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Long-Term Trophic Effect of ACTH on Rat Adrenocortical Cells

An Ultrastructural, Morphometric and Autoradiographic Study

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Summary. The changes occurring in rat adrenocortical cells (zona fasciculata) during an 8 day period of treatment with ACTH, were investigated by morphometric and autoradiographic methods.

The most important ultrastructural change consists in a conspicuous increase in the smooth endoplasmic reticulum, that accounts for about 50% of the total increase of cellular volume. Also the mitochondrial fraction shows a significant increase, which is found to be due both to the increment in the number of mitochondria per cell and to the increase in the mean volume of organelles themselves.

The quantitative autoradiographic data, indicating an increment in the incorporation of 3H-orotate and 3H-leucine into adrenocortical cells of the treated animals, allow us to conclude that the ACTH-induced ultrastructural changes are the morphological expression of a stimulation of the cellular protein synthesis.

Since mitochondria are largely autonomous in the synthesis of their enzymes and structural proteins, it is possible to hypothesize that ACTH also intervenes in the regulation of the mitochondrial protein synthesis.

Key-Words: Adrenal Cortex—ACTH—Autoradiography—Stereology—Rat.

The effects of ACTH on the fine structure of adrenocortical cells have been studied by numerous authors (Sabatini *et al.*, 1962; Schwarz *et al.*, 1962; Nishikawa *et al.*, 1963; Luse, 1967; Kjaerheim, 1968; Idelman, 1970). However, some ultrastructural modifications (open mitochondrial forms, swelling of the smooth reticulum) previously reported as an expression of the rapid action of ACTH in increasing steroidogenesis, may be due to preservation artifacts. In fact, similar ultrastructural alterations are sometimes found in adrenocortical cells of intact animals, as a result of poor fixation (Meneghelli, 1969).

As is well known, ACTH also possesses a long-term effect on the adrenal cortex, by maintaining and stimulating trophism and consequently the steroidogenic capacity of adrenocortical cells.

The aim of this paper is to present a correlated morphometric and autoradiographic study of the trophic action of ACTH on rat adrenocortical cells (*zona fasciculata*).

Materials and Methods

Treatment of Animals. Thirty male albino rats (Wistar-derived), weighing about 300 g, were divided into 5 stocks. Four stocks were given i.p. injections of 30 I.U./kg of ACTH (Sigma) for 1, 2, 4 and 8 consecutive days, respectively. The control stock received only

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i.p. injections of physiological saline. Three hours before the sacrifice each stock was subdivided into two groups, which received i.p. injections of 100 μc of 3H-orotate and 3H-leucine (New England Nucl. Corp.), respectively. The animals were sacrificed by cervical dislocation. The rats and their respective left adrenal glands were weighed and the relative adrenal weight was calculated. As the mean specific weight of adrenal glands (evaluated by pyknometry) does not vary during the entire experimental period, the relative adrenal weight is also an estimation of the relative adrenal volume.

Electron Microscopy. Fragments of the right adrenal gland of each rat, were fixed in 5% glutaraldehyde in cacodylate buffer (Sabatini *et al.*, 1963), postfixed in 1% OsO_4 in phosphate buffer (Millonig, 1961), and embedded in Dow Epoxy Resin (D.E.R.) according to Lockwood (1964).

The zona fasciculata was examined on toluidine blue stained thick sections (Trump *et al.*, 1961). At this level adjacent thin sections were cut with LKB III ultramicrotomes. The thin sections were counterstained by the method of Karnovsky (1961) or Reynolds (1963) and observed in a Hitachi HU 11 electron microscope.

Morphometry. From each rat 6 tissue blocks, randomly selected, were examined. The percent cellular volume occupied by mitochondria, lipid droplets, smooth endoplasmic reticulum (s.e.r.) (membrane space), and lysosomes was calculated on prints at a magnification of 24000 (120 micrographs/stock) with methods of "differential point counting" (Weibel, 1969). The membrane profile concentration (i.e., μ^2 of s.e.r., including Golgi apparatus/μ^3 of cell) was estimated on prints at a magnification of 40000 (120 micrographs/stock) by the "crossing method" (Loud, 1962). The average number of mitochondria per unit volume of cytoplasm was calculated according to Weibel *et al.* (1969). The surface to volume ratio (s/v) of mitochondria was measured by Chalkley's method (for references, see Underwood, 1970). The absolute amout of organelles in the individual adrenocortical cell was calculated by determining the average cellular volume on microphotographs at a magnification of 1250 (80 microphotographs/stock), with methods analogous to those we have previously described (Nussdorfer, 1970).

Autoradiography. The autoradiography was performed using 0.5 μ thick sections according to Nussdorfer *et al.* (1969). The specimens were coated by dipping them into Ilford L4 liquid emulsion and were left to incubate in a dessicator for 25 days. The incorporation of the tracers into adrenocortical cells was calculated by the method of the "mean grain count" (Rogers, 1968).

Statistical Treatment of Results. Student *t*-test was used for the statistical evaluation of the data. The difference between two mean values was considered significant if the probability of error (P) was found to be less than 0.05.

Results

Ultrastructural and Morphometric Data

After 2, 4 and 8 days of ACTH administration, the relative adrenal weight of the treated animals is significantly greater than that of the controls (Table 1).

