

11372

# Acta endocrinologica

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Volume 60  
January-April 1969

PERIODICA · COPENHAGEN

Eigentum der  
Universitäts-Bibliothek  
München

Printed in Denmark  
by Bogtrykkeriet Forum  
Copenhagen

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II. Medizinische Klinik der Universität München, Deutschland  
(Prof. Dr. Dr. G. Bodechtel)

SUBCELLULAR DISTRIBUTION  
AND ARTERIO-VEINUS DIFFERENCES OF  
SYNTHETIC  $^3\text{H}$ - $\beta^{1-23}$ -CORTICOTROPHIN-23-AMIDE-ACETATE  
IN THE ADRENAL OF RATS

By

*O. A. Müller and P. C. Scriba\**

ABSTRACT

1. Synthetic  $^3\text{H}$ - $\beta^{1-23}$ -corticotrophin-23-amide-acetate ( $^3\text{H}$ -ACTH) was given as a single dose by means of a 95 s infusion into the thoracic aorta of hypophysectomized rats. Distribution of radioactivity was determined in adrenal subcellular fractions prepared by homogenization and differential centrifugation: 3, 12, 40 and 120 min after the start of the  $^3\text{H}$ -ACTH infusion. Radioactivity in nuclear fractions, given as a % of total adrenal CPM rose from 27 % (3 min) to 37 % (40 and 120 min,  $P < 0.0025$ ). These results may indicate ACTH penetration through the outer cell membrane and interaction with adrenal nuclei. Microsomal radioactivity (18 %) at 3 min after the start of  $^3\text{H}$ -ACTH infusion appeared to be in excess of the contribution of this fraction to the total adrenal weight and decreased significantly with time (120 min: 3 %,  $P < 0.0005$ ). These observations are discussed with regard to mechanisms of action of ACTH.
2. When synthetic  $^3\text{H}$ - $\beta^{1-23}$ -corticotrophin-23-amide-acetate was infused into the thoracic aorta of rats, the differences in the radioactivity of the plasma from the aorta and from the adrenal vein were significantly higher ( $P < 0.0125$ ) in hypophysectomized than in sham-operated rats, presumably indicating in the latter the saturation by endogenous ACTH of adrenal receptors for ACTH.

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Supported by Deutsche Forschungsgemeinschaft. Presented in part at the International Symposium on Protein and Polypeptide Hormones, Liège (*Scriba & Müller* 1968).

\* Herrn Prof. Dr. Dr. G. Bodechtel zum 70. Geburtstag gewidmet.

Experiments involving hypophysectomy of rats over prolonged periods have shown dissociations of some ACTH-effects on the adrenal from the stimulation of adrenal steroidogenesis by ACTH. From day 1 to day 14 after hypophysectomy, *corticosterone synthesis* gradually became less sensitive to ACTH stimulation, but stimulations of the *adrenal blood flow* and *ascorbic acid output* were obtained on day 4 to day 7 (Stachelin *et al.* 1965). The normal expected increase of adrenal *cyclic 3',5'-AMP* content after i. v. ACTH administration on day 4 to 9 (Butcher & Sutherland 1968) and stimulation of *aminoacyl-transferase* by i. m. or i. v. ACTH treatment on day 7 to 14 after hypophysectomy were also observed (Scriba & Kluge 1968). The latter enzyme, the activity of which was doubled by treatment of rats with ACTH, is rate limiting for *in vitro*  $^{14}\text{C}$ -glycine incorporation into protein by adrenal 15 000 g supernatants (Scriba & Reddy 1965) and by adrenal polysomes (Scriba & Fries 1967). Adrenal *NADPH levels* and *NADPH-regenerating enzymes* were maintained for 24 h after hypophysectomy, whereas corticosterone secretion fell to baseline output within 30–60 min (Harding & Nelson 1964 *a, b*). Finally 30 h after hypophysectomy, the adrenal *aminoacyltransferase* was found to be elevated, due to the stress of hypophysectomy and was refractory to ACTH treatment at the time when corticosterone secretion was at a baseline, but still responsive to ACTH (Scriba *et al.* 1967; Scriba & Kluge 1968).

