Journal of Steroid Biochemistry, 1974, Vol. 5, pp. 925–933. Pergamon Press. Printed in Great Britain.

INTERACTIONS OF ACTH WITH ITS ADRENAL RECEPTORS: SPECIFIC BINDING OF $ACTH_{1-24}$, ITS *O*-NITROPHENYL SULFENYL DERIVATIVE AND $ACTH_{11-24}$

J. M. SAEZ, A. M. MORERA, A. DAZORD and P. BATAILLE

Unité de Recherches Endocriniennes et Métaboliques chez l'Enfant, INSERM, U. 34, Hôpital Debrousse, 29 Chemin Soeur Bouvier, 69322 Lyon Cedex 1, France

SUMMARY

$ACTH_{1-24}$, its *o*-nitrophenyl sulfenyl derivative and $ACTH_{11-24}$ were iodinated by the chloramine T method and purified on carboxymethylcellulose. Enzymatic digestion shows that all the iodine was incorporated in the form of monoiodotyrosine. The three ACTHs bound to adrenal crude membranes (pellet 20,000 *g*) prepared from normal human, rat and sheep adrenals. In the presence of an excess of membrane protein more than 60% of the radioactivity was bound specifically.

The binding of ^{125}I-$ACTH_{1-24}$ to its adrenal receptor is very rapid; it is temperature dependent, inhibited by ions and by the pancreatic trypsin inhibitor. $ACTH_{1-24}$ and its *o*-nitrophenyl sulfenyl derivative inhibited identically the binding of ^{125}I-$ACTH_{1-24}$. $ACTH_{11-24}$ and $ACTH_{1-10}$ were only, respectively, 15% and 6%, as active as $ACTH_{1-24}$ in displacing the bound ^{125}I-$ACTH_{1-24}$.

The binding of ^{125}I-$ACTH_{11-24}$ is more inhibited by $ACTH_{1-24}$ than by $ACTH_{11-24}$. $ACTH_{1-10}$ at high concentrations (10^{-4} M) had no effect.

The adenyl cyclase of the same adrenal preparations was stimulated by $ACTH_{1-24}$; NPS–$ACTH_{1-24}$ stimulated adenyl cyclase activity but inhibited the action induced by $ACTH_{1-24}$ in the same way as $ACTH_{11-24}$ which was without effect on adenyl cyclase. On the other hand, $ACTH_{1-10}$ stimulated adenyl cyclase activity and its action was additional to $ACTH_{1-24}$.

It can be concluded that the 1–10 N-terminal sequence of ACTH, in addition to being the biological active site of the molecule, contributes to binding and increases the affinity of ACTH for its receptor.

At present it is generally accepted that most and perhaps all the effects of ACTH on the adrenals are the result of the interaction of this hormone with the cell membrane. The derivatives ACTH–cellulose, ACTH–agarose and ACTH–polyacrylamide are incapable of penetrating inside the cells, yet they are all capable of producing the effects of ACTH[1–3]. The existence of specific binding sites for ACTH have already been demonstrated in fractions of bovine adrenals rich in plasma membranes[4, 5] and in soluble fractions from tumors of the adrenals in mice[6].

This paper describes a comparative study of the binding kinetics of $^{125}ACTH_{1-24}$ to particulate fractions prepared from rat, sheep, and human adrenals. Several factors influencing this binding have been studied. Furthermore, observation of the displacement of the binding of $^{125}ACTH_{1-24}$ by its analogues, and of the binding of ^{125}I-$ACTH_{11-24}$ and ^{125}I-NPS-$ACTH_{1-24}$ have enabled us to establish certain relations between the structure of the hormone and its binding to receptor. Similarly we have studied the effect of these ACTH analogues on adenyl cyclase in the same subcellular preparations.

EXPERIMENTAL PROCEDURE

Materials

$ACTH_{1-24}$ was provided by CIBA. $ACTH_{11-24}$, $ACTH_{1-10}$ and the *o*-nitrophenyl sulfenyl derivative of $ACTH_{1-24}$ were a gift from Dr. Rittel and Dr.

*Abbreviations

$ACTH_{1-24}$	= corticotropin-(1-24)–tetracosapeptide
$ACTH_{11-24}$	= corticotropin-(11-24)–tetradecapeptide
$ACTH_{1-10}$	= corticotropin-(1-10)–decapeptide
NPS–$ACTH_{1-24}$	= [9-tryptophan (*o*-nitrophenylsulfenyl)]-corticotropin-(1-24)–tetracosapeptide
EGTA	= ethyl glycol-bis (β-amino-ethyl ether) N, N′ tetra-acetic acid
EDTA	= ethylene dinitrilo tetra-acetic acid.

Desaulles (Ciba-Geigy A.G., Basel, Switzerland). Carrier-free Na ^{125}I, ^{125}I-monoiodotyrosine and ^{125}I-diiodotyrosine were obtained from Saclay, France. Microfine silica, Quso G-32 was obtained from the Philadelphia Quartz Co. (U.S.A.), talc tablets from Gold Leaf Pharmacol Co. Inc. (U.S.A.). Carboxymethyl cellulose microgranular from Whatman Biochemicals Ltd., England, and bovine albumin, leucine–aminopeptidase type V, from Sigma Chemical Co. (U.S.A.). Other chemicals were of reagent grade.

Membrane preparation

Crude adrenal membranes (pellet 20,000 g) from human, ovine and rate adrenals were prepared as described elsewhere[8]. Highly purified ovine adrenal membranes, verified by electron microscopy, were obtained using the method of Finn *et al.*[5].

5′-Nucleotidase was determined according to Heppel *et al.*[7]. Adenyl cyclase activity was measured as described alsewhere[8].