Table 1. *Changes in relative adrenal weight after ACTH administration*

Duration of treatment	Relative adrenal weight (mg/100 g body weight)	Standard error (± SE)	Increase in % (Δv)	Level of significance (P)
Control	14.26	± 0.52	—	—
1 day	15.00	± 0.51	5.2	0.1
2 days	16.40	± 0.61	15.0	0.05
4 days	19.08	± 0.73	33.8	0.05
8 days	20.40	± 0.73	43.0	0.01

Table 2. *Synopsis of morphometric parameters of rat adrenocortical cells after ACTH administration*

Duration of treatment		Control			
		1 day	2 days	4 days	8 days
Volume of Cells					
μ^3	1680 ± 92.1	1980 ± 103.2	2292 ± 128.2	2790 ± 166.2	3353 ± 213.5
Δv	—	17.2	24.5	66.0	99.8
P	—	0.05	0.01	0.01	0.005
Volume of nuclei					
μ^3	130 ± 5.1	143.7 ± 5.2	162.3 ± 5.8	181 ± 6.0	209.1 ± 6.1
Δv	—	10.5	24.8	39.3	60.7
P	—	0.05	0.05	0.01	0.01
Diameter of nuclei					
μ	6.28 ± 0.246	6.50 ± 0.235	6.77 ± 0.260	6.98 ± 0.261	7.36 ± 0.267
Δl	—	3.5	7.8	11.2	16.7
P	—	0.05	0.05	0.01	0.01
Volume of mitochondrial fraction					
μ^3	635.5 ± 31.4	676.2 ± 30.7	830.7 ± 48.1	1038.2 ± 56.0	1256.2 ± 86.1
Δv	—	6.4	30.7	64.7	97.7
P	—	0.1	0.05	0.05	0.01
Volume of lipid fraction					
μ^3	121 ± 10.7	195.5 ± 16.1	213 ± 17.5	210 ± 15.9	314.2 ± 23.7
Δv	—	61.5	76.0	73.0	151.1
P	—	0.01	0.01	0.01	0.001
Volume of lysosomial fraction					
μ^3	15.5 ± 1.30	14.9 ± 1.42	17.2 ± 2.5	15.5 ± 1.8	19.7 ± 2.4
Δv	—	−3.9	11.3	0	27.1
P	—	no significant	0.1	no significant	0.05
Membrane space					
μ^3	778.2 ± 48.2	950.1 ± 62.1	1068 ± 60.2	1345.8 ± 89.9	1554.5 ± 101.1
Δv	—	22.1	37.2	72.9	99.9
P	—	0.01	0.01	0.005	0.005
Surface of smooth reticulum					
μ^2	7750 ± 501.2	9182 ± 600.3	10649 ± 623.8	13900 ± 874.1	15720 ± 1002.1
Δs	—	18.5	37.4	79.3	102.8
P	—	0.01	0.01	0.005	0.005

The volume of the cells of the zona fasciculata increases significantly as early as the 1st day of treatment and is almost doubled by the 8th day of ACTH administration (Table 2). Furthermore, also the volume and diameter of nuclei increase significantly, though to a lesser extent than the cellular volume, from the 2nd day of hormonal treatment (Table 2). The nuclei of adrenocortical cells