Dissociations of ACTH-effects on the adrenal led to the assumption that there may be more than one adrenal receptor for ACTH, or alternatively multiple sequences of relatively independent effects with one common primary interaction in the mechanism of ACTH-action. At present, many authors favour the view, that ACTH interacts with the outer cell membrane and gives rise to increased formation of cyclic 3',5'-AMP as the common primary effect to which all further actions of ACTH on the adrenal gland may possibly be attributed, thus implying that ACTH does not enter the adrenal cell (Butcher & Sutherland 1968; Garren 1968; Schwyzer 1968). The purpose of this study was the further elucidation of the mechanisms of action of ACTH by analysis of the adrenal subcellular distribution of radioactivity after *in vivo* intraaortic infusion of synthetic  $^3\text{H}$ - $\beta^{1-23}$ -corticotrophin-23-amide-acetate into hypophysectomized rats.

## MATERIAL AND METHODS

Synthetic  $\beta^{1-23}$ -corticotrophin-23-amide-acetate\* labelled with tritium, and named here  $^3\text{H}$ -ACTH, was purified from unspecific radioactivity, using dextran-gel-filtration as described previously (*v. Werder et al.* 1968). Solutions of purified  $^3\text{H}$ -ACTH (0.1 v HCl) stored at  $-18^\circ\text{C}$  initially contained 3% and after 11 days, 9.5% of unspecific

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\* The generous supply of synthetic  $^3\text{H}$ - $\beta^{1-23}$ -corticotrophin-23-amide-acetate ( $^3\text{H}$ -ACTH) by Farbwerke Hoechst AG is gratefully acknowledged.

radioactivity (*v. Werder* 1968).  $^3\text{H}$ -ACTH solutions were therefore freshly made up once a week and thawed immediately before use. A single dose of purified  $^3\text{H}$ -ACTH [1.1–1.65  $\mu\text{c}$  = 300–450 mU (biological activity according to *Vogel* 1965) = 3–4.5  $\mu\text{g}$  in 1.0 ml saline (0.025 N HCl)], was continuously infused for 95 s into the thoracic aorta of rats, hypophysectomized 90 min previously by the parapharyngeal route (male Sprague-Dawley albino rats, 180–250 g, Fa. Gassner, Ottobrunn). Both adrenals were removed, carefully cleaned from adherent tissue, weighed and homogenized in the cold for  $3 \times 5$  s (Potter-Elvehjem, Teflon pestle) at 20 mg adrenal per ml medium (0.25 M sucrose, 0.08 M KCl, 0.01 M  $\text{MgCl}_2$ , 0.05 M tris-HCl buffer pH 7.6) within 5 min (*Müller* 1968). To maintain constant homogenizing conditions, a homogenizing glass was selected that had a space between the Teflon pestle and the glass of between 0.06 and 0.14 mm (*Fa. Braun-Melsungen*, personal communication). To reduce further the variation in the homogenizing conditions, the same Teflon pestle was used throughout the study. The preparation of subcellular fractions was achieved by differential centrifugation at  $+3^\circ\text{C}$  of homogenates: *nuclear fraction* (15 min  $\times$  1000 g), *mitochondrial fraction* (10 min  $\times$  15 000 g), *microsomal fraction* (90 min  $\times$  105 000 g) and *soluble fraction* (supernatant). Examination by light microscope revealed that the nuclear fractions of rats adrenals contained 76% intact nuclei, 7% ruptured nuclei and 14% nuclei with adherent cellular material, 3% erythrocytes and practically no unbroken cells (*Müller* 1968). The results of the colorimetric determinations of RNA and DNA (*Dische* 1955; *Schneider* 1945; *Scriba & Reddy* 1965) in subcellular fractions are given in Table 1. Without further purification, subcellular fractions were solubilized with NCS reagent, model 190620 (Nuclear-Chicago GmbH, Frankfurt) and the radioactivity was assayed by liquid scintillation counting at 24% efficiency, using the internal standard method for quenching correction. Loss of radioactivity due to adsorption of  $^3\text{H}$ -ACTH to centrifuge tube walls (*v. Werder* 1968) was less than 8% (*Müller* 1968). For the analysis of differences of synthetic  $^3\text{H}$ - $\beta^{1-23}$ -corticotrophin-amide concentrations in the plasma of rats, blood was continuously collected from the adrenal veins after careful ligation of non-adrenal veins for an 8 min period after the start of the  $^3\text{H}$ -ACTH infusion (300–450 mU for 95 s). During the same period, 0.05 ml blood samples were drawn at 30 s intervals from a second catheter inserted into the abdominal aorta, starting 30 s after the start of the  $^3\text{H}$ -ACTH infusion into the thoracic aorta. Samples

Table 1.

