MITOCHONDRIAL DAMAGE IN EXPERIMENTAL CONGENITAL ADRENAL HYPERPLASIA

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INTRODUCTION

Injection of the synthetic cyanoketosteroid (2 α-cyano-4,4,17 α-tri-methyl-androst-5-en-17β-ol-3-one) into pregnant rats leads to adrenal hyperplasia and abnormal genital development in the offspring (Goldman, Bongiovanni, and Yakovac, 1966). The effects observed depend on

the time of administration and correlate well with the appearance of maximum 3β-hydroxy steroid dehydrogenase[1] (3β-HSD) activity in the developing adrenal cortex and gonads. The changes in rat fetuses due to cyanoketosteroid are identical to those in human infants with an inherited deficiency of 3β-HSD (Bongiovanni, 1961). In both rats and humans, inactivity of 3β-HSD blocks steroid synthesis at the point where pregnenolone and 17-hydroxypregnenolone are converted to progesterone and 17-hydroxyprogesterone (Samuels and Uchikawa, 1967). Active glucocorticoids cannot be synthesized, leading to uninhibited adrenocorticotropin (ACTH) secretion by the pituitary. Secondary hyperplasia of the zona fasciculata and zona reticularis is accompanied by excessive production of 3β, Δ^4 steroids, some of which have weak androgenic activity. Evidence for the role of ACTH in causing adrenal hyperplasia in the experimental situation is that dexamethasone, which suppresses pituitary secretion of ACTH, prevents cyanoketosteroid induction of adrenal hyperplasia in guinea pigs (Castells and Bransome, 1969).

Cyanoketosteroid inhibition in vivo is long-lived (Goldman, Yakovac, and Bongiovanni, 1965; Goldman, 1967) and relatively specific. Neither the 3α- or 17β-steroid dehydrogenase nor the 11- or 21-hydroxylase is affected (Goldman and Winter, 1967). However, in vitro inhibition of bovine and *Pseudomonas testosteroni* Δ^5-3-ketosteroid isomerase and steroid 3α-dehydrogenase has been demonstrated (Goldman, 1968 *a*; Neville and Engel, 1968).

In this report, the ultrastructural appearance of the hyperplastic adrenal cortex of fetal rats is described. Marked swelling and internal disorganization of mitochondria and enlargement of lipid droplets in the zona fasciculata were seen. The findings may be interpreted as a morphological response to excessive endogenous ACTH secretion.

MATERIALS AND METHODS

Goldman's protocol (1966) was closely followed for these studies. Pregnant Sprague-Dawley albino rats were purchased from the Charles River Breeding Laboratories, North Wilmington, Mass. An experimental group of 11 animals received 60 mg/kg body weight of cyanoketosteroid[2] suspended in a vehicle of 90% corn oil and 10% absolute ethanol in one intramuscular injection, while 11 control animals were injected with an equal volume of vehicle. At appropriate times the mothers were rapidly anesthetized with ether, and the fetuses were delivered by hysterotomy. No significant fetal loss or abnormality was detected in the experimental group. Fetuses were promptly weighed, and both adrenals and a biopsy of liver from one member of each litter were dissected and fixed in 3% glutaraldehyde (prepared fresh from a 70% stock solution under Argon gas, as supplied by E. Ladd, Inc., Burlington, Vt.) buffered at pH 7.2 by 0.05 M sodium cacodylate, to give a total osmolarity of 325 milliosmols. Maternal adrenals were weighed and dissected into 1 mm cubes in the same fixative. The adrenals of the remaining fetuses and mothers were excised, weighed, and quick frozen to −70°C in buffered saline for subsequent enzyme analysis. Some unfixed frozen adrenals from each group of fetuses were used for fat stains.

