THE ACTION OF CORTICOTROPIN (ACTH) ON NUCLEIC ACIDS AND SUBCELLULAR ELEMENTS OF THE ADRENAL CORTEX*, ‡

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Hormones are thought to regulate the functions of various tissues by forming complexes with enzymes or their inhibitors and thus inhibiting or enhancing enzymatic activity. The action of the so called trophic hormones of the anterior pituitary gland is of special interest, because they may stimulate hypertrophy and hyperplasia of certain organs such as gonads, adrenals, thyroid, and mammary gland. The study of trophic hormone activity may be expected to reveal some of the fundamental features of normal and pathological growth.

While the mechanisms initiated by trophic hormones may not be the same in each case, some light may be thrown on the nature of the hypertrophy by investigating in some detail subcellular changes induced by a representative hormone of this group. This initial study concerns the effect of administration of corticotropin (ACTH) on the subcellular elements of the adrenal gland. It is known (2, 3) that ACTH leads not only to an increased production of cortical steroids shortly after its application, but also induces the enlargement and increase in weight of the adrenal gland after repeated application.

Materials and Methods

Immature (3 to 5 weeks old) Wistar rats were used in all experiments except when animals with larger or smaller adrenals were needed for the sake of comparison (as in Fig. 1). Each experimental animal received intramuscularly 25 U.S.P. units of corticotropin Armour 1 to 3 times daily. In several experiments of longer duration an equivalent amount of corticotropin-gel Wilson was also injected. The determinations were made on a series of six experimental and six control animals of the same age, sex, and approximately the same body weight. Throughout the whole procedure the adrenal tissues of both groups (twelve glands in each) were handled concomitantly. It must be pointed out that this is indispensable for a reliable comparison, especially in all centrifugation steps. We always compared, therefore, the total amount of tissue from six controls with the corresponding total amount of adrenal tissue from six experimental animals.

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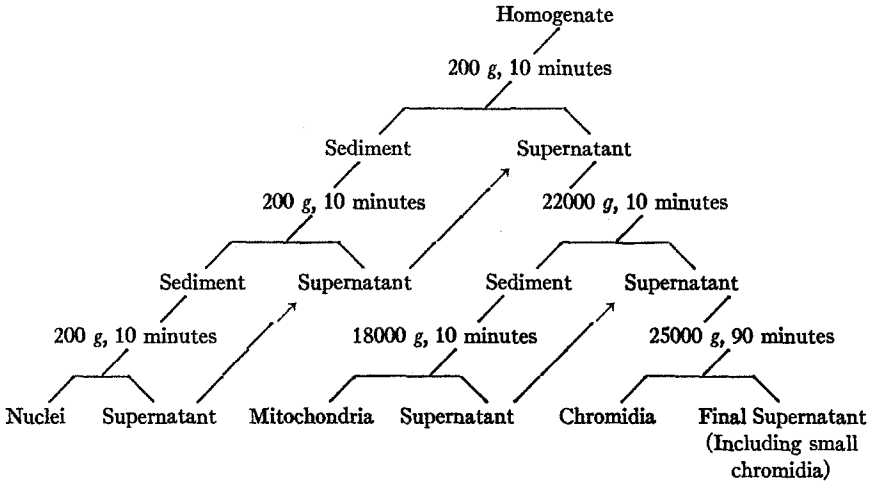
At the end of the experiment, the adrenals, removed under chloroform narcosis, were decapsulated and washed with cold saline. Each group of twelve glands was then blotted with filter paper and weighed on a torsion balance. Subsequently the tissue was homogenized with a glass homogenizer in cold isotonic (0.25 M) sucrose in the proportion of 5 per cent of tissue per milliliter of homogenate. The homogenization of whole adrenal glands, without separating cortex from the medulla, introduces a certain error in results, not exceeding 12 per cent. In a transverse section through the fixed gland, the ratio of both axes of nearly cofocal ellipses corresponding to medulla and cortex is approximately 1:2. The ratio of corresponding volumes of both oblate spheroids is 1:8.

