positive charge is not located on the nitrogen atom conventionally written with a positive charge alongside it; the hydrogen atoms attached to it are only slightly more positive than others in the molecule.

This charge picture emphasizes the naivete of attempts to picture receptor binding sites in terms of a localized negative charge with which the onium cation must bind. A more realistic view is that the receptor site should be negatively charged so that long-range coulombic forces may be used in attracting the overall positive histamine cation but that when close-up the specificity is provided by the steeper dispersion force potential. The charge distribution may reveal something of the complimentary nature of the receptor. It may also be worthwhile to calculate the molecular electrostatic potential of the ion and to investigate the variation of charge density as a function of conformation.

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Adrenocorticotropin. 49.[1] Synthesis and Biological Activity of [2-δ-Aminovaleric acid,5-arginine]adrenocorticotropin-(2–19)

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An adrenocorticotropin analogue, [2-δ-aminovaleric acid,5-arginine]adrenocorticotropin-(2–19), has been synthesized by the solid-phase method and its biological activity has been determined. It was found that substitution of arginine for glutamic acid at position 5 of [2-δ-aminovaleric acid]adrenocorticotropin-(2–19) increased the steroidogenic potency in isolated rat adrenal cells and the lipolytic potency in isolated rat fat cells but decreased the lipolytic potency in isolated rabbit fat cells. The synthetic analogue had only 2% of the melanotropic potency of the parent molecule.

Draper et al.[2,3] reported recently that [Arg^5]-ACTH-(4–10) had a lipolytic activity in rabbit fat cells that was five times greater than that of the natural sequence, ACTH-(4–10). Since there is a good correlation between lipolytic activity and steroidogenic activity,[4] we deemed it of interest to synthesize an adrenocorticotropically active fragment of ACTH to determine if the equivalent amino acid substitution would increase steroidogenic potency. The peptide fragment that we have synthesized is [Ava^2,Arg^5]-ACTH-(2–19) (III, see Figure 1). We have previously shown[5] that [Ava^2]-ACTH-(2–19) (II) possesses a greater in vivo steroidogenic potency than does ACTH-(1–19) (I).

Peptide III was synthesized by the solid-phase procedure[6] as described for the synthesis of I.[7] Treatment of the protected peptide resin with liquid HF,[8,9] followed by purification of the crude peptide by chromatography on Sephadex G-25 and carboxymethylcellulose, gave peptide III which was shown to be highly purified by paper electrophoresis, amino acid analysis, and thin-layer chromatography.

A summary of the biological activities of peptide III is reported in Table I together with that of peptides I and II, for comparison. It may be seen that the substitution of arginine for glutamic acid (III vs. II) significantly increases the steroidogenic activity. The combination of the arginine substitution in position 5 and the substitution of δ-aminovaleric acid for the amino terminal Ser-Tyr residues of peptide I (III vs. I) doubles the steroidogenic potency.

The most remarkable aspect of the biological data is the significant decrease of melanotropic activity while increasing steroidogenic activity (III vs. II) due to the substitution of arginine for glutamic acid. Peptide III possesses approximately 2% of the melanotropic activity of peptide II in both the in vivo and in vitro assays. This contrasts with the general observation[4] that melanotropic activity is equally or less sensitive than steroidogenic activity to structural changes in ACTH peptides. It was reported earlier that amino acid substitutions in ACTH peptides which give analogues possessing only a fraction of the steroidogenic activity also result in either comparable changes[10,11] or moderate decrease[12] of melanotropic activity. The most dramatic example[13,14] is [Trp-(Nps)^9]-ACTH, which has 1% of the steroidogenic activity and 300% of the melanotropic activity of the parent molecule. A comparison of the melanotropic and steroidogenic potencies of peptides III and II shows a complete reversal of this relationship. We believe this marks the first instance thereof.

