J. steroid Biochem. Vol. 22, No. 2, pp. 273–279, 1985
Printed in Great Britain.

0022-4731/85 $3.00 + 0.00

ISOLATED ADRENOCORTICAL CELLS OF THE DOMESTIC FOWL (*GALLUS DOMESTICUS*): STEROIDOGENIC AND ULTRASTRUCTURAL PROPERTIES

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(*Received* 14 *May* 1984)

Summary—Isolated adrenocortical cells from White Leghorn chickens (*Gallus domesticus*) were compared to those from rats (*Rattus norvegicus*). Cells were prepared from collagenase-dispersed adrenal glands of sexually mature male animals. Corticosterone was measured by radioimmunoassay after incubation for 2 h with steroidogenic agents. Of the four ACTH analogues used, three were 6–17 times more potent with rat cells than with fowl cells (potencies were indicated by half-maximal steroidogenic concentrations). However, 9-tryptophan (*O*-nitrophenylsulfenyl) $ACTH_{1\text{-}24}$ was 8 times more potent with fowl cells than with rat cells, thus suggesting that ACTH receptor differences exist between the two cell types. In addition, cAMP analogues were 10 times more potent with rat cells than with fowl cells suggesting that fowl corticosteroidogenesis is less dependent on cAMP than is rat corticosteroidogenesis. At equal cell concentrations, rat cells secreted 20–40 times more corticosterone than did chicken cells when they were maximally stimulated. Although rat cells converted 8 times more pregnenolone to corticosterone than did fowl cells, the half-maximal steroidogenic concentration for pregnenolone-supported corticosterone synthesis was the same for both cell types (about 5 μM). This suggests that fowl cells have lower steroidogenic enzyme content rather than lower steroidogenic enzyme activity. An unusual feature seen in the isolated fowl adrenocortical cells was an abundance of intracellular filaments.

INTRODUCTION

It is generally believed that adrenocortical function of the domestic fowl, as in the mammal, is dependent on the pituitary gland [1, 2]. A classic pituitary-adrenocorticotropic control mechanism in the domestic fowl is indicated by experiments in which plasma corticosterone concentrations were raised by mammalian ACTH preparations [3, 4]. In addition, experiments *in vitro* have shown that domestic fowl adrenal tissue responds to ACTH [5–7]. On the other hand, hypophysectomy of the domestic fowl does not result in the drastic adrenal atrophy and the associated fall in plasma corticosterone that occurs in hypophysectomized rats [1]. Other work suggests that autonomous activity of the adrenal cortex [8] and the action of extrahypophyseal corticotropic substances [3, 9] are involved in the regulation of adrenocortical function in the domestic fowl. Thus, despite some similarities, there appear to be differences in the control of adrenocortical secretion between the bird and the mammal.

In order to compare corticosteroidogenesis in these two vertebrate classes, we have used isolated adrenocortical cells. Although isolated rat cells have been a valuable tool in studying the steroidogenic action of ACTH analogues [10], no studies have been conducted using isolated fowl adrenocortical cells. Whole glands or tissue slices [5–7] have been used. However, the use of isolated cells avoids the problems of interpretation presented by experiments *in vivo* that result from steroid clearance by organs other than the adrenal glands and from indirect effects on the adrenal gland by the agents being tested. In addition, it minimizes the diffusion barriers encountered *in vitro* when using large tissue masses.

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EXPERIMENTAL

Animals

Sexually mature, male White Leghorn chickens (24–30-weeks old) were individually housed at 20 ± 2°C under a 16 h light: 8 h dark photocycle. They were fed a standard commercial diet and water *ad libitum*. Sexually mature (250–300 g) male Sprague–Dawley rats were maintained in a temperature (22°C), light (14 h light: 10 h dark photocycle), and humidity controlled room and were provided feed (Purina Formulab No. 5008) and water *ad libitum*.

Male chickens were quickly killed by cervical dislocation. Rats were anesthetized with ether and killed by sectioning the abdominal aorta. Adrenal glands

were quickly removed, trimmed free of connective tissue and diced into small pieces for digestion.

Cell isolation and incubation

The basic cell isolation and incubation medium was Krebs–Ringer Hepes (4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid) buffer (24.2 mM Hepes, 118.5 mM NaCl, 4.75 mM KCl, 2.54 mM $CaCl_2$, 1.20 mM KH_2PO_4, 1.20 mM $MgSO_4$, pH 7.5) with 0.2% glucose.