The subcellular fractions were separated by differential centrifugation in a refrigerated International centrifuge and in a Servall centrifuge SS-1 at 2–4°. It was not regarded as necessary to separate the so called "poorly sedimented" or "fluffy" layer (4, 5). This fraction, which stains with pyronine Y in distinction to mitochondria (6) was intentionally in-

TABLE I



cluded among other basophilic granules which sedimented after 90 minutes at the top speed of the Servall centrifuge. The centrifugation procedure used is illustrated by the diagram shown in Table I.

The basophilic substance in the intact cell has been shown (7, 8) to occur in the form of "endoplastic reticulum," composed of RNA-rich granules accompanying membrane-bound vesicles and tubules (9, 10). In this study we dealt exclusively with the material obtained from the crushed cells. It was therefore necessary to designate by one term, such as chromidia, the cytoplasmic RNA–rich material obtained by centrifugation. This term has a certain historic justification being used sporadically in the cytology literature of the past for the cytoplasmic basophilic structures stained with pyronin in histological preparations (11–13). The term microsomes is almost comparable but refers to one isolated fraction only and does not relate the particles to their basophilic property.

The purity of nuclear and mitochondrial material was checked microscopically (× 450), using a freshly prepared aqueous mixture of methyl green and pyronine Y (6). Chromidial contamination appears as a pink coloration. It must be noted, however, that the basophilic granules of the adrenal take up pyronine much less avidly than those of other organs, perhaps because of a high content of lipides.

The nucleic acid and protein content of each fraction was determined after precipitating the protein material with an equal volume of 10 per cent trichloracetic acid. Total nitrogen of each fraction was determined by nesslerization. DNA and RNA were extracted from the fractions by the procedure of Schneider (14), measured colorimetrically by reactions with twice recrystallized diphenylamine and orcinol respectively. Standard samples of highly polymerized DNA and purified RNA were prepared following the procedure of Kay and Dounce (15, 16). RNA was prepared from isolated chromidia of rat liver, DNA from isolated nuclei of thymus tissue of immature rats. 4 gm. of thymus tissue (wet weight) yielded 68.1 mg. of purified DNA, 2.4 mg. of which in 100 ml. aqueous solution showed an extinction of 0.462 at 260 mμ wave length. $E_1^{1\ \text{per cent}}_{\text{cm.}}$ at 260 mμ was, therefore, 191 and thus equivalent in purity to the best products of the original procedure of Kay *et al.* (15). All our DNA measurements and the DNA content of counted nuclei were calculated on this basis. 21.4 μg. of this DNA per 1 ml. showed an extinction of 0.100 at 595 mμ in the reaction with diphenylamine. Determinations of RNA were performed in an analogous way, using purified RNA as standard. The ratio of RNA to nitrogen, frequently used in cytochemical work, is referred to in this paper as the basophilic quotient (B.Q.) of a given fraction because this value seems to characterize the cytoplasmic fractions at least as well as the histological staining with pyronine or other basic dyes.

The counting of nuclei was performed on aliquots of homogenized tissue in the Petroff-Hausser bacterial counting chamber at 450 magnification. For this purpose, a sample of adrenal tissue was homogenized in exactly 4 ml. of isotonic sucrose, and an aliquot of this suspension was diluted with a 3 per cent acetic acid and 1 ml. of 0.5 per cent methyl green solution, up to the volume of 25 ml. The mixture was gently rehomogenized to avoid clumping of nuclei as far as possible. Direct counting was used almost exclusively for determination of DNA content of an average adrenal nucleus (see below). All later determinations of the nuclear amounts were calculated by dividing the total amount of DNA found colorimetrically by the amount of DNA found in a nucleus by the procedure described. The amount of DNA per nucleus was assumed to be constant.

Assuming that the nuclei of the adrenal tissue are diploid and most of the cells uninuclear, the nuclear DNA values are valid for the average adrenal cell. The data reported on subcellular elements are, therefore, related first to the total amount of tissue in each series of six experimental and six control animals, secondly to this unit of DNA.

