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STUDIES ON THE ROLE OF PROTEIN SYNTHESIS IN THE REGULATION OF CORTICOSTERONE PRODUCTION BY ADRENOCORTICOTROPIC HORMONE IN VIVO

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The primary event following the administration of adrenocorticotropic hormone (ACTH) is the immediate increase in steroid synthesis by the adrenal gland.[1] Previous studies by numerous investigators suggested that this action of ACTH was the result of specific activation of enzymes.[2, 3] However, studies showing that puromycin or chloramphenicol blocked the stimulation of corticosterone synthesis by ACTH suggested that protein synthesis was involved in this process.[4, 5] But

the importance of these findings in the physiological role of ACTH remained unclear since puromycin did not appear to block the ACTH stimulation of steroid synthesis when administered *in vivo*.[4] Furthermore, Hechter and Halkerston, in a recent review of the action of ACTH, reported studies (which were modifications of the *in vitro* studies of Ferguson) which led them to doubt that the puromycin inhibition of steroidogenesis was caused by its action on adrenal protein synthesis.[6]

In the present communication, studies on the mechanism of the stimulation of adrenal steroidogenesis by ACTH *in vivo* are reported. Evidence is presented which suggests that ACTH regulates corticosterone production by activating specific protein synthesis at a site involving translation of stable template RNA.

Experimental.—In the following studies the effect of ACTH on corticosterone production by rat adrenals *in vivo* was studied using the method of Lipscomb and Nelson.[7] Female rats weighing 200 gm were hypophysectomized by the transaural approach approximately 2 hr prior to their use. At that time baseline corticosterone production was markedly reduced; however, the adrenal glands were still fully responsive to ACTH stimulation.[1, 8] The animals were anesthetized with nembutal. ACTH was injected into one femoral vein, and C^{14}-leucine or C^{14}-uridine was injected into the other. A needle was then threaded through the left adrenal vein almost to its entrance into the adrenal gland, and its entire venous drainage was obtained for 3 min, starting either 7 or 17 min after the injection of ACTH, depending on the experiment. The adrenal glands were then rapidly removed and frozen in solid carbon dioxide until used. Corticosterone in blood and adrenal tissue was extracted with methylene chloride and assayed according to the fluorescence method of Silber *et al.*[9] Incorporation of leucine-C^{14} into adrenal protein was measured by the method of Siekovitz and prepared for liquid scintillation counting as described by Maxwell.[10] The uridine-C^{14} incorporated into adrenal RNA was extracted and prepared for assay of radioactivity as described

TABLE 1

EFFECT OF PUROMYCIN ON ACTH STIMULATION OF STEROIDOGENESIS

Treatment	Corticosterone secretion (μg/3 min), mean ± SEM	C^{14}-Leucine incorporation (d.p.m./100 mg adrenal), mean ±SEM
Control	0.23 ± 0.02 (10)*	7830 ± 161 (10)
ACTH	1.71 ± 0.32 (14)	7410 ± 650 (11)
Puromycin 3 min prior to ACTH	0.83 ± 0.09 (5)	4340 ± 344 (5)
Puromycin 40 min prior to ACTH	0.52 ± 0.11 (8)	990 ± 190 (8)

* Number rats.

Puromycin (30 mg) was administered intraperitoneally, followed by intravenous ACTH (2 milliunits) either 3 or 40 min later. ACTH (2 mU) was also administered to untreated animals, and control rats were similarly injected with saline. Immediately following the ACTH or saline, 4 μc C^{14}-leucine was injected intravenously. Adrenal venous blood was obtained from 7 to 10 min after the injection of ACTH for corticosterone assay. At this time (10 min after ACTH or saline), the adrenals were removed and assayed for C^{14}-leucine incorporation into protein.