Adrenocortical cells were isolated as previously described [11, 12] except that 0.2% collagenase (Type I; Sigma Chemical Co.) and 0.01% lima bean trypsin inhibitor (Worthington Biochemical Corp.) were used instead of 0.25% trypsin. As previously reported [11], few medullary cells appeared in the rat cell suspensions and zona fasciculata cells were predominant. However, when chicken adrenal tissue was used, at least 40% of the cells were medullary cells whereas 60% were adrenocortical cells. Only adrenocortical cells were counted; these cells were easily identified in the light microscope because they contained many lipid droplets.

For incubation, additions to the basic cell medium were: 5.1 mM $CaCl_2$ (7.64 mM final concentration), 0.5% bovine serum albumin (Fraction V; Sigma Chemical Co.), 0.01% lima bean trypsin inhibitor and one of the following agents: purified ostrich $ACTH_{1\text{-}39}$ (osACTH; gift from Dr Ryno Naudé, Department of Biochemistry, University of Port Elizabeth 6000, Republic of South Africa), synthetic human $ACTH_{\alpha\text{-}1\text{-}24}$ (hACTH) (Cortrosyn; Organon Inc.), porcine $ACTH_{1\text{-}39}$ (Grade II; Sigma Chemical Co.), 9-tryptophan (*O*-nitrophenylsulfenyl)-$ACTH_{1\text{-}24}$ (abbreviated in this reported as $Trp(Nps)^9$ $ACTH_{1\text{-}24}$ or npsACTH; gift from Dr William R. Moyle, Department of Obstetrics and Gynecology UMDNJ-Rutgers Medical School, Piscataway, NJ 08854, U.S.A.), 8-bromo-cyclic AMP (8Br-cAMP), cyclic AMP (cAMP) and pregnenolone (Sigma Chemical Co.). ACTH analogues were added to the incubation medium in 0.9% NaCl, pH 2.6, containing 0.1% bovine serum albumin. Cyclic AMP analogues and pregnenolone were made up in the basic cell medium.

Cell concentrations were $225{,}000 \pm 15{,}000$ fowl cells/ml and $208{,}000 \pm 7{,}000$ rat cells/ml (means $\pm$ SE). Incubation volumes (90% cell suspension, 10% test substances) varied from 250 μl to 1.0 ml. Isolated cell suspensions were incubated in plastic culture tubes (12 × 75 mm) in a Dubnoff metabolic shaking bath (66 oscillations/min) at 37°C for 2 h. In each experiment at least 96% of cell were viable after incubation as indicated by trypan blue exclusion. After incubation, cell suspensions were frozen until radioimmunoassay for corticosterone.

Radioimmunoassay for corticosterone

Corticosterone, the major glucocorticoid secreted by rat [12] and chicken [6, 13] adrenocortical cells, was measured by a modification of the radioimmunoassay procedure of Roy *et al.* [14] using specific antibody (Miles Research Products).

Preparation of cells for electron microscopy

After incubation, cells were prepared for electron microscopy essentially as described previously [11]. Briefly, cell suspensions were fixed with a modified Karnovsky's fixative [15] comprised of 2% paraformaldehyde and 2.5% glutaraldehyde in a 0.1 M sodium cacodylate–HCl buffer, pH 7.5. These cells were then packed into cohesive pellets in a microcentrifuge (10,000 *g* for 3 min) according to a modification of the procedure of Malamed [16]. The resulting pellets were placed in the fixative for 2 h and then immersed in a buffer rinse overnight. The pellets were post-fixed in cold buffered 1% OsO_4 for 60 min, rinsed in cold water, and then placed in aqueous 1% uranyl acetate for 1 h. Pellets were dehydrated in ethanol at concentrations up to 95% and then embedded in Epon 812. Ultrathin sections of the pellets were staind with uranyl acetate and lead citrate for subsequent viewing in a Phillips 300 electron microscope.

Analysis of data

Analysis of variance [17] was used to evaluate the data; data are expressed as means $\pm$ SE and were deemed significant when $P \leq 0.05$.

