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UP REGULATION OF CORTICOTROPHIN RECEPTORS BY $ACTH_{1-24}$ IN NORMAL AND HYPOPHYSECTOMIZED RABBITS

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SUMMARY. The number of ACTH binding sites, in adrenal membranes from adult female rabbits, has been measured at different times after hypophysectomy and after $ACTH_{1-24}$ treatment. The receptor number was significantly reduced 192 h after removal of the pituitary gland as compared to intact controls. Conversely, ACTH treatment of intact rabbits enhanced the number of ACTH binding sites, or restored these levels to presurgical values in hypophysectomized animals. These results suggest that ACTH, like other hormones, is able to induce an increase in the number of its own receptors; the physiological significance of such variations remains however to be elucidated.

INTRODUCTION. It is well known that the magnitude of the acute steroidogenic response of the adrenal cortex to ACTH is a function not only of the amount of hormone which reaches the target cells, but also of the plasma levels of ACTH to which the adrenal glands have been chronically exposed for hours or days. The "priming" ability of repeated injections of ACTH, and the decrease in adrenocortical sensitivity to ACTH after hypophysectomy are examples of this phenomenon (1).

The earliest event in ACTH action is thought to be a non covalent interaction with a specific membrane receptor (2, 3). Recently, it has been shown that the burst of ACTH responsiveness in the ovine fetal adrenal, just before parturition, is accompanied by an increase in the number of ACTH binding sites (4).

In order to elucidate the mechanism of corticotrophin receptor regulation, we have studied the effect of hypophysectomy and $ACTH_{1-24}$ injection on the number of ACTH binding sites in the adrenal gland.

MATERIALS AND METHODS.

Animals : Female New Zealand white rabbits 6-8 months old were used. Hypophysectomy was performed by the parapharyngeal route. Hypophysectomized animals were housed at 25±1°C and their diet was supplemented with 0.9% glucose and 0.9% NaCl in drinking water. At the end of the experiments, all hypophysectomized animals were autopsied and sella turcicas were examined to ensure complete hypophysectomy. Sham operations included the entire surgical procedure with the exception of the removal of the gland.

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Experimental procedures : In the first experiment, groups of rabbits were sacrificed at different times after hypophysectomy, intact rabbits served as controls. In the second experiment, animals hypophysectomized 6 days earlier were injected daily, intramuscularly, with 0.25 mg $ACTH_{1-24}$ "immediat" (synacthène) and 1 mg $ACTH_{1-24}$ "retard" (synacthène retard) obtained from Ciba. These animals were killed 48h after the first injection (and therefore 24h after the last one). Intact rabbits were similarly injected and killed 24, 48 or 96h after the first injection.

Tissue collection and membrane preparation : Animals were killed by cervical dislocation between 14.00 and 16.00h, their adrenals were removed immediately and kept in buffer containing 20 mM Tris HCl pH 7.4 and 0.25M sucrose, at 0°C. Crude adrenal membranes were prepared as previously described (4) and kept in liquid N_2 until assayed for ACTH binding activity.

Iodination and assay procedures : Synthetic $ACTH_{1-24}$ was iodinated with Na $[^{125}I]$ (Amersham, England) using chloramine T at a low concentration (2). $[^{125}I]$ $ACTH_{1-24}$ was purified on a carboxymethylcellulose column with an exponential gradient of ammonium acetate (5). Specific activity was about 400 µCi/µg. Membranes were incubated in duplicate in 0.25 ml of 20 mM Tris HCl buffer containing 1% BSA (Sigma) with approximately 10^5 cpm $[^{125}I]$ $ACTH_{1-24}$ and increasing amounts of unlabelled $ACTH_{1-24}$ (18 to 890 picomoles for the assays and 50 µg for assessing non specific binding). Bound and free hormone were separated by centrifugation. Scatchard plots (6) were found to be curvilinear (4, 7, 8), hence the apparent number of binding sites was estimated by extrapolating the slope for high affinity only, as previously discussed (4). Since the physiological variations of receptors/mg protein were small (threefold at the outside), membrane preparations from differently treated animals were always incubated in the course of the same binding experiment to minimize interassay variations. In addition, in each case, a "standard membrane preparation", kept in liquid N_2 between the assays, was also assayed to ensure reproducibility of the estimation of these binding sites; variation coefficient for this control was 10% for 14 subsequent assays.

