Synthesis of Deamino-A[1] Sheep Insulin†

Panayotis G. Katsoyannis* and Clyde Zalut

ABSTRACT: The synthesis and isolation in purified form of an analog of sheep insulin which differs from the parent molecule in that the α-amino group of the A chain has been replaced by hydrogen is described. For the synthesis of this analog, the deamino-A[1] chain of sheep insulin was first synthesized and isolated in the S-sulfonated form. Conversion of the latter material to the sulfhydryl form and combination with the S-sulfonated derivative of the B chain of bovine (sheep) insulin afforded the deamino-A[1] sheep insulin. Isolation of this analog from the combination mixture was effected by chromatography on a carboxymethylcellulose column with an exponential sodium chloride gradient. Deamino-A[1] insulin possesses a potency ranging from 7 to 10 IU per mg, when assayed by the mouse convulsion method.

In the study of the relationship between chemical structure and biological activity in polypeptide and protein hormones, special attention has been given to the role of the various functional groups. This is particularly true in the neurohypophysial hormones where the role of the primary amino, the phenolic hydroxyl and the three carboxamide groups on the biological activities of these molecules has been intensely investigated through the synthesis of a variety of analogs (for reviews, see Schwartz and Livingston, 1964, and Pickering, 1970).

One of the findings from these studies was that the free α-amino group of the neurohypophysial hormones is not essential for the manifestation of the biological effects associated with these hormones (Hope *et al.*, 1962; Kimbrough *et al.*, 1963). It was further shown that replacement of the α-amino group by hydrogen in the structure of these hormones results in considerably higher biological activities than the natural compounds (Hope *et al.*, 1962; Kimbrough *et al.*, 1963).

We have investigated the effect of comparable structural changes on the biological profile of insulin, as part of our program directed toward the understanding of the relationship between chemical structure and biological activity of this hormone (Katsoyannis, 1969; Katsoyannis *et al.*, 1967b, 1971). The present report describes the synthesis and isolation of such a modified molecule related to sheep insulin. This analog differs from the parent molecule in that the α-amino group of the A chain has been replaced by hydrogen.

Experimental Procedures and Results

Materials and Techniques. Preswollen microgranular CM-cellulose (Whatman CM 52/1) and Sephadex G-50 (Pharmacia Uppsala) were used in this investigation. Crystalline bovine zinc insulin was generously provided by Eli Lilly and Co. Sodium tetrathionate was prepared as described by Gilman *et al.* (1946). Amino acid analyses were performed in a Beckman-Spinco amino acid analyzer (Model 120C) equipped with a digital readout system (Model CRS 12AB, Infotronics Corp., Houston, Texas) according to the method of Spackman *et al.* (1958). To calculate the molar ratios, the average micromoles of glutamic acid, glycine, and leucine found was assumed to be equal to the theoretical number of residues in accordance with the known number of each of these residues in the compound to be analyzed. Protein determinations were carried out by the method of Lowry *et al.* (1951). Thin-layer electrophoresis was performed by a method developed in this laboratory (Tometsko and Delihas, 1967) and was carried out with a Wieland-Pfleiderer pherograph (Brinkman Instruments). Determinations of insulin activity were carried out by the mouse convulsion assay method as has been described previously (Katsoyannis and Tometsko, 1966; Katsoyannis *et al.*, 1967b). Optical rotations were taken with a Zeiss photoelectric precision polarimeter. Capillary melting points were determined for all compounds and are not corrected.

