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Investigations on Adrenocortical Mitochondria Turnover

I. Effect of Chronic Treatment with ACTH on the Size and Number of Rat Zona fasciculata Mitochondria*

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Summary. The effects of a chronic administration of ACTH (up to 36 consecutive days) on the mitochondria of the *zona fasciculata* of the rat adrenal cortex were investigated by stereologic techniques. It was found that, while the volume of the mitochondrial compartment significantly increases in relation to the duration of treatment, the size and number of mitochondria display a different pattern. Up to the 9th day of hormone treatment mitochondria significantly increase in volume, whereas their number per cell is only slightly increased. After 12 days of ACTH-treatment there is a tremendous increase in the number of organelles per cell, resulting in a significant decrease in their average volume. After 24 and 36 consecutive days of treatment the number of mitochondria per cell as well as their average volume both show a slight but significantly constant increase.

The hypothesis that ACTH controls the processes of growth and division of adrenal mitochondria is discussed in the light of evidence indicating that mitochondria contain a complete genetic apparatus largely independent of nuclear control.

Key words: Adrenal cortex — Mitochondria — ACTH — Stereology — Electron microscopy.

Introduction

Adrenal cortex mitochondria were the object of particular attention in numerous ultrastructural investigations (see Idelman, 1970, for review), since their unusual fine structure and their role in corticosteroidogenesis were described (Belt *et al.*, 1956).

Various lines of evidence indicate that adrenocortical mitochondrial morphology varies according to the experimental condition investigated. In fact, ACTH was found to induce reorganization of the tubulo-lamellar cristae of foetal, newborn (Kahri, 1966, 1968; Milner, 1973) and adult rat (Armato *et al.*, 1972; O'Hare *et al.*, 1973) as well as of human foetus (Milner *et al.*, 1970) adrenocortical cells cultured *in vitro* into a population of free vesicles, which are the typical feature of the completely differentiated highly functioning *zona fasciculata* mitochondria (Idelman, 1970; Nussdorfer, 1970a). Similar results were obtained by using 3', 5'-cyclic AMP (Milner, 1972; O'Hare *et al.*, 1973), which is thought

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to be involved in the intracellular mediation of ACTH-action (see Robinson *et al.*, 1971, for a review). Furthermore, analogous structural modifications were reported to occur in the mitochondria of hypophysectomized rat adrenocortical cells after ACTH administration (Sabatini *et al.*, 1962; Nishikawa *et al.*, 1963; Idelman, 1970). A significant decrease in the surface of mitochondrial cristae after hypophysectomy and its reversal after treatment with ACTH and cyclic nucleotides, were also demonstrated by stereologic procedures in both *zona glomerulosa* (Nussdorfer *et al.*, 1973, 1974) and *zona fasciculata* (Nussdorfer and Mazzocchi, 1972a, 1973a, b) of the rat adrenals.

In addition to maintain the normal morphology of the adrenocortical mitochondria inner membrane, ACTH was also found to induce increase in volume of these organelles (Yago *et al.*, 1971), and to reverse their hypophysectomy-induced decrease in number (Canick *et al.*, 1972). Previously we have also demonstrated that the chronic treatment with high doses of ACTH induces both increase in size and number of rat *zona fasciculata* mitochondria (Nussdorfer *et al.*, 1971), whereas the administration of corticosterone to intact rats was found to exert opposite effects (Nussdorfer and Mazzocchi, 1970), presumably by inhibiting the hypothalamo-hypophyseal axis.

However, it is to be stressed that mitochondrial proliferation was correlated to a decrease in the mitochondrial average volume both in regenerating rat adrenals (Yago *et al.*, 1972), and in the hypophysectomized rats after ACTH-treatment (Canick *et al.*, 1972), and that mitochondria hypertrophy or gigantism were depicted after hypophysectomy (Volk *et al.*, 1966; Sekiyama *et al.*, 1971; Sharawy *et al.*, 1973).

In conclusion, although there is a good deal of evidence indicating that ACTH controls the processes of growth and division of adrenocortical mitochondria, however, it is to be emphasized that the modalities of this action of ACTH are not still completely settled.

It therefore seemed worthwhile to investigate by stereologic and electron microscopic methods the effects of a very long ACTH administration (up to 36 consecutive days) on the mitochondria of the normal rat adrenal *zona fasciculata*.

Materials and Methods

Treatment of rats. Young adult male albino rats (Wistar-derived) weighing about 200–220 g. were used. Forty two animals were divided into 7 experimental groups, which received i.p. injections of 10 IU/Kg of ACTH (Sigma) for 3, 6, 9, 12, 24 and 36 consecutive days, respectively. Other 24 animals were given only i.p. injections of normal saline and served as a control.

All the rats were maintained on Purina Rat-Mouse chow and tap water *ad libitum*. The rats of each experimental group were killed at appropriate times together with 4 control animals, by cervical dislocation.