Processing for electron microscopy consisted of fixation in glutaraldehyde for 2–3 hr, direct transfer to 1% osmium tetroxide in 0.1 M phosphate for 1 hr, dehydration in ethanol, embedding in Epon 812, sectioning with a diamond knife on a Porter-Blum MT-2 ultramicrotome (Ivan Sorvall Inc., Norwalk, Conn.), and staining of silver sections in uranyl acetate followed by lead citrate. The sections were examined and photographed in a Zeiss 9A electron microscope at direct magnifications of 1700 and 7100. Thick sections (1 μ) from each block were cut with glass knives and stained with toluidine blue, basic fuchsin, or hematoxylin triosine (Lane and Europa, 1964) for light microscopy.

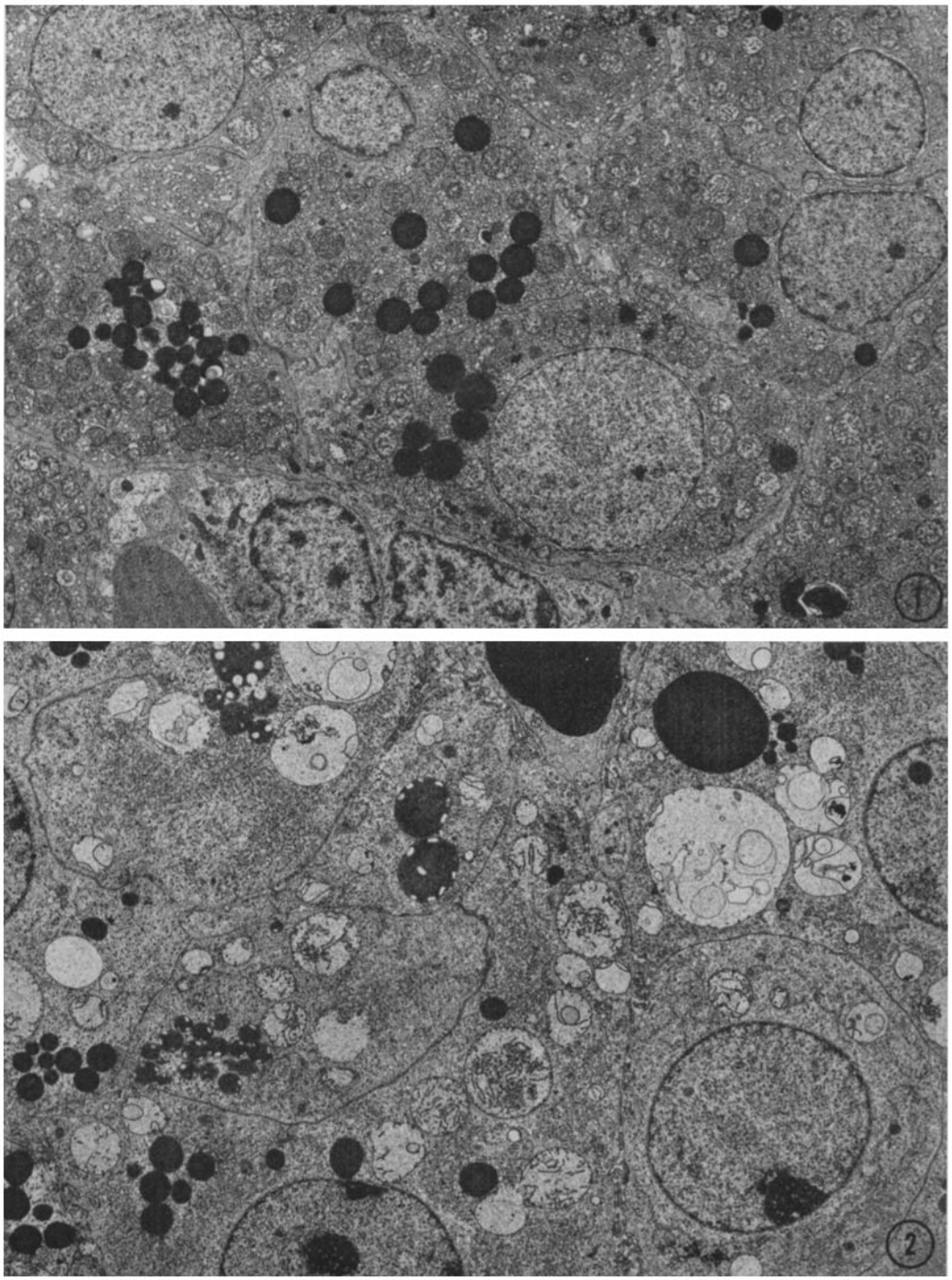
RESULTS

