MOLECULAR PHARMACOLOGY, 12, 789-799

Inhibition of Adrenocorticotropin Effects on Adrenal Cell Membranes by Synthetic Adrenocorticotropin Analogues: Correlation of Binding and Adenylate Cyclase Activation

D. KIRK WAYS,[1] CAROL F. ZIMMERMAN, AND DAVID A. ONTJES

Departments of Pharmacology and Medicine, University of North Carolina School of Medicine, Chapel Hill, North Carolina 27514

(Received November 24, 1975)
(Accepted May 4, 1976)

SUMMARY

WAYS, D. KIRK, ZIMMERMAN, CAROL F. & ONTJES, DAVID A. (1976) Inhibition of adrenocorticotropin effects on adrenal cell membranes by synthetic adrenocorticotropin analogues: correlation of binding and adenylate cyclase activation. *Mol. Pharmacol.*, 12, 789-799.

Human ACTH and a number of ACTH peptide fragments were prepared by solid-phase synthesis. Of the analogues tested, only $ACTH_{1-39}$ and $ACTH_{1-24}$ stimulated rat adrenal membrane adenylate cyclase *in vitro*. Several shorter fragments, including $ACTH_{6-39}$, $ACTH_{9-24}$, $ACTH_{9-19}$ amide, $ACTH_{9-20}$, $ACTH_{9-18}$, and $ACTH_{12-39}$, inhibited stimulation of the enzyme by native bovine ACTH. The inhibition produced by this group of peptides appears to be specific for ACTH-stimulated adrenal adenylate cyclase. Basal and fluoride-stimulated enzyme activity in adrenal and fluoride- or epinephrine-stimulated activity in liver were not inhibited by $ACTH_{9-24}$. To elucidate further the action of the inhibitory peptides, we tested their ability to inhibit the binding of $[^{125}I]ACTH_{1-24}$ to adrenal particles. Peptides having either agonist or antagonist activity in the adenylate cyclase assay were effective competitors for binding. The order of potency according to both adenylate cyclase inhibition and competitive binding affinity was $ACTH_{6-39} > ACTH_{9-19}$ amide $\cong ACTH_{9-24} > ACTH_{9-20} \cong ACTH_{9-18} > ACTH_{12-39}$. The analogues $ACTH_{1-8}$, $ACTH_{9-16}$, and $ACTH_{19-39}$ were inactive in both the adenylate cyclase and binding systems. Comparison of this series of closely related peptides in two independent assay systems should more clearly define the structural requirements for the binding of ACTH to its membrane receptor.

INTRODUCTION

The earliest biochemical event in the action of ACTH upon its target tissue is thought to be a noncovalent interaction with the adrenal cell membrane, leading to the activation of membrane adenylate cyclase and the generation of increased intracellular concentrations of adenosine cyclic 3',5'-monophosphate (1, 2). There is much evidence to support the hypothesis that cyclic AMP is the major intracellular mediator in controlling the rate of steroidogenesis. Only recently has it become possible to make direct observations of ACTH binding to putative receptors. Specific ACTH binding sites have been demonstrated in extracts from adrenal tumors

This investigation was supported in part by Research Grant AM-13587 from the National Institute of Arthritis and Metabolic Diseases and by Grant IN-15P from the American Cancer Society.

[1] Predoctoral Fellow under United States Public Health Service Training Grant AM-05330.

in mice (2, 3) and in membranes from bovine (4) and human adrenal tissue (5, 6). Many studies have been done to elucidate the structural features required in the ACTH molecule for stimulation of adenylate cyclase and of steroidogenesis, but knowledge of the structural requirements for binding is quite limited. An understanding of such relationships would be of value in the design of more effective ACTH inhibitors. We report here the properties of a series of synthetic fragments of ACTH in the inhibition of hormone-stimulated adenylate cyclase in crude adrenal membranes. We have also compared the ability of these peptides to inhibit the membrane binding of radiolabeled ACTH.

MATERIALS AND METHODS

ACTH peptides. Partially purified bovine ACTH (62.4 USP units/mg), donated by Parke Davis and Company, was used as the standard in adenylate cyclase assays. $ACTH_{1-24}$[2] (Synacthen) was obtained from Ciba and used for radioiodination. We prepared synthetic fragments of human ACTH by the solid-phase procedure of Merrifield (7), with the modifications we have previously described (8). The resin used for routine synthesis was a polystyrene-divinylbenzene copolymer with 2% cross-linking (Cyclo Chemical Company), while the resin used to prepare peptides terminating in a carboxamide was a benzhydrylamine derivative of the same polymer (Beckman). Blocking group selection was as follows: *tert*-butyloxycarbonyl for α-amino functions; benzyl ethers for hydroxyl functions of serine; benzyl esters for β- and γ-carboxyl groups of aspartic and glutamic acids; 2-chlorobenzyloxycarbonyl for ϵ-amino functions of lysine (9); tosyl for imidazole functions of histidine and guanidino functions of arginine (10, 11); and 2,6-dichlorobenzyl ethers for hydroxyl functions of tyrosine (9). Dicyclohexylcarbodiimide was used as the coupling reagent except for asparagine, for which we used the *p*-nitrophenyl ester (7). 2-Mercaptoethanol (1%, v/v) was added to the 4 N HCl–dioxane used for all α-amino deblocking subsequent to the coupling of tryptophan. Final side chain deblocking and cleavage of the peptide from the resin were carried out for 30 min at 0° in anhydrous HF (12) containing 10% anisole and 1% 2-mercaptoethanol. After drying the resin and removing excess anisole (8), we extracted the peptide products with 0.05 M acetic acid and recovered them by lyophilization.

