GROWTH AND DIFFERENTIATION OF MITOCHONDRIA

IN THE REGENERATING RAT ADRENAL CORTEX

A Correlated Biochemical and Stereological Approach

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ABSTRACT

Diameters of the circular profiles of spherical mitochondria in parenchymal cells of the zona fasciculata in rat adrenal cortex were measured for intact controls and for the regenerating adrenal cortex on electron micrographs recorded at random. The diameter data were then processed by Bach's method which deals with the sphere size distribution. The structural parameters of the mitochondria were computed with the aid of an electronic computer. The total number of mitochondria in all the parenchymal cells of the zona fasciculata were calculated. The surface area of the inner mitochondrial membrane was then determined stereologically. Biochemical parameters were obtained for the protein, the phospholipid, and the cytochrome P-450 content, per averaged mitochondrion. The number of cytochrome P-450 molecules contained in the inner membrane was determined in terms of the unit surface area and of the unit amount of phospholipid. These correlated biochemical and stereological parameters have led to the following conclusions. (a) The genesis of the mitochondria after the adrenal enucleation is almost completed within 10 days. (b) During the period of mitochondrial proliferation, the mitochondria are small in size and also immature both in the structure and in the function of their inner membrane. (c) These small and immature mitochondria grow through an increase of the phospholipid and protein, and this increase is accompanied by expansion of the area of the membrane surface. (d) An enrichment of the inner membrane with cytochrome P-450 molecules occurs, thus indicating the differentiation of adrenocortical mitochondria. The process of membrane differentiation is not tightly coupled with that of membrane growth.

INTRODUCTION

In a previous paper (1), two of the present writers (M. Seki and S. Sekiyama) reported on electron microscopical changes found in the organelles of parenchymal cells of the zona fasciculata in the regenerating rat adrenal cortex. In the observations described in the above report, the present investigators focussed attention on the histograms of the mitochondrial frequencies which were grouped as a function of the profile diameter. The histograms seem to indicate that at the fifth and also at the 10th day in tissue regeneration, most mitochondria were small in size, but at the 20th

day, most mitochondria were large in size as contrasted with the mitochondria in the intact controls.

The above finding encouraged the writers to make use of the regenerating adrenal cortex to investigate the current biological problem concerning the growth and differentiation of mitochondria (2, 3). They have accordingly performed a computation by the use of Bach's method for the analysis of sphere size distribution (4) and also with other stereological methods (5, 6), to illustrate quantitatively the possible changes in size and number of the mitochondria in the regenerating rat adrenal cortex. The stereological data concerning mitochondria have been correlated with the biochemical data obtained previously (7) as well as currently. This correlation has shed some light on the kinetics of the growth and differentiation of mitochondria.

MATERIALS AND METHODS

Preparation of the Animal Tissues

Male Wistar rats weighing about 250 g were used. Details concerning the methods of treatment of these animals were the same as described previously (7). More than 20 rats were subjected to the operation for adrenal enucleation in the individual experimental groups. At the fifth, 10th, and 20th day after the operation, the wet-weight of the regenerating adrenocortical tissue was checked in each individual rat. For stereological study, two typical rats representing their respective experimental groups were selected. These two animals were those which possessed regenerating adrenocortical tissue, the weight of which was close to the mean weight. These tissues were those selected for the stereological study. The tissues from the remaining animals were pooled together for each group for the determination of the phospholipid contents in the mitochondrial fraction. As intact controls, three rats were sacrificed for the stereological study and 10 rats for the biochemical study.

Light and Electron Microscopy for the Stereological Study

The adrenal tissue was bisected, and one-half of it was fixed for light microscopy in 4% formalin in 0.1 M sodium phosphate buffer (pH 7.4). Embedded in paraffin, this tissue was sectioned semiserially at 4 μ in thickness with 100-μ intervals between consecutive sections, and then stained with hematoxylin and eosin. 20–30 sections were usually obtained from each half of the bisected tissues. Whole-view photographs of these sections were then taken at a primary magnification of 40 (referred to later as level I).