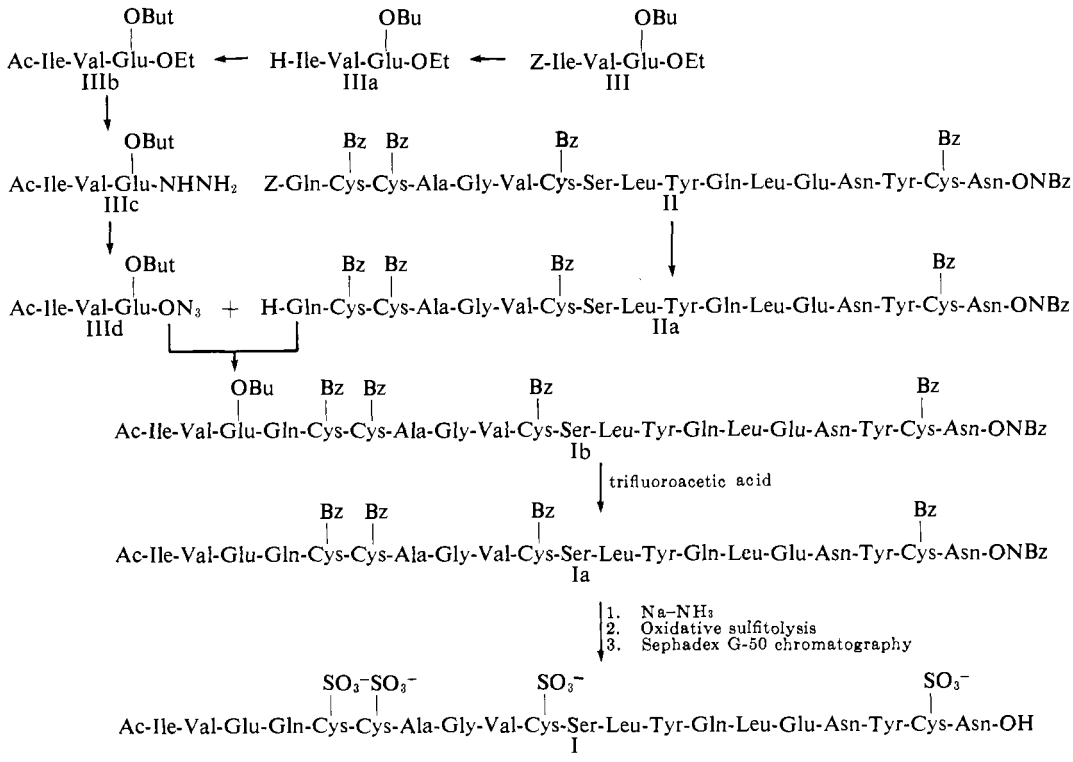
Synthesis of the Deamino-A[1] Sheep Insulin. For the preparation of this analog, the deamino-A[1] chain of sheep insulin in the sulfhydryl form is combined with the S-sulfonated derivative of the B chain of bovine (sheep) insulin. The synthesis of the deamino-A[1] chain in the protected form (Ib) was accomplished by the azide coupling of the partially protected heptadecapeptide derivative (IIa) containing the C-terminal sequence with the N-terminal tetrapeptide fragment (IIId) which has the α-amino group of the N-terminal residue, glycine, replaced by hydrogen (acetyl-tripeptide). The blocking of the secondary functions of the constituent amino acid residues and the synthesis of the various intermediates was accomplished by the standard procedures of peptide chemistry. Removal of the *tert*-butyl ester protecting group from the protected chain Ib was carried out upon treatment with trifluoroacetic acid. Removal of the remaining blocking groups, namely, *S*-benzyl and *p*-nitrobenzyl ester from Ia was effected on exposure to sodium in liquid ammonia (Sifferd and du Vigneaud, 1936). The deblocked deamino-A[1] chain on oxidative sulfitolysis (Bailey and Cole, 1959) was converted to the S-sulfonated derivative (I), which prior to its combination with the S-sulfonated B chain is transformed to the sulfhydryl form. The overall scheme which was used for the construction of the S-sulfonated derivative of the deamino-A[1] chain of sheep insulin is summarized in Chart I.

N-Benzyloxycarbonyl-L-isoleucyl-L-valyl-L-glutamic Acid α-Ethyl-γ-tert-butyl Ester (III). The synthesis of this peptide derivative was accomplished as described previously (Katsoyannis *et al.*, 1963).

† From the Department of Biochemistry, Mount Sinai School of Medicine, City University of New York, New York, New York 10029. *Received October 29, 1971.* This work was supported by the National Institute for Arthritis and Metabolic Diseases, U. S. Public Health Service (AM 12925). For the previous paper of this series, see *Biochemistry 10*, 3884 (1971).

* To whom correspondence should be addressed.

CHART I



*N-Acetyl-*L*-isoleucyl-*L*-valyl-*L*-glutamic Acid α-Ethyl-γ-tert-butyl Ester (IIIb).* A solution of III (5.8 g) in methanol (250 ml) was hydrogenated over 10% palladium–charcoal catalyst (1 g). After 1 hr, the catalyst was filtered off and the filtrate evaporated to dryness *in vacuo*. The residue (IIIa, 4.2 g) was dissolved in a mixture of benzene (40 ml) and chloroform (10 ml) and allowed to react with acetic anhydride (1 ml). After 24 hr at room temperature, the reaction mixture was diluted with ethyl acetate (40 ml) and the precipitated crystalline product was isolated by filtration and washed with ethyl acetate. The dry product was reprecipitated from a suspension in DMF[1] (100 ml) by the addition of 1 N acetic acid, collected, and washed successively with 1 N acetic acid, water, 1 N $NaHCO_3$, and water, and dried; wt 4.4 g (95% based on IIIa used), mp 229–231°. 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, 59.4; H, 8.86; N, 8.7. Found: C, 59.3; H, 8.75; N, 9.0.

