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# THE ULTRASTRUCTURE OF FATTY LIVER INDUCED BY PROLONGED ETHANOL INGESTION

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Recent biochemical evidence indicates that ethanol has a direct as well as an indirect effect on hepatic metabolism.[2-5] One of the principal manifestations of these effects is the accumulation of lipid in liver cells. The mechanism whereby ethanol produces fatty liver has been a matter of considerable debate, and the discrepancies among previous studies probably hinge in no small degree on the different experimental models utilized.

With respect to lipid metabolism, it appears that small, repeated doses of ethanol affect the liver in a manner different from a large, single dose of ethanol. Specifically, a large, single dose causes deposition in the liver of lipid originating from peripheral depots,[6,7] whereas prolonged administration results in the deposition of endogenously synthesized lipids, as well as lipid of dietary origin when it is available.[8,9] Lieber,

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Jones, Mendelson and DeCarli[10] successfully produced fatty livers in rats by prolonged feeding of ethanol incorporated into a diet designed to eliminate the complications of other nutritional variations. They devised a completely synthetic, nutritionally adequate, liquid diet, with the test diet containing ethanol isocalorically replacing sucrose to the extent of 36 per cent of the total calories.

In the present study livers of rats maintained on the above diets were examined for ultrastructural changes which accompany the production of fatty liver, and an attempt is made to correlate the structural features with the metabolic events.

## Material and Methods

The composition of the diets and general experimental procedures have been described in detail by Lieber, Jones and DeCarli.[11] The diets contained approximately 1 Calorie per ml. In the basic control diet, amino acids, including 1.50 mg methionine per ml, constituted 16 per cent of the calories; fats, 43 per cent and carbohydrate in the form of sucrose, 41 per cent. Vitamins, including 0.25 mg choline per ml, and inorganic salts were incorporated into the mixture, and the diet was stabilized with sodium carrageenate. In a pilot study, rats fed the basic diet *ad libitum* grew at a rate similar to rats fed Purina® Rat Chow. In the ethanol-containing diet, 36 per cent of the total calories of the basic diet contributed by sucrose were replaced isocalorically with ethanol. Ninety-five per cent laboratory alcohol was used for this purpose.

Male, litter-mate, Sprague-Dawley rats (Charles River Laboratories, Brookline, Mass.) were pair-fed the control and ethanol-containing diets daily, and they continued to grow. For hepatic lipid analyses, rats were sacrificed at varying intervals up to 42 days.[10,11] Livers for morphologic study were obtained at 6, 16, 20, 22 and 27 days. The number of animals for each time period was 6, 6, 4, 4 and 6, respectively; half the animals at each time period received the ethanol-containing diet and the other half were pair-fed the sucrose control diet. In addition, 2 rats, litter mates of the 6-day animals and fed Purina® Rat Chow *ad libitum*, were sacrificed with the 6-day group.

Portions of the tissues were fixed in neutral buffered formalin for light microscopy. Frozen sections of formalin-fixed tissue were stained with Sudan IV to demonstrate lipid. Paraffin-embedded tissue was sectioned and stained with hematoxylin and eosin.

For electron microscopy, tissue blocks were fixed in pH 7.4 collidine-buffered[12] 1 per cent osmium tetroxide, dehydrated through a graded series of alcohol, followed by propylene oxide, and embedded in Epon 812 according to the method of Luft.[13] Sections cut at approximately 1 $\mu$ from each block were stained with Azur II-methylene blue[14] for light microscopic examination and orientation. At least one block from each time period contained a central vein and portal area in a single section so that the intralobular localization of areas represented in the corresponding electron micrographs could be ascertained. Thin sections were stained with 1 per cent alcoholic uranyl acetate alone or followed by lead citrate[15] or with lead hydroxide[16] alone. They were examined with an RCA EMU-3F electron microscope.

## Results

### *Lipid Analyses*

Details of the lipid analyses have been published by Lieber and coworkers.[10,11] Compared with the controls, total hepatic lipids, triglyc-

erides and cholesterol esters of the ethanol-fed animals were significantly elevated at 4 to 9 days and became further elevated with increasing time. The greatest relative change was in the triglycerides which were increased 8- to 10-fold by 3 to 6 weeks.[10,11]

### LIGHT MICROSCOPY

*Sucrose Control Animals.* Hematoxylin and eosin-stained sections of livers at all time periods were essentially normal. Other sections stained with Sudan IV showed a small and variable amount of fine lipid droplets scattered with a panlobular distribution. This was the usual finding in the livers of rats fed the sucrose control diet, which had a relatively high fat content (43 per cent of calories), although some specimens exhibited practically no lipid droplets.

*Alcohol-Fed Animals.* At 6 days, definite lipid droplets were present, mostly in the central and mid-zones of the lobules. Some livers showed a panlobular distribution. The numbers and sizes of the lipid droplets increased with time, but there were moderate differences between individual animals. No "alcoholic hyalin" was seen.

### ELECTRON MICROSCOPY

*Control Animals.* The liver cells of the chow diet and sucrose control diet animals were similar. It is of importance to note, however, that the granular endoplasmic reticulum of the cells immediately adjacent to the central veins in both groups of animals differed in organization from the granular endoplasmic reticulum of the cells further removed from the central veins. In the latter (Fig. 1), the endoplasmic reticulum generally appeared as flattened cisternae with elongated, narrow profiles, often in stacks, in more or less parallel arrays. In contrast, the granular endoplasmic reticulum of the cells immediately adjacent to the central vein was less well ordered, with irregular dilatations of the cisternae, and occasional vesicular forms (Figs. 2 and 3).

The changes in the granular endoplasmic reticulum in liver cells of alcohol-fed animals, to be discussed below, extended well beyond the immediate pericentral cells.

*Alcohol-Fed Animals. Lipid Droplets.* The lipid droplets at 6 days (Fig. 4) ranged in size up to 6 $\mu$ in diameter, but most of the droplets averaged 1.5 to 2.5 $\mu$. There was a general tendency for the droplets to be distributed near the sinusoidal borders of the liver cells, but they could be found in all portions of the cytoplasm. Close proximity of lipid droplets and mitochondria was common. At 16 days the lipid droplets showed a greater distribution of sizes and ranged up to 12 $\mu$ in diameter. At 20 to 27 days the droplets measured up to 20 $\mu$ in diameter, but there were still a large number of droplets in the 1- to 3-$\mu$ range.