From the second half of the bisected tissue, 10 tissue blocks, which were made from the tissue of the control rats also, were prepared at random. These tissue blocks were fixed for electron microscopy in Millonig's fixative for 2 hr at 4°C, dehydrated in graded alcohol, and embedded in Epon. Silver-to-grey thin sections were cut through the zona fasciculata with a Porter-Blum MT2 ultramicrotome. Electron micrographs were recorded at primary magnifications of 1000 (level II), 2000–4000 (level III), and 10,000 (level IV). 10 tissue blocks from one of the three intact control rats were fixed in 1.5% glutaraldehyde in 0.1 M sodium phosphate buffer (pH 7.4) for 2 hr at 0°C. They were next transferred into a second solution of 1% osmium tetroxide in the same buffer of 0.1 M sodium phosphate (pH 7.4) as used above, and then processed in the same manner as the other tissue blocks mentioned above.

Stereological Sampling Levels and Computations

Four sampling levels were taken essentially in accord with the method of Weibel et al. (5).

LEVEL I: The volume fractions of the histological components of adrenal gland, viz. the zona glomerulosa, zona fasciculata, zona reticularis, and medulla, were measured by the point-counting volumetry (5) on the histological photographs at level I. Each photograph was entirely covered by a point net.

LEVEL II: The planimetric method was used to estimate the volume of the intercellular space and also the space occupied by the nuclei of the parenchymal cells in the zona fasciculata. 10 micrographs at level II were used for the measurements of every group of animals. The combined volume fractions for both the intercellular and the nuclear space have been subtracted from the volume for the whole zona fasciculata, which volume was normalized to 1.00, to give the volume fraction for the cytoplasm of parenchymal cells.

LEVEL III: The shape of the mitochondria in the parenchymal cells of the zona fasciculata in rat adrenal cortex in the present study was assumed to be essentially spherical, because ultrathin sections of these mitochondria show, under the electron microscope, either circular or elliptic profiles with an axial ratio near unity (1, 8, 9–11). On this assumption, diameters of more than 500 mitochondrial profiles were measured on the electron micrographs at level III, both for the three individual control animals and for each of the experimental groups.

When mitochondrial envelope contours were not clearly recognizable due to oblique sectioning, the diameter was then measured to the outermost visible density. Even when this was done, few of the mitochondrial profiles having diameters shorter than 0.2 μ could be detected. Theoretical corrections for the

smallest, unobservable image were then made according to Bach (4). When elliptic profiles with axial ratios smaller than 1.25 were detected, the arithmetic means of both the major and the minor axes were taken as the circle diameter. Elongated profiles (defined in the present study as those profiles which have axial ratios larger than 1.26) were occasionally discovered at the 5th day of regeneration, but were not included in the diameter measurements. The validity for assuming that the mitochondria in the parenchymal cells of the zona fasciculata were spheres and the validity of the sampling procedures were checked by determining the distribution of axial ratios.

Using the pooled raw data of circle diameters, a computation was performed with an electronic computer (TOSBAC 3400) on a program drawn up according to Bach's method. In brief, the formulae used were as follows,

$$N_v = \frac{1}{T} \sqrt{\frac{2}{\pi} \sum_{i=0}^{n} p_i f\left(\sqrt{2\pi} \frac{r_i}{T}\right)},$$

$$\bar{R} = \frac{1}{2}\left(\frac{N_A}{N_V} - T\right),$$

$$\bar{V} = 2T^2\bar{R} + (2\bar{R} + T)(\pi[S^2 + \bar{r}]^2 - 2T\bar{r}),$$

$$\bar{S}_o = 8\left(\frac{N_A}{N_V}\bar{r} - T\bar{R}\right),$$

and $V_v = N_v \cdot \bar{V}$,

where N_v is the number of mitochondria per 1000 μ^3 of cytoplasm, $\bar{R}$ the mean radius in μ, $\bar{V}$ the mean volume in μ^3, $\bar{S}_o$ the mean surface area of the outer envelope membrane, V_v the volume fraction occupied by the mitochondrial population in the cytoplasm, T the section thickness, π the circular constant, p_i the observed frequency of circles within the i-th class of circle radius of r_i, $f\left(\sqrt{2\pi}\dfrac{r_i}{T}\right)$ a function related to the Gauss error function, $\bar{r}$ the mean of observed circle radii, and s^2 its variance. The section thickness T was set at 0.06 μ as a constant in the formulae, since the micrographs were recorded on silver-to-grey thin sections (12).