RNA and DNA-determinations in subcellular fractions of rat adrenals: Results are given as  $\mu\text{g}$  nucleic acid per mg adrenal (mean  $\pm$  S. E. M.,  $N = 5$ ). There was no DNA detectable in 4 out of 5 supernatants (1000 g). Purified nuclei of rat liver have an RNA/DNA ratio of 0.23 (according to *Siebert* 1966), for total adrenal homogenates a ratio of 1.2 has been reported (*Munro et al.* 1962).

	Total Homogenate	1000 g Supernatant	1000 g Sediment
RNA	7.52 $\pm$ 0.39	3.92 $\pm$ 0.74	3.10 $\pm$ 0.13
DNA	6.56 $\pm$ 0.56	(0.10 $\pm$ 0.10)	6.36 $\pm$ 0.80
$\frac{\text{RNA}}{\text{DNA}}$	1.19 $\pm$ 0.13	—	0.52 $\pm$ 0.07

of 0.075 ml plasma from the adrenal venous blood, or pooled aortic blood were solubilized with NCS and counted as mentioned above.

Statistical analysis of the data was performed by Student's *t*-test and the results are given as the mean  $\pm$  S. E. M. (Diem 1960).

## RESULTS

### *The aortic infusion of synthetic $^3\text{H}$ - $\beta^{1-23}$ -corticotrophin-23-amide-acetate*

#### *I. The increases of corticosterone release into the plasma of the adrenal vein of the rat*

Increases in corticosterone release from the adrenal caused by a single dose (300–450 mU) of  $^3\text{H}$ -ACTH given as an aortic infusion for 95 s were assayed fluorimetrically (Scriba *et al.* 1966) and are given in Table 2 for the different experimental groups.

#### *II. The subcellular distribution of synthetic $^3\text{H}$ - $\beta^{1-23}$ -corticotrophin-23-amide-acetate in the adrenals of rats*

The subcellular distribution of  $^3\text{H}$ -ACTH was determined 3 min (group I, N = 19), 12 min (group II, N = 17), 40 min (group III, N = 17) and 120 min (group IV, N = 17) after the start of the  $^3\text{H}$ -ACTH infusion. Subcellular distribution of the radioactivity 3 to 120 min after  $^3\text{H}$ -ACTH infusion, is shown

*Table 2.*

Increases in corticosterone secretion into the plasma of adrenal vein of rats during and after infusion of synthetic  $^3\text{H}$ - $\beta^{1-23}$ -corticotrophin-23-amide-acetate:  $^3\text{H}$ -ACTH, given as a single dose by infusion of 95 s, stimulated corticosterone release into the adrenal vein during the first three minutes after the start of  $^3\text{H}$ -ACTH infusion (group I). Maximal stimulation was noted in the other groups, 4 to 11 min after the start of  $^3\text{H}$ -ACTH infusion. Corticosterone release at 120 min after the start of  $^3\text{H}$ -ACTH infusion was found to be still elevated above the baseline secretion, which was 0.007  $\mu\text{g}/\text{min}$  90 min after hypophysectomy, but the former was not regularly checked.

Group	I	II	III	IV
Collection period of adrenal venous blood (min after start of $^3\text{H}$ -ACTH infusion)	0–3 N = 13	4–11 N = 17	4–11 N = 17	4–11 N = 17
Increase of corticosterone secretion $\mu\text{g}/\text{min}$	0.059 $\pm$ 0.008	0.373 $\pm$ 0.028	0.292 $\pm$ 0.028	0.395 $\pm$ 0.038



in Fig. 1 (CPM) and Fig. 2 (% of total adrenal radioactivity (= 100 %)), which was calculated as the sum of radioactivity in the subcellular fractions).

