Status Spongiosus of CNS and Hepatic Changes Induced by Cuprizone (Biscyclohexanone Oxalyldihydrazone)

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STATUS SPONGIOSUS has been found in the brain in various neurologic disorders.[1] Although, at the light microscopic level, the status spongiosus is similar in appearance among the disorders, it differs considerably at the ultrastructural level. In some it is due to the markedly swollen astrocytes,[2] in others, to the intramyelinic vacuoles,[3,4] and in still others, to the intra-axonal vacuoles.[5]

Experimental production of this status has been achieved by the administration of various chemical substances to animals.[6-12] Cuprizone (biscyclohexanone oxalyldihydrazone), a chelator used as a reagent for copper analysis,[13] is one of them. By oral administration, it produced severe status spongiosus, most extensive in the cerebellar white matter and the brain stem of mice.[11] There were also focal areas of myelin loss. An interesting aspect of this condition was the prominence of the cells with large pale nuclei resembling Alzheimer's Type II glia cells in the affected areas. Alzheimer's glia cells are usually found in the liver disease.[1] The presence of these cells, therefore, suggested some disturbance of hepatic function in association with status spongiosus in these Cuprizone-treated mice. The combination of the status spongiosus and Alzheimer's glia cells is also found in spongy degeneration of the brain (Van Bogaert and Bartland type [14]) or Canavan's disease.[15] Therefore, we feel that ultrastructural studies of the CNS and the liver in this particular condition could be of help in understanding spongy degeneration of the brain (SD).

Materials and Methods

Weanling Swiss-Webster male mice were used. Of the total of 40 mice, 10 controls were fed only Rockland Mouse/Rat Diet (Rockland Co., Monmouth, Ill.). The remaining 30 were fed a mixture of 0.5 gm. Cuprizone (G. Frederick Smith Chemical Co., Columbus, Ohio) per 100 gm. of diet and water given ad lib. Twelve mice

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fed with Cuprizone and 3 controls were used for ultrastructural study. The remaining ones were used for preliminary light microscopic study. The test mice were studied at 3 days, 5 days, and thereafter when clinical signs developed. For histochemical study of the liver, 1 control mouse and 5 test mice were sacrificed at 3, 7, 9, and 13 days and were used for the study of the oxidative enzymatic activity. The technique has been described previously.[16]

Intraperitoneal injection of 1 ml. of 1% trypan blue was given to 2 mice when signs of encephalopathy developed.

Ether-anesthetized mice were sacrificed by intracardiac perfusion with 5% glutaraldehyde in 0.067 M phosphate buffer, pH 7.38. For electron microscopy of CNS, tissue was obtained from the right half of the pons, the midbrain, or from both, and from the cerebellar white matter. Facing blocks and the left half were processed for the light microscopic study. The tissue was minced and immediately postfixed in Dalton's fixative for 90 min., dehydrated in graded alcohol and propylene oxide, and embedded in Araldite or Epon. Several pieces of the liver from the glutaraldehyde perfused or nonperfused test and control mice were used for the ultrastructural study. The liver pieces were minced and fixed in Palade's veronal-buffered osmium for 90 min., and processed as above. Thin sections stained with lead citrate and uranyl acetate were examined in the Siemens Elmiskop IA and RCA EMU-3C.

Results

The mice given the Cuprizone diet became inactive at 1–2 weeks. Growth retardation, compared to the controls, and weakness of the posterior limbs were the chief clinical signs. Most of the mice died within 3 weeks when Cuprizone feeding continued.

Gross Observation

No significant abnormalities were apparent on gross examination of the brain and the liver. Those mice given injections of trypan blue, when the signs fully developed, did not show any staining of the brains even after the whole body turned blue.

Light Microscopy

Central Nervous System. In a mouse sacrificed 3 days after the start of the experiment, significant changes were absent. Some mice sacrificed at 5 days, before clinical signs appeared, had only a slight sponginess of the midbrain and pontine tegmentum. At this stage Alzheimer's Type II glia were already apparent. The mice with fully developed signs revealed diffuse status spongiosus in the brain stem, largely in the tegmentum of the pons and midbrain (Fig. 1), and to a lesser degree in the diencephalon (particularly in the thalamic nuclei), as well as in the cerebellar and cerebral white matter. The severity of the changes varied but was present in all the mice. Severely affected ones showed vacuoles even in the deep cortex. Higher magnification revealed that the sponginess was due to the presence of many large

vacuoles surrounded and occasionally traversed by the myelinated fibers and to the vacuolated clear cytoplasm of glial cells. The nuclei of these cells were enlarged, and their chromatin was scattered. Occasionally, the vacuoles were at the periphery of the neuronal perikarya. A variety of stains—hematoxylin and eosin, periodic acid-Schiff, Bodian, and Heidenhain's stains—failed to stain the vacuoles, as did Sudan IV on frozen section.

Liver. In general, the histologic architecture was well preserved. The nuclei of the hepatocytes varied in size slightly, and binucleated hepatocytes were occasionally seen.