Crude deblocked peptides were desalted and partially purified on 2.5 × 100 cm columns of Bio-Gel P-2 (Bio-Rad) for molecular weights less than 2000, or on Bio-Gel P-6 for molecular weights above 2000. For further purification of the peptide products, we used 1 × 15 cm columns of carboxymethyl cellulose according to the procedures of Yamashiro and Li (11). All synthetic peptides were characterized by the determination of amino acid composition after hydrolysis for 20 hr in 6 N HCl at 110° (13). The content of tryptophan was measured spectroscopically (14). Thin-layer chromatograms of 10–50-μg samples were run on 250-μm cellulose plates (Analtech, Inc.) in 1-butanol–acetic acid–water, upper phase, 4:1:5 (system I), and 1-butanol–pyridine–acetic acid–water, 4:1:1:2 (system II). High-voltage paper electrophoresis in pyridine–acetate buffer, pH 3.7 (11) was performed at 6000 V for 4 hr. Electrophoretic R_F values were calculated based on comparison with an L-lysine standard with R_F = 1.00.

Adrenal membrane particles. Adrenal glands from 250-g male Holtzman rats were excised under ether anesthesia, trimmed of surrounding tissue, quartered, and homogenized with a Teflon pestle. The

[2] Synthetic analogues of human adrenocorticotropic hormone have been abbreviated by the use of subscript notation to indicate the portion of the sequence included in the peptide fragment. The complete sequence of human ACTH is

Ser-Tyr-Ser-Met-Glu-His-Phe-Arg-Trp-Gly-
1 5 10
Lys-Pro-Val-Gly-Lys-Lys-Arg-Arg-Pro-Val-
15 20
Lys-Val-Tyr-Pro-Asn-Gly-Ala-Glu-Asp-Glu-
25 30
Ser-Ala-Glu-Ala-Phe-Pro-Leu-Glu-Phe
35

homogenate was first centrifuged at 4° for 10 min at 600 × *g* to remove unbroken cells and nuclei. The supernatant was then centrifuged for 1 hr at 40,000 × *g*, and the pellet was resuspended in an appropriate buffer for measurements of adenylate cyclase activity or ACTH binding.

Adenylate cyclase assay. We used a modification of the procedure of Schorr and Ney (15) to measure the conversion of [^{32}P]ATP to [^{32}P]cAMP[3] by aliquots of adrenal membrane suspension containing approximately 50 μg of protein as determined by the method of Lowry *et al.* (16). As described by Schorr and Ney, the final concentrations of components in the 70-μl assay mixture were 2 mM ATP, 3 mM $MgSO_4$, 2 mM cAMP, 10 mM theophylline, and 40 mM Tris, pH 7.4. Phosphoenolpyruvate, 8 mM, and 0.3 unit of pyruvate kinase per assay tube were included as an ATP-generating system. Each tube contained 1–2 μCi of [α-^{32}P]ATP (International Chemical and Nuclear Corporation) and 0.05 μCi of [^{3}H]cAMP, which served as an internal recovery standard. All assay tubes were run in duplicate. After a 20-min incubation at 30°, the reaction was stopped by addition of 1 ml of a cold solution containing 100 μg of ATP and 50 μg of cAMP. Blank tubes were not incubated, but were treated immediately with the stopping solution and kept at 4°. [^{32}P]cAMP and [^{3}H]cAMP were recovered by chromatography on neutral alumina according to Salomon *et al.* (17).

Preparation of radiolabeled ACTH. $ACTH_{1-24}$ was lightly iodinated with ^{125}I by mixing 20 μg of peptide with 1 mCi of carrier-free Na^{125}I (New England Nuclear) in 400 μl of 0.01 M sodium phosphate, pH 7.4. After the addition of 6 μg of Chloramine-T in 60 μl of buffer, the solution was mixed for 20 sec, and the reaction was stopped by the addition of 10 mg of Quso G-23 (Philadelphia Quartz Company) in 1.0 ml of buffer. The suspension was mixed for 2 min and centrifuged. The Quso pellet was washed by mixing with 2.0 ml of deionized water, followed by centrifugation. Radiolabeled peptide was then extracted from the Quso by mixing the pellet with 2.0 ml of 1% acetic acid–40% acetone (v/v). The supernatant extract was stored in aliquots at −20° before use. In some cases aliquots of the [^{125}I]ACTH were further purified on carboxymethyl cellulose according to Lefkowitz *et al.* (2), but this procedure was not routinely used, since it did not usually enhance the proportion of radioactivity which could be specifically bound to adrenal particles. The Quso-purified tracer had a specific activity of approximately 100 μCi/μg, corresponding to an average iodine content of about 0.2 mole of iodine per mole of peptide. Hence the iodinated product probably consisted chiefly of monoiodinated and noniodinated hormone (5). Chromatoelectrophoresis, as described by Yalow and Berson (18), showed the tracer to be free both of unreacted ^{125}I and of damaged hormone.

A larger quantity of $ACTH_{1-24}$ (1.0 mg) was iodinated in the same fashion, using similar proportions of Chloramine-T (282 μg) and Na^{127}I (45 μg). This product, when purified on Quso and carboxymethyl cellulose, had a specific activity of approximately 15 units/mg in the adenylate cyclase assay. Hence the iodination procedure led to a partial, but not complete, loss of biological activity.

