

# ULTRASTRUCTURE, STEROIDOGENIC POTENTIAL, AND ENERGY METABOLISM OF THE SNELL ADRENOCORTICAL CARCINOMA 494

## A Comparison with Normal Adrenocortical Tissue

G. L. KIMMEL, F. G. PÉRON, A. HAKSAR, E. BEDIGIAN,  
W. F. ROBIDOUX, JR., and M. T. LIN

From the Worcester Foundation for Experimental Biology, Shrewsbury, Massachusetts 01545. Dr. Kimmel's present address is the Research Triangle Institute, Research Triangle Park, North Carolina 27709.

### ABSTRACT

Electron microscope studies were carried out with the adrenocortical carcinoma 494 and normal adrenal cortex tissue. The mitochondria of the tumor cells showed marked differences when compared with mitochondria from fasciculata cells of the normal adrenal cortex. These differences were primarily related to mitochondrial number and crista structure.

Corticosterone production in isolated tumor cells was extremely low and neither ACTH nor dibutyryl cyclic AMP had any stimulatory effect. Normal adrenal cells showed at least a tenfold increase under identical conditions. In the presence of corticosteroid precursors the amount of corticosterone produced by the tumor cells was much less than that produced by normal cells.

The results indicate a reduced capacity for  $11\beta$ -hydroxylation in the tumor mitochondria and a possible reduced capacity for biosynthetic steps before the  $11\beta$ -hydroxylation reaction. Glycolysis in isolated tumor cells was also lower than in normal cells.

Isolated tumor mitochondria oxidized succinate normally with a good degree of coupling with phosphorylation. However, unlike normal adrenal mitochondria, the tumor mitochondria showed little or no oxygen uptake with other Krebs cycle substrates. These data suggest that the tumor mitochondria may be lacking in the flavoprotein dehydrogenases responsible for the oxidation of NADH and NADPH, although other components of the respiratory chain may be intact.

### INTRODUCTION

The adrenocortical carcinoma 494, originally discovered in the rat by Snell and Stewart (1), has been studied by several laboratories (2-7) to determine its steroid biosynthetic potential and compare it with that in the normal adrenal. Ney et al. (3) noted that the tumor was able to utilize

exogenous substrates for corticosteroid production. However, the rate of synthesis was greatly reduced (2, 3). This was especially true of the  $11\beta$ -hydroxylation of 11-deoxycorticosterone (DOC)<sup>1</sup> to corticosterone (2).

The effect of adrenocorticotropin (ACTH) and adenosine-3',5'-monophosphate (CAMP) on the tumor has also been studied. Although ACTH was unable to stimulate steroidogenesis in this tissue (3), Schorr et al. (4, 6) demonstrated that the trophic hormone was capable of stimulating the tumor adenylyl cyclase to produce fairly large amounts of CAMP. The only abnormality appeared to be in the lack of specificity for ACTH. Unlike the adenylyl cyclase in the normal adrenal cortex, which is stimulated only by ACTH, the tumor adenylyl cyclase was stimulated by a number of other trophic hormones (e.g., thyroid-stimulating hormone, luteinizing, and follicle-stimulating hormone). From these results and the finding that large concentrations of exogenous CAMP failed to stimulate corticosteroidogenesis in vitro (3), it was concluded that reduced steroidogenesis in the tumor was due primarily to a defect(s) beyond the formation of CAMP (4).

Corticosteroidogenesis in the normal adrenal gland is a process dependent on several steps. Fractionation studies showed some of these to occur in the mitochondrial fraction and others to occur in the microsomal fraction (8, 9). Therefore, it is possible that the reduced rate of steroidogenesis in the tumor may be a result of a defect(s) in one or more of the cell organelles. The present study was designed to correlate the ultrastructure of the rat adrenocortical carcinoma 494 with its corticosteroidogenic potential and to make an overall comparison with the normal rat adrenal cortex. The findings of an alteration in the tumor mitochondrial structure and the biosynthetic steps associated with it give evidence that the abnormal steroidogenesis of the tumor can be largely attributed to abnormal mitochondrial function. In addition, our results demonstrate a lowered capacity for specific mitochondrial oxidations in the tumor which suggest a possible lack of certain enzymes in the respiratory chain.

<sup>1</sup>The generic names of substances for which trivial names have been given in the paper are: Pregnenolone,  $3\beta$ -hydroxy-5-pregnene-20-one; progesterone, 4-pregnene-3,20-dione; DOC, 11-deoxycorticosterone, 21-hydroxy-4-pregnene-3,20-dione; corticosterone,  $11\beta$ ,21-dihydroxy-4-pregnene-3,20-dione;  $11\beta$ -OH-progesterone,  $11\beta$ -hydroxy-4-pregnene-3,20-dione.

## MATERIALS AND METHODS

### *Animals*

The rat adrenocortical carcinoma 494 was transplanted and maintained in a manner similar to that described by Ney et al. (3). Young male Sprague-Dawley rats (Charles River Breeding Laboratories, Inc., Wilmington, Mass.), weighing 50-70 g, received viable tumor tissue subcutaneously through a small incision near the base of the tail. The tumor tissue for all studies was taken 4-6 wk after the initial transplant. The atrophic adrenals from the tumor-bearing animal were also removed at this time. Normal male rats of the same age were used as a source for normal adrenal tissue. Adrenal tissue was also taken for electron microscope examination from tumor-bearing and control rats which had received long-term ACTH treatment. Treatment consisted to three daily subcutaneous injections (morning, noon, and evening) of ACTH (Acthar gel, 40 USP U/ml, Armour Pharmaceutical Co., Chicago, Ill.), for a 2-wk period.

### *Preparation of Tissue for Electron Microscopy*

Tissues for ultrastructural studies were fixed in 5% glutaraldehyde (pH 7.2) for 2 h at room temperature. This was followed by postfixation in 2% OsO<sub>4</sub> (pH 7.2) for 1.5 h. Both fixatives were made in 0.1 M PO<sub>4</sub> buffer containing CaCl<sub>2</sub>. Samples were then dehydrated in a graded series of aqueous ethanol solutions and embedded in Epon 812 by the method of Luft (10). Ultrathin sections were cut on a Porter-Blum MT2-B ultramicrotome (Ivan Sorvall, Inc., Newtown, Conn.), and stained with uranyl acetate and lead citrate. Sections were viewed with a Zeiss EM9S-2 electron microscope.