The animals and their respective right adrenal glands were weighed and the "relative adrenal weight" (*i.e.*, mg of adrenal per 100 g of body weight) was calculated. As the mean specific weight of adrenals (evaluated by pyknometry) does not show significant variations during the animal treatment, the "relative adrenal weight" is also an estimation of the "relative adrenal volume".

Electron Microscopic Techniques. Fragments of the left adrenal of each rat were fixed in 3% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.2) (Sabatini *et al.*, 1963), postfixed in 1% OsO_4 in 0.1 M phosphate buffer (pH 7.2) (Millonig, 1961), and embedded in an epoxy resin (Lockwood, 1964).

Thick sections were cut with LKB III ultramicrotome, stained with toluidine blue (Trump *et al.*, 1961), and observed with the light microscope in order to facilitate selection of the middle portion of the *zona fasciculata*. Thin sections were counterstained with lead hydroxyde (Karnovsky, 1961), and examined in the Hitachi HU-12 electron microscope.

Sampling Procedures. For each rat 3 tissue blocks, containing the *zona fasciculata*, were examined. Each of these blocks was sectioned at 0.5 μ for light microscopy and at 600–800 Å for electron microscopy. At low magnification, 2 technically perfect series of thick and thin sections for each block were selected. Three light micrographs were recorded from each series of thick sections at a magnification of 1250 (18 light micrographs for each animal). From each series of thin sections, 6 electron micrographs at a final magnification of 18000 (36 electron micrographs for each rat), and 4 electron micrographs at a final magnification of 36000 (24 for each rat) were recorded for stereologic assessments.

Stereologic Procedures. The percentage of cell volume occupied by the nuclei, mitochondria, lipid droplets, and "membrane space" (*i.e.*, the cellular space occupied by the membranes of endoplasmic reticulum, including Golgi apparatus) (Loud, 1962) was estimated by the method of "differential point counting" (Weibel, 1969) on the prints at a magnification of 18000. The concentration of endoplasmic reticulum and mitochondrial cristae (*i.e.*, μ^2 of endoplasmic reticulum membranes and mitochondrial cristae per μ^3 of cell) was evaluated by the "crossing method" (Loud, 1962) on the prints at a magnification of 36000. The absolute amount (volume and surface) of the various organelles in the individual adrenocortical cell was obtained by determining the average cell volume on the light micrographs at a magnification of 1250, with the same indirect approach we have described previously (Nussdorfer, 1970b).

The single mitochondrion average volume and the number of mitochondria per cell were evaluated by the following method, based on these three assumptions: (1) adrenal mitochondria are randomly distributed through the cytoplasm; (2) the size distribution of mitochondria is continuous throughout each zone; (3) the shape of mitochondria in the parenchymal cells of the rat *zona fasciculata* is essentially spherical, since ultrathin sections of these organelles show, circular or elliptical profiles with an axial ratio to unity (average value: 1.133).

On the prints at a magnification of 18000 the % of tissue explored (about 120 μ^2 per electron micrograph) occupied by the parenchymal cells was evaluated by differential point counting. On the same prints the number of mitochondrial sections per mm^2 of parenchymal tissue surface (Na) and the diameter distribution of mitochondrial profiles were determined. When elliptical profiles were encountered the arithmetic means of the major and minor axes were taken as the circle diameter. Applying Schwartz's transformation for spheres (1934) the actual mean diameter of mitochondria ($\overline{D}_M$) was calculated from the diameter distribution of the mitochondrial profiles. The number of mitochondria per mm^3 of tissue (Nv) was evaluated by De Hoff's-Rhines' method (De Hoff *et al.*, 1961):

$$Nv = Na/(\overline{D}_M)$$

The number of mitochondria per cell (Nc) was calculated by the following formula:

$$Nc = Nv \cdot Vc, \text{ where Vc is the average volume of cells}$$

expressed in mm^3. By dividing the mean volume of the mitochondrial compartment per Nc, the average volume of the single mitochondrion was obtained. From table 3, it is apparent that this value fits well with the volume of a sphere of diameter $\overline{D}_M$.

Statistical Evaluation of the Data. The data obtained from each rat were averaged and the standard deviation from the mean was calculated. The mean values for individual animals were then averaged per experimental group and the standard error was determined. The degree of variability in the intraanimal determinations as compared to the intra-group means by the analysis of variance was found to be not significant ($p > 0.6$–0.9). The statistical comparison of group-averages of the specific parameters was performed by means of Student's t-test. The difference between two mean values was considered significant if the probability of error (p) was found to be less than 0.05. The average values of the morphometric parameters of the 7 control groups were pooled, since the degree of variability in the intra-group determinations as compared to the inter-group means by the analysis of variance was found to be not significant ($p > 0.7$).