The size distribution of spherical mitochondria as a continuous distribution was obtained by the use of the following equation:

$$N_v = \sum_{i=0}^{n} \frac{p_i - 2h_i \sum_{i=i+1}^{n} p_j \beta_{j,i}}{T + 2h\beta_{i,i}},$$

where P_i is the calculated theoretical frequency of spheres within the i-th class of sphere radius, h the width of radius class, and the parameters, $\beta_{j,i}$ and $\beta_{i,i}$, the constants to be determined for each of the grouped classes (4).

The mitochondrial volume fraction V_v was determined also by point-counting volumetry applied to the micrographs at level III and referred to later as the V_v (point).

LEVEL IV: In the present study the outer mitochondrial membrane was defined as the outer envelope, while the inner membrane was defined as the inner envelope and the cristal membranes, the latter being often identified as vesicular or tubular cristae. These definitions are consistent with studies made by Yoshimura et al. (8) and by Satre et al. (13). The average surface area of the inner envelope was thus assumed to be the same as that of the outer membrane ($\bar{S}_o$).

The average surface area of the inner membrane was determined according to Baudhuin and Berthet (cf. equation 7 of reference 6). In brief, the procedure used here was as follows. 50 mitochondrial profiles at level IV were used for counting the number of intersections between the outer membrane and the grid lines, the latter being superimposed on the micrographs. The same 50 profiles were also used for counting the number of intersections between the cristal membrane and the grid lines. The ratio of the number of intersections for the cristal membrane to the number for the outer membrane was then determined. The average surface area of the cristal membrane was calculated by multiplying the mean of the ratio by the $\bar{S}_o$-values which were obtained independently at level III.

Biochemical Methods

The phospholipid contents were determined in the mitochondrial fraction which was isolated from the tissue homogenates as previously described (14). Phospholipid was extracted according to the method of Fleischer et al. (15), and the amount of lipid phosphorus was determined according to Inoue and Oikawa (16). The amount of phosphorus was multiplied by a factor of 25.3 to obtain the amount of phospholipid. The protein was determined by the method of Lowry et al. (17).

RESULTS

Cytoplasmic Volume

The volume fractions of the histological components of all zones, except the zona fasciculata, contribute only in a limited extent to the composition of adrenal cortex in both the intact controls and the regenerating tissues (Table I). By use of the planimetric method applied to the micrographs at level II, it was learned that about 85% of the volume of the zona fasciculata was occupied by the cytoplasm of parenchymal cells throughout the

TABLE I

Volume Fractions of Histological Components of Adrenal Gland in Intact Controls and in Adrenal-Enucleated Rats

Values were obtained by the point-counting volumetry at level I.

Values shown in the parentheses indicate the volume fractions of cortical components in the adrenal cortex which is normalized as 1.000.

Histological components	Intact controls	Days of regeneration		
		5	10	20
Whole gland	1.00	1.00	1.00	1.00
Capsule	0.002	0.347	0.155	0.044
Zona glomerulosa	0.190 (0.193)	0.0 (0.0)	0.083 (0.098)	0.084 (0.088)
Zona fasciculata	0.650 (0.677)	0.653 (1.00)	0.762 (0.902)	0.869 (0.912)
Zona reticularis	0.115 (0.120)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)
Medulla	0.040	0.0	0.0	0.0