RESULTS

Species differences in maximal corticosterone production with steroidogenic agents

At equal cell concentrations, rat cells secreted 20–40 times more corticosterone than did chicken cells when maximally stimulated by ACTH analogues (Fig. 1) or cyclic AMP analogues (Fig. 2) or when provided with a maximal steroidogenic concentration of pregnenolone (Fig. 2). With 10 μM pregnenolone (3.17 μg/ml), a maximal steroidogenic concentration (Fig. 2), about 76% was converted to corticosterone (2.40 μg/ml) by rat cells, whereas only 10% was converted to corticosterone by chicken cells (0.33 μg/ml).

Species differences in the relative potencies of ACTH analogues

Figure 1 shows the effects of various ACTH analogues on corticosterone production. In fowl and rat cells, corticosterone production was induced over approximately two log orders of ACTH analogue concentration.

The data of Fig. 1 indicated differences among the ACTH analogues in their potencies for stimulating corticosterone production. Potencies were quantified using equations presented by Sayers *et al.*[12]. These equations permitted calculation of the half-maximal steroidogenic concentrations of the ACTH analogues: the lower the values the greater the potencies

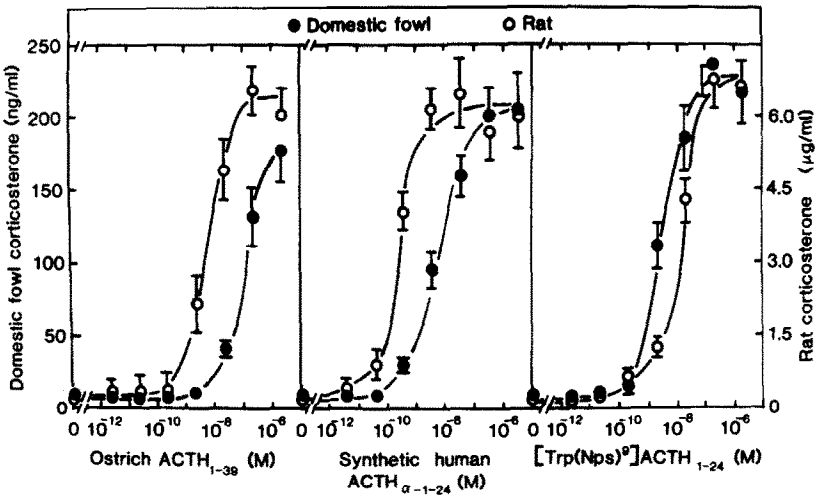

Fig. 1. Corticosterone production by isolated domestic fowl and rat adrenocortical cells in response to ACTH analogues. Each symbol represents the mean of corticosterone values from nine cell suspensions (three suspensions from each of three experiments). SE are presented by bars.

(Table 1). The order of the potencies in domestic fowl cell preparations was npsACTH = hACTH > osACTH, and for rat cell preparations was hACTH > osACTH > npsACTH.

The effects of the analogues on fowl and rat cells were different. Table 1 shows that the avian peptide osACTH was 17 times more potent with rat cells than it was with chicken cells: hACTH was similarly more potent (13 times) and so was porcine $ACTH_{1-39}$ (6.5 times; data not shown in Table 1). In contrast, however, npsACTH was 8 times more potent with chicken cells than it was with rat cells.

Species differences in the relative potencies of cAMP analogues

In both chicken and rat adrenocortical cell suspensions 8Br-cAMP was about 50 times more potent than cAMP in stimulating steroidogenesis (Fig. 2). The maximal steroidogenic concentrations for 8Br-cAMP and cAMP, respectively, were 0.1 and 5.0 mM with rat cells, and 1.0 and 50 mM with chicken cells. Thus, each analogue was 10 times more potent with rat cells than with domestic fowl cells.

Species differences in the conversion of the precursor pregnenolone to corticosterone

Rat cells converted nearly 8 times more pregnenolone to corticosterone than did fowl cells when provided with a maximal steroidogenic concentration of precursor in the absence of other steroidogenic agents (*cf.* Results: *Species differences in maximal corticosterone production with steroidogenic agents*). However, the half-maximal steroidogenic concentration of precursor was the same for both cell types (about 5 μM).