Preparation of labelled ACTH

$ACTH_{1-24}$, $ACTH_{11-24}$ and NPS–$ACTH_{1-24}$ were labelled by ^{125}I-Na by the method described by Lefkowitz *et al.*[6]. After each addition of chloramine T, the incorporation of ^{125}I was measured by precipitation with 10% trichloroacetic acid and absorption on Quso, until 80% of the radioactivity was bound to proteins. The reaction product was put on a carboxymethyl cellulose column (1 × 13 cm) previously equilibrated with 0·01 M ammonium acetate (pH 4·5). After passing 200 ml of this buffer through the column, it was eluted, in 3 ml fractions, with an exponential gradient of ammonium acetate buffer [lower reservoir 200 ml of 0·01 M (pH 4·5), upper reservoir 350 ml of 0·6 M (pH 5·2)]. For the $ACTH_{1-24}$ and NPS–$ACTH_{1-24}$, after recovering 130 fractions (390 ml), the buffer in the upper reservoir was replaced by a 0·8 M ammonium acetate buffer (pH 5·2). The fractions corresponding with the peak of radioactivity were pooled and diluted by half with 20 mM Tris–HCl buffer (pH 7·4) containing 2% albumin. This material was divided into 1 ml fractions and stored at −20°C.

Binding measurement of ^{125}I-ACTH to membranes

The membranes were incubated in 0·25 ml of 20 mM Tris–HCl buffer (pH 7·4) containing 1% albumin and ^{125}I-ACTH. Incubation temperatures and times are detailed in figures and tables. At the end of the incubation, the medium was layered over 1 ml of 0·25 M sucrose in 20 mM Tris–HCl buffer (pH 7·4) containing albumin and centrifuged immediately in plastic conical tubes at 50,000 *g* for 10 min. at 0°C. After aspiration of the supernatant, the tip of the centrifuge tube was cut off and counted. For kinetic studies, 0·1 ml of the incubation mixture was layered over 0·2 ml of the last buffer described, contained in a plastic micro-test tube and centrifuged in a Beckman micro-centrifuge as described by Rodbell *et al.*[9]. All binding determinations were performed in triplicate. Three additional samples were also measured in the presence of unlabelled ACTH (200 μg per sample). The latter estimated the non-specific binding which was subtracted in each instance. All experiments reported were repeated at least three times to ensure the reproducibility of results.

Leucine–aminopeptidase degradation of iodinated ACTH was performed according to the procedure of Lefkowitz *et al.*[6]. At the end of incubation, a 0·2 ml aliquot was applied on Whatman 3 MM chromatography paper. Appropriate standards (^{125}I-monoiodotyrosine, ^{125}I-diiodotyrosine and Na ^{125}I) were applied on reference strips at each side of the sample. The chromatogram was developed for 8 h at 30° in the system butanol–acetic acid–water (4:1:5 v/v) as described by Izzo *et al.*[10]. Radioactivity was detected either by scanning the paper in a windowless flow counter or by cutting the paper into 1 cm pieces and counting them in a well-type scintillation counter. Another 0·5 ml aliquot was applied to a Sephadex G-50 column (0·9 × 60 cm) equilibrated with 20 mM Tris–HCl buffer (pH 7·4) and calibrated with $ACTH_{1-24}$, ^{125}I-monoiodotyrosine and Na ^{125}I.

RESULTS

Purity of iodinated ACTH

The elution profiles of the three labelled ACTHs ($ACTH_{1-24}$, NPS–$ACTH_{1-24}$ and $ACTH_{11-24}$) from the carboxymethyl cellulose column is shown in Fig. 1. The iodinated $ACTH_{1-24}$ was separated almost

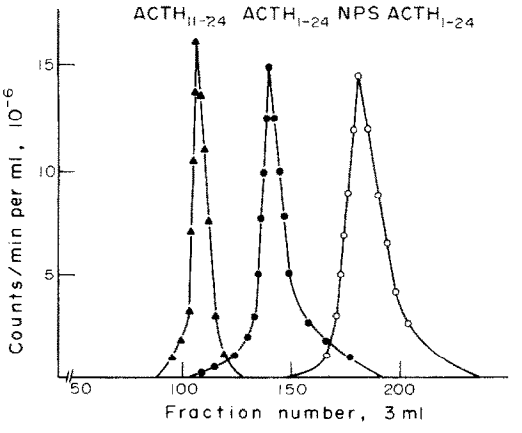

Fig. 1. Elution profiles of ^{125}I-$ACTH_{11-24}$, ^{125}I-$ACTH_{1-24}$ and ^{125}I-NPS–$ACTH_{1-24}$ on carboxymethylcellulose column as described under Methods: 100 μg of each ACTH were iodinated separately with 1·5 mCi of Na ^{125}I carrier free.

completely from non-iodinated $ACTH_{1-24}$ (data not shown). The iodinated $ACTH_{1-24}$ stimulated the adenyl cyclase activity of subcellular preparations of adrenals. However, to obtain the same stimulation, it required about twice as much iodinated $ACTH_{1-24}$ than non-iodinated $ACTH_{1-24}$. Comparable results were reported by Lefkowitz *et al.*[6]

Owing to insufficient quantities of unlabelled hormone, we were unable to check whether iodinated and non-iodinated NPS–$ACTH_{1-24}$ were separated on a carboxymethylcellulose column. However the following observation suggests that they were separated. After purification on a column and in the presence of 0·1 mg of membrane protein, the percentage of binding of ^{125}I-$ACTH_{11-24}$ and ^{125}I-NPS–$ACTH_{1-24}$ was similar to that of ^{125}I-$ACTH_{1-24}$ (60–75%). On the other hand, if the purification was made by adsorption to Quso G-32[11], only 20–30% of the radioactivity was bound to the membranes.

The specific activity of the ^{125}I-$ACTH_{1-24}$ varied from 300 to 450 μCi/μg. The specific activity that would be obtained if the hormone was labelled at one ^{125}I atom/molecule hormone is about 500 μCi/μg[11]. Therefore the iodinated and non-iodinated ACTH were probably not separated completely. The specific activity of ^{125}I-NPS–$ACTH_{1-24}$ and ^{125}I-$ACTH_{1-24}$ were of the same order of magnitude as ^{125}I-$ACTH_{1-24}$.

After the column chromatography, the three iodinated ACTHs showed on electrophoresis on paper[11] a single peak of radioactivity that remained at the origin.