*N-Acetyl-*L*-isoleucyl-*L*-valyl-γ-tert-butyl-*L*-glutamic Acid Hydrazide (IIIc).* A solution of IIIb (3.6 g) in methanol (150 ml) was treated with hydrazine hydrate (4 ml). After 24 hr at room temperature, the precipitated product was collected, washed with cold methanol and water, and dried: wt 3.1 g (89%), mp 288° dec, $[\alpha]_D^{26}$ −34.0° (*c* 1, dimethyl sulfoxide).

Anal. Calcd for $C_{22}H_{41}N_5O_6$: C, 56.1; H, 8.70; N, 14.9. Found: C, 56.3; H, 8.84; N, 15.1.

*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 (Deamino Sheep A[1] Chain S-Sulfonate).* (I). *N*-Benzyloxycarbonyl-L-glutaminyl-*S*-benzyl-L-cysteinyl-*S*-benzyl-L-cysteinyl-L-alanylglycyl-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 ester (II, 1 g), whose synthesis was described in a previous paper (Katsoyannis *et al.*, 1966), was dissolved in trifluoroacetic acid (15 ml) containing water (0.3 ml) and HBr was passed through the solution for 1.5 hr at 0°. Addition of anhydrous ether to the reaction mixture caused the precipitation of the partially protected heptadecapeptide IIa. This product was isolated by centrifugation, washed with ether, and dried over KOH *in vacuo*. To a solution of this material in DMF (50 ml), containing triethylamine (0.4 ml) and cooled to 0°, was added the acetyl-tripeptide azide IIId prepared as follows. A suspension of *N*-acetyl-L-isoleucyl-L-valyl-γ-*tert*-butyl-L-glutamic acid hydrazide (IIIc, 0.5 g) in DMF (35 ml) cooled to −15° was brought into solution by the addition of 2 N HCl (3.5 ml) and mixed with $NaNO_2$ (30 mg) dissolved in cold water (0.5 ml). After stirring for 5 min at −10°, the reaction mixture was poured into cold, half-saturated NaCl solution (150 ml) and the precipitated solid azide IIId was filtered, washed with cold water, and dried for 1 hr over P_2O_5 at 0° *in vacuo*. This azide was then added to the solution of the heptadecapeptide derivative prepared as described previously. The reaction was allowed to proceed at 0° with stirring over a period of 48 hr during which time it became necessary to add additional DMF (50 ml) to control the increasing viscosity of the reaction solution. The reaction mixture was then poured into methanol (500 ml) containing acetic acid (0.5 ml). The precipitated protected eicosapeptide Ib was isolated by filtration, washed successively with methanol, 50% aqueous methanol, and water, and dried; wt, 0.95 g (85%). A solution of this material in trifluoroacetic acid (20 ml) was stored at room temperature

[1] Abbreviations used are: DMF, dimethylformamide; Z, benzyloxycarbonyl; Bu, *tert*-butyl; NBz, *p*-nitrobenzyl; Et, ethyl; Bz, benzyl; N_3, azide; Ac, acetyl.

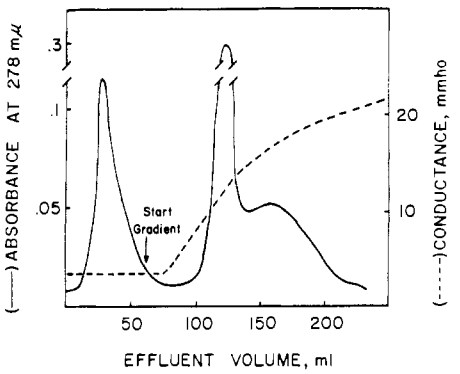


FIGURE 1: Chromatography of a combination mixture of deamino-A^1 chain of sheep insulin and B chain of bovine (sheep) insulin on a 0.9 × 23 cm CM-cellulose column with acetate buffer (pH 3.3; Na^+ 0.024 M) and an exponential NaCl gradient. The column effluent was monitored by a Gilford recording spectrophotometer and by a conductivity meter (Radiometer, Copenhagen). Two combination mixtures (see Experimental Procedures and Results) were processed. The deamino-A^1 sheep insulin (slowest moving component; 150–200 ml of effluent) was recovered as the hydrochloride (1.1 mg, specific activity 7–10 IU).

