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MITOCHONDRIAL 11 β HYDROXYLATION AND ADRENAL MORPHOLOGY IN RATS BEARING A MAMMOTROPIC, ACTH-SECRETING PITUITARY TUMOR

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SUMMARY

The function and structure of adrenal mitochondria has been studied in rats bearing an ACTH, growth hormone and prolactin secreting tumor (MtT-F4). Basal levels of 11 β hydroxylation of deoxycorticosterone (DOC) in incubated mitochondria were higher in MtT-F4 than in controls ($P < 0{\cdot}001$). The addition of NADH and calcium to controls increased the conversion of DOC to corticosterone ($P < 0{\cdot}001$) but no change was observed in MtT-F4. With addition of NADPH plus calcium, the increase in controls was of a much higher magnitude than that produced in MtT-F4 ($P < 0{\cdot}05$). Calcium alone diminished the high levels of hydroxylation in MtT-F4. Measurement of mitochondrial swelling showed that in the presence of albumin and at 37°C swelling rates in MtT-F4 and controls were similar; however, large amplitude swelling developed in MtT-F4 when albumin was omitted and temperature was lowered to 25°C. ATPase activity was not different in mitochondria from both groups of rats either in the presence or absence of magnesium. The depressive effect of calcium, an uncoupler of oxidative phosphorylation, showed that some form of energy was needed for the high rate of basal hydroxylation in MtT-F4. The diminished response to the nucleotides indicated that probably both the P 450 hydroxylation chain and the transhydrogenase enzyme are impaired in MtT-F4. Electron microscopy of whole adrenal cortex showed a general appearance of hyperfunction. Mitochondria of zona glomerulosa contained inclusions of high electron density, and the outer membrane showed a multillaminar appearance. In zona fasciculata mitochondria showed normal structure, but different electronic density and individual variation in the number of cristae. In contrast, mitochondria of zona reticularis showed normal morphology.

INTRODUCTION

ACTH has a regulatory effect upon steroid hydroxylations and on metabolic functions of adrenal mitochondria[1–5]. In this regard, we have previously shown that in rats bearing transplants of a pituitary tumor that secretes mainly ACTH, in addition to growth hormone and prolactin (MtT-F4), there was an increase in basal levels of [11β]-hydroxylation in incubated adrenal mitochondria. Stimulation of this enzymatic system by the addition of Krebs cycle intermediates *in vitro* was considerably reduced in mitochondria from MtT-F4 rats, when compared to normal mitocondria[6].

The present studies were designed to further examine the adrenal mitochondrial function in rats bearing the MtT-F4. In addition, we have carried out

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Trival names of steroids refer to the following compounds: 18-hydroxycorticosterone = 11 β,20,21-trihydroxy-18,20-epoxy-4-pregnen-3-one; 18-hydroxydeoxycorticosterone = 20,21-dihydroxy-18,-20-epoxy-4-pregnen-3-one; pregnenolone = 3β-hydroxy-5-pregnen-20-one.

electron microscopic examinations to establish whether the biochemical changes found in this and in our former work could be correlated to morphological alterations in mitochondria and other cytoplasmic organelles of the rat adrenocortical cell.

MATERIALS AND METHODS

Fischer rats were kept under a controlled light schedule and fed standard rat chow and water *ad libitum*. In rats bearing the mammotropic pituitary tumor (Furth's MtT-F4) used in these experiments, the plasma levels of ACTH, growth hormone and prolactin were found to be 6000, 30 and 50 times higher than normal [7]. The tumor was transplanted into the hind legs of rats [8], and after 3–6 weeks of the tumor growth, the animals were killed by decapitation. The adrenals were removed, trimmed of fat and surrounding tissue and weighed. The adrenals from normal rats and tumor bearing rats were homogenized in 0·25 M sucrose. The homogenate was spun at 900 *g* for 10 min at 4°C in a B-20 International Equipment refrigerated centrifuge to sediment the nuclear fraction; the supernatant was centrifuged at 5000 *g* for 20 min to sediment the mitochondria. The mitochondrial pellet was washed once with sucrose and resedimented at the same speed. The particles were suspended in 50 mM Tris buffer pH 7·4 and aliquots were used for the experiments described below.

The measurement of [11β]-hydroxylation was carried out as described previously [6]. Briefly, the reaction mixture consisted of 50 mM Tris buffer pH 7·4, 5 mM $MgCl_2$, 0·1% bovine serum albumin (BSA), 50 μg deoxycorticosterone (DOC) and 0·2–0·3 mg mitocondrial protein in a total volume of 1 ml. Incubation was carried out at 37°C for 10 min in air; at the end of this period the medium containing the mitochondria was extracted with dichloromethane, the extract washed with 0·1 N NAOH and water, and the quantity of corticosterone present was determined by fluorescence in 65% sulfuric acid–ethanol in an Aminco-Bowman spectrofluorometer. Where indicated, NADPH (500 μg), NADH (500 μg), and calcium (11 mM) were added to the incubation. Results were expressed as μg of corticosterone formed/10 min/mg protein.

