QUANTITATIVE CHANGES IN MOUSE LIVER ULTRASTRUCTURE FOLLOWING CORTISONE AND INSULIN ADMINISTRATION

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Summary

Linear analysis of whole cell montages provided the basis for determining the following in mouse liver parenchymal cells: the cytoplasmic volume fraction of mitochondria, lysosomes, lipid, glycogen, and cytoplasmic matrix; the membrane profile concentration; and mitochondrial number and dimensions (including a shape coefficient). Included are a number of studies of the validity of the methods used, some new statistical considerations, and a new, more objective method of determining mitochondrial shape.

The ultrastructural changes in liver parenchymal cells following cortisone and insulin administration and in subsequent recovery periods following the cessation of hormone treatment were defined. Observed decreases in the mitochondrial volume fractions in the cortisone-treated series were no longer evident when corrections were made for the observed cell volume changes. Lipid and glycogen accumulation, which was responsible for cell hypertrophy, was observed in most experimental groups. Decreased membrane profile concentrations were originally observed in most groups; correction of these values for cell hypertrophy, however, showed an initial increase in this parameter after cortisone treatment which was then followed by a decrease after a 3-day recovery period. The mean mitochondrial volume increased up to fourfold in the cortisone-treated series and to a much lesser extent in the insulin-treated series. The increase in mean mitochondrial volume was balanced by a decrease in the number of mitochondria per cell such that the total cell mitochondrial volume remained relatively constant. It is suggested that the changes in mitochondrial volume and number were a result of mitochondrial fusion. These changes were temporary. Ten days after the hormone treatment the cells were only slightly different from the control cells.

I. Introduction

It is only relatively recently that the methods of “linear analysis”, which have been used for a long time for the analysis of petrological sections, were applied to the cytometric analysis of electron micrographs.

The aim of the methods as applied to electron micrographs is to evaluate in situ the relative (or absolute) volume of the different cytoplasmic components. In early studies, only the cytoplasmic volume fraction of mitochondria was estimated (Clawson et al. 1958; Hudson, Lazarow, and Hartmann 1961). Loud, Barany, and Pack (1964) presented methods based on linear analysis of electron micrographs for the determination of mitochondrial dimensions and endoplasmic reticulum concentration as well as the volume fraction of cytoplasmic components.

Much interest has been concentrated on mitochondrial dimensions and number per cell. The methods used for the estimation of these parameters have varied. The use of in situ analysis of electron micrographs has been limited. Isolated liver mitochondria in suspension have been examined by phase-contrast microscopy (Novikoff 1957), by use of a Coulter particle counter (Glas and Bahr 1966), and by

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analysis of micrographs of these isolated mitochondria (Baudhuin and Berthet 1967) by the method of analysis proposed by Wicksell (1925). In such preparations, heterogeneous cell populations including not only hepatic parenchymal cells but also extraparenchymal cells such as endothelial cells, Kupffer cells, bile duct cells, and blood cells have been considered; hence the variation in any parameter from parenchymal cell to parenchymal cell cannot be calculated. Loud and co-workers have been the main exponents of in situ analysis of electron micrographs of not only normal livers but also experimental livers (Loud 1961, 1962, 1968; Loud, Barany, and Pack 1964; Kimberg, Loud, and Wiener 1968; Wiener et al. 1968). Their methods of determining mitochondrial dimensions depend on a priori assumptions about mitochondrial shape.

The major problem in determining the dimensions of any discrete organelle is the adoption of an adequate shape model. Biological variation can give a non-uniformity in the shape of organelles. The model adopted has to account for the majority of mitochondria and give a minimal error in the calculation of the mean mitochondrial volume in any cell. In the present study, a shape coefficient was determined for mitochondria using the mean length of sampling line traversing the mitochondria. With linear analysis this is the only parameter readily obtainable from the micrographs. This shape coefficient estimate obviates an a priori assumption of mitochondrial shape and allows a more accurate determination of the mitochondrial dimensions. The calculation of such a shape coefficient does not, unfortunately, eliminate all the problems inherent in estimating three-dimensional parameters from two-dimensional micrographs of biological material.