[^{125}I]$ACTH_{1-24}$ binding assay. Adrenal particles (10–50 μg of protein) suspended in 100 μl of 50 mM Tris, pH 7.5, were added to 12 × 75 mm polystyrene culture tubes. Approximately 0.5 ng of [^{125}I]ACTH (80,000 cpm) freshly diluted in 100 μl of buffer was then added, followed by 200 μl of buffer containing 0.5% bovine serum albumin and the desired concentration of unlabeled ACTH peptide. After mixing, incubations were routinely conducted in triplicate for 10 min at 4°. The incubations were stopped by layering 200 μl of the incubation mixture over 200 μl of cold Tris buffer in a 400-μl polyethylene tube (Beckman). These tubes were immediately centrifuged for 4 min in a Beckman Microfuge. After aspiration of the supernatant, the tips of the tubes, containing the pellet, were cut off and placed in vials for counting.

[3] The abbreviations used are: cAMP, adenosine cyclic 3′,5′-monophosphate; NPS, *o*-nitrophenylsulfenyl.

Counts bound "nonspecifically" to the particles were determined in all experiments by adding an excess of unlabeled $ACTH_{1-24}$ (0.1 mM) to some incubation tubes. This binding, which was subtracted from the total counts bound in incubation mixtures containing varying concentrations of ACTH analogues, ranged from 0.1% to 2.0% of total counts added. Batches of tracer could usually be stored for 2 weeks before a rise in nonspecific binding indicated the need for a new preparation.

RESULTS

Chemical characteristics of synthetic ACTH analogues. Table 1 lists the ACTH peptides prepared by solid-phase synthesis and describes the behavior of the partially purified products on paper electrophoresis and thin-layer chromatography. Products were chromatographically homogeneous, except where indicated in the table. Contaminants, when present, appeared to constitute less than 10% of the total ninhydrin-staining material. Amino acid compositions were generally within 15% of theoretical values. The content of tryptophan, measured spectroscopically, ranged from 0.8 to 1.3 residues/molecule in the tryptophan-containing analogues.

Effects of ACTH analogues on adenylate cyclase. All solid-phase peptides were assayed for their ability to stimulate adrenal membrane adenylate cyclase *in vitro* at multiple concentrations from 0.1 to 100 μM. Native bovine ACTH was the assay standard. Synthetic human $ACTH_{1-39}$ had an adenylate cyclase-stimulating potency equivalent to 144 ± 4.7 units/mg (SE). The activity of $ACTH_{1-24}$ (Ciba) in this assay

TABLE 1

Characteristics of synthetic ACTH peptides

Peptide	R_F values[a]			Amino acid composition of acid hydrolysate[b]
	System I	System II	Electrophoresis	
$ACTH_{19-39}$	0.75	0.70	0.24	Lys 1.0(1), Asx 2.0(2), Ser 0.8(1), Glu 4.5(4), Pro 3.1(3), Gly 1.0(1), Ala 3.1(3), Val 1.9(2), Leu 1.0(1), Tyr 0.9(1), Phe 2.0(2)
$ACTH_{12-39}$	0.58	0.53	0.55	Lsy 2.9(3), Arg 1.9(2), Asx 2.0(2), Ser 1.0(1), Glu 4.3(4), Pro 4.5(4), Gly 1.8(2), Ala 3.1(3), Val 3.1(3), Leu 0.9(1), tyr 0.8(1), Phe 1.8(2)
$ACTH_{6-39}$	0.56	0.50	0.66	Lys 4.2(4), His 1.2(1), Arg 3.3(3), Asx 1.8(2), Ser 0.8(1), Glu 4.1(4), Pro 4.0(4), Gly 3.0(3), Ala 2.9(3), Val 2.7(3), Leu 1.0(1), Tyr 0.8(1), Phe 3.1(3), Trp 1.3(1)
$ACTH_{1-39}$	0.56	0.30	0.60	Lys 4.2(4), His 1.2(1), Arg 3.2(3), Asx 1.9(2), Ser 3.1(3), Glu 5.2(5), Pro 4.1(4), Gly 2.9(3), Ala 2.9(3), Val 2.7(3), Met 0.8(1), Leu 1.0(1), Tyr 1.9(2), Phe 3.2(3), Trp 1.2(1)
$ACTH_{9-24}$	0.48	0.45	0.87	Lys 3.8(4), Arg 2.0(2), Pro 3.3(3), Gly 1.8(2), Val 2.8(3), Tyr 1.0(1), Trp 1.0(1)
$ACTH_{9-20}$	0.44	0.45 (0.56)[c]	0.92	Lys 2.9(3), Arg 2.2(2), Pro 1.7(2), Gly 1.8(2), Val 2.2(2), Trp 0.8(1)
$ACTH_{9-19}$ amide	0.45	0.34	0.94	Lys 3.1(3), Arg 1.9(2), Pro 2.0(2), Gly 2.1(2), Val 0.9(1), Trp 0.9(1)
$ACTH_{9-18}$	0.45	0.27	0.99	Lys 3.0(3), Arg 1.8(2), Pro 1.0(1), Gly 1.8(2), Val 1.2(1), Trp 1.0(1)
$ACTH_{9-16}$	0.46	0.43	0.88	Lys 3.3(3), Pro 1.1(1), Gly 1.8(2), Val 0.9(1), Trp 0.9(1)
$ACTH_{9-14}$	0.60	0.66	0.61	Lys 1.1(1), Pro 0.9(1), Gly 2.2(2), Val 0.9(1), Trp 1.3(1)
$ACTH_{1-8}$	0.67 (0.38)[c]	0.61 (0.30)[c]	0.63	His 0.8(1), Arg 1.0(1), Ser 1.0(1), Glu 1.0(1), Tyr 1.0(1), Phe 1.0(1), Met 1.0(1)

[a] Peptides were localized by ninhydrin staining. See the text for details of the chromatographic procedures.

[b] Theoretical values are indicated in parentheses.