TABLE I: Amino Acid Composition[a] of the S-Sulfonated Deamino-A^1 Chain of Sheep Insulin.

Amino Acid	Theory	Found: After 24-hr Hydrolysis	Found: After 72-hr Hydrolysis
Aspartic acid	2	2.0	2.0
Serine	1	0.9	0.8
Glutamic acid	4	4.0	3.9
Glycine	1	1.0	1.0
Alanine	1	1.0	1.1
Half-cystine	4	2.8[b]	2.7[b]
Valine	2	1.7	2.0
Isoleucine	1	0.7	0.9
Leucine	2	2.0	2.1
Tyrosine	2	1.8[b]	1.6[b]

[a] Number of amino acid residues per molecule. [b] Uncorrected for destruction.

for 30 min and then filtered through a sintered-glass filter to remove any traces of impurities. Dilution of the filtrate with anhydrous ether caused the precipitation of the partially protected eicosapeptide derivative Ia; wt 0.9 g. Amino acid analysis of an acid hydrolysate showed the following composition expressed in molar ratios: $Asp_{2.0}Ser_{0.9}Glu_{3.9}Gly_{1.0}Ala_{1.1}$-$Val_{1.6}Ile_{0.6}Leu_{2.0}Tyr_{1.6}$S-BzCys$_{3.7}$, in good agreement with the values expected from theory. The low values of Val and Ile are due to the well known resistance to acid hydrolysis of Ile-Val.

The reduction of Ia (180 mg) with sodium in liquid ammonia (200 ml) and the sulfitolysis of the reduced product was accomplished by the procedure described previously (Katsoyannis *et al.*, 1966). After evaporation of the ammonia, the residue was dissolved in 8 M guanidine hydrochloride (20 ml) and to this solution adjusted to pH 8.9 with acetic acid was added sodium sulfite (600 mg) and sodium tetrathionate (300 mg). The reaction mixture was stirred at 25° for 4 hr and then dialyzed in a Visking 18/32 dialyzing tubing at 4° for 24 hr. Upon lyophilization of the dialysate, the deamino-A^1 chain *S*-sulfonate (I) was obtained as a white powder; wt 160 mg. For purification, the lyophilized material was chromatographed on a Sephadex G-50 column (fine grade, bead form; 2.4 × 50 cm) equilibrated and eluted with 5% acetic acid. The elution pattern of this column, as determined by monitoring the effluent by a Gilford recording spectrophotometer, indicated the presence of a single peak. Lyophilization of the effluent gave the purified final product I as a white fluffy material; wt 135 mg (85% based on Ia used).

Amino acid analysis of the purified product after acid hydrolysis gave the composition shown in Table I in a very good agreement with the theoretically expected values. On thin-layer electrophoresis in 0.5 N acetic acid (pH 2.9) and 3500 V and in 0.01 N NH_4HCO_3 (pH 10.0) and 3500 V the synthetic material moved as a single component (Pauly reaction).

S-Sulfonated Derivative of the B Chain of Bovine Insulin. This compound, which is identical with the corresponding sheep insulin derivative (Sanger and Tuppy, 1951a,b; Brown *et al.*, 1955), was prepared as described previously (Katsoyannis *et al.*, 1967c).

Isolation of Insulin Analog Produced by Combination of Synthetic Sheep Deamino-A^1 Chain and Natural Bovine (Sheep) B Chain (Deamino-A^1 Sheep Insulin). The synthesis of this insulin analog was carried out by interaction of the sulfhydryl form of the deamino-A^1 chain with the S-sulfonated form of the B chain (Katsoyannis and Tometsko, 1966; Katsoyannis *et al.*, 1967a). In a typical combination experiment, 20 mg of deamino-A^1 chain was converted to the sulfhydryl form and allowed to react with 5 mg of S-sulfonated B chain. The combination mixture was subsequently treated as described previously (Katsoyannis *et al.*, 1967a,b). Isolation of the insulin analog was accomplished by chromatography on a 0.9 × 23 cm CM-cellulose column with an exponential NaCl gradient as was described in detail in a previous report (Katsoyannis *et al.*, 1967a,b). Chromatography of two combination mixtures, each corresponding to the amounts of materials indicated above, gave the pattern shown in Figure 1. The insulin analog is eluted with application of the NaCl gradient and is the slowest moving material. A similar situation exists with the chromatographic pattern of natural insulin, all synthetic and half-synthetic insulins (Katsoyannis *et al.*, 1967b). The effluent containing the insulin analog was concentrated in a rotary evaporator to approximately 10 ml and the protein material was isolated *via* picrate as the hydrochloride (1.1 mg) as was described previously (Katsoyannis *et al.*, 1967b).