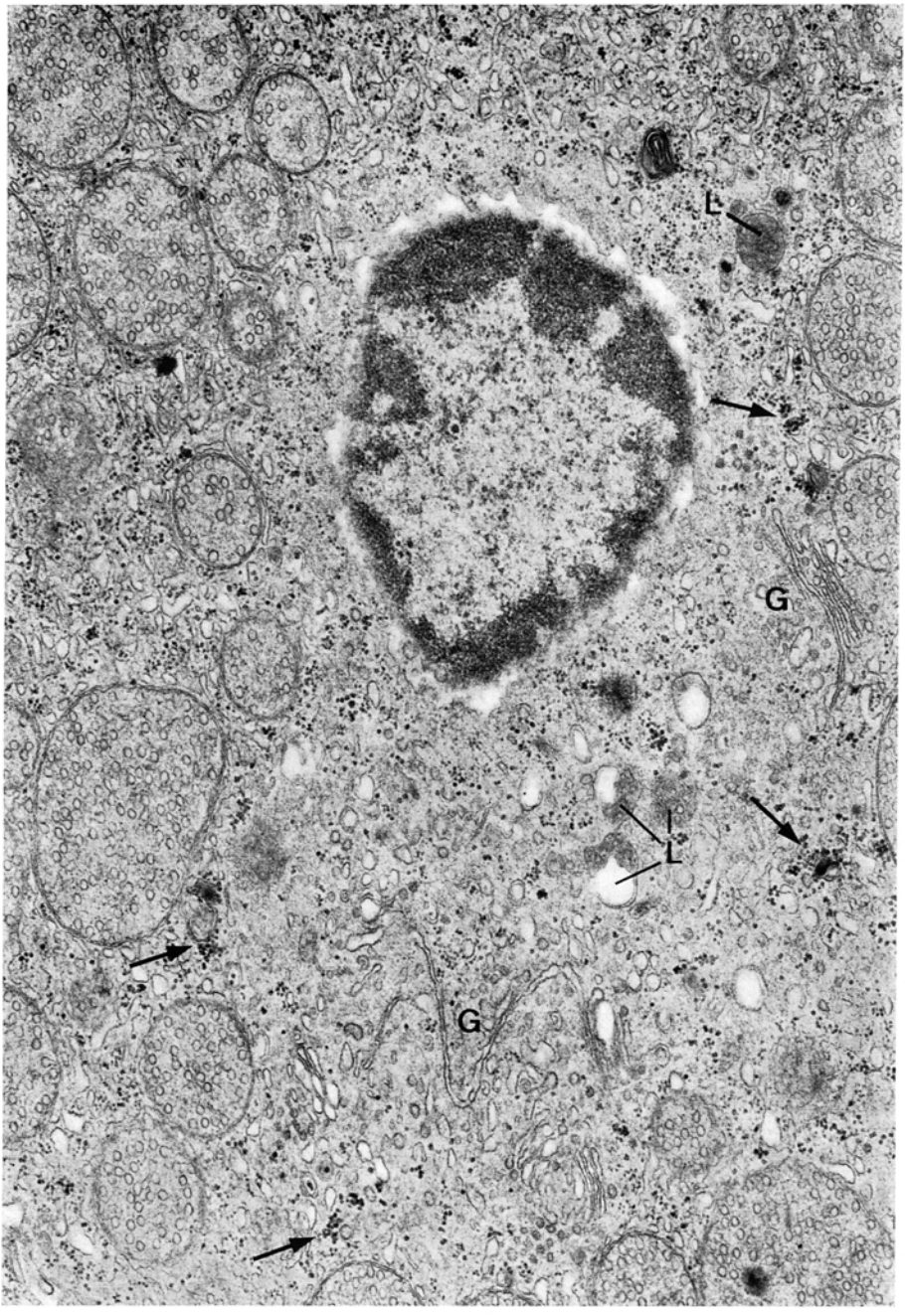


Fig. 1. Cell of the zona fasciculata of a control rat adrenal cortex. The smooth endoplasmic reticulum is well developed. In the cytoplasm there is a sparse storage of glycogen (arrows). *G* Golgi apparatus; *L* Lysosome. × 20200