### *Cell Preparation and Incubations*

Cells were isolated from the normal adrenal as previously described by this laboratory (11, 12). Isolated tumor cells were prepared both with and without the use of proteolytic enzymes. Tumor cells were isolated using trypsin and collagenase following a procedure identical to that for normal adrenal cell isolation. For tumor cells isolated without enzymes, viable tumor tissue was placed in a 50-ml erlenmeyer flask containing 20 ml Krebs-Ringer bicarbonate buffer plus glucose (KRBG) (11, 12). The flask was gassed with 95% O<sub>2</sub>-5% CO<sub>2</sub>, and the tissue stirred for 15-20 min at 37°C using a magnetic stirrer. This was repeated six to eight times, with fresh KRBG each time. The first two fractions were discarded due to a large amount of contamination with red blood cells. The remaining fractions were then centrifuged and concentrated according to the procedure for normal adrenal cell isolation. All isolated cells demonstrated an ultrastructure identical to that of their respective intact tissue. Similar results have been reported by Sharma et al. (13)

for normal adrenal cells and by Sharma and Hashimoto (5) for trypsinized adrenal tumor cells. There were no apparent structural differences between cells isolated with enzymes and those isolated in buffer alone.

All experiments were carried out using tumor cells isolated without enzymes, except in the case where cells isolated with and without enzymes were compared. Incubations of the isolated cells were always carried out in KRBG containing 5 mg/ml BSA. Lima bean trypsin inhibitor (1 mg/ml) was also added when cells were isolated with trypsin and collagenase. Incubations were carried out in 10-ml beakers in a final volume of 1.5 ml with shaking in a Dubnoff metabolic incubator (60 rpm) at 37°C for 2 h under an atmosphere of 95% O<sub>2</sub>-5% CO<sub>2</sub>.

ACTH used in the incubation studies was USP corticotropin reference standard distributed by United States Pharmacopeial Convention, Inc. Dibutyryl cyclic-3', 5'-AMP (dbCAMP) and NADPH were obtained from Sigma Chemical Co., St. Louis, Mo.; trypsin and lima bean trypsin inhibitor from Worthington Biochemical Corp., Freehold, N. J.; collagenase from Gallard-Schlesinger Chemical Mfg. Corp., Carle Place, N. Y.; and bovine serum albumin (BSA) fraction V powder, Pentex, from Miles Laboratories Inc. Kankakee, Ill. All other chemicals were of reagent grade purity obtained from various sources.

### *Steroid Measurement*

Corticosterone was measured essentially by the method of Silber et al. (14). Incubation sample aliquots and corticosterone standards were dissolved in 13% EtOH and extracted with 4 ml of methylene dichloride. After removal of the ethanolic phase, 3 ml of 80% aqueous H<sub>2</sub>SO<sub>4</sub> were added to the methylene dichloride extract. After shaking, the fluorescence of the acid phase was measured on an Aminco-Bowman spectrophotofluorometer (American Instrument Co., Travenol Laboratories, Inc., Silver Spring, Md.), 50 min after addition of the acid. Appropriate blank values were subtracted for any interfering fluorescence contributed by pregnenolone or 11 $\beta$ -OH progesterone.

Pooled incubation samples were also measured for DOC. After the addition of radiolabeled DOC (<0.001  $\mu$ g, specific activity, 40 Ci/mmol, New England Nuclear, Boston, Mass.) as a recovery standard, the samples were extracted thoroughly with methylene dichloride. After evaporation of most of the organic solvent under a stream of N<sub>2</sub>, the concentrated extracts were run on paper chromatograms in the Bush B<sub>3</sub> system (15). The  $\Delta^4$ -3-ketosteroids on the dried chromatograms were visualized under UV lights and tentative identification was made by comparison with known standards. A narrow strip from each chromatogram was immersed in blue tetrazolium reagent (16) for the detection of steroids containing  $\alpha$ -ketol-reducing side chains; i.e., corticosterone and DOC. If a blue spot corresponding to DOC was observed, the remaining area on the chromatogram was

eluted with ethanol and DOC measured quantitatively by the blue tetrazolium assay (16).

### *Oxygen Uptake Studies*

Mitochondria from the tumor tissue and normal adrenal were isolated by a procedure described previously (17). The one exception was that only four to eight passes of a loose-fitting pestle of the Potter-Elvehjem homogenizer were made for homogenization of the tissue. Although not usually done for preparing mitochondria from normal adrenals (17), this was necessary to avoid excessive breakage of the apparently fragile tumor mitochondria. Electron microscope examination revealed isolated fractions rich in intact mitochondria in both normal and tumor preparations. However, there was a degree of breakdown of the internal matrix and breaking of the outer membrane in some of the tumor mitochondria. Oxygen utilization of the incubated mitochondria was measured polarographically using a vibrating microplatinum electrode in conjunction with an Oxygraph, model KM (Gilson Medical Electronics, Inc., Middleton, Wis.) as previously described (18, 19).

### *Pyruvate and Lactate Measurements*

Incubations of normal and tumor cells were also carried out for pyruvate and lactate production. The effect of ACTH and dbCAMP were investigated, and arsenite was used as a blocking agent for pyruvate-lactate utilization. Pyruvate and lactate were measured by following the oxidation-reduction of NADH/NAD<sup>+</sup> at 340 nm with lactate dehydrogenase as previously described (12, 20).

## RESULTS

### *Ultrastructure*

**NORMAL ADRENAL TISSUE:** The zona fasciculata of the normal adrenal gland showed ultrastructural features (Fig. 1) identical to those reported by others (21-24). The mitochondria were numerous and closely packed within the cell. They were circular or oval in shape and contained packed vesicular cristae. Lipid droplets were abundant and distributed irregularly in almost all cells. An agranular endoplasmic reticulum and free ribosomes were also apparent throughout the cytoplasm.

