et al., 1959) which we confirmed, already shows that transglutaminase is a ubiquitous enzyme with particularly high activities occurring in liver, lung, spleen, kidney, muscle, and placenta. It is probably of the utmost physiological protective significance that normal blood does not contain any transamidase at all. In this tissue such activity arises only during the process of coagulation through the limited proteolysis of the fibrin stabilizing factor zymogen (factor XIII) by thrombin (Lorand and Konishi, 1964; Lorand *et al.*, 1968b).

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Adrenocorticotropin. Solid-Phase Synthesis of α^{1-19}-Adrenocorticotropic Hormone, Alanyl-α^{1-19}-adrenocorticotropic Hormone, and Prolyl-α^{1-19}-adrenocorticotropic Hormone and Their Adrenocorticotropic Activity*

James Blake, Kung-Tsung Wang, and Choh Hao Li†

ABSTRACT: α^{1-19}-Adrenocorticotropic hormone nonadecapeptide, alanyl-α^{1-19}-adrenocorticotropic hormone eicosapeptide, and prolyl-α^{1-19}-adrenocorticotropic hormone eicosapeptide have been synthesized by the solid-phase method. A comparison of the *in vivo* steroidogenic potencies of the above peptides indicates that a one-residue extension of the peptide chain at the amino terminal lowers, but does not destroy, steroidogenic activity of α^{1-19}-adrenocorticotropic hormone.

Structure–activity studies on α-ACTH[1] and related peptides have, in part, been directed toward elucidating the importance of the free amino-terminal group for steroidogenic potency (Waller and Dixon, 1960; Lebovitz and Engel, 1963; Dixon, 1962). Synthetic peptides in which the amino-terminal serine has been replaced by proline, sarcosine, and

* From The Hormone Research Laboratory, University of California, San Francisco, California 94122. *Received September 20, 1971.* This work was supported in part by grants from the National Institute of General Medical Sciences (GM-2907), the Geffen Foundation, and the Allen Foundation. K.-T. W. is a Fulbright Scholar for 1969–1971 and on leave from the Department of Chemistry, National Taiwan University, Taiwan. Paper XLI in the Adrenocorticotropin series. For paper XL, see Li and Hemmasi (1972).

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[1] All the asymmetric amino acids occurring in the peptides mentioned in this paper are of the L configuration. Abbreviations used are: ACTH, adrenocorticotropic hormone; Boc, *tert*-butyloxycarbonyl; Bpoc, 2-(biphenyl)isopropyloxycarbonyl.

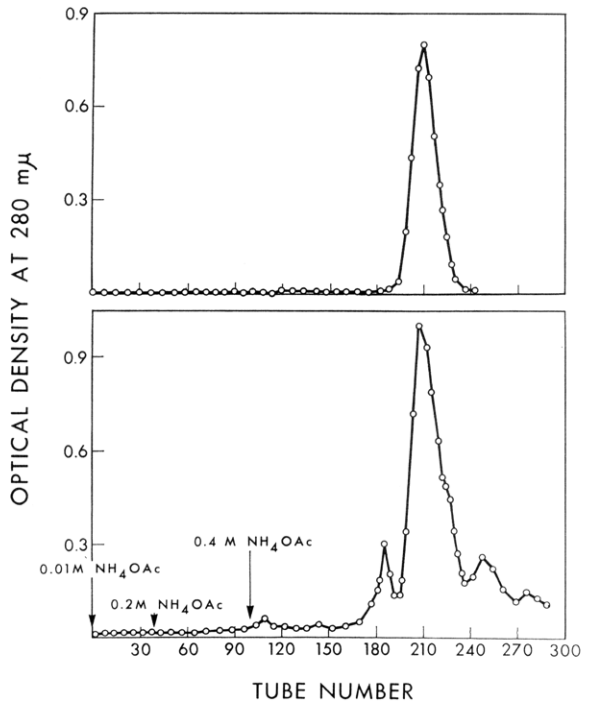

FIGURE 1: Lower: CM-cellulose chromatography (column, 1.0 × 55 cm) of crude nonadecapeptide I. The initial buffer was 0.01 M ammonium acetate, pH 4.5. After 40 tubes (4 ml/tube), a gradient with respect to pH and salt concentration was started by introducing 0.2 M ammonium acetate buffer of pH 6.7 through a 500-ml mixing flask containing the starting buffer. Later the gradient was increased by substituting 0.4 M ammonium acetate at the indicated position. Upper: rechromatography of the major peak under the same conditions.