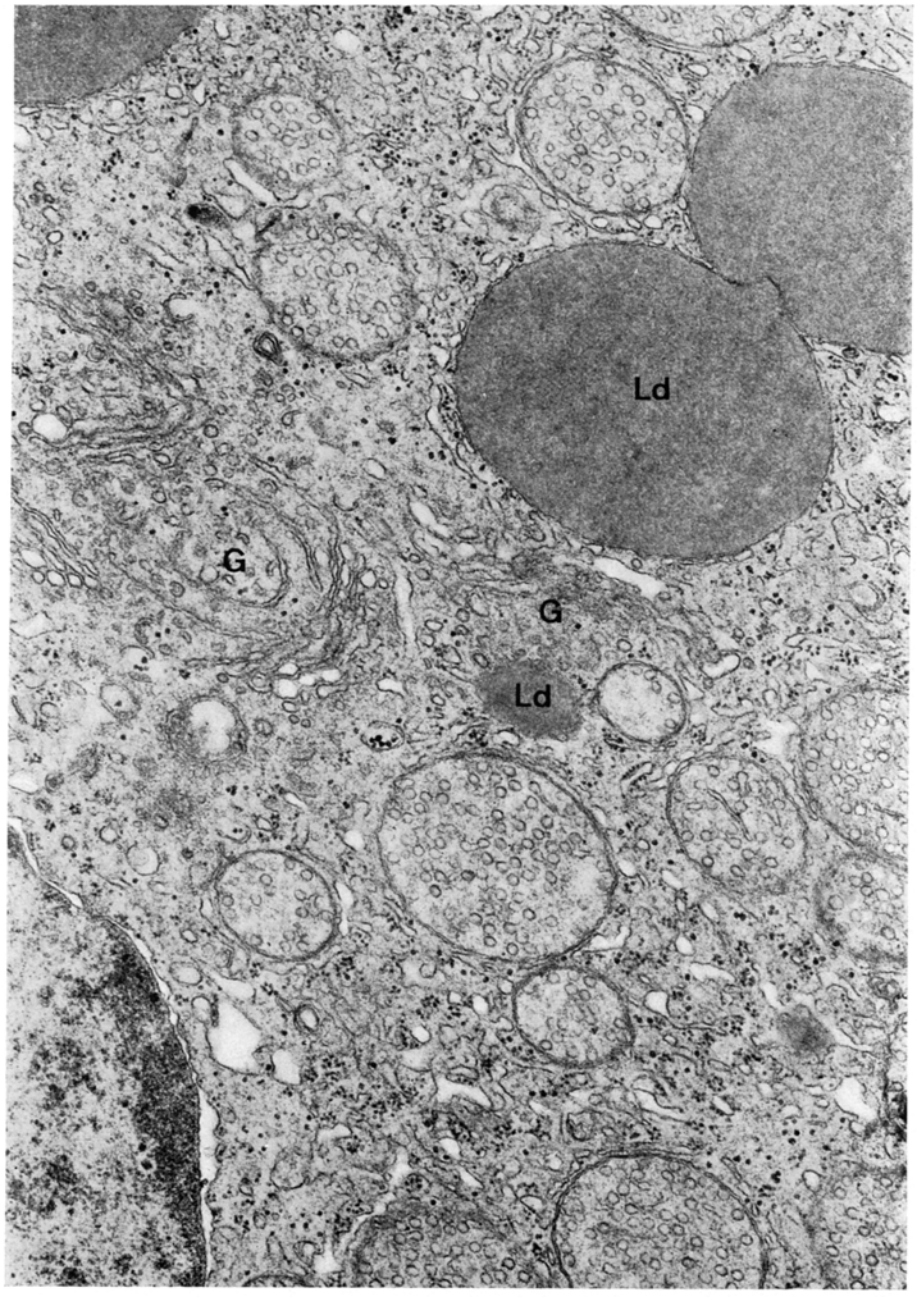


Fig. 2. Cell of the zona fasciculata of rat adrenal cortex after 4 days of treatment with ACTH. The smooth endoplasmic reticulum is very abundant and the Golgi apparatus (*G*) is prominent. Numerous lipid droplets (*Ld*) are also present. × 27000

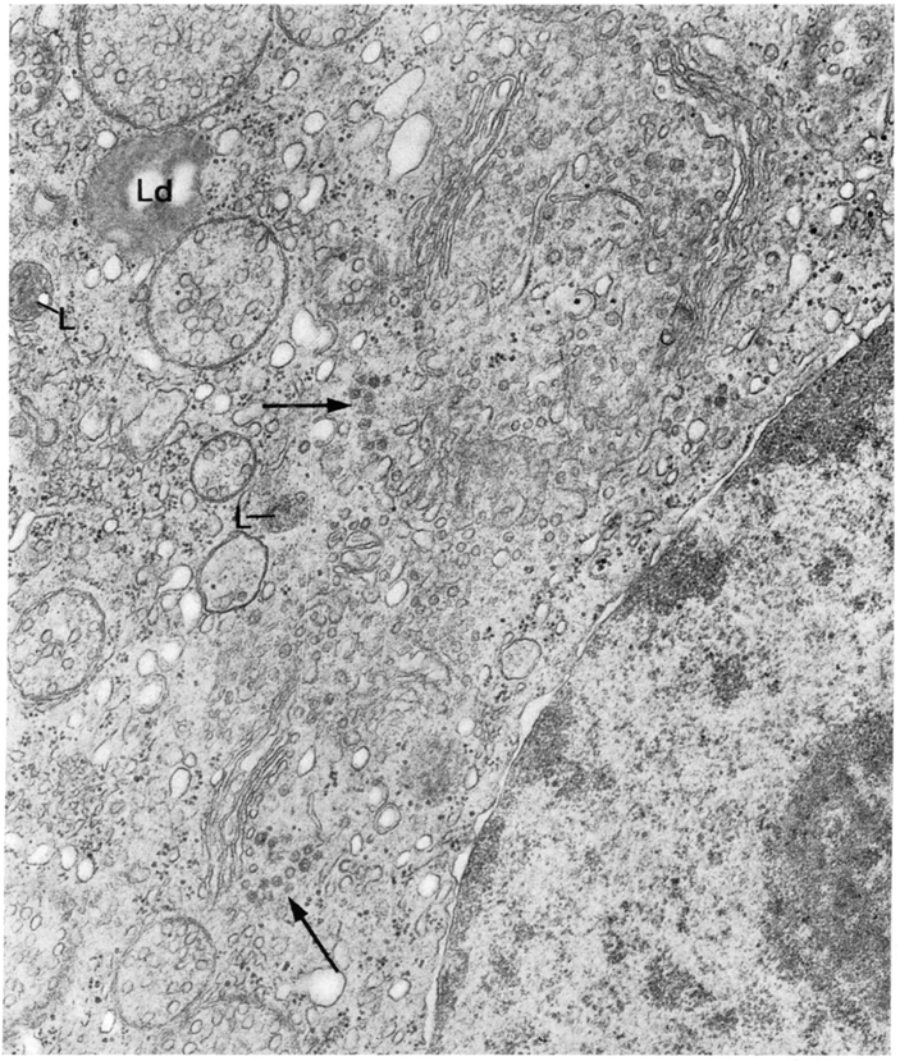


Fig. 3. Cell of the zona fasciculata of rat adrenal cortex after 8 days treatment with ACTH. The Golgi apparatus shows an hypertrophic appearance. It consists of flat saccules and vesicles, of which many are coated (arrows). The coated vesicles seem to arise from the dilated extremities of the golgian saccules. *Ld* lipid droplet; *L* lysosome. × 23500

of the treated animals do not show qualitative ultrastructural variations in comparison with those of the controls. Mitoses are either absent or extremely rare in the zona fasciculata, both in the treated and in the control rats.