**TUMOR TISSUE:** The ultrastructure of the adrenocortical carcinoma tissue is shown in Fig. 2. The most striking intracellular change from that of the normal adrenal involves the mitochondria. The number of mitochondria per cell in the tumor was noticeably less than that of normal fasciculata cells (25% that of mitochondria from normal rat adre-

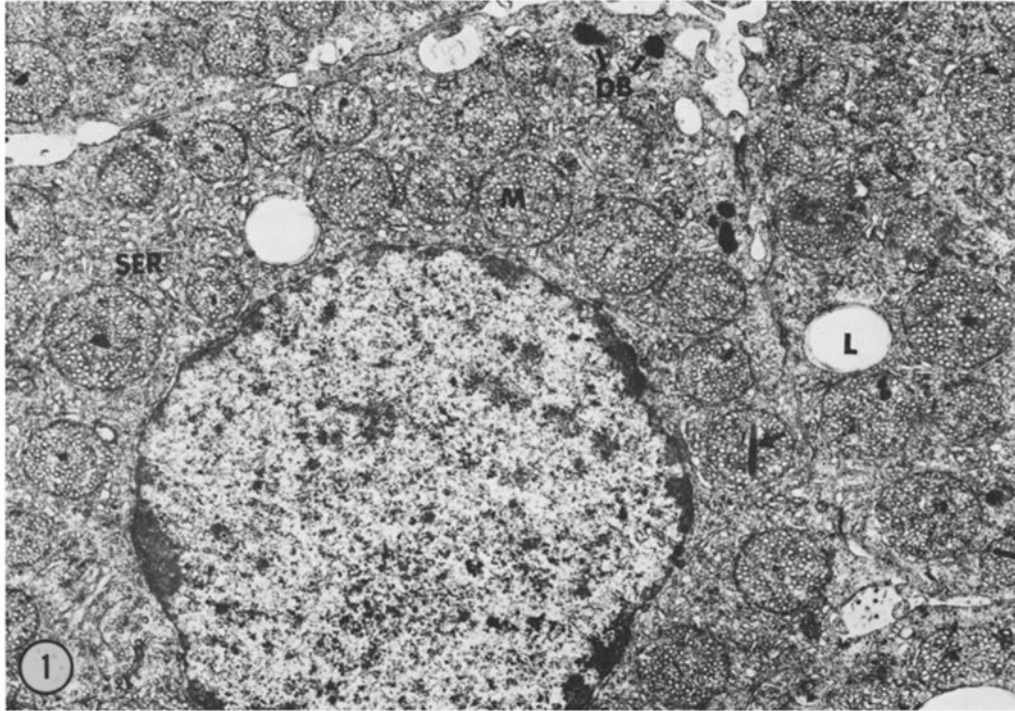


FIGURE 1 Section from fasciculata region of the normal rat adrenal cortex. Note the numerous mitochondria (*M*) with vesicular cristae, and the preponderance of smooth endoplasmic reticulum (*SER*). Other features of the cell include lipid droplets (*L*), dense bodies (*DB*), and numerous free ribosomes. Arrow identifies one of the many intramitochondrial inclusions.  $\times 10,000$ .

nal, based on milligrams of protein/original tissue wet weight)<sup>2</sup>. Many of the mitochondria were elongated and generally showed a much less uniform shape. However, the most striking change was the predominance of sparse, lamella-shaped cristae. Many of the tumor cells contained round to oval vesicles, each possessing an electron-lucid matrix. In many cases these vesicles were so large and numerous that they occupied almost all of the cytoplasmic space. Occasionally, the membrane of one or more of these vesicles was seen to be continuous with the outer nuclear membrane. The endoplasmic reticulum of the tumor cells was dilated and often granular, and free ribosomes, often occurring in clusters, were quite apparent.

**ADRENAL TISSUE FROM TUMOR-BEARING ANIMAL:** The zona fasciculata of the atrophied adrenal gland from the tumor-bearing animal showed marked ultrastructural differences from both the normal adrenal and the adrenocortical tumor (Fig. 3). The cells appeared smaller, and the nuclear to cytoplasmic ratio appeared to increase. The nuclei often presented an irregular appear-

ance, in contrast to the generally oval nuclei of the tumor and normal adrenal cells.

The mitochondria of adrenal cells from tumor-bearing rats were less numerous and of less uniform shape than those found in normal fasciculata cells. Mitochondria from the atrophied adrenals had lost their characteristic vesicular cristae and developed lamellar cristae similar to those in tumor mitochondria. The mitochondrial matrix was more dense and few intramitochondrial inclusions were seen. Further, many mitochondria appeared to be associated with the formation of membranous whorls within the atrophied adrenal cells. The smooth endoplasmic reticulum of the atrophied adrenals was less developed than in normal fasciculata cells. Lipid droplets were present, and lysosome-like dense granules were numerous. The observations are not unlike those found in adrenocortical tissue from hypophysectomized animals (24).

**LONG-TERM ACTH TREATMENT:** The *in vitro* results of Ney et al. (3) on corticosterone production by the adrenal from the tumor-bearing