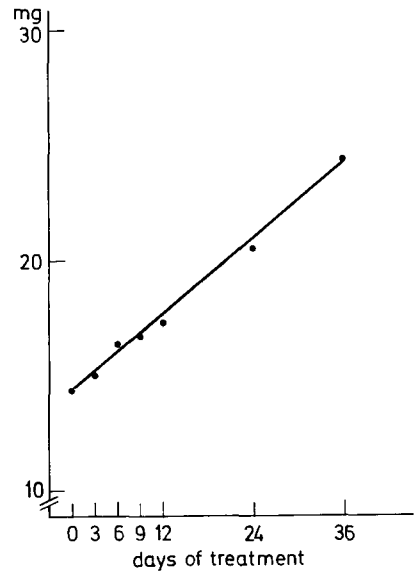


Fig. 1. Changes in relative adrenal weight as function of the number of days of ACTH-treatment

Table 1. Relative adrenal weight of ACTH-treated rats

Duration of ACTH treatment	Relative adrenal weight (mg/100 g b.w.)	Standard error (± SE)	Increase in % (Δ)	Level of significance (p)
Control (24)	14.41	1.48	—	—
3 Days (6)	15.06	1.54	4.5	N.S.
6 days (6)	16.32	1.66	13.3	< 0.01
9 days (6)	16.80	1.65	16.6	< 0.01
12 days (6)	17.39	1.70	20.7	< 0.01
24 days (6)	20.62	2.26	43.1	< 0.01
36 days (6)	24.43	2.73	69.5	< 0.01

Animals were treated as described in the text. The number of animals in each group is indicated in parentheses. N. S., not significant.

Results

After ACTH-treatment the "relative adrenal weight" is found to be significantly greater than that of the control animals (Table 1). From Fig. 1, it appears that this parameter increases linearly with the duration of treatment.

The volume of cells of the *zona fasciculata* increases significantly in the treated rats. Also the volume of adrenocortical nuclei displays an analogous behaviour, although the percentual increase of this parameter is quite lesser than that of the cell volume (Table 2). The nuclei of the treated animals do not show any

Table 2. Synopsis of morphometric parameters of adrenocortical cells of ACTH-treated rats

Duration of ACTH Treatment	Volume of cells	Volume of Nuclei	Volume of Lipid Compartment	Membrane space	Surface of SER
	μ^3	μ^3	μ^3	μ^3	μ^2
Control (24)	1710.2±205.2	122,6±13.5	140.9±15.6	842.2± 91.8	8590.4±1030.8
3 Days (6)	1941.1±213.5	129.6±13.7	139.1±15.7	953.4±104.8	9724.7±1089.0
Δ%	13.5	5.7	—1.3	13.2	13.2
p	<0.01	N.S.	N.S.	<0.01	<0.01
6 days (6)	2185.6±262.3	145.6±16.4	152.7±18.2	1054.2±125.5	11174.5±1407.9
Δ%	27.8	18.7	8.4	25.2	30.1
p	<0.01	<0.01	<0.02	<0.01	<0.01
9 days (6)	2369.5±281.9	146.4±17.6	204.7±26.5	1118.4±139.8	12079.5±1453.3
Δ%	38.5	19.4	45.1	40.6	40.6
p	<0.01	<0.01	<0.01	<0.01	<0.01
12 days (6)	2331.2±283.5	135.3±14.3	195.2±24.4	1109.9±127.9	11531.1±1382.7
Δ%	34.6	2.2	38.5	31.8	34.2
p	<0.01	<0.01	<0.01	<0.01	<0.01
24 days (6)	2600.3±364.0	155.2±17.5	234.8±31.2	1234.1±149.5	13451.6±1748.6
Δ%	52.0	26.6	66.6	46.5	56.6
p	<0.01	<0.01	<0.01	<0.01	<0.01
36 days (6)	2923.7±394.6	160.1±18.2	284.9±35.3	1395.2±156.3	14649.6±1919.2
Δ%	70.9	30.6	102.2	65.6	70.5
p	<0.01	<0.01	<0.01	<0.01	<0.01

Animals were treated as described in the text. The number of animals in each group is indicated in parentheses. Δ%, percentual change in respect to the control group; p, level of significance of the difference between the control and ACTH-treated groups; N.S., not significant

qualitative ultrastructural variations. Mitoses are extremely rare both in the control and in 3, 6 days ACTH-treated rats. After 12 days of hormone administration mitoses seem to be increased in number.

Compared with the controls, adrenocortical cells of ACTH-treated rats display only slight qualitative ultrastructural variations, that are completely analogous to those we have previously described (Nussdorfer *et al.*, 1971; Nussdorfer and Mazzocchi, 1972a): the Golgi apparatus shows an hypertrophic appearance and contains numerous vesicles many of which are coated; large portions of the cytoplasm are filled by a meshwork of closely packed interconnected tubules of the smooth endoplasmic reticulum (SER); numerous are the *caveolae* or coated pits at the plasma membrane; an elaborate microvillous pattern protrudes into the subendothelial space.