After 3 days' feeding of Cuprizone, the most distinctive feature was the presence of small vacuoles within the hepatocytes. These vacuoles became larger after 7 days and occupied almost the entire cytoplasm of hepatocyte (Fig. 2). These vacuoles measured up to 20 μ in the greatest dimension. Some nuclei appeared indented owing to the presence of such vacuoles (arrow, Fig. 2). After 13 days of feeding, the size of the vacuoles became smaller than those seen at 7 days, and the ratio of vacuole to cytoplasm also decreased. Succinic dehydrogenase and NADH tetrazolium reductase activities were strong in the edges of these vacuoles in all the mice except for those sacrificed at 3 days.

Electron Microscopy

Central Nervous System. The most prominent changes were: (1) large vacuoles within the myelin sheaths, resembling those observed in triethyltin intoxication; and (2) swollen glial cells with large amounts of cytoplasm and many clear vacuoles (Fig. 3). When the vacuoles formed in the myelin sheaths, the splitting of the sheaths always occurred at the intraperiod lines and usually at the outer loops (Fig. 4). Multiloculated vacuoles were often formed in the myelin sheaths (Fig. 3 and 4). However, the thickness of each layer of the sheaths did not appear to differ from that of the normal state. On only rare occasions, degeneration of the myelin was observed. Swollen glia were observed in both perivascular and intervascular areas. Their cytoplasm was electron-lucent, and the cytoplasmic organelles tended to be clustered near the nuclear membrane. The vacuoles were bound by a single membrane (Fig. 3 and 5). There was no apparent relation between known cytoplasmic organelles and the vacuoles. Some vacuoles were so large that they occupied almost the entire cytoplasm. Microtubules of 180–230 A diameter, some of which had dense central cores, were randomly scattered in the clear cytoplasm of many cells (Fig. 5). The microtubules were occasionally clustered around the centrioles. Smooth

endoplasmic reticulum was proliferated in some of the glia. Collections of small vesicles, dense bodies (about 0.5 μ in diameter), and myelin figures were also observed (Fig. 5), but glial fibers or glycogen granules were not found in any of the cells. Their nuclei were swollen, and their chromatin was scattered. The mitochondria were only slightly enlarged, with small vesicles in their cristae. These glia were often found as satellites of the neurons arrayed in rows in the white matter. Frequently the glia were juxtaposed to the myelin sheaths, and at times a thin layer of myelin formed the cellular wall. Some were attached to the blood vessels. Rarely, there were degenerating neuronal processes containing abnormal mitochondria and dense bodies (Fig. 3). The neuronal perikarya did not appear significantly changed, although large vacuoles often compressed them. The endothelial and perithelial cells appeared essentially normal. The extracellular spaces were not dilated.

Liver.[17] Numerous vacuoles seen by the light microscope proved to be mitochondria of various sizes (Fig. 6). The largest ones were up to 20 μ in diameter, but the smallest mitochondria were comparable with normal mitochondria in size. However, even these normal-sized mitochondria lacked mitochondrial granules, and the stroma of mitochondria was less electron-dense. The cristae of the mitochondria were normal in caliber, but the number was increased. They appeared short in giant mitochondria, and most of the cristae were seen along the inner border of mitochondrial membranes (Fig. 6 and 7). In rare instances, cristae were long and appeared to be located deeper in the matrix. Some megamitochondria suggested the fusion of smaller ones (Fig. 7A).

In most hepatocytes parallel arrays of rough endoplasmic reticulum were no longer seen as parallel to each other. They ran along the surface of disfigured mitochondria (Fig. 6 and 7). Distinct large glycogen area, as seen in normal liver, was not present at 7 days of feeding, but a smaller glycogen area was still evident. There was a marked increase of free ribosomes throughout the cytoplasm. Mild proliferation of smooth endoplasmic reticulum was present at 7 days.

At 14 days outer and inner membranes of most mitochondria became wavy (Fig. 8). Many cristae showed club-shaped dilatation, with accumulation of moderately dense homogeneous material (Fig. 8). In other areas, there was almost complete loss of cristae (Fig. 8 and 9). Such areas of mitochondria, bordered only by single membranes, appeared to be protruding outward from the regular contours of the mitochondria. High resolution study of the transitional zone failed to identify clearly whether the loss of one membrane was inner or outer (Fig. 9, inset).

In the hepatocytes of animals fed 13 days there was marked proliferation of smooth endoplasmic reticulum (SER) (Fig. 10). Free ribosomes were dispersed within SER (Fig. 10). The SER was slightly dilated and mostly contained moderately dense, homogeneous material. In some cisternae of SER there were round bodies of much higher density (Fig. 10, arrow). In some instances, there appeared to be a continuation of the outer membrane of a mitochondrion with that of SER. In general, there was an increase of autophagic vacuoles containing various elements of cytoplasm. Mitochondrial remnants were, however, rarely seen within these autophagic vacuoles. There was mild degree of fatty phanerosis at 14 days.