Other methods : Proteins were determined by the method of Lowry et al. (9). DNA was assayed according to Morimoto et al. (10).

RESULTS

Fig. 1 shows the time course of specific binding of $[^{125}I]$ $ACTH_{1-24}$ to rabbit adrenal membranes after incubation at 37°, 20° and 4°C. At 37° and 20°C maximal binding occurred in less than 10 min and was followed by a decrease which was more rapid at 37° than at 20°C. Incubation of membranes at 4°C resulted in a plateau of maximal binding between 20 and 80 min; these conditions were therefore chosen in the subsequent experiments, since the plateauing of ligand binding for extended incubation periods indicated a minimal degradation rate of both hormone and receptor and a steady state occupation of the latter, ensuring an accurate titration. Specific binding of $[^{125}I]$ $ACTH_{1-24}$ was directly dependent upon membrane protein concentration up to 0.1 mg/ml. Under optimal conditions, more than 70% of the iodinated $ACTH_{1-24}$ molecules could be specifically bound, non specific binding being less than 5%; for standard assays, protein concentrations of about 0.07 mg/ml were used, since they are

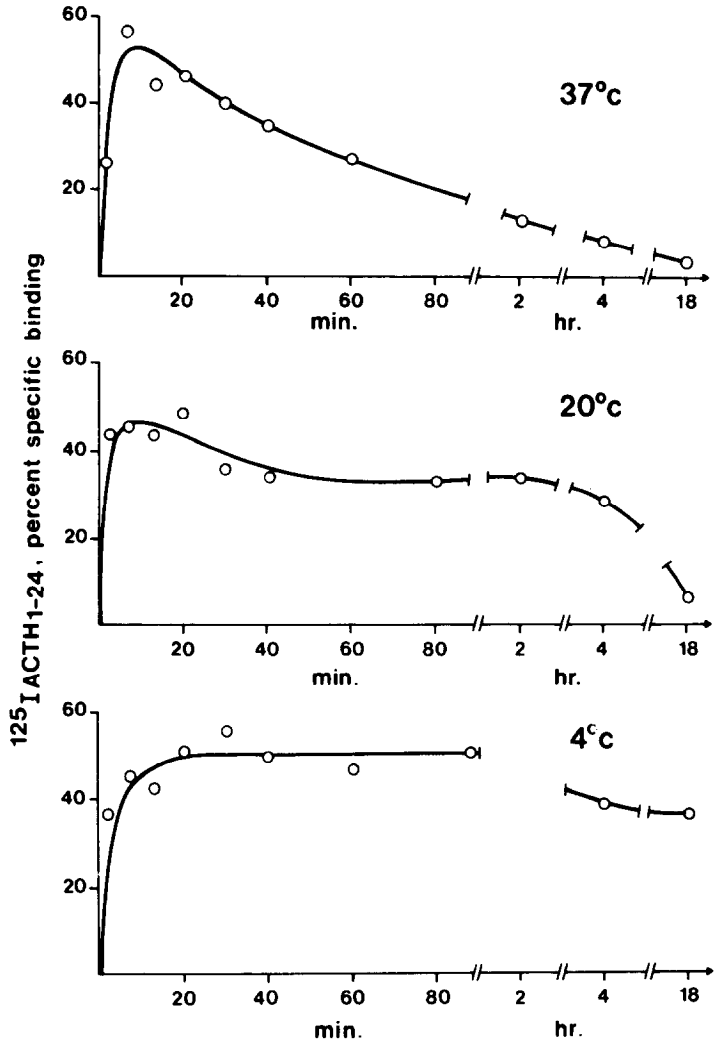


Figure 1: Time course of specific binding of [^{125}I]ACTH$_{1-24}$ at 37°, 20° and 4°C to rabbit adrenal membranes. Membranes (0.13 mg/ml) were incubated with 3.10^{-10}M [^{125}I] ACTH$_{1-24}$ in the absence and in the presence of excess of unlabelled ACTH$_{1-24}$. The latter was used to determine the non specific binding which has been subtracted from each experimental point.

well within the range where binding is proportional to the amount of membranes present. [^{125}I]ACTH$_{1-24}$ bound to rabbit adrenal membranes was displaced in a dose dependent manner by ACTH$_{1-24}$ and porcine ACTH$_{1-39}$ (Sigma), whereas LH, PRL, GH, insulin in large excess were without effect (data not shown). The affinity of native ACTH$_{1-24}$ for the receptors, assessed by the method of Latner et al. (11), was similar to that of the iodinated species (data not shown).