TABLE II

Volume of Cytoplasm in All the Parenchymal Cells of Zona Fasciculata

Parameters	Units	Intact controls	Days of regeneration		
			5	10	20
Adrenal weight*	mg/head	33.4±1.15 (13)	11.1±0.77 (27)	17.3±0.85 (22)	21.0±0.98 (22)
Volume of zona fasciculata	μ^3/head	21.7×10^9	7.2×10^9	13.2×10^9	17.9×10^9
Volume of cytoplasm of all the parenchymal cells	μ^3/head	19.0×10^9	6.3×10^9	11.2×10^9	15.5×10^9

* Mean ± standard error of the mean. Number of animals in each individual group is shown in the parentheses.

duration of tissue regeneration (87.5% in intact controls, 87.3% in the experimental group at the 5th day, 85.1% at the 10th day, and 85.0% at the 20th day, respectively). The total cytoplasmic volume of all parenchymal cells in the zona fasciculata per individual rat was determined as follows: the average wet-weight of the adrenal gland was multiplied by the value of the volume fraction of the zona fasciculata shown in Table I, and this product was multiplied by the value of the cytoplasm in the parenchymal cells just mentioned above, thus yielding the total cytoplasmic volume listed in Table II. In these calculations the specific weight of the tissue components was assumed to be unity. It is to be noted here that the time-course of adrenal weight recovery in the present study was almost the same as that reported previously (7).

Geometrical Parameters of Mitochondria and Validity of Computation

Table III summarizes the results of the computation of the geometrical parameters of the mito-

chondria. Some comments seem to be necessary with regard to the validity of the use of Bach's method. The present writers wish to enumerate their comments but they are restricting them to the mitochondrial population of the 5th day of regeneration, because on this 5th day some elongated mitochondria were scattered among the spherical ones, thus presenting a most complex situation among the animal groups.

The writers have classified the mitochondrial profiles at the 5th day period into several groups according to their axial ratios. The profiles with axial ratios between 1.00 and 1.05 (referred to later as circular) constituted 60% of the total profiles of 523, those with ratios between 1.06 and 1.15 (practically circular) 24%, those with ratios between 1.16 and 1.25 (elliptic) 11%, and those with ratios larger than 1.26 (elongated) 5%. The largest axial ratio was about 2.0 in the present study.

According to the nomograph presented by Hennig and Elias (18), when oblong ellipsoids with the axial ratio of 2.0 are cut at random, those ellipses with axial ratios larger than 1.26 constitute

TABLE III

Number and Size of Mitochondria in Intact Controls and in the Regenerating Rat Adrenal Cortex

Parameters	Intact controls	Days of regeneration		
		5	10	20
N_v	716	764	547	291
$\bar{R}$ (μ)	0.476 ± 0.0047	0.348 ± 0.0047	0.413 ± 0.0065	0.579 ± 0.0069
$\bar{V}$ (μ^3)	0.580 ± 0.021	0.258 ± 0.0096	0.364 ± 0.0093	0.983 ± 0.0364
V_v	0.416	0.197	0.199	0.286
V_v (point)	0.370	0.220	0.235	0.297

Values shown in this table have been obtained by Bach's method, except those of V_v (point) which have been obtained by the point-counting volumetry. The N_v-values have been expressed in terms of the number of mitochondria per 1000 μ^3 of the cytoplasm of the parenchymal cells in the zona fasciculata. The values for $\bar{R}$ and $\bar{V}$ indicate the mean ± standard error of the mean calculated on the basis of the corresponding N_v-values (see Bach [4] for details of these calculations). All the differences between $\bar{R}$- and $\bar{V}$-values were statistically significant ($p < 0.05$) when tested by Cochran's correction of the t test (19). The variability within group was estimated in the control group by the use of the values computed separately for each of the three intact rats, and the results were as follows: N_v, 694 ± 19; $\bar{R}$, 0.469 ± 0.014 μ; and $\bar{V}$, 0.617 ± 0.026 μ^3 (mean ± standard error of the mean). Then, all the diameter data of the three control rats were combined to obtain the values listed in this table. Mean values thus obtained are slightly different from those listed just above due to an additional correction for the smallest, unobservable profiles that was necessary when the data were combined.
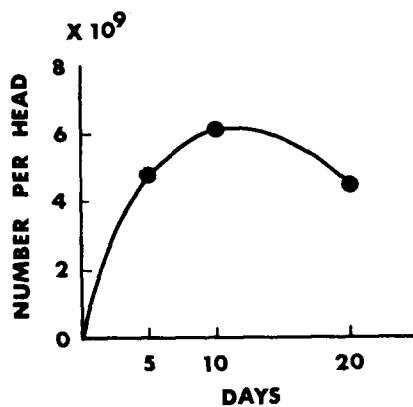