Histologic differences between the adrenal cortices of treated and control rats were limited to the zona fasciculata of fetuses treated on the 18th or 19th day of gestation and examined on the 21st day. By light microscopy, the zone was enlarged due to cellular hyperplasia, and the cytoplasm of the cells contained enlarged fat droplets and clear vacuoles that proved by electron microscopy to be swollen mitochondria. An abundance of fat was demonstrated by Sudan IV stains of frozen sections. No alterations in the architecture of the glands or the other zones were detected.

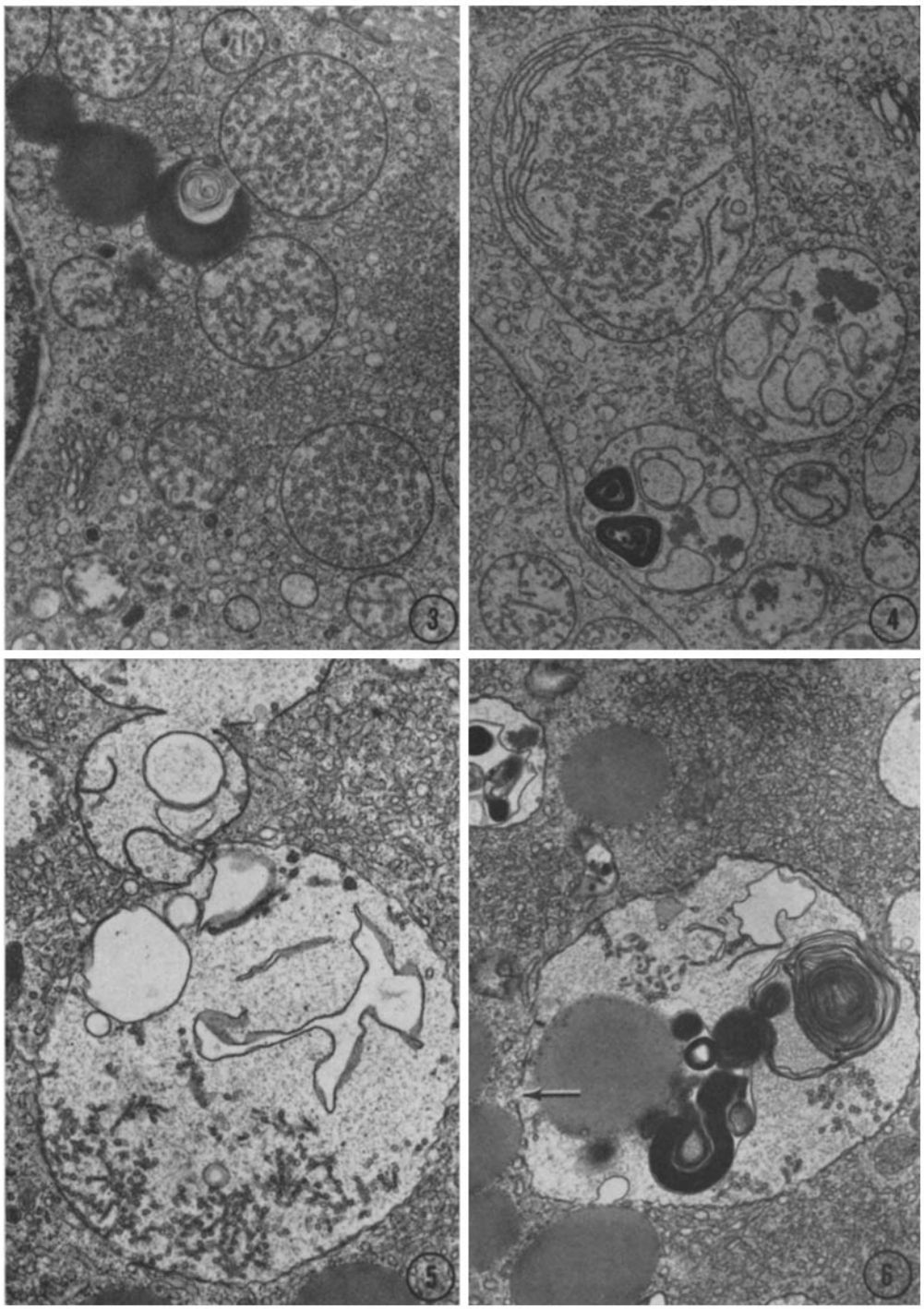
Electron microscopy of the zona fasciculata from treated fetuses revealed striking abnormalities of the mitochondria (Figs. 1–6) consisting of