Also surprising in the biological data is the decrease in lipolytic activity in isolated rabbit fat cells (III vs. II) upon the substitution of arginine for glutamic acid in contrast to the result[2,3] obtained for the equivalent substitution in ACTH-(4–10). The most likely explanation for this difference probably relates to the different chain lengths of the peptide pairs and to the difficulties in extrapolating the results obtained for a small peptide to a larger peptide. In agreement with our observation is another recent result in this laboratory. The substitution of arginine for glutamic acid at position 8 (within the Met-Glu-His-Phe-Arg-Trp-Gly sequence) of camel β_2-melanotropin gives a peptide possessing 16% of the melanotropic activity and 41% of the lipolytic activity of the native peptide.[15,16] It is of interest that the decrease in lipolytic activity in rabbit fat cells qualitatively parallels the decrease in melanotropic activity in agreement with a previous suggestion[4,17] that the hormonal receptors in rabbit fat cells and frog melanophores are similar.

Finally, the lipolytic activity in isolated rat fat cells (III vs. II) shows an increase in potency due to the substitution of arginine for glutamic acid. This parallels the increase in steroidogenic potency and is also in agreement with a previous suggestion[4,17] that the hormonal receptors of rat

H-Ser-Tyr-Ser-Met-Glu-His-Phe-Arg-Trp-Gly-Lys-Pro-Val-Gly-Lys-Lys-Arg-Arg-Pro-OH
1 3 5 7 9 11 13 15 17 19

I, ACTH-(1-19)

H-Ava-Ser-Met-Glu-His-Phe-Arg-Trp-Gly-Lys-Pro-Val-Gly-Lys-Lys-Arg-Arg-Pro-OH
3 5 7 9 11 13 15 17 19

II, [Ava^2]-ACTH-(2-19)

H-Ava-Ser-Met-Arg-His-Phe-Arg-Trp-Gly-Lys-Pro-Val-Gly-Lys-Lys-Arg-Arg-Pro-OH
3 5 7 9 11 13 15 17 19

III, [Ava^2,Arg^5]-ACTH-(2-19)

Figure 1. Structure of peptides I-III.

Table I. Biological Activity

	Steroidogenesis in vitro[a]	Lipolysis in vitro		Melanotropic stimulating act.	
		Rat[a]	Rabbit[b]	In vitro (units/mmol)[c]	In vivo (nmol)[d]
ACTH-(1-19) (I)	1.00	1.00		23×10^7 ($7-30 \times 10^7$) [3]	0.1
[Ava^2]-ACTH-(2-19) (II)	1.26 (0.80-2.22) [2]	0.57 (0.52-0.63) [2]	1.00	10×10^7 ($3-23 \times 10^7$) [3]	0.1
[Ava^2,Arg^5]-ACTH-(2-19) (III)	2.19 (1.36-3.75) [2]	1.58 (1.03-2.47) [1]	0.27 (0.10-0.56) [2]	0.2×10^7 ($0.01-0.52 \times 10^7$) [3]	5

[a] Potency was measured against ACTH-(1-19) standard. Numbers in parentheses represent the 95% confidence limits; numbers in brackets represent the number of assays. [b] Potency of peptide III was measured against peptide II standard. [c] Potency measured against sheep ACTH standard whose potency was 45×10^7 units/mmol. [d] Dose required to produce a change of melanophore index in *Rana pipiens* from 1+ to 3+ within 1 h.

fat cells and rat adrenal cells are similar.

Experimental Section

H-Ava-Ser-Met-Arg-His-Phe-Arg-Trp-Gly-Lys-Pro-Val-Gly-Lys-Lys-Arg-Arg-Pro-OH (III). In a previous publication[12] we described the synthesis of a protected nonadecapeptide resin corresponding to the sequence of [9-β-(1-naphthylalanine)]-ACTH-(1-19). Midway through that synthesis a portion (1.18 g, 0.24 mmol of peptide) of Boc-Gly-Lys(*o*-BrZ)-Pro-Val-Gly-Lys(*o*-BrZ)-Lys(*o*-BrZ)-Arg(Tos)-Arg(Tos)-Pro-resin was removed and subjected to the following reaction procedure: (a) washed with three 12-ml portions of methylene chloride; (b) treatment with 15 ml (total volume) of trifluoroacetic acid–methylene chloride (1:1) for 15 min; (c) washed with three 12-ml portions of methylene chloride, three 12-ml portions of methylene chloride–ethanol (2:1), and three 12-ml portions of methylene chloride; (d) treatment with 12 ml of methylene chloride and 0.8 ml of diisopropylethylamine for 5 min; (e) washed with six 12-ml portions of methylene chloride; (f) treatment with 0.75 mmol of *tert*-butyloxycarbonylamino acid symmetrical anhydride in 8 ml of methylene chloride for 30 min; (g) addition of 0.2 ml of diisopropylethylamine to the above coupling mixture and shaking for an extra 15 min; (h) washed with three 12-ml portions of methylene chloride and three 12-ml portions of ethanol. After eight cycles the protected octadecapeptide resin was dried under vacuum.