*Endoplasmic Reticulum.* The granular endoplasmic reticulum showed marked alterations in the centrilobular cells at all the time periods from 6 to 27 days. At 6 days (Figs. 4 and 5) the cytoplasm was packed with small, circular or oval vesicular profiles measuring from 0.2 to 0.5 $\mu$. At later time periods, there was considerably more variation in the sizes and shapes of the vesicles, particularly at 16 days (Figs. 6 and 7). Some of the vesicles were agranular, but most had ribosomes on the surface. The ribosomes tended to be spaced irregularly on the membranes and varied considerably in number from vesicle to vesicle (Figs. 6 and 7). The vesicles in general contained pale, flocculent densities, but a few contained denser, rounded bodies ranging up to 0.1 $\mu$ in diameter, generally becoming more prominent with time (Figs. 5 and 9). These structures were related in no special way to the large lipid droplets. In the mid-zones, most of the granular endoplasmic reticulum was vesicular, although some elongated forms persisted. The periportal cells contained elongated profiles as well as some vesicular endoplasmic reticulum.

The smooth endoplasmic reticulum was widely distributed in the centrilobular cells at 6 days, scattered among the vesicles. There were rare instances of fern-like arrangements of smooth membranes in close association with smooth endoplasmic reticulum of the usual configuration (Fig. 5). By 16 days, some of the centrilobular and mid-zonal cells contained large clusters of closely packed smooth endoplasmic reticulum (Fig. 6). In the periportal cells the smooth endoplasmic reticulum was more dispersed and less conspicuous. At subsequent time periods there was still a moderate amount of smooth endoplasmic reticulum dispersed generally throughout the cytoplasm.

*Mitochondria.* At 6 days the mitochondria exhibited moderate pleomorphism similar to the controls. At 16 days (Fig. 7) the centrilobular mitochondria began to show some enlargement, shortening of cristae, pallor of the matrix, and in some cells, a decrease or almost complete absence of matrix granules. In spite of the general tendency towards enlargement, there were still many small mitochondria less than 1 $\mu$ in diameter. This variation in size was also present at all subsequent time periods. The changes in the cristae and matrix generally prevailed regardless of the size of the mitochondrion. The mitochondrial changes extended into the mid-zones of the lobules. At 20 to 22 days, there was increased pallor of the matrix and shortening of the cristae in the centrilobular and mid-zones. Some bizarre-shaped mitochondria were encountered (Fig. 10). Longitudinal sections through some of the cristae showed a faint, axial, fibrillar density (Fig. 11). Occasional dilated cristae contained several wavy or helical fibrils, orientated more or less parallel to the long axis of the cristae (Fig. 12). Occasional mitochondria

showed an increased separation between the inner and outer membranes. Discontinuities of the outer mitochondrial membrane were occasionally seen. At 27 days, centrilobular mitochondria exhibited moderate to marked pallor of the matrix (Fig. 8). Intracristal filaments were more numerous. Discontinuities of the outer membrane were more obvious at this time, and in rare instances, wavy or helical fibrillar material was seen between the inner and broken outer membranes (Fig. 8, *inset*).

*Other Structures.* The appearance of the Golgi zones varied considerably without any particular relationship to the duration of feeding or the quantity of lipid in the particular cell. Some Golgi zones (Fig. 6) contained numerous small, rounded densities in the vesicles and cisternae; others did not. Microbodies were similar to those in controls in appearance and number, as were the lysosomes. The bile canaliculi showed no significant variations from the controls. No structures resembling "alcoholic hyalin" [17,18] were seen.

## Discussion

The metabolic effects of ethanol are many, and these effects have been well summarized in the recent reviews previously cited.[2-5] The present system was designed to study the effects of continuous ethanol ingestion in moderate doses over a period of days while supplying adequate calories and nutrients.

After 6 days of this regimen, the accumulation of lipid in the hepatic cells was well established, as demonstrated morphologically and biochemically. Among the organelles of the hepatic cell, the earliest and most profound alteration was of the endoplasmic reticulum, which became vesiculated to the point of resembling a microsomal preparation. The cells most affected were in the centrilobular zone, with some extension into the midzone. An unusual observation at 6 days was that of a fern-like structure of smooth membranes associated with the smooth endoplasmic reticulum. The distinct increase in clusters of smooth endoplasmic reticulum at 16 days suggested that the fern-like structures might be a form of smooth endoplasmic reticulum in a rapidly proliferating phase. They were not observed after 6 days.

It is worthy of particular note that the pattern of lipid accumulation followed the same general zonal distribution as the changes in the endoplasmic reticulum. It appears probable that this coincident distribution reflects some direct relationships. Using a variation of this model, Lieber and Spritz [8,9] showed that a major portion of the lipids in the resultant fatty liver contained endogenously synthesized fatty acids, and when available, dietary fatty acids. Other work has shown that ethanol increases the hepatic synthesis of fatty acids [19,20] as well as triglycer-

ides.[21-23] Increased esterification of fatty acids to triglycerides by liver homogenates[23,24] as well as microsomal preparations[23] from animals pretreated with ethanol has been demonstrated. The changes in the endoplasmic reticulum, therefore, may well be a manifestation of the accelerated synthesis of fatty acids and triglycerides by the hepatic cell.

Alterations of the endoplasmic reticulum and ribosomes with decreased protein synthesis and lipid accumulation have been demonstrated in rat livers in carbon tetrachloride intoxication,[25,26] where the primary lesion appears to involve the ribosomes.[27] It has been hypothesized that decreased protein synthesis leads to lipid accumulation because of diminished formation of lipoprotein for the release of triglyceride from the cell. Similar associations between impaired protein or lipoprotein synthesis and the production of fatty liver have been made in other experimental conditions, including intoxications with ethionine,[28] yellow phosphorus[29] puromycin,[30] and orotic acid feeding.[31,32] In ethanol intoxication, however, there is evidence that triglyceride release is not impaired, and in fact hypertriglyceridemia is common.[2-5,10] Seakins and Robinson[33] found no change in the incorporation of 1-$C^{14}$-leucine into liver and plasma proteins of rats at 2½ and 14 hours following the administration of a single dose of ethanol. In liver perfusion studies[4] decreased lipoprotein release was demonstrated only at very high concentrations of ethanol in the perfusate (400 mg per 100 ml), but amino acid incorporation into liver protein was still achieved. Ashworth, Johnson and Wrightsman[34] in a combined biochemical and morphologic study demonstrated in intact rats that amino acid incorporation into total hepatic protein and serum albumin and globulin was actually increased in animals given a single, large dose of ethanol 5½ to 7½ hours previously; the endoplasmic reticulum and mitochondria were intact. Protein metabolism in the present model has not yet been studied.