[1] 3 hydroxysteroid: NAD oxidoreductase (EC 1.1.1.51).

[2] Dr. Gordon O. Potts of the Sterling-Winthrop Research Institute generously provided the cyanoketosteroid.



FIGURES 1 and 2 Zona fasciculata of adrenal cortex int he fetal rat, 21 days of gestation. Fig. 1 shows the control rat, injected with vehicle 72 hr earlier. There are numerous relatively uniform spherical mitochondria and deeply osmiophilic fat droplets, both averaging 1 μ in diameter. Fig. 2 shows cyanoketone injection 72 hr earlier. There are fewer but larger mitochondria, the largest of which has a diameter of 5 μ. There is also a loss of cristae in the mitochondria, leading to rarefaction. Fat droplets are variable in size and appearance, the largest reaching 4 μ in diameter and others showing numerous round to oval clear areas. Uranyl acetate and lead citrate. $\times$ 4000.



FIGURES 3–6 Zona fasciculata of adrenal cortex in the fetal rat, 21 days of gestation. Fig. 3 shows the control fetus. Mitochondria contain evenly distributed vesicular cristae of uniform diameter. The remaining cytoplasm contains some fat droplets, numerous vesicles of agranular endoplasmic reticulum, numerous free ribosomes, Golgi vesicles, and a few vesicles filled with dense, membranous material. Figs. 4–6 represent cyanoketone injection. In Fig. 4, the large mitochondrion retains a relatively normal density of small vesicular cristae plus some long tubular forms. Some irregularly shaped, moderately dense inclusions are present within five of the mitochondria, and one also contains two very dense, whorled membrane aggregates resembling myelin figures. Fig. 5 indicates marked swelling and rarefaction of mitochondria, which are closely aligned or fused. The clear intramitochondrial vacuoles may be dilated cristae or internally herniated inner membrane. Fig. 6 represents swollen mitochondria showing myelin figures, inclusion of a lipid droplet, and focal loss of continuity of the external limiting membrane (arrow). Uranyl acetate and lead citrate. × 13,000.

(*a*) swelling of most mitochondria in most cells, with occasional giant forms attaining a diameter of 5 μ; (*b*) apparent reduction in the number of mitochondria per cell; (*c*) reduction of the number of cristae in most mitochondria, including those with normal diameter, below what might be accounted for by swelling alone; (*d*) irregularities of the external unit membrane with either internal herniation or external protrusion; (*e*) inclusions of spherical droplets having an ill defined limiting membrane and osmiophilia typical of fat droplets; (*f*) inclusions of densely osmiophilic, irregularly contoured granular material; (*g*) formation of complex, dense membranous whorls resembling myelin figures; (*h*) focal interruption of the outer double membrane; and (*i*) close approximation of two or three mitochondria with fusion of their membranes. Fat droplets in these cells also differed from normal (Figs. 1, 2). Their size varied from 0.5 to 4 or 5 μ and many showed scalloping of their margins to give the appearance of a half or quarter moon. Although most droplets were homogeneously osmiophilic, as in normal cells, some of them contained small round to oval clear areas. There were no alterations in the agranular endoplasmic reticulum that is normally so conspicuous in these cells, or in the Golgi complex or other cellular constituents.

