SYNTHETIC INSULIN ANALOGUES

by

Clyde Zalut

A dissertation submitted to the Graduate Faculty in Biomedical Sciences in partial fulfillment of the requirements for the degree of Doctor of Philosophy, Mount Sinai Medical School of the City University of New York.

1971

This manuscript has been read and accepted for the Graduate Faculty in Biochemistry in satisfaction for the dissertation requirement for the degree of Doctor of Philosophy.

6/23/71
date

P G Katsoyannis
Chairman of Examining Committee

6/23/71
date

Executive Officer

P G Katsoyannis

P. G. Katsoyannis

A. C. Trakatellis

S. B. Koritz

Supervisory Committee

The City University of New York

FORWARD

The author would like to thank Miss Scheibe, Mrs. Liu and Miss Klimaski for technical assistance and constructive criticism.

Dr. (Laban) Katsoyannis played a very significant role, which I am still assessing.

To my wife, as usual, my silent gratitude.

TABLE OF CONTENTS

Page

FOREWORD.....iii

LIST OF TABLES.....vi

LIST OF CHARTS AND FIGURES.....vii

I. INTRODUCTION

A. Purpose of Investigation.....1

B. Approach to the Problem.....3

1. Nomenclature.....3

2. Materials and Methods.....3

C. Synthesis of the A Chain Analogues.....5

1. Deamino Sheep A Chain S-Sulfonate.....5

2. Destetrapeptide A^{1-4} Human and Sheep A Chain S-Sulfonates.....7

D. Synthesis of the B Chain Analogue.....9

1. Synthesis of the Destripeptide B^{28-30} Bovine B Chain S-Sulfonate.....9

E. Synthesis, Isolation and Characterization of the Insulin Analogues.....13

1. General Procedure.....13

2. Characterization of the Insulin Analogues.....16

II. EXPERIMENTAL

A. Synthesis of the Deamino Sheep A Chain S-Sulfonate.....28

1. N-Acetyl-L-isoleucyl-L-valyl-L-glutamic Acid-α-Ethyl-γ-t-Butyl Ester.....28

Page

2. N-Acetyl-L-isoleucyl-L-valyl-γ-t-butyl-L-glutamic Acid Hydrazide.....29

3. N-Acetyl-L-isoleucyl-L-valyl-L-glutamyl-L-glutaminyl-S-sulfo-L-cysteinyl-S-sulfo-L-cysteinyl-L-alanylglycyl-L-valyl-S-sulfo-L-cysteinyl-L-seryl-L-leucyl-L-tyrosyl-L-glutaminyl-L-leucyl-L-glutamyl-L-asparaginyl-L-tyrosyl-S-sulfo-L-cysteinyl-L-asparagine.....29

B. Synthesis of the Destetrapeptide A^{1-4} Sheep A Chain S-Sulfonate.....31

C. Synthesis of the Destetrapeptide A^{1-4} Human A Chain S-Sulfonate.....33

D. Synthesis of the Destripeptide B^{28-30} Bovine B Chain S-Sulfonate.....33

E. Synthesis, Isolation and Characterization of the Insulin Analogues.....36

1. Deamino A^{1} Sheep Insulin.....36

2. Destetrapeptide A^{1-4} Sheep and Porcine Insulins.....39

3. Destripeptide B^{28-30} Bovine and Human Insulins.....42

III. RESULTS AND DISCUSSION.....45

IV. SUMMARY.....51

ABBREVIATIONS.....52

BIBLIOGRAPHY.....55

LIST OF TABLES

The following tables give the amino acid composition of the designated compound.

Page

I. Deamino Sheep A Chain S-Sulfonate.....8

II. Destetrapeptide A^{1-4} Sheep A Chain S-Sulfonate.....11

III. Destetrapeptide A^{1-4} Human A Chain S-Sulfonate.....12

IV. Destripeptide B^{28-30} Bovine B Chain S-Sulfonate.....14

V. Deamino A^{1} Sheep Insulin.....19

VI. Destetrapeptide A^{1-4} Sheep Insulin.....20

VII. Destetrapeptide A^{1-4} Porcine Insulin.....21

VIII. Destripeptide B^{28-30} Bovine Insulin.....22

IX. Destripeptide B^{28-30} Human Insulin.....23

LIST OF FIGURES AND CHARTS

Page

FIGURES:

1. Structure of sheep insulin.....2

2. CM-cellulose chromatography (4.5 x 50 cm column) in 8M urea of: (I) Destripeptide B^{28-30} bovine B chain S-sulfonate (30 mg); (II) B-SSO_3^- (50 mg).....15

3. Thin layer electrophoresis of: a) Natural bovine insulin; b) Deamino A^1 sheep insulin; 0.5 N acetic acid, 3500 v, 14 min.....24

4. Thin layer electrophoresis of: a) Natural bovine insulin; b) Deamino A^1 sheep insulin 0.01 N NH_4HCO_3, pH 10.0, 3500 v, 14 min.....25

5. Thin layer electrophoresis of: a) Natural bovine insulin; b) Destetrapeptide A^{1-4} porcine insulin; c) destetrapeptide A^{1-4} sheep insulin 0.5 N acetic acid, 3500 v, 13 min.....26

6. Thin layer electrophoresis of: a) destripeptide B^{28-30} bovine insulin; b) natural bovine insulin; 0.5 N acetic acid, 3400 v, 15 min.....27

7. CM-cellulose chromatography (0.9 x 23 cm column), with an exponential NaCl gradient, of two combination mixtures containing the deamino A^1 sheep insulin.....38

8. CM-cellulose chromatography (0.9 x 23 cm column), with an exponential NaCl gradient, of two combination mixtures containing the destetrapeptide A1-4 sheep insulin.....40

9. CM-cellulose chromatography (0.9 x 23 cm column), with an exponential NaCl gradient, of two combination mixtures containing the destetrapeptide A^{1-4} porcine insulin.....41

10. CM-cellulose chromatography (0.9 x 23 cm column), with an exponential NaCl gradient, of a combination mixture containing the destripeptide B^{28-30} bovine insulin (assayed 75 IU).....43

11. CM-cellulose chromatography (0.9 x 23 cm column), with an exponential NaCl gradient, of a combination mixture containing the destripeptide B^{28-30} human insulin (assayed 90 IU).....44

CHARTS:

I. Synthetic scheme for the preparation of deamino sheep A chain S-sulfonate.....6

II. Synthetic scheme for the preparation of destetrapeptide A^{1-4} sheep and porcine A chain S-sulfonates.....10

III. Cleavage and resynthesis of insulin: sulfitolysis of insulin produces A-SSO_3^- and B-SSO_3^- (1); A-SSO_3^- is converted to the sulfhydryl form with mercaptoethanol (2); the sulfhydryl A chain reacts with the B-SSO_3^- to produce insulin.....17

I. INTRODUCTION

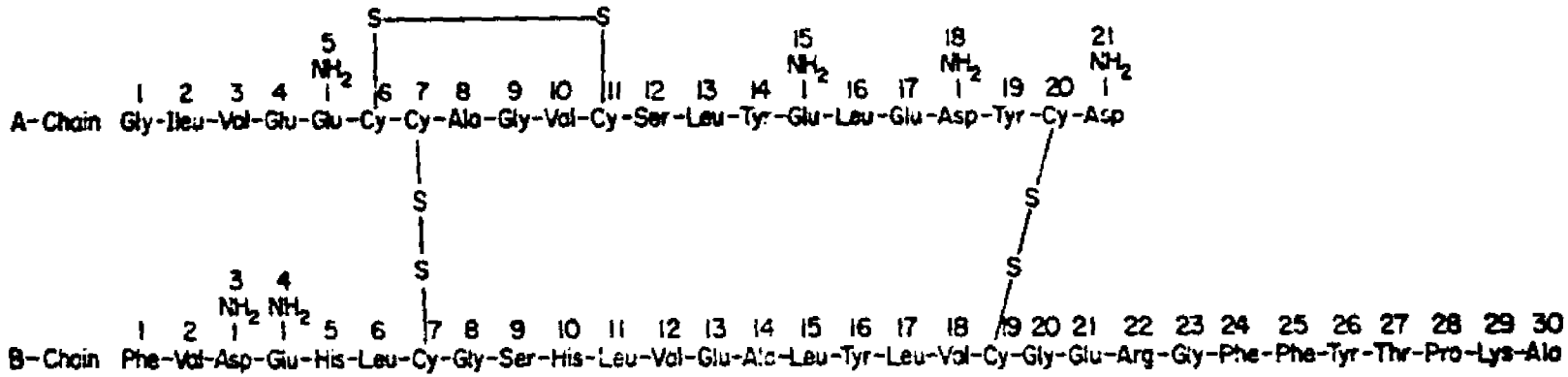
A. Purpose of the Investigation

The significance of insulin in the treatment of diabetes and its primary role in protein studies are well documented (1,2,3). With the structure of insulin known (4, 5,6), Figure 1, and the chemical synthesis well worked out (3,7), the goal remains to determine the relationship between insulin's structure and its physiological function. By making various synthetic insulin analogues and then studying their biological activity it is hoped that a pattern will evolve elucidating this relationship. The possibility exists that insulin analogues may be produced with more desirable properties (e.g. higher specific acitivity, longer acting, etc.), than the natural insulin.

Therefore, the present investigation has the following purposes:

1. To chemically synthesize different insulin analogues.
2. To evaluate the latter's biological activity and
3. To draw preliminary conclusions regarding the structure-activity relationship of insulin.

Figure 1



The Structure of Sheep Insulin

B. Approach to the Problem

1. Nomenclature

The nomenclature used in this work is based essentially on the rules given in E. Schröder and K. Lübke (8). The amino acid sequence of the sheep insulin molecule has been numbered, starting at the N-terminus as shown in Figure 1. In the discussion which follows, these numbers will be used when reference is made to a particular sequence of amino acids in the insulin molecule. Thus, A^{1-21} means a peptide having the amino acid sequence of the A chain of sheep insulin. Similarly, B^{1-9} represents a peptide derivative containing the first nine amino acids from the N-terminus of the B chain.

2. Materials and Methods

In this investigation only the L-isomers of commercially available amino acids were used. All melting points were determined with a Capillary Melting Point Apparatus and are uncorrected. For paper chromatography the protected peptides were deblocked on exposure to 2N HBr in acetic acid. Serine containing peptides were deblocked by treatment with HBr in TFA. For paper chromatography Whatman No. 1 paper was used: R_f^1 values refer to the Partridge (9) system; R_f^2 values refer to the system (10) 1-butanol-pyridine-acetic acid-water (30:20:6:24) and are expressed as a multiple of the distance traveled by a histidine marker.

The chromatograms were developed with ninhydrin spray or by developing the Pauly (11) color reaction depending on the nature of the peptides. Thin layer electrophoresis as described by Tometsko and Delihas (12) was performed with a Wieland-Pfleiderer pherograph (Brinkman Instruments). Preswollen microgranular CM-cellulose (Whatman CM 52/1) as well as G-15 and G-50 Sephadex (Pharmacia Uppsala) were used in this investigation. Washing of these resins and preparation of the corresponding columns are described in detail by Katsoyannis et al. (13,14). Monitoring of column effluents was done with a Gilford recording spectrophotometer at 278 mμ.

Optical rotations were taken with a Zeiss photoelectric precision polarimeter. The elemental analyses were performed by a commercial laboratory. The amino acid analyses of acid hydrolysates were carried out by the method of Spackman, Stein and Moore (15) with a Beckman-Spinco amino acid analyzer, Model 120C . Enzymatic analyses were carried out with LAP according to the method of Hill and Smith (16) using a crystalline enzyme (Worthington), and with APM (Henley and Co.) according to the method of Pfleiderer et al. (17). For taking infrared spectra 1 mg of sample was thoroughly mixed with 250 mg of KBr and converted to a pellet under 18,000 pounds of total load pressure with a Carver laboratory press, Model B. The infrared spectrum was taken with a Perkin-Elmer 457.

The S-sulfonated A and B chains (A-SSO_3^- and B-SSO_3^-) of bovine or porcine insulin were obtained by sodium sulfite, sodium tetrathionate sulfitolysis of insulin as outlined in Katsoyannis et al. (18).

All insulins synthesized throughout this investigation were made by interaction of the sulfhydryl form of the specific A chain with B-SSO_3^- as described by Katsoyannis and Tometsko (19). Recovery of the insulin analogues from the combination mixtures followed exactly the procedures outlined by Katsoyannis et al. (14,20). Biological assays were carried out by the mouse convulsion method (19).

Protein determination was performed by the method of Lowry et al. (21).