Rates of mitochondrial swelling were measured as follows: the mitochondrial suspension (0·2 mg protein per tube) was added to tubes containing 50 mM Tris buffer pH 7·4 and 5 mM $MgCl_2$. To one set of tubes was added 0·1% BSA and the mixture was incubated at 37°C; a second set did not receive BSA and was left standing at room temperature. In both cases total volume was 1 ml. At 0, 5, 15, 30 and 60 min the reaction mixture was transferred to cuvettes and read at 520 mμ in a Beckman DBG spectrophotometer. The expansion in mitochondrial volume after incubation produced a decrease in the absorption at 520 mμ [9] which was taken as a measure of swelling.

Adenosínetriphosphatase (ATPase, ATP phosphohydrolase, EC No. 3.6.1.4) activity of mitochondrial preparations was assayed in 50 mM Tris buffer pH 7·4, 0·1% BSA, 2·2 mM ATP and 0·2–0·3 mg of protein, total volume 1 ml. One set of tubes contained 5 mM $MgCl_2$ (medium A) whereas the other did not (medium B). The mixture was incubated with shaking in air at 37°C for 5 min and the incubation stopped by addition of 10% trichloroacetic acid; after centrifugation, the content of inorganic phosphate in the supernatant was determined by the method of Lowry and Lopez [10]. Dinitrophenol (DNP) was added at 0·1 mM. Results were expressed as μmoles Pi released in 5 min per mg protein.

Fixation of the tissue for electron microscopy was performed in the cold room (5°C) as follows: Immediately after decapitation of the animals, the body was perfused through the thoracic aorta with 2–3% glutaraldehyde in Milloning buffer pH 7·2–7·4[11]. The perfusion head was held 90–100 cm above the rat's body. Initially, the perfusion flow was kept at 2–5 ml/min for 2–5 min, followed by 1–3 ml/min for 30 min. The first 10 ml perfused were warmed at 25°C. At the end of the perfusion, the adrenals were removed, cut into small pieces and left for 7 h in a medium similar to the one used for perfusion. The material was then sequentially washed with buffered glutaraldehyde containing 6·5% glucose, postfixed in 1% osmium tetroxide and block stained in 2% aqueous uranyl acetate. The tissue was dehydrated in graded steps of ethanol and embedded in Epon 812[12]. Sections 500 Å thick were cut on a LKB model III Ultratome and mounted in naked grids, with the exception of the serial sections, which were mounted in bar ti grids on carbon membranes. The mounted sections were finally post-stained with lead citrate according to the method of Reynolds[13]. Examination of the tissue was carried out in a Siemens Elminskop I or a JEOL 100 B/4 operated at 80 kV.

The following chemicals were purchased from Sigma (St. Louis, Mo. U.S.A.): NADPH (tetrasodium salt), NADH (disodium salt), ATP (disodium salt) and DNP (grade III). All other chemicals employed were reagent grade.

RESULTS

1. *Rates of* [11β]-*hydroxylation.* The [11β]-hydroxylation of DOC to corticosterone in incubated mitochondrial preparations from normal and MtT-F4 adrenals was studied under basal conditions (i.e. no cofactors added) and after the addition of NADH plus calcium, NADPH plus calcium or calcium alone to the medium. The response to pyridine nucleotides was assessed in the presence of calcium since it is known that mitochondria shows poor permeability towards exogenous pyridine nucleotides unless a swelling agent like calcium is also present [14].

In confirmation of previous findings, basal levels of [11β]-hydroxylation were substantially higher in MtT-F4 than in controls (Figs. 1–3, column I vs III). When NADH plus calcium were added to the medium (Fig. 1), control mitochondria doubled the conversion of DOC to corticosterone (column I vs II); in contrast, Consequently, [11β]-hydroxylation in normal mitochondria additioned of NADPH plus calcium was significantly higher than in MtT-F4 studied under identical conditions (column II vs IV).

however, the increase induced in controls by NADPH (Fig. 2, column II vs I) was of a much higher magnitude than that observed in MtT-F4 (column IV vs III). NADH did not modify the hydroxylation rates in MtT-F4 (Fig. 1, column III vs IV). When NADPH plus calcium were used to stimulate hydroxylations, both control and MtT-F4 increased their basal levels of enzyme activity (Fig. 2);

Figure 3 shows the results obtained when the medium contained 11 mM calcium but no pyridine nucleotides. In MtT-F4 mitochondria, the effect of calcium was to diminish the basal levels of [11β]-hydroxylation (column IV vs III); contrarily, no change was observed in controls (column II vs I). Thus, in the presence of calcium the conversion of DOC to corticosterone in MtT-F4 compared to controls was not significantly different (Fig. 3, column II vs IV).