Stein and Stein[35] found essentially no changes in the endoplasmic reticulum or mitochondria up to 16 hours after a single large dose of ethanol. Pfeiffer[36] found only minimal changes in the endoplasmic reticulum of the liver cells after maintaining rats on a normal diet and water containing 10 per cent ethanol for 200 days. No biochemical data were reported in the study.

In electron microscopic studies of livers of rats during the first few days of choline deficiency[37,38] mitochondria show no significant changes, and the endoplasmic reticulum tends to undergo generalized dilatation and some vesiculation. The changes, therefore, do not parallel those of the present model.

Mitochondrial alterations, localized principally in the centrilobular cells, were first seen at 16 days and increased with continued ethanol

feeding. Swelling, distortions, decreased density of the matrix, and loss of matrix granules occur in a multitude of situations [see review by Trump and Ericsson[39]]. Swollen cristae containing wavy or helical fibrils have been noted under less general circumstances, namely, in the livers of protein-deficient rats by Svoboda and Higginson[40] and in the pancreatic acinar cell of starved salamanders by Sprenk and Herman.[41] There is no obvious common factor to explain the existence of the wavy or helical fibrils. Mitochondrial structure, however, is similarly affected in all three instances. They may arise by an alteration in the normal intermolecular bonding of the phospholipids and proteins which compose mitochondrial membranes, permitting a rearrangement of these components into helical fibrils, a mechanism alluded to by Svoboda and Higginson.[40] The helical fibrils associated with the outer limiting membranes probably arise by a similar mechanism.

Extremely large mitochondria which they interpreted as representing "alcoholic hyalin" were recently described by Porta, Hartroft and de la Iglesia.[42] No "alcoholic hyalin" was detected, however, by either light or electron microscopy in this study. Svoboda and Manning[43] detected no abnormalities in the mitochondrial structure of liver cells of rats given single daily doses of ethanol by intragastric tube 5 days weekly for 6 weeks. In this regard, their finding is similar to the effect of just one dose of ethanol,[34,35] and emphasizes one dissimilarity between the previous models and the present one.

The functional significance of the mitochondrial changes cannot be stated with certainty, but decreased metabolism of radioisotope-labeled palmitate and chylomicrons was demonstrated by Lieber and associates[8,9] in a variation of this model. It has also been shown that there is decreased oxidation of fatty acids,[19,20] decreased pyruvate oxidation,[44] and decreased succinate oxidation in male rats.[44] French[45] observed a histochemical "shift" of maximal succinic dehydrogenase and choline dehydrogenase activities from the periphery of the lobule to the centrilobular zone following prolonged ethanol ingestion by rats, and suggested that this may have been due to an "unmasking" of enzymatic activities by an increase in the permeability of the mitochondrial membrane to phenazine methosulfate.

The granules present in the cytoplasmic vesicles are of undetermined composition. Bruni and Porter[46] presented a strong argument in favor of similar granules in the endoplasmic reticulum of normal livers being condensed protein, and cast doubt on interpretations by others that the granules are lipid. In at least the instance of fatty liver following the feeding of orotic acid,[32] however, the material within the granular endoplasmic reticulum appears clearly to be lipid. It seems not unlikely that these

granules may be constituted of proteins or lipids or both, depending upon the prevailing conditions of the study.

Finally, the observation that in the normal chow-fed and sucrose control diet-fed animals the granular endoplasmic reticulum of the cells immediately adjacent to the central vein differs significantly from that of cells further removed from the central vein must be emphasized. In the former, the granular endoplasmic reticulum is not arranged in parallel arrays of flat cisternae, but, instead, is less well organized and exhibits irregular dilatations and often vesiculation. These configurations can easily be mistaken for pathologic changes, when, in fact, they are normal variations within the liver lobule.

## Summary

Livers of rats maintained on a completely synthetic, nutritionally adequate, liquid diet containing ethanol showed lipid accumulation in the centrilobular cells with marked changes in the endoplasmic reticulum. The alterations in the endoplasmic reticulum were readily evident after 6 days, and were characterized by a replacement of the elongated profiles by abundant vesicular structures, with or without ribosomes on their surfaces.

Mitochondrial changes appeared at 16 days and were progressive. They consisted of enlargement with shortening of the cristae, pallor of the matrix, decreased numbers or absence of matrix granules, and the appearance of bizarre shapes and intracristal helical fibrils. There occasionally was widening of the space between the inner and outer membranes, and rarely helical fibrils were present between these membranes.

Possible functional counterparts of these morphologic changes are discussed, especially in relation to the previously described alterations in hepatic lipid metabolism produced by ethanol, mainly increased lipogenesis and decreased fatty acid oxidation.

The difference in the appearance in the normal liver lobule of the granular endoplasmic reticulum of hepatic cells immediately adjacent to the central vein from that in the hepatic cells in the remainder of the lobule is stressed.

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[ *Illustrations follow* ]

## Legends for Figures

Except where otherwise indicated the preparations were stained with uranyl acetate and lead citrate.

Fig. 1. Sucrose control diet, 6 days. Hepatic cell, fourth from a central vein. The granular endoplasmic reticulum appears as elongated, flat profiles in stacks. These may be compared with the cells immediately adjacent to a central vein (Fig. 3). Mitochondria vary moderately in size and shape. A sinusoid containing an erythrocyte is at the top. Near the sinusoidal border of the hepatic cell are several irregularly rounded, pale spaces representing lipid droplets which had been dissolved out in the processing. Two more such droplets are at the bottom of the micrograph. × 5,000.