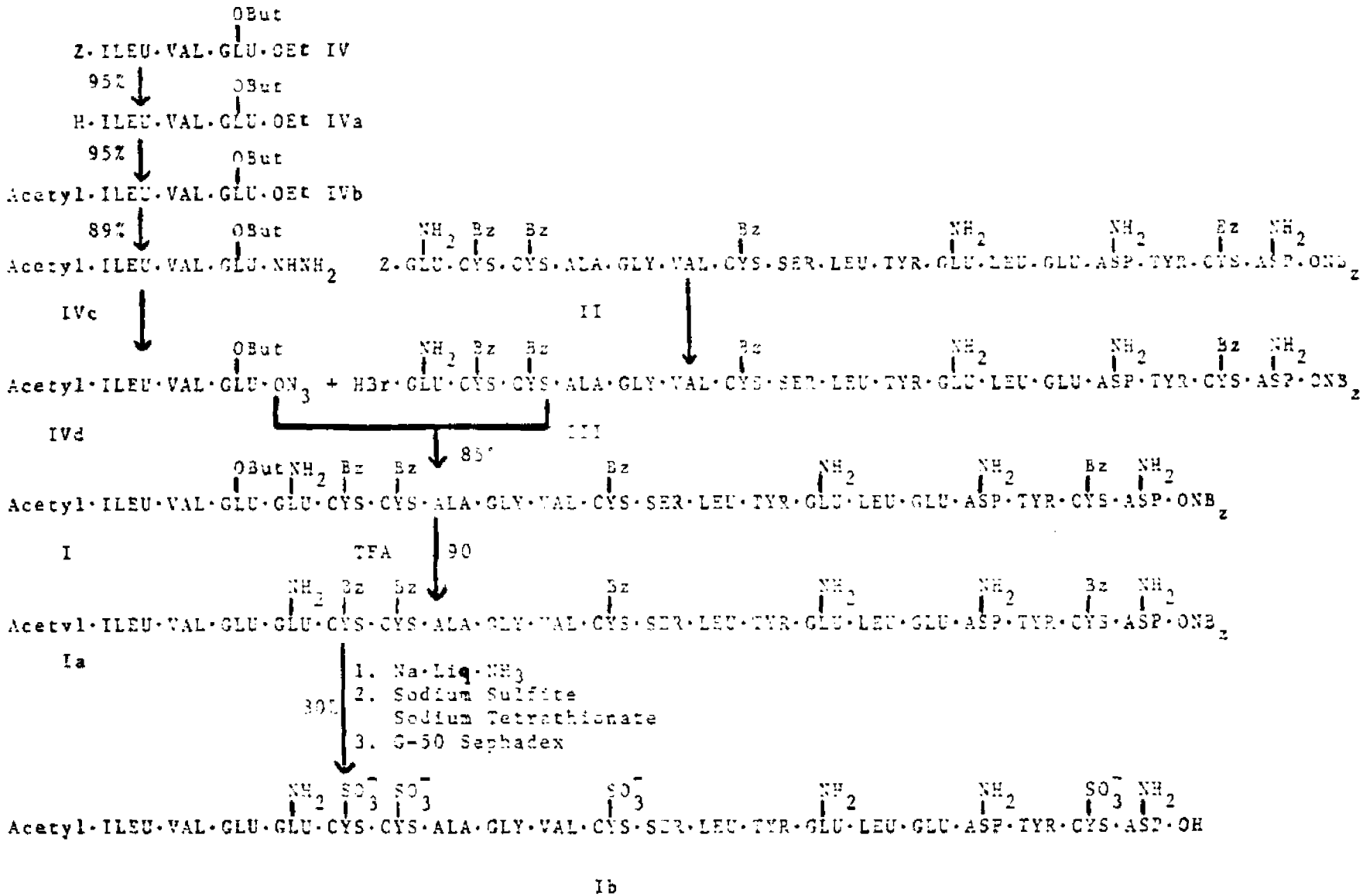
Bovine and porcine insulins were kindly and generously supplied by the Eli Lilly Company.

C. Synthesis of the A Chain Analogues

1. Deamino Sheep A Chain S-Sulfonate (Ib)

The protected sheep A chain lacking the N-terminal amino group (I) (Chart I) was made by decarbobenzoxylating the known heptadecapeptide (13), N-carbobenzoxy-L-glutaminyl-S-benzyl-L-cysteinyl-S-benzyl-L-cysteinyl-L-alanyl-glycyl-L-valyl-S-benzyl-L-cysteinyl-L-seryl-L-leucyl-L-tyrosyl-L-glutaminyl-L-leucyl-L-glutamyl-L-asparaginyl-L-tyrosyl-S-benzyl-L-cysteinyl-L-asparagine p-nitrobenzyl

CHART I


Z·ILEU·VAL·GLU·OEt IV
95%
H·ILEU·VAL·GLU·OEt IVa
95%
Acetyl·ILEU·VAL·GLU·OEt IVb
89%
Acetyl·ILEU·VAL·GLU·NHNH2
IVc
Z·GLU·CYS·CYS·ALA·GLY·VAL·CYS·SER·LEU·TYR·GLU·LEU·GLU·ASP·TYR·CYS·ASP·ONBz
II
Acetyl·ILEU·VAL·GLU·ON3 + HBr·GLU·CYS·CYS·ALA·GLY·VAL·CYS·SER·LEU·TYR·GLU·LEU·GLU·ASP·TYR·CYS·ASP·ONBz
IVd
III
85°
Acetyl·ILEU·VAL·GLU·GLU·CYS·CYS·ALA·GLY·VAL·CYS·SER·LEU·TYR·GLU·LEU·GLU·ASP·TYR·CYS·ASP·ONBz
I
TFA
90
Acetyl·ILEU·VAL·GLU·GLU·CYS·CYS·ALA·GLY·VAL·CYS·SER·LEU·TYR·GLU·LEU·GLU·ASP·TYR·CYS·ASP·ONBz
Ia
30%
1. Na·Liq·NH3
2. Sodium Sulfite
Sodium Tetrathionate
3. G-50 Sephadex
Acetyl·ILEU·VAL·GLU·GLU·CYS·CYS·ALA·GLY·VAL·CYS·SER·LEU·TYR·GLU·LEU·GLU·ASP·TYR·CYS·ASP·OH
Ib

ester (II) with HBr in TFA, and coupling the ensuing product III with an excess of the azide tripeptide N-acetyl-L-isoleucyl-L-valyl-γ-t-butyl-L-glutamic acid azide (IVd). The latter azide (IVd) was made from a series of reactions starting with the tripeptide (22), N-carbobenzoxy-L-isoleucyl-L-valyl-L-glutamic acid-α-ethyl-γ-t-butyl ester (IV), as shown in Chart I. The formation of N-acetyl-L-isoleucyl-L-valyl-L-glutamic acid-α-ethyl-γ-t-butyl ester (IVb) by decarbobenzoxylating IV via hydrogenation, followed by treatment with acetic anhydride, was carried out under conditions known to give the optically pure product (23). The desired product Ib was obtained from I according to the usual procedures, i.e. deprotection by TFA and sodium in anhydrous liquid ammonia (24), followed by sulfitolysis with sodium sulfite and sodium tetrathionate (13). The over-all reaction sequence and the yield at each synthetic step are shown in Chart I. Amino acid analysis of an acid hydrolysate of the synthetic material Ib gave a composition in molar ratios in excellent agreement with the theoretically expected values, Table I. Thin layer electrophoresis at two pH values gave a single Pauly-positive spot, indicative of the presence of a single component.

2. Destetrapeptide A^{1-4} Sheep and Porcine (Human) A Chain S-Sulfonates (IIa and Va, respectively)

TABLE I: Amino Acid Composition[a] of the Deamino Sheep A Chain S-Sulfonate

Amino Acid	Theory	Found
Aspartic acid	2	2.0
Serine	1	0.9
Glutamic acid	4	4.0
Glycine	1	1.0
Alanine	1	1.0
Valine	2	1.7
Isoleucine	1	0.7
Leucine	2	2.0
Tyrosine	2	1.8
Cysteine	4	b

a. Number of amino acid residues per molecule.

b. Not determined.

IIa and Va were obtained from the known protected heptadecapeptides II and V, respectively (13,25) by sodium in anhydrous liquid ammonia treatment followed by sulfitolysis and purification on G-50 Sephadex as shown in Chart II. Amino acid analyses of acid hydrolysates and of LAP digests of the synthetic materials IIa and Va gave a composition in molar ratios in excellent agreement with the theoretically expected values, Tables II and III, respectively.

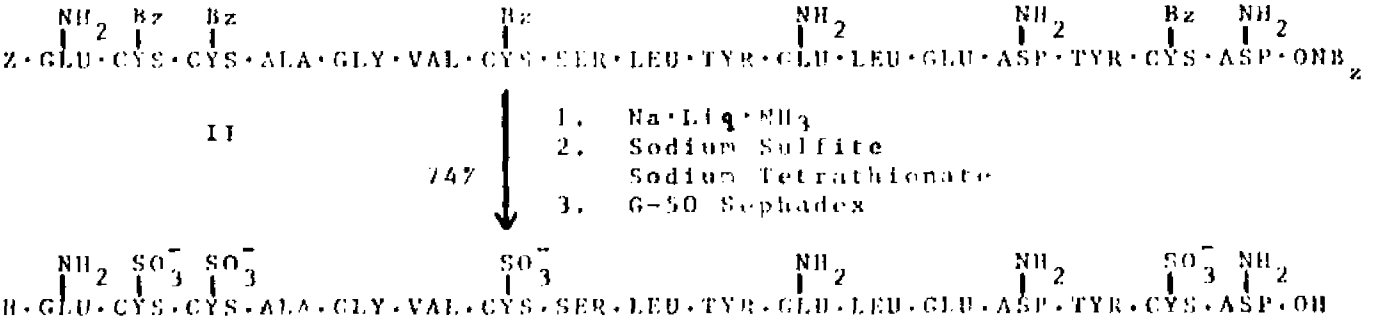
Thin layer electrophoresis at two pH values gave a single Pauly-positive spot for both IIa and Va, indicative of the presence of a single component.

D. Synthesis of the B Chain Analogue

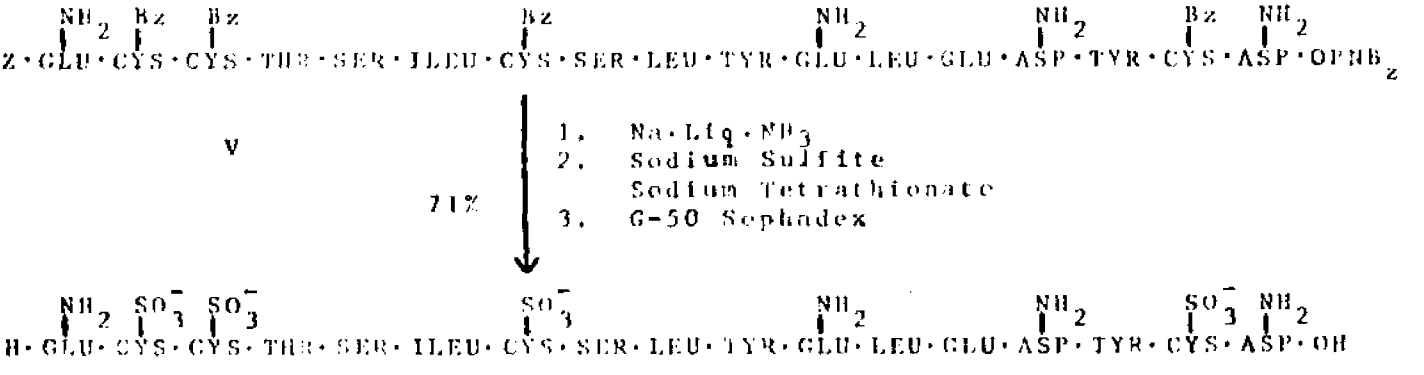
1. Synthesis of the Destripeptide B^{28-30} Bovine B Chain S-Sulfonate

The destripeptide B^{28-30} chain was prepared by reduction of beef insulin with sodium in liquid ammonia, followed by oxidative sulfitolysis with sodium sulfite and sodium tetrathionate. The $B\text{-}SSO_3^-$ derivative was isolated from the reaction mixture by continuous flow electrophoresis as described by Katsoyannis et al. (18). Further purification was obtained by column chromatography in 8M urea on CM-cellulose. Amino acid analysis of the destripeptide B^{28-30} chain after acid hydrolysis gave a composition expressed in

CHART II



IIa



Va

TABLE II: Amino Acid Composition[a] of the Destetrapeptide A^{1-4} Sheep A Chain S-Sulfonate

Amino Acid	Acid Hydrolysis		Enzymatic Hydrolysis (LAP)	
	Theory	Found	Theory	Found
Aspartic acid	2	1.9		
Glutamine			2 }	Emerge at the same
Asparagine			2 }	position; not determined
Serine	1	0.9	1	1.0[b]
Glutamic acid	3	3.1	1	1.1
Glycine	1	1.0	1	1.0
Alanine	1	1.0	1	1.0
Valine	1	1.0	1	1.0
Leucine	2	2.0	2	2.1
Tyrosine	2	1.9	2	2.0
Cysteine	4	d		
S-Sulfocysteine			4	4.0[c]

a. Number of amino acid residues per molecule.

b. Separated from glutamine and asparagine in a 30° chromatographic run.

c. Eluted from the long column of the Beckman-Spinco analyzer after 26 ml of effluent.

d. Not determined.