Ultrastructure of isolated domestic fowl adrenocortical cells

Figures 3 and 4 show section through isolated adrenocortical cells of the domestic fowl after incubation with hACTH for 2 h. These cells retained the typical characteristics of steroid secreting cells: mitochondria with tubular infoldings of their inner mitochondrial membranes and numerous lipid droplets. There was an abundance of filaments, a feature rarely seen in rat adrenocortical cells. Microtubules also were seen in the cytoplasm. All these features were common in fowl cells incubated with or without hACTH.

Table 1. Comparison of half-maximal steroidogenic concentrations of ACTH analogues for isolated domestic fowl and rat adrenocortical cells

Adrenocortical cell	Ostrich $ACTH_{1-39}$(M)	Synthetic Human $ACTH_{\alpha-1-24}$(M)	Trp $(Nps^9)ACTH_{1-24}$(M)
Domestic fowl	$(1.1 \pm 0.2) \times 10^{-7}$	$(3.1 \pm 0.5) \times 10^{-9}$	$(2.6 \pm 0.5) \times 10^{-9}$
Rat	$(6.6 \pm 2.0) \times 10^{-9}$	$(2.4 \pm 0.6) \times 10^{-10}$	$(2.1 \pm 0.3) \times 10^{-8}$

The data comprising Fig. 1 were plotted using equations modified from those presented by Sayers *et al.*[12]. The data for each ACTH analogue closely approached linearity as indicated by their coefficients of correlation measured by linear regression; no value was less than 0.84. The equations permitted the calculation of the half-maximal steroidogenic concentration of each analogue (slopes of plots). Each value represents the mean ± SE of the half-maximal steroidogenic concentration of ACTH analogue from three experiments.

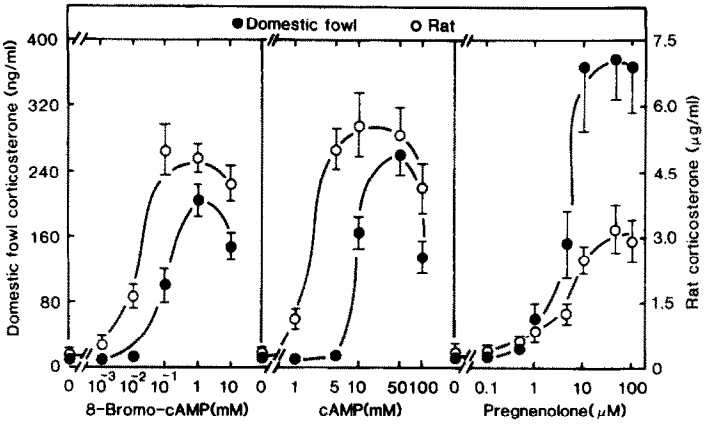

Fig. 2. Corticosterone production by isolated domestic fowl and rat adrenocortical cells in response to cAMP analogues and exogenous pregnenolone. Each symbol represents the mean of corticosterone values from nine cell suspensions (three suspensions from each of three experiments). SE are represented by bars.

DISCUSSION

The domestic fowl provides a readily available source of non-mammalian adrenocortical cells for comparison with commonly studied mammalian cells. Both fowl and rat adrenal glands produce corticosterone predominantly [6,12,13], thus providing a common index for the comparison of steroidogenic responses. This comparison revealed at least three differences between corticosteroidogenesis in the domestic fowl and in the rat.

First, the evidence suggests a difference in the function of the ACTH receptor as indicated by the strikingly greater potencies of osACTH, hACTH and porcine ACTH with rat cells than with fowl cells (Fig. 1; Table 1 and data under Results). The number of amino acid residues of these analogues does not account for their relative potencies because osACTH and porcine ACTH have 39 residues, whereas hACTH and npsACTH have 24 residues. In addition, assuming that the biological activity of ACTH analogues residues in the NH_2-terminal region of the hormone [10], it would have been expected that among the ACTH analogues, npsACTH, an ACTH