The adrenocortical cells of the ACTH-treated animals display only slight ultrastructural variations, that can be detected without the aid of morphometric methods (Figs. 1, 2). After administration of ACTH, the Golgi apparatus is greatly developed and occupies a large portion of the cytoplasmic area. There are numerous

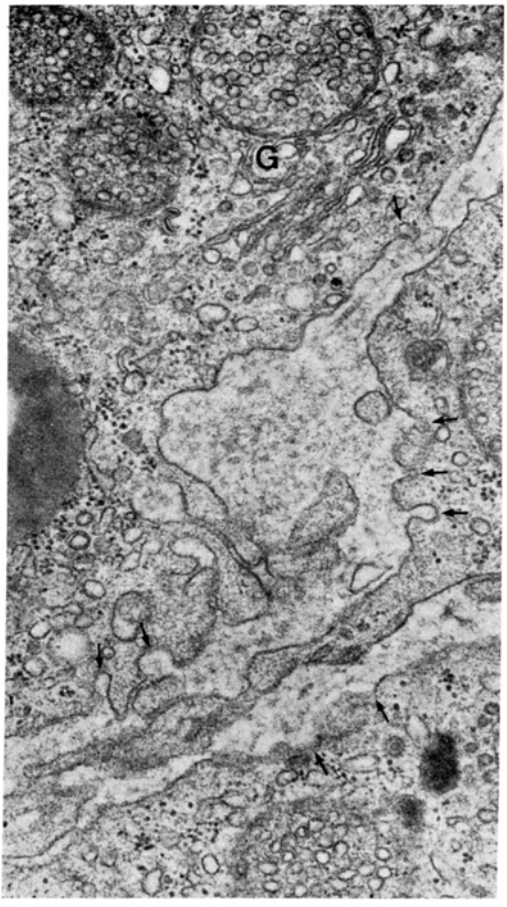


Fig. 4. Cell of the zona fasciculata of rat adrenal cortex after 8 days of treatment with ACTH. Numerous coated pits or caveolae at the plasma membrane of adrenocortical cells are found after hormonal treatment (arrows). *G* Golgi apparatus. × 25000

coated vesicles, which arise from the dilated extremities of the golgian *cisternae* (Fig. 3). Several coated pits or caveolae at the plasma membrane can also be seen (Fig. 4). The s.e.r. appears as a meshwork of closely interconnected tubules and fills large portions of cytoplasm, lacking in any other subcellular organelle (Fig. 5).

The volume of the mitochondrial fraction increases significantly in relation to the duration of ACTH treatment (Table 2), as also do the mitochondrial diameter and volume and the number of mitochondria per cell (Table 3). For all the times of experiment, the volumetric increment of the mitochondrial fraction is about 32–35% due to the increase in the mean volume of organelles themselves and about 65–68% due to the increase in the average number of mitochondria per cell. The surface-to-volume ratio of mitochondria, on the other hand, does not show any significant variation (Table 3).

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

Duration of treatment	Control	1 day	2 days	4 days	8 days
Number per cell					
	1376 ± 41.1	1550 ± 43.9	1790 ± 50.6	1946 ± 62.1	2030 ± 79.9
P	—	0.05	0.05	0.01	0.01
Diameter					
(μ)	0.960 ± 0.011	0.951 ± 0.009	0.958 ± 0.010	1.010 ± 0.012	1.062 ± 0.012
P	—	no significant	no significant	0.05	0.05
Volume					
(μ^3)	0.462 ± 0.031	0.455 ± 0.030	0.462 ± 0.032	0.538 ± 0.058	0.628 ± 0.069
P	—	no significant	no significant	0.05	0.05
Surface to volume ratio					
	1.099 ± 0.021	1.109 ± 0.030	1.059 ± 0.028	1.067 ± 0.022	1.052 ± 0.017
P	—	no significant	no significant	no significant	no significant

The volume of lipid fraction and the surface and volume (membrane space) of s.e.r. also increase significantly in relation to the number of days of animal treatment (Table 2). The volume of the lysosomial fraction, however, reveals a slight but significant increase only at the 8th day of ACTH administration (Table 2).

If the data shown in Table 2 are plotted on a graph, as function of the number of days of animal treatment, it may be noted that the increment in the various morphological parameters is not linearly related to the duration of treatment (Figs. 6, 7). In fact, about 67–80% of the total increment in volume of cells and other subcellular organelles has already occurred by the 4th day of the hormone administration.

Fig. 8 shows that the increment in cell volume is about 50% due to the increase in s.e.r. (membrane space), while the increases in volume of nuclei, lipid fraction, and of mitochondrial fraction account for about 5, 15 and 29%, respectively, of the total volumetric increase of adrenocortical cells.

Autoradiographic Data

The incorporation of 3H-orotate and 3H-leucine is significantly increased in adrenocortical cells of treated animals (Fig. 9). The increment in the tracer's incorporation is in relation to the duration of ACTH administration. It must be noted, however, that this relation is not linear; in fact, about 65% and 90% of the total increment in the 3H-orotate and 3H-leucine incorporation, respectively, has already occurred by the 4th day of animal treatment.

The specificity of this effect of ACTH is confirmed by the lack of increase (or the slight decrease) in the incorporation of the tracers in the capsular fibroblasts.