TABLE III: Amino Acid Composition[a] of the Destetrapeptide A^{1-4} Human A Chain S-Sulfonate

Amino Acid	Acid Hydrolysis		Enzymatic Hydrolysis (LAP)	
	Theory	Found	Theory	Found
Aspartic acid	2	1.9		
Glutamine			2 }	Emerge at the same
Asparagine			2 }	position; not determined
Serine	2	1.8	2	1.9[b]
Threonine	1	0.9	1	1.0
Glutamic Acid	3	3.0	1	1.0
Isoleucine	1	1.0	1	1.0
Leucine	2	2.0	2	2.0
Tyrosine	2	1.9	2	2.0
Cysteine	4	d		
S-Sulfocysteine			4	4.2[c]

a. Number of amino acid residues per molecule.

b. Separated from glutamine and asparagine in a 30° chromatographic run.

c. Eluted from the long column of the Beckman-Spinco analyzer after 26 ml effluent.

d. Not determined.

molar ratios in good agreement to the theoretically expected values for the natural B-SSO_3^- except that one residue each of thr, pro, lys and ala was missing, Table IV. Previous studies (26,27,28) using reducing conditions of sodium in anhydrous liquid ammonia, as used above, have shown that the tripeptide pro-lys-ala is cleaved from the C-terminal end of the B chain, and at the same time the adjacent threonine is chemically altered so that it does not show up in the amino acid analysis.

A single Pauly-positive spot was obtained with the destripeptide B^{28-30} chain on thin layer electrophoresis at pH 10, indicative of a single component. A comparison of this B-SSO_3^- derivative with the natural bovine B-SSO_3^- showed: very little difference in chromatographic pattern in 8M urea on CM-cellulose, Figure 2; identical thin layer electrophoresis mobility in NH_4HCO_3 buffer, pH 10; and similar infrared patterns.

E. Synthesis, Isolation and Characterization of the Insulin Analogues

1. General Procedure

The detailed procedures for the synthesis, isolation, and characterization of insulin described by Katsoyannis et al. (14,20) were used unchanged for the insulin analogues. Briefly, the mercaptoethanol reduced A chain, in excess, was

TABLE IV: Amino Acid Composition[a] of the S-Sulfonated Destripeptide B^{28-30} Chain of Bovine Insulin

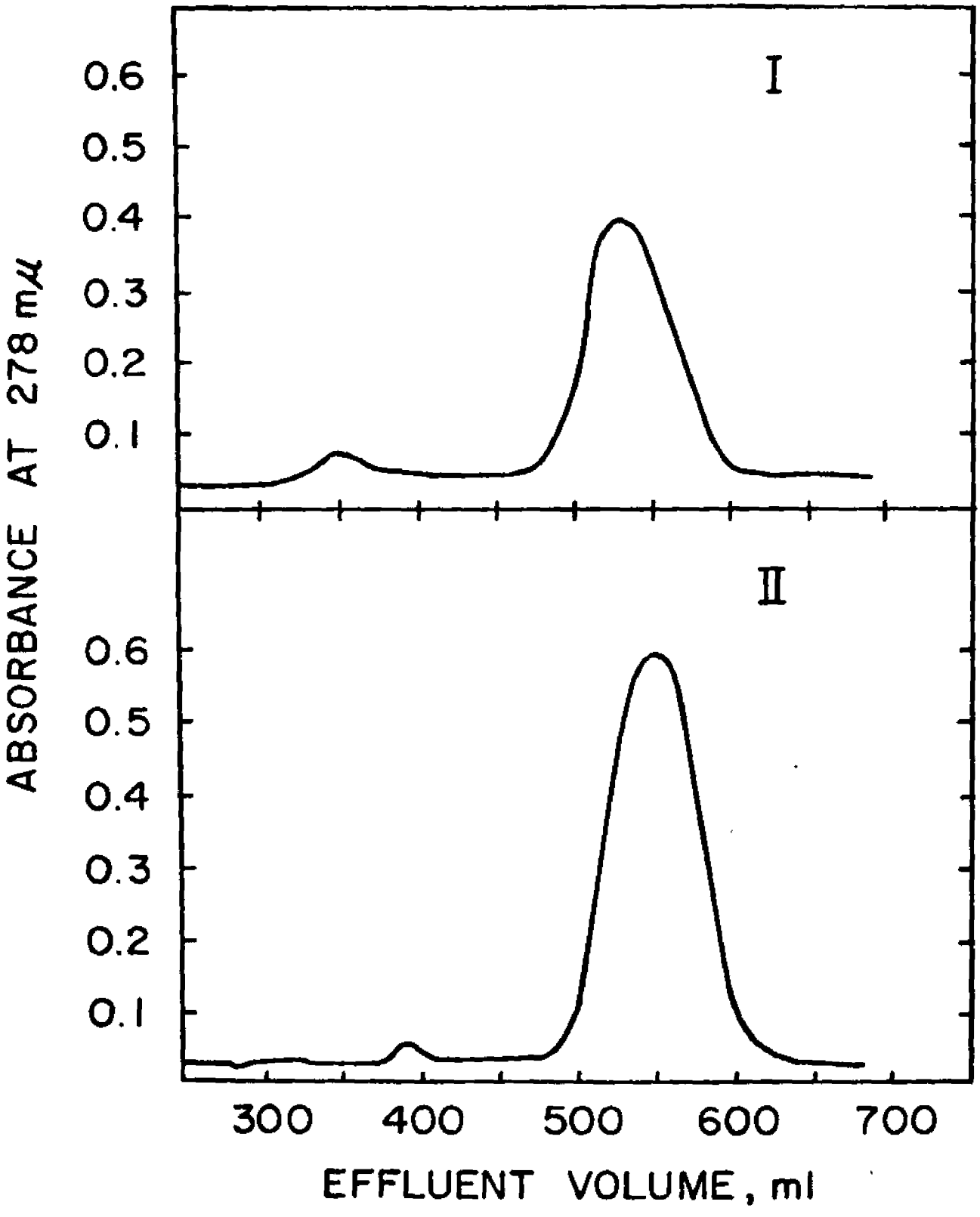
	Acid Hydrolysis	
Amino Acid	Theory	Found
Lysine	0	Trace
Histidine	2	2.0
Arginine	1	1.0
Aspartic acid	1	1.0
Serine	1	0.9
Threonine	1	0.1[b]
Glutamic acid	3	3.0
Proline	0	0
Glycine	3	3.0
Alanine	1	1.0
Valine	3	2.9
Leucine	4	3.8
Tyrosine	2	1.9
Phenylalanine	3	3.0
Cysteine	2	c

a. Number of amino acid residues per molecule.

b. Amount present as threonine.

c. Not determined.

Figure 2


I
II
0.6
0.5
0.4
0.3
0.2
0.1
ABSORBANCE AT 278 mμ
300
400
500
600
700
EFFLUENT VOLUME, ml

combined with the B-SSO_3^- (Chart III). From this combination mixture the insulin analogue and other by-products were precipitated as the picric acid salts and eventually separated by CM-cellulose chromatography with an exponential NaCl gradient. The insulin analogue from the chromatographic effluent was isolated as the hydrochloride via the picrate.

2. Characterization of the Insulin Analogues

Amino acid analysis was run on each isolated hydrochloride, and its molar ratios compared to that theoretically expected for the predicted insulin analogue. Each hydrochloride was also tested for biological activity as compared to natural insulin using the mouse convulsion assay. The insulin analogues were then subjected to thin layer electrophoresis to test for homogeneity and also to compare their mobility with the natural hormone. It should be noted that in each case the insulin analogue was found to be the slowest moving component eluted by the NaCl gradient from the CM-cellulose column. A similar situation exists with the chromatographic pattern of natural insulin, all synthetic, half-synthetic and hybrid

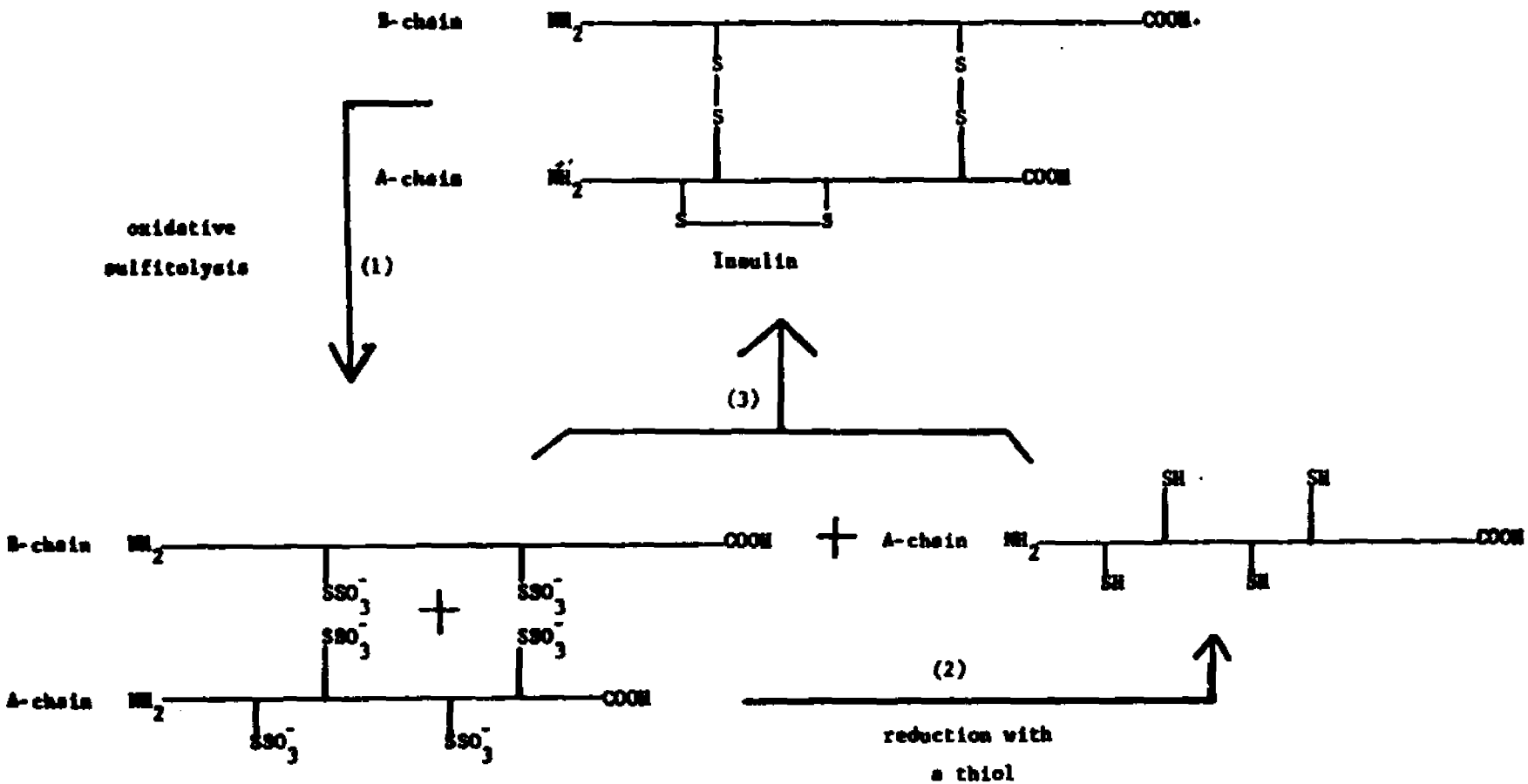

B-chain
A-chain
oxidative
sulfitolysis
(1)
Insulin
(3)
B-chain
A-chain
(2)
reduction with
a thiol

Chart III

insulins (20). As can be seen (Tables V-IX) the molar ratios of each insulin analogue obtained from amino acid analysis of its acid hydrolyzate are in very close agreement to the theoreticallly expected values.

Each analogue on thin layer electrophoresis moved as a single component (Pauly reaction), Figures 3-6. Tests using the mouse convulsion method showed that: the destripeptide B^{28-30} bovine insulin has a specific activity ranging from 22-28 IU/mg; the destripeptide B^{28-30} human insulin 17 IU/mg and the deamino A^{1} sheep insulin 7-10 IU/mg. The destetrapeptide A^{1-4} sheep and porcine insulins showed no biological activity.

TABLE V: Amino Acid Composition[a] of the Deamino A[1] Sheep Insulin

Amino Acid	Theory	Found
Lysine	1	1.1
Histidine	2	1.8
Arginine	1	1.1
Aspartic Acid	3	2.9
Threonine	1	0.9
Serine	2	1.9
Glutamic Acid	7	6.9
Proline	1	1.1
Glycine	4	4.2
Alanine	3	2.9
Valine	5	4.6
Isoleucine	1	0.6
Leucine	6	6.0
Tyrosine	4	b
Phenylalanine	3	2.9
Cysteine	6	b

a. Number of amino acid residues per molecule.

b. Not determined.

TABLE VI: Amino Acid Composition[a] of the Destetrapeptide A^{1-4} Sheep Insulin

Amino Acid	Theory	Found
Lysine	1	1.0
Histidine	2	1.7
Arginine	1	1.1
Aspartic Acid	3	2.8
Threonine	1	0.9
Serine	2	1.9
Glutamic Acid	6	6.1
Proline	1	1.1
Glycine	4	4.0
Alanine	3	2.9
Valine	4	3.8
Leucine	6	5.9
Tyrosine	4	b
Phenylalanine	3	2.7
Cysteine	6	b

a. Number of amino acid residues per molecule.

b. Not determined.