Fig. 2B shows that the number of ACTH binding sites, expressed per mg protein, in rabbit adrenals fell quickly after hypophysectomy (from 3.79±

0.18 nmoles/mg to 2.68±0.30 nmoles/mg at 1h; $p < 0.01$) then increased to return to the control level after 24h. A second decrease was observed from 96h onwards, which became significant at 192h (2.77±0.19 nmoles/mg; $p < 0.01$). A similar pattern was observed when the number of binding sites was expressed per 2 adrenal glands instead of proteins (Fig. 2C). The fall at 192h (8.69±1.03 nmoles vs 32.7±3.21 nmoles; $p < 0.001$) being amplified because of the involution of the glands and the resulting decrease in crude membrane proteins (3.15±0.36 mg/ 2 adrenals vs 8.59±0.67 in control animals; $p < 0.001$). Intermediary values were found when the number of receptors was correlated with the amounts of DNA (results not shown), since the total DNA content of the adrenal glands decreased from 0.455±0.050 mg (controls) to 0.296±0.050 (192h after hypophysectomy), hence less than the protein decrease.

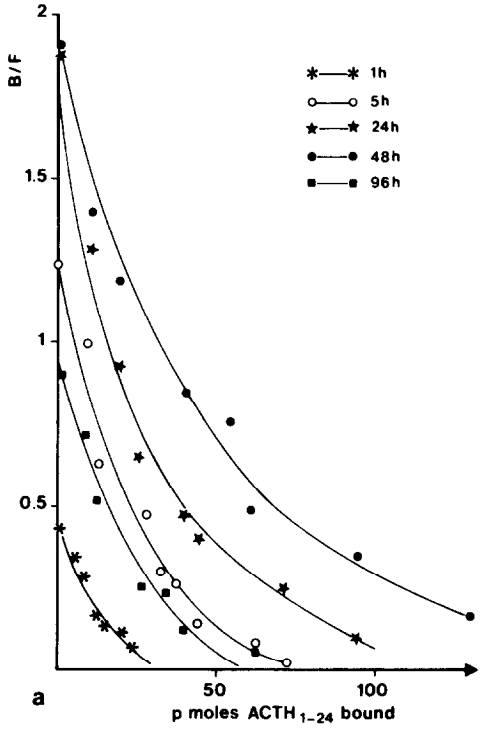


Figure 2: Effect of hypophysectomy on the number of ACTH receptors in the female rabbit adrenals. The values (specific binding) were obtained by Scatchard analysis of the competition data (see panel A).
- Panel B: values expressed as nmoles $ACTH_{1-24}$ per mg membrane protein
- Panel C: values expressed per 2 adrenals

Each point represents the mean (± SEM) for the number of animals indicated at the bottom of each bar.
a: $p < 0.01$ vs controls
b: $p < 0.001$

