Tucker for their technical help in the immunoassays and bioassays. We are grateful to A. Erenea, C. Garcia, R. Wolbers, B. Lerch, and R. Kaiser for their excellent technical assistance. We thank Dr. R. Burgus for the amino acid analyses and Dr. N. Ling for the mass spectra. This research is supported by AID (Contract No. AID/csd 2785), the Ford Foundation, and the Rockefeller Foundation grants to Dr. Roger Guillemin, whom we gratefully acknowledge.

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Adrenocorticotropin.† 45. Synthesis and Steroidogenic Activity of [1-Alanine]adrenocorticotropin-(1–20)-amide, [1-D-Alanine]adrenocorticotropin-(1–20)-amide, and [[1-^{14}C]Alanine]adrenocorticotropin-(1–20)-amide

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Three biologically active peptides, [1-alanine]-α-adrenocorticotropin-(1–20)-amide (I), [1-D-alanine]-α-adrenocorticotropin-(1–20)-amide (II), and [[1-^{14}C]alanine]-α-adrenocorticotropin-(1–20)-amide (III), have been synthesized by the solid-phase method. All of the synthetic peptides exhibited high steroidogenic potency, and peptide II was shown to have prolonged steroidogenic activity *in vivo*.

The synthesis of the NH_2-terminal nonadecapeptide of α-ACTH and its analogs[2,3] has recently been achieved by the solid-phase method.[4] This communication reports the synthesis and adrenal-stimulating activity of [Ala^1]-α-ACTH-(1–20)-NH_2 (I), [D-Ala^1]-α-ACTH-(1–20)-NH_2 (II), and [^{14}C-Ala^1]-α-ACTH-(1–20)-NH_2 (III). The structure of I is shown in Figure 1.

The decision to synthesize these peptides was based upon several considerations. (i) Since the NH_2-terminal amino acid in ACTH is serine,[5] whose hydroxyl group must be protected in the solid-phase method, and since the side chain of serine does not contribute significantly to the steroidogenic activity of the hormone,[6,7] alanine was used to replace serine at the NH_2 terminus. (ii) The synthesis of II was carried out in order to determine whether D-alanine would enhance the biological activity as does D-serine.[8] (iii) It is desirable to synthesize a peptide whose biological potency is as close as possible to natural ACTH. Since α-ACTH-(1–19)-amide possesses greater steroidogenic activity than its free acid,[9] we decided to synthesize the amide instead of the free acid. (iv) For synthesis of a peptide amide, it was advisable to employ a phenyl ester linkage of the peptide to the polystyrene resin as demonstrated by our success in the synthesis of α-melanotropin[10] whose COOH-terminal amino acid is valine. Since the 20th amino acid in ACTH is valine,[5] we decided to synthesize I instead of the nonadecapeptide amide. Finally, for convenience in handling radioactive materials, it was deemed advisable to attach the radioactive label in the NH_2-terminal alanine.

Peptides I–III were synthesized in a manner analogous to that used for α-melanotropin.[10] The Boc-Val-O-C_6H_4-CH_2CO_2-resin was subjected to the standard procedure of the solid-phase method.[4] The side-chain protecting groups are described in the Experimental Section, but we note here the use of the *tert*-butyl group for protection of the side chain of glutamic acid. Although N^{α}-Boc protection

†For paper 44, see ref 1. All asymmetric amino acids occurring in the peptides mentioned in this paper are of the L configuration unless otherwise indicated. Abbreviations used are: ACTH, adrenocorticotropic hormone; Boc, *tert*-butyloxycarbonyl; Bpoc, 2-(*p*-biphenylyl)isopropyloxycarbonyl; DCC, dicyclohexylcarbodiimide; CMC, carboxymethylcellulose.

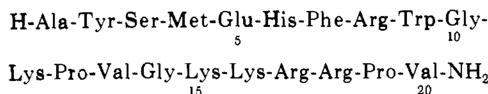

Figure 1. Amino acid sequence of [Ala[1]]-α-ACTH-(1-20)-NH_2.

was used in the first 13 cycles, the remaining residues (His, Glu, Met, Ser, Tyr) with the exception of alanine were coupled as their N^α-Bpoc derivatives in order to prevent deblocking of the *tert*-butyl ester during N^α deprotection. After coupling of the alanine residue (Boc derivative), the peptide resin was treated first with trifluoroacetic acid to remove the *tert*-butyl group and then with ammonia at −20° to give the protected peptide amide. Treatment of the protected peptide amide with liquid HF at 0°[11,12] was followed by purification of the crude product by gel filtration on Sephadex G-25. The highly purified products were obtained by chromatography on carboxymethylcellulose.[13] Figure 2 shows the CMC chromatograms for peptide III.