After incubation of the three iodinated ACTHs with leucine aminopeptidase (70 units/ml) for 6 h at 37°, more than 80% of the radioactivity was eluted from the G-50 column in the same elution volume as monoiodotyrosine and had the same polarity as this compound on paper chromatography (Fig. 2). No radioactivity was detected as diiodotyrosine after chromatography on the column or paper. These results suggest that the iodine was incorporated as monoiodotyrosine.

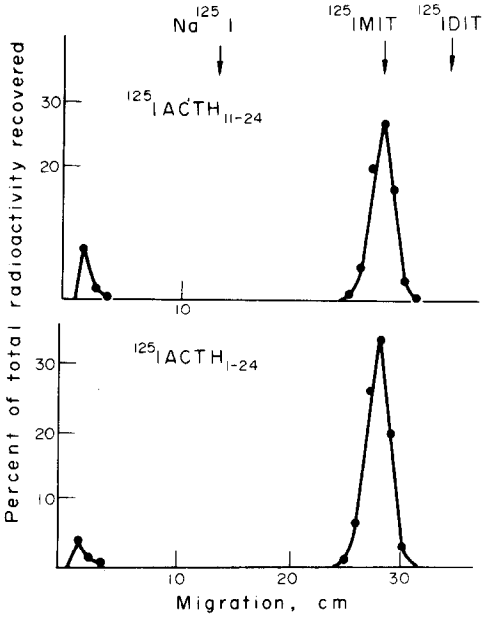

Fig. 2. Paper chromatography patterns of ^{125}I-$ACTH_{1-24}$ (bottom) and ^{125}I-$ACTH_{11-24}$ (top), degradation products after treatment with leucine aminopeptidase. Vertical arrows denote the mobility of Na^{125}I, ^{125}I-monoiodotyrosine and ^{125}I-diiodotyrosine. Both ACTH remain at the origin of the chromatogram.

Binding of labelled ACTH *to adrenal preparations from rat, sheep and man*

With the freshly labelled ACTHs in low concentration (10^{-10} M) and in the presence of 0·6 mg of protein (ml of crude membrane) of the three species, 60–75% of the radioactivity was bound at 4° (total binding).

Table 1. Distribution of adenyl cyclase, 5′-nucleotidase and ACTH-binding activities in different subcellular fractions of sheep adrenal. All determinations were made using the same amount of protein (100 μg)

	Adenyl cyclase			5′-Nucleotidase	
	Basal	ACTH 10^{-5} M	NaF 12 mM	Inorganic phosphate liberated	Binding*
	Cyclic AMP (pmoles/20 min/mg protein)‡			(μmoles/h/mg protein)	(pmoles/mg protein)‡
Whole homogenate	193 ± 8	301 ± 13	522 ± 22	2·5	12 ± 1·1
800 *g*	192 ± 7	252 ± 11	545 ± 20	0	8 ± 0·9
20,000 *g*	274 ± 10	452 ± 14	1162 ± 40	5·5	23 ± 1·4
105,000 *g*	156 ± 6	177 ± 8	940 ± 39	7·9	6 ± 0·8
Supernatant (105,000 *g*)	17 ± 1	24 ± 2	42 ± 4	0	—
Purified membranes*	364 ± 12	600 ± 15	4749 ± 60	32·5	30 ± 1·6
Purified mitochondria*	7 ± 1	5 ± 1	16·7 ± 3	0	0·4 ± 0·2

* Prepared by the method of Finn *et al.*[5].

* Specific binding was determined at 4° for 30 min in the presence of 3×10^{-8} M ^{125}I-$ACTH_{1-24}$ as described under Methods.

‡ Mean ± 1 S.D. of 6 measurements.

Table 2. Effects of several factors on binding of ^{125}I-ACTH$_{1-24}$. Rat adrenal crude membranes (0·6 mg/ml) were incubated 30 min at 4° with ^{125}I-ACTH$_{1-24}$ in the conditions indicated in the table. Results for specific binding obtained under control conditions (61% of the radioactivity added) was given an arbitrary value of 100

		EDTA 8 mM	EGTA 8 mM
Control	100	100	100
+ $MgCl_2$ 5 mM	60	92	—
+ $MnCl_2$ 5 mM	43	88	—
+ $CaCl_2$ 5 mM	51	93	95
+ Glucagon 10^{-5} M	100		
+ Insulin 10^{-5} M	100		
+ PGE_1 10^{-5} M	100		
+ Prolactin 10^{-5} M	100		

When 200 μg of ACTH$_{1-24}$ was added to the incubation medium, the binding was reduced to 1–3% (non-specific binding). As the labelled ACTH aged, the total binding of label reduced and the non-specific binding increased. In all the experiments described in this paper, the iodinated ACTH was used within three weeks of its labelling with a total binding greater than 50% and a non-specific binding below 7%.

The distribution of ACTH binding sites and the activities of adenyl cyclase and 5'-nucleotidase in the different subcellular fractions are given in Table 1. The binding and enzymatic activities of the 20,000 *g* pellet are about twice that of the whole homogenate. The purified membranes isolated by the method of Finn *et al.*[5] have 5'-nucleotidase activity and NaF stimulatable adenyl cyclase five times greater than the 20,000 *g* pellet, but the purification of basal and ACTH stimulatable adenyl cyclase is less. These results suggest that during the purification (with discontinuous and continuous gradient) by the method of Finn *et al.*[5], the improvement of the quality of the membranes, proved by electron microscopy, is not accompanied by an equal improvement of all the enzyme activities related to the membranes.

The binding of the three labelled ACTHs with the adrenal preparations was specific and displaced only by ACTH$_{1-24}$ and certain of its analogues (see below). Insulin, glucagon, ovine prolactin, prostaglandins PGE_1 and PGE_2 in concentrations of 10^{-5} M were without effect (Table 2).