No changes were seen in the cells of the zona glomerulosa or intermediate zone in these treated fetuses, and the mitochondria of the connective tissue cells, adrenal medullary cells, and hepatic cells were also unremarkable. Very rarely, enlarged mitochondria with normal cristae were seen in the zona fasciculata of fetuses treated on day 20 and examined on the 22nd day, and in the adrenals of treated mothers. Some mitochondria in these tissues tended to approach and indent one another and occasionally fat droplets were enclosed by mitochondrial membranes, but most of these cells were indistinguishable from controls. The differences in response of the fetuses on day 20 and those injected on days 18 or 19 were surprising in view of an approximately equivalent adrenal weight gain of 50% in each group of fetuses. Similarly, a 25% increase in the ratio of maternal adrenal to body weight was not associated with significant morphological changes.

DISCUSSION

Mitochondrial vacuolization and loss of cristae, as well as indentation of one mitochondrion by another, have been described in adrenal cortical cells of adult rats treated with large doses of ACTH or with a variety of inhibitors of corticosteroid synthesis (Luse, 1967; Levine and Skelton, 1967). The effects of metapirone[3] and amphenone[4] in rats were more severe when ACTH was also given (Schwartz and Suchowsky, 1963). Hypophysectomy, on the other hand, reduced the degree of mitochondrial hypertrophy and cavitation in rats treated with aminoglutethimide, but the effects returned promptly when ACTH was added (Racela, Azarnoff, and Svoboda, 1969).

The above considerations raise the question as to whether the change observed in the fetal adrenals in the present study may have been mediated by ACTH. Other changes known to be induced by ACTH, such as an increased quantity and dilatation of agranular endoplasmic reticulum (AER) and reduction in the amount of lipid in cells of the zona fasciculata (Yamori, Matsuura, and Sakamoto, 1961; Borowicz, 1965; Luse, 1967), were not observed. However, the fact that these fetal rat cells showed only the mitochondrial component of the ACTH response might be explained by the inactivation of 3β-HSD. Localization of the enzyme in the microsomal fraction of adrenal homogenates (Beyer and Samuels, 1956) suggested that the agranular endoplasmic reticulum might be altered, as it is the dominant constituent of adrenal microsomes (Christensen and Gillim, 1969). There was no evidence of structural or quantitative abnormality of the AER but electron microscopy cannot resolve possible alterations in the conformation of an enzyme or its relation to membranes.

Lipid accumulation is readily understood as a secondary phenomenon due to the inhibition of the sequence of reactions leading from cholesterol to progesterone. Mitochondrial alterations, on the other hand, are not so easily explained. A variety of substances that cause mitochondria to swell, including ACTH, also enhance pregnenolone synthesis (Koritz, 1968). Noting this correlation, Koritz (1968) postulated that the primary effect of ACTH might be to enhance mitochondrial permeability to pregnenolone, with secondary hyperactivity of mitochondrial desmolases. Excessive ACTH secretion in combination with pregnenolone retention, therefore, might well produce exactly the kind of morphologic changes in mito-

[3] 2-methyl-1,2-bis[3-pyridyl]-1-propanone.
[4] 3,3-di-[*p*-aminophenyl]-butanone-2.