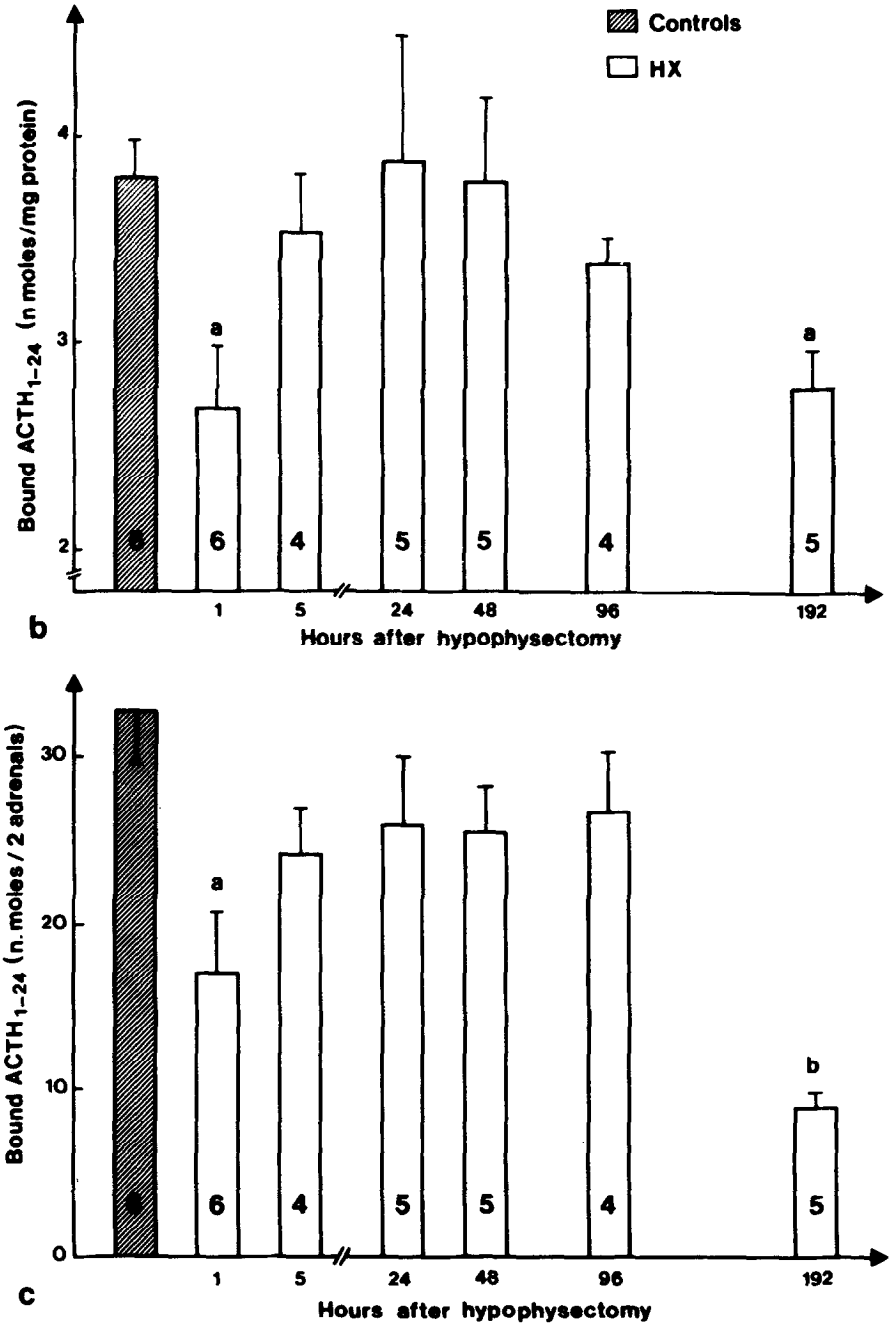


Figure 2b and 2c

No significant variations of the dissociation constant were observed (K_D= 1.7-2.5.10^{-7} M) at any stage (fig. 2A).

The decrease observed at 1h did not seem to result from the operative stress, because sham operated animals killed 1 h after operation exhibited a

number of ACTH binding sites similar to control animals (3.49±0.36 nmoles/mg protein).

The effect of $ACTH_{1-24}$ injections, to both intact and hypophysectomized rabbits, is shown in Fig. 3. A 24h treatment significantly enhanced the number of binding sites in intact animals to 5.86±0.39 nmoles/mg protein ($p < 0.001$ vs controls, fig. 3A). Such an increase was also observed, and even amplified, when the number of ACTH receptors was expressed per 2 adrenals (fig. 3B). Up to 48h no significant increase of DNA had taken place. These changes persisted for up to 96h of treatment, but at that period, DNA had increased to a considerable extent (from 0.455±0.050 mg to 1.084±0.044 mg).

The reduced binding observed after 8 days of hypophysectomy was prevented or even reversed, by a 48h treatment with $ACTH_{1-24}$ of rabbits hypophysectomized for 6 days (fig. 3A, B), the value obtained (4.76±0.46 nmoles/mg protein) being not significantly different from intact animals. It must be emphasized that