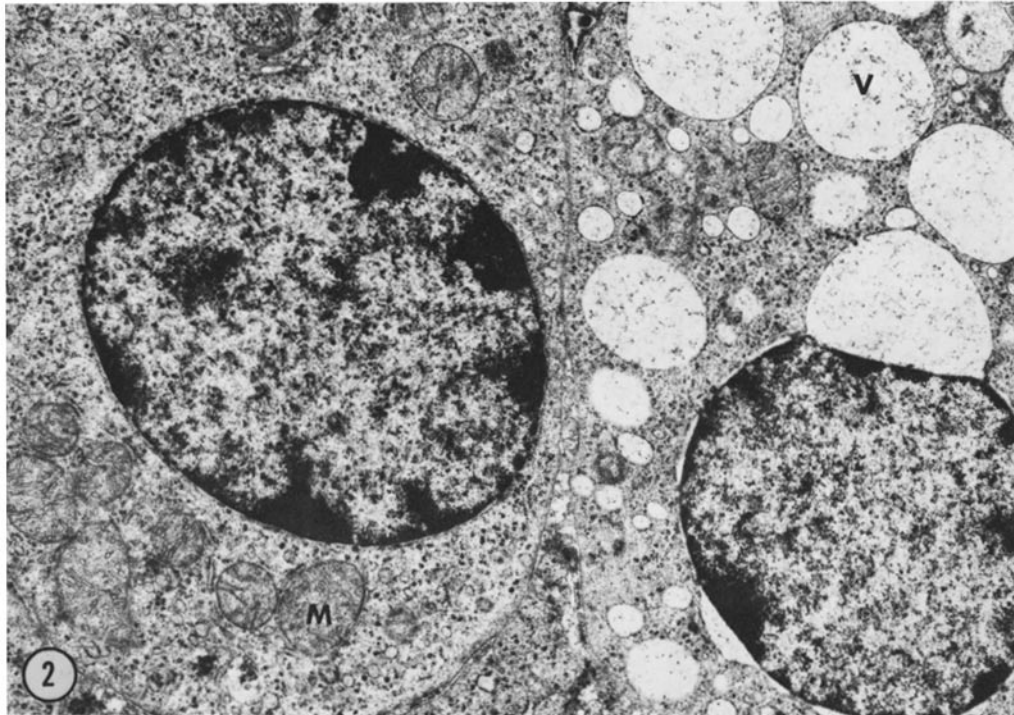


FIGURE 2 Micrograph of the Snell adrenocortical carcinoma 494. Mitochondria (*M*) are relatively scarce and possess lamellar cristae. Clusters of ribosomes and rough endoplasmic reticulum fill the cytoplasm. Some cells are filled with large electron-lucid vacuoles (*V*).  $\times 8,100$ .

rat demonstrated that the decreased production of steroid by this adrenal returned to normal levels after pretreatment with ACTH. To study any ultrastructural changes which might occur after ACTH administration, we injected both normal and tumor-bearing rats with ACTH over a period of 2 wk. At the end of the treatment period the adrenals of both groups were examined.

The changes in ultrastructure of the normal adrenal observed between the control (Fig. 1) and ACTH-treated (Fig. 4) rats in this study are in accordance with previous findings (25-27). These include, most noticeably, an increase in the number of mitochondria, lipid droplets, and smooth endoplasmic reticulum. In addition, the normal adrenal glands after ACTH treatment had increased to about five times their normal size.

The change in the adrenal from the tumor-bearing animal was striking after ACTH (Fig. 5). The cells appeared to regain their normal size and nuclear to cytoplasmic ratio. The nuclei presented a normal round to oval appearance. The mitochondria were of a normal shape and size, and they filled the cytoplasm. The mitochondrial cristae had

regained their vesicular character. The lipid droplets became noticeably larger, equal to those in the normal adrenal after ACTH treatment. The adrenals had become greatly enlarged, the wet weight rising from approximately 8 mg/pair before ACTH to about 100 mg/pair after the 2-wk period of injections.

#### *Steroidogenesis*

We found that the adrenocortical tumor can be dissociated into isolated cells without the use of proteolytic enzymes. This is a property not found in most other tissues, and it permitted a comparison of the steroidogenic capacity of cells isolated with and without proteolytic enzymes. When incubated with various steroid precursors, there was little or no difference in the steroidogenesis of the cells regardless of the method used for isolation, indicating that the use of trypsin and other proteolytic enzymes is unnecessary in the case of this adrenal tumor.

Table I shows the corticosterone production by isolated cells from the normal adrenal and the

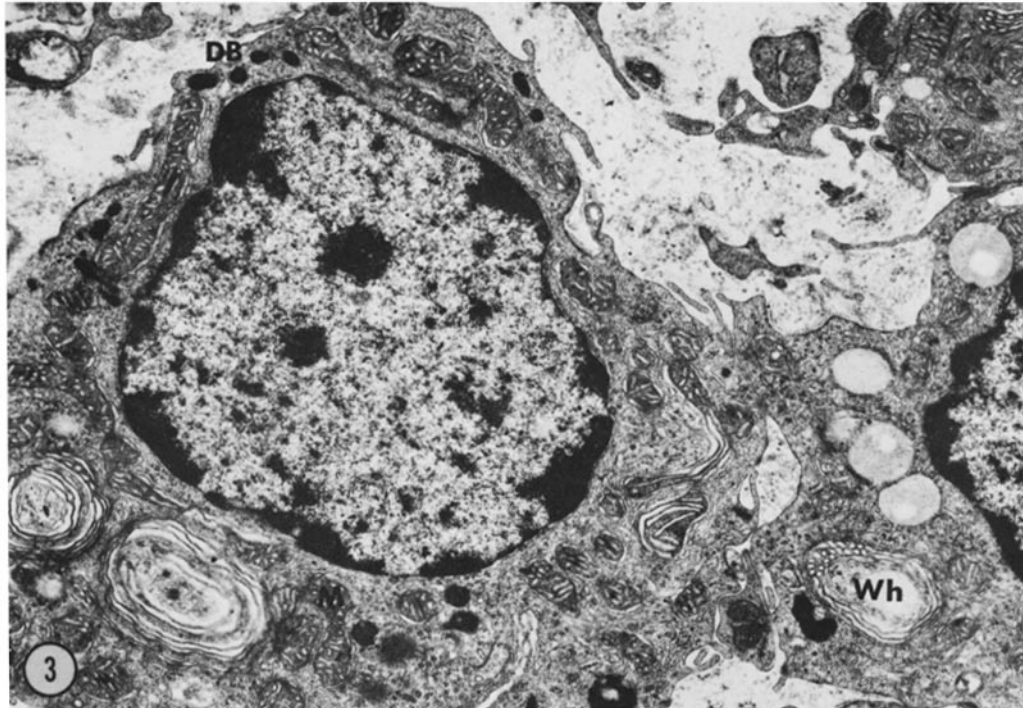


FIGURE 3 Adrenal fasciculata region from the atrophied adrenal of the tumor-bearing rat. The cells are smaller and contain fewer mitochondria (*M*) than normal fasciculata cells. The mitochondria no longer exhibit vesicular cristae and are often seen associated with whorls of membranes (*Wh*). Small dense bodies (*DB*), probably lysosomes, are evident within these cells.  $\times 8,100$ .

adrenal tumor. As noted in Materials and Methods, normal cells were isolated using trypsin-collagenase, the tumor cells in buffer alone. The normal adrenal cells synthesize only negligible amounts of corticosterone in control incubations. This is in agreement with our previously published data (20). Addition of ACTH or dbCAMP produced at least a 10-fold increase in the levels of corticosterone. The addition of corticosterone precursors, pregnenolone, DOC, and  $11\beta$ -OH-progesterone, also led to a marked increase in corticosterone production. Progesterone, however, seemed to be utilized less readily than other precursors.