From the quantitative viewpoint (Tables 2, 3), the volume of the mitochondrial and lipid compartments and "membrane space", as well as the surface of SER membranes and mitochondrial cristae are found to be significantly increased in relation to the duration of ACTH-treatment. If the morphometric parameters are plotted on a graph as function of the number of days of hormone administration, it may be noted that, at variance with the "relative adrenal weight",

Table 3. Synopsis of morphometric parameters of adrenocortical mitochondria of ACTH-treated rats

Duration of ACTH treatment	Volume of mitochondrial compartment μ^3	Surface of mitochondrial cristae μ^2	Diameter of mitochondria μ	Volume of mitochondria μ^3	Number of mitochondria per cell
Control (24)	601.4± 73.9	4330.1± 477.1	0.956±0.078	0.494±0.040 [0.457]	1215.9±128.5
3 days (6)	718.6± 80.5	5317.6±600.8	0.992±0.084	0.552±0.044 [0.511]	1299.6±130.2
Δ%	19.5	22.8	3.8	11.7	6.8
p	<0.01	<0.01	N.S.	<0.01	<0.02
6 days (6)	856.6±102.8	6424.5± 770.8	1.051±0.084	0.657±0.058 [0.607]	1303.4±133.1
Δ%	42.4	48.4	9.9	32.9	7.2
p	<0.01	<0.01	<0.01	<0.01	<0.02
9 days (6)	903.4±117.4	6752.2± 871.0	1.112±0.089	0.703±0.064 [0.720]	1284.5±128.5
Δ%	50.2	55.9	16.3	42.3	5.6
p	<0.01	<0.01	<0.01	<0.01	<0.05
12 days (6)	891.2± 99.7	6326.1± 765.1	0.921±0.075	0.449±0.031 [0.409]	1981.9±201.4
Δ%	48.2	46.1	—3.7	—9.2	62.9
p	<0.01	<0.01	N.S.	<0.01	<0.01
24 days (6)	974.6±128.6	7012.8± 911.6	0.944±0.078	0.462±0.038 [0.441]	2106.6±214.5
Δ%	62.1	61.9	—1.3	—6.5	73.2
p	<0.01	<0.01	N.S.	<0.01	<0.01
36 days (6)	1081.7±129.1	7896.4±1026.2	0.981±0.079	0.502±0.041 [0.493]	2152.2±228.5
Δ%	79.8	82.4	2.6	1.7	76.9
p	<0.01	<0.01	N.S.	N.S.	<0.01

Animals were treated as described in the text. The number of animals in each group is indicated in parentheses. The numbers in square brackets indicate the volume of a sphere of diameter $\overline{D}_M$. Δ%, percentual change in respect to the control group; p, level of significance of the difference between the control and ACTH-treated groups; N.S., not significant.

their increase is not linearly related to the duration of treatment (Figs. 2, 3). In fact, about 54–63% of the total increment has already occurred by the 9th day of ACTH administration.

The morphometric parameters for the mitochondria of control and ACTH-treated groups are shown in table 3. It is evident that, while the volume of the mitochondrial compartment shows a constant significant increase with the duration of the treatment, the average volume (and diameter) of single mitochondrion does not display an analogous pattern. In fact, mitochondria significantly increase in volume up to the 9th day of hormone treatment, whereas after 12 days of ACTH-administration they show a dramatic volumetric decrease,

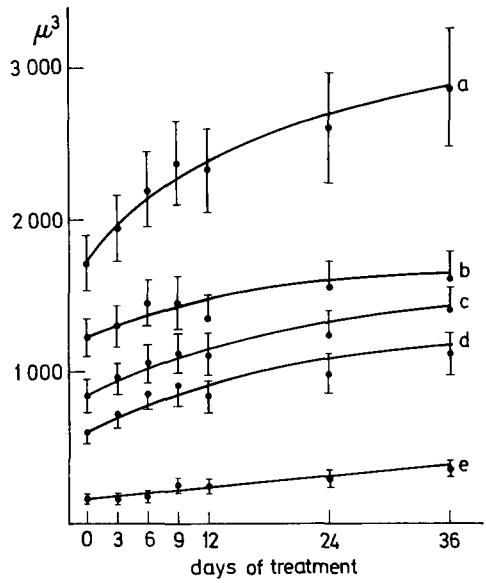


Fig. 2. Changes in the various morphometric parameters of rat adrenocortical cells as function of the number of days of ACTH-treatment. Standard errors are indicated. *a*, volume of cells; *b*, volume of nuclei (×10); *c*, membrane space; *d*, volume of mitochondrial compartment; *e*, volume of lipid compartment