a) The yield of radioactivity in both adrenals, calculated as a % of infused radioactive ACTH, was low and showed a continuous decline ( $0.134 \pm 0.037$  to  $0.058 \pm 0.012$  to  $0.050 \pm 0.007$  and to  $0.034 \pm 0.003$  %) with time (respectively 3, 12, 40 and 120 min after the start). Presumably, the low yield of radioactivity from  $^3\text{H}$ -ACTH depends on the small contribution of adrenal blood flow (approx. 0.45 %) to the cardiac output of rats (Granwiler 1965). Using natural ACTH (Richards & Sayers 1951) or  $^{131}\text{I}$ -ACTH (Cats & Kassenaar 1957 a, b) at comparable doses, less than 1 % of the biological activity or the radioactivity was found in the adrenals.

b) In nuclear fractions (15 min  $\times$  1000 g), a remarkably large amount of radioactivity was found (Figs. 1 and 2). The possibility that the radioactivity

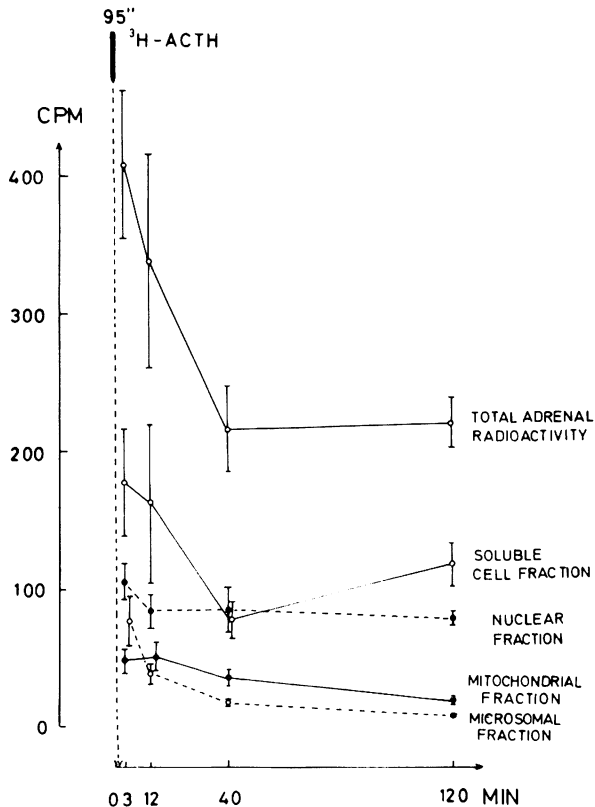


Fig. 1.

Total rat adrenal radioactivity and the CPM detected in the subcellular fractions: The results are given as CPM (Mean  $\pm$  S. E. M.), and the time as min after the start of  $^3\text{H}$ -ACTH aortic infusion for 95 s.

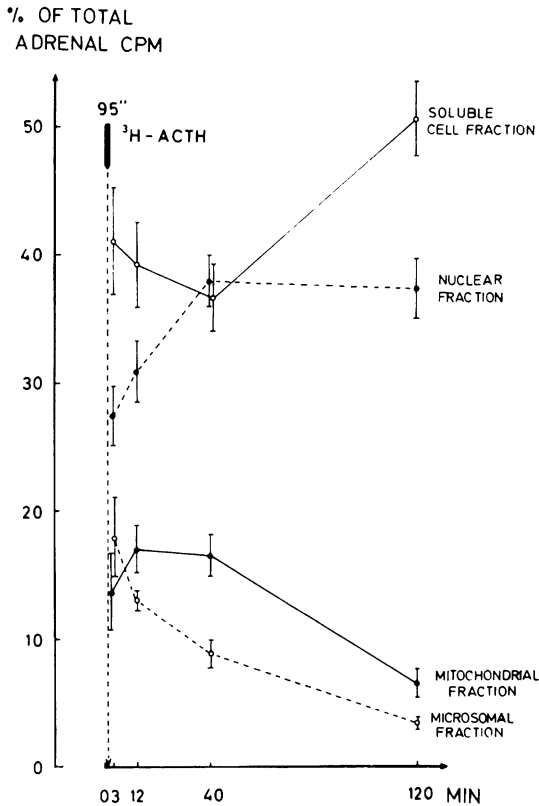


Fig. 2.