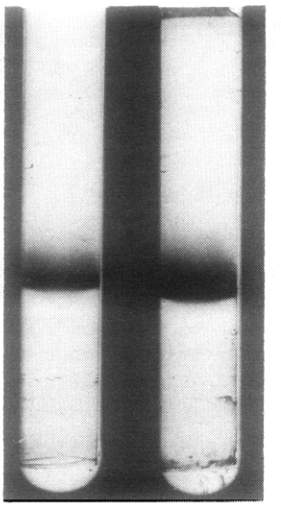

FIGURE 2: Gel electrophoresis of peptide I on polyacrylamide gel at pH 4.5. Left, 5 μg of peptide I; right, 10 μg of peptide I. Peptide bands were detected by staining with Amido-Schwarz dye.

γ-aminobutyric acid (Geiger *et al.*, 1969; Fujino *et al.*, 1970), thereby altering the nature and/or position of the amino group, have been shown to possess 50–150% of the biological activity of the peptide containing the natural sequence. The 51% activity reported for α^{2-23}-ACTH doeicosapeptide amide (Geiger *et al.*, 1964) in which the amino-terminal serine has been deleted from the natural sequence, indicates that displacement of the amino group is not critical for steroidogenic activity.

As a further clarification of the role of the NH_2-terminal serine in steroidogenic activity we now report the solid-phase synthesis of alanyl-α^{1-19}-ACTH eicosapeptide (II) and prolyl-α^{1-19}-ACTH eicosapeptide (III)—peptides which contain the natural nonadecapeptide sequence, but in which the amino group has been displaced away from the rest of the molecule. In addition, as an internal check of the above analogs and the solid-phase method, we also report the solid-phase synthesis of α^{1-19}-ACTH nonadecapeptide (I) which has already been synthesized by conventional procedures (Li *et al.*, 1960, 1964).

The synthesis of α^{1-19}-ACTH nonadecapeptide (I), H-Ser-Tyr-Ser-Met-Glu-His-Phe-Arg-Trp-Gly-Lys-Pro-Val-Gly-Lys-Lys-Arg-Arg-Pro-OH, followed the standard solid-phase procedure of Merrifield (1964). Amino acids were successively coupled to prolyl-resin as their N^{α}-Boc or N^{α}-Bpoc derivatives by activation with dicyclohexylcarbodiimide (Sheehan and Hess, 1955). Side chains were protected as follows: serine, *O*-benzyl; tyrosine, *O*-benzyl; glutamic acid, γ-benzyl ester; lysine, N^{ϵ}-benzyloxycarbonyl; histidine, N^{im}-*tert*-butyloxycarbonyl; arginine, N^{G}-tosyl. The last two deserve brief comment. We have observed that the use of the standard N^{G}-nitro protection for the side chain of arginine in solid-phase synthesis can lead to the formation of ornithine-containing peptides which are not readily separable from the desired product (Yamashiro *et al.*, 1971). However, the N^{G}-tosylarginine residue, which is also readily deblocked in liquid HF (Mazur and Plume, 1968), gives no such side products. The coupling of histidine was achieved with N^{α}-Bpoc-N^{im}-Boc-histidine (Yamashiro *et al.*, 1971); the succeeding amino acid residues of γ-benzylglutamic acid and methionine were also coupled as their Bpoc derivatives. The strategy herein was to minimize the deblocking of the histidine side chain during N^{α}-deprotection and thus reduce the possibility of a resulting amino acid acylated imidazole undergoing an acyl shift to a proximate amino terminal during neutralization. Although such a shift has yet to be reported, the reactivity of acylimidazoles toward amines (Paul and Anderson, 1960) suggests that the possibility does exist. Also, since deblocking of the N^{α}-Bpoc group resulted in partial deblocking of the N^{im}-Boc group (Yamashiro *et al.*, 1971), and we judged that the danger, if any, of an acyl shift should decrease with increasing distance between the terminal amino group and the imidazole moiety of histidine we returned to the more convenient N^{α}-Boc protection for the coupling of the last three amino acids.