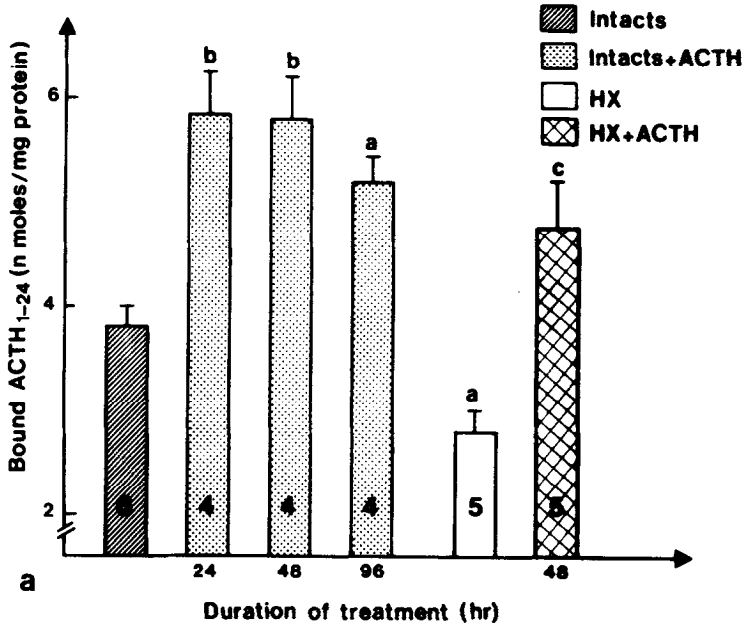
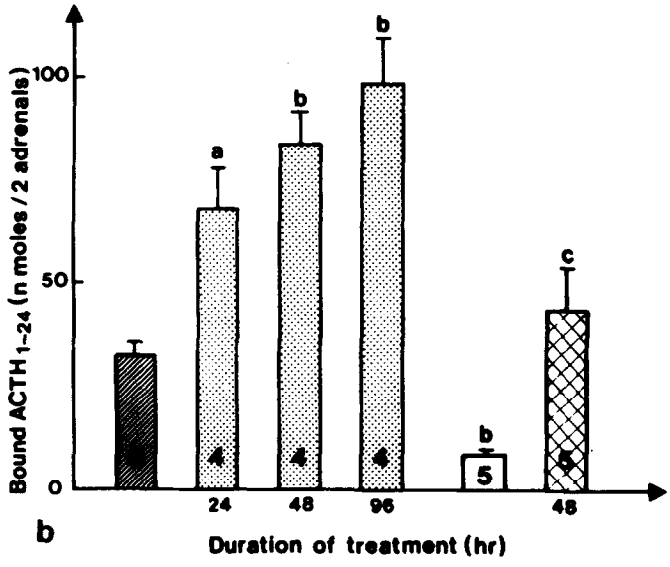


Figure 3: Effect of $ACTH_{1-24}$ treatments on the number of ACTH receptors (mean ± SEM) in adrenal membranes from intact and 8 days hypophysectomized rabbits.

- Panel A: values expressed per mg membrane protein
- Panel B: values expressed per 2 adrenals.

Receptor affinity was the same in control and $ACTH_{1-24}$ stimulated groups (panel C)

a: $p < 0.01$ vs intact controls
b: $p < 0.001$
c: $p < 0.01$ vs hypophysectomized controls and not different from intact animals.



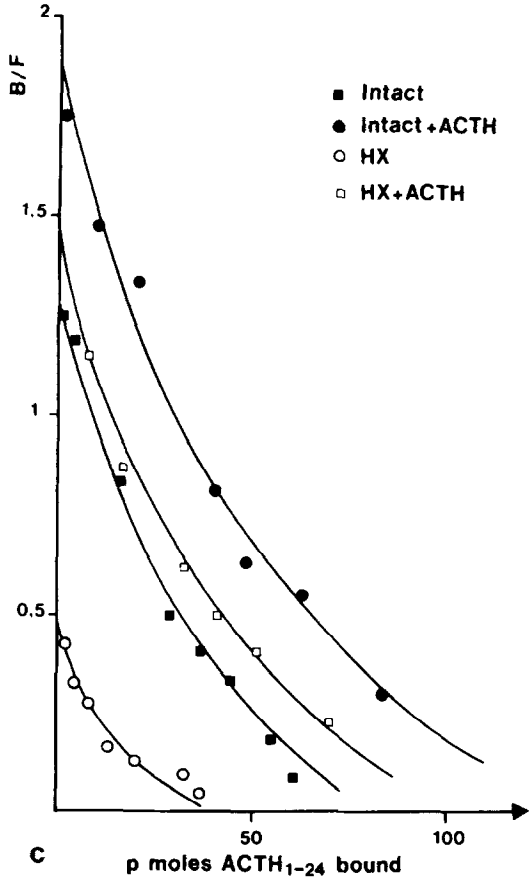


Figure 3b and 3c

under our experimental conditions, this increase of receptors occurred without any simultaneous increase in DNA, as opposed to the results of Bransome (12), who reported an ACTH induced increase of DNA as early as 24h after the onset of treatment, with dexamethasone "suppressed" guinea-pigs.