OBSERVATIONS

The deoxyribonucleic acid content of an average adrenal nucleus was determined in three independent experiments, each time with a different amount of tissue, part of which was used for counting nuclei, the rest for colorimetric measurement of DNA. In each experiment fifteen independent counts were made. Because no published data on the DNA content of adrenal nuclei were found, the counts are summarized in Table II. The amount of DNA for an average adrenal nucleus, 5×10^{-12} gm., is close to the value indicated by Vendrely (17) but lower than that of Thomson *et al.* (18) for diploid nuclei of various tissues of the rat.

To characterize the difference between the increase in weight induced by ACTH and the normal growth of the gland, the cell content of adrenals of different size and weight from untreated rats 24 hours to 3 months old, was compared with that of adrenals of similar weight from ACTH-treated animals. In untreated animals there is an almost perfect linear relationship be-

tween the weight of the gland and the number of cells, except in quite young animals (Fig. 1). Thus if the organ doubles in weight, the number of cells increases 100 per cent. In very young rats, 10 days old or younger, the cells were found to be relatively more numerous per unit of weight. Fractionation revealed that the cytoplasm of these smaller cells is relatively poor in RNA-rich cytoplasmic granules (chromidia). The adrenals enlarged by repeated injections of ACTH, however, did not show this simple relationship between increase in weight and the number of cells. Following ACTH administration relatively fewer cells were found in the unit of weight, suggesting that the increment represents primarily growth of the cytoplasm. The effect of ACTH is illustrated in Fig. 1 by crosses above the line marking the physiological growth of the gland. As can be seen from the haphazard location of the crosses,

TABLE II

The Deoxyribonucleic Acid Content of an Average Adrenal Nucleus

Experiment	Total wet weight of tissue	Amount used for DNA determination	DNA found	Amount used for counting nuclei	No. nuclei $\times 10^6$	DNA calculated in total tissue	No. nuclei $\times 10^6$ calculated in total tissue	DNA per nucleus and cell $\times 10^{-12}$ g
	mg.	mg.	mg.	mg.		mg.	mg.	
I	140	70	0.276	70	56	0.552	112	4.92
II	380	190	1.024	190	200	2.048	400	5.12
III	318	238	1.200	79.5	80	1.600	320	5.0
Average								5.0×10^{-12} g

the cytoplasm of an average adrenal cell can be developed to a greater or lesser degree, independently of cell division.

An increase in total weight of adrenal tissue can be detected usually within 5 to 7 hours after only one or two injections of ACTH. A more conspicuous manifestation of ACTH action is the increase of RNA. Thus in the homogenates of three experimental series (eighteen animals) the total RNA had already increased some 15 to 25 per cent compared to the control, 2 to 3 hours after administration of 25 units of ACTH to each animal. In this period of time no measurable change in the amount of protein was found. Later, *i.e.* 5 to 6 hours after ACTH administration, the rise in RNA still persisted, or was even more marked, being accompanied also by an increase in protein. The homogenates obtained in these experiments were separated by differential centrifugation into subcellular fractions. Two typical experiments out of several are illustrated in Table III. The effect seems to be concentrated in sedimentable chromidia ("fluffy layer" plus microsomes) which increased both in RNA and in mass. The relative increase in RNA exceeded the increase in mass so that the basophilic quotient of this fraction became significantly

higher. On the other hand there seemed to be no change in ribonucleoprotein particles of the final supernatant. The mitochondrial material was greater after a single injection of ACTH but this increment was smaller than in chromidia and the basophilic quotient of mitochondria remained unchanged.

The effect of a single administration of ACTH is reversible and in the course of 12 to 24 hours it disappeared completely. If the administration of ACTH