TABLE VII: Amino Acid Composition[a] of the Destetrapeptide A^{1-4} Porcine Insulin

Amino Acid	Theory	Found
Lysine	1	1.1
Histidine	2	2.0
Arginine	1	1.0
Aspartic Acid	3	3.0
Threonine	2	1.8
Serine	3	2.8
Glutamic Acid	6	6.0
Proline	1	0.9
Glycine	3	3.0
Alanine	2	1.9
Valine	3	2.9
Isoleucine	1	0.9
Leucine	6	5.9
Tyrosine	4	b
Phenylalanine	3	2.7
Cysteine	6	b

a. Number of amino acid residues per molecule.

b. Not determined.

TABLE VIII: Amino Acid Composition[a] of the Destripeptide B^{28-30} Bovine Insulin

Amino Acid	Theory	Found
Lysine	0	0
Histidine	2	2.1
Arginine	1	1.0
Aspartic acid	3	3.0
Threonine	1	0[b]
Serine	3	2.7
Glutamic acid	7	7.1
Proline	0	0
Glycine	4	4.1
Alanine	2	2.1
Valine	5	4.6
Isoleucine	1	0.7
Leucine	6	5.9
Tyrosine	4	c
Phenylalanine	3	2.9
Cysteine	6	c

a. Number of amino acid residues per molecule.

b. Amount present as threonine.

c. Not determined.

TABLE IX: Amino Acid Composition[a] of the Destripeptide B^{28-30} Human Insulin

Amino Acid	Theory	Found
Lysine	0	0.2
Histidine	2	1.9
Arginine	1	1.0
Aspartic acid	3	3.0
Threonine	2	1.0[b]
Serine	3	2.7
Glutamic acid	7	7.0
Proline	0	0
Glycine	4	4.0
Alanine	1	1.3
Valine	4	3.7
Isoleucine	2	1.5
Leucine	6	6.0
Tyrosine	4	c
Phenylalanine	3	2.9
Cysteine	6	c

a. Number of amino acid residues per molecule.

b. Amount present as threonine.

c. Not determined.

Figure 3

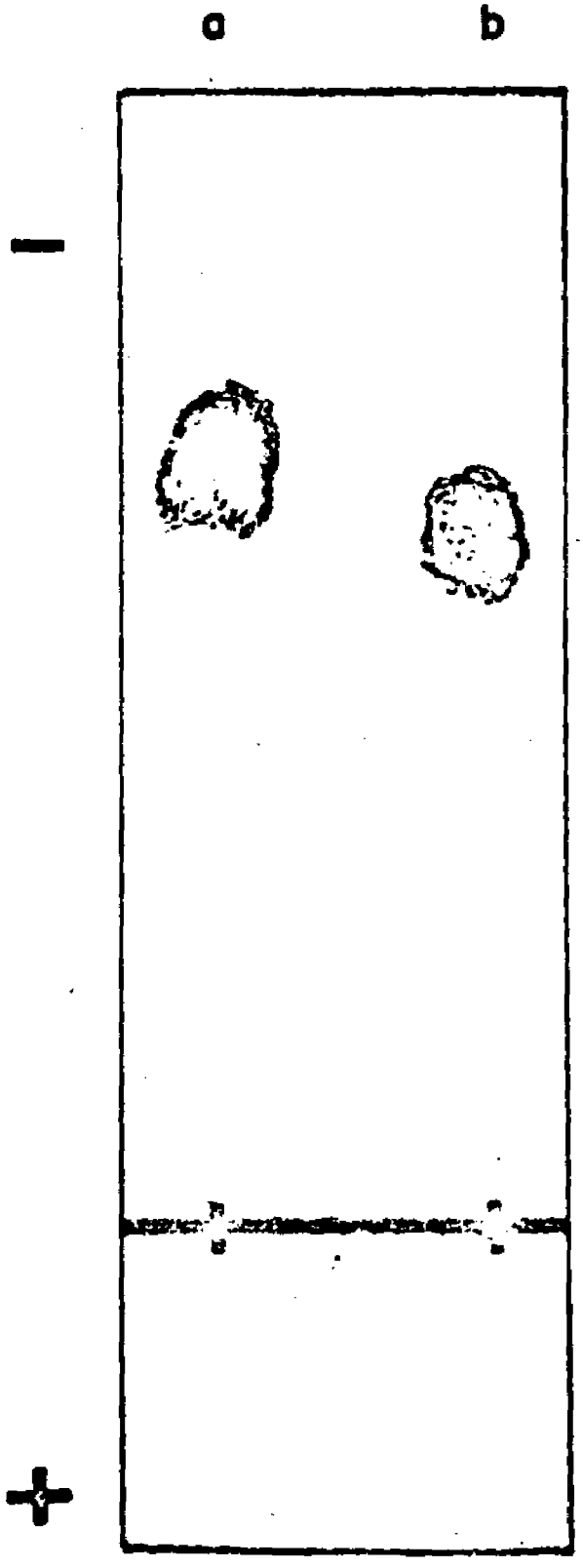


Figure 4

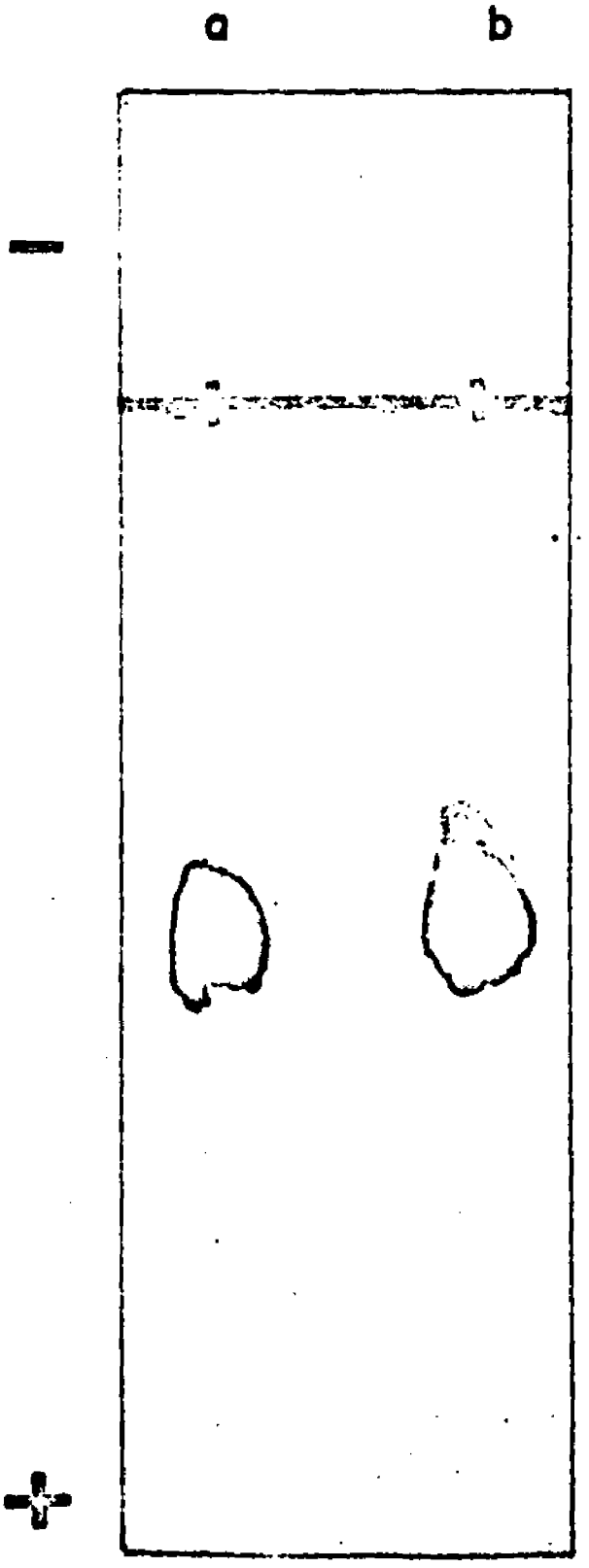


Figure 5

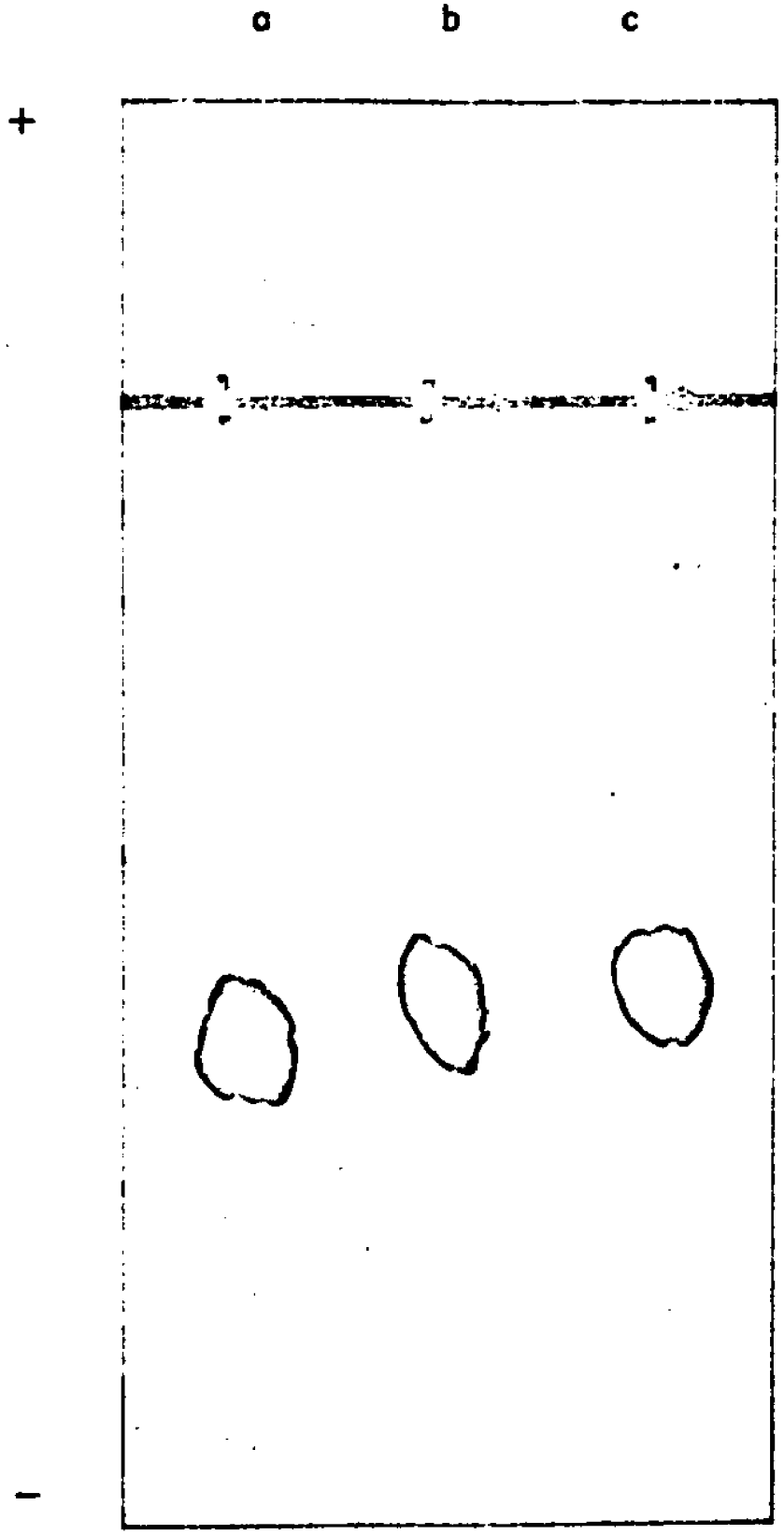
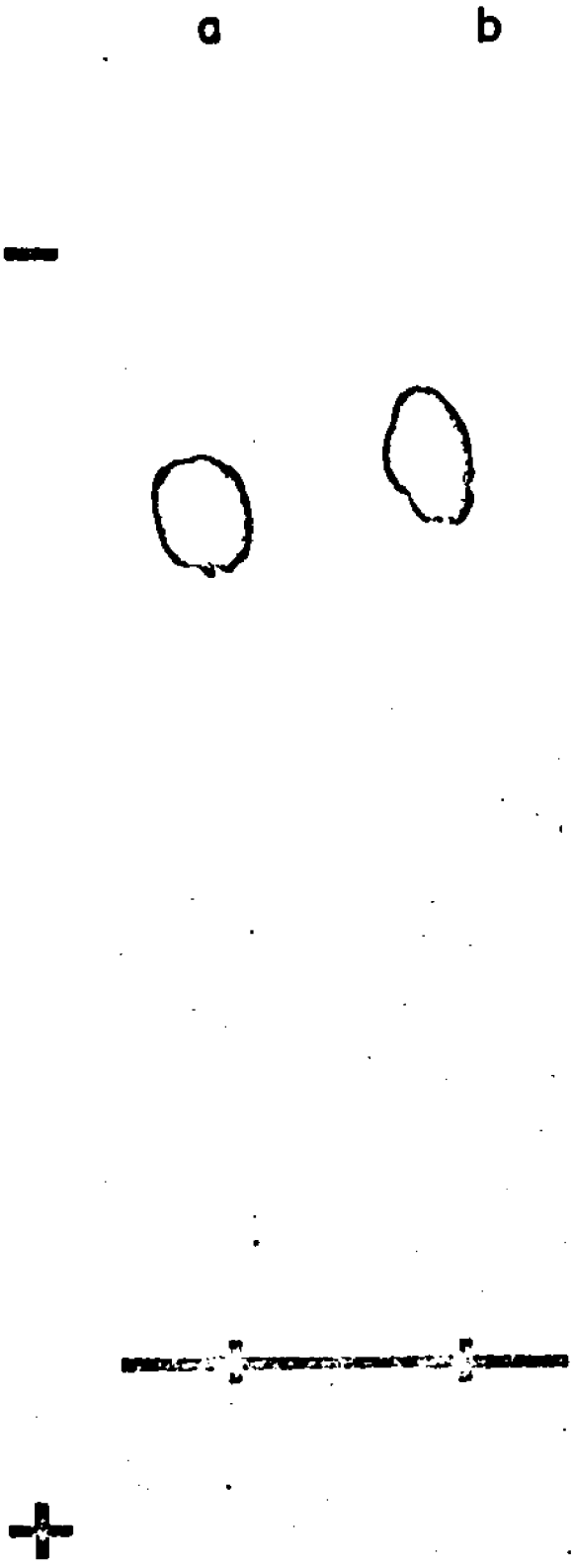