2. *Rates of mitochondrial swelling.* Figure 4 presents results of experiments in which the swelling rate was plotted vs incubation time from 0 to 60 min. In the

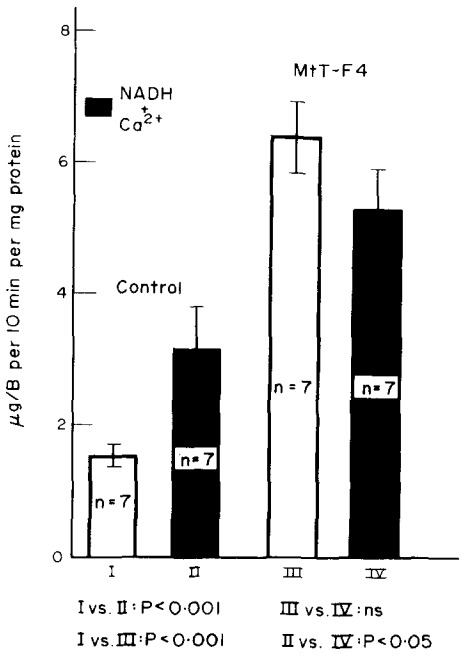

Fig. 1. 11β Hydroxylation of deoxycorticosterone in adrenal mitochondria: response to NADH and calcium. The assay system for [11β]-hydroxylation consisted of 50 mM Tris buffer pH 7·4, 5 mM $MgCl_2$, 0·1% BSA, 50 μg deoxycorticosterone and 0·2–0·3 mg protein, total volume 1 ml. After incubation for 10 min at 37°C, the resulting corticosterone was extracted with dichloromethane and measured by fluorimetry. The amount of NADH added was 500 μg/tube; calcium was 11 mM. Column I: basal levels of hydroxylation in controls; II: effect of NADH plus calcium in controls; III: basal levels of hydroxylation in MtT-F4; IV: effect of NADH plus calcium in MtT-F4. Results expressed as μg corticosterone (B) formed /10 min/mg protein (mean S.D.; n = number of observations).

figure, the ordinate expresses the difference in absorption at 520 mμ between normal and MtT-F4 mitochondria [− Δ OD 520 (c − MtT-F4)]. Thus, if rates of swelling were identical in both normal and MtT-F4 mitochondria, a horizontal straight line would be expected, whereas a curve with a negative slope would result if MtT-F4 mitochondria swelled more than the controls.

The upper curve in Fig. 4 shows the results obtained in a Tris-$MgCl_2$-BSA medium at 37°C: in this condition, little swelling was observed, although the shape of the curve suggests that MtT-F4 mitochondria swelled slightly more than the controls. When the incubation was performed at 20°C without albumin (lower curve) it is seen that a marked swelling of MtT-F4 adrenal mitochondria in the first 30 min bent the curve towards negative values. After 30 min, and up to 60 min, the rate of swelling in both types of subcellular particles was similar and a flat curve was obtained.

3. *Measurement of ATPase activity.* The results of determination of ATPase activity are shown in Table 1. Both normal and MtT-F4 mitochondria showed comparable enzyme activity (i.e. ~ 1 μmole Pi released in 5 min/mg protein) when incubated in a 50 mM Tris − 0·1% BSA in the presence of magnesium (Medium A). When magnesium ions were omitted (Medium B), the activity of the

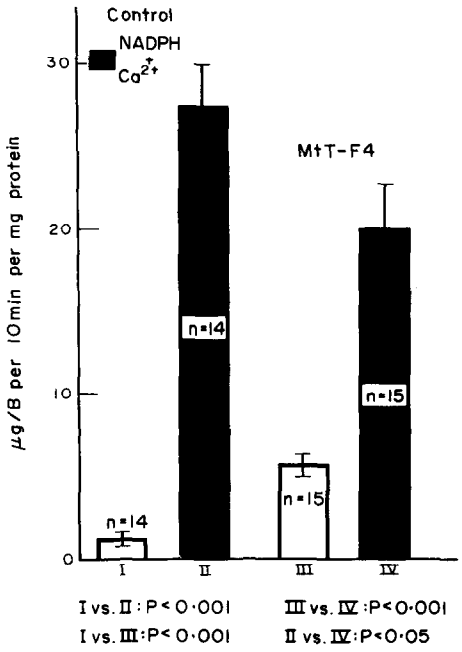

Fig. 2. 11β Hydroxylation of deoxycorticosterone in adrenal mitochondria: response to NADPH and calcium. Assay of [11β]-hydroxylation as described in Fig. 1. The amount of NADPH added was 500 μg/tube; calcium was 11 mM. Column I: basal levels of hydroxylation in controls; II: effect of NADPH plus calcium in controls; III: basal levels of hydroxylation in MtT-F4; IV: effect of NADPH plus calcium in MtT-F4. Results expressed as μg corticosterone (B) formed /10 min/mg protein (mean ± SD; n = number of observations).

enzyme showed a substantial decrease; as in the previous case, the levels obtained with normal mitochondria (0·32 μmoles Pi) were in the range of values produced by MtT-F4 mitochondria (0·51 μmoles Pi), the differences not being statistically significant ($P = 0·05$).