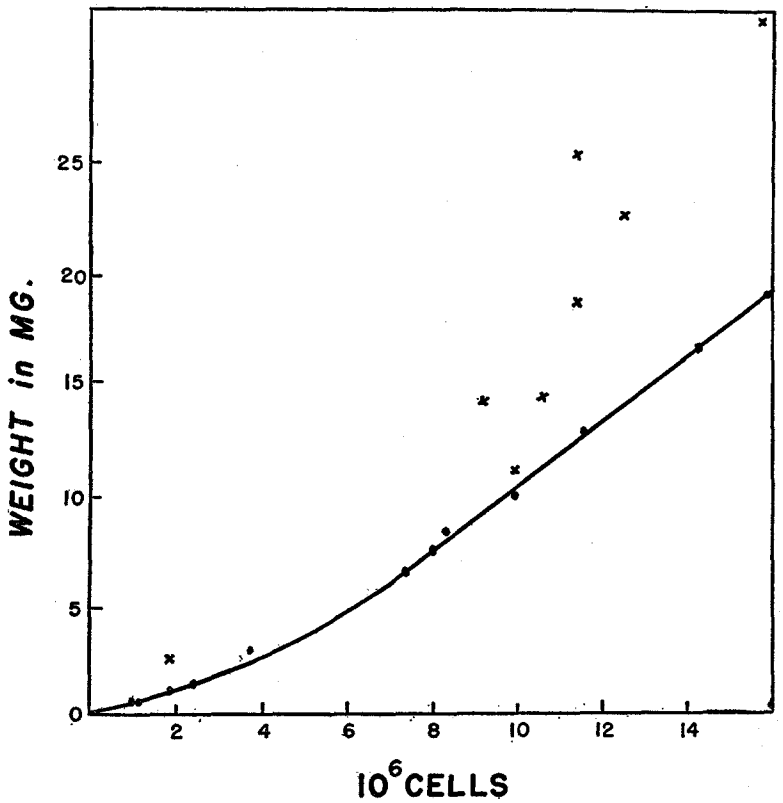


FIG. 1. Relationship between number of cells and weight of adrenal gland in control animals (dots) and after administration of ACTH (crosses).

was continued, further increase of cytoplasmic substance was observed in all three cytoplasmic fractions, but was again most pronounced in the sedimentable chromidia. There the RNA increase exceeded the increase in mass. The mass of the mitochondrial fraction increased further, but its basophilic quotient remained unchanged as in the short time experiments. The increase of cytoplasmic substance after administration of 260 and 425 units is shown in Table IV. Enlargement of the gland is due primarily to increase in mass of the average cell while the number of cells increases only a little after 260

TABLE III

Cytoplasmic Effects after a Single Administration of ACTH

Experiment	Amount of tissue	Treatment	Homogenate		Mitochondria			Large chromidia			Small chromidia and supernatant		
			RNA	Nitrogen	RNA	Nitrogen	B. Q.	RNA	Nitrogen	B. Q.	RNA	Nitrogen	B.Q.
	mg.		*mg.*	*mg.*	*mg.*	*mg.*		*mg.*	*mg.*		*mg.*	*mg.*	
	233	Control	2.5	3.0	—	—	—	—	—	—	—	—	—
1	236	40 units	2.9	3.1	—	—	—	—	—	—	—	—	—
		3 hrs.	+16%	+3%									
	116	Control	—	—	0.135	0.385	0.35	0.300	0.239	1.24	0.656	0.500	1.18
2	118	25 units	—	—	0.137	0.414	0.32	0.330	0.252	1.31	0.678	0.520	1.12
		5 hrs.											
	258	Control	—	—	0.206	0.771	0.27	0.440	0.414	0.96	1.260	0.752	1.6
3	285	60 units	—	—	0.226	0.805	0.28	0.605	0.474	1.28	1.240	0.788	1.6
		5 hrs.			+9%	+4%	—	+37%	+14%	—	—	+4%	—

In none of these three experiments was there any difference in the DNA content between control and experimental samples. In example 3 the nuclei of both samples were analyzed for RNA and protein nitrogen content. No difference was found. This is in contrast to data in column 2, Table V, where 60 units administered over a longer period of time produced changes in the number of nuclei and in their content.