The following procedure was used to prepare the symmetrical anhydrides[18,19] of the *tert*-butyloxycarbonylamino acids. To a solution of 1.5 mmol of *tert*-butyloxycarbonylamino acid in 5.5 ml of methylene chloride at 0° was added 0.75 mmol of dicyclohexylcarbodiimide in 0.62 ml of methylene chloride. The mixture was stirred for 15 min at 0° and filtered rapidly and the precipitate was washed with approximately 2 ml of methylene chloride. The combined filtrate was added to the peptide resin at step f.

After the introduction of tryptophan in the peptide resin, 0.1 ml of β-mercaptoethanol[20] was added in step b. Side-chain protecting groups were as follows: Ser, *O*-benzyl; Arg, N^G-*p*-toluenesulfonyl; His, N^{im}-Boc.

A portion of the protected octadecapeptide resin (0.7 g) was treated with 2 ml of anisole and 10 ml of liquid HF for 40 min at 0°. The HF was evaporated at 0°, the peptide mixture resin was dried under vacuum, and then 15 ml of trifluoroacetic acid was added. After stirring for 10 min, the mixture was filtered and the filtrate was evaporated to a residue that was distributed between 20 ml of 0.2 N acetic acid and 10 ml of ether. The aqueous layer was washed further with 10 ml of ether and it was then evaporated to approximately 2-ml volume and chromatographed on Sephadex G-10. Material corresponding to the single peptide peak, obtained by elution with 0.5 N acetic acid, was combined, evaporated to approximately 2-ml volume, and chromatographed on Sephadex G-25 in 0.5 N acetic acid. Lyophilization of the fractions corresponding to the main peak gave 70 mg of crude peptide III. Chromatography on carboxymethylcellulose as previously described[7] for peptide I, followed by rechromatography in the same system, gave 8 mg of peptide III (76% peptide content by ultraviolet absorption at 280 nm, 3% yield based on starting *tert*-butyloxycarbonylprolyl resin).

Paper electrophoresis of peptide III (400 V, 3 h) at pH 3.7 and 6.9 gave single ninhydrin-positive, Pauly-positive, chlorine-positive spots at R_f^{Lys} 1.03 and 0.92, respectively. Paper electrophoresis at pH 11.0 (0.01 M sodium carbonate) gave a single ninhydrin-positive, Pauly-positive spot at R_f^{Arg} 1.85. Thin-layer chromatography of peptide III on silica gel in the system 3.4% trichloroacetic acid in *sec*-butyl alcohol–water (14.5:5.5) gave a single ninhydrin-positive, chlorine-positive spot at R_f 0.3. Amino acid analysis[21] on an acid hydrolysate gave $Lys_{3.0}His_{1.1}Arg_{3.8}Ser_{0.9}Pro_{2.1}Gly_{1.9}Val_{1.0}Met_{0.9}Phe_{1.0}Ava_{1.0}$. A sample (0.5 mg) of peptide III was dissolved in 0.25 ml of Tris buffer, pH 8.5, and treated with 10 μg of trypsin and 10 μg of chymotrypsin for 22 h at 37°. The solution was boiled for 15 min, cooled, and treated with 20 μg of leucine aminopeptidase for 44 h at 37°. Amino acid analysis of the digest gave $Trp_{0.9}Lys_{2.9}His_{1.0}Arg_{3.1}Pro_{1.7}Gly_{1.8}Val_{1.0}Phe_{1.0}$. There was less than 0.1 residues of Ser and Met, and Ava was not detectable. The low values for these amino acids, and Arg, are due to the resistance of the Ava-Ser-Met-Arg sequence to enzymatic digestion.[5] The ultraviolet spectrum of peptide III in 0.001 N HCl was the same as that of synthetic H-Met-Glu-His-Phe-Arg-Trp-Gly-OH,[22] indicating that the tryptophan residue was intact.