At 0·1 mM, DNP had no discernible effect whether tested in the presence or absence of Mg (Table 1). In further investigations, not shown in Table 1, DNP was also tested at 0·4 and 0·8 mM: in one experiment a slight stimulatory effect of DNP was obtained with both normal and MtT-F4 mitochondria, whereas in a second experiment DNP failed to activate ATPase activity in the two preparations.

4. *Electron microscopic findings in adrenals from tumor bearing rats.* In rats bearing MtT-F4, the cells of the outermost layer of the adrenal were present in clusters and identification of the zona glomerulosa was difficult by light microscopy. Nevertheless, the ultrastructure of nuclei and cytoplasm of these cells was not altered. Some mitochondria showed a typical spherical or elongated structure with parallel tubular cristae and electrodense matrix; however, abnormal mitochondria were also seen, presenting inclusions of high electron density, lost of cristae and multilaminar alterations in the outer membrane (Fig. 5). Smooth endoplasmic reticulum (SER) showed normal morphology and occupied a large portion of the cytoplasm. Rough endoplasmic reticulum was scarce although of normal appearance, and some free ribosomes were also seen (Fig. 5). Multilaminar bodies were observed near the mitochondria (Fig. 5).

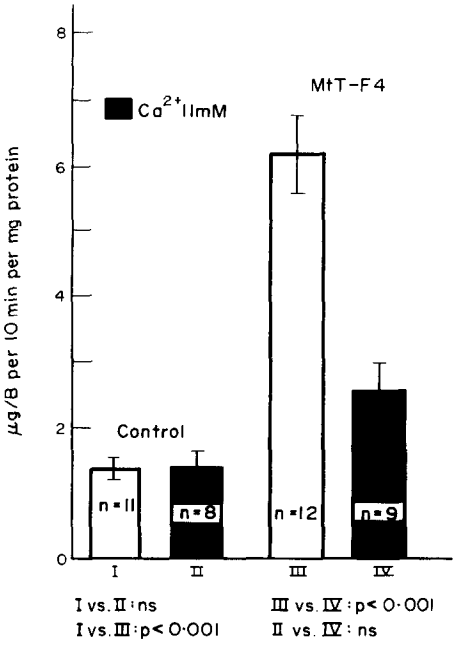

Fig. 3. 11β Hydroxylation of deoxycorticosterone in adrenal mitochondria: response to calcium (11 mM). Assay of [11β]-hydroxylation as described in Fig. 1. Column I: basal levels of hydroxylation in controls; II: effect of calcium in controls; III: basal levels of hydroxylation in MtT-F4; IV: effect of calcium in MtT-F4. Results expressed as μg corticosterone (B) formed /10 min/ mg protein (mean $\pm$S.D.; n = number of observations).

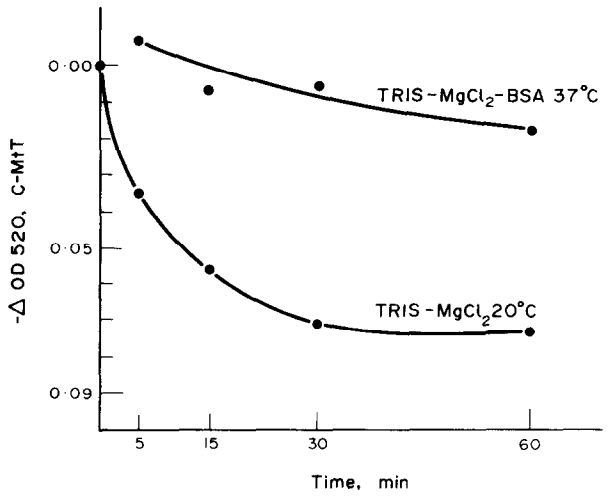

Fig. 4. Measurement of mitochondrial swelling: the mitochondrial suspension (0·2–0·3 mg protein) was incubated in 50 mM Tris buffer, 5 mM $MgCl_2$, 0·1% BSA at 37°C (upper curve) or in 50 mM Tris buffer and 5 mM $MgCl_2$ at 20°C (lower curve). At the times indicated in the abscissa, the o.d. at 520 mμ was registered in the spectrophotometer. The values for MtT-F4 adrenal mitochondria were subtracted from the controls; to ordinate in the figure expresses the difference in absorption at 520 mμ between normal and MtT-F4 mitochondria. The curves were drawn using the mean values from three separate experiments.