Corticosterone production by tumor cells was extremely low when compared to that by normal cells. Neither ACTH nor dbCAMP had any stimulating effect on the tumor cells. Among the corticosterone precursors,  $11\beta$ -OH-progesterone was utilized most efficiently followed by DOC and pregnenolone. Progesterone was again the least efficient precursor. It is clear from Table I that in the tumor cells, corticosterone levels, after a 2-h

incubation in the presence of DOC, progesterone, and pregnenolone, were only about 1.5–2.5% of those in normal cells, whereas with  $11\beta$ -OH-progesterone, they were as much as 13%.

The results described above suggested that a major defect in the steroidogenic pathway in the tumor was at the  $11\beta$ -hydroxylation step which takes place in the mitochondria and is the last step in the conversion of DOC to corticosterone. If this were the case, DOC might be expected to accumulate when the tumor cells were incubated with pregnenolone or progesterone. Table II shows the qualitative results after chromatographic analysis of the incubation media after incubation with these steroids and other substances. Normal cells did not produce detectable levels of corticosterone under control conditions, but did so under all other conditions tested. DOC, however, was only qualitatively detectable in chromatograms of samples to which the steroid precursors, pregnenolone and progesterone, had been added. The tumor cells also showed detectable levels of DOC in chromatographed samples containing the steroid precursors,

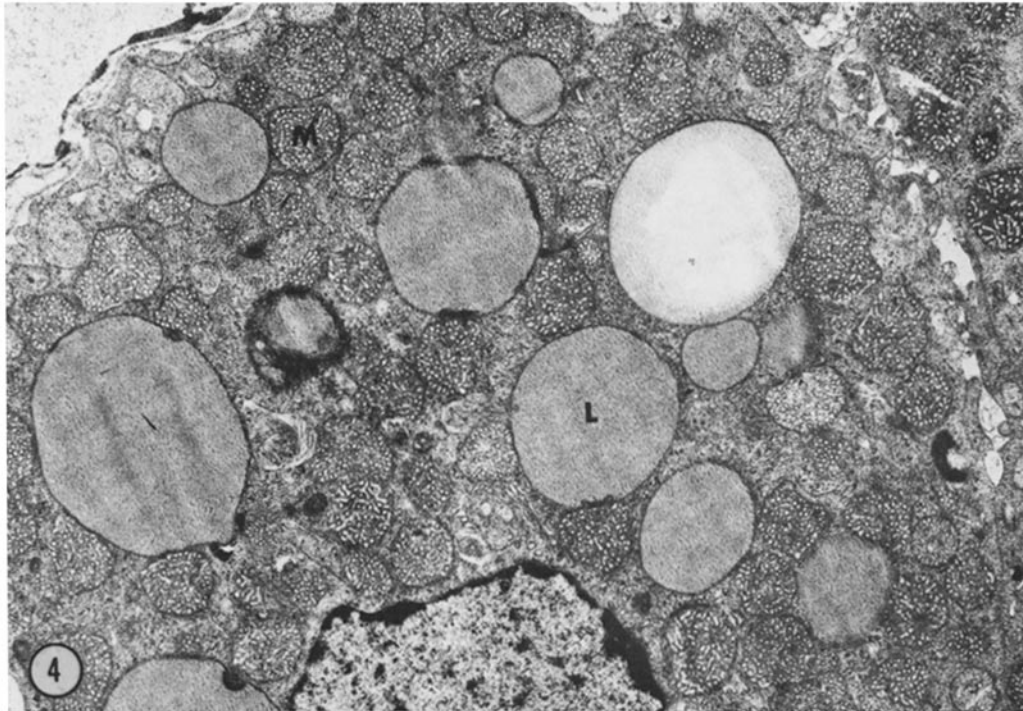


FIGURE 4 Rat adrenal from animal treated with ACTH. Note the close packing of mitochondria (*M*) and the vesicular appearance of mitochondrial cristae. Lipid droplets (*L*) are numerous and generally larger than in nonstimulated adrenal fasciculata cells.  $\times 8,100$ .

but did not demonstrate any qualitatively detectable corticosterone production under any of the other conditions tested. The eluted DOC regions from the chromatograms with detectable levels of DOC were quantitatively assayed by the blue-tetrazolium procedure. These values coupled with corticosterone levels (quantitative) provided a DOC to corticosterone ratio (Table II). The ratios for the tumor cells were 15–25 times higher than those for the normal cells, indicating a significant build up of DOC in the tumor cells. It must be mentioned that actual levels of DOC were lower in the tumor cell incubations than in those of the normal cell, indicating a decreased production of DOC or its increased metabolism by a pathway not leading to corticosterone. At present, however, we have been unable to detect any other  $\Delta^4$  unconjugated 3-ketosteroid besides DOC which might be a metabolic product of the tumor cell incubations.

**OXYGEN UPTAKE STUDIES:** Isolated mitochondria were studied for their ability to utilize oxygen and produce corticosterone. As shown in Fig. 6 A, the oxygraph trace obtained with adrenal

mitochondria from normal, nonstressed rats (normal mitochondria) was as expected when succinate was the oxidizable substrate (18, 28). A P/O ratio of about 1.9 was obtained after the addition of 200 nmol of ADP, whereas 10.5 nmol of corticosterone were formed per minute per milligram mitochondrial protein after all exogenous ADP had been utilized. Addition of 2,4-dinitrophenol (2,4-DNP) led to a large increase in oxygen uptake, and as is usual, to an inhibition of corticosterone production brought about by the uncoupling effect of this substance (17). The picture presented by tumor mitochondria incubated with succinate was similar in terms of  $O_2$  consumption as well as to the degree of coupling of oxidation with phosphorylation, as indicated by a P/O ratio of about two (Fig. 6 B). On the other hand, it is clear that after the addition of DOC, little  $11\beta$ -hydroxylation of this steroid took place. What little corticosterone synthesis occurred with DOC was completely abolished by the addition of 2,4-DNP. Although not shown in the figures, addition of the respiratory chain inhibitors Antimycin A and KCN in similar experiments inhibited succinate-supported  $O_2$  uptake in