chondria found in response to cyanoketosteroid and other postcholesterol inhibitors of steroidogenesis. Large mitochondria were described in the adrenal cortex of human fetuses at 6–17 wk gestation (Ross, 1962), at a time when 3β-HSD activity is known to be low (Bloch, 1968). An increase in the size and number of adrenal mitochondria in human fetuses treated with intra-amniotic injections of ACTH 6–7 hr earlier was described by Johannisson (1968). The observation by Volk and Scarpelli (1966) that female adult rats have giant mitochondria in the zona fasciculata 2 wk after hypophysectomy might appear to contradict the hypothesis that ACTH stimulation is responsible for enlargement of mitochondria, but not enough is known about sequential changes in enzyme activity of the adrenal deprived of ACTH to interpret this finding. However, the fact that only females show mitochondrial gigantism is interesting in view of the ability of estrogens to inhibit 3β-HSD (Goldman, 1968 *b*).

In the present study, mitochondrial alterations were limited to the zona fasciculata, with sparing of adrenal medulla, fibroblasts, endothelial cells, and hepatocytes. Therefore, cyanoketone is not a general toxin for mitochondria. Also, the possibility that the structural changes might represent an artifact of tissue processing is remote. Unresponsiveness of the mitochondria in the zona glomerulosa may be cited as additional evidence of ACTH involvement in the production of the morphological changes, as the outer zone probably does not react to ACTH (Griffiths and Glick, 1966). Furthermore, mitochondrial structure in the two zones of untreated fetuses as well as adults is strikingly different, as lamellar cristae are found in the zona glomerulosa and tubulovesicular cristae in the fasciculata (Luse, 1967). Such structural differences must reflect different metabolic capability and susceptibility.

It is difficult to explain why the hyperplastic adrenals of 22-day fetuses were relatively unaffected 48 hr after treatment or why the maternal adrenals, which also were large, had minimal ultrastructural alteration. Further studies with varying dose schedules and serial sampling of the tissues are in progress.

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REFERENCES

GOLDMAN, A. S., A. M. BONGIOVANNI, and W. C. YAKOVAC. 1966. Production of congenital adrenal cortical hyperplasia, hypospadias, and clitoral hypertrophy (Adrenogenital Syndrome) in rats by inactivation of 3β-hydroxysteroid dehydrogenase. *Proc. Soc. Exp. Biol. Med.* **121**:757.

BONGIOVANNI, A. M. 1961. Unusual steroid pattern in congenital adrenal hyperplasia: deficiency of 3β-hydroxy dehydrogenase. *J. Clin. Endocrinol. Metab.* **21**:860.

SAMUELS, L. T., and T. UCHIKAWA. 1967. Biosynthesis of adrenal steroids. *In* The Adrenal Cortex. A. B. Eisenstein, editor. Little, Brown and Co. Inc., Boston. 61.

CASTELLS, S., and E. D. BRANSOME, JR. 1969. Comparison of the effects of ACTH and an inhibitor of 3β-hydroxysteroid dehydrogenase on the synthesis of adrenal microsomal proteins. *J. Clin. Endocrinol. Metab.* **29**:539.

GOLDMAN, A. S., W. C. YAKOVAC, and A. M. BONGIOVANNI. 1965. Persistent effects of a synthetic androstene derivative on activities of 3β-hydroxysteroid dehydrogenase and glucose-6-phosphate dehydrogenase in rats. *Endocrinology.* **77**:1105.

GOLDMAN, A. S. 1967. Experimental congenital adrenocortical hyperplasia: persistent postnatal deficiency in activity of 3β-hydroxy-steroid dehydrogenase produced *in utero*. *J. Clin. Endocrinol. Metab.* **27**:1041.

GOLDMAN, A. S., and J. S. D. WINTER. 1967. Activities of various steroidogenic enzymes in two forms of experimental adrenal hyperplasia. *J. Clin. Endocrinol. Metab.* **27**:1717.

GOLDMAN, A. S. 1968 *a*. Further studies of steroidal inhibitors of Δ^5, 3β-hydroxysteroid dehydrogenase and $\Delta^5 - \Delta^4$, 3 ketosteroid isomerase in *Pseudomonas testosteroni* and in bovine adrenals. *J. Clin. Endocrinol. Metab.* **28**:1539.