The homogeneity of the products was shown by paper electrophoresis at two pH's and amino acid analyses of acid and enzymic hydrolysates. In the case of [^{14}C-Ala[1]]-α-ACTH-(1-20)-NH_2, a radioactivity scan of the electrophoretic pattern showed a single symmetric peak.

The peptides were assayed for *in vivo* steroidogenic activity[14] and the results are summarized in Table I. The activities of peptides I and III compared favorably to the activity of α-ACTH-(1-19)-NH_2[7] synthesized by the solution method. By the same assay procedure, α-ACTH-(1-19)-NH_2 was found to possess 140 units/mg.[15] Peptide II appeared to have the same potency as peptides I and III. This was lower than we expected because of the reported very high activity of [D-Ser[1],Nle[4],Val[25]]-α-ACTH-(1-25)-NH_2);[8] however, this apparent discrepancy was resolved by a comparative time study as shown in Figure 3. Although peptides I and II produced equal amounts of corticosterone in 5 min (the time on which the activities in Table I are based), peptide II was producing significantly more corticosterone than peptide I after 10 and 20 min. The prolonged steroid production for peptide II was presumably due to its greater resistance to destruction by enzymatic digestion. This result lends additional support to the explanation generally accepted for the increase in steroidogenic activity associated with the substitution of the amino terminal serine by D-serine,[8] β-alanine,[16] or α-aminoisobutyric acid[17] in peptides related to ACTH. Apparently these amino acids increase the lifetime of the peptide in the biological system by virtue of the resistance which they confer toward digestion by aminopeptidases.

Table I. Biological Activity of the Synthetic Peptides

Peptide	*In vivo* steriodogenesis,[a] units/mg
[Ala[1]]-α-ACTH-(1-20)-NH_2 (I)	142 (87–224) [3]
[D-Ala[1]]-α-ACTH-(1-20)-NH_2 (II)	153 (89–240) [3]
[^{14}C-Ala[1]]-α-ACTH-(1-20)-NH_2 (III)	150 (102–216) [2]

[a]Activity was measured against highly purified α_s-ACTH [B. T. Pickering, R. N. Andersen, P. Lohmar, Y. Birk, and C. H. Li, *Biochim. Biophys. Acta*, **74**, 763 (1963)] with a potency of 100 units/mg. Numbers in parentheses represent the 95% confidence limits; numbers in brackets represent the number of assays.

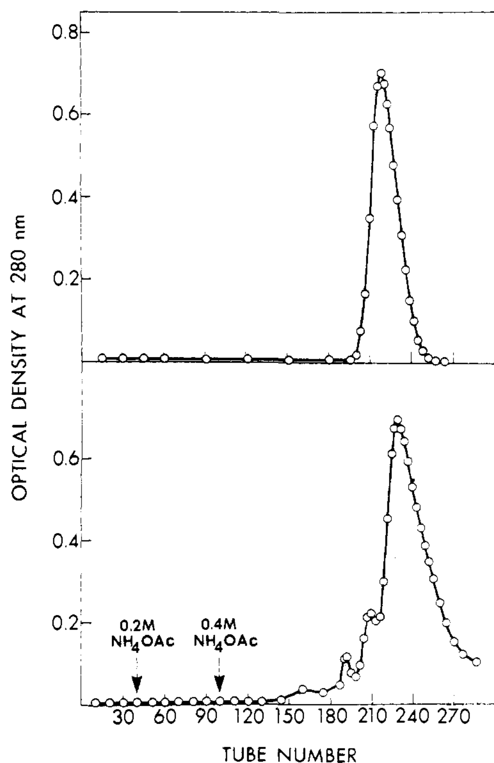

Figure 2. Lower: carboxymethylcellulose chromatography (column 1 × 55 cm) of crude peptide III. The initial buffer was 0.01 *M* ammonium acetate, pH 4.5. After 40 tubes, 4 ml/tube, a gradient with respect to pH and salt concentration was started by introducing 0.2 *M* ammonium acetate buffer of pH 6.7 through a 500-ml mixing flask containing 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.

Experimental Section

tert-**Butyloxycarbonyl-L-[^{14}C]alanine.** A solution of L-[^{14}C]alanine (250 μCi, 0.0017 mmol) in 5 ml of 0.01 *N* hydrochloric acid was evaporated to dryness and the residue was dissolved in 1.2 ml of water and 0.9 ml of dioxane. Unlabeled alanine, 44.5 mg (0.5 mmol), and 27.4 mg of magnesium oxide were added and the mixture was stirred for 10 min at 45°. Then 0.2 ml of Boc azide was added and the mixture was stirred for 18 hr at 45°. The reaction mixture was evaporated to dryness and the residue was dissolved in 5 ml of water. After an addition of alkali to raise the pH to 9, the aqueous solution was washed with two 3-ml portions of ether. The aqueous layer was cooled to 0°, saturated with sodium chloride, and acidified to pH 3 by the addition of hydrochloric acid. The product was extracted with two 10-ml portions of ethyl acetate and the combined organic extracts were washed with