In the present study, changes in the volume fraction of cytoplasmic components, mitochondrial dimensions, and membrane profile concentration were determined in mouse liver parenchymal cells following cortisone and insulin administration and in subsequent recovery periods following cessation of hormone treatment. Wherever possible, standard errors were calculated for the various estimated parameters in each cell to indicate the variability in each cell of the parameter considered. The results thus provide a basis for determining variations within and between the different experimental groups.

II. Materials and Methods

(a) Treatments

Liver samples from the following experimental groups of female albino mice fed ad libitum were used in this study:

(1) Control mice;
(2) CR1 mice—these received daily subcutaneous injections of 2.5 mg cortisone acetate (Merck, Sharp, & Dohme; 25 mg/ml in saline suspension) for 4 days, and were killed 24 hr after the final injection;
(3) CR3 mice—received 4-day cortisone treatment as described above but were allowed a 3-day recovery period before being killed;
(4) CR5 mice—4-day cortisone treatment with a 5-day recovery period;
(5) CR10 mice—4-day cortisone treatment with a 10-day recovery period;
(6) IR1 mice—received daily subcutaneous injections of 0.5 units of protamine zinc insulin (Commonwealth Serum Laboratories; 40 units/ml diluted with 0.85% NaCl immediately prior to injection) for 2 days, and were killed 24 hr after the final injection;
(7) IR3 mice—2-day insulin treatment as described above but allowed a 3-day recovery period before being killed;
(8) IR10 mice—2-day insulin treatment with a 10-day recovery period.
(b) Electron Microscopy

Tissue pieces no larger than 1 mm$^3$ were fixed at 4°C for 2 hr in Palade’s fluid. Sections with silver interference colours were cut with an LKB ultratome. They were stained on the grid for 1 hr with uranyl acetate (saturated solution in 50% ethanol) and examined under an Hitachi HS7 electron microscope.

(c) Quantitative Analysis of Cytoplasmic Components

Whole cell montages with a final magnification of 14,040 or 18,900 were used for the quantitative analysis of cytoplasmic components. Selection of a cell for this quantitative analysis required that the nucleus be present and not be obviously tangentially cut (as judged by diameter and by the appearance of the nuclear envelope). Ten liver parenchymal cells of each experimental type were chosen according to these criteria so that no more than three cells were taken from any one liver block and at least four livers in each series were represented. There was no indication from the surrounding components in the sections that any of the 10 cells considered in each type were immediately adjacent to either central or portal veins but the proximity of these cells to central and portal veins above and below the plane of section was not known. The assumption that the cells studied were distributed mainly in the mid-regions of the lobule was thus only moderately certain. Sampling lines consisting of parallel lines 20 mm apart, which provided the basis for estimating the volume fraction of cytoplasmic components, mitochondrial dimensions, and endoplasmic reticulum concentration, were drawn onto the montages in both the horizontal and vertical direction. This method of linear sampling is not random in the true sense but rather is systematic with a random start. The validity of this type of sampling was investigated in relation to the randomness of the sampling and its reproducibility.

(i) Volume Fraction of Cytoplasmic Components

The volume fraction composition of the cytoplasm was estimated from the fraction of the total sampling line that passed over the various cytoplasmic components. The cytoplasmic components measured in this way were mitochondria, lysosomes, lipid, and glycogen. Glycogen-containing areas were defined as cytoplasmic regions which were relatively devoid of other cytoplasmic components. There was normally no trouble in the subjective definition of the edges of such areas. The main bulk of the cytoplasm exclusive to these formed bodies was termed cytoplasmic matrix. It consisted of rough and smooth endoplasmic reticulum, ribosomes, and ground cytoplasm. The volume fraction of this cytoplasmic matrix was calculated by difference after determining the volume fraction of the other cytoplasmic components.

The adequacy of the sampling method for determining the volume fraction of the cytoplasmic components was evaluated by determining the volume fraction of mitochondria in a cell by both systematic and more randomized sampling. For a random sample, parallel lines were drawn in one direction only onto the cell montage at 2-mm intervals and numbered consecutively. Twenty-five of these numbered lines were then chosen at random using a table of random numbers. The cytoplasmic volume fraction of mitochondria was determined from these 25 lines and compared with that obtained by the systematic sampling procedure on lines 20 mm apart. There was very little difference in the mitochondrial volume fraction determined by the two methods (random sample $0.1251 \pm 0.0142$, cf. systematic sample $0.1319 \pm 0.0110$, the standard error being determined as for a ratio of normal variates as described below).