Figure 6



II. EXPERIMENTAL

A. Synthesis of the Deamino Sheep Insulin A Chain S-Sulfonate (Ib) (Chart I)

1. N-Acetyl-L-isoleucyl-L-valyl-L-glutamic acid-α-ethyl-γ-t-butyl ester (IVb)

N-Carbobenzoxy-L-isoleucyl-L-valyl-L-glutamic acid-α-ethyl-γ-t-butyl ester (IV, 5.8 g) (22) was dissolved in methanol (250 ml) and hydrogenated for one hr over 10% palladium-charcoal catalyst (1 g). The catalyst was filtered off and the filtrate evaporated to dryness in vacuo. The residue (IVa), dissolved in a mixture of benzene (40 ml) and chloroform (10 ml), was reacted with acetic anhydride (1 ml). After 24 hr at room temperature ethyl acetate (40 ml) was added to the reaction mixture. The crystalline precipitate was collected by filtration and washed with ethyl acetate. The dried product was partially dissolved in DMF (100 ml) and reprecipitated with dilute acetic acid, filtered and washed successively with dilute acetic acid, water, 1N $NaHCO_3$, and water, and dried; wt 4.4 g (90%) mp 229-31°. A sample for analysis was recrystallized from ethanol; melting point unchanged. $[\alpha]_D^{26°}$ -29.4° (c 1, DMF).

Anal Calcd for $C_{24}H_{43}N_3O_7$: C, 59.38; H, 8.86; N, 8.66.
Found: C, 59.25; H, 8.75; N, 9.03.

2. N-Acetyl-L-isoleucyl-L-valyl-γ-t-butyl-L-glutamic acid hydrazide (IVc)

A solution of IVb (3.6 g) in methanol (150 ml) was treated with hydrazine hydrate (4 ml). Within a short time a precipitate was formed, which after 24 hr at room temperature was filtered, washed with cold methanol, then water until neutral and dried; wt 3.1 g (89%); mp 288° dec; $[\alpha]_D^{26°}$ -34.0° (c 1, DMSO).

Anal Calcd for $C_{22}H_{41}N_5O_6$: C, 56.05; H, 8.70; N, 14.86.
Found: C, 56.31; H, 8.84; N, 15.14.

3. N-Acetyl-L-isoleucyl-L-valyl-L-glutamyl-L-glutaminyl-S-sulfo-L-cysteinyl-S-sulfo-L-cysteinyl-L-alanylglycyl-L-valyl-S-sulfo-L-cysteinyl-L-seryl-L-leucyl-L-tyrosyl-L-glutaminyl-L-leucyl-L-glutaminyl-L-asparaginyl-L-tyrosyl-S-sulfo-L-cysteinyl-L-asparagine (Deamino Sheep A Chain S-Sulfonate) (Ib)

Protected heptadecapeptide II (1 g) was dissolved in TFA (15 ml) containing water (0.3 ml) and HBr was passed through this solution for 1 1/2 hr at 0°. The heptadecapeptide hydrobromide III was then precipitated with anhydrous ether, centrifuged, washed several times with anhydrous ether, and dried over KOH in vacuo. This material was dissolved in DMF (50 ml) containing Et_3N (0.4 ml), cooled to 0°, and allowed to react with the tripeptide azide IVd, which was prepared as follows. A suspension of N-acetyl-L-isoleucyl-L-valyl-γ-t-butyl-L-glutamic acid hydrazide (IVc, 500 mg) in DMF (35 ml) was cooled to -15° and brought

into solution by the addition of 2N HCl (3.5 ml). To this solution was added sodium nitrite (74 mg) dissolved in cold water (0.5 ml). After 5 min at -15° the reaction mixture was added to half-saturated sodium chloride solution (150 ml) and the precipitated azide was filtered off, washed with cold water, and dried at 4° for 1 hr over P_2O_5 *in vacuo*. This azide was subsequently added to the heptadecapeptide solution above. The reaction mixture was stirred at 4°, adding from time to time DMF (50 ml) to prevent it from gelling. After 48 hr the mixture was poured into methanol (500 ml) containing acetic acid (0.5 ml). The crude, precipitated, protected sheep A chain analogue I was isolated by filtration, washed successively with absolute methanol, 50% aqueous methanol, and water, and dried; wt 0.95 g (85%).

A solution of this material in TFA (20 ml) was stored at room temperature for 30 min and then filtered through a sintered glass filter to remove traces of impurities. Dilution of the filtrate with anhydrous ether caused the precipitation of the partially protected analogue Ia; wt 0.9 g.

Amino acid analysis of an acid hydrolysate showed the following composition expressed in molar ratios: $Asp_{2.0}$ $Ser_{0.9}Glu_{3.9}Gly_{1.0}Ala_{1.1}Val_{1.6}Ileu_{0.6}Leu_{2.0}Tyr_{1.6}$Cysteine$_{0.5}$ S-benzylcysteine $_{3.2}$ in good agreement with the values expected from theory. The reduction of Ia (180 mg) was carried out with sodium in anhydrous liquid ammonia as

described previously. Sulfitolysis with sodium sulfite (600 mg) and sodium tetrathionate (300 mg) followed by dialysis and then lyophilization yielded the sheep A chain S-sulfonate lacking the N-terminal amino group (Ib) as a white powder; wt 160 mg. Sephadex G-50 chromatography of this synthetic material in 5% acetic acid gave a single peak pattern on continuous monitoring at 278 mμ. Lyophilization of the effluent gave the purified analogue Ib; wt 135 mg (80% based on Ia used).

Amino acid analysis of an acid hydrolysate gave the composition expressed in molar ratios shown in Table I. On thin layer electrophoresis both in 0.5 N acetic acid, 3500 v and in 0.01 N NH_4HCO_3, pH 10, 3500 v the synthetic material Ib moved as a single component (Pauly reaction).

B. Synthesis of the Destetrapeptide A^{1-4} Sheep A Chain S-Sulfonate (IIa)(Chart II)

The thoroughly dry, protected heptadecapeptide II (200 mg) (13) was dissolved in anhydrous liquid ammonia (150 ml) in a 250 ml round bottom flask fitted for magnetic stirring. The reaction was carried out at the boiling point of the solution. Cleavage of the protective groups was accomplished by adding very small pieces of sodium into the solution until a faint blue color appeared throughout. The blue color was allowed to persist for 1 min and was then discharged by the addition of a few crystals of

ammonium chloride. The solution was evaporated at atmospheric pressure to about 10 ml and dried from the frozen state. The residue was dissolved in 8M guanidine hydrochloride (20 ml) containing acetic acid (0.5 ml). The pH of the solution was adjusted to 8.9 with ammonium hydroxide, and sodium sulfite (680 mg) then added. After stirring the solution for 15 to 30 min at room temperature freshly prepared sodium tetrathionate (320 mg) was added.

The reaction mixture was stirred for 4 hr at room temperature then dialyzed against four changes of distilled water (4 l each) at 4° for 24 hr. Visking 18/32 dialysis tubing was used. The dialyzate was then lyophilized; wt 165 mg.

The lyophilized material (165 mg) was dissolved in 5% acetic acid (5 ml) and applied to a Sephadex G-50 column (fine grade bead form; 2.4 x 50 cm), which had been equilibrated with 5% acetic acid. The effluent was continuously monitored with a Gilford recording spectrophotometer at 278 mμ. The chromatographic pattern obtained indicated the presence of a single component, which was isolated by lyophilization of the effluent; wt 130 mg (74%, based on II used). Amino acid analysis of an acid hydrolysate and of an LAP digest gave the compositions expressed in molar ratios shown in Table II.

On thin layer electrophoresis in 0.5 N acetic acid, 3400 v, and in 0.01 N NH_4HCO_3, pH 10.0, 2900 v, the synthe-

tic material IIa moved as a single component (Pauly reaction).

C. Synthesis of the Destetrapeptide A^{1-4} Human A Chain S-Sulfonate (Va) (Chart II)

The procedures used here were identical to those used to obtain IIa. From the known protected heptadecapeptide V (200 mg) (25) purified human A chain S-sulfonate analogue Va was obtained; wt 125 mg (71%, based on V used). Amino acid analysis data both for an acid hydrolysate and an LAP digest is given in Table III. On thin layer electrophoresis, carried out at two pH values as described previously, the synthetic material Va moved as a single component (Pauly reaction).

D. Synthesis of the Destripeptide B^{28-30} Bovine B Chain S-Sulfonate

The classical reduction procedure of Sifferd and du Vigneaud (24) was employed for the cleavage of insulin to its individual chains. Essentially the method used for deblocking the protected synthetic insulin A chains was followed (13,22). In a typical experiment thoroughly dry crystalline bovine zinc insulin (250 mg) was dissolved in anhydrous liquid ammonia (200 ml) in a 500 ml round bottom flask fitted for magnetic stirring. The reaction was car-

ried out at the boiling point of the solution. Reduction was accomplished by adding small pieces of sodium into the solution until a faint blue color appeared throughout. The blue color was allowed to persist for 30 seconds and was then discharged by the addition of ammonium chloride (ca 2 g). The solution was evaporated at atmospheric pressure to about 10 ml and dried from the frozen state. The reduced product thus obtained was sulfitolyzed using sodium sulfite and sodium tetrathionate. Briefly, this material was dissolved in 8M quanidine hydrochloride (25 ml) containing acetic acid (0.5 ml) and to this solution adjusted to pH 8.9 with dilute ammonium hydroxide was added sodium sulfite (1 g) and freshly prepared sodium tetrathionate (500 mg). The reaction mixture was stirred for 16 hr at room temperature and then dialyzed against five changes of distilled water (4 l each) at 4° for 20 hr. A Visking 18/32 dialyzing tubing was used. The dialyzate was adjusted to pH 5.0 and the precipitated product was separated by centrifugation. Lyophilization of the precipitate afforded a preparation enriched in B-SSO_3^-. The latter was purified by continuous flow electrophoresis, which was accomplished at 2400 v, 100-130 ma and 5° using as a supporting buffer a 0.12% NH_4HCO_3 solution adjusted to pH 7.8 with NH_4OH. The electrode rinse of the separator was a solution of 0.315% NH_4HCO_3. The tubes containing the B-SSO_3^- derivative were pooled and lyophilized to yield the S-sulfonated chain as a

white fluffy material, 80 mg. See Katsoyannis et al. (18) for a detailed description of the above techniques. Further purification of this B-SSO_3^- derivative was achieved by chromatography on the 4.5 x 50 cm CM-cellulose column using 8M urea-acetate buffer, pH 4.1. The chromatographic pattern obtained is shown in Figure 2-I. For removing the urea, the fractions containing the chain material were titrated with concentrated HCl to pH 3.0 and then placed on a 4 x 60 cm G-15 Sephadex column equilibrated with 5% (v/v) acetic acid. The pooled effluent containing the chain material was concentrated in a rotary evaporator (30°) to about 10 ml and mixed with 15 ml of saturated picric acid solution. After 24 hr at 2° the precipitated picrate was isolated by centrifugation and washed with half-saturated picric acid solution. Separation of the picric acid salts from the chain material was readily accomplished upon chromatography of its picrate on the 1.2 x 50 cm Sephadex G-15 column equilibrated with 0.05 M NH_4HCO_3. The B-SSO_3^- derivative was recovered as a white fluffy material upon lyophilization of the effluent. The amino acid analysis of this material after acid hydrolysis gave a composition in molar ratios shown in Table IV. The purified product possessed the specific rotation of $[\alpha]_D^{25}$ -95.4° (c 0.1, 0.5 N acetic acid) compared to $[\alpha]_D^{25}$ -95.8° $\pm$ 4.3° (c 0.1, 0.5 N acetic acid) which was found for natural bovine B-SSO_3^-. On high voltage thin layer electrophoresis in 0.01 N NH_4HCO_3

adjusted to pH 10 with NH_4OH and 3400 v the purified material exhibited a single Pauly-positive spot, and had the same mobility as natural bovine B-SSO_3^-. Finally no difference was detected in the infrared spectra of the above product and natural bovine B-SSO_3^-.