TABLE 2

EFFECT OF CYCLOHEXIMIDE ON ACTH STIMULATION OF STEROIDOGENESIS

Treatment	Corticosterone secretion (μg/3 min), mean ±SEM	C^{14}-Leucine incorporation (d.p.m./100 mg adrenal), mean ±SEM
Control	0.27 ± 0.02 (10)*	7920 ± 405 (11)
ACTH	1.64 ± 0.45 (11)	9335 ± 662 (11)
Cycloheximide 3 min prior to ACTH	0.30 ± 0.02 (6)	248 ± 49 (6)
Cycloheximide 40 min prior to ACTH	0.16 ± 0.05 (7)	170 ± 31 (7)

* Number rats.

The experimental procedure is the same as described for Table 1 with the exception that cycloheximide (10 mg) was administered intraperitoneally in place of puromycin.

by Weiss,[11] and assayed in a low background Nuclear-Chicago gas-flow counter.

Materials.—L-leucine-C^{14} (uniformly labeled), 223 mc/mmole, and uridine-2-C^{14}, 30.0 mc/mmole, were obtained from the New England Nuclear Corporation; actinomycin D and cycloheximide were from the Cancer Chemotherapy Division of the National Institutes of Health; puromycin was from the Nutritional Biochemical Corporation; and U.S.P. adrenocorticotropic hormone, 1 unit/mg, was from the United States Pharmacopoeia Co.

Results.—Inhibition of adrenal protein synthesis and steroidogenesis with puromycin and cycloheximide: When 30 mg puromycin was injected intraperitoneally as few as 3 min prior to the intravenous administration of 2 mU ACTH, the stimulation of steroidogenesis measured 10 min later was inhibited by approximately 60 per cent. This inhibition was increased when puromycin was injected 40 min prior to ACTH administration (Table 1). That puromycin in these experiments inhibited adrenal protein synthesis was demonstrated by the following. Immediately after the intravenous injection of ACTH, C^{14}-leucine was injected intravenously also, and 10 min later the glands were rapidly removed, frozen, and subsequently prepared for assay of radioactivity as described in *Methods*. As shown in Table 1, when puromycin was administered intraperitoneally 3 min prior to ACTH, the incorporation of C^{14}-leucine into adrenal protein was partially inhibited, and when puromycin was injected 40 min prior to ACTH, the inhibition was even greater.

However, considering the possibility that puromycin inhibited this phenomenon by a mechanism other than its known inhibition of protein synthesis,[12, 13] the effect of cycloheximide on this process was studied in similar experiments. Cycloheximide was selected because it is structurally different from puromycin and has been shown to inhibit protein synthesis at a similar site, but by a different mechanism.[14] As shown in Table 2, cycloheximide was even more effective in suppressing adrenal protein synthesis and steroidogenesis when administered intraperitoneally either 3 or 40 min prior to ACTH. With puromycin and cycloheximide, the degree of inhibition of the steroidogenic response to ACTH correlated well with the extent of inhibition of adrenal protein synthesis as measured by C^{14}-amino acid incorporation into adrenal protein.

The possibility that these findings reflected an inhibition by puromycin and cycloheximide of steroid release from the adrenals into the venous blood rather than a suppression of steroid synthesis was considered. This was ruled out since, as shown in Table 3, corticosterone did not accumulate in the adrenal glands at a time when secretion into the adrenal venous blood was markedly inhibited by cycloheximide.

Inhibition of adrenal RNA synthesis with actinomycin D: Because it has been shown that the induction of specific protein synthesis in bacterial[17] and in various mammalian systems apparently requires previous RNA synthesis,[18–20] the effect of actinomycin D (e.g., ref. 21) on the stimulation of steroidogenesis by ACTH was studied.

In the following studies actinomycin D (1 mg) was injected intraperitoneally 2 hr prior to the intravenous administration of ACTH (2 mU) and uridine-C^{14} (15 μc). Twenty minutes after injecting ACTH, the adrenals were rapidly removed and prepared for assay of uridine-C^{14} incorporation into adrenal RNA (*Methods*).

TABLE 3

EFFECT OF CYCLOHEXIMIDE ON CORTICOSTERONE CONTENT OF ADRENALS AFTER STIMULATION BY ACTH

Treatment	Corticosterone content (μg) of adrenals per rat, mean ±SEM
Control	0.28 ± 0.07 (4)*
ACTH	0.70 ± 0.15 (3)
Cycloheximide 40 min prior to ACTH	0.33 ± 0.08 (4)

* Number rats.