[c] R_F of a minor contaminant.

TABLE 2

Effects of synthetic ACTH peptides on adrenal particles in vitro

Peptide	Approximate activity *in vitro*	Concentration required for 50% inhibition of native ACTH in adenylate cyclase assay	Concentration required for 50% inhibition of [^{125}I] $ACTH_{1-24}$ binding
		μM	*μM*
$ACTH_{1-39}$ (solid phase)	144 ± 4.7		9.3
$ACTH_{1-24}$ (Ciba)	148 ± 19		1.3
$ACTH_{6-39}$	0	<1	0.25
$ACTH_{9-24}$	0	4.6	3.3
$ACTH_{9-19}$amide	0	6.9	1.0
$ACTH_{9-18}$	0	24	15
$ACTH_{9-20}$	0	40	25
$ACTH_{12-39}$	0	>500[a]	50
$ACTH_{9-16}$	0	NA[b]	NA[c]
$ACTH_{9-14}$	0	NA[b]	NA[c]
$ACTH_{1-8}$	0	NA[b]	NA[c]
$ACTH_{19-39}$	0	NA[b]	NA[c]

[a] Inhibition of 15% was seen at a concentration of 1 mM.

[b] Not active at concentrations up to 500 μM.

[c] Not active at concentrations up to 100 μM.

was 148 ± 19 units/mg. None of the shorter solid-phase fragments of ACTH were active in stimulating adenylate cyclase at concentrations of 100 μM or less (Table 2).

We tested each ACTH fragment for its ability to inhibit the activation of adenylate cyclase by native ACTH. In the inhibition assays, bovine ACTH standard was added at a fixed concentration of 1 μM, a level at which 50–70% maximal stimulation of adenylate cyclase occurred. The concentration of ACTH fragments added to incubation tubes containing 1 μM ACTH ranged from 1 μM to 1 mM. Figure 1 depicts the data from a typical inhibition experiment. $ACTH_{6-39}$, which had been ineffective as an agonist, was a very potent ACTH antagonist, showing an inhibition effect of over 50% at an equimolar concentration (1 μM). Complete inhibition did not occur with increasing concentrations of this analogue, up to 100 μM. The reason for the persistence of some adenylate cyclase activity at higher inhibitor concentrations is unclear, but it is possible that the inhibitor may have exerted weak agonist effects under the experimental conditions. The fragments $ACTH_{9-24}$ and $ACTH_{9-19}$ amide were next in the order of inhibitory potency, producing 50% inhibition at 6–7 times the concentration of native ACTH. $ACTH_{9-18}$ and $ACTH_{9-20}$ were still weaker inhibitors, causing 50% inhibition only at concentrations 20–40 times that of ACTH. $ACTH_{12-39}$ had a very

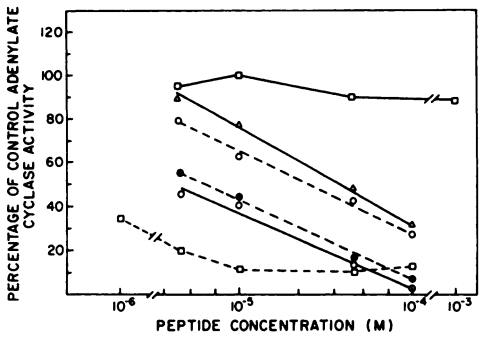

FIG. 1. *Inhibition in vitro by synthetic ACTH fragments of effects of bovine ACTH on adrenal membrane particles*

Details of the adenylate cyclase assay are given in the text. Basal enzyme activity was 63.1 pmoles of cAMP per milligram of protein during 20 min, and enzyme activity in the presence of 1 μM ACTH was 308 pmoles/mg of protein during 20 min. Basal enzyme activity was subtracted from all values. Activity in the presence of ACTH represents a control activity of 100%. Experimental concentrations of ACTH analogues, plotted on the abscissa, were determined from quantitative amino acid analysis of peptide samples after acid hydrolysis. □——□, $ACTH_{12-39}$; △——△, $ACTH_{9-20}$; ○- - -○, $ACTH_{9-18}$; ●- - -●, $ACTH_{9-19}$ amide; ○——○, $ACTH_{9-24}$; □- - -□, $ACTH_{6-39}$.

slight, but significant,[4] inhibitory activity, producing 15% inhibition at the highest concentration tested. Other analogues, including $ACTH_{1-8}$, caused no significant difference in the control level when present in concentrations up to 500 μM (Table 2). Such analogues appear to lack the ability either to mimic or to antagonize the effects of ACTH upon adrenal adenylate cyclase *in vitro*.

To learn more about the specificity of the inhibitory phenomenon, we examined the effects of $ACTH_{9-24}$ upon adenylate cyclase activity stimulated under a variety of other conditions. First we tested the analogue at a concentration of 100 μM for its ability to block the stimulation of adenylate cyclase by sodium fluoride in adrenal membranes. We then looked for inhibition of fluoride-stimulated and epinephrine-stimulated activity in crude rat liver cell membranes. As shown by the data in Table 3, the analogue failed to inhibit basal or fluoride-stimulated cyclase in adrenal, and had no effect on fluoride or epinephrine-stimulated liver cyclase activity.