The reproducibility of the sampling method was further examined by placing a grid of parallel lines (20 mm apart) onto the cell montage at various orientations from 0 to 180° at 30-degree intervals. The data showed no significant difference in the mitochondrial volume fraction at the various grid positions. The mean volume fraction of these six positions was $0.1491 \pm 0.0077$. The coefficient of variation of this mean was 5·20%, indicating a high reproducibility.

The standard error of the mitochondrial volume fraction in each cell was estimated as for a ratio of normal variates (Quenouille 1952). A value for the approximate standard deviation of the mitochondrial volume fraction, $p$, in each cell is given by:

$$S.D. (p) = p ((V_x\bar{\bar{x}} + (V_y\bar{\bar{y}}) - [Cov(x,y)(\bar{\bar{x}})])^{1}$$
where $x$ is the length of the individual sampling lines traversing mitochondria, $y$ is the length of the individual sampling lines, and $V$ and Cov are variance and covariance respectively. The mitochondrial volume fraction, $p$, is $\Sigma x/\Sigma y$.

(ii) Mitochondrial Dimensions

The equations which are used in the definition of the dimensions of mitochondria are contingent upon shape assumption. Shape characteristics can be determined empirically from the transection lengths in the electron micrographs. For bodies of any shape, providing they are not re-entrant, a shape characteristic $Q$ is defined as

$$ Q = \frac{(\bar{x}^2)}{2(\bar{y})^2}, $$

where $\bar{t}$ in this context is the length of the sampling line traversing the individual mitochondria (Case, De Hoffman, and Placzek 1953). For various shapes, $Q$ has a specific value; for example, for spheres $Q = 0.5625$, for cylinders of infinite length and circular cross section $Q = 0.6667$, and for oblate spheroids $Q = 0.28125 \log_e(2a/b)$ where $a$ and $b$ are major and minor axes respectively.

Mitochondrial dimensions were evaluated by equations presented by Case, De Hoffman, and Placzek (1953). For spheres of radius $a$, the mean transection diameter ($\bar{t}$) = $4a/3$, thus the diameter $(2a) = 3\bar{t}/2$. The mitochondrial volume then is $4\pi a^3/3$. These calculations give the mean diameter and volume of mitochondria in any one cell. The standard error of mitochondrial diameter and volume for an estimate of within-cell error in these parameters may be determined as follows (Robinson, personal communication). The variance of the radius $(Va)$ is $(0.75)^2 (Vf)$. The standard error of the mitochondrial diameter then is twice that for the radius $a$. The variance of the volume $(V)$ is $16\pi a^4(Va)$; the standard error then being $4\pi a^2$(S.E. of $a$). These variance estimates are based on the normal distribution of the transection diameter ($\bar{t}$). A normal distribution of the transection diameters in cells was in fact observed; for control cell 1, for example, $x^2$ calculated to test the "goodness of fit" of the observed distribution with the theoretical normal distribution was $14 \cdot 80$ (13 degrees of freedom, $0 \cdot 40 > P > 0 \cdot 30$), indicating a normal distribution of this parameter.

The number of mitochondria $(N)$ per unit volume of cytoplasm was estimated from $N = p/V$ where $p$ is the mitochondrial volume fraction of the cytoplasm and $V$ is the mean mitochondrial volume. Cell volume necessary in the estimation of total number of mitochondria per cell was determined from light microscopic preparations stained in Groat's haematoxylin (20 min) and 0.1% acid fuschin in 0.1n HCl (3–4 min) followed by three washes in 0.1n HCl. The volume of the cells was calculated for prolate ellipsoids ($V = \pi ab^2/3$, where $a$ and $b$ are minor and major diameters respectively). The use of light microscopic preparations for volume determination was necessitated by the small number of cells which can be sampled in electron microscopic preparations and by the virtual impossibility of determining section plane in such thin sections. Large numbers of cells must of course be studied to determine volumes because of the inhomogeneity of the cell volumes in rodent liver as a consequence of the presence of different ploidy classes. Cell shrinkage, which may reach 50%, was not taken into account in the volume calculations; but since this is probably a systematic error the comparisons remain valid.