Time course of binding of ^{125}I-ACTH$_{1-24}$ to adrenal crude membranes

The binding of ^{125}I-ACTH$_{1-24}$ to its receptor is a very rapid process (Fig. 3). For this reason, the kinetics of the binding during the first few minutes of the interaction is difficult to measure with precision. During the first10 min of the incubation, the binding at 37° is higher than that observed at 4°. At 37° the maximum binding was reached in 10 min. This maximum is followed by a very rapid diminution (Fig. 3). After 24 h the total binding is similar to the non-specific binding. At 4° equilibrium is reached after 20 min and maintained for 1 h. After 24 h, the binding was about 40% of that seen at 20 min.

These differences observed in the binding kinetics at 4° and 37° are similar in the three species and could be due either to the degradation of the hormone or a denaturation of the receptor, or both of these effects. Figure 4 shows that the pre-incubation of the receptor at 4° for 4 h did not change the capacity of the binding,

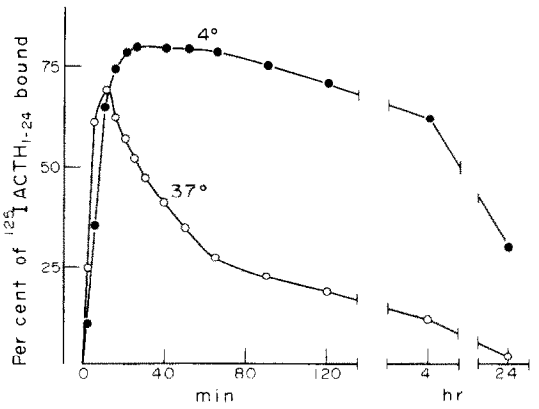

Fig. 3. Time course of binding of ^{125}I-ACTH$_{1-24}$ to crude human adrenal membranes. Membrane protein (0·35 mg/ml) in 6 ml of 20 mM Tris–HCl (pH 7·4), containing 1% albumin and 1×10^{-9} ^{125}I-ACTH$_{1-24}$ were incubated at 4° or 37°. At the indicated times triplicate aliquots (0·1 ml) were withdrawn and the total bound ^{125}I-ACTH$_{1-24}$ determined as described under Methods. The non-specific binding for both temperatures, from 5 min to 24 h, represented 1·5–2% of the total binding (data not shown).

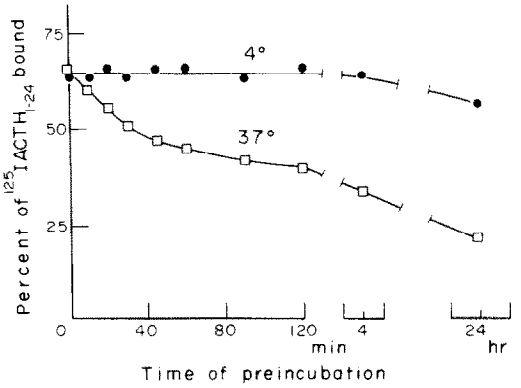

Fig. 4. Effects of membrane preincubation on binding of ACTH. Human crude adrenal membranes (2·5 mg/ml) in 20 mM Tris–HCl (pH 7·4) were preincubated at 4° and 37°. At the time indicated an aliquot of 0·1 ml was taken and incubated 30 min with 18,000 counts/min. of ^{125}I-ACTH$_{1-24}$ (total volume 0·25 ml). Specific binding of membrane preincubated at 4° and 37° was measured at 4° as described under Methods.

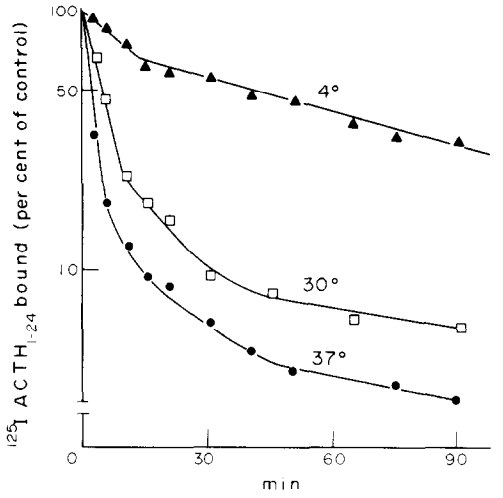

Fig. 5. Time course of dissociation of ^{125}I-ACTH bound to human adrenal membranes, Membranes (1·7 mg/ml) were incubated at 4° in 6 ml of 20 mM Tris–HCl and 3·2 × 10^{-9} M ^{125}I-ACTH$_{1-24}$. After 30 min, 2 ml of Tris–HCl containing 4 mg of ACTH$_{1-24}$ were added. At the indicated times triplicate aliquots (0·1 ml) were withdrawn and the bound ^{125}I-ACTH$_{1-24}$ determined as described under Methods.

to the contrary, preliminary incubation at 37° caused a diminution, which increased with time. However, this denaturation of the receptor seems not to be sufficient to explain the binding kinetics observed at 37° (compare Figs. 3 and 4). The second factor that contributes to the explanation of this phenomenon is the existence of a degradation of the hormone which is more rapid at 37° than at 4° (unpublished data).

The dissociation of the ACTH-receptor complex is a process which varies as a function of temperature (Fig. 5). At 4° the dissociation is composed of at least two slopes whose apparent rates of dissociation are in the order of 5 × 10^{-4} s^{-1} and 1 × 10^{-5} s^{-1}. At 30° and 37° the dissociation is more rapid and more complex.

Effect of membrane protein concentration

The binding of ACTH is a function of the concentration of the membrane proteins (Fig. 6). At the two temperatures at which they were studied, 4° and 37°, 20 and 9% of ^{125}I-ACTH$_{1-24}$ were bound, respectively, in the presence of 40 μg membrane protein/ml. The non-specific binding with this concentration of protein is 1%, similar to that seen in the absence of membranes. At 4°, the maximum binding is obtained with a concentration of 0·8 mg protein/ml, whilst at 37° it was obtained with a lower concentration of the protein. At 4° and 37° the non-specific binding is a linear function of the protein concentration, consequently, the specific fixation decreases when high concentrations of proteins are used. This is most evident at 37°.