Amino acid analysis of the insulin analog after acid hydrolysis gave a composition expressed in molar ratios in good agreement with the theoretically expected values (Table II). On thin-layer electrophoresis in 0.5 N acetic acid (Figure 2) and in 0.01 N NH_4HCO_3 (Figure 3) the synthetic analog moved as a single component (Pauly reaction). This insulin analog, by the mouse convulsion assay method, was found to possess a potency of 7–10 IU/mg. Crystallization of this material was not attempted.

Discussion

The significance of the various functional groups of insulin to its biological activity has been pursued in several laboratories through the preparation of derivatives obtained by chemical modifications of the natural hormone with

TABLE II: Amino Acid Composition[a] of the Deamino-A^1 Sheep Insulin.

Amino Acid	Calcd	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
Half-cystine	6	4.4[b]
Valine	5	4.6
Isoleucine	1	0.6
Leucine	6	6.0
Tyrosine	4	2.9[b]
Phenylalanine	3	2.9

[a] Number of amino acid residues per molecule. [b] Uncorrected for destruction.

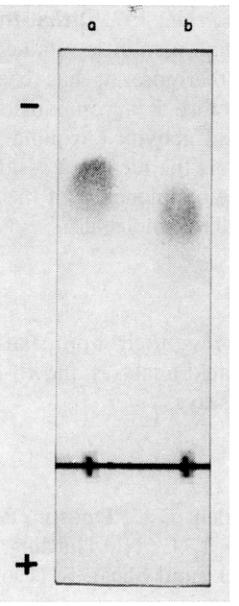


FIGURE 2: Thin-layer electrophoresis of natural bovine insulin (a) and synthetic deamino-A^1 sheep insulin (b): 0.5 N acetic acid, 3500 V, and 14 min.

specific reagents (Africa and Carpenter, 1970; Levy and Carpenter, 1967, 1970; Bromer *et al.*, 1967). This approach, however, is limited with respect to the modifications which can be introduced, and has the disadvantage that the reagents used for modifying functional groups lack specificity and often lead to a mixture of products. Although the product heterogeneity has been overcome in a few cases (see, for example, Africa and Carpenter, 1970), it appears that the approach which involves the chemical synthesis of insulin analogs not only permits an unlimited variety of modifications but also the isolation of these analogs in pure form. The synthetic approach thus appears to be the method of choice in structure–activity studies (Katsoyannis, 1969; Katsoyannis *et al.*, 1967b).

In this report, the synthesis and biological activity of deamino-A^1 sheep insulin, an analog lacking the α-amino group present in the A chain of the hormone are described. This permitted us to evaluate the extent to which the free amino group in the A chain is necessary for the manifestation of the biological activity of insulin as determined by the mouse convulsion assay method. It was found that deamino-A^1 sheep insulin possesses a potency of 7–10 IU/mg, which is approximately 35% of the biological activity of insulin (25 IU/mg). This substantial loss of activity resulting from the replacement of the amino group of the A^1 residue by hydrogen sharply contrasts with the finding that even total elimination of the B^1 residue does not affect the biological activity of the hormone (Brandenburg, 1969). On the other hand, removal of both the A^1 and B^1 residues, glycine and phenylalanine, respectively, from the insulin molecule (Brandenburg and Ooms, 1968; Africa and Carpenter, 1970) produces an analog that exhibits only about 10% of the hormonal activity of the native protein. On this basis, it may be deducted that the A^1 residue is important for the biological activity of insulin. We have now demonstrated that even a modification of the A^1 residue results in a substantial decrease in biological activity.