Treatment of the protected nonadecapeptide-resin with liquid HF (Sakakibara *et al.*, 1967; Lenard and Robinson, 1967), and purification of the crude product by chromatography on Sephadex G-25 and carboxymethylcellulose (Peterson and Sober, 1956) gave peptide I (Figure 1). The product was shown to be homogeneous by paper electrophoresis and gel electrophoresis (Figure 2), and its amino acid composition was as expected (see the Experimental Section). Enzymatic digestion of peptide I with trypsin and chymotrypsin gave an electrophoresis pattern (Figure 3) identical with that obtained from the nonadecapeptide synthesized by conventional techniques (designated as peptide I-C). Also, peptides I and I-C were identical in their behavior on carboxymethylcellulose (see Li *et al.*, 1964 for the behavior of I-C on carboxymethylcellulose) and in their optical rotations.

Peptides II and III were obtained by coupling Boc-alanine and Boc-proline, respectively, to the nonadecapeptide-resin, and working up the product as described for peptide I.

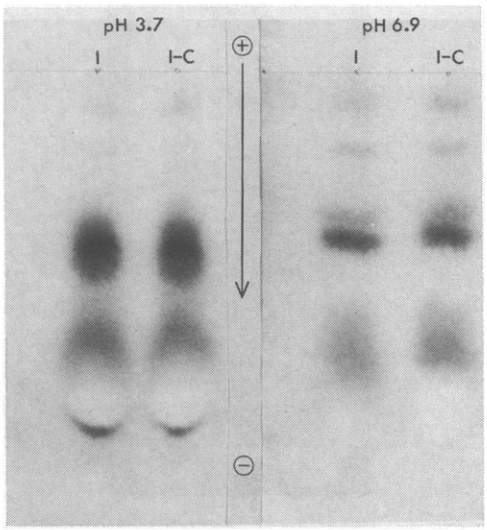

FIGURE 3: Paper electrophoresis at pH 3.7 and 6.9 of the trypsin–chymotrypsin digests of peptides I and I-C.

The *in vivo* steroidogenic activities of peptides I, II, III, and I-C relative to that of α_s-ACTH (Li *et al.*, 1955), as determined by a published procedure (Vernikos-Danellis *et al.*, 1966), are listed in Table I. The significant retention of activity in peptides II and III indicates that the position of the amino-terminal group relative to the remainder of the molecule is not critical for biological activity, in agreement with the results of Geiger *et al.* (1964), but it is essential for full activity of ACTH. Also of note are the comparable activities of peptides I and I-C which indicate that the solid-phase procedure is applicable for the synthesis of biologically active nonadecapeptide.

Experimental Section

Seryl-tyrosyl-seryl-methionyl-glutamyl-histidyl-phenylalanyl-arginyl-tryptophyl-glycyl-lysyl-prolyl-valyl-glycyl-lysyl-lysyl-arginyl-arginyl-proline (*I*). Boc-prolyl-resin (4.62 g, 1.10 mmoles of proline) was prepared by the standard reaction of chloromethylated polystyrene with the triethylammonium salt of Boc-proline in refluxing ethanol (Merrifield, 1964) and treated as follows: (1) wash with three 40-ml portions of methylene chloride; (2) deblocking by shaking with 55 ml (*total* volume) of trifluoroacetic acid–methylene chloride (1:1) for 20 min; (3) wash with three 40-ml portions of methylene chloride, three 40-ml portions of ethanol–chloroform (1:1), and three 40-ml portions of chloroform; (4) neutralize by shaking with 40 ml of chloroform and 3 ml of triethylamine for 5 min; (5) wash with three 50-ml portions of chloroform and three 40-ml portions of methylene chloride; (6) add 4.4 mmoles of N^{α}-Boc-N^{G}-tosylarginine (Ramachandran and Li, 1962) in 30 ml of methylene chloride and 4 ml of dimethylformamide and shake for 10 min; (7) add 4.4 mmoles of dicyclohexylcarbodiimide in 5 ml of methylene chloride and shake for 3 hr; (8) wash with four 40-ml portions of dimethylformamide and four 50-ml portions of ethanol.