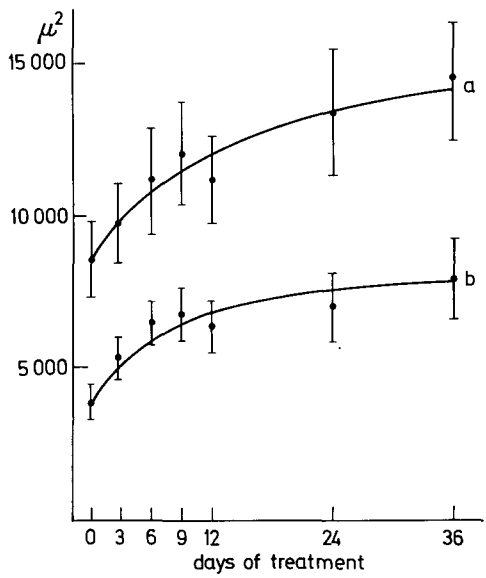
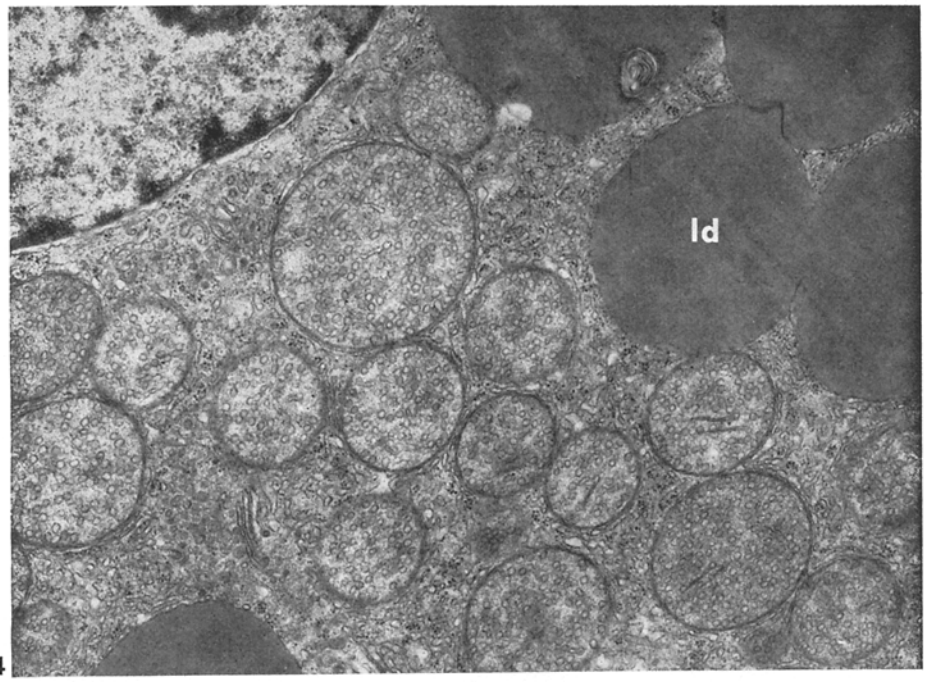


Fig. 3. Changes in surface of SER (*a*) and mitochondrial cristae (*b*) of rat adrenocortical cells as function of the number of days of ACTH-treatment. Standard errors are indicated

which is significant as compared to the average volume of controls. The average mitochondrial volume again increases (significantly in the respect to the value found in 12 days ACTH-treated rats) after 24 and 36 days of hormonal treatment.



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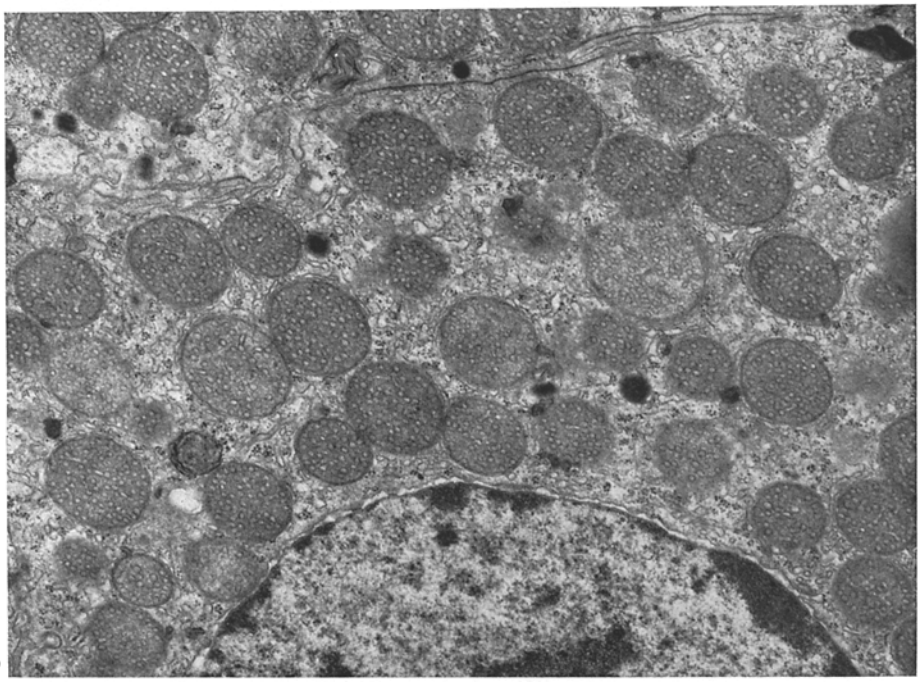