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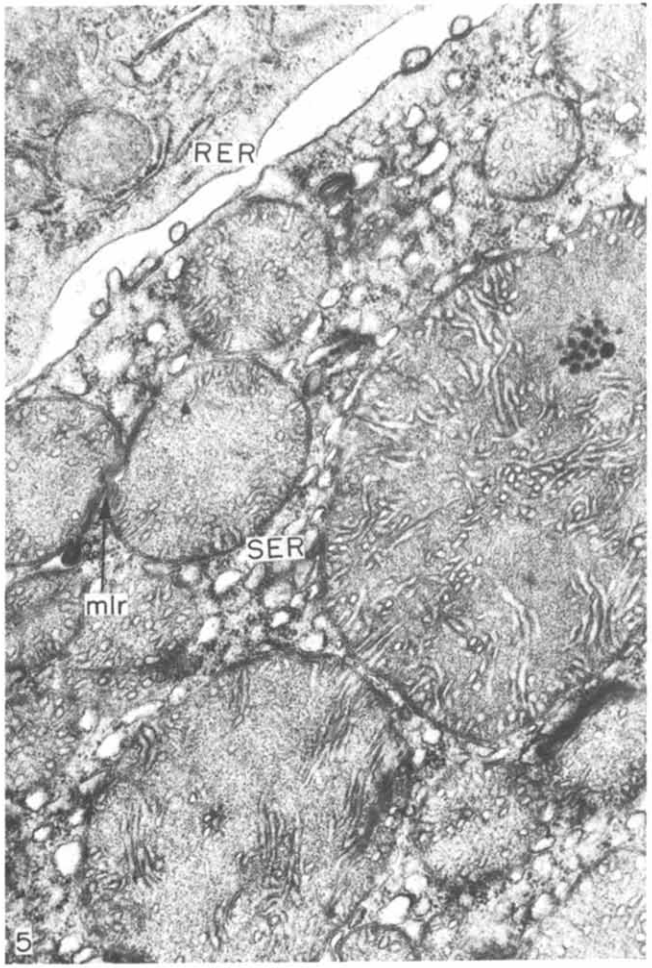

Fig. 5. Zona glomerulosa of adrenal gland from rat with MtT-F4. Cytoplasm showing mitochondria with electrodense matrix, inclusions of high electronic density and multilaminar (mlr) formation in the membranes. Rough (RER) and smooth (SER) endoplasmic reticulum appeared normal. magnification × 52,000.

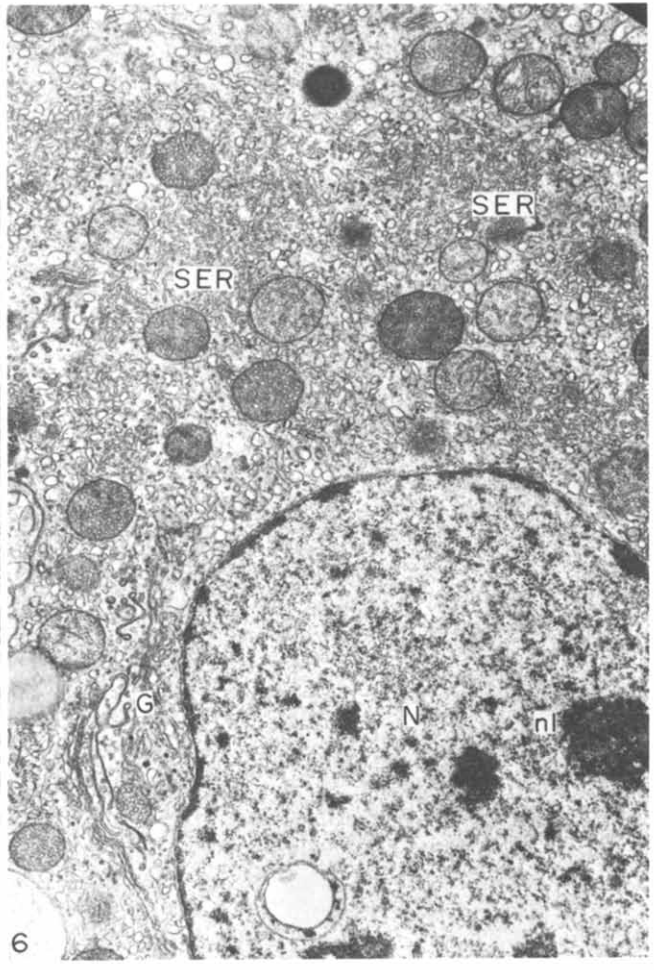

Fig. 6. A low power view of cell from zona fasciculata, of a rat with MtT-F4 showing hypertrophy of smooth endoplasmic reticulum (SER) and mitochondria of different structure and electronic density. Nucleus (N) with perinuclear chromatin and nucleolus (nl), and the Golgi apparatus (G) in juxtaposition are also seen. magnification × 10,000.