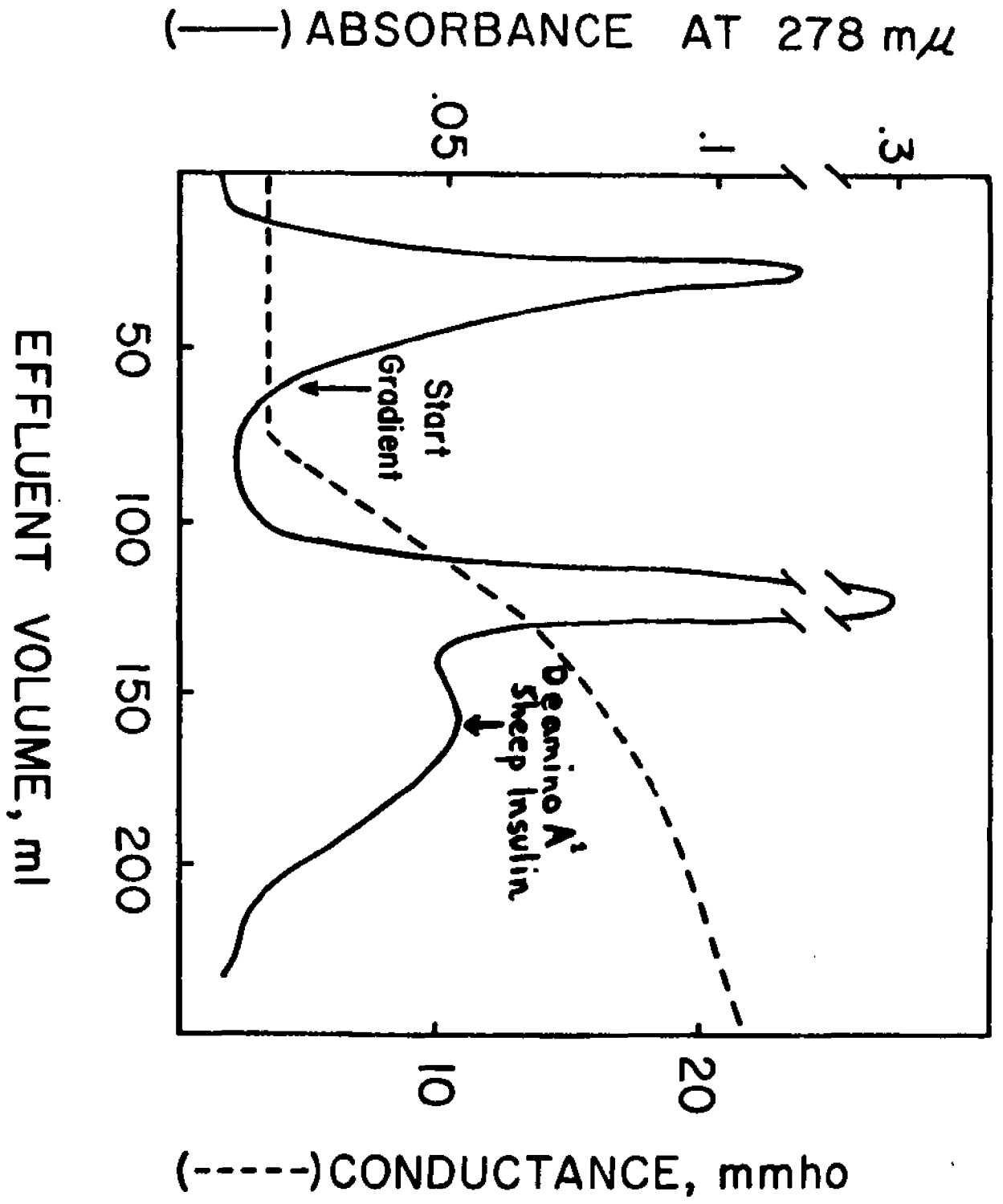
E. Synthesis, Isolation and Characterization of the Insulin Analogues

1. Deamino A^1 Sheep Insulin

This analogue was synthesized from the corresponding chains according to the method outlined by Katsoyannis and Tometsko (19). In a typical experiment an aqueous solution of 20 mg deamino A chain S-sulfonate was treated at pH 5.0 with 2-mercaptoethanol for 6 min at 100°. The reaction mixture was cooled to 5°, extracted with ethyl acetate to remove the 2-mercaptoethanol and the ensuing reduced A chain was allowed to react with 5 mg of bovine B-SSO_3^- at pH 9.6. After 18 to 22 hr at 2° acetic acid was added to the reaction mixture to give a final concentration of 10% (v/v), and the resulting solution was mixed with an equal volume of saturated picric acid solution. After 24 hr at 2° the precipitated picrate was isolated by centrifugation and washed once with a 5 ml portion of a half-saturated picric acid solution. This solid was dissolved in a few drops of acetone-water (4:1 by volume) and the resulting solution was mixed with 40 ml of cold, dry acetone containing 4 drops of

concentrated HCl. The precipitated white product was allowed to settle for 2 hr at 2°, collected by centrifugation and washed successively with dry acetone and dry ether. Separation of insulin from this precipitate was accomplished as follows. The precipitate (which consists of the hydrochlorides of the various products formed by the combination of the A and B chains) was suspended in 1 ml of acetate buffer (pH 3.3; Na^+, 0.04 M) and any insoluble material removed by centrifugation, and then washed with 2 ml of the same buffer. If more than one recombination mixture was needed (usually two were used), each mixture was treated separately up to this point. The combined supernatants were placed on the 0.9 x 23 cm CM-cellulose column and chromatographed using a NaCl gradient as described by Katsoyannis et al. (20). The effluent was monitored continuously at 278 mμ and also by a Radiometer conductivity meter (Copenhagen). Figure 7 illustrates the chromatographic pattern obtained using two recombination mixtures of the deamino sheep A chain. The insulin analogue is eluted with application of the NaCl gradient and is the slowest moving material. The effluent containing the insulin analogue was concentrated in a rotary evaporator (20-25°) to approximately 10 ml and mixed with an equal volume of saturated picric acid solution. The precipitated picrate was collected by centrifugation and converted to the hydrochloride by the method of Randall (29). Amino acid analysis of an acid hydrolysis of this product

Figure 7


(——) ABSORBANCE AT 278 mμ
.05
1
3
EFFLUENT VOLUME, ml
50
100
150
200
Start Gradient
β-amino A¹ Sheep Insulin
10
20
(-----) CONDUCTANCE, mmho

gave the molar ratios shown in Table V.

This hydrochloride was assayed by the mouse convulsion method and had a specific activity of 7-10 IU/mg.

Finally, on thin layer electrophoresis both at pH 10, 0.01 N NH_4HCO_3, 3500 v and 0.5 N acetic acid, 3500 v, this material moved as a single component and had a mobility slightly different from that of the natural bovine hormone; Figures 4 and 3, respectively.

2. Destetrapeptide A^{1-4} Sheep and Porcine Insulins

Essentially the same procedures as above were used for combination of the chains and isolation of the analogues. Figures 8 and 9 show the chromatographic pattern obtained using two recombination mixtures for each analogue. In each case the insulin analogue was found to be the slowest moving component eluted by the NaCl gradient. Amino acid analysis of the destetrapeptide A^{1-4} sheep and porcine insulin hydrochlorides is given in Tables VI and VII, respectively. Quantitites up to 40 micrograms per mouse of each of the above insulin analogues produced no convulsions in several groups of mice tested (5 to 10 mice per group). Both insulin analogues on thin layer electrophoresis in 0.01 N NH_4HCO_3, pH 10, 3500 v, did not move from the origin, whereas in 0.5 N acetic acid, 3500 v, both moved as a single component (Pauly reaction) having the same mobility, which was slightly different from natural bovine insulin, Figure 5.

Figure 8

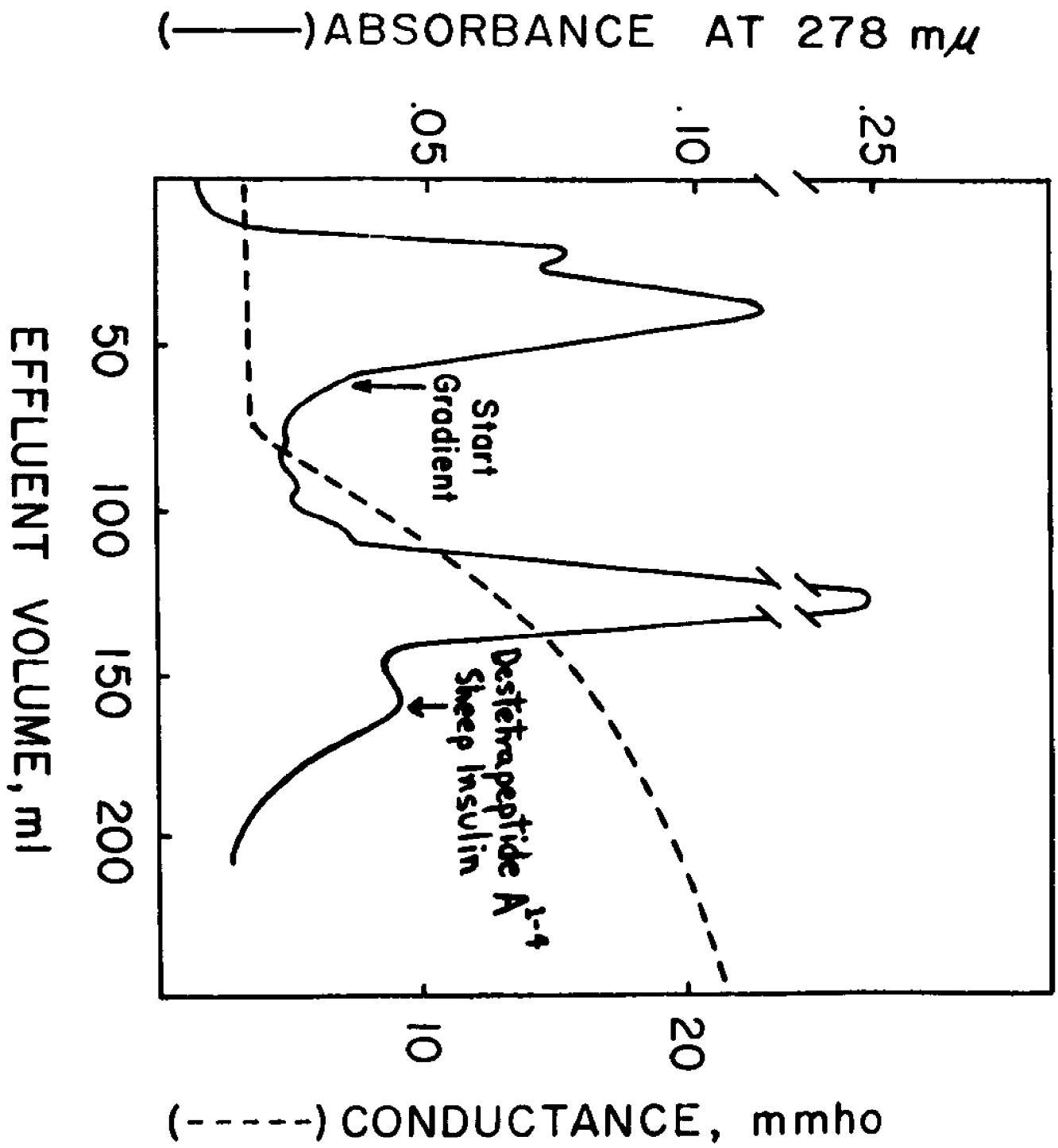
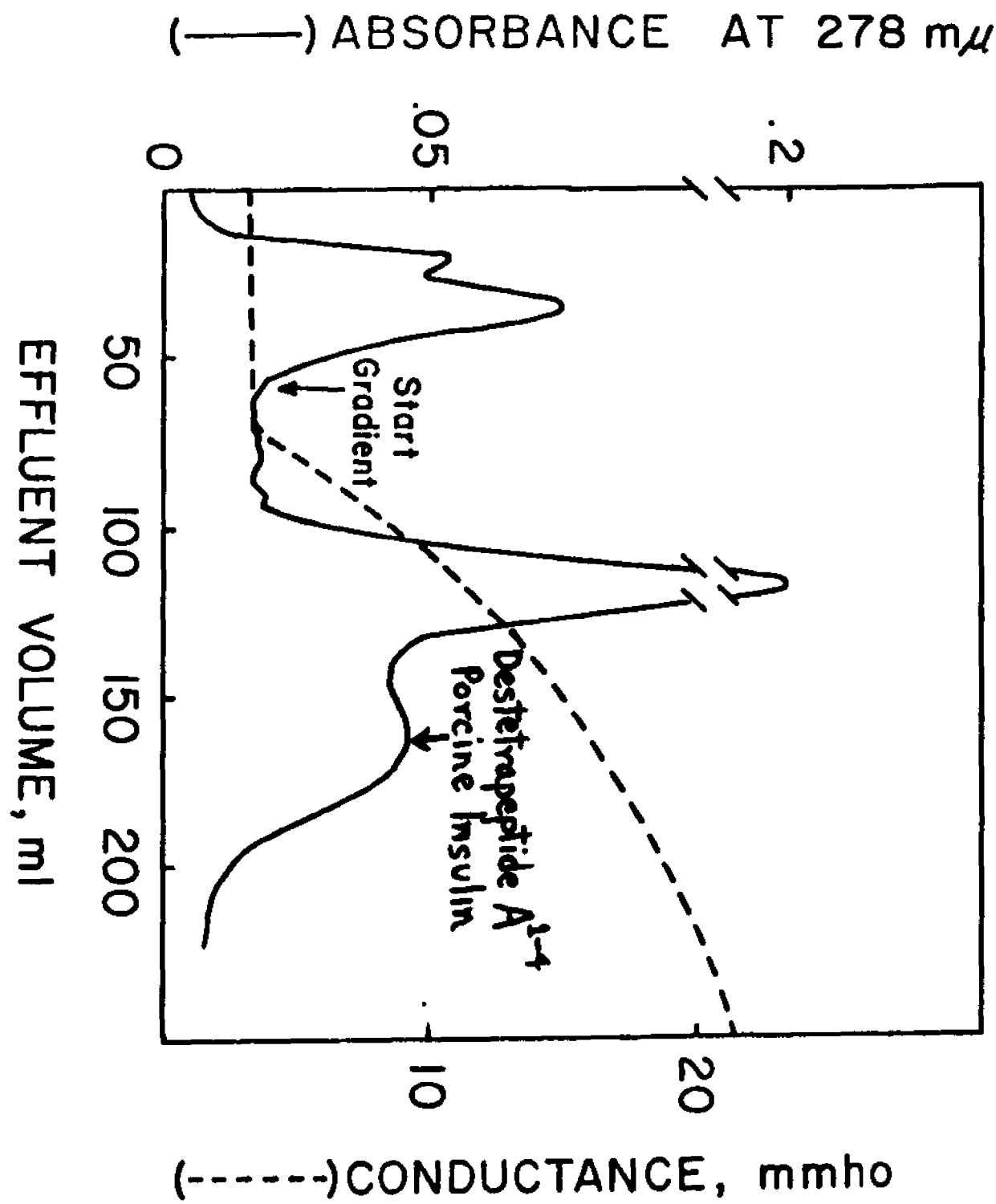