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Fig. 4. Cortical cell from the rat *zona fasciculata*. After 9 days of ACTH-treatment, mitochondria display an hypertrophic appearance. Lipid droplets (*ld*) are numerous. ×16000

Fig. 5. Cortical cells from the rat *zona fasciculata*. At the 12th day of ACTH administration, mitochondria are more numerous and seem to be decreased in size. ×16000

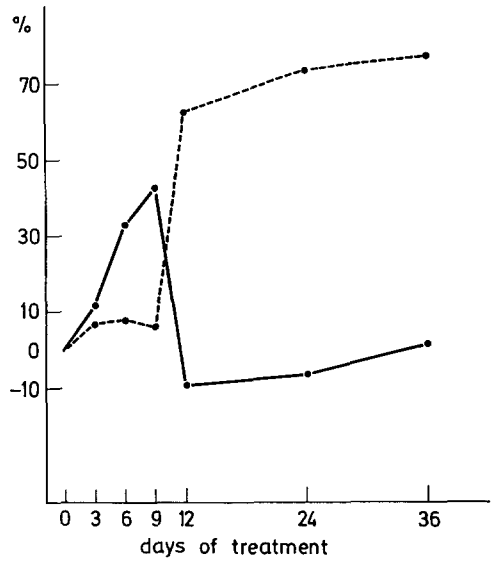

Fig. 6. Graph showing the % changes in the average volume of single mitochondrion (continuous line), and in number of mitochondria per cell (hatched line) as function of the number of days of ACTH-treatment

The number of mitochondria per cell is slightly, but significantly increased in the 3, 6 and 9 days ACTH-administered rats (about 6.5%), while it shows a very conspicuous increase after 12 days of treatment (63%). Difference in mitochondrial volume and number can also roughly be noted in the electron micrographs (Figs. 4, 5). The difference between the behaviour of two parameters (volume and number of mitochondria), is clearly demonstrated in Fig. 6, in which it can be seen that at the 12th day of ACTH treatment there is a cross between the curve of mitochondrial volume and that of number of organelles per cell.

It must be noted that in the electron micrographs of adrenocortical cells from 9 days ACTH-injected rats there is an unusual number of images suggesting mitochondrial division (Fig. 7).

Discussion

The effect of ACTH on the adrenal cortex has been widely investigated at optical level (for review, see Deane, 1962), and the bulk of evidence indicates that ACTH induces hypertrophy of the adrenal gland principally by enlargment of the *zona fasciculata-reticularis*. However, there is not complete agreement as to the mechanism of this hypertrophy. In the present investigation we have shown that the increase in the "relative adrenal weight" is linearly related to the duration of hormone treatment, while the rate of increment in the cell volume significantly falls with the number of days of ACTH administration. This suggests that the ACTH-induced adrenal hypertrophy is not only due to cell hypertrophy, but also to cell proliferation. The increased number of mitoses found in the *zona fasciculata* of 9–12 days ACTH-treated rats, and the increased DNA content

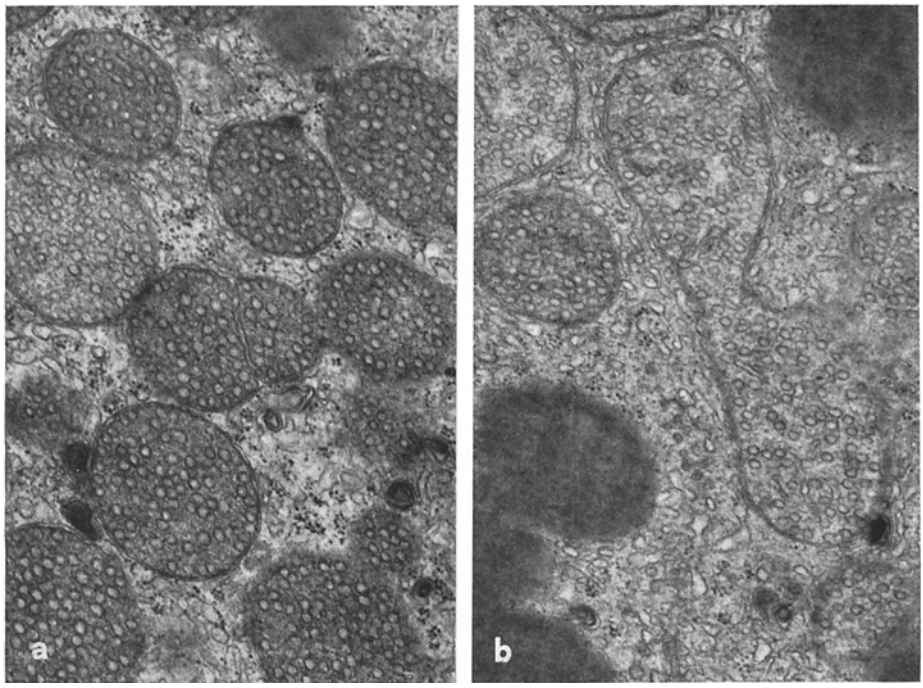

Fig. 7a and b. In 9 days ACTH-treated adrenocortical cells an unusual number of images suggesting mitochondrial division are noted: some mitochondria contain elongated areas of the inner membrane which seem to form a complete partition (a), some others show close constrictions at their middle portion (b). ×25000

of intact and hypophysectomized rat adrenals after ACTH administration (Farese, 1968; Pfeiffer *et al.*, 1972) lend support to this possibility.