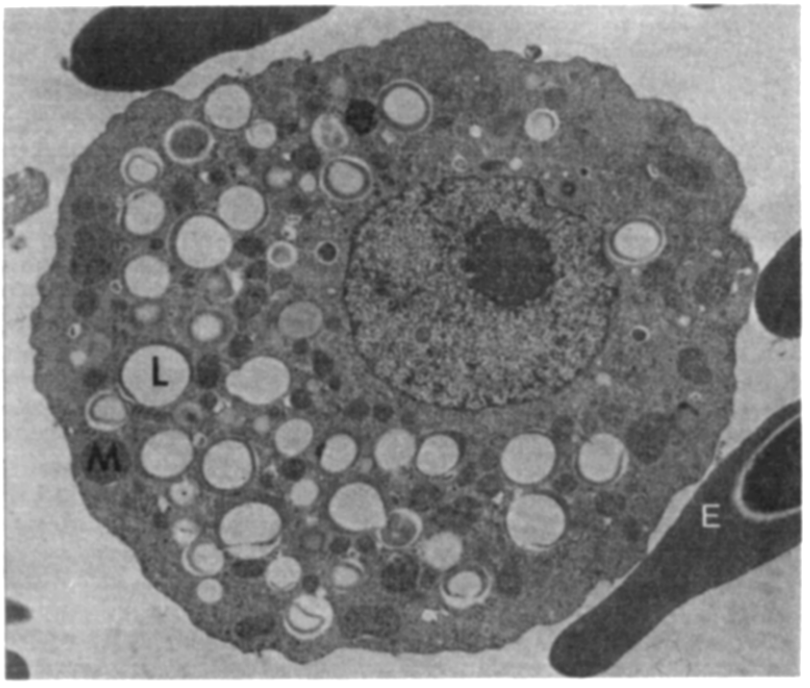

Fig. 3. Section through an isolated adrenocortical cell of the domestic fowl after incubation for 2 h with ACTH. (M), mitochondrion; (L), lipid droplet (most of the lipid has been extracted during the dehydration steps); (E) erythrocyte, ×8,800.

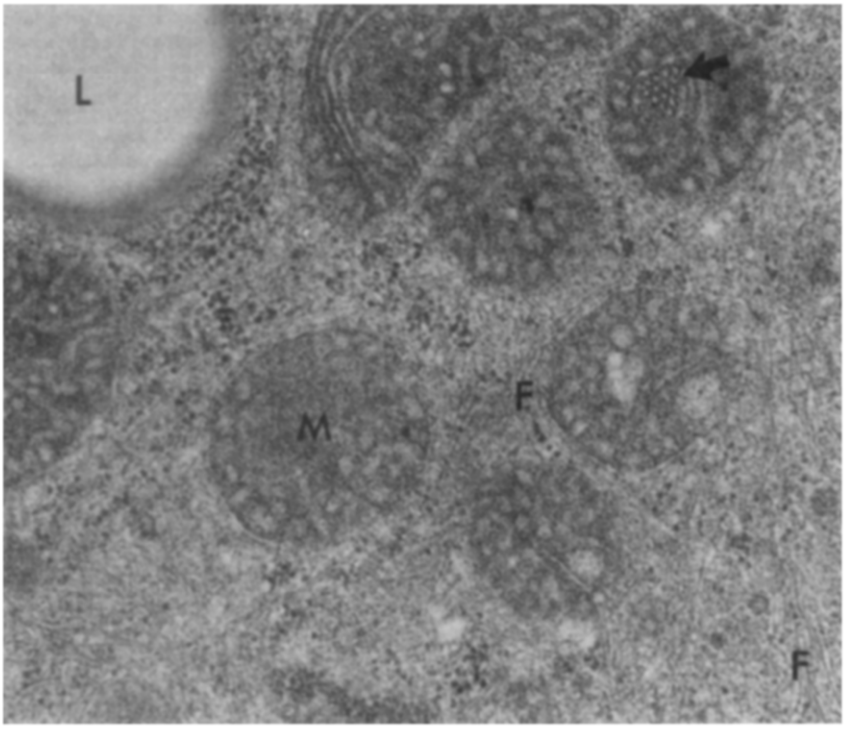

Fig. 4. Section through part of an isolated adrenocortical cell of the domestic fowl after incubation for 2 h with ACTH (M), mitochondrion; (L), lipid droplet; (F), cytoplasmic filaments. The arrow points to an intramitochondrial stack of tubules, ×60,000.

analogue that is modified in the ninth position of the NH_2-terminal region [18], would have had the lowest potency with fowl cells as was the case with rat cells. However, npsACTH and hACTH had equivalent potencies in the chicken cell preparation, and npsACTH was 8 times more potent with fowl cells than it was with rat cells (Table 1). These data suggest that fowl cells have a lower receptor specificity for ACTH-like molecules than do rat cells.