Characteristics of [^{125}I]$ACTH_{1-24}$ binding to adrenal particles and displacement of binding by ACTH fragments. The time course of the binding of [^{125}I]$ACTH_{1-24}$ to adrenal particles is shown in Fig. 2. Maximum binding at 4° occurred within 2 min, the earliest time at which measurements could be made. Total binding declined slowly with continued incubation, falling to about 70% of the initial value after 70 min. At 25° (not shown) total binding reached a similar initial peak but declined more rapidly with time, falling by more than 50% in less than 30 min. The decline in binding with continued incubation appeared to be due to degradation of the tracer rather than to degradation of ACTH binding sites, since preliminary incubation of the adrenal membranes in buffer for 2 hr at 25° did not impair their ability to bind fresh tracer. Labeled ACTH dissociated immediately from the adrenal particles upon acidification of the medium with HCl or addition of an excess of unlabeled ACTH (Fig. 2). Specific binding of tracer was directly dependent upon adrenal particle concentrations between 10 and 50 μg of protein per 400-μl incubation volume. At concentrations greater than 50 μg of protein per tube, further increases in specific binding were not seen. Under optimal conditions, specific binding of 20–30% of the total added counts could be obtained, and nonspecific or nondisplaceable binding was less than 1% of the total added counts. When membranes containing bound tracer were separated from the incubation medium and treated with 1.0 N HCl, approximately 60% of the eluted tracer could be rebound to an excess of fresh membranes, indicating that binding itself was independent of the process of tracer degradation.

To determine whether the binding phenomenon was specific for adrenal membranes, we compared the binding of [^{125}I]$ACTH_{1-24}$ to membrane particles prepared identically from several different tissues. Incubation was carried out with tracer (150,000 cpm) and membranes (50 μg of protein) from rat adrenal, kidney, lung, and liver. Excess $ACTH_{1-24}$ (100 μM) was added to control tubes and the nonspecific binding thus measured was subtracted from total binding to calculate specific binding. The results are shown in Fig. 3. Although the non-adrenal tissues all displayed some nonspecific adsorption of the tracer, only the adrenal particles showed binding which was specifically displaceable with unlabeled $ACTH_{1-24}$. In the case of liver and lung particles, tracer binding actually showed a slight increase in the presence of unlabeled hormone. Hence small negative numbers were calculated to represent specific binding.

To characterize further the properties of the adrenal membrane binding sites, we performed equilibrium experiments using [^{125}I]$ACTH_{1-24}$ and unlabeled $ACTH_{1-24}$. A relatively low adrenal membrane concentration was selected to enhance the sensitivity of the competitive binding phenomenon. A Scatchard plot of the equilibrium data is shown in Fig. 4. The relationship between the bound to free ratio and total ACTH bound is not linear over a wide range of ACTH concentrations, indicating that not all binding sites behave identically as saturation occurs. The apparent

[4] $p < 0.05$ by Student's t-test; $N=6$.

TABLE 3

Effects of $ACTH_{9-24}$ on adenylate cyclase stimulated by agents other than ACTH

Tissue and conditions for stimulation	Adenylate cyclase activity
	pmoles cAMP/mg protein/20 min ± SE
Adrenal membranes[a]	
Basal	36.8 ± 1.2
Basal + $ACTH_{9-24}$ (0.1 mM)	39.0 ± 0.4
NaF (10 mM)	61.0 ± 3.0
NaF (10 mM) + $ACTH_{9-24}$ (0.1 mM)	73.6 ± 0.5[b]
Liver membranes[a]	
Basal	31.3 ± 2.1
NaF (10 mM)	109.0 ± 8.5
NaF (10 mM) + $ACTH_{9-24}$ (0.1 mM)	101.3 ± 3.3
Epinephrine (1 mM)	42.4 ± 2.7
Epinephrine (1 mM) + $ACTH_{9-24}$ (0.1 mM)	40.0 ± 1.6

[a] Prepared by the method for obtaining adrenal membrane particles described in the text.

[b] Significantly different from NaF (10 mM) alone; $0.01 < p < 0.05$.

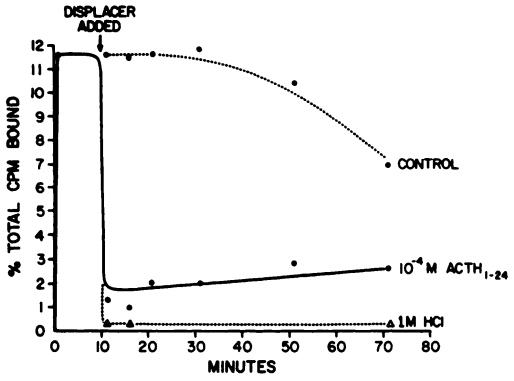

FIG. 2. *Time course of binding and displacement of [^{125}I]$ACTH_{1-24}$*

Each incubation tube contained 14 μg of membrane protein in 300 μl of buffer. At zero time 80,000 cpm of [^{125}I]$ACTH_{1-24}$ were added to all tubes, and bound counts were immediately determined in triplicate tubes as described in the text. Ten minutes after addition of the tracer, 100 μl of buffer were added to one group of control tubes, 100 μl of 400 μM ACTH in buffer were added to a second group of tubes, and 100 μl of 4 M HCl, to a third group of tubes. Three tubes from each group were centrifuged at the times indicated, and bound counts were determined.

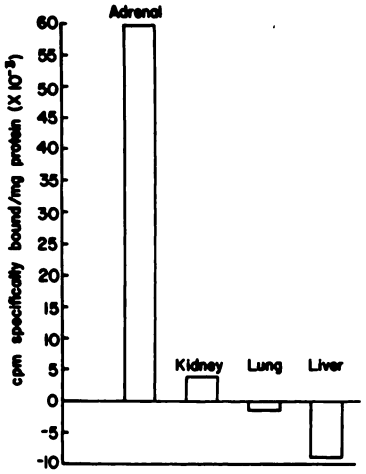

FIG. 3. *Specific binding of [^{125}I]$ACTH_{1-24}$ to membranes from adrenal and non-adrenal tissues*

Membrane particles from rat kidney, lung, and liver were prepared as described for rat adrenal.