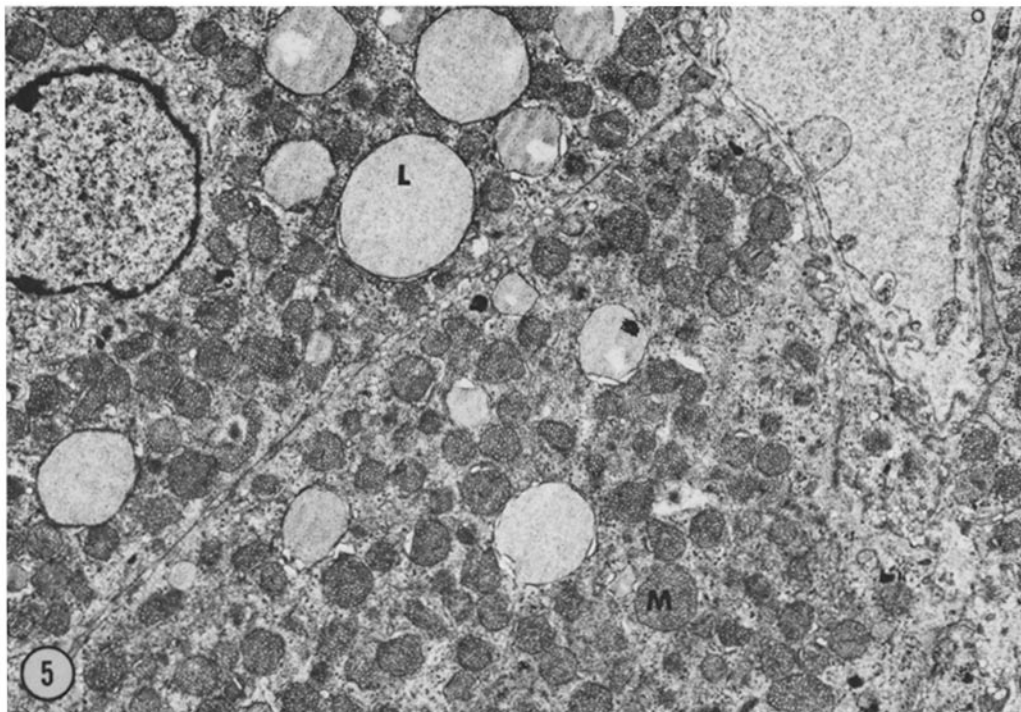


FIGURE 5 Fasciculata region from tumor-bearing rat treated with ACTH. Cells appear similar to normal rat adrenocortical cells. Note vesicular cristae of the mitochondria.  $\times 5,000$ .

TABLE I  
*Corticosterone Levels in Cells Isolated from the Normal Adrenal and Adrenal Tumor*

Additions	Normal	Tumor
Control	1.01 $\pm$ 0.17 (8)*	0.07 $\pm$ 0.01 (8)
ACTH 800 $\mu$ U/ml	11.99 $\pm$ 1.20 (8)	0.07 $\pm$ 0.01 (8)
ACTH 1,600 $\mu$ U/ml	13.56 $\pm$ 0.79 (8)	0.08 $\pm$ 0.01 (8)
dbCamp 500 $\mu$ M	13.98 $\pm$ 0.45 (8)	0.06 $\pm$ 0.01 (8)
Pregnenolone 80 $\mu$ g/ml	36.64 $\pm$ 1.88 (8)	0.50 $\pm$ 0.05 (8)
Progesterone 80 $\mu$ g/ml	7.14 $\pm$ 0.62 (8)	0.17 $\pm$ 0.01 (8)
DOC 80 $\mu$ g/ml	37.17 $\pm$ 1.55 (8)	0.61 $\pm$ 0.07 (8)
11 $\beta$ -OH-Progesterone 20 $\mu$ g/ml	37.08 $\pm$ 1.86 (7)	4.79 $\pm$ 0.18 (8)

\* Mean  $\pm$  standard error (N).

Either  $1.5\text{--}2.3 \times 10^6$  (normal) or  $1.0\text{--}1.5 \times 10^6$  (tumor) cells per beaker were incubated 2 h at 37°C. Results are expressed as micrograms corticosterone/ $10^6$  cells.

both the control and tumor mitochondria. Corticosterone production was also completely inhibited by these two substances in the control and tumor mitochondria.

Other experiments carried out with the Krebs-cycle acids  $\alpha$ -ketoglutarate, malate, and isocitrate showed expected  $O_2$  uptake rates and substrate-supported steroid 11 $\beta$ -hydroxylation of DOC in normal mitochondria (18, 29). On the other hand,

little or no  $O_2$  uptake or corticosterone production was observed with these substrates when tumor mitochondria were incubated, regardless of whether  $P_i$  or ADP were added singly or together to the incubation medium. As will be discussed later, these observations could indicate a relative paucity in the tumor of some flavoprotein enzymes in both the respiratory chain and the cytochrome P450 chain involved with steroid hydroxylations.



TABLE II  
Qualitative Analysis of Pooled Samples for Steroid Production and their Resultant DOC-to-Corticosterone Ratio

	Normal			Tumor		
	B*	DOC	DOC:B	B*	DOC	DOC:B
Control	-	-		-	-	
ACTH 1600 $\mu$ U/ml	+	-		-	-	
dbCamp 500 $\mu$ M	+	-		-	-	
Pregnenolone 80 $\mu$ g/ml	+	+	0.4	-	+	10.7
Progesterone 80 $\mu$ g/ml	+	+	2.5	-	+	37.0

Steroids separated by paper chromatography and analyzed with blue tetrazolium.

\* B, Corticosterone.

+, visible spot; -, no visible spot after treatment with the blue tetrazolium reagent.