NEVILLE, A. M., and L. L. ENGEL. 1968. Inhibition of α- and β-hydroxy-steroid dehydrogenase and steroid Δ-isomerase by substrate analogues. *J. Clin. Endocrinol. Metab.* **28**:49.

LANE, B. P., and D. L. EUROPA. 1964. Differential staining of ultrathin sections of epon-embedded tissues for light microscopy. *J. Histochem. Cytochem.* **13**:579.

LUSE, S. 1967. Fine structure of adrenal cortex. *In* The

Adrenal Cortex. A. B. Eisenstein, editor. Little, Brown and Co. Inc., Boston. 1.

LEVINE, A. J., and F. R. SKELTON. 1967. A light and electron microscopic study of hyaline droplet and vacuole formation in the adrenal glands of rats treated with methylandrostenediol. *Amer. J. Pathol.* **51**:831.

SCHWARTZ, W., and G. K. SUCHOWSKY. 1963. Die wirkung von metopiron und amphenon β auf die nebennierenrinde der ratte. *Virchows Arch. Abt. A Pathol. Anat.* **337**:270.

RACELA, A., JR., D. AZARNOFF, and D. SVOBODA. 1969. Mitochondrial cavitation and hypertrophy in rat adrenal cortex due to aminoglutethimide. *Lab. Invest.* **21**:52.

YAMORI, T., S. MATSUURA, and S. SAKAMOTO. 1961. An electron microscopic study of the natural and stimulated adrenal cortex in the rat. *Z. Zellforsch. Mikrosk. Anat.* **55**:179.

BOROWICZ, J. W. 1965. Some ultrastructural changes in adrenal cortical cells of rats after hypophysectomy and following ACTH administration. *Beitr. Pathol. Anat. Allg. Pathol.* **132**:441.

BEYER, K. F., and L. T. SAMUELS. 1956. Distribution of steroid 3β-ol- dehydrogenase in cellular structures of the adrenal gland. *J. Biol. Chem.* **219**:69.

CHRISTENSEN, A. K., and S. W. GILLIM. 1969. The correlation of fine structure and function in steroid secreting cells, with emphasis on those of the gonads. *In* The Gonads. K. W. McKerns, editor. Appleton-Century-Crofts Inc., New York. 415.

KORITZ, S. B. 1968. On the regulation of pregnenolone synthesis. *In* Functions of the Adrenal Cortex. K. W. McKerns, editor. Appleton-Century-Crofts Inc., New York. **1**:27.

ROSS, M. H. 1962. Electron microscopy of the human fetal adrenal cortex. *In* The Human Adrenal Cortex. A. R. Currie, T. Symington, and J. K. Grant, editors. The Williams and Wilkins Co., Baltimore. 558.

BLOCH, E. 1968. Fetal adrenal cortex: function and steroidogenesis. *In* Functions of the Adrenal Cortex, K. W. McKerns, editor. Appleton-Century-Crofts Inc., New York. **2**:721.

JOHANNISSON, E. 1968. The foetal adrenal cortex in the human. *Acta. Endocrinol.* **58** (Suppl.):130.

VOLK, T. L., and D. G. SCARPELLI. 1966. Mitochondrial gigantism in the adrenal cortex following hypophysectomy. *Lab. Invest.* **15**:707.

GOLDMAN, A. S. 1968 *b*. Production of congenital adrenocortical hyperplasia in rats by estradiol-17β and inhibition of 3β-hydroxy-steroid dehydrogenase. *J. Clin. Endocrinol. Metab.* **28**:231.

GRIFFITHS, K., and D. GLICK. 1966. Determination of the 11β-hydroxylase activity in microgram samples of tissues; its quantitative distribution in the rat adrenal, and the influence of corticotrophin. *J. Endocrinol.* **35**:1.