TABLE IV

*Cytoplasmic Effect of ACTH after Longer Administration**

Experiment	Weight of tissue	Total No. nuclei in 12 glands $\times 10^6$	Average weight of gland	No. nuclei per gland $\times 10^6$	Mitochondria			Large chromidia			Small chromidia (Final supernatant)		
					RNA	N	B.Q.	RNA	N	B.Q.	RNA	N	B.Q.
	mg.		*mg.*										
Control	116.4	96	9.7	8.0	138	460	0.3	245	225	1.04	595	580	1.03
					1.43	**4.72**		**2.55**	**2.34**		**6.2**	**6.0**	
Experiment, 260 units	222	129 (+34%)	18.5 (+90%)	10.8 (+35%)	192 (+40%)	670 (+45%)	0.29	495 (+102%)	345 (+53%)	1.43	1034 (+73%)	1100 (+90%)	0.94
					1.49	**5.2** (+8%)		**3.83** (+50%)	**2.68** (+14%)		**8.01** (+29%)	**8.5** (+40%)	
Experiment, 425 units	468	244 (+154%)	39 (+300%)	20.3 (+153%)	418 (+200%)	1393 (+200%)	0.3	1450 (+490%)	762 (+240%)	1.9	1655 (+178%)	2020 (+24%)	0.8
					1.71 (+20%)	**5.71** (+20%)		**5.94** (+130%)	**3.12** (+38%)		**6.78** (+10%)	**8.27** (+37%)	

* Total amounts are given in micrograms; amounts per cell micrograms $\times 10^6$. Amounts per cell are set in bold-faced type. Increases are put in parentheses.

units. Nevertheless the subsequent cellular divisions are a limiting factor for the relative increase of cellular mass and cytoplasmic fractions. For example the data in Table IV show that although the total increase in mass of the sedimentable chromidia after administration of 425 units is about four times as great as that after injection of 260 units, the increase per cell is considerably smaller. This is also true of the increase in the mitochondrial fraction.

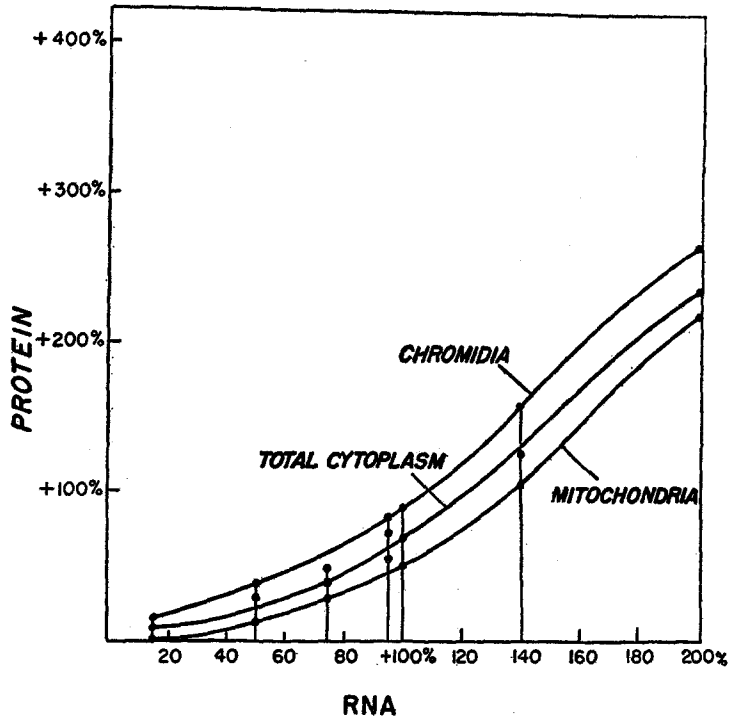


FIG. 2. Relationship between relative increase in cytoplasmic RNA and cytoplasmic fractions in adrenal gland after ACTH administration.

The time curves of the increase in mass of mitochondria and of sedimentable chromidia suggest that the two processes are synchronized. Thus there seems to be no increase in the mitochondrial system without a corresponding increase in chromidia. Only during the first hours after a single injection of ACTH was there difficulty in detecting the formation of mitochondrial substance. In all other instances the increase in the mass of the chromidial system was parallel to an increase in mitochondrial mass and both appeared to depend in the same way on the total increase in cytoplasmic RNA. Two analogous curves were obtained when the increase of mitochondrial and chromidial mass was

plotted against the increase of cytoplasmic RNA (Fig. 2). Apparently the formation of mitochondrial protein is closely related to the increase in cyto-