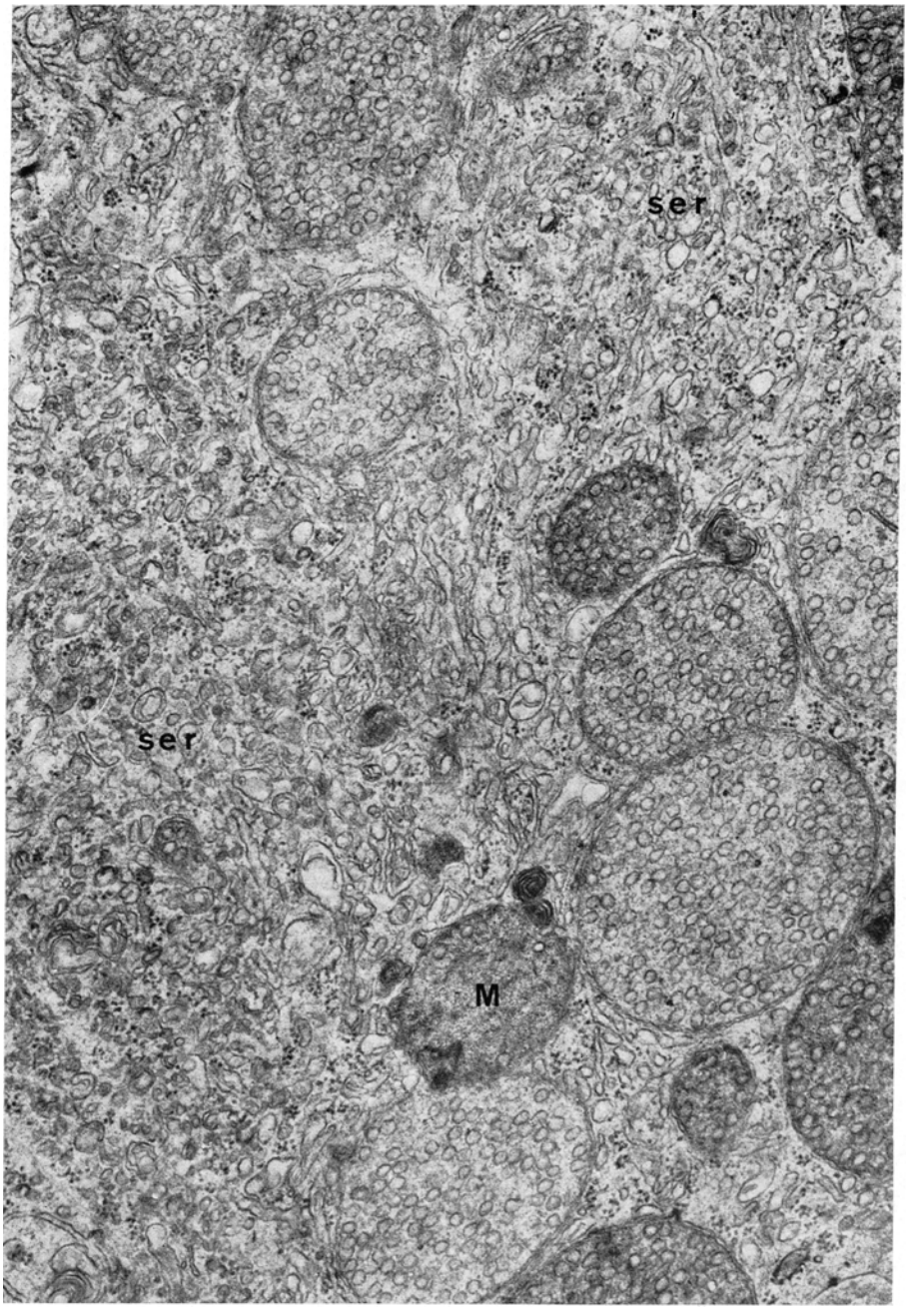
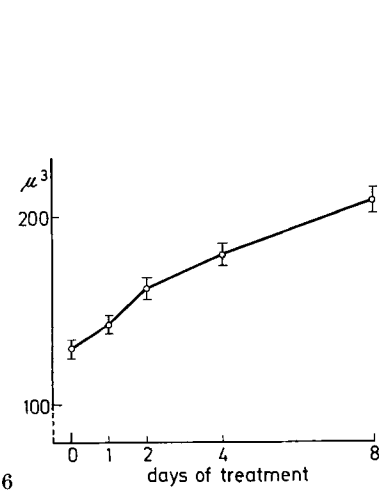


Fig. 5. Cell of the zona fasciculata of rat adrenal cortex after 8 days of treatment with ACTH. The smooth endoplasmic reticulum is hypertrophic and occupies a large zone of cytoplasm, lacking of any other organelle. *M* indicates a mitochondrion in degeneration. × 28000



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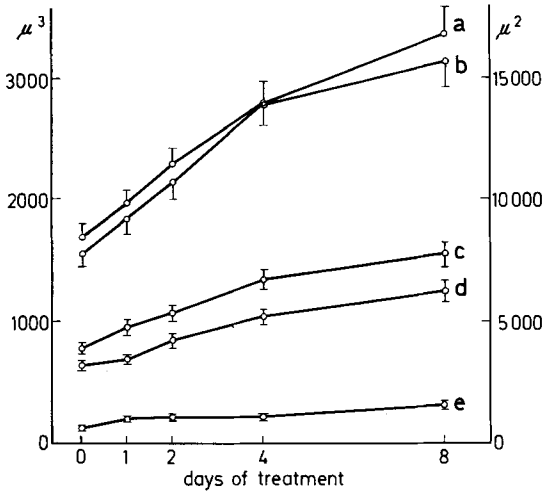