K_d of the binding sites of higher affinity is approximately 3.5×10^8 M^{-1}, and the number of these sites is approximately 230 pmoles/mg of membrane protein.

To determine which portions of the ACTH sequence were required for the binding phenomenon, we compared the synthetic ACTH fragments for their ability to inhibit the binding of tracer to adrenal membranes. In these experiments the peptide fragments were added in concentrations ranging from 10 μM to 100 μM. Specific binding in the presence of the peptide was expressed as a percentage of the specific binding seen in the absence of peptide. The results of typical binding inhibition experiments are shown in Figs. 5 and 6. In Fig. 5 a series of analogues with progressive shortening from the amino terminus are compared with $ACTH_{1-24}$. Several of the synthetic peptides were inhibitors of binding, with the degree of inhi-

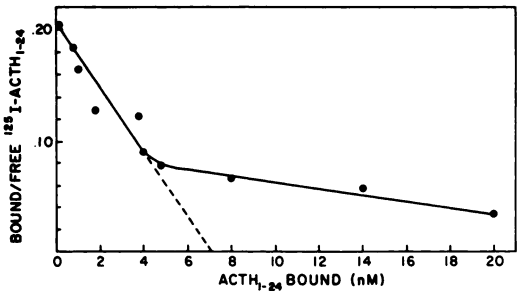

FIG. 4. *Scatchard plot for binding of ACTH$_{1-24}$ to adrenal membranes*

Unlabeled ACTH$_{1-24}$ was added at concentrations varying from 1 nM to 10 μM to tubes containing 12 μg of membrane protein, followed by the addition of 80,000 cpm of [^{125}I]ACTH as described in the text. To calculate the total amount of ACTH$_{1-24}$ bound, it was assumed that the binding behavior of unlabeled ACTH$_{1-24}$ was the same as that of the tracer. If a straight line is fitted by least squares to the seven points representing the lowest quantities of bound ACTH, the association constant K_d of the higher-affinity binding sites is approximately 3.5×10^8 M^{-1}. The concentration of these sites is approximately 230 pmoles/mg of membrane protein.

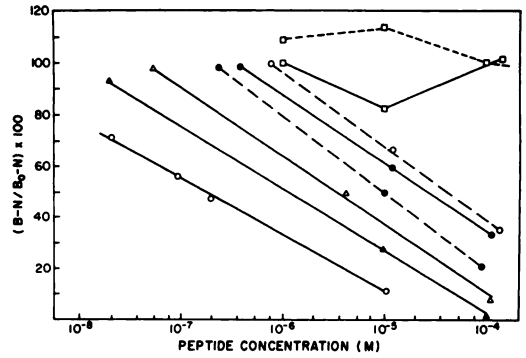

FIG. 5. *Inhibitory effects of ACTH analogues on binding of [^{125}I]ACTH to adrenal membranes*

The ordinate represents the percentage of control binding, where B and B_0 are counts per minute bound in the presence and absence, respectively, of unlabeled peptide, and N refers to nonspecifically bound counts. Molar concentration estimates for the bovine ACTH standard are based on the assumption of a theoretical specific activity of 150 units/mg for the pure hormone (19). Experimental concentrations of the synthetic analogues are based on quantitative amino acid analysis. ○——○, ACTH$_{6-39}$; ▲——▲, ACTH$_{1-24}$; △——△, ACTH$_{9-24}$; ●- - -●, ACTH$_{1-39}$ (synthetic); ●——●, bovine ACTH; ○- - -○, ACTH$_{12-39}$; □——□, ACTH$_{19-39}$; □- - -□, ACTH$_{1-8}$.

bition being proportional to log peptide concentration. Synthetic ACTH$_{6-39}$ was the most potent inhibitor of binding. With this compound, 50% inhibition occurred at a concentration of 250 nM. The ACTH$_{1-24}$ standard was somewhat less potent, causing a comparable degree of inhibition at a concentration of 1.3 μM. Next in order of inhibitory potency were ACTH$_{9-24}$, ACTH$_{1-39}$ (synthetic), and ACTH$_{12-39}$. The analogues ACTH$_{19-39}$ and ACTH$_{1-8}$ were without inhibitory effects. In Fig. 6 the results of deletions from the sequence between residues 14 and 24 are examined. The ACTH$_{1-24}$ standard produced 50% inhibition at 1.4 μM, a value similar to that obtained from the experimental data in Fig. 4. The shorter analogue ACTH$_{9-19}$ amide was at least as potent as ACTH$_{1-24}$, while ACTH$_{9-24}$ was slightly less potent. ACTH$_{9-20}$ and ACTH$_{9-18}$ were much less potent than either the ACTH$_{1-24}$ standard or the closely related ACTH$_{9-19}$ amide. ACTH$_{9-16}$ and ACTH$_{9-14}$ were unable to inhibit binding at the concentrations tested. The data from all binding inhibition experiments are summarized in Table 2.