FIGURE 1 Mitochondrial proliferation during adrenocortical regeneration. The mean value for the total number of mitochondria in all the parenchymal cells of the zona fasciculata in an intact individual rat was 13.6 × 10⁹.

about 65% of the total section profiles and those with smaller ratios about 35%. In other words, the frequency of ellipses with axial ratios smaller than 1.26 would be only a little more than half the frequency of those with larger axial ratios. In view of this, the degree of contribution of elongated mitochondria at the 5th day to the elliptic or practically circular profiles may be considered negligible, and so no correction was made for the diameter data with regard to the elongated mito-

chondria. No further study of the biological significance of the elongated mitochondria was made.

As mentioned earlier, the mean of major and minor axes was taken as the diameter for the elliptic or practically circular profile. This procedure was expected to result in a fair approximation, because some artifacts, e.g. the possible local compression of ultrathin sections, may be responsible, at least partly, for the observed frequency of elliptic profiles. Further, the volume fraction of mitochondria in the cytoplasm determined by the point-counting method was roughly comparable with the corresponding value obtained by Bach's method as shown in Table III. This favors the assumption that the mitochondria are essentially spherical. It is therefore considered that Bach's method may be applie tdo the writers' objectives.

Proliferation and Size Distribution of Mitochondria

The total number of the mitochondria in all the parenchymal cells of the zona fasciculata of adrenal cortex in the individual rat has been calculated by multiplying the total cytoplasmic volume by the N_v-values, as shown in Fig. 1. The mitochondrial proliferation seems to continue up to the 10th day.

The dotted histograms in Fig. 2 show the size distribution of the reconstructed mitochondria in
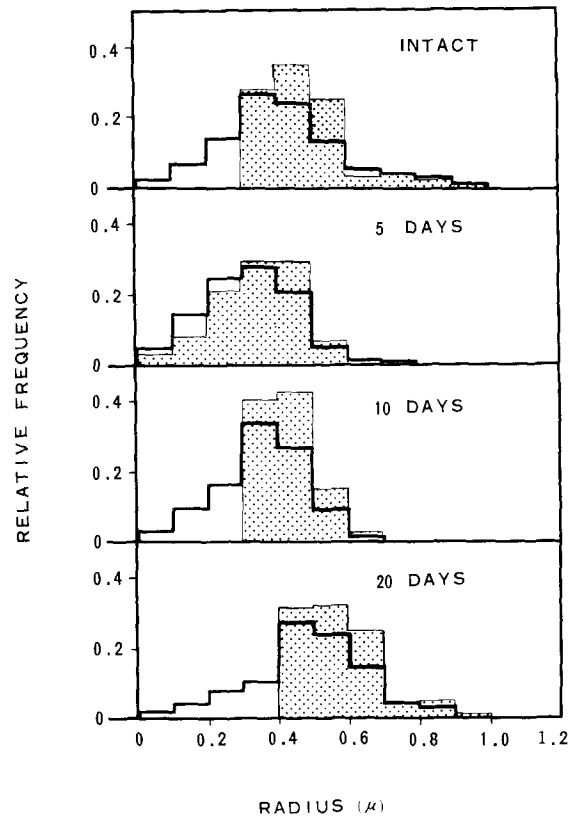
FIGURE 2 Size distribution histograms for the reconstructed mitochondrial population in intact controls and in the regenerating adrenal cortex. The histograms with heavy lines show the relative frequency of circles of mitochondrial profiles on the electron micrographs, the values being corrected on a theoretical basis (see text). The dotted histograms show the relative frequency of three-dimensionally reconstructed mitochondria as the function of radii. The histograms have been constructed on the combined data taken from three rats in the control or from two rats in the experimental groups.