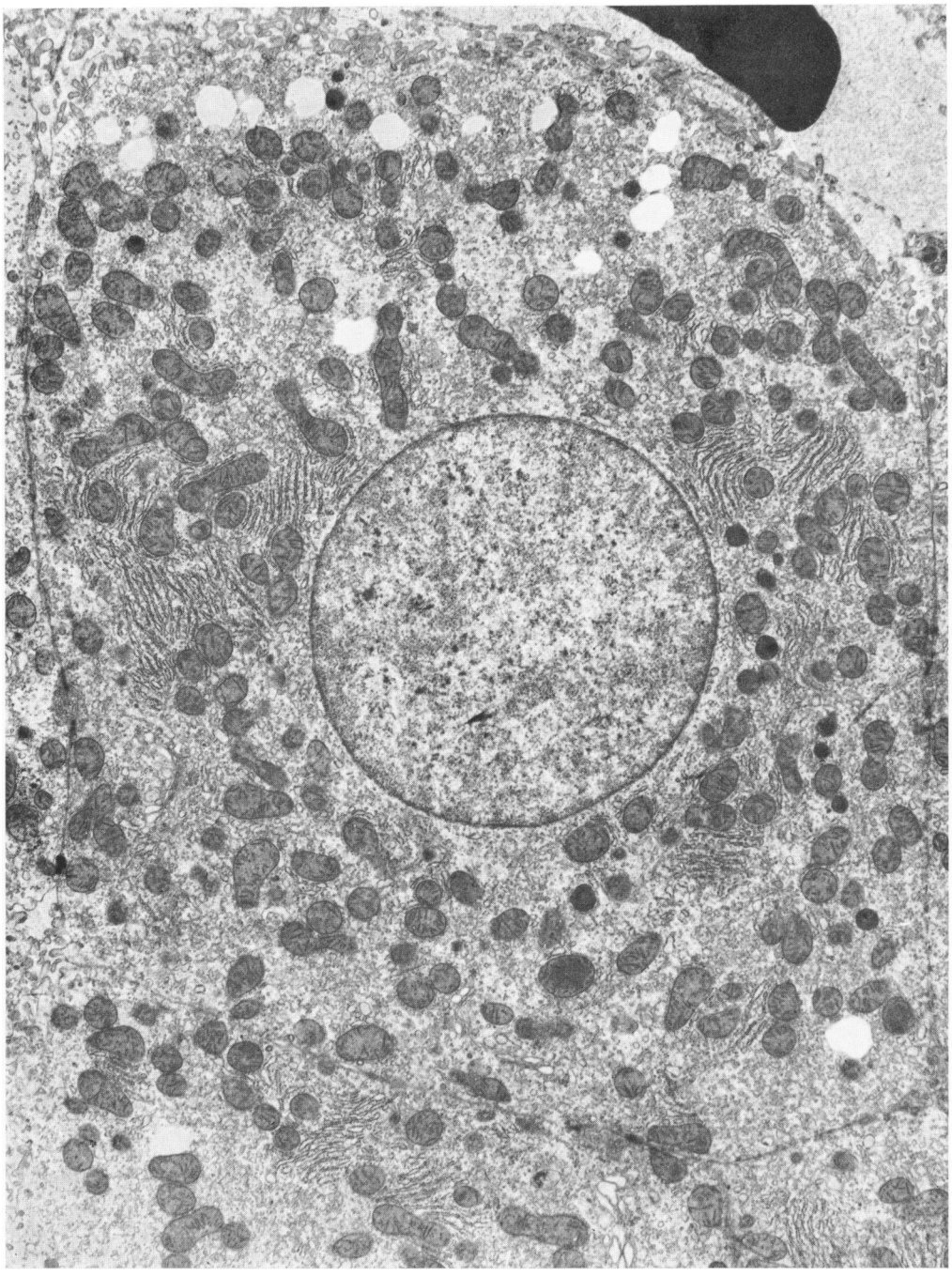

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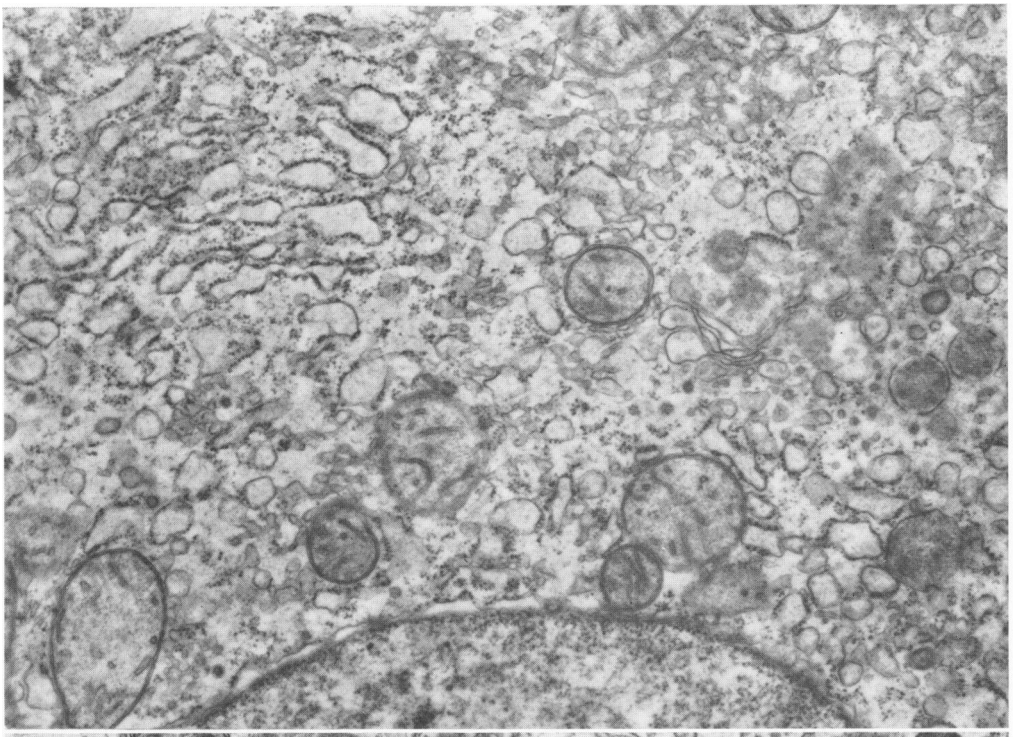
FIG. 2. Chow control diet. Portion of a hepatic cell immediately adjacent to a central vein. The cisternae of the granular endoplasmic reticulum are short and irregularly dilated, and some vesicular profiles are present. This contrasts with that of cells further removed from the central vein, in which the profiles tend to be flat, elongated, and usually in well-ordered stacks. × 17,000.