TABLE V

Effect of ACTH on the Nuclei of the Adrenal Gland

Experiment	ACTH units	Average weight of gland	Total No. nuclei in 12 glands $\times 10^6$	Nuclei per gland $\times 10^6$	Increase	RNA			Nitrogen		
						Total	Per nucleus $\times 10^{-12}$	Increase	Total	Per nucleus $\times 10^{-12}$	Increase
		mg.				*mg.*			*mg.*		
Control	0	11.5	106.8	8.8	0	0.151	0.142	0	0.390	3.7	0
Experiment	25	14.0	106.8	8.8	0	0.153	0.142	0	0.390	3.7	0
Control	0	12.7	120.0	10.0	0	0.111	0.92	0	0.390	3.25	0
Experiment	60	15.6	134.0	11.2	+12%	0.143	1.07	+15%	0.485	6.62	+11%
Control	0	10.1	100.0	8.5	0	0.120	1.2	0	0.260	2.6	0
Experiment	108	18.0	110.0	9.1	+10%	0.168	1.53	+27%	0.365	3.32	+27%
Control	0	14.6	132.0	11.0	0	0.150	1.22	0	0.400	3.03	0
Experiment	260	32.3	165.0	13.7	+25%	0.242	1.46	+18%	0.868	5.38	+77%
Control	0	9.7	96.0	8.0	0	0.111	1.15	0	0.328	3.4	0
I	265	18.5	130.0	10.8	+35%	0.233	1.79	+55%	0.660	5.07	+46%
II	360	23.0	169.0	14.2	+75%	0.235	1.39	+20%	0.767	4.54	+33%
III	428	29.0	224.0	18.6	+133%	0.299	1.24	+7%			

TABLE VI

Effect of ACTH on the Metabolism of Adrenal Gland

ACTH units	Weight of tissue	Total No. of cells in 12 glands $\times 10^6$	Respiration			Anaerobic glycolysis			Succinoxidase pH 7.4		
			c.mm. O_2/hr.	Q_{O_2}	Cell Q_O $\times 10^{-6}$	c.mm. CO_2/hr.	$Q\frac{CO_2}{N_2}$	Cell CO_2 Q_{N_2} $\times 10^{-6}$	c.mm. O_2/hr.	Q_{O_2}	Cell Q_{O_2} $\times 10^{-6}$
	mg.										
0	152	140	133	4.3	0.95	135	4.5	0.96	224	7.4	1.6
110 (4 days)	312	172	214	4.6	1.24	243 (+80%)	4.0	1.4	506 (+126%)	8.1	3.0

plasmic RNA as is also the chromidial protein. A difference should be noted, however, in the behavior of the three separated cytoplasmic fractions after ACTH administration; whereas the basophilic quotient of the chromidial fraction increased significantly, that of the final supernatant seemed to have a

tendency to decrease and the quotient of the mitochondria remained unchanged.

Prolonged administration of ACTH leads not only to cytoplasmic growth but also to more frequent cellular divisions. The data, shown in Table V, indicate changes in mass and RNA content of isolated nuclei. By comparison with Tables III and IV it can be seen that although increases in nuclear RNA and protein content were detected, they occurred at a much slower rate than the corresponding increases in cytoplasmic constituents.

TABLE VII

Succinoxidase Activity of Adrenal Gland after ACTH Administration

Tissue slices, 1 hour, 37°C., 100 per cent O_2.

ACTH units	Time	Weight of tissue	O_2 consumed	pH	Q_{O_2}	Increase
	hrs.	*mg.*	*mm.*			
0	—	142	130	7.1	4.6	—
22.5	7	147	269.1	7.1	9.2	+100%
0	—	41	65	7.4	8.1	—
25.0	5	41	110	7.4	13.7	+69%
0	—	116	268	7.6	11.6	—
25.0	4½	147	426	7.6	14.6	+26%

In order to manifest the increase of succinoxidase activity after ACTH even in short time experiments, the pH of the Ringer solution was raised from 7.1 (7 hours) to 7.6 (4.5 hours). In tissue slices the succinate ions permeate freely into cells at higher pH (optimum pH 8.0). The difference in succinate activity in controls is thus not an expression of different succinoxidase levels but of different working conditions.