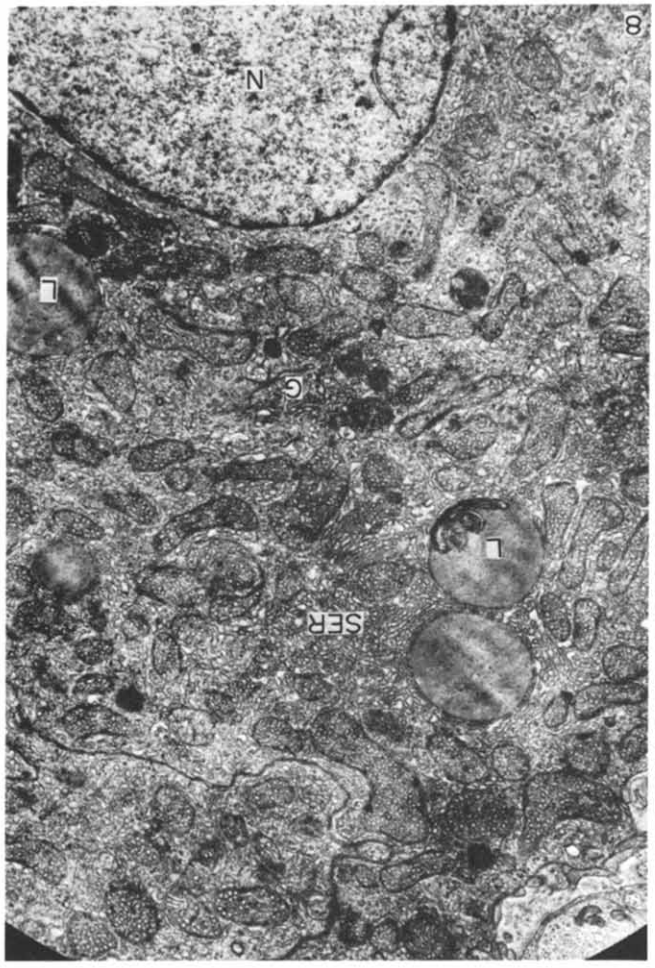

Fig. 7. Cell of the zona fasciculata, from rat bearing the MtT-F4. High power magnification of Golgi apparatus (G) in juxtaposition, showing electron dense material inside the dictiosomal cisternae and coated vesicles (cv). The smooth endoplasmic reticulum contained electron dense material inside its cisternae. Polyribosomes (pr) are also seen. magnification × 40,000.

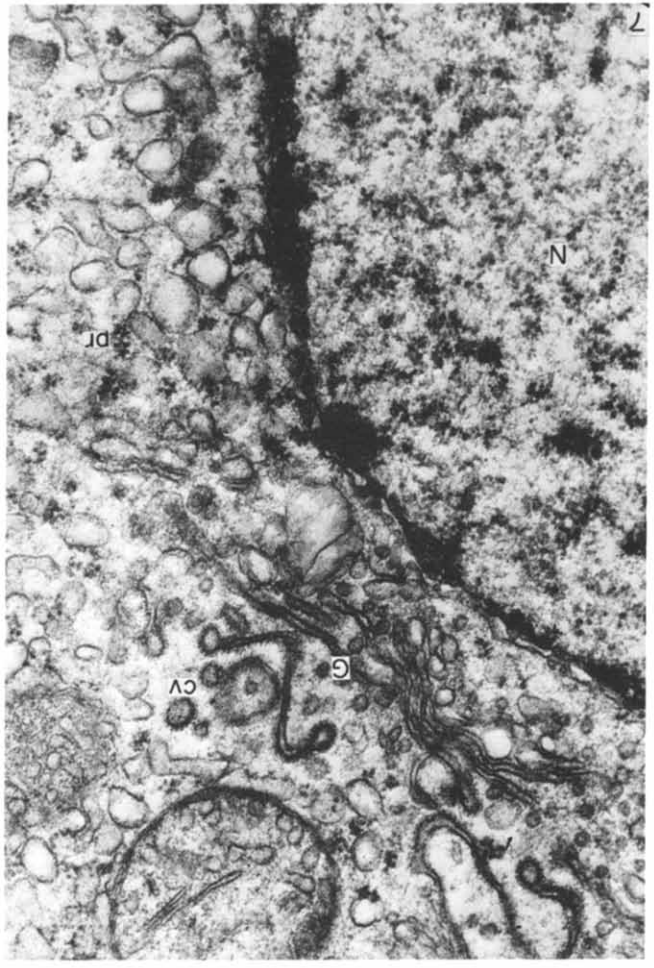

Fig. 8. Cytoplasm of zona reticularis cell from adrenal or rat with MtT-F4. Lipid inclusions (L), smooth endoplasmic reticulum (SER) and Golgi apparatus (G). magnification × 10,000.