A solution of 0.31 mg of peptide III and 6 μg of chymotrypsin in 70 μl of Tris buffer was incubated at 37° for 17 h. A parallel digestion was conducted on peptide II. Both digests were submitted to paper electrophoresis at pH 6.9, and peptide spots were detected by ninhydrin, Pauly, Ehrlich, and Sakaguchi reagents. The peptide patterns were the same for both peptides except that the Pauly positive (histidine containing) peptide obtained from the digestion of peptide III had a greater mobility than the corresponding peptide obtained from the digestion of peptide II; R_f^{Lys} for the two peptides was 0.53 and 0.13, re-

spectively. The relative mobilities of these two peptides are consistent with the structures of the expected histidine-containing peptides from a chymotryptic digestion—H-Ava-Ser-Met-Arg-His-Phe-OH and H-Ava-Ser-Met-Glu-His-Phe-OH from peptides III and II, respectively.

Biological Assay. In vitro steroidogenesis in isolated rat adrenal cells was measured by the method of Moyle et al.[14] In vitro lipolytic activity in isolated rat and rabbit fat cells was measured by the method of Ramachandran and Lee.[23] In vitro melanotropic activity was determined by the method of Shizume et al.[24] Potency was measured against highly purified sheep ACTH[25] standard. In vivo melanotropic activity was determined by the method of Hogben and Slome.[26]

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Pharmacological Activity of Nitroxide Analogues of Dichloroisoproterenol and Propranolol

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Spin-labeled analogues of dichloroisoproterenol and propranolol were synthesized. It was found that the K_D's of both probes for the β-adrenergic receptors of frog erythrocytes were about 30-fold higher than the K_D's previously reported for the parent antagonists. Thus the introduction of a bulky nitroxide moiety in place of the isopropyl group on the amino nitrogen is associated with a decrease in affinity for the β-adrenergic receptors. Nonetheless, the affinity of the spin-labeled propranolol would appear to be within a range compatible with EPR measurements.

In recent years, the use of nitroxides as free-radical probes in the study of drug interactions has received considerable attention.[1-3] In particular, spin-labeled probes of acetylcholine[4,5] have been developed to study the topography of the muscarinic cholinergic receptor[6] and acetylcholinesterase.[7,8] Furthermore, we have observed that in order to "map" the surface of the receptor, the dissociation constant of a spin-labeled probe must be no greater than 1×10^{-5} M. Recently, Sinha and Chignell[9] reported the synthesis of a spin-labeled analogue of dichloroisoproterenol (**2**) and propranolol (**4**). In their article, they reported that **2** and **4** inhibit, at 6.65×10^{-5} M, the isoproterenol-stimulated adenylate cyclase of fat cell membranes by 19 and 41%, respectively. Unfortunately, the authors[9] did not publish the dissociation constant for either agent. With this in mind, we felt that it might be advantageous to repeat their experiments to determine the feasibility of using these probes to study the topography of the β-adrenergic receptors.

In our experience with sodium cyanoborohydride reductive aminations,[10] the use of high-molecular-weight amines or hindered amines has given unsatisfactory yields of the desired products. For example, Sinha and Chignell[9] have reported a 36% yield of 4-[[2-hydroxy-3-(1-naphthalenyloxyl)propyl]amino]-2,2,6,6-tetramethyl-1-piperidinyloxyl (**4**) and presumably a low yield of 4-[[2-(3,4-dichlorophenyl)-2-hydroxyethyl]amino]-2,2,6,6-tetramethyl-1-piperidinyloxyl (**2**) following the method discussed in the literature.[10,11] However, we observed that reaction of 3,4-dichlorophenyloxirane (**1**) with 4-amino-2,2,6,6-tetramethyl-1-piperidinyloxy gave better than an 80% yield of the desired product **2**. In addition, we were able to obtain 85% of **4** by reacting (1-naphthalenyloxyl)methyloxirane with the precursor spin-labeled probe,