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Fig. 6. Changes in volume of nuclei of rat adrenocortical cells after ACTH administration. Standard errors are indicated

Fig. 7. Changes in morphological parameters of rat adrenocortical cells, as function of number of days of ACTH administration. Standard errors are indicated. *a* s.e.r. surface; *b* cellular volume; *c* membrane space; *d* volume of mitochondrial fraction; *e* volume of lipid fraction

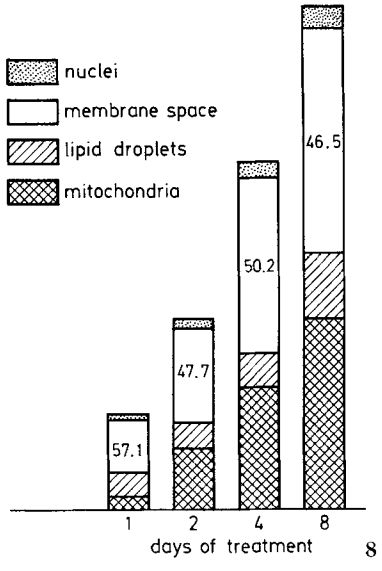

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Fig. 8. Histogram demonstrating the total increase (Δv) in volume of rat adrenocortical cells after 1, 2, 4 and 8 days of ACTH treatment. The extent to which nuclei, membrane space, mitochondria and lipid droplets are involved in the ACTH-induced increase in cellular volume is indicated

Discussion

In attempting to elucidate the mechanisms underlying the steroidogenic response of adrenocortical cells to ACTH, numerous investigations were performed (for comprehensive references, see Haynes, 1968). Nevertheless, there is considerable disagreement as to the mechanism of action of this hormone.

Numerous investigations indicate that ACTH (via 3',5'-AMP) stimulates the intramitochondrial hydroxylation of cholesterol and its conversion into pregnenolone (Stone *et al.*, 1954; Creange *et al.*, 1966). Moreover, numerous lines of evidence indicate that ACTH also possesses a general stimulatory effect on the protein synthesis of adrenocortical cells both in vivo (Fiala *et al.*, 1956; Bran-

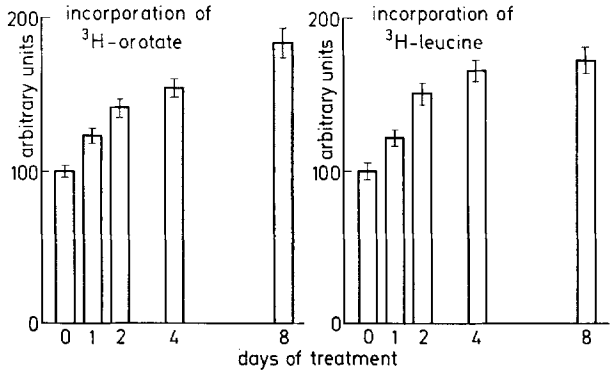

Fig. 9. Histograms showing the increase in the incorporation of 3H-orotate and 3H-leucine into the rat adrenocortical cells, induced by ACTH administration. Standard errors are indicated

some *et al.*, 1961, 1963; Farese *et al.*, 1963) and in vitro (Bransome *et al.*, 1964; Farese, 1966, 1968, 1969; McKerns, 1968).

Our morphometric data indicate that the trophic effect of ACTH on rat adrenocortical cells in vivo mainly consists in a significant increase in the cell volume, about 50% of which is due to the increase of s.e.r. membranes. Since numerous enzymes of the steroid-synthesis are localized in the microsomic fraction (Srere *et al.*, 1948; Dorfman *et al.*, 1965; Samuels *et al.*, 1967; Péron *et al.*, 1968; Inano *et al.*, 1969), this suggests that the increase of s.e.r. in adrenocortical cells of ACTH-treated rats is the morphological expression of the increased hormone synthesis. This is also supported by the demonstration, in rat hepatocytes, of a direct correlation between the quantity of s.e.r. and the activity of enzymes contained in the microsomic fraction (Stäubli *et al.*, 1969). The volumetric increase in lipid fraction, which mainly consists of cholesterol undergoing utilization (Lanman, 1962; Wolman, 1964; Luse, 1967), may be correlated to the increment of s.e.r., since it is here that the enzymes of cholesterol synthesis are localized (Dorfman *et al.*, 1965). The increase of the lipid fraction is also in agreement with the results of in vitro studies, indicating that ACTH can effect an increase in the rate of synthesis of cholesterol from acetate and glucose (Dexter *et al.*, 1967; Kowal, 1969).

The significant increase in volume of the mitochondrial fraction of adrenocortical cells of ACTH-treated rats, also agrees with an accelerated rate of steroid-synthesis. In fact, it is well known that some enzymes of steroid-synthesis are localized at the level of mitochondrial *cristae* (Dorfman *et al.*, 1965; Harding *et al.*, 1968; Simpson et al., 1968; Satre *et al.*, 1969; Dodge *et al.*, 1970).