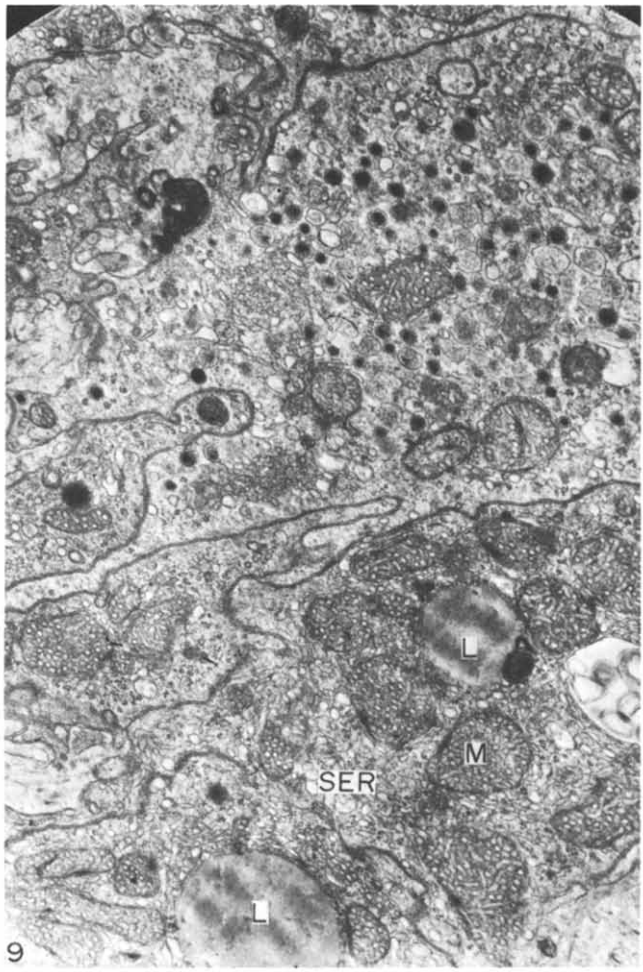

Fig. 9. Zona reticularis cell contacting the medulla of adrenal from rat with MtT-F4. Normal mitochondria (M), lipid inclusions (L), smooth endoplasmic reticulum (SER) and its cisternae. magnification × 21,000.

Table 1. Determination of adenosinetriphosphatase (ATPase) activity in adrenal mitochondria from normal rats and from rats bearing the MtT-F4 tumor

	n	Medium A	Medium A +DNP	Medium B	Medium B +DNP
			(μmoles Pi/5 min/mg protein)		
Controls	8	1·06 ± 0·23	1·09 ± 0·21	0·32 ± 0·07	0·43 ± 0·04
MtT-F4	9	1·03 ± 0·16	1·05 ± 0·18	0·51 ± 0·05	0·48 ± 0·04
p value		NS	NS	= 0·05	NS

Assay of ATPase: Medium A: 50 mM Tris buffer pH 7·4, 5 mM $MgCl_2$, 0·1% BSA, 2·2 mM ATP and 0·2–0·3 mg protein. Medium B: the same without $MgCl_2$. DNP: 0·1 mM. Incubation was carried out at 37°C for 5 min and stopped by 10% trichloroacetic acid; after centrifugation, the inorganic phosphate released into the supernatant was determined by the method of Lowry and Lopez [10].

The zona fasciculata was increased in size. The ultrastructure showed cells with hypertrophic SER forming interconnected cisternae and occupying most of the cytoplasmic space (Fig. 6). In the paranuclear zone of the cytoplasm, the SER presented a discrete electrodense material inside the cisternae (Fig. 7). Mitochondria were round with abundant tubulo–vesicular cristae which varied in number from one organelle to another; the electronic density was also not uniform, and according to this appearance there were light and dark mitochondria (Fig. 6). The cytoplasm contained a large number of ribo and polysomes. The Golgi apparatus was seen in a paranuclear position as well as in other areas of the cytoplasm; it was composed of the typical dictiosomal cisternae placed in parallel and contained a discrete electrodense material. Some of the Golgi vesicles presented a nap-like electron dense coating, both when bound to the cisternae or when free (Fig. 7). The cell nuclei contained abundant chromatin placed in a regular sequence although the inner side of the cariotheca showed condensations of chromatin. One or two nucleoli were frequently seen (Fig. 6). At high magnification it was possible to observe the classical membrane structure of the cariotheca as well as a condensed perinuclear space. The outer side of the membrane composing the paranuclear space was free of ribosomes. Nuclear pores were frequently observed (Fig. 7).

In contrast to the changes found in the mitochondria or SER of the zona glomerulosa and fasciculata, the cells of the zona reticularis appeared normal on electron microscopy. In all cases, the animals used for morphological studies were rats bearing the tumor for 5–6 weeks (Figs. 8 and 9).

DISCUSSION

Several explanations may be offered for the high basal levels of [11β]-hydroxylation found in rats with the MtT-F4. First, there might be an accumulation of pyridine nucleotides or Krebs cycle intermediates inside the abnormal organelle which support the conversion of DOC to corticosterone; second, the specific activity of the enzyme might be increased due to some modification in the molecule induced by ACTH, and third, there might be an increase in the synthesis of the enzyme. Which of these possibilities is valid is not known at the present time.

The findings on [11β]-hydroxylation supported by NADPH and calcium indicates that rats subjected to prolonged action of tumor ACTH show an im-

pairment in the response to overloading of the P 450 chain, inasmuch as NADPH is the first carrier of the hydroxylation chain[15]. Brownie *et al.*[16] have suggested that chronic ACTH action on the adrenal decreases cytochrome P 450.