(iii) Endoplasmic Reticulum Concentration

The calculation of this quantity involves a determination of the cumulative length of these membranes per unit area of cytoplasm (membrane profile concentration) from the frequency with which the membranes intersect the superimposed sampling lines (Loud 1962). The following relationship (based on Buffon's "needle problem") is used:

$$ \text{membrane profile concentration} = \text{membrane length in microns} / \mu^2 \text{ of cytoplasm} $$

$$ = (\pi c/2L)(M/1000), $$

where $c$ is the number of intersections between endoplasmic reticulum and the superimposed sampling lines, and $L$ is the total length of the sampling line. The factor $M/1000$, where $M$ is the magnification, expresses the result in microns. In the crossing count, tangential contacts were also counted. Obliquely sectioned membranes were included if they could be readily identified.
Fig. 1.—General cytoplasm of control liver parenchymal cell.

Fig. 2.—General cytoplasm of CR3 liver parenchymal cells showing heterogeneity of enlarged mitochondria, glycogen, and some lipid accumulation.

Fig. 3.—General cytoplasm of CR10 liver parenchymal cell. Glycogen accumulation is still evident; mitochondrial volume is no longer increased.
Fig. 4.—Juxtaposition of mitochondria (arrows) indicating fusion of mitochondria is shown here in a CR$_3$ liver parenchymal cell, and is also seen in CR$_1$, IR$_3$, and IR$_{10}$ livers.

Fig. 5.—General cytoplasm of IR$_{10}$ liver parenchymal cell showing enlarged mitochondria and glycogen accumulation.
The method will clearly give somewhat of an underestimate because of failure to include membranes sectioned at high degrees of obliquity. No distinction was made between rough and smooth endoplasmic reticulum in the present work.

(d) Statistical Analyses

Student’s t-test was used for differences between means and F-test for inhomogeneity of the variances of control and experimental groups. Analyses of variance were conducted for between-liver differences in each experimental group for any one parameter.

III. Results

Following cortisone administration, the liver parenchymal cells, in contrast to the control cells (Fig. 1), showed glycogen and lipid accumulation, which was still evident after a 10-day recovery period (Figs. 2 and 3). The mitochondria in CR1 and CR3 livers were enlarged, often juxtaposed, and showed some irregular profiles (Figs. 2 and 4); in CR10 livers, the mitochondria were no longer enlarged (Fig. 3). In the insulin-treated series, especially in IR3 and IR10 livers, glycogen accumulation and some enlarged mitochondria were evident (Fig. 5). Lipid accumulation was less marked.

(a) Cytoplasmic Volume Fractions

The mean volume fraction of the cytoplasmic components in the control and experimental liver parenchymal cells is shown in Table 1. Cell volumes were estimated to determine whether the observed decreases in the volume fractions indicated a true reduction in amount or a changed cytoplasmic concentration of a component. The mean percentage of the control cell volume for the experimental groups is recorded in Table 1. The observed cell volume increases approximately paralleled the combined lipid and glycogen volume fractions in the CR1, CR3, and IR3 livers; in CR10 and IR10 livers, however, the cell volume was normal but the combined lipid and glycogen volume fraction was 12 and 17% respectively. The mean mitochondrial volume fraction and cytoplasmic matrix volume fraction corrected for changes in cell volume are shown in Table 1. For both parameters, student’s t-test showed no significant difference between the mean control value and each experimental value (in each case, \( P > 0.05 \)). Homogeneity of the cytoplasmic matrix volume fraction in the different experimental series does not indicate a constancy of the components forming this fraction. Within the different experimental series the proportions of ground cytoplasm and rough and smooth endoplasmic reticulum in this volume fraction varied. Changes in the total endoplasmic reticulum concentration were estimated quantitatively.

(b) Mitochondrial Dimensions

The mean mitochondrial dimensions for each series are recorded in Table 2. The total number of mitochondrial transections from which these parameters are derived for the 10 cells in each series ranged from 969 to 1301.

For most cells the observed shape coefficient \( (Q) \) closely approximated the theoretical values for spheres \( (Q = 0.5625) \). Student’s t-test showed that only the mean \( Q (0.6102) \) in CR3 livers differed significantly from the mean \( Q (0.5685) \) observed in control livers \( (t = 4.34, P < 0.01) \). This does not imply that all mito-
chondria in all cells from CR3 livers were non-spherical in shape. There were only two cells in the CR3 sample which had high values for Q, namely 0.6591 and 0.6383; the remaining eight cells had a Q value for mitochondrial shape within the range observed in other series which did not differ significantly from the control series.