terms of relative frequency as a function of the radius. In a particular case the size distribution of the spheres can be obtained by multiplying the distribution of observed radii of circles by a constant factor. The common distribution between the above two was termed "self-reproducing distribution" by Bach (4). The x^2-test for a consistency between the histograms with heavy line in Fig. 2 and the theoretical ones (not shown in this figure) computed from the self-reproducing distribution indicated that the discrepancy between them was rather large. Thus, the self-reproducing distribution is excluded from the possible types of distribution of mitochondria.

Biochemical Parameters for the Single Mitochondrion

The calculations shown in Table IV were made on the following assumptions. (a) The amounts of

mitochondrial protein in the zona fasciculata have been calculated by multiplying the mitochondrial protein recovered from the homogenates of whole gland (but without capsules) by the corresponding values on the histological volume fraction (cf. values in the parentheses of Table I). (b) It could be assumed that cytochrome P-450 can not be localized in significant amounts in adrenal medulla (20). Although there have been reported some differences in the concentration of this cytochrome in the mitochondria prepared from the different histological layers in ox- and pig-adrenal cortex (20), the concentrations of this cytochrome in reference to mitochondrial protein have been assumed to be the same throughout the layers of adrenal cortex. (c) Similarly, the concentration of mitochondrial phospholipid per unit amount of protein has been assumed to be uniformly homogeneous throughout the adrenal cells.

TABLE IV

Amounts of Protein, Phospholipid, and Cytochrome P-450 in an Averaged Mitochondrion

Parameters	Units	Intact controls	Days of regeneration		
			5	10	20
Protein					
Average amount for whole adrenal*	μg/mg	23.0	5.4	15.7	14.4
Average amount in the individual rat	μg/head	768	59.9	272	300
Amount in the zona fasciculata	μg/head	500	59.9	245	274
Amount in an averaged mitochondrion	μg	36.8×10^{-9}	12.5×10^{-9}	40.2×10^{-9}	60.9×10^{-9}
Phospholipid					
Ratio to protein	—	0.355	0.273	0.301	0.294
Amount in an averaged mitochondrion	μg	13.1×10^{-9}	3.4×10^{-9}	12.1×10^{-9}	17.9×10^{-9}
Cytochrome P-450					
Concentration*	mμmoles/μg protein	1.68×10^{-3}	0.09×10^{-3}	0.84×10^{-3}	1.01×10^{-3}
Amount in the adrenal cortex	mμmoles/head	1.29	0.0054	0.23	0.30
Amount in the zona fasciculata	mμmoles/head	0.87	0.0054	0.21	0.27
Amount in an averaged mitochondrion	mμmoles	64.0×10^{-12}	1.1×10^{-12}	34.4×10^{-12}	60.0×10^{-12}
Number of molecules in an averaged mitochondrion	—	38,500	660	20,700	36,000

* These values have been taken from our previous paper (7).

Based on any of these assumptions the values for the regenerating rat adrenal cortex would be reasonable, because the zona fasciculata in these tissues occupies the overwhelming portion (90–100%) of the adrenal cortex as seen in Table I. Moreover, the differences between the results of these calculations are large enough to exceed significantly the limitations due to the errors occasioned by these assumptions.