Rat adrenal subcellular radioactivity given as a % of the total adrenal radioactivity (CPM): Time (min) after the start of  $^3\text{H}$ -ACTH aortic infusion, which lasted for 95 s.

in the nuclear fraction, averaging 54 % of total wet weight of both adrenals (= approx. 27 mg), was due to contamination with soluble cell fraction can be excluded, since the latter was diluted approx. 1:50 during the homogenization – centrifugation procedure. The initial (3 min) radioactivity in the nuclear fraction may be artificially high because of possible, e.g. microsomal contaminations. However, the radioactivity of the latter fraction decreased significantly with time (comparison of the 3 min value with that of the 40 or 120 min ( $P < 0.0025$ )). The total nuclear radioactivity (CPM) was constant from 12 to 120 min (Fig. 1), but showed a relative increase (%) with time (Fig. 2). The relative values obtained at 40 and 120 min were significantly higher than those found at 3 min ( $P < 0.0025$ ), or 12 min ( $P < 0.05$ ).

c) Mitochondrial radioactivity (CPM) at 120 min (Fig. 1) was significantly

lower ( $P < 0.005$ ) than the CPM at 3, 12 and 40 min, whereas no significant difference was found for the other time periods.

d) A more clearcut decrease of radioactivity (Fig. 1) was observed in the *microsomal fraction* of rat adrenals (3 to 12 min:  $P < 0.05$ ; 12 to 40 min:  $P < 0.005$ ; 40 to 120 min:  $P < 0.0005$ ), corresponding to a continuous decline of the relative amount of total radioactivity (Fig. 2). Microsomal radioactivity was surprisingly high at 3 min. Since microsomal material may well be lost to nuclear and mitochondrial fractions during cell fractionation, microsomal radioactivity appears at this time to be in excess of the microsomal contribution to cell volume, as judged from weight measurements.

e) The decrease in radioactivity (CPM) in the *soluble cell fraction* parallels the decrease in total adrenal radioactivity (Fig. 1) and probably represents the removal of label from the adrenals. The relative amount of the total radioactivity (Fig. 2) therefore remained approx. constant from 3 to 40 min. Slight increases ( $P < 0.05$ ) of radioactivity (CPM) in the soluble cell fraction at 120 min, compared to the 40 min value may be due to decreases in the microsomal and mitochondrial counts. There was no significant change in total adrenal radioactivity from 40 to 120 min.

### *III. Aortic-adrenal venous differences in the concentrations of synthetic $^3\text{H}$ - $\beta^1$ - $^{23}$ -corticotrophin-23-amide-acetate in the plasma*

There are technical difficulties in the study of adrenal a-v differences of  $^3\text{H}$ -ACTH. Since blood from the adrenal arteries may not be obtained directly the principle of intermittent sampling of aortic blood (a sample of 0.05 ml per 30 s) had to be chosen, as previously described (see Methods). With this procedure, a mean aortic  $^3\text{H}$ -ACTH level from 30 s to 8 min after the start of  $^3\text{H}$ -ACTH infusion was obtained, and could be compared with the value obtained from the continuous adrenal vein effluent from 0 to 8 min. Continuous arterial sampling for 8 min was not possible, since the blood loss would have been too high. No correction for the time delay in the appearance of  $^3\text{H}$ -ACTH in the adrenal vein due to retardation on passage through adrenal vessels was made. It was assumed, that plasma  $^3\text{H}$ -ACTH levels are representative for whole blood levels, and that aortic  $^3\text{H}$ -ACTH levels are identical with the levels in the adrenal arteries. The a-v differences of  $^3\text{H}$ -ACTH radioactivity across the adrenal were calculated and are given in Table 3. Hypophysectomized rats were found to have a positive and sham-operated rats a negative mean a-v difference (Table 3). For comparison of adrenal a-v differences in hypophysectomized and sham-operated rats, the artificially negative a-v difference in the latter group was arbitrarily made zero, since it cannot be less than zero. The difference in the means of a-v differences (434.2 CPM), from hypophysectomized and sham-operated rats is significant ( $P < 0.0125$ ). Since the amounts of plasma collected from adrenal veins were practically equal in both groups, differences in the

Table 3.

Radioactivity (CPM) in the aorta and the adrenal vein and a-v differences after infusion of synthetic  $^3\text{H}$ - $\beta^{1-23}$ -corticotrophin-23-amide-acetate in hypophysectomized and sham-operated rats: Results are given as the mean  $\pm$  S. E. M. of CPM per 0.075  $\mu\text{ml}$  plasma from aortic and adrenal-venous blood, collected from 0-8 min as described under Methods.  $^3\text{H}$ -ACTH infusion was started at 0 min and lasted 95 s.