FIG. 3. Sucrose control diet, 6 days. Portion of a hepatic cell immediately adjacent to a central vein. The granular endoplasmic reticulum is similar to that in the chow-fed control hepatic cell (Fig. 2). Cells further removed from the central vein (Fig. 1) showed the usual configuration of flat, elongated profiles of granular endoplasmic reticulum, usually in stacks. × 17,000.



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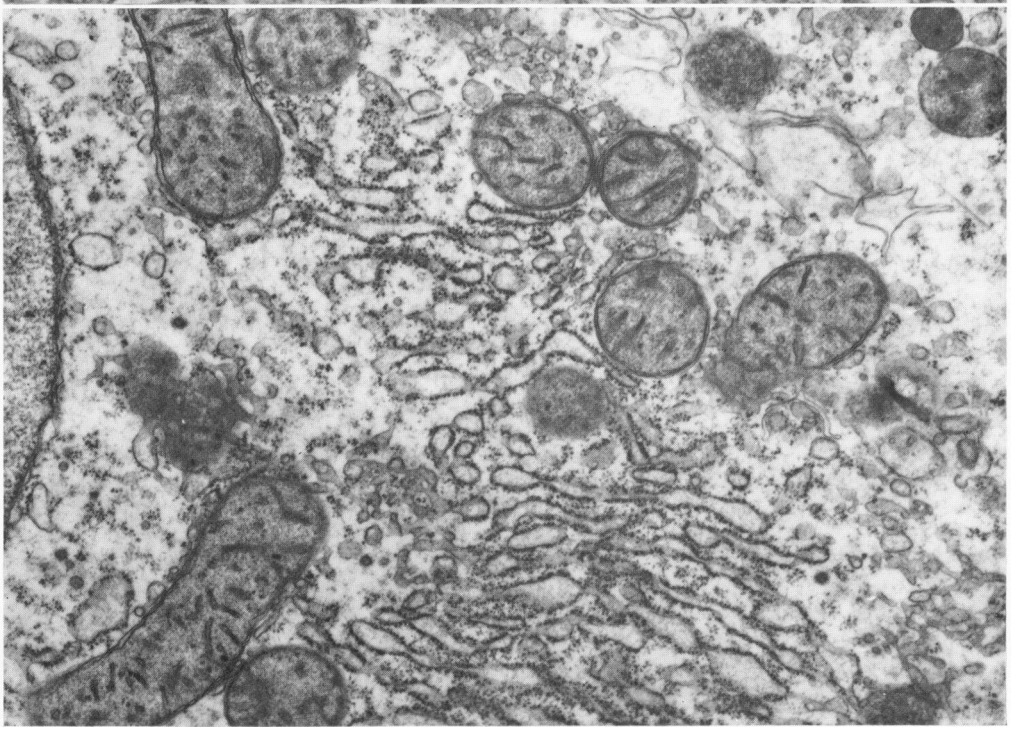

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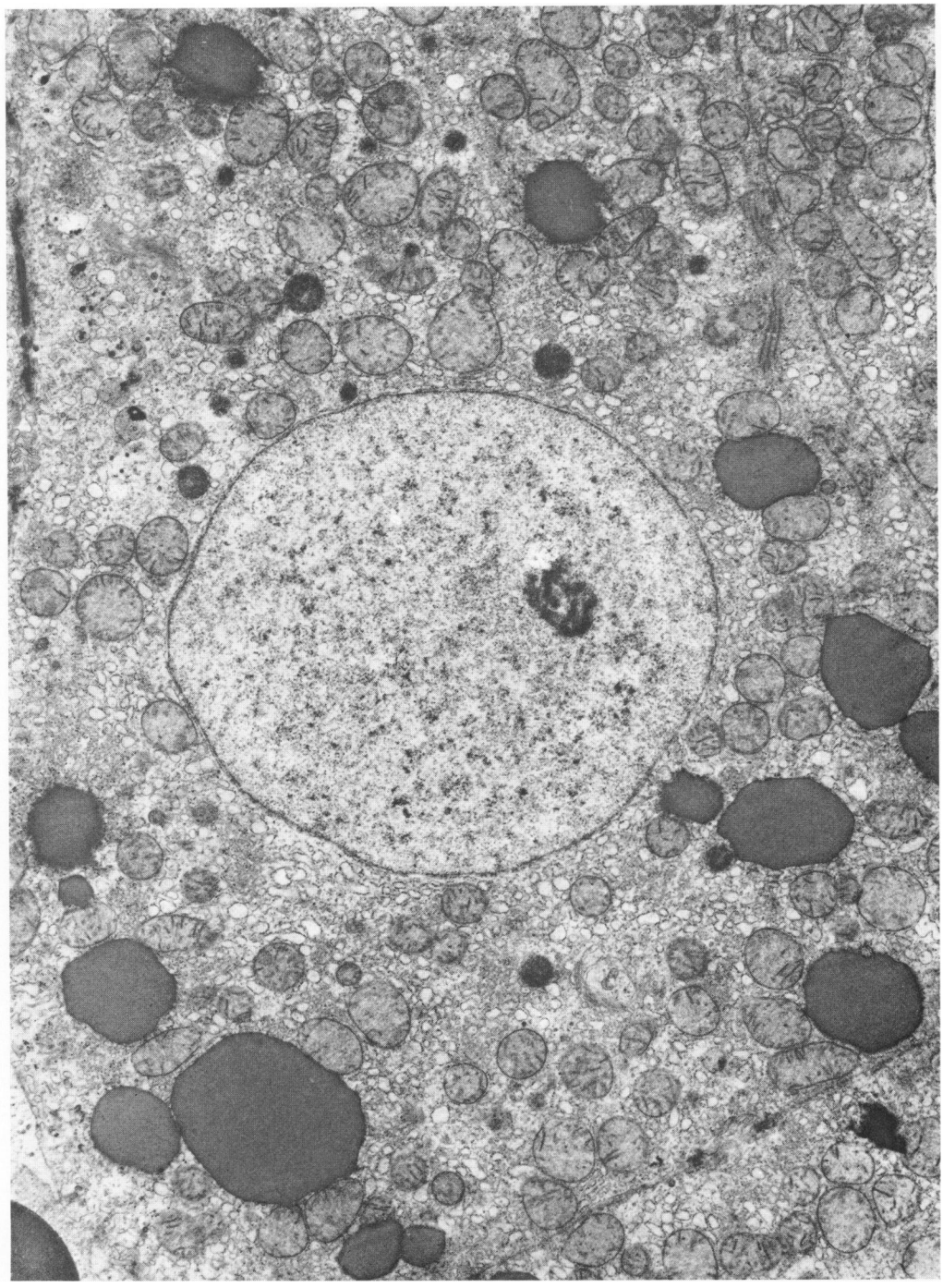