(——)ABSORBANCE AT 278 mμ
.05
.10
.25
EFFLUENT VOLUME, ml
50
100
150
200
Start Gradient
Desoctapeptide A1-4 Sheep Insulin
10
20
(- - - - -) CONDUCTANCE, mmho

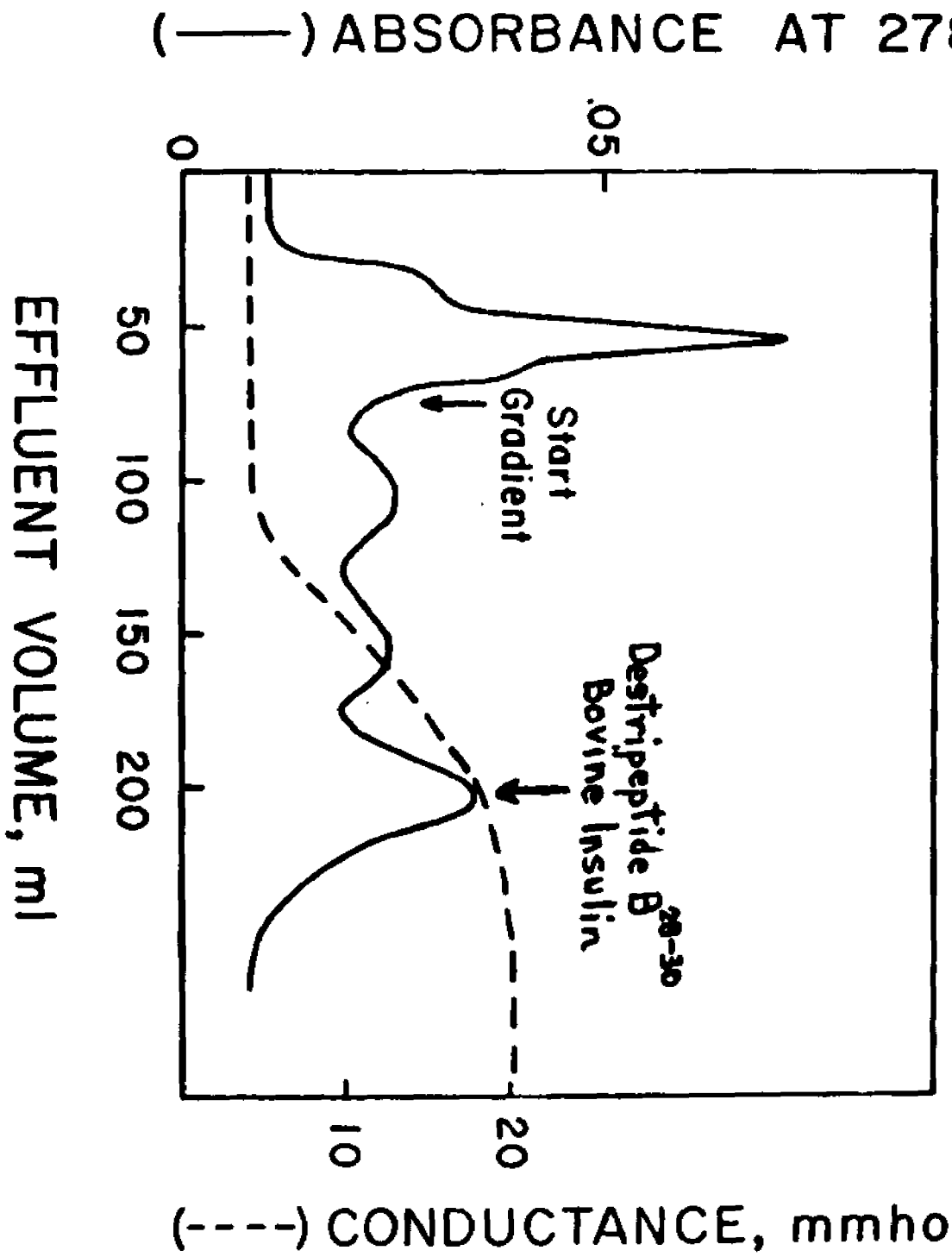
Figure 9


(——) ABSORBANCE AT 278 mμ
0
.05
.2
EFFLUENT VOLUME, ml
50
100
150
200
Start Gradient
Desoctapeptide A1-4 Porcine Insulin
10
20
(------) CONDUCTANCE, mmho

3. Destripeptide B^{28-30} Bovine and Human Insulins

The same procedures as used above were again utilized for combination of the chains and isolation of the insulin analogues. Figure 10 shows the chromatographic pattern obtained from combination mixtures of the destripeptide B^{28-30} chain with bovine A chain (75 IU). The last component eluted with the NaCl gradient was found to be the destripeptide B^{28-30} bovine insulin, as confirmed by its amino acid analysis shown in Table VIII. The weight of the insulin analogue hydrochloride isolated was 1.1 mg. Thin layer electrophoresis of this material (0.5 N acetic acid, 3500 v) revealed a single component (Pauly reaction) having a mobility slightly different from that of the natural hormone, Figure 6. Finally, this analogue when assayed by the mouse convulsion technique possessed a specific activity of 22-28 IU/mg. Figure 11 shows the chromatographic pattern obtained from combination mixtures of the destripeptide B^{28-30} chain with human (porcine) A chain (90 IU). Again the last component eluted with the NaCl gradient was found to be the destripeptide B^{28-30} human insulin as confirmed by its amino acid analysis shown in Table IX. The weight of insulin analogue hydrochloride recovered was 1.8 mg. This human insulin analogue possessed a specific activity of 17 IU/mg.

Figure 10


(——) ABSORBANCE AT 278 mμ
0
.05
EFFLUENT VOLUME, ml
50
100
150
200
Start Gradient
Destripeptide B 28-30 Bovine Insulin
10
20
(----) CONDUCTANCE, mmho

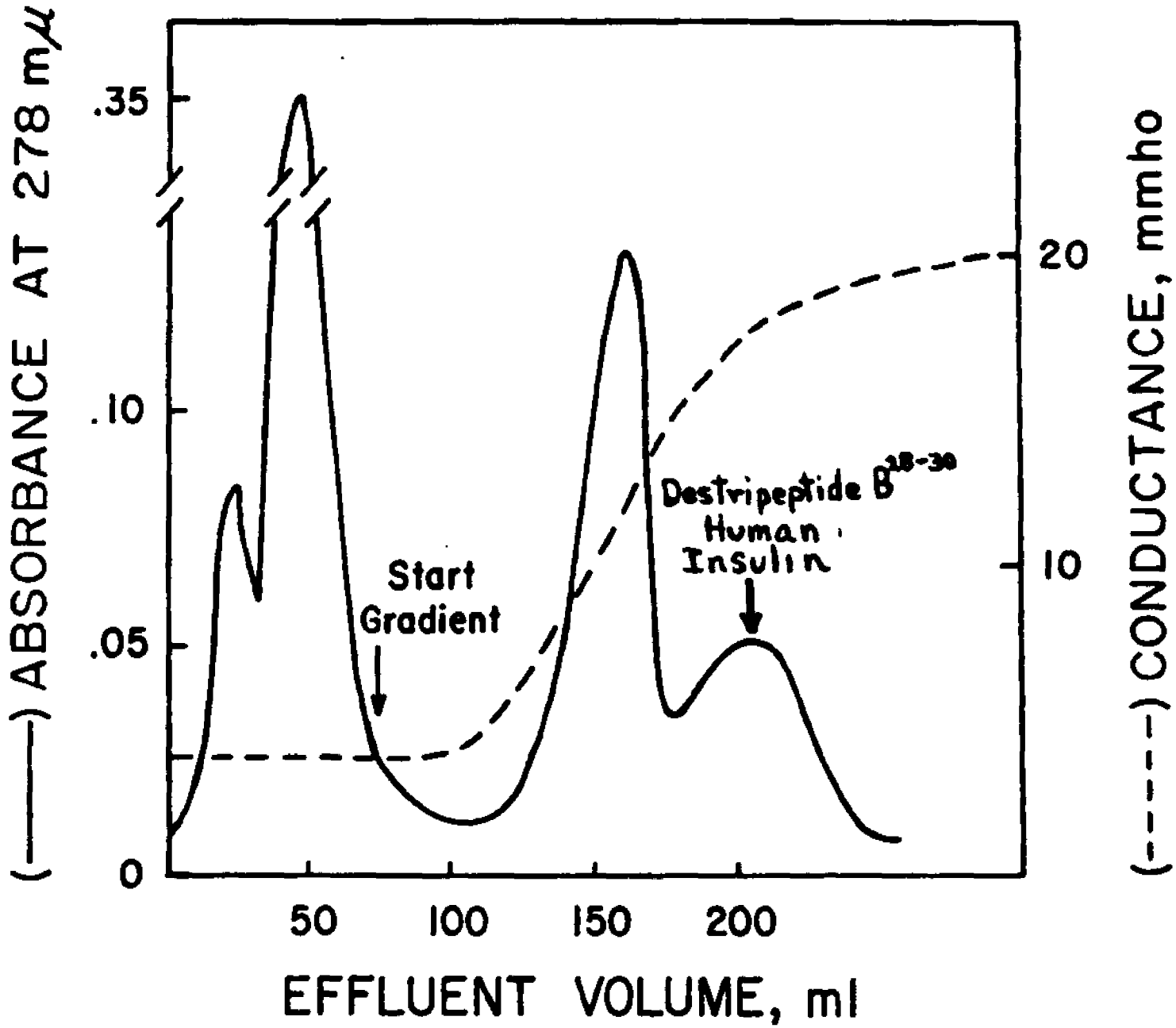

(——) ABSORBANCE AT 278 mμ
.35
.10
.05
0
20
10
(- - - -) CONDUCTANCE, mmho
Start Gradient
Destripeptide B(28-30) Human Insulin
50
100
150
200
EFFLUENT VOLUME, ml

Figure 11

III. RESULTS AND DISCUSSION

Investigations of the relationship of chemical structure to biological activity in the insulin field have been carried out in several laboratories (3,7). An approach which provided information regarding structure-activity relationships involved chemical modification of various functional groups of the insulin molecule (30,31,32,33,34). Equally interesting results were obtained by enzymatic modification of the insulin molecule. This approach consists in the selective removal of one or more amino acid residues from the amino or carboxyl termini of the insulin chains with the aid of various proteolytic enzymes (7,30).

The development of methods, however, for preparing purified A and B chains and the development of highly efficient techniques, both for their combinations (14,19) and the isolation of the insulin thus produced (19,20) has opened unlimited possibilities for the synthesis of insulin analogues and for study of structure-activity relationships. To this end a number of analogues of the A and B chains have been synthesized, submitted to combination experiments with natural B and A chains, respectively and the combination mixtures tested for biological activity (35-42). Biological evaluation of combination mixtures of A and B chains, however, is insufficient for drawing meaningful conclusions regarding structure-activity relationships. Generation of

a certain amount of biological activity by combination of modified A and B chains might indicate that either the chains have combined very efficiently and the product, "insulin analogue", is not very active or that the chains do not combine efficiently but the analogue is highly active. Obviously then, isolation of an analogue in purified form is a prerequisite for meaningful conclusions regarding structure-activity studies.