	Aorta	Adrenal vein	A-V Difference
Sham-operation N = 11	2154.6 $\pm$ 246.2	2442.4 $\pm$ 330.2	-287.7 $\pm$ 120.9
Hypophysectomy N = 14	2656.0 $\pm$ 126.1	2509.6 $\pm$ 153.7	+146.5 $\pm$ 126.3

adrenal blood flow cannot explain the higher adrenal a-v differences for  $^3\text{H}$ -ACTH in hypophysectomized animals.

#### DISCUSSION

Increased a-v differences in  $^3\text{H}$ -ACTH in hypophysectomized rats as compared with sham-operated animals, support the concept of a specific uptake of  $^3\text{H}$ -ACTH by the adrenal. This interpretation implies the hypothesis of saturation of the adrenal receptor sites by endogenous ACTH. Endogenous ACTH is increased in sham-operated rats, as indicated by the high corticosterone secretion (Müller 1968), due to stress in both previous sham-operation and laparotomy in these rats. With the large doses of  $^3\text{H}$ -ACTH used, dilution of specific radioactivity through endogenous ACTH in sham-operated rats does not appear to be a likely explanation for the lower a-v differences.

Preparation of adrenal subcellular fractions by homogenization and differential centrifugation can produce fractions of only relative purity, particularly if the fractions have not been washed. The so-called nuclear fraction is most likely to contain fragments of the outer-cell membrane. The contamination of the nuclear fraction with outer-cell membrane material remains presumably constant at different time intervals after ACTH. According to this assumption, the amount of radioactivity found in the »nuclear« fraction is probably not only due to contamination with outer-cell membrane material, because of the distinct patterns of only limited stimulation of corticosterone synthesis and in contrast, the relative increase of »nuclear« radioactivity with time. The concept of ACTH accumulating in the nuclei with time is supported by the recent demonstration of TSH in thyroid nuclei by means of the fluorescent-antibody technique (Greenspan & Hargadine 1965; Blum *et al.*

19967). These authors, using this technique for an immunoassay of TSH, have noted immunoreactive material in adrenal nuclei, which was not seen after absorption with synthetic ACTH of their antiserum to impure TSH.

So far, no answer can be given as to whether the radioactivity in e. g. nuclear fractions still represents  $^3\text{H}$ -ACTH or fragments of the synthetic  $^3\text{H}$ -ACTH used in this study. Unspecific redistribution of  $^3\text{H}$ -ACTH present in the adrenal during the homogenization-centrifugation procedure is unlikely in view of the distinct distribution patterns, obtained at various times after  $^3\text{H}$ -ACTH administration. This argument also applies to possible tritium exchange reactions. Further, subcellular distribution of porcine  $^3\text{H}$ -ACTH added *in vitro* to bovine adrenal slices or to homogenates of slices was markedly different in pattern. However, the studies of these authors are not directly comparable with the results reported here, because in these studies high concentrations of ACTH added to the incubation medium, differences in the homogenization-centrifugation procedure and other unknown factors may also be responsible for differences in the results (*Nishizawa et al.* 1965).

The results of this study support the view that ACTH may penetrate the cell membrane and accumulate intracellularly, though definite evidence is lacking. Confirmation of the penetration of ACTH into the cell would induce the study of intracellular binding sites. It is conceivable that the mechanism of action of ACTH also involves membrane interactions at intracellular sites. Possibly these interactions also have cyclic 3',5'-AMP as »second messenger« (*Butcher & Sutherland* 1968). Hence, the demonstration of similarities of many adrenal responses to ACTH and to cyclic 3',5'-AMP does not rule out the possibility of intracellular ACTH interactions. From the time pattern of the appearance of intracellular radioactivity it seems conceivable that »nuclear ACTH« might rather be involved in long-term effects of the hormone, e. g. the so-called trophic actions. In contrast, the immediate corticosterone response of the adrenal to ACTH may be due to outer-cell membrane interaction.

#### ACKNOWLEDGEMENT

The authors would like to thank Dr. L. A. Menahan for his most helpful discussion of this paper.

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Received on July 10th, 1968.