40 min after the intraperitoneal administration of 10 mg cycloheximide, 2 mU ACTH was injected intravenously. This was compared with untreated rats which were injected intravenously with 2 mU ACTH and with saline-injected controls. 10 min after the administration of ACTH, the animals were sacrificed, the adrenal glands rapidly removed, homogenized, and corticosterone was extracted with methylene chloride and assayed as described in *Methods*.

TABLE 4

EFFECT OF ACTINOMYCIN D ON ACTH STIMULATION OF STEROIDOGENESIS

Treatment	Corticosterone secretion (μg/3 min), mean ±SEM	C^{14}-Uridine incorporation (cpm/100 mg adrenal), mean ±SEM
Control	0.34 ± 0.01 (10)*	84 ± 20 (10)
ACTH	1.12 ± 0.16 (10)	79 ± 14 (10)
Actinomycin 2 hr prior to ACTH	1.67 ± 0.27 (11)	7 ± 1 (11)

* Number rats.

Two hr after the administration of actinomycin D (1 mg) intraperitoneally to rats, 2 mU ACTH was injected intravenously. This was immediately followed by 15 μc uridine-C^{14}, also administered intravenously. From 17 to 20 min after the administration of ACTH (for 3 min) adrenal venous blood was obtained and assayed for corticosterone as described in *Methods*. The animals were sacrificed (i.e., 20 min after ACTH and C^{14}-uridine administration), and the adrenals were removed and assayed for radioactive incorporation into RNA as described in *Methods*. This group was compared with animals which were treated with ACTH (no actinomycin D pretreatment) and saline controls.

In addition, corticosterone was extracted from the adrenal venous blood obtained during the last 3 min of the experiment (17–20 min after ACTH). As shown in Table 4, although RNA synthesis was almost completely inhibited by actinomycin D, the steroidogenic response to ACTH was entirely intact. Similar results were obtained using orotic acid-C^{14} as the RNA precursor, but incorporation into RNA was less than with uridine.

Studies suggesting regulation of steroidogenesis by synthesis of a protein with a rapid rate of turnover: Since the above studies indicated that the steroidogenic response to ACTH was dependent upon protein synthesis, the following studies were performed to elucidate further this relationship. As shown in Figure 1, when 50 mU ACTH were injected intravenously, corticosterone synthesis increased within 3 min, reached a maximum in 10–15 min, and maintained this level for at least 50

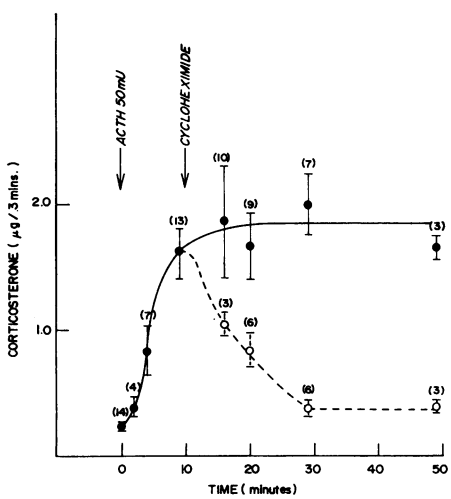

FIG. 1.—Stimulation of corticosterone by ACTH and its decline following cycloheximide administration. ACTH (50 mU) was injected intravenously. Corticosterone was obtained from the adrenal venous blood which was collected for 3-min periods, as described in *Methods*, immediately prior to the times indicated above. Cycloheximide (10 mg) was injected intraperitoneally to a group of these rats 10 min after the administration of ACTH, and adrenal venous blood was similarly obtained and assayed for corticosterone, 5, 10, 20, and 40 min after cycloheximide treatment. At each point the number of rats used and the standard error of corticosterone secretion are indicated. The solid line is the ACTH alone; the dotted line follows cycloheximide treatments.