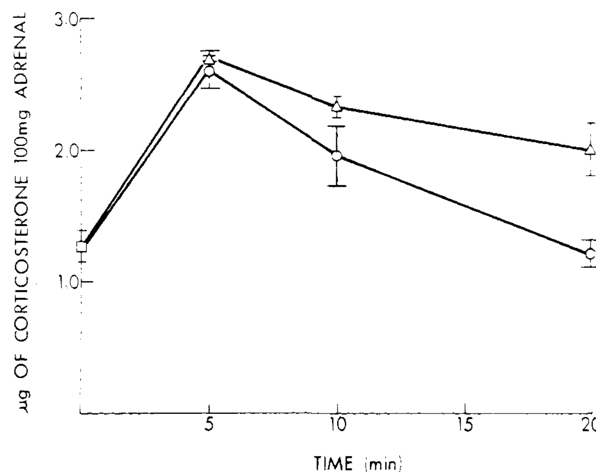

Figure 3. Amount of corticosterone in the adrenal glands of hypophysectomized rats as a function of time after the injection of 3 ng of peptide: [Ala[1]]-α-ACTH-(1-20)-NH_2 (--○--); [D-Ala[1]]-α-ACTH-(1-20)-NH_2 (--△--); basal level of corticosterone (--□--). Each point represents the mean of four rats; the vertical lines indicate the standard error in each determination.

water, dried, and evaporated to an oily residue. Petroleum ether (1 ml) was added and the residue was stored at 4° overnight. The crystalline product that developed was washed with cold petroleum ether and dried to yield 70 mg (0.37 mmol) of *tert*-butyloxycarbonyl-L-[^{14}C]alanine, mp 80–82° (reported 80–82°,[18] 83–84°[19]). Thin-layer chromatography on silica gel in the system chloroform–methanol (1:1, v/v) gave a single chlorine-positive spot. Radioactivity measurement by liquid scintillation showed the product to contain 470 μCi/mmol.

[Ala^1]-α-ACTH-(1-20)-NH$_2$. The *tert*-butyloxycarbonyl valyl ester of *p*-hydroxyphenylacetoxy resin[10] (4.31 g, 0.82 mmol of valine) was allowed to react with acetic anhydride as previously described[10] and treated by the following steps: (a) washed with three 50-ml portions of methylene chloride; (b) treated with 50 ml (total volume) of trifluoroacetic acid–methylene chloride (1:1, v/v) for 15 min; (c) washed with three 50-ml portions of methylene chloride; (d) washed with three 50-ml portions of ethanol-chloroform (2:3, v/v); (e) washed with three 50-ml portions of chloroform; (f) treated with 50 ml of chloroform and 2.5 ml of triethylamine for 5 min; (g) washed with three 50-ml portions of chloroform; (h) washed with three 50-ml portions of methylene chloride; (i) treated with 3.4 mmol of *tert*-butyloxycarbonyl proline in 22 ml of methylene chloride for 10 min; (j) addition of 3.4 mmol of DCC in 6 ml of methylene chloride and shook for 3.5 hr; (k) washed with three 50-ml portions of dimethylformamide and three 50-ml portions of ethanol.

Side-chain protecting groups were as follows: Arg, N^G-*p*-toluenesulfonyl; Lys, N^ϵ-*p*-bromobenzyloxycarbonyl;[20] Ser, *O*-benzyl; Tyr, *O*-benzyl; His, N^{im}-Boc; Glu, γ-*tert*-butyl ester. N^α protection was by the Boc group, except for Tyr, Ser, Met, Glu, and His, which were protected by the N^α-Bpoc group. Deblocking of the N^α-Bpoc group was effected in 0.05 *N* HCl in chloroform as previously described.[10] Deblocking of the N^α-Boc group in the presence of tryptophan was accomplished in the presence of 0.2 ml of 2-mercaptoethanol.[21]

The peptide resin was dried after coupling of the tyrosine residue. An aliquot of the peptide resin was coupled with *tert*-butyloxycarbonyl alanine by the procedure described above and washed with dimethylformamide, ethanol, and methylene chloride. The peptide resin was, in succession, treated with trifluoroacetic acid–methylene chloride (1:1, v/v) for 30 min, washed with methylene chloride, chloroform–ethanol, and chloroform, treated with triethylamine in chloroform, washed with chloroform and ethanol, and then dried.