DISCUSSION

We undertook these studies to learn more about the structural requirements for the binding of ACTH to its receptor on the adrenal cell membrane. As a means of testing the effectiveness of our procedures for the synthesis of ACTH analogues, we synthesized and purified a peptide containing the entire sequence of human ACTH. Yamashiro and Li (11) previously reported the solid-phase synthesis of human ACTH. Their product, after extensive purification, showed an activity of 150 units/mg in bioassays *in vivo*. Our product was synthesized by a similar method and shows similar chemical characteristics, although it was subjected to less extensive purification. Its biological activity in the adenylate cyclase assay *in vitro* approaches the activity to be expected for native ACTH. We believe, therefore, that our solid-phase technique is capable of producing ACTH analogues in which valid structure-function comparisons may be made. Of all the shorter fragments produced and studied,

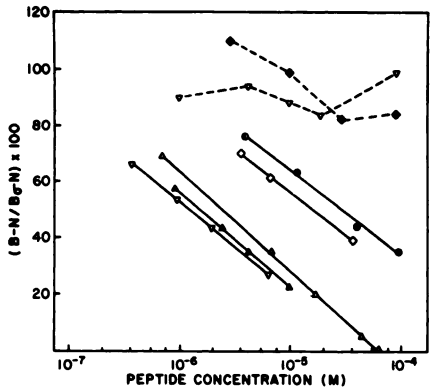

FIG. 6. *Inhibitory effects of ACTH analogues on [^{125}I]ACTH binding to adrenal membranes*

▽——▽, $ACTH_{9-19}$ amide; ▲——▲, $ACTH_{1-24}$; △——△, $ACTH_{9-24}$; ◇——◇, $ACTH_{9-18}$; ◐——◐, $ACTH_{9-20}$; ▽- - -▽, $ACTH_{9-16}$; ◆- - -◆, $ACTH_{9-14}$. Unrelated hormones that failed to inhibit binding at concentrations of 100 μM include bovine parathyroid hormone, human calcitonin, angiotensin II, L-epinephrine, and L-norepinephrine.

none had detectable agonist activity in the adenylate cyclase assay. Although we found stimulation of adenylate cyclase *in vitro* by $ACTH_{6-39}$, this peptide might have weak agonist effects in other assay systems. Fujino *et al.* (20) have reported that a similar peptide, $ACTH_{6-23}$ amide, had a potency of 0.1 unit/mg in a steroidogenic assay *in vivo*.

Other laboratories have observed that certain ACTH analogues devoid of steroidogenic activity can inhibit the effects of ACTH *in vitro*. Seelig and Sayers (21) reported that $ACTH_{11-24}$ is an inhibitor of ACTH-stimulated adenylate cyclase and of corticosterone secretion in isolated rat adrenal cortical cells. Hofmann and coworkers (22) found that analogues in which the tryptophan in position 9 is replaced by phenylalanine or *N*-methyltryptophan are inactive in the stimulation of adenylate cyclase in bovine adrenal membranes but inhibit ACTH binding, producing 50% inhibition at molar excesses of 5 or 10:1. Hofmann *et al.* (4) also reported that a biologically inactive ACTH analogue, $ACTH_{11-20}$ amide, can inhibit the binding of a biologically active analogue, ([^{14}C]Phe)(Gln^5)-$ACTH_{1-20}$ amide, to adrenal membrane particles. Recently Saez and associates (5) have confirmed that $ACTH_{11-24}$ is a weak inhibitor of ACTH-stimulated adenylate cyclase *in vitro*. In addition, they have reported that the *o*-nitrophenylsulfenyl derivative of $ACTH_{1-24}$ is both a weak agonist and an inhibitor of ACTH (5). Both $ACTH_{11-24}$ and [NPS-Trp^9]-$ACTH_{1-24}$ were capable of displacing [^{125}I]$ACTH_{1-24}$ from binding to crude adrenal membranes, while $ACTH_{1-10}$ was not. The implication of all this work has been that the structural requirements for biological activity and for receptor binding are not identical.

The binding phenomenon that we observed with crude rat adrenal membrane particles has many of the characteristics to be expected in the binding of ACTH to putative hormone receptors. We have shown that binding is rapid, reversible, and specifically inhibited only by ACTH analogues having either agonist or antagonist effects in our bioassay system *in vitro*. The concentration range of unlabeled $ACTH_{1-24}$ required for the displacement of [^{125}I]ACTH in our binding system is similar to that initially reported by Lefkowitz *et al.* (2) and by Saez *et al.* (5).

In spite of the strong evidence that [^{125}I]ACTH binding is functionally related to the control of adenylate cyclase activity in our membrane preparations, we doubt that the binding sites involved are entirely identical with the physiologically active receptors for ACTH in the intact adrenal cell. In broken membrane preparations the total number of hormone binding sites exceeds the number of receptors believed to function in the intact cell (3). In similar fashion, the total cAMP levels attainable with ACTH stimulation of adrenal tissue far exceed the levels required for maximal stimulation of steroidogenesis (1). Seelig and Sayers (21) noted that in intact, isolated rat adrenal cells the concentration of ACTH required to produce half-maximal cAMP production is 35 times greater than the concentration required for half-maximal stimulation of steroidogenesis. On the basis of their studies of several ACTH analogues in the isolated cell system, these authors proposed a model having an excess of functional receptor-aden-

ylate cyclase complexes, over and above those needed to induce maximum steroidogenesis.

In the membrane preparations used in this study, there is an excellent correlation between the potencies of various analogues in the inhibition of binding and of adenylate cyclase. Table 2 summarizes these inhibitory properties in terms of the concentrations required for 50% inhibition of both binding and enzyme activation. Numerical values derived from the two assays should not necessarily be identical for a given analogue, since the conditions for the assays are different. However, both assays show a similar order of potency for the inhibitory analogues: $ACTH_{6-39}$ > $ACTH_{9-19}$ amide ≅ $ACTH_{9-24}$ > $ACTH_{9-20}$ ≅ $ACTH_{9-18}$ > $ACTH_{12-39}$. The analogues $ACTH_{1-8}$, $ACTH_{9-16}$, and $ACTH_{19-39}$ were inactive in both systems. In the binding assay, $ACTH_{1-24}$ was more effective than either native porcine or synthetic human $ACTH_{1-39}$. Lefkowitz *et al.* (2) had previously observed that $ACTH_{1-24}$ was moderately more effective than porcine ACTH in binding to an extract of mouse adrenal adenoma membranes. The biological activities of synthetic $ACTH_{1-24}$ and porcine ACTH are approximately the same (19).