Surface Area and Cytochrome P-450 Content of Inner Membrane

An increase in the surface area of the outer and inner membranes per averaged mitochondrion was clearly detected at level IV (Table V). The number of molecules of cytochrome P-450 per unit surface area of the inner membrane at the 5th day was only about 6% of the intact control level (Table VI). Apparently, this value then quickly increased to the same percentage as in the intact control level at the 10th day, followed by a decrease until the 20th day. If the number of molecules is expressed in reference to the unit-amoutn of phospholipid, the degree of loading of cyto-
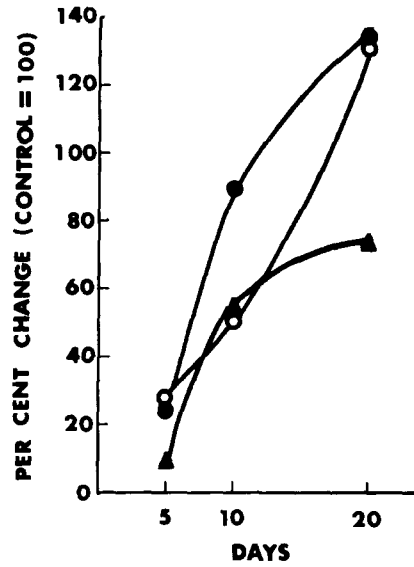

Figure 3 A comparison between membrane growth and differentiation in an averaged mitochondrion. ——●——, amount of phospholipid; ——O——, surface area of the whole inner membrane; ——▲——, the cytochrome P-450 content per unit-amount of phospholipid.

Table V

Measurement of Inner Membrane Surface Area in an Averaged Mitochondrion

		Days of regeneration		
Parameters	Intact controls	5	10	20
Inner envelope (same as $\bar{S}_o$) (μ^2)	3.108	1.765	2.328	4.521
Intersection ratio*	7.95 ± 0.40	3.59 ± 0.19	5.38 ± 0.40	7.17 ± 0.40
Surface area of cristal membrane (μ^2)	24.7	6.34	12.5	32.4
Total surface area of inner membrane (μ^2)	27.7	8.1	14.8	36.9

* Ratio of the number of intersections occurring between the superimposed grid lines and the cristal membrane to those occurring between the grid lines and the outer envelope membrane. Mean ± standard error of the mean.

Table VI

Cytochrome P-450 Content in the Inner Membrane

			Days of regeneration		
Parameters	Units	Intact controls	5	10	20
Number of molecules per unit surface area	μ^{-2}	1390	81	1400	980
Number of molecules per unit amount of phospholipid	$(10^{-9} \mu g)^{-1}$	2940	190	1700	2010

chrome P-450 at the 10th day is then shown to be far below the control level. In Fig. 3 the per cent of increase in the inner membrane surface area and of the phospholipid in a single mitochondrion are compared with the per cent of increase in the cytochrome P-450 content per unit-amount of phospholipid.

DISCUSSION

Statistical Evaluation of Results

The statistical control of the present data, especially of the geometrical parameters presented in Table III, raises some difficulties because of the limited number of animals subjected to the stereological analysis. The variability due to individual animals within a group was estimated only for the control group (see the values listed in the legend to Table III) but not for the experimental groups. Therefore, the significance of the differences in the mean values between groups could not be established statistically with respect to the variability of individual animals. The only statistical evaluation that could be made in the present study was on the differences between the mitochondrial populations as shown in Table III. A second difficulty arises from the fact that tissues for the stereological analysis in the experimental groups had been selected from near the mean weight of the observed weight increase in the regenerating adrenals.

In spite of these difficulties all differences in the mean values found among all animal groups could be expected to be large enough to exceed significantly the variability within the control group, because this variability was minimal whatever fixative was used. However, the writers are still aware that some reservations about drawing any conclusions from the present study are in order. They are aware too that a rigorous procedure of random sampling would be required for studies of a more detailed character. In view of the above, the writers are here pointing out only those aspects of their results that involve qualitative large changes.

Proliferation of Mitochondria

The mitochondrial proliferation apparently becomes completed earlier than the increase in the cytoplasmic volume (compare Table II and Fig. 1). The increase in the cytoplasmic volume may represent, at least partly, the rate of cellular proliferation, because the amount of DNA in the

regenerating adrenal cortex has been known to increase during the 20 day period (7). Speculation, therefore, can be made that the mitochondrial proliferation proceeds at a rate higher than the rate of cellular proliferation. The fact that the N_v-value was quite high at the 5th day yet considerably low thereafter is in concordance with this speculation. Nevertheless, further details on the kinetics of mitochondrial proliferation are yet to be studied.