**Table 1**

**Mean Volume Fraction of Cytoplasmic Components**

<table>
<thead>
<tr>
<th>Cytoplasmic Component</th>
<th>Mean Volume Fraction for Treatment Series:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
</tr>
<tr>
<td>Mitochondria</td>
<td>0.2058</td>
</tr>
<tr>
<td>Standard error</td>
<td>0.0164</td>
</tr>
<tr>
<td>Lysosome</td>
<td>0.0103</td>
</tr>
<tr>
<td>Standard error</td>
<td>0.0014</td>
</tr>
<tr>
<td>Lipid</td>
<td>0.0095</td>
</tr>
<tr>
<td>Standard error</td>
<td>0.0026</td>
</tr>
<tr>
<td>Glycogen</td>
<td>0.0153</td>
</tr>
<tr>
<td>Standard error</td>
<td>0.0056</td>
</tr>
<tr>
<td>Cytoplasmic matrix</td>
<td>0.7591</td>
</tr>
<tr>
<td>Standard error</td>
<td>0.0165</td>
</tr>
</tbody>
</table>

**Table 2**

**Mean Mitochondrial Parameters ± Standard Errors of the 10 Cells in Each Experimental Group**

<table>
<thead>
<tr>
<th>Treatment Group</th>
<th>Shape Coefficient (Q)</th>
<th>Diameter (μ)</th>
<th>Volume (μ³)</th>
<th>No. of Mitochondria/μ³ of Cytoplasm</th>
<th>No. of Mitochondria per Cell*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.5685 ± 0.0045</td>
<td>1.1404 ± 0.0516</td>
<td>0.8191 ± 0.1113</td>
<td>0.2770 ± 0.0330</td>
<td>754</td>
</tr>
<tr>
<td>CR1</td>
<td>0.5712 ± 0.0078</td>
<td>1.2741 ± 0.0855</td>
<td>1.2473 ± 0.3140</td>
<td>0.1455 ± 0.0198</td>
<td>486</td>
</tr>
<tr>
<td>CR3</td>
<td>0.6102 ± 0.0084</td>
<td>1.7601 ± 0.1673</td>
<td>3.5481 ± 0.7381</td>
<td>0.0939 ± 0.0232</td>
<td>208</td>
</tr>
<tr>
<td>CR5</td>
<td>0.5702 ± 0.0054</td>
<td>1.6958 ± 0.1271</td>
<td>2.9964 ± 0.7029</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CR10</td>
<td>0.5749 ± 0.0065</td>
<td>1.1353 ± 0.0608</td>
<td>0.8901 ± 0.1382</td>
<td>0.2490 ± 0.0288</td>
<td>680</td>
</tr>
<tr>
<td>IR1</td>
<td>0.5880 ± 0.0054</td>
<td>1.0908 ± 0.0476</td>
<td>0.7048 ± 0.0898</td>
<td>0.2673 ± 0.0302</td>
<td>663</td>
</tr>
<tr>
<td>IR3</td>
<td>0.5838 ± 0.0065</td>
<td>1.2541 ± 0.0748</td>
<td>1.1328 ± 0.1926</td>
<td>0.2233 ± 0.0325</td>
<td>580</td>
</tr>
<tr>
<td>IR10</td>
<td>0.5653 ± 0.0156</td>
<td>1.2360 ± 0.0568</td>
<td>1.0475 ± 0.1420</td>
<td>0.2030 ± 0.0221</td>
<td>562</td>
</tr>
</tbody>
</table>

* Number of mitochondria per cell estimated using the cell volumes determined from light microscopic preparations. If shrinkage in these preparations is taken into account the number of mitochondria per cell could be up to twice that in the above estimate.
A greater heterogeneity of mitochondrial profiles was evident in some CR3 cells (Fig. 2); in the control and other experimental groups the majority of profiles were circular, with few cylindrical or filiform profiles. The frequency distribution of mitochondrial profile eccentricity (major:minor diameter) showed that in all groups more than 80% of the observed mitochondria had an eccentricity ratio in the range 1.00 to 1.59. CR3 differed from other groups in that 3.7% of the mitochondria had an eccentricity greater than 3.10.