Africa and Carpenter (1970) have suggested that removal of the A^1 and B^1 residues results in conformational changes of the molecule which indirectly affect the active site of insulin so that its biological activity is diminished but not eliminated. In view of the data presented in this report, it would appear that only the A^1 residue is involved. It is interesting to note that the A^1 residue, unlike the residue B^1, is invariant in insulin sequences from various species (Smith, 1966). Furthermore, according to the three-dimensional structure of insulin this residue is on the surface of the molecule in all polymeric states of the hormone and probably contributes to the stabilization of its tertiary structure (Adams *et al.*, 1969; Blundell *et al.*, 1971a,b). The existing evidence suggests (for

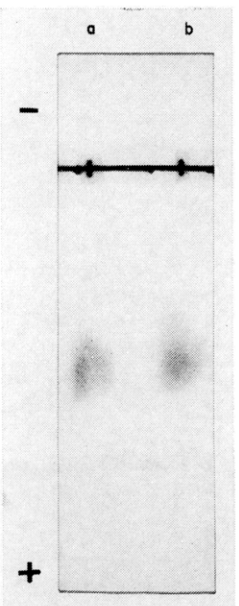


FIGURE 3: Thin-layer electrophoresis of natural bovine insulin (a) and synthetic deamino-A^1 sheep insulin (b): 0.01 N NH_4HCO_3, pH 10, 3500 V, and 14 min.

review, see Blundell *et al.*, 1971b) that the general molecular structure of crystalline insulin is retained when this protein is in solution. Furthermore, it has been indicated that a change in this structure is accompanied by a proportional decrease in biological activity (Arquilla *et al.*, 1969). These considerations support the idea that residue A^1 may be critically involved in the maintenance of the molecular structure required for a fully active molecule.

Acknowledgments

The authors express their appreciation to Miss Karin Scheibe for amino acid analyses and to Mrs. Mei-Ying Liu for the biological assays.

References

Adams, M. J., Blundell, T. L., Dodson, E. J., Dodson, G. G., Vijayan, M., Baker, E. N., Harding, M. M., Hodgkin, D. C., Rimmer, B., and Sheat, S. (1969), *Nature (London) 224*, 491.

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

Arquilla, E. R., Bromer, W. W., and Mercola, D. (1969), *Diabetes 18*, 193.

Bailey, J. L., and Cole, R. D. (1959), *J. Biol. Chem. 234*, 1733.

Blundell, T. L., Cutfield, J. F., Cutfield, S. M., Dodson, E. J., Dodson, G. G., Hodgkin, D. C., Mercola, D. A., and Vijayan, M. (1971a), *Nature (London) 231*, 506.

Blundell, T. L., Dodson, G. G., Dodson, E. J., Hodgkin, D. C., and Vijayan, M. (1971b), *Recent Progr. Hormone Res. 27*, 1.

Brandenburg, D. (1969), *Hoppe-Seyler's Z. Physiol. Chem. 350*, 741.

Brandenburg, D., and Ooms, H. A. (1968), *Protein Polypeptide Horm. Proc. Int. Symp., 1968*, 482.

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

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

Gilman, A., Philips, F. S., and Koelle, E. S. (1946), *Amer. J. Physiol. 164*, 348.

Hope, B. D., Murti, V. V. S., and du Vigneaud, V. (1962), *J. Biol. Chem. 237*, 1563.

Katsoyannis, P. G. (1969), *Diabetes, Proc. Congr. Int. Diabetes Fed., 6th, 1967, 172*, 379.

Katsoyannis, P. G., Fukuda, K., and Tometsko, A. (1963), *J. Amer. Chem. Soc. 85*, 1681.

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

Katsoyannis, P. G., Tometsko, A., Zalut, C., and Fukuda, K. (1966), *J. Amer. Chem. Soc. 88*, 5625.

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

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

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

Katsoyannis, P. G., Zalut, C., Harris, A., and Meyer, R. J. (1971), *Biochemistry 10*, 3884.

Kimbrough, Jr., R. D., Cash, W. D., Branda, L. A., Chan, W. Y., and du Vigneaud, V. (1963), *J. Biol. Chem. 238*, 1411.

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

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

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

Pickering, B. T. (1970), *in* Pharmacology of the Endocrine System and Related Drugs: The Neurohypophysis, Vol. 1, Heller, H., and Pickering, B. T., Ed., New York, N. Y., Pergamon Press, p 81.

Sanger, F., and Tuppy, H. (1951a), *Biochem. J. 49*, 463.

Sanger, F., and Tuppy, H. (1951b), *Biochem. J. 49*, 481.

Schwartz, I. L., and Livingston, L. M. (1964), *Vitamins Hormones 22*, 261.

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

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

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

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