Coupling of valine was achieved with 6.6 mmoles of Boc-valine. After the coupling of Boc-valine and Boc-proline, the peptide-resin was treated with 40 ml of dimethylformamide, 2 ml of acetic anhydride, and 1 ml of *N*-methylmorpholine for 20 min. After the introduction of tryptophan into the resin, deblocking of Boc groups with trifluoroacetic acid was accomplished in the presence of 0.5 ml of β-mercaptoethanol (Marshall, 1968). The methionine (Sieber and Iselin, 1968), glutamic acid (Wang and Merrifield, 1969), and histidine (Yamashiro *et al.*, 1971) residues were coupled as their N^{α}-Bpoc derivatives, and deblocking of the resulting peptides was achieved by two treatments with 0.04 N HCl in chloroform as previously described (Blake and Li, 1971).

TABLE I: Biological Activity of Synthetic α^{1-19}-ACTH, Alanyl-α^{1-19}-ACTH, and Prolyl-α^{1-19}-ACTH

Peptides	Adrenocorticotropic Potency[a] (units/mg)
I-C, α^{1-19}-ACTH	111 (83–144) [3]
I, α^{1-19}-ACTH	92 (76–122) [3]
II, Ala-α^{1-19}-ACTH	59 (40–81) [2]
III, Pro-α^{1-19}-ACTH	35 (20–50) [2]

[a] The potency for sheep ACTH was assumed to be 100 units/mg; the numbers in parentheses represent the 95% confidence limits; the numbers in brackets represent the number of assays.

A portion (0.5 g, 0.082 mmole) of the dried nonadecapeptide-resin was treated with 1.5 ml of anisole and 12 ml of liquid HF for 1 hr at 0°. After evaporation at 0°, the resin was dried under vacuum, and stirred 5 min with 5 ml of trifluoroacetic acid. The mixture was filtered, the resin was washed with trifluoroacetic acid, and the filtrate was evaporated to a residue which was distributed between 30 ml of 0.2 N acetic acid and 15 ml of ether. The aqueous layer was washed twice more with ether and was evaporated to a volume of *ca.* 10 ml. Chromatography on Sephadex G-25 (2.5 × 133 cm, 0.5 N acetic acid elution), followed by rechromatography of the major peak, gave 66 mg of peptide. Chromatography on carboxymethylcellulose followed by rechromatography of the major peak, tubes 197–222 (Figure 1), gave 18 mg of peptide I (peptide content 81% as determined by ultraviolet spectrum, 8% yield based on Boc-prolyl-resin); $[\alpha]^{25}D$ −83° (*c* 0.3, 0.1 N acetic acid) [lit. (Li *et al.*, 1964) $[\alpha]^{25}D$ −84.9° (*c* 0.5, 0.1 N acetic acid)].

Amino acid analysis (Spackman *et al.*, 1958) of an acid hydrolysate gave $Lys_{3.0}His_{1.0}Arg_{2.8}Ser_{1.7}Glu_{1.0}Pro_{2.1}Gly_{2.0}$-$Val_{1.0}Met_{0.9}Tyr_{1.0}Phe_{1.0}$. Measurement of the ultraviolet spectrum in 0.1 N sodium hydroxide (Beaven and Holiday, 1952) gave Tyr:Trp = 1.02. The ultraviolet spectrum of peptide I in 0.001 N HCl was identical with that of peptide I-C. Paper electrophoresis in pyridine–acetate buffer (pH 3.7, 400 V, 4 hr) gave one ninhydrin-positive, Pauly-positive spot at R_F 0.90 (relative to lysine). Paper electrophoresis in collidine–acetate buffer (pH 6.9, 400 V, 4 hr) gave one ninhydrin-positive. Pauly-positive spot at R_F 0.74. Electrophoresis on polyacrylamide gel at pH 4.5 showed one band (Figure 2).