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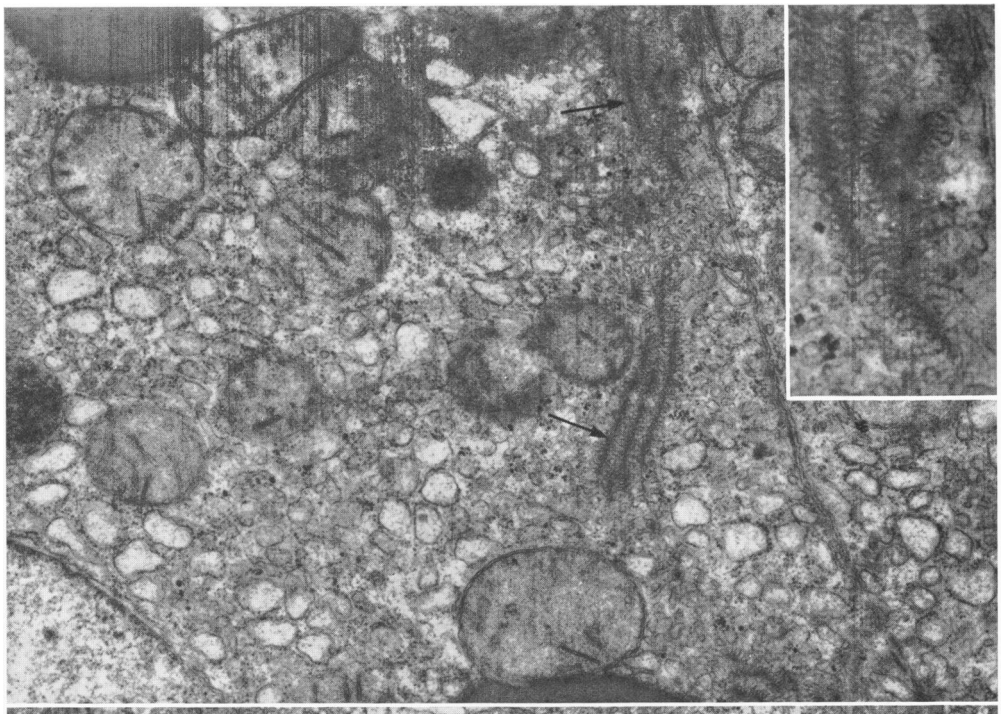
FIG. 4. Ethanol-containing diet, 6 days. A hepatic cell contains large lipid droplets and vesicular endoplasmic reticulum, characteristic of the centrilobular cells. Lead hydroxide stain. × 6,800



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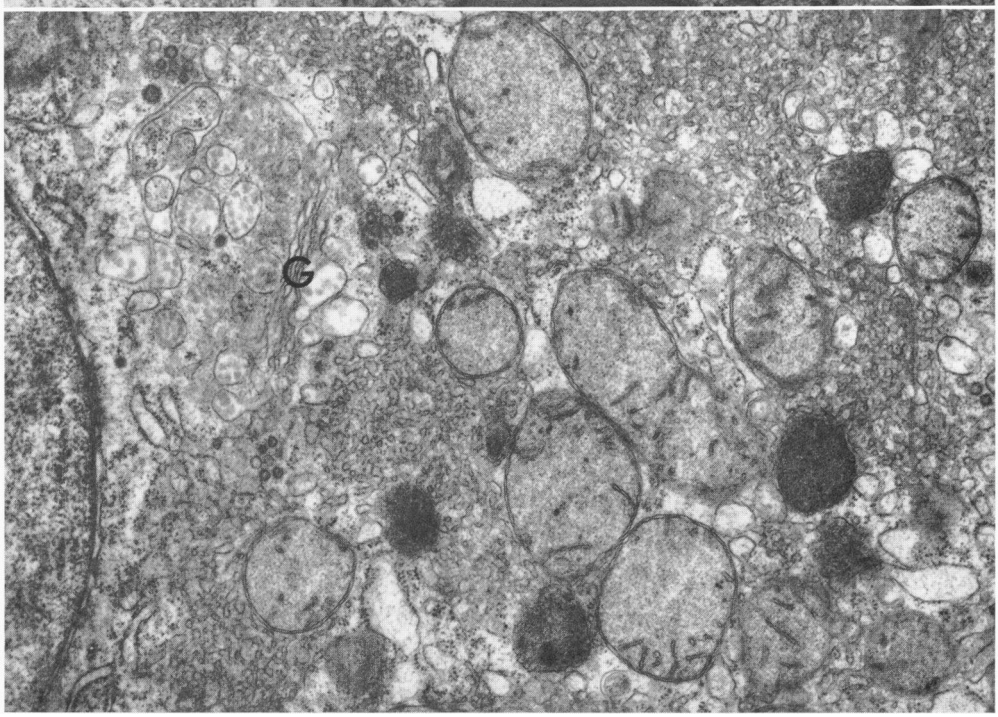