Our morphometric data concerning the effect of ACTH on the various subcellular compartments of rat adrenal *zona fasciculata*, are largely analogous to those reported in earlier contributions (Nussdorfer *et al.*, 1971; Nussdorfer and Mazzocchi, 1972a), and, therefore, will be discussed here only briefly.

Stereologic findings show that the most conspicuous ACTH-induced structural changes involve increase in volume of mitochondrial compartment and "membrane space", as well as in surface of SER and mitochondrial cristae. This fits well with the biochemical evidence showing that the enzymes of the steroid-synthesis are located in both these subcellular organelles (see for review, Dorfman *et al.*, 1965; Tamaoki, 1973). Since ACTH, 3', 5'-cyclic AMP and hypophysectomy were found to induce changes in activity of some of these enzymes as well as in the mitochondrial content of cytochrome P-450 both *in vivo* (Griffiths *et al.*, 1967; Kimura, 1969; De Nicola *et al.*, 1973; De Nicola, 1973; Pfeiffer *et al.*, 1972), and *in vitro* (Kowal, 1967, 1969), we can reasonably assume that the increase in surface of SER membranes and mitochondrial cristae are associated with a corresponding quantitative variation of the enzymes of steroid-synthesis contained in it.

On the ground of evidence indicating that ACTH and 3', 5'-cyclic AMP enhance the nuclear (Farese, 1968; McKerns, 1968; Nussdorfer and Mazzocchi, 1971a, 1972b) and mitochondrial (Nussdorfer and Mazzocchi, 1971a, 1972b)

DNA-dependent RNA synthesis by adrenocortical cells, it is possible to hypothesize that the mechanism underlying the trophic action of ACTH and its physiologic intracellular mediator on the rat *zona fasciculata* involves an integrated stimulation of both nuclear and mitochondrial protein synthesis at the transcription level. The ACTH-induced increase in volume of adrenocortical nuclei also agrees with an enhanced nuclear function (Merkle, 1968; Mitro *et al.*, 1970).

The significant increase in volume of lipid compartment after ACTH-treatment, already previously described in chronically stimulated adrenocortical cells (Magalhães *et al.*, 1969; Nussdorfer *et al.*, 1971; Nussdorfer and Mazzocchi, 1972a), could be correlated according to Christensen (1965) with the increase in SER membranes. In fact, it was found that in SER are located the enzymes involved in the synthesis of cholesterol (Dorfman *et al.*, 1965), which is an important component of the lipid droplets (Moses *et al.*, 1969; Sand *et al.*, 1972; Frühling *et al.*, 1973), and ACTH was reported to stimulate the synthesis of cholesterol from acetate and glucose (Dexter *et al.*, 1967; Kowal, 1969; Sharma *et al.*, 1972). However, in the rat only 10–15% of cholesterol is synthesized in adrenocortical cells (Ichii *et al.*, 1967), and, therefore, the possibility subsists that, according to previous contributions (Dexter *et al.*, 1970; Armato *et al.*, 1972), the increase in volume of lipid compartment can be due, at least in part, to the ACTH-stimulated uptake of cholesterol from the bloodstream.

As to the hypertrophy of the Golgi apparatus in ACTH-treated rat adrenocortical cells, it should be recalled that, even if a specific role of this organelle in corticosteroid synthesis and secretion is not known at present, a large body of data indicates that the Golgi apparatus may be integral to steroidogenesis (Nussdorfer *et al.*, 1971, 1973; Nussdorfer and Mazzocchi, 1972a; for further details on this topic, see Christensen *et al.*, 1969). However, the possibility that the Golgi apparatus hypertrophy can be simply due to the general stimulation of adrenocortical cell metabolism cannot be disregarded at present. Also the number of *microvilli* and *caveolae* at the plasma membrane seems to be closely related to the functional activity of adrenocortical cells (Christensen *et al.*, 1969; Nussdorfer *et al.*, 1971, 1973; Shelton *et al.*, 1971; Nussdorfer and Mazzocchi, 1972a).

In conclusion it is possible to assume that these structural changes represent the morphologic expression of the ACTH-induced enhancement of the growth and steroidogenic capacity of the *zona fasciculata* cells of the rat adrenal cortex.

In addition to regulate the growth of adrenocortical cells *in toto*, ACTH is found to exert striking effects on the size and number of adrenocortical mitochondria, which are dependent on the duration of hormonal treatment.