Also puzzling is the fact that an avian ACTH preparation, osACTH [19] had only about 2.6% of the potency of the mammalian analogues, hACTH, porcine ACTH and npsACTH, when incubated with chicken cells (Table 1). However, the low potency of osACTH with chicken cells might be explained by recent evidence which suggests that the ostrich and the domestic fowl arose from different ancestral stocks during the course of avian evolution [20].

Second, fowl corticosteroidogenesis appeared less dependent on cAMP-mediated processes than did rat corticosteroidogenesis. Cyclic AMP analogues were 10 times more potent with rat cells than with fowl cells (Fig. 2). However, the fowl cells were similar to the rat cells in that for each cell preparation, 8Br-cAMP was about 50 times more potent than cAMP. This species difference in cAMP potency is consistent with other work [21].

Third, a difference in steroidogenic enzyme function was apparent. This was indicated by comparison of the maximal corticosterone production induced in each cell type by steroidogenic agents: rat adrenocortical cells produced 20 to 40 times more corticosterone than did domestic fowl cells (Figs 1 and 2). In addition, with 10 μM pregnenolone, a maximal steroidogenic concentration, rat cells secreted 8 times more corticosterone than did fowl cells (Fig. 2). A deficiency either in enzyme activity or content in the chicken cells could explain this difference. However, the half-maximal steroidogenic concentration of pregnenolone, a measure of enzyme activity, was roughly the same for both cells types (about 5 μM), thus arguing against a defect in enzyme activity. Accordingly, the data suggest that chicken cells have a lower steroidogenic enzyme content, at the step for the conversion of pregnenolone to progesterone or a site beyond this step. A comparatively low enzyme content would explain why domestic fowl cells were less responsive than were rat cells to steroidogenic agents (Figs 1 and 2).

Whereas, the rat adrenocortical cell preparations were essentially free of medullary cells [15], the domestic fowl cell preparations contained 60% adrenocortical cells and 40% medullary cells. Thus, it can be argued that the responses of domestic fowl cell preparations were not strictly comparable to the rat cell preparations. However, our preliminary work with isolated fowl adrenocortical cells purified on a continuous Percoll (Pharmacia Fine Chemicals) den-

sity gradient, has provided data similar to those reported here.

Adrenocortical response *in vitro* can be altered by prior stress *in vivo* [22]. Thus, it can be argued that the ether-anaesthetized rats were subjected to stress leading to alterations in adrenocortical cells response *in vitro*, whereas the chickens killed by cervical dislocation were relatively free of stress. However, prior stress *in vivo* is accompanied by diminished adrenocortical tissue response to ACTH *in vitro* [22]. Thus, an even greater difference in the response of isolated domestic fowl and rat adrenocortical cells than that shown by our work might be expected if stress in the rat were reduced.

There was excellent preservation of the ultrastructure of the isolated fowl adrenocortical cells judging by comparison with the appearance of these cells *in situ* [23,24]. As is typical of mammalian steroid secreting cells *in situ* [25] and *in vitro* [11], characteristic features were mitochondria with tubular infoldings of their inner membranes and many lipid droplets (Figs 3 and 4). However, fowl cells showed an abundance of intracellular filaments (Fig. 4) of a sort rarely seen in rat adrenocortical cells. Our qualitative observations revealed no apparent ultrastructural differences between cells incubated with and without ACTH. Such differences have been reported for isolated rat adrenocortical cells incubated for 2 h, but these were revealed by quantitative methods [26].

Our work is the first reported on isolated adrenocortical cells of the domestic fowl. It has shown that fowl corticosteroidogenesis differs from rat corticosteroidogenesis at at least three sites: (1) the ACTH receptor (2) cAMP-dependent processes and (3) the enzymes responsible for the conversion of pregnenolone to progesterone or those responsible for subsequent steps. Thus, the use of these cells in further studies may lead to new information on (1) the evolution of the ACTH receptor, (2) the evolution of the dependence of corticosteroidogenesis on cAMP and (3) the evolution of steroidogenic enzymes in the adrenal cortex.

Acknowledgements—We thank J. Gibney for expert technical assistance. This work was supported by New Jersey American Heart Association Chapters and the New Jersey Agricultural Experiment Station.

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