min. Surprisingly, however, when cycloheximide was administered during maximum steroidogenesis, i.e., 10 min after ACTH, the rate of steroid synthesis rapidly decreased, returning to baseline levels in 20 min. Figure 2 shows a semilogarithmic plot of these data which illustrates that the decline followed first-order kinetics with a $T-{}^1/_2$ of approximately 8 min. These findings demonstrate that the initial stimulation of steroidogenesis by ACTH as well as continued steroid synthesis involve a cycloheximide-sensitive process. Since it was shown that cycloheximide inhibited adrenal protein synthesis, it may be inferred from the above that cycloheximide inhibited the synthesis of a protein with a rapid turnover, the level of which determined the rate of steroidogenesis. This hypothetical protein is therefore in some way a regulator, perhaps a rate-limiting enzyme, of steroid biosynthesis. The following preliminary studies on the site of action of this regulator protein in the steroid synthetic pathway indicate that the pathway between Δ5-pregnenolone and corticosterone is intact after cycloheximide treatment. As shown in Table 5, when cycloheximide was administered intraperitoneally 15 min prior to sacrifice, and the adrenals rapidly removed, quartered, and incubated in buffer which also contained this antibiotic, the stimulation of corticosterone synthesis by ACTH *in vitro* was suppressed. However, Δ5-pregnenolone added to this incubation markedly stimulated corticosterone secretion despite the cycloheximide treatment. Previous studies suggested that ACTH acted at a site in the pathway of steroid biosynthesis between cholesterol and Δ5-pregnenolone.[22] The above findings indicate that the cycloheximide-sensitive step, presumably the ACTH-stimulated protein, also acts at a locus prior to Δ5-pregnenolone.

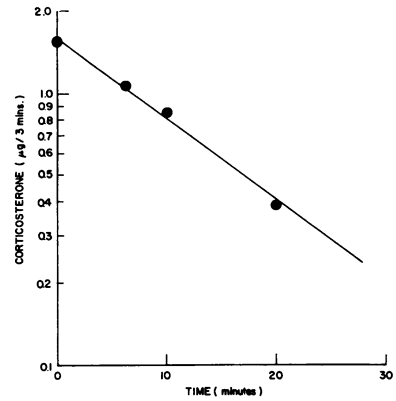

FIG. 2.—The data from Fig. 1 depicting the decline in corticosterone secretion after the administration of cycloheximide are plotted on a semilogarithmic scale.

Because recent studies have shown that certain compounds administered *in vivo* can elicit increased enzyme levels by blocking degradation of enzyme,[23] the possibility that ACTH acted by this mechanism was considered, and ruled out by the following experiment. As described above, animals were given a maximum stimulating dose of 50 mU of ACTH,

TABLE 5

EFFECT OF PREGNENOLONE ON THE PATHWAY OF CORTICOSTERONE BIOSYNTHESIS AFTER INHIBITION BY CYCLOHEXIMIDE

Treatment	Corticosterone (μg/100 mg/hr) mean ±SEM	
Control	5.3 ± 0.7	(7)*
ACTH	36.4 ± 5.4	(6)
Cycloheximide + ACTH	10.7 ± 2.4	(6)
Cycloheximide + Δ5-pregnenolone	27.4 ± 6.7	(8)
Δ5-pregnenolone	20.0 ± 4.1	(9)

* Number rats.

Two hr after hypophysectomy, cycloheximide (10 mg) was administered intraperitoneally to rats 15 min prior to sacrifice. The adrenal glands were rapidly removed, quartered, and placed in Krebs-Ringer bicarbonate buffer, pH 7.4. Cycloheximide (250 μg) was then added to the medium and the flasks were preincubated for 30 min. Following this the medium was removed and replaced with 2 ml fresh buffer and 250 μg cycloheximide. These flasks were then divided into two groups, (1) in addition to cycloheximide, 0.5 unit ACTH was added; (2) in addition to cycloheximide, 40 μg pregnenolone were added. The flasks were then incubated for 2 hr. These were compared with adrenals obtained from untreated rats which were similarly preincubated for 30 min in buffer with no additions. Subsequently, the medium was removed, and as above, the buffer was replaced and the flasks were divided into three groups, as follows: *controls*, no additions; *ACTH*, 0.5 unit added; *pregnenolone*, 40 μg added. All flasks were then incubated for 2 hr after which the adrenals were homogenized in the incubation medium and corticosterone was extracted with methylene chloride and assayed by the fluorescence method of Silber *et al.*[9]