Finally one can expect that ACTH will also influence the metabolic activity because it stimulates growth of the cytoplasm. Perry and Cummings (19), using the reduction of tetrazolium salts, observed an increase in succinoxidase activity of the gland following the injection of ACTH. As shown in Table VI the increase in both respiration and anaerobic glycolysis after several injections of ACTH (110 units in the course of 4 days) was of about the same magnitude and approximately parallel to the increase in the weight of the gland. Therefore when comparison was made on the basis of weight only (such as is expressed in Q_{O_2} and $Q_{CO_2}^{N_2}$ values) no significant change was observed. On the other hand the increase in respiration and glycolysis was immediately apparent when comparison was made per cell. Manometric measurements of respiration and succinoxidase activity in adrenal slices after a single administration of ACTH, 4 to 7 hours after injection, showed a con-

spicuous increase in both, against a regular but small increase in weight of tissue. In these experiments the succinoxidase activity usually showed a much higher increase than would correspond either to the increase in the weight of the gland or to the increase in mitochondria (Table VII).

DISCUSSION

The data presented do not establish the identity of the primary reaction which starts the chain of the cytoplasmic growth induced by ACTH. However, they seem to indicate clearly that ACTH acts as a growth factor on the cytoplasm of the adrenal cortex with the *sedimentable chromidia* apparently the main target of this action. This is evident from the fact that the basophilic quotient of the chromidial fraction is the only one which increases strikingly after ACTH administration. The centrifugal force possible with the Servall SS-1 centrifuge could not remove all particulate matter from the final supernatant and thus separate it completely from the macromolecular nucleoprotein of this fraction. Nevertheless the behavior of the basophilic quotient of the final supernatant fraction in ACTH-induced growth may indicate a different role of chromidial and macromolecular nucleoprotein systems. In any case growth of the adrenal tissue induced by ACTH appears initially as an increase of cytoplasmic RNA, followed or accompanied by an increase of chromidial and mitochondrial protein, largely independent of cell division. No effect on nuclear protein and nuclear RNA is detected in the early stages and the enlargement of the gland in the beginning seems to be due to an increase of cytoplasm, without any increase in the number of cells. Later, after repeated administration of ACTH, there is also an increase in nuclear RNA and mass, followed by cellular divisions. These observations indicate that the nucleus may not be the main center for protein syntheses as claimed by Caspersson (20), but that synthesis of cytoplasmic material may depend to a large extent directly on cytoplasmic granules. Moreover there are two observations supporting the view that mitochondria may develop with some dependence on the chromidial system of the cell. These are, first, that the formation of mitochondrial protein is synchronized with the increase of chromidial basophilic quotient, and second, that the increase in chromidial and mitochondrial protein has an analogous form of dependence on cytoplasmic RNA. The clarification of the nature of this dependence is, of course, beyond the scope of methods used in this investigation.

We have seen that the initial phase of this growth process is an increase in amount of cytoplasmic granules, followed by increase in nuclei and cell division. Hence, it is perhaps more accurate to assume that a circular causal mechanism between mutually interdependent systems is active rather than that any particular system of subcellular granules has a directing role in

protein synthesis. As in so many physiological processes, Cannon's concept of homeostasis (21) can also be useful for interpretation of the difference between physiologic and pathologic growth processes. For example the cell divisions which eventually occur may be interpreted as an achievement of a new nucleus-plasma equilibrium disturbed by a physiological stimulus such as, in our experiments, a trophic hormone.

An objection could be raised against any definite interpretation of data related to an average cell on the ground that the basic assumption of even distribution of ACTH action on the cell population may be an oversimplification of events. However, since the gland grows evenly under ACTH stimulation there seems to be no reason to suppose that only certain cells out of the whole population are sensitive to ACTH. Thus it seems that the data related to an average cell give in this instance an adequate, although necessarily a statistical, picture of adrenal growth induced by ACTH.

SUMMARY

It was shown experimentally that ACTH causes an increase of cytoplasmic RNA and subcellular granules of the adrenal cortex shortly after administration. This effect was found to be concentrated primarily on the basophilic cytoplasmic granules and to be largely independent of cell division. The increase of nuclear RNA, DNA, and nuclear mass followed that of the cytoplasm. The observations indicate also that the mass increase of mitochondrial and chromidial systems is closely related to the increase in cytoplasmic, largely chromidial RNA.

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