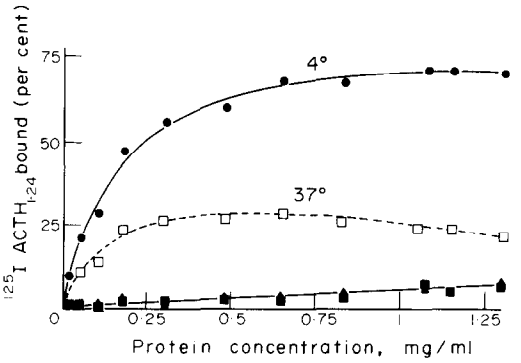

Fig. 6. Binding of ^{125}I-ACTH$_{1-24}$ to crude rat adrenal membranes as a function of protein concentration. Membranes in 0·25 ml of 20 mM Tris–HCl (pH 7·4) containing 1% albumin and 4 × 10^{-8} M ^{125}I-ACTH$_{1-24}$ were incubated 30 min. Total binding at 4° (●—●) and at 37° (□- - -□) and non-specific binding at 4° (▲—▲) and at 37° (□—□) were measured as described under Methods.

Effect of various ions on ACTH *binding*

The binding of ACTH$_{1-24}$ is inhibited by several ions (Fig. 7). Divalent cations are more active than monovalent cations. The power of a salt to inhibit the binding of ACTH seems to depend not only on cations but also on anions. Ammonium sulphate and Na_2SO_4 caused more inhibition than NH_4Cl and NaCl, but by contrast the inhibition induced by $MgCl_2$ is greater than that produced by $MgSO_4$. NaCl, KCl and NH_4Cl produced the same inhibition. The binding of ^{125}I-ACTH$_{11-24}$ and ^{125}I-(NPS–ACTH$_{1-24}$) is inhibited in a similar fashion to that

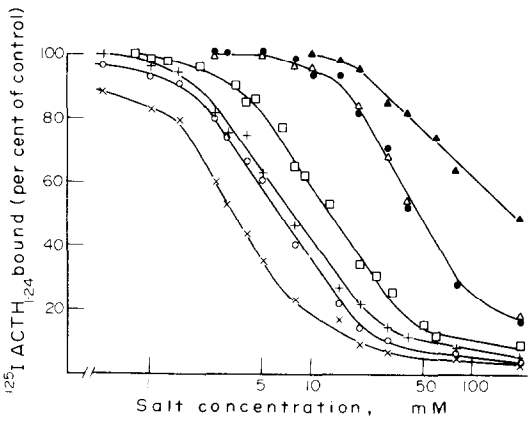

Fig. 7. Effects of concentration of various ions on total binding of ^{125}I-ACTH to crude sheep adrenal membranes. Membrane proteins were incubated 30 min at 4° in 20 mM Tris–HCl, without albumin, containing 1 × 10^{-9} M ^{125}I-ACTH$_{1-24}$ and the indicated concentrations of salts. The pH was adjusted to 7·4 in all experiments. In these experiments the total and the non-specific binding without salts were 65% and 4%, respectively. $MnCl_2$ (×), $CaCl_2$ (○), $MgCl_2$ (×), $MgSO_4$ (□), Na_2SO_4 (△), $(NH_4)_2SO_4$ (●), NaCl, KCl or NH_4Cl (▲).

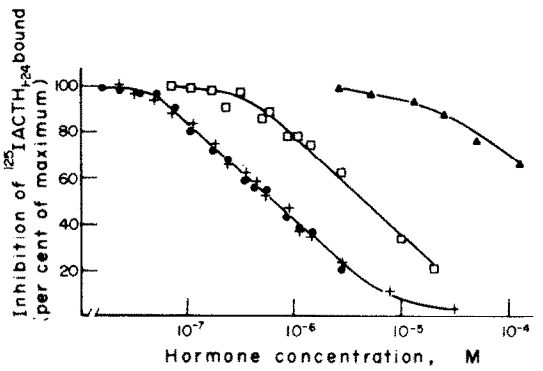

Fig. 8. Displacement of ^{125}I-ACTH$_{1-24}$ bound to crude sheep adrenal plasma membranes by ACTH analogues. Membranes were incubated 30 min at 4° in 0·25 mg of 20 mM Tris–HCl (pH 7·4) containing 1% albumin and 4×10^{-10} M ^{125}I-ACTH$_{1-24}$ and the indicated concentrations of unlabelled ACTH analogues: NPS–ACTH$_{1-24}$ (●), ACTH$_{1-24}$ (×), (ACTH$_{11-24}$ (□) and ACTH$_{1-10}$ (▲). In these experiments the total binding was 71%.

of ^{125}I-ACTH$_{1-24}$ (data not shown). EDTA or EGTA caused the inhibitions produced by divalent cations to disappear (Table 2).

Displacement of ^{125}I-ACTH$_{1-24}$, ^{125}I-NPS–ACTH$_{1-24}$ *and* ^{125}I-ACTH$_{11-24}$ *bound to adrenal membranes by various* ACTH *analogues*

The binding of ^{125}I-ACTH$_{1-24}$ is displaced in a similar fashion by ACTH$_{1-24}$ and NPS–ACTH$_{1-24}$. On the other hand, the displacement produced by ACTH$_{11-24}$ and ACTH$_{1-10}$ at the same molarity is about 6 and 15 times less, respectively, than that produced by ACTH$_{1-24}$ (Fig. 8). The displacement of the binding of ^{125}I-NPS–ACTH$_{1-24}$ by other analogues