In partial disagreement with the findings of previous investigations (Nussdorfer *et al.*, 1971), in which, however, doses of ACTH 3 times higher than those used in the present study were administered, up to the 9th day of treatment the major effect of ACTH seems to consist in the mitochondria hypertrophy, which involves increase in surface of both the outer and inner membranes. In fact, the ACTH-induced increase in volume of mitochondrial compartment is about 80% due to the increase in the mitochondrion average volume, and only 20% due to the increase in number of organelles per cell.

It is now current knowledge that mitochondria contain a complete genetic apparatus which is largely independent of nuclear control (for review, see Roodyn

et al., 1968; Ashwell *et al.*, 1970). Mitochondrial protein synthesis seems to be almost exclusively concerned with the structural proteins of the inner membrane (Clark-Walker *et al.*, 1967; Neupert *et al.*, 1967, 1968; Yu *et al.*, 1968; Tzagoloff *et al.*, 1973, for comprehensive review), while the outer membrane synthesis is directed by the nuclear genetic apparatus (Clark-Walker *et al.*, 1967; Neupert *et al.*, 1967, 1968; Yu *et al.*, 1968). On the ground of evidence showing that 12 hs after ACTH administration the incorporation of 3H-uridine into adrenal mitochondrial compartment is significantly enhanced (Nussdorfer and Mazzocchi, 1971a), and that selective inhibitors of the mitochondrial protein synthesis (chloramphenicol and ethidium bromide, Kahri, 1970; Milner, 1972a), as well as corticosterone (Kahri, 1973), which was found to inhibit directly adrenocortical mitochondria RNA synthesis (Nussdorfer and Mazzocchi, 1971b), inhibit the ACTH-induced differentiation of mitochondria in rat adrenal cells in tissue culture, it is possible to suggest that the mechanism of the ACTH-induced mitochondrial hypertrophy might involve the integrated stimulation of both nuclear and mitochondrial protein synthesis.

At the 12th day of hormonal stimulation a tremendous increase in the number of mitochondria per cell is found. Since it now appears well demonstrated that mitochondria are capable of replication by division (for extensive review concerning this phenomenon, see Tandler *et al.*, 1969), we could assume that in the presence of ACTH mitochondria increase in size by growth until their diameter is larger than 1–1.2 μ, and then they divide and begin the growth cycle again. This interpretation might well explain the net decrease in the average volume of mitochondria found at this experimental time.

These findings could also suggest that during the first 9 days of ACTH treatment mitochondrial growth is coupled with an accelerated synthesis of mitochondrial DNA, resulting in the duplication of the genetic apparatus in hypertrophied mitochondria. Previous data from this laboratory have shown that adrenocortical mitochondrial population synthesizes DNA at a constant rate independent of the cellular stage (Nussdorfer and Mazzocchi, 1971a), because each individual mitochondrion possesses a genetic duplication cycle, which is not in phase with that of other mitochondria (Guttes *et al.*, 1967; Roodyn *et al.*, 1968). On this ground it is conceivable that ACTH by accelerating the growth and DNA synthesis of adrenal mitochondria induces at least a partial synchronization of the genetic duplication cycle of these organelles, most of which (about 50–55%) dramatically divide between the 9th and 12nd day of treatment. The slight but significant increase in the number of mitochondria found after 3 days of hormone administration, could be interpreted to be the result of the ACTH-induced accelerated duplication of that portion of mitochondrial population which was already in "S" phase at onset of hormone treatment. In fact, about 5–6% of the mitochondrial population was shown to incorporate 3H-thymidine in intact rat adrenocortical cells (Nussdorfer and Mazzocchi, 1971a).

Furthermore, it must be recalled that stimulation of mitochondrial DNA synthesis seems to be not the primary effect of ACTH. In fact, evidence indicates that 12 hs after ACTH administration, mitochondrial DNA synthesis, at variance with mitochondrial RNA synthesis, does not display any significant increase (Nussdorfer and Mazzocchi, 1971a). It is possible, therefore, to hypothesize

that ACTH-induced increase in mitochondrial protein synthesis (and/or nuclear protein synthesis) could trigger mitochondrial DNA duplication, for example, by activating mitochondrial DNA-polymerase.

After 24 and 36 consecutive days of ACTH-treatment the number of mitochondria per cell as well as the average volume of organelles both show a slight but significantly constant increase. This can be the result of the balance of two processes: (1) division of hypertrophied mitochondria, and (2) hypertrophy of the newformed mitochondria.

In conclusion, the present findings seem to suggest the following sequential steps in the ACTH-induced stimulation of adrenal mitochondria turnover:

(1) stimulation of nuclear and mitochondrial RNA and protein synthesis;

(2) stimulation of the growth and DNA duplication of adrenal mitochondria;

(3) division of hypertrophied mitochondria.

Pulse labelling experiments with 3H-thymidine after various days of ACTH administration as well as investigations on the duration of the duplication cycle of adrenocortical mitochondria are in progress to gain further insight into this problem.

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