Receptor affinity was the same in intact or hypophysectomized controls and $ACTH_{1-24}$ stimulated groups (fig. 3C).

DISCUSSION.

Adrenal corticotrophin receptors are unusual in at least two of their properties : the very low affinity for the stimulating peptide (K_D about 10^{-7}M) as compared to circulating levels of the hormone (ranging from 10^{-11}M to 10^{-9}M), and the exceedingly high levels of binding sites (2-4 nmoles/mg protein) 2 to 3 orders of magnitude higher than for most known hormone receptors. It has even been questioned (13) as to whether the binding sites titrated were of any physiological significance. However, the K_D of the receptor/hormone binding and the apparent K_M of adenylate cyclase activation by the hormone are well correlated (2, 3, 8, 14 and unpublished results), whereas minute amounts of $_cAMP$ (hence of hormone bound to its receptor) appear sufficient for a maximal stimulation of steroidogenesis (15).

Hence the present data, and the results of a preceding paper (4), which tend to correlate the levels of corticotrophin receptors with the responsive state of the adrenal cortex and with the availability of circulating ACTH seem to be relevant to the problem of the functional specificity of these binding sites: a close coincidence has been reported (4) between the burst of ACTH responsiveness in the ovine fetal adrenal, immediately before parturition, and a three fold increase of corticotrophin receptor levels per cell. It was then suspected that ACTH itself might be the signal which triggers the "up regulation" of its own receptors: the present work indicates that this might indeed be the case, since corticotrophin, among a number of hormones tested, is the sole one which either increases its receptor levels in the intact animals, or restores these levels to presurgical values in the hypophysectomized animals (Fig. 3). "Self regulating" systems of hormones provoking an increase in the level of their own receptors have been reported for prolactin (16, 17), oestradiol (18) and angiotensin (19), and are commonly admitted as providing one of the means through which a maintenance of the cells responsiveness to the hormone is secured (20). In the present work, the striking increase of corticotrophin receptors after 48 hours treatment, both in the intact and in the hypophysectomized animals, occurs without any variation in DNA, which strongly suggests that it is the number of receptors per cell, and not the number of cells which is increased by the hormone. In addition,

these results are closely correlated with recent data (21) where it has been shown that chronic ACTH treatment to rats, enhances the ability of cortical cells to synthesize $_{c}$AMP in response to ACTH.

It might of course be questioned, whether a two or three fold fluctuation of receptor levels could at all be critical in a system in which the total number of binding sites exceeds by far the amount of hormone which might ever be bound. This question is in bearing with the fact that steroidogenesis is severely impaired within very short periods folowing hypophysectomy, but that no significant decrease in the stimulation of adenylate cyclase by corticotrophin could be demonstrated within the same delays after removal of the pituitary gland (22). This should locate the bottleneck reaction which is sensitive to ACTH, at a step distal to $_{c}$AMP. In a recent work, however, the decay of the steroidogenic response after hypophysectomy closely matches our own data on the loss of corticotrophin receptors (see ref. 23, fig. 1), but in this case, the activity of adenylate cyclase was not reported. A thorough reconsideration of the correlations, in the same animals, between receptor levels, adenylate cyclase stimulation and steroidogenesis, after hypophysectomy thus seems necessary.

Corticotrophin receptors are about the only receptors located in the plasmalemma, for which the classical "down regulation" viz. the loss of receptors under the action of the specific hormone (for a review see 24) has never been established. The onset and time course of this event is usually much faster than the subsequent "up regulation" and both phenomena cannot presently be considered as strictly symetrical regulatory mechanisms. In the present work, the loss of receptors one hour after hypophysectomy (fig. 2) could possibly be due to a burst of pituitary hormones, including ACTH, at the moment the gland is removed. This hypothesis seems relevant to the fact that the phenomenon could be mimicked by injecting crude extracts from single rabbit pituitary glands into control animals (results not shown). Hitherto, it was however not possible to observe the same effect by injecting even large amounts of purified ACTH, which casts some doubt on the identity of the pituitary factor involved.

In conclusion, the present results suggest that ACTH, like other hormones, might induce an "up regulation" of its own receptors, which lends new support to the physiological significance of these binding sites. Whether the number of these receptors could be limiting for the response of cortical cells to ACTH remains however to be elucidated.

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