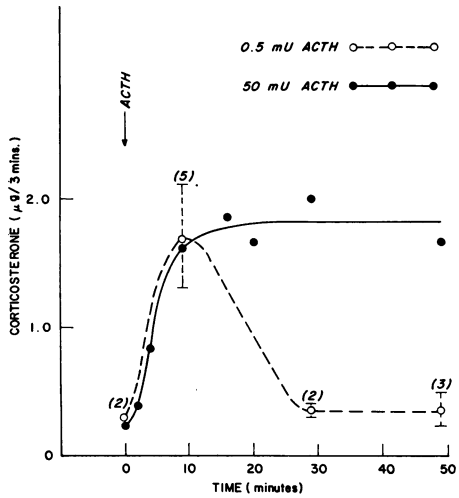

FIG. 3.—Comparison of stimulation of corticosterone by different doses of ACTH. The curve illustrating corticosterone secretion after the administration of 50 mU ACTH is the same as that shown in Fig. 1. The secretion of corticosterone that followed the intravenous injection of 0.5 mU ACTH was measured at 0, 10, 30, and 50 min after treatment. The procedure was similar to that described in Fig. 1 and *Methods*. At each point the number of rats and the standard error of the corticosterone determination are indicated.

and 10 min later protein synthesis was blocked by the injection of cycloheximide. If ACTH acted by blocking *degradation* of a protein which regulated steroidogenesis, then a second injection of ACTH after cycloheximide should have prevented the fall in corticosterone which was demonstrated in Figures 1 and 2. However, in fact, an additional injection of 50 mU of ACTH (immediately after cycloheximide) failed to modify the rapid fall-off in steroid production which was caused by cycloheximide.

Mechanism determining the duration and maximum rate of steroidogenesis: Previous studies have shown that the stimulation of steroidogenesis is dose-dependent between 0.06 and 0.50 m ACUTH.[1] However, as illustrated in Figure 3, 0.5 and 50 mU ACTH increased corticosterone secretion to the same level. Furthermore, it was shown that the rate of steroid synthesis could not be increased beyond this level, because when a second injection of 50 mU ACTH was administered, during maximum stimulation, no additional increase in steroidogenesis occurred.

However, as also shown in Figure 3, the duration of the stimulated steroidogenesis was clearly dose-dependent. Thus, since the half-time of ACTH in the circulation of the rat has been shown to be approximately 2 min,[24] the smaller dose would be expected to maintain plasma ACTH levels for a much shorter period than the larger one. These findings indicate that steroid synthesis is not only stimulated but also sustained by ACTH. Since it was proposed that ACTH stimulates steroidogenesis by initiating the synthesis of a rapidly turning-over protein, therefore, in line with the above data, it appears that the continued presence of ACTH is required for continued synthesis of this protein.

These findings also suggested the following mechanism to account for the factors which limit the maximum rate of steroidogenesis reached after the injection of at least 0.5 mU ACTH, as distinguished from the duration of maximum steroidogenesis which was considered above (Fig. 3). The following calculation suggests that the rate of steroidogenesis obtained after maximal ACTH stimulation is dependent on a rate of synthesis determined by a fixed amount of mRNA and the high degradation rate of the regulator protein stimulated by ACTH. If it is assumed that after ACTH administration the rise in steroidogenesis is directly related to the synthesis of a regulator protein, then in the differential equation

$$\frac{d(RP)}{dt} = K_1 \cdot \frac{\text{ACTH}}{\alpha + \text{ACTH}} - K_2\,[RP],$$