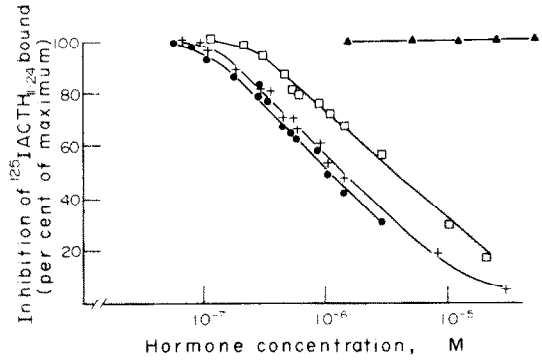

Fig. 9. Displacement of ^{125}I-ACTH$_{11-24}$ bound to crude sheep plasma adrenal membranes by ACTH analogues: membranes were incubated 30 min at 4° in 0·25 ml of 20 mM Tris–HCl (pH 7·4) containing 1% albumin and 3×10^{-10} M ^{125}I-ACTH$_{11-24}$ and the indicated concentrations of unlabelled ACTH analogues: NPS–ACTH$_{1-24}$ (●), ACTH$_{1-24}$ (×), ACTH$_{11-24}$ (□) and ACTH$_{1-10}$ (▲). In these experiments the total binding was 67%.

of ACTH is similar to that of ^{125}I-ACTH$_{1-24}$ (data not shown). The binding of ^{125}I-ACTH$_{11-24}$ is displaced by ACTH$_{1-24}$ and NPS$_{1-24}$, but the displacement of these two peptides is greater than that of ACTH$_{11-24}$. On the other hand, ACTH$_{1-10}$ at high concentrations (5×10^{-4} M) was without effect (Fig. 9). The results of Figs. 10 and 11 and similar experiments show that the apparent dissociation constant for both ^{125}I-ACTH$_{1-24}$ and ^{125}I-NPS–ACTH$_{1-24}$ is about 6×10^{-7} M and for ^{125}I-ACTH$_{11-24}$ about 4×10^{-6} M. It must be pointed out that all these values are only rough since we have not taken into consideration the degradation of the hormone which even at 4° is important (unpublished data).

Effects of ACTH *analogues on the adenyl cyclase activity*

The stimulation of adrenal cyclase by certain analogues and fragments of ACTH$_{1-24}$ have been studied (Table 3). ACTH$_{11-24}$ has no stimulatory effect on adenyl cyclase, whilst ACTH$_{1-10}$ and NPS–ACTH$_{1-24}$ significantly increase the basal activity but far less than ACTH$_{1-24}$. The significant inhibitory effect of ACTH$_{11-24}$ on the stimulation induced by ACTH$_{1-24}$ is easily explained by the fact that it displaces bound ^{125}I-ACTH$_{1-24}$ without stimulating adenyl cyclase. The fact that NPS–ACTH$_{1-24}$ inhibits the stimulation induced by ACTH$_{7-24}$ can also be explained, if it is assumed this analogue of ACTH has a stimulatory action of adenyl cyclase well below that of ACTH$_{1-24}$, but an affinity for the receptor identical to ACTH$_{1-24}$ and greater than ACTH$_{11-24}$.

The results for ACTH$_{1-10}$ are a little more complex to interpret. The stimulatory effect on adenyl cyclase is in agreement with the findings in isolated cells[12]. However, Seelig *et al.*[13] found in isolated adrenal cells that ACTH$_{1-10}$ acts as a competitive antagonist to ACTH$_{1-39}$ with respect to corticosterone production.

Table 3. Action of analogues and fragments of ACTH$_{1-24}$ on the adenyl cyclase activity of particulate preparations of rat adrenals (20,000 g pellet)

	Cyclic AMP (pmoles/mg protein/20 min)
Basal activity	280 ± 15*
ACTH$_{1-24}$ 10^{-5} M	915 ± 50†
NPS–ACTH$_{1-24}$ 10^{-5} M	380 ± 33†
ACTH$_{11-24}$ 10^{-4} M	279 ± 20
ACTH$_{1-10}$ 10^{-4} M	364 ± 27†
ACTH$_{1-24}$ (10^{-5} M) + ACTH$_{11-24}$ (10^{-5} M)	800 ± 30‡
ACTH$_{1-24}$ (10^{-5} M) + NPS–ACTH (10^{-5} M)	645 ± 40‡
ACTH$_{1-24}$ (10^{-5} M) + ACTH$_{1-10}$ (10^{-4} M)	1100 ± 70‡

* mean ± SD (12 observations).
† $P < 0{\cdot}001$ compared to basal activity.
‡ $P < 0{\cdot}01$ compared to ACTH$_{1-24}$ 10^{-5} M.

Our results showed that the stimulation induced by maximal concentration of $ACTH_{1-10}$ and $ACTH_{1-24}$ were additive.* $ACTH_{1-10}$ displaces 15 times less bound ^{125}I-$ACTH_{1-24}$ than $ACTH_{1-24}$ at the same molarity; but it has a protective effect on the degradation of ^{125}I-$ACTH_{1-24}$ which is similar to that of $ACTH_{1-24}$ (unpublished data). The additive effect that we found could be a reflection of the inhibition by $ACTH_{1-10}$ of the degradation of $ACTH_{1-24}$ and the weak capacity of this fragment to displace bound $ACTH_{1-24}$. Furthermore, the fact that the degradation of $ACTH_{1-24}$ by intact isolated cells is less than by subcellular fractions (unpublished data) probably explains the difference between Seelig's results and ours.

DISCUSSION

The biosynthesis of corticosteroids seems to be under the control of many hormonal factors[14]. The first step in the mechanism of action of these hormones seems to be the attachment of the hormone to specific sites localized on the cell surface. In animals, a specific binding to the particulate fractions of the adrenals has been shown for angiotensin[15], ACTH[4–6], prostaglandins[8], prolactin[16, 17] and insulin[18]. Nevertheless, the role of the last two hormones in the control of adrenal steroidogenesis has not yet been defined.

Previously, ACTH binding to adrenal preparations has been studied in only two species, mice[6, 20] and oxen[4, 5]. Our studies have shown the existence of these receptors in the adrenals of rat, sheep and man, but the number of apparent sites varies from one species to another. It is of interest that with the same type of preparation we have seen evidence for distinct binding sites for ACTH, insulin and prostaglandins (PGE_1 and PGE_2) in both human and ovine adrenals[8, 18].