The mitochondrial volume was only significantly different from the control mitochondrial volume in CR3 and CR5 livers ($P < 0.01$); in all other cases, $P > 0.05$. Decreases in the number of mitochondria per cell were evident in all experimental series, the most marked decreases being in CR1 and CR3 livers.

(c) Membrane Profile Concentration

Significant decreases in the endoplasmic reticulum concentration per cell were observed after cortisone and insulin administration (Table 3). Student's $t$-test showed that only CR10 and IR10 livers did not differ significantly from the control liver cells ($P > 0.05$). Variance ratio test ($F$-test) for homogeneity of control and experimental variances showed no significant difference between control and each experimental series at $P(0.05)$ except for CR10 liver cells in which $0.05 > P > 0.01$.

<table>
<thead>
<tr>
<th>Treatment Group</th>
<th>Membrane Profile Conc. ± S.E.</th>
<th>$t$-test*</th>
<th>$F$-test†</th>
<th>Variance Ratio ($F$)‡</th>
<th>Corrected Membrane Profile Conc.</th>
<th>$t$-test*</th>
<th>Change as Fraction of Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>4.77 ± 0.15</td>
<td></td>
<td></td>
<td></td>
<td>0.47 ($P &gt; 0.05$)</td>
<td>4.77 ± 0.15</td>
<td></td>
</tr>
<tr>
<td>CR1</td>
<td>4.13 ± 0.22</td>
<td>2.30 ($P &gt; 0.05$)</td>
<td>2.13 ($P &gt; 0.05$)</td>
<td>0.86 ($P &gt; 0.05$)</td>
<td>5.79 ± 0.31</td>
<td>2.93 ($0.05 &gt; P &gt; 0.01$)</td>
<td>1.21</td>
</tr>
<tr>
<td>CR4</td>
<td>2.01 ± 0.20</td>
<td>8.45 ($P &lt; 0.01$)</td>
<td>1.71 ($P &gt; 0.05$)</td>
<td>4.39 ($P &gt; 0.05$)</td>
<td>3.40 ± 0.26</td>
<td>4.50 ($P &lt; 0.01$)</td>
<td>1.29</td>
</tr>
<tr>
<td>CR10</td>
<td>4.83 ± 0.31</td>
<td>0.21 ($P &gt; 0.05$)</td>
<td>3.94 ($0.05 &gt; P &gt; 0.01$)</td>
<td>0.42 ($P &gt; 0.05$)</td>
<td>4.83 ± 0.31</td>
<td>0.01</td>
<td></td>
</tr>
<tr>
<td>IR1</td>
<td>4.04 ± 0.12</td>
<td>3.72 ($P &lt; 0.01$)</td>
<td>1.77 ($P &gt; 0.05$)</td>
<td>3.06 ($P &gt; 0.05$)</td>
<td>4.04 ± 0.12</td>
<td>0.01</td>
<td></td>
</tr>
<tr>
<td>IR2</td>
<td>3.86 ± 0.24</td>
<td>3.21 ($P &lt; 0.01$)</td>
<td>2.37 ($P &gt; 0.05$)</td>
<td>1.37 ($P &gt; 0.05$)</td>
<td>4.24 ± 0.28</td>
<td>1.66 ($P &gt; 0.05$)</td>
<td>0.89</td>
</tr>
<tr>
<td>IR10</td>
<td>4.04 ± 0.26</td>
<td>0.39 ($P &gt; 0.05$)</td>
<td>2.34 ($P &gt; 0.05$)</td>
<td>1.31 ($P &gt; 0.05$)</td>
<td>4.64 ± 0.26</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Student's $t$-test for difference between means.
† $F$-test for homogeneity of variance of control and experimental series.
‡ Variance ratio of membrane profile concentration for between-liver differences in each series.

Thus the variation between cells was similar in all series. Analysis of variance showed no significant between-liver differences in the membrane profile concentration in any series; in each series $P > 0.05$ (Table 3). After correction of the observed membrane profile concentration for cell volume changes a significant biphasic change became apparent in the cortisone-treated series (Table 3).