The Small-Sized Mitochondria

The geometrical parameters that describe the size of reconstructed mitochondria have been found to be very small during the mitochondrial proliferation period (Table III). The average amounts of total protein and phospholipid per individual mitochondrion are also significantly lower than in the intact controls (Table IV), and the inner membrane is not fully developed with reference to either its surface area (Table V) or cytochrome P-450 content per unit-amount of phospholipid (Table VI). These sets of evidence indicate that mitochondria are considerably small in size with not fully developed membrane structures during their proliferation period. Indeed, more than 30% of the members of the mitochondrial population at the 5th day consisted of the smallest types with radii shorter than 0.3 μ (Fig. 2). Corresponding to these facts would be the functional aspect of a very low level of cholesterol sidechain-cleavage during the corresponding experimental period of tissue regeneration, and also the considerably low level of plasma corticosterone after the excessive doses of adrenocorticotropin (ACTH) administered to the adrenal-enucleated rats (7).

Mitochondrial Growth

That these smaller mitochondria do grow to a larger size is evidenced from the continuous increases in the total amounts of protein and phospholipid in an averaged mitochondrion. The mitochondrial growth is observable during and after the mitochondrial proliferation period; the averaged geometrical parameters at the 20th day overshot the control level (Table III). In addition, the size distribution histograms for mitochondria have clearly shown a shift of the whole population to the right, along with the period of tissue regeneration (Fig. 2). The smallest mitochondria detected at the 5th day grew at the fastest rate, and

their rapid disappearance from the size distribution histogram reflects the peak with its relative frequency of more than 0.40 at the 10th day, the latter being the highest of all histograms throughout the animal groups.

The increase in the amount of total phospholipid per single mitochondrion is roughly ascribed to the increase in the amount of inner membrane phospholipid, since the outer membrane contributes only about 10% of the total surface area of the whole mitochondrial membrane (Table V). Thus, the per cent changes in phospholipid content and total surface area of the inner membrane should be parallel with one another throughout the membrane growth process. However, this was not the case at the 10th day, when the per cent of phospholipid content greatly exceeded the degree of increase in the membrane surface area (Fig. 3). Thus, it is possible that the membrane growth observed here is a complex process involving phospholipid loading up to the preexisting membrane followed by membrane expansion.

The phospholipid-to-protein ratio in mitochondria has been shown to be modified by environmental conditions by Luck (21) in experiments with *Neurospora*. Indeed, this ratio was subjected to a considerable change in the adrenocortical mitochondria during their proliferation and growth periods (Table IV).

Mitochondrial Differentiation

The establishment (13, 22) of the writers' finding, viz. that the inner membrane contains cytochrome P-450 as one of the constituent components of the steroid hydroxylating enzyme system (23), substantiates that the increase in the amount of this cytochrome in the averaged mitochondrion (Table IV) may be taken as an indication of the enrichment of the enzyme system. The amount of cytochrome *a* (24) and the degree of activity of 2,4-dinitrophenol–stimulated ATPase (25) have been known to be minimal in adrenocortical mitochondria. Further, it is quite difficult to isolate steroid hydroxylases at present. Therefore, cytochrome P-450 (per unit-amount of phospholipid) is considered to be one of the best materials for following the study of the inner membrane differentiation. It is to be noted that the membrane differentiation as defined here is not tightly coupled with the process of membrane growth (Fig. 3).

In conclusion, the present results seem to be consistent with the hypothesis that the inner mito-chondrial membrane first grows by incorporating phospholipid into the preexisting membrane and then, secondly, differentiates by assembling the constitutive functional molecules, as suggested by Getz (26) in a recent review article on lipids in membrane development.

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