the synthesis constant, K_1, includes the conditions necessary for synthesis. The fraction ACTH/(α + ACTH) approaches unity when the administered dose of ACTH is 0.5 mμ or greater, as was used in the present studies (Fig. 3). The degradation is represented by K_2 [RP], where K_2 is the degradation constant, and [RP] the level of regulator protein. At the steady state, when $dRP/dt = 0$, $K_1 = K_2$ [RP], i.e., at the maximum level of steroidogenesis, the rate of synthesis is equal to the amount of degradation. From this equation it appears that in order to increase the maximum rate of steroidogenesis, the synthesis constant, K_1, must be increased. In microorganisms it has been suggested that the induction of protein synthesis results from an increased synthesis of mRNA.[25] However, in the present studies it has been shown that the ACTH stimulation of steroidogenesis was not suppressed by doses of actinomycin D which markedly inhibited RNA synthesis (Table 4). Therefore, once the maximum rate of steroidogenesis was induced by 0.5 mU ACTH, presumably, preformed mRNA is optimally utilized and even 50 mU ACTH does not further increase steroidogenesis. Thus, under these conditions K_1 apparently includes mRNA and remains constant. However, since it has been indicated that the rate of degradation, K_2, is extremely high, as shown by the rapid decline in stimulated steroidogenesis after protein synthesis was blocked by cycloheximide, it is suggested that the maximum rate of steroid synthesis reached after ACTH administration is determined by the high degradation K_2 [RP].

Discussion and Summary.—The mechanism of the stimulation of corticosterone by ACTH *in vivo* has been studied. The data suggest the following.

(1) The findings that puromycin and cycloheximide inhibited the stimulation of steroidogenesis by ACTH in these studies and that chloramphenicol was shown to act similarly *in vitro*[5] indicated that protein synthesis was involved in this phenomenon.

(2) Because actinomycin D which inhibited RNA synthesis for at least 2 hr had no effect on either adrenal protein synthesis or the ACTH stimulation of steroid synthesis, it indicated that adrenal messenger RNA was stable and that synthesis of mRNA did not occur after ACTH treatment. The possibility that the specific mRNA was not inhibited by the actinomycin D treatment should be considered. However, this is extremely unlikely, considering the marked inhibition of uridine incorporation into RNA, and the previous demonstration that the amount of actinomycin D used in these experiments, administered *in vivo*, completely suppressed the synthesis of all moieties of RNA with the exception of a small amount of transfer RNA in rat liver.[26] These findings are in line with previously reported *in vitro* studies.[4]

(3) Since the inhibition of protein synthesis at a time of maximal steroidogenesis resulted in a rapid decline of steroid synthesis with a $T-1/2$ of 7–10 min, steroidogenesis is probably regulated by a protein with a rapid rate of turnover.

(4) Since ACTH stimulated corticosterone synthesis without increasing adrenal protein synthesis in general, as measured by amino acid incorporation into adrenal protein, and since the decline in stimulated steroid synthesis after cycloheximide treatment showed first-order kinetics, it suggested that ACTH stimulated a specific protein regulator of steroidogenesis.

(5) The finding that the duration of stimulated steroidogenesis was determined by the dose of ACTH administered indicated that the maintenance of maximum

steroidogenesis was dependent upon the continued presence of ACTH.

The implications of these data are, of course, dependent on the assumption that the observations which were obtained by the use of inhibitors of RNA and protein synthesis were the result of these specific demonstrated actions, rather than from some other unknown side effects of these antibiotics.

Thus, in summary, it appeared that ACTH stimulated corticosterone biosynthesis by acting at the level of translation of specific mRNA. This resulted in the synthesis of a rapidly turning-over protein which in turn regulated steroid synthesis. Continued synthesis of this protein depended upon continued presence of ACTH.

The rapid turnover of mRNA's in bacteria led to the hypothesis that enzyme induction was regulated at the level of the gene through the synthesis of specific mRNA's.[25] However, in higher organisms where, as shown in the present study, mRNA appears to be relatively stable,[27-29] it is not surprising that recent studies of enzyme induction have indicated that inhibition of enzyme synthesis occurred at the level of the translation of, rather than synthesis of, mRNA.[28]

The present studies imply that in the presence of stable mRNA, the changing rates of steroidogenesis that follow stimulation by ACTH are regulated by the synthesis of a rapidly turning-over protein.

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