IV. DISCUSSION

The accumulation of glycogen and lipid in liver parenchymal cells reflects altered carbohydrate and lipid metabolism in both cortisone- and insulin-treated livers and at various recovery periods subsequent to the cessation of hormone
administration. This lipid and glycogen accumulation, which accounts for most of the cell hypertrophy observed in the experimental series, caused no compression or distortion of the regions containing the other cytoplasmic components. The glucocorticoid-induced glycogen accumulation is known to be an enzyme-dependent \textit{de novo} synthesis involving an increase in the amount and activity of a number of enzymes participating in gluconeogenesis and amino acid transamination, together with an increase in glycogen synthetase activity and decreased peripheral glucose utilization (Greengard, Weber, and Singhal 1963; Morewaki and Landau 1963; Weber 1963; Ashmore, Wagle, and Uete 1964; Nichol and Rosen 1964; Landau 1965). Insulin exerts its metabolic effects by an integrative action on the biosynthesis of key rate-limiting enzymes by acting as an inducer of glycogen synthetase, glycolytic enzymes (glucokinase, phosphofructokinase, and pyruvate kinase), and the strategic enzymes of fatty acid synthesis resulting in glucose utilization, glycogenesis, glycolysis, and lipogenesis (Weber et al. 1967).

A significant decrease in the membrane profile concentration was observed in the CR\textsubscript{1}, CR\textsubscript{3}, IR\textsubscript{1}, and IR\textsubscript{3} livers. After correction for cell hypertrophy these decreases were lessened and an increase was evident in CR\textsubscript{1} liver cells. These changes were only temporary; the membrane profile concentration in CR\textsubscript{10} and IR\textsubscript{10} did not differ significantly from the control value. The significance of these hormone-induced fluctuations in the membrane profile concentration is not known. Wiener et al. (1968) reported that following 6-day cortisone treatment the rough endoplasmic reticulum is decreased by approximately two-thirds and the smooth endoplasmic reticulum by approximately one-half in rat liver parenchymal cells in various regions of the lobule, the decrease in total endoplasmic reticulum in the various regions of the lobule being 30–45\%. In the present study, after cortisone administration for 4 days, the endoplasmic reticulum concentration was increased by about 21\% if allowance was made for all cell volume changes; in the CR\textsubscript{3} livers the endoplasmic reticulum was decreased by approximately 28\%.

Following cortisone and insulin administration there were no marked changes in the ultrastructure of mitochondria. The integrity of the mitochondrial envelope and crests was maintained and there was no apparent change in the density of the mitochondrial matrix. On the other hand there was morphological evidence of fusion of mitochondria (Fig. 4). Two mitochondria appeared to have become apposed. The outer membrane of the mitochondrial envelope became continuous around the apposed mitochondria, and the two inner membranes, at least for some time after fusion occurred, were complete, appearing as a crista which completely traversed the mitochondrion. The mitochondrial quantitative data indicated that the administration of cortisone and insulin resulted directly or indirectly in a redistribution of the total cell mitochondrial volume in terms of the number and size of mitochondria in the liver parenchymal cells. This redistribution was only temporary. In the CR\textsubscript{1}, CR\textsubscript{3}, IR\textsubscript{3}, and IR\textsubscript{10} experimental series there was an increase in the mean mitochondrial volume and a decrease in the number of mitochondria per cell (Table 2). These volume changes were significant only in CR\textsubscript{3} and CR\textsubscript{5} livers ($P < 0.01$). The maximal change in number and volume was observed in CR\textsubscript{3} livers. The total cell mitochondrial volume remained relatively constant in these experimental series. The mitochondrial volume fractions were not significantly different in the various
experimental series after correction for cell volume changes (Table 1). Such constancy
of the total cell mitochondrial volume indicated that there was no loss or gain of
mitochondrial material; cortisone and insulin administration resulted only in its
redistribution. The quantitative data and the qualitative morphological observations
indicated that the observed number decrease was not associated with mitochondrial
degeneration and that the observed mean volume increase was thus unlikely to be
due to mitochondrial synthesis. It was, however, consistent with the fairly unequivocal
morphological evidence of mitochondrial fusion.