The kinetics of the binding of ACTH to its receptor is temperature dependent (Fig. 3). At 37° the maximum binding is reached in 19 min followed by a very rapid decline. This is the consequence of degradation of the hormone (unpublished data) and modification of the receptors (Fig. 4). This later process could be due either to a diminution of its affinity or a reduction of its capacity or both these effects. Lefkowitz *et al.*[19] studied the rate of association of ACTH to a soluble extract of murine adrenal tumour as a function of temperature. They found at 37° there was no state of equilibrium; however, the total binding at 37° during the first hour of incubation was always greater than that observed at 1°. The difference between these results and ours could have been due to less degradation of the hormone in their preparation than in our preparations.

The apparent dissociation constant found for $ACTH_{1-24}$ is similar to that found by Finn *et al.*[5] and Lefkowitz *et al.*[6] and corresponds well with the concentration of hormone which gives half maximal stimulation of adenyl cyclase[6, 21, 22]. However, these concentrations are much greater than those which are required for eliciting a half maximal response with isolated adrenal cells[12, 13, 23–25] and the physiological concentration of this hormone in the blood[11]. Lefkowitz *et al.*[19] using low concentrations of labelled ACTH demonstrated the sites of high affinity (9×10^{-11} M). Using the same concentration of labelled ACTH we could not confirm these high affinity sites. As indicated above, the source of adrenal material was dissimilar in these two series which could explain the discordances.

A quantitative difference between the affinity of the hormone for intact and ruptured cells has been observed in the binding of oxytocin to the toad bladder [26, 27]. It has been suggested that the reduced affinity could be due to a deterioration of the receptor during homogenization. The difference in the affinity of ACTH for intact cells and cell fractions may be due to the same mechanism, as well as hormone degradation that is more pronounced in the fractionated material than in whole cells (unpublished data).

The role of ions in the binding of several peptide hormones to their receptors is well known. Lefkowitz *et al.*[19] have shown that Ca^{2+} inhibits the binding of ACTH but this inhibition does not seem to be exclusive for Ca^{2+} since other divalent ions produce a similar inhibition (Fig. 7). However, the inhibition produced by Ca^{2+} could be specific for this ion plays an important role in the action of ACTH on steroidogenesis in intact cells[28] and on adenyl cyclase in cell homogenates [19, 29].

Studies of the structure–action relation of ACTH in several laboratories[4, 5, 30–33] have shown that the peptide sequences necessary for the binding and biological action of this hormone are localized in different parts of the molecule. The N-terminal sequence is essential for its biological action, the sequence[11–24] C-terminal is important for binding. Our study suggests that the sequence 1–10 N-terminal in addition to being the "active" site of the molecule, should contribute to binding, since the affinity of $ACTH_{11-20}$ amide[4, 5] and $ACTH_{11-24}$ (Fig. 9) for the adrenal receptor is much weaker than that of $ACTH_{1-24}$.

* Maximal stimulation of adenyl cyclase was obtained with $ACTH_{1-24}$ 10^{-5} M. Further increase of $ACTH_{1-24}$ leads to a reduced stimulation, which might be explained by the accumulation of degradation products of $ACTH_{1-24}$. This former phenomenon (reduced stimulation of adenyl cyclase) does not occur, if we add increasing concentrations of $ACTH_{1-10}$ ($10^{-6} - 10^{-4}$ M) in the presence of $ACTH_{1-24}$ 10^{-5} M.

The displacement curves we have obtained (Figs. 8 and 9) suggests the existence of a common site having a different affinity for $ACTH_{1-24}$ and $ACTH_{11-24}$. These results confirm the findings of Seelig *et al.*[12, 13] who showed that $ACTH_{11-24}$ has no biological action and behaves as an antagonist of $ACTH_{1-39}$. Meanwhile, this group of workers showed $ACTH_{11-24}$ at very high concentration is also an antagonist of $ACTH_{1-10}$. However, we were unable to demonstrate a displacement of the binding of ^{125}I-$ACTH_{11-24}$ by $ACTH_{1-10}$.

The biological activity of ACTH *in vitro* and *in vivo* is not significantly diminished until the length of the chain is reduced to less than 1–18 of N-terminal [12, 22, 30]. The blockade of ε-amino group of lysines in positions 11, 15 and 16 or their replacement by other non-basic amino acids is associated with a marked reduction of biological activity[4, 5, 30, 34]; this was interpreted as due to a diminution of affinity. All the modifications of the primary structure of the sequence 4–10 are accompanied by a diminution of biological activity[4, 22, 30, 33] which has been interpreted as diminution of the excitation of the "active centre". The fact that the affinities of (phe^9)-$ACTH_{1-20}$ amide[4] and NPS-$ACTH_{1-24}$ were similar to those of $ACTH_{1-20}$ amide and $ACTH_{1-24}$, respectively, although their biological activities were far lower [4, 12, 25] favours this theory.

At present, it is not possible to be sure whether reduction of biological activity which accompanies the other modification of the primary structure of the sequence 4–10 is due to a diminution of the excitation of the "active centre" or a reduced binding, or to both. A comparative study of action and binding by these analogues is needed to answer this question.

Acknowledgements—The authors are most grateful to Drs. P. Freychet and H. Bornet for helpful suggestions in preparing the manuscript. We are indebted to Drs. W. Rittel and P. A. Desaulles for the generous gift of various ACTH analogues, and to Dr. Y. Guichard for the electron microscopy of our subcellular preparations. We thank Dr. J. Bertrand for his continued interest and encouragement in this work. The secretarial assistance of Miss J. Bois is also appreciated.

This work was supported in part by CNRS grant no. 429904.