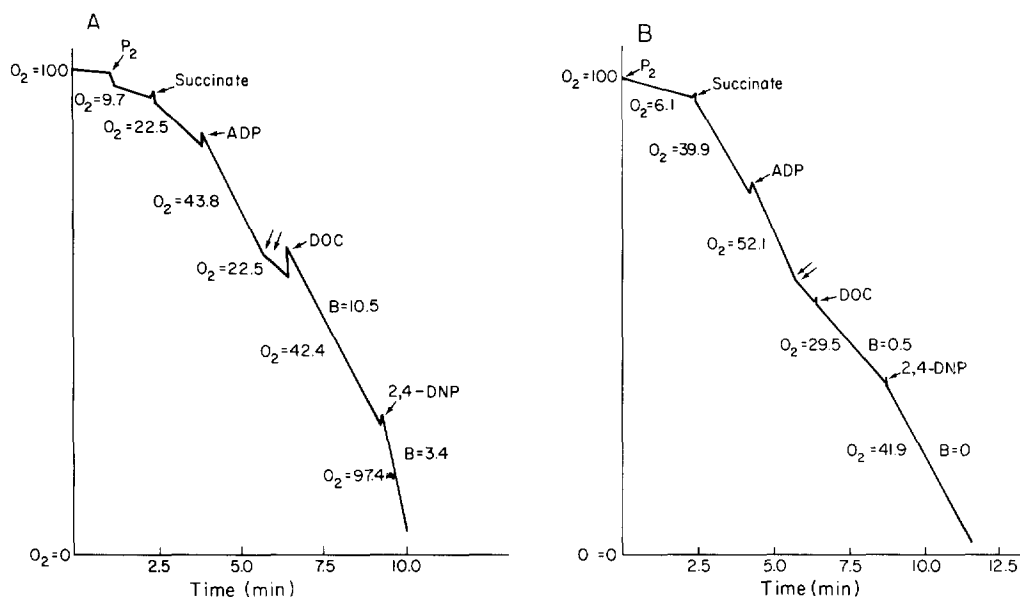


FIGURE 6 A and 6 B Traces of O<sub>2</sub> uptake measured polarographically at 37°C. The ordinate is a relative scale and represents 199 nmol O<sub>2</sub> in 1.00 ml buffer at 37°C. Approximately 0.70 mg mitochondrial protein was added to 1.00 ml buffer, pH 7.4, containing 14 mM Tris-HCl, 16 mM KCl, 16 mM NaCl, 55 mM nicotinamide, 250 mM sucrose, 1.5 mM inorganic phosphate (P<sub>i</sub>), and 0.15% bovine serum albumin (BSA) in final concentrations. 0.05 ml succinate (5  $\mu$ mol), 0.02 ml ADP (0.2  $\mu$ mol), 0.01 ml DOC (0.10  $\mu$ mol), and 0.01 ml 2,4-DNP (0.05  $\mu$ mol) were added as indicated above the traces by arrows. Corticosterone (B) and O<sub>2</sub> values above and below the lines, respectively, are the rates of corticosterone production and O<sub>2</sub> utilized expressed as nmol/mg mitochondrial protein/min incubation. Double arrows show points at which all ADP added has been utilized (38). P<sub>2</sub> indicates the addition of 0.10 ml of mitochondrial suspension. 6 A, control mitochondria; 6 B, tumor mitochondria.

**PYRUVATE LACTATE ACCUMULATION:** Table III compares the glycolytic response of tumor and normal adrenal cells to ACTH and dbCAMP. The doses of the two stimulating agents used are known to be maximally stimulating for normal adrenal cells (12, 20). Under these conditions the increases in pyruvate and lactate accumu-

lation due to both ACTH and dbCAMP were much smaller in tumor cells than in normal cells. This could not have been due to increased metabolism of pyruvate in the mitochondria by the enzyme pyruvate dehydrogenase, because in the presence of arsenite (which inhibits pyruvate dehydrogenase) the increases in pyruvate and lactate in

TABLE III  
Effect of ACTH, dbCAMP, and Arsenic on Pyruvate and Lactate Levels in Tumor and Normal Adrenal Cell Suspensions

Additions	Normal		Tumor	
	Pyruvate	Lactate	Pyruvate	Lactate
None	52.1 ± 6.4	392.2 ± 18.9	37.7 ± 1.3	154.7 ± 10.7
ACTH 2 mU/ml	152.6 ± 18.4 (100.5)	536.1 ± 56.2 (143.9)	54.9 ± 1.7 (19.2)	172.0 ± 5.0 (17.3)
dbCamp 500 μM	126.6 ± 12.0 (74.5)	534.8 ± 35.4 (142.6)	44.7 ± 1.3 (9.0)	179.3 ± 9.9 (24.6)
Arsenite 2.0 mM	220.9 ± 13.6 (168.8)	595.4 ± 11.2 (202.2)	88.2 ± 1.8 (52.5)	234.2 ± 5.3 (79.5)
ACTH 2 mU/ml + Arsenite 2 mM	229.6 ± 9.6 (177.5)	703.6 ± 17.2 (311.4)	83.1 ± 3.3 (47.4)	267.9 ± 11.2 (113.2)
dbCAMP 0.5 mM + Arsenite 2 mM	248.2 ± 14.2 (196.1)	620.5 ± 7.6 (228.3)	86.7 ± 2.9 (51.0)	252.4 ± 17.6 (97.7)

Each value expressed as nM/10<sup>6</sup> cells/2 h incubation time, is the mean ± SE (N = 4). Values in parenthesis denote the net increase over the incubated control. The incubations were carried out for 2 h at 37°C under 95% O<sub>2</sub>-5% CO<sub>2</sub>, and pyruvate and lactate were measured on the total incubate; i.e., cells + medium.

the tumor cells were again much lower than those in the normal adrenal cells.

#### DISCUSSION

In the normal adrenal gland ACTH stimulates membrane adenylyl cyclase which promotes the conversion of ATP to CAMP. This cyclic nucleotide, in turn, increases the synthesis of corticosterone by stimulating the conversion of cholesterol to pregnenolone in the following sequence: cholesterol <sup>1</sup> pregnenolone <sup>2</sup> progesterone <sup>3</sup> DOC <sup>4</sup> corticosterone. Reactions nos. 1 and 4 are exclusively associated with the mitochondria in adrenal cells, whereas reactions nos. 2 and 3 occur in the cell cytosol and microsomes, respectively (8, 9). This sequence is severely limited in the adrenocortical carcinoma 494 (1-5), apparently at a step(s) beyond the formation of CAMP (4). It is also limited in the adrenal from the tumor-bearing animal (3).

Ney et al. (3) showed that the in vitro corticosteroidogenesis of the adrenal from the tumor-bearing animal was stimulated by ACTH treatment. In the present study long-term ACTH treatment restored a normal ultrastructure to this adrenal. These results suggest that the limited steroidogenic capacity of this gland is directly related to reduced circulating levels of ACTH. Such a reduction could be in response to a negative feedback of high circulating levels of corticosterone as reported by Ney et al. (3) and also observed in our laboratory. Although the tumor on a per cell basis produces

very little corticosterone, apparently the great mass of the tumor tissue can produce relatively high circulating levels of corticosterone.

