently information is contained in the amino acid sequence of the two chains which partially directs this synthesis. If one attempts to determine the relationship between structure and biological activity by modifying the amino acid residues in the two synthetic chains, one faces an ambiguity when the two chains are condensed. Any changes in the biological activity of the final product may have resulted from improper matching of the chains rather than to the modification of a particular amino acid residue located in a correctly paired disulfide-bond structure. Until such time as an

unambiguous method of connecting the two chains is devised, it would appear that modifications of the insulin molecule, such as those presented here, which start with the intact structure are likely to yield the most interpretable data.

Acknowledgments

We wish to thank Dr. E. L. Grinnan of Eli Lilly and Co. for arranging for the bioassays and Dr. R. E. Chance for transmitting the details of the ion-exchange chromatography of insulin on DEAE Sephadex prior to publication. We wish to acknowledge the technical aid of Miss Kathryn A. Tinker and the performance of the amino acid analysis by Mrs. Joan Hafeez and Miss Annalisa Layton.

References

Africa, B., and Carpenter, F. H. (1966), *Biochem. Biophys. Res. Commun. 24*, 113.

Anfinsen, C. B., Sela, M., and Tritch, H. (1956), *Arch. Biochem. Biophys. 65*, 156.

Battersby, A. R., and Robinson, J. C. (1955), *J. Chem. Soc.*, 259.

Beecham, A. F. (1954), *J. Am. Chem. Soc. 76*, 4615.

Bodánszky, M. (1955), *Nature 175*, 685.

Bromer, W. W., and Chance, R. E. (1967), *Biochim. Biophys. Acta 133*, 219.

Carpenter, F. H. (1958), *Arch. Biochem. Biophys. 78*, 539.

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

Carpenter, F. H., and Baum, W. E. (1962), *J. Biol. Chem. 237*, 409.

Carpenter, F. H., and Chrambach, A. (1962), *J. Biol. Chem. 237*, 404.

Carpino, L. A., Giza, C. A., and Carpino, B. A. (1959), *J. Am. Chem. Soc. 81*, 955.

Doolittle, R. F. (1965), *Biochem. J. 94*, 742.

Fraenkel-Conrat, H. (1953), *Biochim. Biophys. Acta 10*, 180.

Fraenkel-Conrat, J., and Fraenkel-Conrat, H. (1950), *Biochim. Biophys. Acta 5*, 89.

Goldberger, R. F., and Anfinsen, C. B. (1962), *Biochemistry 1*, 401.

Hanby, W. E., and Waley, S. G. (1950), *J. Chem. Soc.*, 3239.

Harfenist, E. J. (1953), *J. Am. Chem. Soc. 75*, 5528.

Harfenist, E. J., and Craig, L. C. (1952), *J. Am. Chem. Soc. 74*, 3083.

Hofmann, K., Schmiechen, R., Wells, R. D., Wolman, Y., and Yanaihara, N. (1965), *J. Am. Chem. Soc. 87*, 611.

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

Kostka, V., and Carpenter, F. H. (1964), *J. Biol. Chem. 239*, 1799.

Kung, Y.-T., Du, Y.-C., Huang, W.-T., Chen, C.-C., Ke, L.-T., Hu, S.-C., Jiang, R.-Q., Chu, S.-Q., Niu, C.-I., Hsu, J.-Z., Chang, W.-C., Cheng, L.-L., Li, H.-S., Wang, Y., Loh, T.-P., Chi, A.-H., Li, C.-H., Shi, P.-T., Yieh, Y.-H., Tang, K.-L., and Hsing, C.-Y. (1965), *Sci. Sinica (Peking) 14*, 1710.

Levy, D., and Carpenter, F. H. (1966), *J. Am. Chem. Soc. 88*, 3676.

Ludwig, M. L., and Byrne, R. (1962), *J. Am. Chem. Soc. 84*, 4160.

Marglin, A., and Merrifield, R. B. (1966), *J. Am. Chem. Soc. 88*, 5051.

Marzotto, A., Pajetta, P., and Scoffone, E. (1967), *Biochem. Biophys. Res. Commun. 26*, 517.

Meienhofer, J., Schnabel, E., Bremer, H., Brinkhoff, O., Zabel, R., Stroka, W., Klostermeyer, H., Brandenburg, D., Okuda, T., and Zahn, H. (1963), *Z. Naturforsch.18b*, 1120.

Merigan, T. C., Dreyer, W. J., and Berger, A. (1962), *Biochim. Biophys. Acta 62*, 122.

Mills, G. L. (1953), *Biochem. J. 53*, 37.

Schoellman, G., and Shaw, E. (1963), *Biochemistry 2*, 252.

Schröder, E., and Klieger, E. (1964), *Ann. Chim. Liebigs 673*, 208.

Schröder, E., and Lübke, K. (1965), The Peptides, Vol. I, New York, N. Y., Academic, p 37.

Schwyzer, R., and Keppler, H. (1961), *Helv. Chim. Acta 44*, 1991.

Scoffone, E., Marchiori, A. M., Tamburro, A. M., and Rocchi, R. (1964), *Gazz. Chim. Ital. 94*, 695.

Smith, L. F. (1966), *Am. J. Med. 40*, 662.

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

Tesser, G. I., and Nivard, R. J. F. (1964), *Rec. Trav. Chim. 84*, 53.

Virupaksha, T. K., and Tarver, H. (1964), *Biochemistry 3*, 1507.

Wang, S. S., and Carpenter, F. H. (1965), *J. Biol. Chem. 240*, 1619.

Young, J. D., and Carpenter, F. H. (1961), *J. Biol. Chem. 236*, 743.