A portion of the eicosapeptide resin (0.79 g, 0.097 mmol) was suspended in 14 ml of dimethylformamide and the mixture was cooled to −20°. Ammonia was bubbled into the stirring mixture for 3.5 hr; measurement of OD_{280} indicated 0.041 mmol of peptide had been cleaved from the resin. The mixture was filtered and the filtrate evaporated to a residue which was treated with 2.2 ml of anisole and 10 ml of liquid HF for 40 min at 0°. Evaporation of HF at 0° gave a residue that was dissolved in trifluoroacetic acid and evaporated again. The resultant residue was distributed between 20 ml of 0.2 *N* acetic acid and 10 ml of ether; the aqueous layer was washed with two more portions of ether and then evaporated to *ca.* 5-ml volume and chromatographed on Sephadex G-25 in 0.5 *N* acetic acid. Material corresponding to the major peak was isolated and lyophylized. The crude eicosapeptide was chromatographed on carboxymethylcellulose (Figure 2, lower) and the major peak was rechromatographed (Figure 2, upper) to give 24.5 mg of peptide I (peptide content 79%, with 8% yield based on starting *tert*-butyloxycarbonyl valyl resin).

Paper electrophoresis in collidine acetate buffer (pH 6.9, 400 V, 3.5 hr) showed one ninhydrin-positive, Pauly positive spot at R_f 0.73 (Lys standard); paper electrophoresis in pyridine acetate buffer (pH 3.7) showed a single spot at R_f 0.94. Amino acid analysis[22] of an acid hydrolysate gave $Lys_{2.8}His_{0.9}Arg_{3.1}Ser_{0.9}Glu_{1.0}Pro_{2.0}Gly_{2.0}Ala_{1.0}Val_{2.0}Met_{1.0}Tyr_{0.9}Phe_{1.0}NH_{3\ 0.9}$. A solution of 0.75 mg of peptide I in 0.4 ml of Tris buffer (pH 8.5, 0.01 *M* Mg^{2+}) was treated with 15 μg of trypsin and 15 μg of chymotrypsin for 24 hr at 37°. The solution was then heated in boiling water for 15 min, cooled, and treated with 30 μg of leucineaminopeptidase for 44 hr at 37°. Amino acid analysis of the digest gave $Trp_{1.0}Lys_{3.2}His_{1.0}Arg_{3.0}Ser_{1.1}Glu_{1.0}Pro_{2.2}Gly_{2.0}Ala_{1.1}Val_{2.1}Met_{0.9}Tyr_{1.0}Phe_{1.0}$.

[D-Ala^1]-α-ACTH-(1-20)-NH$_2$ (II). Peptide II was obtained from the protected nonadecapeptide resin by the same method used to obtain peptide I, except the final coupling was with *tert*-butyloxycarbonyl-D-alanine.[23] The yield was 23 mg of peptide II (peptide content 76%, with 7% yield based on starting *tert*-butyloxycarbonyl valyl resin). Paper electrophoresis at pH 6.9 and 3.7 gave single spots at R_f 0.69 and 0.95, respectively. Amino acid analysis of an acid hydrolysate gave $Lys_{3.2}His_{1.0}Arg_{3.0}Ser_{0.9}Glu_{1.0}Pro_{2.1}Gly_{2.1}Ala_{1.0}Val_{1.8}Met_{0.9}Tyr_{0.9}Phe_{0.9}$. Amino acid analysis (on the long column) of an enzyme digest as described above for peptide I gave $Ser_{1.0}Glu_{1.0}Pro_{2.4}Gly_{2.0}Ala_{0.1}Val_{2.1}Met_{0.9}Tyr_{0.1}Phe_{1.0}$.

[^{14}C-Ala^1]-α-ACTH-(1-20)-NH$_2$ (III). Peptide III was obtained from the protected nonadecapeptide resin by the same method used to obtain peptide I, except the final coupling was with *tert*-butyloxycarbonyl[^{14}C]alanine. The yield was 23 mg of peptide III (peptide content 78%, with 9% yield based on starting *tert*-butyloxycarbonyl valyl resin). Paper electrophoresis at pH 6.9 and 3.7 gave single spots at R_f 0.77 and 0.91, respectively. Before spraying with ninhydrin, the electrophoretic pattern was also scanned for radioactivity with the Nuclear-Chicago actigraph III and the result was a single symmetric peak. In both cases the position of the radioactivity peak corresponded to the ninhydrin-positive spot. Amino acid analysis of an acid hydrolysate gave $Lys_{3.0}His_{0.9}Arg_{3.0}Ser_{0.8}Glu_{1.0}Pro_{2.2}Gly_{2.0}Val_{2.0}Met_{1.0}Tyr_{0.9}Phe_{0.9}$. Radioactivity measurement by liquid scintillation showed peptide III to contain 410 μCi/mmol.

Acknowledgments. We wish to thank W. F. Hain, S. Liles, J. D. Nelson, and D. Key for their able technical assistance. This work was supported in part by grants from the National Institute of Health (GM-2907), the Allen Foundation, and the Geffen Foundation.

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