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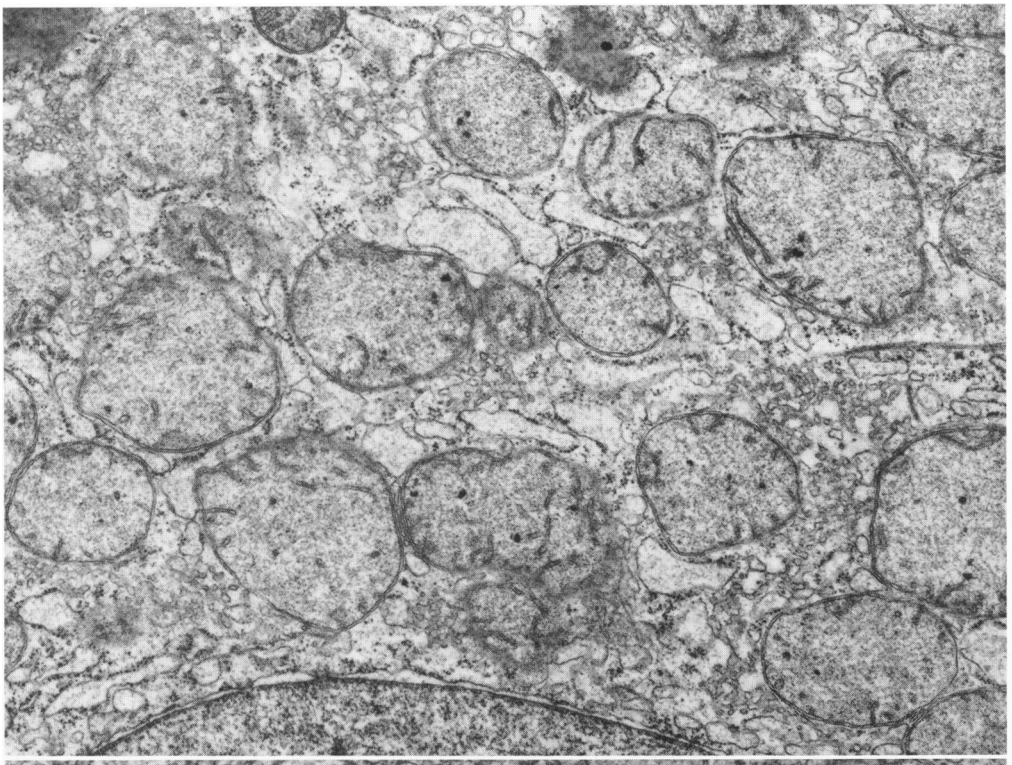
FIG. 5. Ethanol-containing diet, 6 days. Higher magnification of a portion of the cell shown in Figure 4. In addition to vesicular endoplasmic reticulum, there are fern-like structures (arrows) near the cell membrane associated with smooth endoplasmic reticulum of the usual configuration. Inset is one of these structures at higher magnification. Lead hydroxide stain. × 17,600. *Inset,* × 38,000.

FIG. 6. Ethanol-containing diet, 16 days. Large clusters of smooth endoplasmic reticulum are prominent. The Golgi apparatus (G) near the nucleus has many vesicles filled with pale granules. × 17,200.

FIG. 7. Ethanol-containing diet, 16 days. The vesicles of granular endoplasmic reticulum in this centrilobular cell vary considerably more in appearance than at 6 days. The ribosomes are irregular in number and distribution. Some of the mitochondria are larger than normal. × 17,000.

FIG. 8. Ethanol-containing diet, 27 days. Vesicular endoplasmic reticulum remains a prominent feature of the centrilobular cell. Mitochondrial swelling, pallor of the matrix, shortening of the cristae, and diminution or absence of matrix granules are evident (M). A few small mitochondria (m) are still present. Disruptions of mitochondrial membranes (arrows) are common, and one such mitochondrion (lower center and inset) exhibits two helical fibrils (arrow) between the inner membrane and broken outer membrane. × 17,600. *Inset,* × 48,000.



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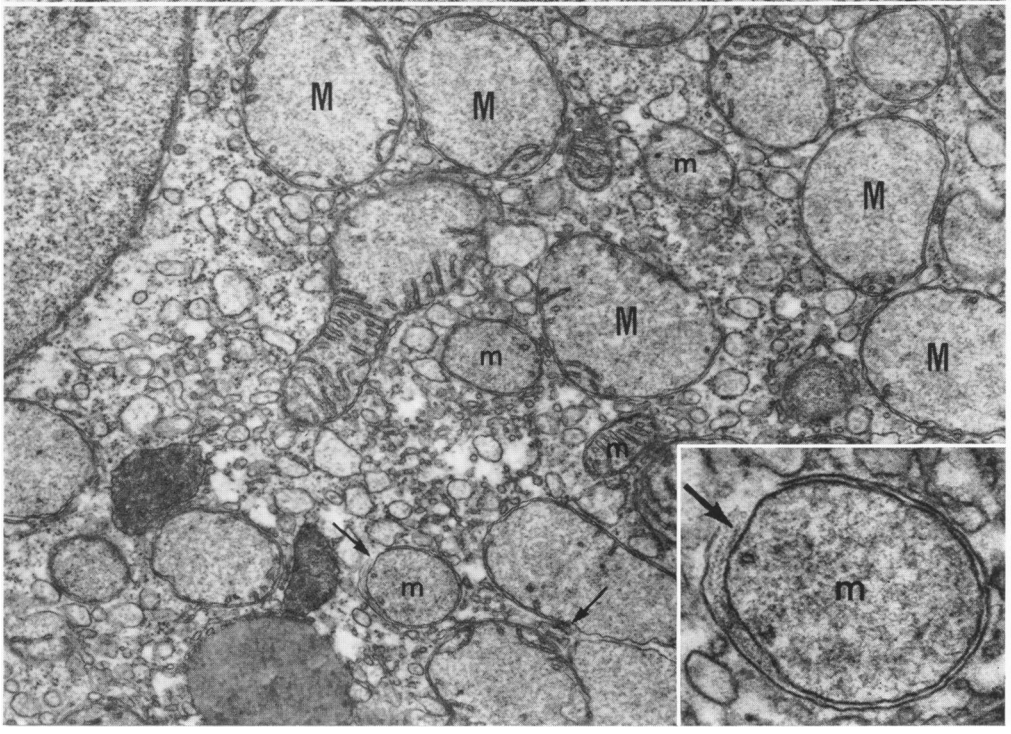