Peptides I and I-C (0.5 mg) were treated separately with 0.3 ml of Tris buffer (pH 8.5, 0.01 M Mg^{2+}) and 10 μg each of trypsin and chymotrypsin at 37° for 24 hr. Paper electrophoresis of the digests at pH 3.7 and 6.9 gave identical peptide patterns (Figure 3) for I and I-C. The digest of peptide I was heated in boiling water for 15 min, cooled, and treated with 25 μg of leucine aminopeptidase for 48 hr at 37°. Amino acid analysis of the digest gave $Ser_{2.2}Tyr_{1.0}Met_{1.0}$-$Glu_{1.0}His_{1.0}Phe_{1.0}Arg_{1.8}Trp_{1.0}Gly_{1.4}Lys_{1.5}Pro_{0.0}Val_{0.4}$. The low

values of the amino acids located in the carboxyl half of peptide I are due to the well-known resistance to enzymatic digestion of peptide bonds involving proline.

Alanyl-seryl-tyrosyl-seryl-methionyl-glutamyl-histidyl-phenylalanyl-arginyl-tryptophyl-glycyl-lysyl-prolyl-valyl-glycyl-lysyl-lysyl-arginyl-arginyl-proline (II). A portion (0.7 g, 0.11 mmole) of the Boc-nonadecapeptide-resin was deblocked with trifluoroacetic acid–methylene chloride (1:1), neutralized with triethylamine, and coupled with 1 mmole of Boc-alanine and dicyclohexylcarbodiimide as described in the procedure above. After being washed and dried, the eicosapeptide resin (0.61 g) was treated with 1.8 ml of anisole and 12 ml of liquid HF for 1 hr at 0°. The mixture was evaporated, dried, and worked up as described for the synthesis of peptide I. The second chromatography on carboxymethylcellulose gave 14.1 mg of peptide II (peptide content 78%, 5%, yield based on Boc-prolyl-resin).

Amino acid analysis of an acid hydrolysate gave $Lys_{3.1}His_{1.0}Arg_{3.2}Ser_{1.7}Glu_{1.0}Pro_{2.0}Gly_{2.0}Ala_{1.0}Val_{1.0}Met_{1.0}Tyr_{0.9}Phe_{1.0}$. Measurement of the ultraviolet spectrum in 0.1 N sodium hydroxide gave Tyr:Trp = 0.97. Paper electrophoresis at pH 3.7 gave one ninhydrin-positive, Pauly-positive spot at R_F 0.89; paper electrophoresis at pH 6.9 gave one ninhydrin-positive, Pauly-positive spot at R_F 0.73. Electrophoresis on polyacrylamide gel at pH 4.5 showed one band.

Prolyl-seryl-tyrosyl-seryl-methionyl-glutamyl-histidyl-phenylalanyl-arginyl-tryptophyl-glycyl-lysyl-prolyl-valyl-glycyl-lysyl-lysyl-arginyl-arginyl-proline (III). A portion (0.7 g, 0.11 mmole) of the Boc-nonadecapeptide resin was treated as described for the preparation of peptide II, except that Boc-proline was substituted for Boc-alanine. The second chromatography on carboxymethylcellulose gave 16.6 mg of peptide III (peptide content 83%, 6% yield based on Boc-prolyl-resin). Amino acid analysis of an acid hydrolysate gave $Lys_{2.9}His_{1.0}Arg_{3.0}Ser_{1.7}Glu_{1.0}Pro_{3.0}Gly_{2.0}Val_{1.0}Met_{1.0}Tyr_{0.9}Phe_{1.0}$. Measurement of the ultraviolet spectrum in 0.1 N sodium hydroxide gave Tyr:Trp = 1.04. Paper electrophoresis at pH 3.7 showed one ninhydrin-positive, Pauly-positive spot at R_F 0.87; paper electrophoresis at pH 6.9 showed one ninhydrin-positive, Pauly-positive spot at R_F 0.69. Electrophoresis on polyacrylamide gel at pH 4.5 showed one band.

Acknowledgments

We thank J. D. Nelson, W. F. Hain, D. Gordon, and S. Lyles for their able technical assistance.

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