When the hydroxylation activity was tested by addition of NADH plus calcium, the conversion of DOC to corticosterone in controls increased above basal levels, whereas no change was found in MtT-F4. NADH is not a direct hydrogen donor to the P 450 chain but it reduces intramitochondrial $NADP^+$ by an energy-dependent transhydrogenase, which suggests that this enzyme is faulty in MtT-F4. This would also account for the small response to the Krebs cycle intermediates found previously[6], in view of the fact that they support hydroxylations in the rat adrenal by making more NADH available to the transhydrogenase [17]. The observation that 11 mM calcium returned the high basal activity of the [11β]-hydroxylase to normal in MtT-F4 probably indicates that this reaction was maintained by some source of energy, the supply of which was blocked by the uncoupling effect of calcium.

Koritz and Kumar[18] have shown that mitochondria isolated from the adrenals of ACTH-treated rats swelled abnormally upon exposure to swelling agents. In our studies, when incubation conditions were identical to those used for measurement of [11β]-hydroxylation, the swelling rate of MtT-F4 mitochondria did not differ greatly from controls. This suggests that an altered permeability was not responsible for the changes in hydroxylation found in MtT-F4. The marked swelling developed by MtT-F4 mitochondria incubated at 20°C in the absence of albumin indicated an abnormal interplay of regulatory factors of mitochondrial swelling-contraction. Among these factors, it is known that free fatty acids which are released from membrane phospholipids, could cause expansion of mitochondrial volume in an albumin-free medium[19].

Cammer and Estabrook[20] reported in bovine adrenal mitochondria a deficiency in the activity of the enzymes required for ATP hydrolysis. We found no correlation between changes in [11β]-hydroxylation and activity of these enzymes. The lack of response to 0·1 mM DNP is partly in agreement with those authors. They found poor stimulation of the adrenal ATPase by DNP when compared to that of liver mitochondria.

The ultrastructural alterations (i.e. lose of cristae, multilaminar aspect of outer membrane) of the zona glomerulosa mitochondria may explain previous findings of decreased aldosterone and 18-hydroxycorticosterone synthesis and decreased aldosterone secretion in rats bearing the tumor[6]. We do not know if ACTH is directly responsible for these changes or whether the large amounts of DOC produced by these adrenals suppress the glomerulosa. In the fasciculata, the presence of mitochondria with different electronic density indicates the coexistence of a heterogeneous population, of normal and defficient organelles. Since we incubated mitochondria isolated from the whole gland, the particles sedimenting at 5000 *g* were probably composed of mitochondria from (a) abnormal glomerulosa, (b) normal and abnormal fasciculata, and (c) normal reticularis. It is possible that the diminished effect of the pyridine nucleotides was due to a lack of response of the altered organelles. On the other hand, the high basal levels of 11β hydroxylation as well as the increase in corticosterone and 18-hydroxydeoxycorticosterone synthesis observed in rats with the MtT-F4[6] may indicate that the remaining normal mitochondrial population was responsive to tumor ACTH.

Consistent with observations on ACTH-stimulated adrenals[21], we have seen

quantitative changes in the SER of the zona fasciculata. Since the enzymes responsible for the conversion of pregnenolone to progesterone (3 β-ol dehydrogenase isomerase) and of progesterone into DOC (21 hydroxylase) are located in the SER, the increase in amount of this cellular structure supports our assumption that the high secretion of DOC in rats bearing the MtT-F4 is due to an effect of ACTH on microsomal enzymes[6]. Regarding the alterations in the Golgi complex from these hyperplastic adrenals, there is no evidence to permit reasonable speculation on the role of the Golgi in steroidogenesis.

Nickerson *et al.*[22] have also studied the adrenal ultrastructure in rats bearing the MtT-F4. While we confirm their findings on the morphology of the SER, we were not able to show a uniform reduction in the number of cristae of mitochondria from zona fasciculata reported by them. Rather, we have observed the simultaneous coexistence of normal and abnormal mitochondria in the same preparation. The configuration of the latter was not as drastically disrupted as found by Nickerson *et al.*[22]. Moreover, the results obtained in MtT-F4 adrenals for ATPase activity and mitochondrial swelling (measured under conditions similar to those used for [11β]-hydroxylation) indicate that some function in these mitochondria were unchanged. Finally, the fixation of the adrenal tissue by perfusion might have contributed to the better preservation of mitochondria for the electron microscopic studies.

We should like to postulate that our findings in rats bearing the ACTH-secreting tumor are probably due to adrenal overstimulation[23]. Although the molecular mechanism by which ACTH induces these changes is not yet apparent, it has been demonstrated that ACTH controls the growth and division[24], protein, lipid and nuclei acid metabolism[3–5] of adrenal mitochondria. Further studies might elucidate the role of the tropic hormone in the last stages of the stress reaction.

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