This investigation has dealt with the isolation in purified form of three A chain insulin analogues and two B chain insulin analogues. The key intermediates in the synthesis of the three A chain analogues were the known protected human and sheep heptadecapeptides A^{5-21} (V and II respectively, Chart II). Removal of the protective groups by sodium in liquid ammonia reduction, followed by sulfitolysis yielded the destetrapeptide A^{1-4} human and sheep A chain S-sulfonates. The deamino sheep A chain S-sulfonate was obtained as above after first forming the partially protected deamino A^{1} chain (Ia, Chart I), starting from the sheep heptadecapeptide (II, Chart I). Each A chain analogue was purified by chromatography on G-50 Sephadex. Amino acid analysis after acid hydrolysis and enzymatic digestion with LAP, as well as thin layer electrophoresis established the chemical and stereochemical homogeneity of the synthetic compounds.

The destetrapeptide A^{1-4} sheep and porcine insulins

and deamino A^1 sheep insulin were produced by combining each of the above purified A chain analogues with natural bovine B-SSO_3^-. Separation of the insulin analogue from other products of the combination mixture was accomplished by CM-cellulose chromatography. In these chromatographic separations, each insulin analogue is the slowest moving component and is eluted at the same position where the natural hormone emerges when chromatographed under the same conditions. This was determined by amino acid analysis and biological assay of the material contained in the effluent of each peak represented in the chromatogram, Figures 7 to 9. As shown in Tables V through VII in each case the synthetic material purified by CM-cellulose chromatography gave an amino acid composition after acid hydrolysis in very good agreement to the theoretically expected values for the corresponding insulin analogue. Thin layer electrophoresis further indicated that each of these analogues was homogeneous. The electrogram of the destetrapeptide A^{1-4} sheep and porcine insulins is shown in Figure 5. The electrograms of the deamino A^1 sheep insulin are shown in Figures 3 and 4. Both the destetrapeptide A^{1-4} sheep and porcine insulin analogues were found to be biologically inactive by the mouse convulsion assay method, whereas the deamino A^1 sheep insulin had a specific activity of 7-10 IU/mg.

The key intermediate in the synthesis of the B chain

insulin analogues mentioned previously is a B chain which lacks the C-terminal tripeptide sequence. This destripeptide B^{28-30} chain is prepared by the sodium in liquid ammonia reduction of bovine insulin. This treatment results in the splitting of the disulfide bridges of insulin and, in addition, causes the cleavage of the B chain between the threonine and proline residues at positions 27-28. The amino acid analysis of an acid hydrolysate of the isolated destripeptide B-SSO_3^- (Table IV) indicates that the cleavage of the threonyl-proline bond is almost quantitative. The molar ratios of pro, lys and ala, which occupy the C-terminal tripeptide sequence in the B chain, indicate only traces of these amino acid residues are present in the acid hydrolysate of that chain. The elimination of the C-terminal tripeptide sequence, pro-lys-ala, results in the formation of a new C-terminal amino acid residue, threonine. It has been shown, however (27), that the sodium-liquid ammonia cleavage of acyl-proline bonds leads to the formation of multiple forms of the new C-terminal amino acid residue which in this case is threonine. It appears that the predominant forms of the new C-terminus are the corresponding amino alcohol and amino aldehyde derivatives. Since either of these derivatives will be decomposed upon acid hydrolysis, the acid hydrolysate of the destripeptide B-SSO_3^- contains only traces of threonine (Table IV). The destripeptide B^{28-30} bovine and human insulin analogues which were produced by the com-

bination of the destripeptide B^{28-30} chain with the corresponding A chains were isolated by the previously described CM-cellulose method. Again it was found that both analogues are eluted at the position where the natural hormone emerges in this chromatographic system. As shown in Tables VIII and IX amino acid analyses of the isolated hydrochlorides of these insulin analogues after acid hydrolysis, gave amino acid compositions expressed in molar ratios in very good agreement with the theoretically expected values. On high voltage thin layer electrophoresis both analogues exhibited single Pauly-positive spots. The electrogram of destripeptide B^{28-30} bovine insulin is shown in Figure 6. The specific activity of this latter analogue ranged from 22 to 28 IU/mg as compared to the specific activity of 23-25 IU/mg found in our laboratory for the natural bovine, sheep and porcine insulins. The specific activity of the destripeptide B^{28-30} human insulin, however, was 70% of that of the natural hormone, namely 17 IU/mg.

It can be seen from the data presented here that each of the A and B chain analogues did combine with its natural counterpart to form an insulin analogue. The low or nonexistant biological activity of the A chain insulin analogues described in this investigation, namely the destetrapeptide A^{1-4} sheep and porcine insulins and deamino A^{1} sheep insulin indicates that there are one or more amino acid residues in the first four positions of the A chain,

which play an essential role in the biological behavior of insulin as manifested by the mouse convulsion method. The high biological activity of the destripeptide B^{28-30} human and bovine insulins indicates that the three amino acid residues at B chain positions 28, 29 and 30 are not necessary for biological activity of insulin. It also further implies that an intact threonine residue at position B^{27} in the parent molecule is not necessary for biological activity of the hormone, since the new C-terminus of both active analogues contains an altered derivative of that amino acid. Finally this investigation has shown that the B chain of insulin can be shortened considerably at the carboxyl end without affecting the biological activity of the hormone. At this moment we have no explanation for the lower specific activity of the destripeptide B^{28-30} human insulin as compared to both the destripeptide B^{28-30} bovine insulin analogue or natural insulin. Thus the chemical synthesis and isolation in purified form of several insulin analogues has been accomplished and their biological activity determined. The pattern relating structure to function has, I believe, been started.

IV. SUMMARY

The synthesis and isolation in a highly purified form of the S-sulfonated derivatives of the deamino A chain and destetrapeptide A^{1-4} chain of sheep insulin and the destetrapeptide A^{1-4} chain of procine (human) insulin are described. Each of these A chain analogues was combined with natural B chain of bovine insulin to form deamino A^{1} sheep insulin and destetrapeptide A^{1-4} sheep and porcine insulins, which were isolated as the hydrochlorides. The former analogue had a specific activity of 7 to 10 IU/mg, whereas the latter two analogues were biologically inactive by the mouse convulsion assay method.

The preparation from natural bovine insulin of the S-sulfonated derivative of the destripeptide B^{28-30} chain is also described. Combination of the latter compound with the sulfhydryl form of the bovine or porcine (human) insulin A chains afforded the destripeptide B^{28-30} bovine or human insulins respectively, which were isolated and characterized. The bovine insulin analogue possessed a specific activity ranging from 22-28 IU/mg, whereas the human insulin analogue has a potency of 17 IU/mg.

ABBREVIATIONS

Ala = alanine

APM = aminopeptidase M

$\overset{NH_2}{\underset{|}{Asp}}$ = asparagine

Arg = arginine

A-SSO_3^- = A chain S-sulfonate

B-SSO_3^- = B chain S-sulfonate

Cys = cysteine

Glu = glutamic acid

$\overset{NH_2}{\underset{|}{Glu}}$ = glutamine

Gly = glycine

His = histidine

Ileu = isoleucine

LAP - leucine aminopeptidase

Leu = leucine

Lys = lysine

Phe = phenylalanine

Ser = serine

Thr = threonine

Tyr = tyrosine

Val = valine

B_z = benzyl

NB_z = p-nitrobenzyl ester

$NHNH_2$ = hydrazide

OBut = tert-butyl ester

OEt = ethyl ester

ON_3 = azide

SO_3^- = sulfonate

Z = carbobenzoxy

DMF = dimethylformamide

DMSO = dimethylsufloxide

Et_3N = triethylamine

HBr = hydrogen bromide (hydrobromic acid)

HCl = hydrochloric acid

KOH = potassium hydroxide

NaCl = sodium chloride

NH_3 = ammonia

NH_4OH = ammonium hydroxide

ca = about

cm = centimeter

g = gram

hr = hour

IU = international unit

l = liter

M = molarity

ma = milliampere

mg = milligram

min = minute

ml = milliliter

mp = melting point

mμ = millimicron

$[\alpha]_D$ = specific rotation - D line of sodium

v = volt

wt = weight

Anal = analysis

Calcd = calculated

BIBLIOGRAPHY

1. Klostermeyer, H., and Humble, R. E., Angew. Chem. Intern. Ed. Engl. 5, 807 (1966).

2. Grodsky, G. M., and Forsham, P. H., Ann. Rev. of Physiol. 28, 347 (1966).

3. Trakatellis, A. C. and Schwartz, G. P., Progress in the Chemistry of Organic Natural Products 26, 120 (1968).

4. Brown, H., Sanger, F. and Kitai, R., Biochem. J. 60, 556 (1955).

5. Harris, J. I., Sanger, F. and Naughton, M. A., Arch. Biochem. 65, 427 (1956).

6. Nicol, D. S. H. W., and Smith, L. F., Nature 187, 483 (1960).

7. Lübke, K., and Klostermeyer, H., Adv. Enzymol. 33, 445 (1970).

8. Schröder, E. and Lübke, K., The Peptides, Academic Press, Inc., New York, 1966.

9. Partridge, S. M., Biochem. J. 42, 238 (1948).

10. Waley, S. G., and Watson, G., ibid. 55, 328 (1953).

11. Stewart, J. M., and Young, J. D., Solid-Phase Peptide Synthesis, p. 53, Freeman, San Francisco, 1969.

12. Tometsko, A. and Delihas, N., Anal. Biochem. 18, 72 (1967).

13. Katsoyannis, P. G., Tometsko, A., Zalut, C., and Fukuda, K., J. Am. Chem. Soc. 88, 5633 (1966).

14. Katsoyannis, P. G., Trakatellis,A. C., Johnson, S., Zalut, C., and Schwartz, G., Biochemistry 6, 2642 (1967).

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

16. Hill, R. L., and Smith, E. L., J. Biol. Chem. 228, 577 (1957).

17. Pfleiderer, G., Celliers, P. G., Stanilovic, M., Wachsmuth, E. D., Determann, H., and Braunitzer, G., Biochem. Z. 340, 552 (1964).

18. Katsoyannis, P. G., Tometsko, A., Zalut, C., Johnson, S., and Trakatellis, A. C., Biochemistry 6, 2635 (1967).

19. Katsoyannis, P. G., and Tometsko, A., Proc. Natl. Acad. Sci. U.S. 55, 1554 (1966).

20. Katsoyannis, P. G., Trakatellis, A. C., Zalut, C., Johnson, S., Tometsko, A., Schwartz, G., and Ginos, J., Biochemistry 6, 2656 (1967).

21. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J., J. Biol. Chem. 193, 265 (1951).

22. Katsoyannis, P. G., Tometsko, A., and Zalut, C., J. Am. Chem. Soc. 88, 5622 (1966).

23. Smart, N. A., Young, G. T., and Williams, M. W., J. Chem. Soc. 3902 (1960).

24. Sifferd, R. H., and du Vigneuad, V., J. Biol. Chem. 108, 753 (1935).

25. Katsoyannis, P. G., Tometsko, A. and Zalut, C., J. Am. Chem. Soc. 89, 4505 (1967).

26. Benisek, W. F., and Cole, R. D., Biochem. Biophys. Res. Comm. 20, 655 (1965).

27. Benisek, W. F., Raftery, M. A., and Cole, R. D., Biochemistry 6, 3780 (1967).

28. Katsoyannis, P. G., Zalut, C., Tometsko, A., Tilak, M., Johnson, S., and Trakatellis, A., J. Am. Chem. Soc. in press (1971).

29. Randall, S. S., Biochim. Biophys. Acta 90, 472 (1964).

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

31. Bromer, W. W., Sheehan, K. S., Berns, A. W., and Arquilla, E. R., Biochemistry 6, 2378 (1967).

32. Levy, D. and Carpenter, F. H., Biochemistry 6, 3559 (1967).

33. Levy, D., and Carpenter, F. H., Biochemistry 9, 3215 (1970).

34. Africa, B., and Carpenter, F. H., Biochemistry 9, 1962 (1970).

35. Weber, U.,Schneider, F., Köhler, P., Weitzel, G., Z. Physiol. Chem. 348, 947 (1967).

36. Weber, U., Hörnle, S., Grieser, G., Herzog, K.-H., and Weitzel, G., Z. Physiol. Chem. 348, 1715 (1967).

37. Weber, U., Hörnle, S., Köhler, P., Nagelschneider, G., Eisele, K., and Weitzel, G., Z. Physiol. Chem. 349, 512 (1968).

38. Weber, U., and Weitzel, G., Z. Physiol. Chem. 349, 1431 (1968).

39. Weber, U., Herzog, K.-H., Grossmann, H., Hörnle, S. and Weitzel, G., Z. Physiol. Chem. 350, 1425 (1969).

40. Hörnle, S., Weber, U., and Weitzel, G., Z. Physiol. Chem. 349, 1428 (1968).

41. Weitzel, G., Eisele, K., Zollner, H., and Weber, U., Z. Physiol. Chem. 350, 1480 (1969).

42. Weitzel, G., Weber, U., Eisele, K., Zollner, H. and Martin, J., Z. Physiol. Chem. 351, 263 (1970).