The observed relative increase in the mean mitochondrial volume was 1·52 in
CR1 livers and 1·38 and 1·28 in IR3 and IR10 livers respectively. This change in
mean mitochondrial volume indicated that in these liver types not all mitochondria
have undergone fusion. The ratio of fused to unfused mitochondria was less in IR3
and IR10 than in the CR1 livers. In each series the mean fraction of mitochondrial
volume fusing (f) can be estimated from \( f = 2(R - 1)/R \), where \( R = V_f/V_o \), \( V_f \) being
the mean mitochondrial volume after fusion and \( V_o \) the original mean mitochondrial
volume. It was thus estimated that the fraction of mitochondrial volume fusing was
0·67 in cortisone-treated livers, half the mitochondrial population being unfused and
half consisting of fused mitochondria. In IR3 and IR10 the fraction of mitochondrial
volume fusing was 0·55 and 0·44 respectively. On the basis of only a single fusion
accounting for the increased mitochondrial volume the theoretical number decrease
would be 33\% in CR1, 27\% in IR3, and 22\% in IR10. These numbers agree with the
deduced number decreases of 35\%, 23\%, and 25\% in CR1, IR3, and IR10 respectively.
The relative mean mitochondrial volume increase in CR3 and CR5 livers was 4·33
and 3·66 respectively. The mean number of mitochondria forming the enlarged
mitochondria in CR3 livers was four, and thus these mitochondria were the mean
result of three fusions. Within each cell in the CR3 and CR5 livers a range in the
extent of mitochondrial fusion was evident and included mitochondria which had not
fused, had undergone a single fusion, or had undergone multiple fusions. The
mitochondria in CR3 and CR5 livers then formed a heterogeneous population
distributed in volume classes, the mean of each class being a multiple of the control
mitochondrial volume. This heterogeneity of the mitochondrial population in CR1,
CR3, and CR5 livers is reflected in the results of the variance ratio test (\( F \)-test) for the
homogeneity of variances in the control and experimental series and of the analysis
of variance for between-liver differences within each experimental series; in both
cases, \( P < 0·01 \) for CR1, CR3, and CR5. The cell-to-cell variation in the mitochondrial
volume in these series indicates an asynchrony in the mitochondrial response to the
administered cortisone resulting in between-cell variation in the rate and extent of
fusion. This between-cell heterogeneity in the mitochondrial volume was accompanied
by a heterogeneity in the absolute number of mitochondria per cell. Mitochondrial
fusion resulted in not only a within-cell heterogeneity of mitochondrial volume but
also of mitochondrial shape which was most evident in CR3 livers (Fig. 2). Subsequent
division of the enlarged mitochondria may have taken place to produce the mito-
chondria in CR10 liver cells which showed no significant difference in size or number
from mitochondria in control liver cells. CR5 livers gave evidence of mitochondrial
division (Fig. 6). It is yet to be resolved whether such a division of these mitochondria
takes place along the original line of fusion. Division of mitochondria may not be the
only factor responsible for this return to the typical control values; but after a 10-day recovery period the normal half-life of mitochondria (Fletcher and Sanadi 1961; Bailey, Taylor, and Bartley 1967) may become an influential factor. In IR10 livers, the mean mitochondrial volume was less than that of IR3 but still greater than the control volume. These values do not, however, differ significantly from the control mean mitochondrial volume ($P > 0.05$).

As early as 1955, Lowe, Mackinney, and Sarkaria had shown by phase-contrast microscopy a decrease of 66·7% in the number of mitochondria per cell following 5 days of cortisone treatment. Kimberg, Loud, and Wiener (1968) and Wiener et al. (1968), working concurrently on the cortisone-induced changes in mitochondria, showed by quantitative analysis a decrease in mitochondrial number of about 70% in peripheral and mid-zonal regions and about 13% in the central regions of the lobule with a parallel increase in the mitochondrial volume of about 300 and 34% respectively following 6 days of cortisone treatment. It was suggested that these changes may be due to either mitochondrial fusion or failure to divide following mitochondrial growth. In the present study the mitochondrial number decrease and volume increase were 35 and 52% respectively in the CR1 livers and 72 and 330% respectively in CR3 livers.

The functional significance of mitochondrial fusion is not known. There is no apparent change in mitochondrial structure following cortisone and insulin administration. The fusion may be a consequence of altered enzyme activity. Since fusion is observed some time after the initial hormone injection and in fact is most pronounced in the mitochondria of liver cells where hormone treatment has been suspended for 3 days, it is suggested that the observed mitochondrial fusion is a secondary or indirect effect of cortisone and insulin administration and as such may even be influential in restoring normal metabolism to the liver cells.
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VI. References


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