We draw several conclusions regarding the structural requirements for ACTH binding to adrenal membranes. Residues 1–5, although contributing to biological activity, seem to do nothing to promote binding. There is even reason to suspect that deletion of the first 5 residues may enhance binding affinity. $ACTH_{6-39}$ is very potent both in the inhibition of [^{125}I]$ACTH_{1-24}$ binding and in the inhibition of adenylate cyclase stimulation *in vitro*. Residues 6–8 are not essential for binding, but their removal clearly decreases binding affinity. Thus a 5–10-fold higher concentration of $ACTH_{9-24}$ than of $ACTH_{6-39}$ was required to produce similar levels of inhibition. Further deletion of residues 9–11 all but destroyed binding capability. $ACTH_{12-39}$ was a very weak inhibitor of both binding and adenylate cyclase. We have found no evidence that the amino-terminal octapeptide by itself can interact with adrenal receptors, since $ACTH_{1-8}$ inhibited neither binding nor stimulation of adenylate cyclase.

From the carboxyl terminus, residues 25–39 may be removed without ill effects upon either binding or biological activity. Further carboxyl-terminal shortening as far as residue 18 is still consistent with binding activity, but deletion of the basic residues 17 and 18 leads to a marked loss of affinity. In contrast to $ACTH_{9-18}$, $ACTH_{9-16}$ was found to be devoid of binding activity. It is known that replacement of a negatively charged carboxyl terminus with a neutral carboxamide in this basic region of the sequence can lead to considerable enhancement of biological activity (23). As one might expect from existing structure activity data, we found that $ACTH_{9-19}$ amide has a higher binding affinity than either $ACTH_{9-18}$ or $ACTH_{9-20}$. Thus our data provide direct evidence for the importance of cationic charge in membrane binding. It seems likely to us that the ACTH receptor contains a negatively charged region, and that ionic forces may play an important role in ACTH-receptor interaction.

REFERENCES

1. Grahame-Smith, D. B., Butcher, R. W., Ney, R. L. & Sutherland, E. W. (1967) *J. Biol. Chem.*, 242, 5535–5541.
2. Lefkowitz, R. J., Roth, J., Pricer, W. & Pastan, I. (1970) *Proc. Natl. Acad. Sci. U. S. A.*, 65, 745–752.
3. Lefkowitz, R. J., Roth, J. & Pastan, I. (1971) *Ann. N. Y. Acad. Sci.*, 185, 195–209.
4. Hofmann, K., Wingender, W. & Finn, F. M. (1970) *Proc. Natl. Acad. Sci. U. S. A.*, 67, 829–836.
5. Saez, J. M., Dazord, A., Morera, A. M. & Bataille, P. (1974) *J. Steroid Biochem.*, 5, 925–933.
6. Saez, J. M., Dazord, A. & Gallet, D. (1975) *J. Clin. Invest.*, 56, 536–547.
7. Merrifield, R. B. (1965) *Science*, 150, 178–185.
8. Ontjes, D. A., Roberts, J. C., Hennessy, J. F., Burford, H. J. & Cooper, C. W. (1973) *Endocrinology*, 92, 1780–1785.
9. Erickson, B. W. & Merrifield, R. B. (1972) in *Chemistry and Biology of Peptides* (Meienhofer, J., ed.), pp. 191–195, Ann Arbor Science Publishers, Ann Arbor, Mich.
10. Sakakibara, S. & Fujii, T. (1969) *Bull. Chem. Soc. Jap.*, 42, 1466.

11. Yamashiro, D. & Li, C. H. (1973) *J. Am. Chem. Soc.*, 95, 1310–1315.
12. Sakakibara, S., Shimonishi, Y., Kishida, Y., Okada, M. & Sugihara, H. (1967) *Bull. Chem. Soc. Jap.*, 40, 2164–2167.
13. Spackman, D. H., Moore, S. & Stein, W. H. (1958) *Anal. Chem.*, 30, 1190–1206.
14. Edelhoch, H. (1967) *Biochemistry*, 6, 1948–1954.
15. Schorr, I. & Ney, R. L. (1971) *J. Clin. Invest.*, 50, 1295–1300.
16. Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.*, 193, 265–275.
17. Salomon, Y., Londos, C. & Rodbell, M. (1974) *Anal. Biochem.*, 58, 541–548.
18. Yalow, R. S. & Berson, S. A. (1968) *J. Clin. Invest.*, 47, 2725–2751.
19. Ney, R. L., Ogata, E., Shimizu, N., Nicholson, W. E. & Liddle, G. W. (1964) in *Proceedings of the Second International Congress of Endocrinology*, Pt. II, pp. 1184–1191, Excerpta Medica, Amsterdam.
20. Fujiino, M., Hatanaka, C. & Nishimura, O. (1971) *Chem. Pharm. Bull. (Tokyo)*, 19, 1066–1068.
21. Seelig, S. & Sayers G. (1973) *Arch. Biochem. Biophys.*, 154, 230–239.
22. Hofmann, K., Montibeller, J. A. & Finn, F. M. (1974) *Proc. Natl. Acad. Sci. U. S. A.*, 71, 80–83.
23. Ramachandran, J., Chung, D. & Li, C. H.(1965) *J. Am. Chem. Soc.*, 88, 2696–2707.