In the tumor the mitochondrial ultrastructure was markedly altered and the number of mitochondria per cell in the tumorous tissue was seen to be considerably lower than that normally found in adrenal fasciculata cells. In addition, large electron-lucid vesicles were quite often present within the cell. While the mitochondrial origin of these was not determined, the vesicles share some of the characteristics of the aminoglutethamide-induced abnormal mitochondria reported by Racela et al. (30).

In light of the altered crista structure and reduced corticosteroidogenesis of the tumor, it is possible that the vesicular conformation of rat adrenocortical mitochondrial cristae in some way enhances the ability of mitochondria to perform the associated steroidogenic conversion. If this is the case, then the lamellar cristae would not provide the optimum conditions for cholesterol side-chain cleavage and the 11β-hydroxylation reactions. In the tumor, our results and those of others (2, 31) showed that this latter enzymatic conversion was severely limited. However, Sharma and Brush (31) reported that the cells of this adrenocortical tumor are capable of converting cholesterol to DOC, indicating that tumor mitochondria possess the capacity to convert cholesterol to pregnenolone. If this is indeed the case, then it would certainly appear that the structural

alterations in the tumor mitochondria can be associated only with events leading to the  $11\beta$ -hydroxylation of DOC to corticosterone, and not with the conversion of cholesterol to pregnenolone. Further studies on this question would provide a better understanding of tumor and mitochondrial function.

The fact that in the tumor 21-hydroxylation of  $11\beta$ -OH-progesterone to corticosterone was altered to a much lesser extent than  $11\beta$ -hydroxylation of DOC to corticosterone gives additional evidence that the major defect in the adrenocortical carcinoma is mitochondrial in origin because the 21-hydroxylase enzyme is associated with the microsomal component of the fasciculata cells (8). The lack of corticosterone production from progesterone via  $11\beta$ -OH-progesterone can be explained by the fact that the conversion of progesterone to  $11\beta$ -OH-progesterone is dependent on the mitochondrial  $11\beta$ -hydroxylase system (32).

The oxygen studies also showed the tumor mitochondria to be grossly abnormal in terms of  $O_2$  utilization as a result of oxidation of Krebs-cycle substrates like isocitrate, malate, and  $\alpha$ -ketoglutarate. The finding that succinate was efficiently oxidized and indeed led to efficient coupling of oxidation to phosphorylation, makes it highly probable that these mitochondria have a full complement of succinate dehydrogenase enzyme, coenzyme(s) Q, and cytochromes required for  $O_2$  utilization and ATP production. The finding that the other above-mentioned Krebs-cycle substrates were not utilized for  $11\beta$ -hydroxylation of DOC or  $O_2$  uptake suggests that the tumor mitochondria are lacking in flavoprotein enzymes required for NADH and NADPH oxidation (respiratory chain NADH dehydrogenase and the NADPH dehydrogenase of the steroid hydroxylation chain). This is supported by the fact that exogenous NADPH additions did not lead to the usual conversion of DOC into corticosterone as seen in normal mitochondria (16). On the other hand, we cannot assume at the moment that the respective mitochondrial dehydrogenases responsible for substrate oxidation (e.g., malate dehydrogenase) are present or were functional in the tumor mitochondria. Experiments are being carried out to investigate these questions.

Abnormal metabolic function of the tumor tissue was also apparent by a lower activity of the Embden-Meyerhof pathway. This was manifested by a lower production of lactate and pyruvate in the incubated tumor cells as compared with that

found in normal adrenal cells. Whereas ACTH, dbCAMP, and arsenite additions led to the usual large accumulation of lactate and pyruvate (20) in the incubated normal cells, stimulation of glycolysis by ACTH and dbCAMP or inhibition of pyruvate oxidation by arsenite was much less in the tumor cells. As mentioned above, these results suggest an abnormal metabolic function in the tumor. However, we cannot categorically rule out that the decrease in Embden-Meyerhof pathway activity (as reflected by lowered pyruvate and lactate production) is due to glycolysis being channeled through other pathways, rather than being totally blocked. One of these pathways would involve the conversion of glyceraldehyde-3-phosphate to glycerol-3-phosphate via dihydroxyacetone phosphate. Should glycerol-3-phosphate be one of the main products of glucose metabolism in the tumor tissue besides pyruvate (or lactate), it is conceivable that the glycerophosphate shuttle provides a mechanism for transporting cytoplasmic reducing equivalents into the mitochondria. The product of this reaction, reduced FAD (the flavin coenzyme of mitochondrial glycerol-3-phosphate dehydrogenase), could then be oxidized via CoQ and the respiratory chain to yield ATP necessary for growth. It would be desirable to know whether ATP production in the tumor cell arises mainly as a result of glycolysis *per se* or via a mechanism utilizing the glycerophosphate shuttle. In any case, unlike the virtual absence of the cytosol NAD-linked glycerol-3-phosphate dehydrogenase in a wide variety of cancer tissues (33-35), we have found a normal degree of activity of NAD as well as a very high activity of the mitochondrial counterpart, FAD-linked enzyme, in tumor cells<sup>2</sup>. Further experiments are in progress to test the physiological significance of the above findings.

In conclusion, it is clear that the reduced corticosteroidogenesis of the Snell adrenocortical carcinoma is, at least in part, due to reduced mitochondrial function. This specifically involves a lowered capacity for  $11\beta$ -hydroxylation and could possibly be linked to a lack of or reduction in the certain mitochondrial enzymes necessary for normal oxidation. However, we do not wish to suggest that this is the only cellular function that could be altered in the tumor cell. A number of other steps

<sup>2</sup> F. G. Péron, A. Haksar, M. T. Lin, D. Kupfer, G. Kimmel, and W. Robidoux, Jr. Manuscript in preparation.

in steroidogenesis (e.g., CAMP-dependent protein kinase activation [36] or intracellular pooling of steroid precursors and products [37]), may be involved. The relative importance of these steps in steroidogenesis will be realized only with additional investigation into the function of both the normal and tumorous adrenal.

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