REFERENCES

1. Schimmer B. C., Ueda K. and Sato G. H.: *Biochem. biophys. Res. Commun.* **32** (1968) 806–810.
2. Civen M.: *Symp. Metabolic Regulation.* Academic Press, New York (1972) p. 313
3. Richardson M. C. and Schulster D.: *J. Endocr.* **55** (1972) 127–139.
4. Hofmann K., Wingender W. and Finn F. M.: *Proc. natn. Acad. Sci. U.S.A.* **67** (1970) 829–836.
5. Finn F. M., Widnell C. C. and Hofmann K.: *J. biol. Chem.* **247** (1972) 5695–5703.
6. Lefkowitz R. F., Roth J., Pricer W. and Pastan I.: *Proc. natn. Acad. Sci. U.S.A.* **65** (1970) 745–752.
7. Heppel L. A. and Hilmoe R. J.; *J. biol. Chem.* **188** (1951) 665–669.
8. Dazord A., Morera A. M., Bertrand J. and Saez J. M.: *Endocrinology* (in press).
9. Rodbell M., Kraus H. M., Pohl S. L. and Birnbaumer L.: *J. biol. Chem.* **246** (1971) 1861–1871.
10. Izzo J. L., Roncone A., Izzo M. J., Foley R. and Bartlett J. W.: *J. biol. Chem.* **247** (1972) 1219–1226.
11. Berson S. and Yalow R.: *J. clin. Invest.* **47** (1968) 2725–2751.
12. Seelig S. and Sayers G.: *Archs. biochem. Biophys.* **154** (1972) 230–239.
13. Seelig S., Sayers G., Schwyzer R. and Schiller P., *Fed. Eur. biochem. Soc. Lett.* **19** (1971) 232–234.
14. Mulrow P. C., *Ann. Rev. Physiol.* **34** (1972) 409–424.
15. Lin S. Y. and Goodfriend T.: *Am. J. Physiol.* **218** (1970) 1319–1328.
16. Friesen H., Tolis G., Shin R., Hwang P. and Hardy J.: *Int. Symp. on Human Prolactin*, Brussels. Excerpta Medica, Amsterdam (1973) p. 11.
17. Turkington R. W., Frantz W. L. and Majunder G. C., *Int. Symp. on Human Prolactin*, Brussels. Excerpta Medica, Amsterdam (1973) p. 41.
18. Saez J. M., Dazord A., Gallet D. and Bertrand J.: *C.r. hebd. Séanc. Acad. Sci. Paris* **276** (1973) 2039–2042.
19. Lefkowitz R. J., Roth J. and Pastan I.: *Ann. N.Y. Acad. Sci.* **185** (1971) 195–209.
20. Wolfsen A. R., McIntyre H. B. and Odell W. D.: *J. clin. Endocrinol. Metab.* **34** (1972) 684–689.
21. Tauton D., Roth J. and Pastan I.: *J. biol. Chem.* **244** (1969) 247–253.
22. Ide M., Tanaka A., Nakamura M. and Kabayashi T.O.: *Archs. Biochem. Biophys.* **149** (1972) 189–196.
23. Kitabchi A. E. and Sharma R. L.: *Endocrinology* **88** (1971) 1109–1116.
24. Schwyzer R., Schiller P., Seelig S. and Sayers G.: *Fed. Eur. biochem. Soc. Lett.* **19** (1971) 229–231.
25. Moyle W. R., Kong Y. C. and Ramachandran J.: *J. biol. Chem.* **248** (1973) 2409–2417.
26. Bär H. P., Hechter O., Schwartz I. L. and Walter R.: *Proc. natn. Acad. Sci. U.S.A.* **67** (1970) 7–18.
27. Roy C., Bockaert J., Rajerison R. and Jard S.: *Fed. Eur. biochem. Soc. Lett.* **30** (1973) 329–332.
28. Haksar A. and Peron F. G.: *Biochem. biophys. Res. Commun.* **47** (1972) 445–450.
29. Kelly L. A. and Koritz S. B.: *Biochim. biophys. Acta* **237** (1971) 141–155.
30. Ramachandran J. and Li C. H.: *Adv. Enzymol.* **29** (1967) 391–477.
31. Tesser G. I. and Rittel W.: *Recl. Trav. chim. Pays-Bas Belg.* **88** (1969) 553–561.
32. Fujino M., Hatanaka C. and Nishimura O.: *Chem. Pharm. Bull.* **19** (1971) 1066–1069.
33. Tesser G. I., Maier R., Schenkel-Hulliger L., Barthe P. L., Kamber B. and Rittel W.: *Acta Endocr., Copenh.* **74** (1973) 56–66.
34. Geiger R. and Schroder H. G.: *Hoppe-Seyler'. Z. physiol. Chem.* **354** (1972) 156–162.

DISCUSSION

Jensen:

You have evidence that the 1–10 sequence is contributing very significantly to the binding to the membrane receptor but where is the part of the molecule that is responsible for the biological activity located?

Saez:

The 1–10 sequence is the biologically active part and this sequence contributes to the binding since the affinity of $ACTH_{11-24}$ is about ten times lower than the affinity of $ACTH_{1-24}$. On the other hand, you saw on one of the slides that ACTH 1–10 can displace bound $ACTH_{1-24}$ but cannot displace $ACTH_{11-24}$. I think this is an argument for saying that ACTH 1–10 contributes to the binding.

Jensen:

So the binding and the active centre seem to be different?

Saez:

The most important part of the molecule for binding is the sequence 11–24 but this sequence has no biological action.

Ungar:

On one of the slides you had a $CaCl_2$ concentration of 15 mM. I'm wondering why you used such a high concentration.

Saez:

Well, we can start to get an inhibition at 1 mM but you get almost complete inhibition between 10 and 15 mM. At 1 mM you have about 10% decrease of the binding.

Ungar:

Do you see an increase in activation with lower concentrations of calcium?

Saez:

With EDTA and EGTA there is no modification of the binding.

Grant:

I suppose because you were labelling the ACTH fragments that they were highly purified because the peptides which you get very often contain small blocks used in the synthesis. Can you confirm that your peptides were in fact quite pure?

Saez:

Well, the peptide was a gift from Dr. Rittel from Basel and they purified the peptide extensively before he gave it to me.

Crabbé:

I wonder if you've had the chance to try this interesting ACTH analog (C-41795) which has been developed by the crew of chemists at Ciba, Basel. It is an octa-deca peptide with, among the characteristics, a d-serine at the C-1 terminal which exerts a surprisingly long action after intravenous injections.

Saez:

No, we haven't had the chance.