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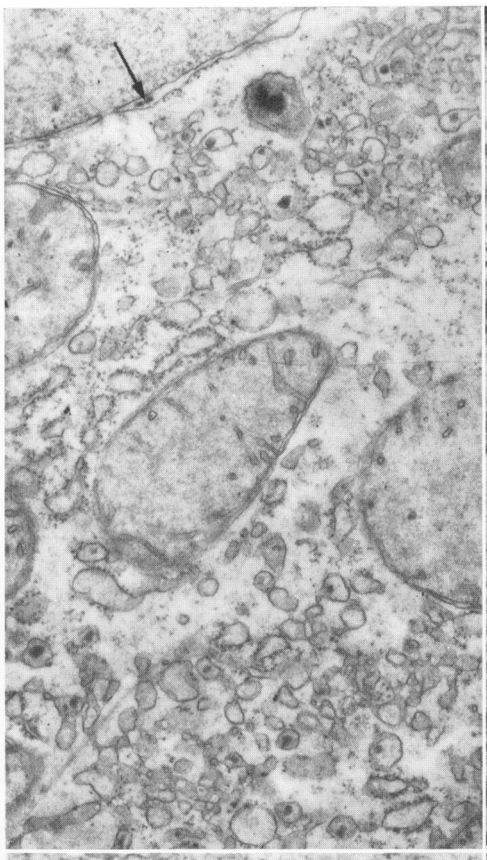
FIG. 9. Ethanol-containing diet, 27 days. Dense bodies or granules are present within cytoplasmic vesicles. These increased in number with increasing time on the diet. Similar granules between the inner and outer membranes of the nuclear envelope (arrow) were rare. × 21,000.

FIG. 10. Ethanol-containing diet, 20 days. A "tadpole"-shaped mitochondrion, one of the more unusual shapes encountered. The "tail" contains oval profiles of membranes, probably derived from cristae. Uranyl acetate stain. × 21,000.

FIG. 11. Ethanol-containing diet, 22 days. A mitochondrion with a filamentous density (arrow) in a crista, possibly an early stage of formation of helical fibrils. × 28,000.

FIG. 12. Ethanol-containing diet, 20 days. Mitochondrion with helical fibrils within a dilated crista. × 44,000.



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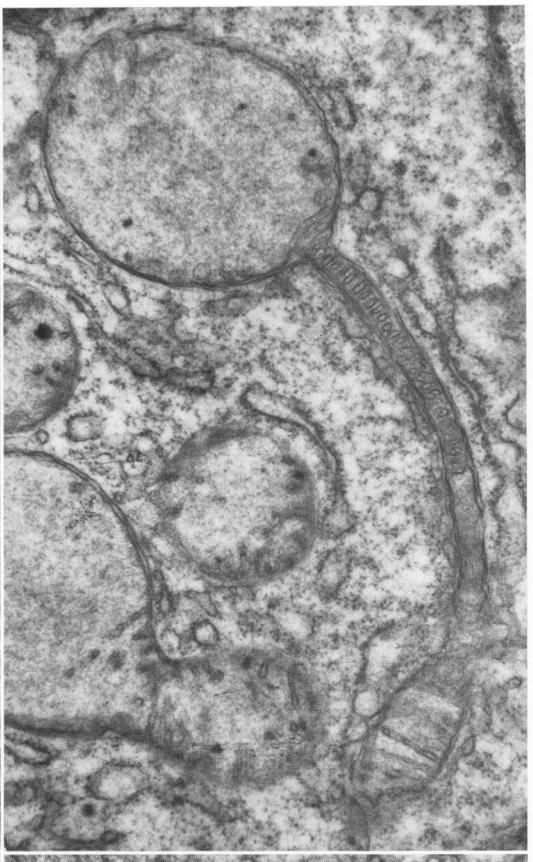

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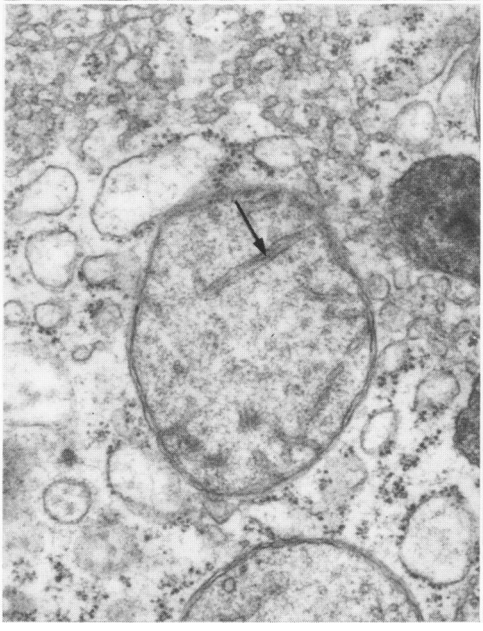

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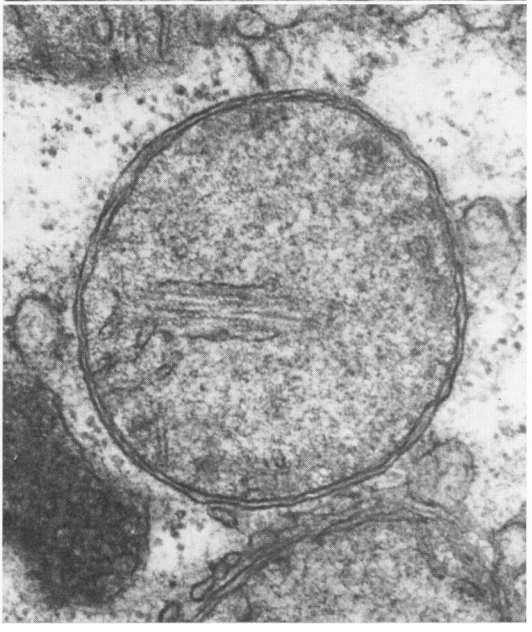

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