Regarding the mechanism of increase of s.e.r. membranes, it is thought to consist of either (1) increased synthesis of new membranes or/and (2) slowed-down katabolism of them. Our autoradiographic data support the 1st possibility. In fact, they show a significant increase in the protein synthesis in adrenocortical cells of ACTH-treated rats.

The volumetric increment of nuclei resulting from ACTH administration, is also in keeping with a stimulation of nuclear functionality (Mitro *et al.*, 1970). In addition, the presence of numerous coated pits at the plasma membrane of adrenocortical cells of ACTH-treated rats is also in agreement with an accelerated protein synthesis, since it indicates an increase in the cellular uptake of polypeptide materials (Christensen *et al.*, 1969).

It may therefore be stated that the effects of ACTH on s.e.r. are mediated by the stimulation of protein synthesis (synthesis of enzymes and structural proteins) in adrenocortical cells.

The increase in volume of the mitochondrial fraction in adrenocortical cells of treated rats deserves some further discussion. In fact, it cannot be due to the increased rate of protein synthesis dependent upon the stimulation of nuclear RNA production by ACTH, since it is well known that mitochondria are able to synthesize DNA and RNA autonomously (for references, see Roodyn *et al.*, 1968). In the description of results it has been seen that the increase in the mitochondrial fraction is due to the increment both in the number of mitochondria per cell and in the average volume of the organelles themselves. Considering that the mitochondrial surface-to-volume ratio remains unchanged for the entire duration of experiment, an increase in the surface of mitochondrial cristae might also be assumed. It is therefore necessary to put forward the work hypothesis that ACTH intervenes in the regulation of the intramitochondrial synthesis of RNA and DNA. This hypothesis is also supported by the results of a recent investigation carried out by Kahri (1970) on adrenocortical cells in vitro.

As previously described, the increase in the incorporation of tracers and the increase in volume of various morphological parameters are not related linearly to the duration of ACTH treatment. In fact, the rate of increment falls with the number of days of ACTH administration. This finding can easily be explained by taking into account that ACTH increases the hematic and intracellular concentration of corticosteroid-hormones. In fact, it has been demonstrated that corticosteroids inhibit protein synthesis and trophism of adrenocortical cells in vivo, not only indirectly by inhibiting the hypothalamo-hypophysial-adrenal axis, but also directly (short negative feed-back mechanism) (Nussdorfer and Mazzocchi, 1970a, b). Moreover, since the increase in volume of the mitochondrial fraction is not a linear function of the duration of animal treatment, it must be assumed, according to Burrow's in vitro experiments (Burrow, 1969), that corticosteroids directly inhibit the mitochondrial protein synthesis. However, in previous experiments (Nussdorfer *et al.*, 1970a) we had not been able to show any direct action of corticosteroids on adrenocortical mitochondria of hypophysectomized ACTH-treated rats. In the light of the results of the present study, this fact might be explained by assuming that the katabolic turnover of adrenocortical mitochondrial fraction is much slower than that of s.e.r. membranes. The duration of corticosteroid treatment might therefore have been too short to allow any appreciation of a significant quantitative alteration of the mitochondrial fraction.

In the description of results it has been said that the Golgi apparatus appears hypertrophic in adrenocortical cells of the treated animals. An enlargement of the Golgi apparatus has always been reported as an expression of hyperactivity

of the steroid-secreting cells (Reese *et al.*, 1938; McDonald *et al.*, 1965, 1969; Kjaerheim, 1968). Since no function of the Golgi apparatus in steroid synthesis and secretion is yet known, this finding is difficult to explain. According to Long and Jones (1967), it may be thought that the Golgi apparatus might be a site of steroid-conjugation preparatory to hormone secretion. Alternatively, since it is known that the Golgi complex plays a role in the formation of the lysosomial digestive apparatus (de Duve, 1963; Novikoff *et al.*, 1963, 1964), it is also possible to hypothesize that the hypertrophy of this organelle is expression of the accelerated synthesis of lysosomial hydrolases in response to the increased katabolic turnover of the subcellular organelles. This hypothesis is also supported by the slight but significant increase in volume of the lysosomial fraction at the 8th day of ACTH administration. It must also be noted that this hypothesis does not necessarily conflict with that of Long *et al.* (1967).

In conclusion, the results of the present study indicate that the mechanism of the trophic action of ACTH on rat adrenal cortex consists in the stimulation of the protein synthesis (intra- and extramitochondrial protein synthesis) in adrenocortical cells, the morphological expression of which is the increase in volume of the various cellular organelles (nucleus, mitochondria, s.e.r., etc.) with a consecutive increment in volume of cells and of the gland itself.

Since the ACTH-induced rise of corticosterone concentration in the rat blood stream, can be detected within 15–30 min (Shima *et al.*, 1969), it is difficult to admit that this rapid tropic action of ACTH is dependent upon the stimulation of the synthesis of enzymes of corticosteroidogenesis. According to Koritz's hypothesis (Hirshfield and Koritz, 1964; Koritz, 1968), it may be considered that the mechanism underlying the rapid response of adrenocortical cells to ACTH, consists in an increase of the mitochondria permeability to pregnenolone. The long-term trophic action of ACTH, which we have shown to be due to the stimulation of the cellular synthesis of enzymes and structural proteins, would enable the adrenocortical cells to mantain for long periods the increased rate of hormone production and secretion.

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