Discussion

Our interest was first aroused by the histologic similarity of the spongy degeneration (SD) of the brain in man and that produced by Cuprizone in mice. In the course of our study, however, it became clear that the ultrastructural findings of these two conditions were different. Intramyelinic vacuoles were observed in both but have also been seen in some other conditions such as triethyltin intoxication [4] and isonicotinic acid hydrazid-induced encephalopathy.[18] They were always formed between the intraperiod lines and therefore can be considered to be analogous to the embryonic extracellular space since the intraperiod line is formed by apposition of the external surface of the plasma membrane of the myelin forming cells.[19] These vacuoles were, however, not continuous to the extracellular space.[4,20] Also, the study using radioactive sulfate suggested no appreciable alteration of the brain extracellular space in triethyltin edema.[4]

In SD, swollen astrocytes with "watery" cytoplasm contained abnormal mitochondria. Their cristae were distorted and crystalline arrangements were seen in the matrix.[3,21] Similar mitochondria were not observed in our mice. Furthermore, in SD, neither astrocytes nor oligodendroglia showed large vacuoles, as seen in the mice, although some astrocytic cytoplasm contained many small vesicles. In our mice, almost all glia in the lesions were swollen. Since their cytoplasm was electron-lucent, with vacuolation, the identification of the cell types was extremely difficult. The presence, however, of the cytoplasmic microtubules without glial fibers and the close contact with the myelin sheaths seen in some of the cells in the white matter suggest that not only astrocytes but oligodendroglia were affected.

The changes in the oligodendroglia in response to the tissue injury were well studied by light microscopy by Penfield and Cone [22] and by

Ferraro and Davidoff.[23] Maxwell and Kruger [24] reported ultrastructural changes in the oligodendroglia after ionizing radiation. They described swollen oligodendroglia with clear patches in the cytoplasm and with cytoplasmic organelles clustered around the nucleus, leaving a relatively clear space between the organelles and the plasma membrane. Similar changes were seen in our Cuprizone-treated mice. Vacuolation of the oligodendroglia has also been observed in hereditary ataxia of the rabbit[25] in methionine sulfoximine intoxication,[26] and in experimental allergic encephalomyelitis,[27] and may well be a later or more severe changes of "acute swelling" of the oligodendroglia.

Swollen astrocytic cytoplasm has been well documented in various conditions, but the formation of large cytoplasmic vacuoles is rather unusual. These glial changes may possibly be due to severe metabolic disturbances resulting in altered permeability of the cell membrane. Subsequent influx of excessive fluid in the cytoplasm may be responsible for the formation of the vacuoles. As seen in some of the glia, the vacuoles enlarged to occupy almost the entire cytoplasm and perhaps eventually resulted in cellular disintegration. Formation of the intramyelinic vacuoles in this particular condition, therefore, could be explained by the metabolic disturbance of the oligodendroglia unlike the effect in triethyltin intoxication,[4] in which oligodendroglia were normal, and such an explanation may not be tenable.

The changes seen in the liver can be divided into two phases: initial enlargement of mitochondria, and subsequent proliferation of smooth endoplasmic reticulum. Mitochondrial enlargement is indeed a common denominator in any type of cellular injury.[28] In these nonspecific mitochondrial swellings, the degree of the enlargement was mild. In several other experimental conditions, the degree of enlargement appears to be more than slight. In essential fatty-acid deficiency [29] and in partial or complete starvation,[30-32] the hepatic mitochondria showed an approximately 50-fold increase in volume. In riboflavin-deficient mouse liver, the enlargement was calculated as about a 125-fold increase in volume.[33] In Cuprizone-treated liver, mitochondria appeared to be much larger than those seen in riboflavin-deficient cases, although the degree of enlargement was not calculated.

The mechanisms whereby this enlargement takes place have not been established. Several hypotheses have been presented in various experimental conditions. Tandler *et al.*[33] postulated that the alteration of phospholipid constituents of mitochondrial membrane might be responsible for megamitochondria seen in riboflavin-deficient mice. They cited their biochemical studies which showed profound alteration of

phospholipids. The postulated mechanisms whereby megamitochondria were produced from the alteration of phospholipids were divided into two. The first was the loss of membrane integrity, producing selective inflow of certain materials including water. The second was the infusion of mitochondria due to the altered surface adhesiveness created by the changes of phospholipids of the mitochondrial membrane. Others claimed that megamitochondria were produced as the result of compensatory hypertrophy. Wilson and Leduc[29] favored this view from their study on essential fatty acid deficiency, in which there was uncoupling of oxidative phosphorylation. Cuprizone is a chelating agent for copper and is known as a strong inhibitor of the beef liver mitochondrial amine oxidase,[34] which, in all likelihood, is a copper protein complex. Therefore, other copper-protein enzymes such as cytochrome C oxidase could also be affected directly by Cuprizone. With a copper-deficient diet, cytochrome oxidase activity was diminished to 37% of the control value in the rat liver. The electron micrographs showed mild enlargement of mitochondria.[35] It is likely that amine oxidase also was inhibited in that study.[35] If one compares mitochondria seen in dietary copper deficiency and in Cuprizone treatment, one is struck by the remarkable difference in the size of mitochondria between two conditions, while in both conditions activities of the same copper enzymes were apparently diminished. Whether this difference in magnitude of mitochondrial response is due to the difference of severity of enzyme inhibition or whether there are some additional factors involved in Cuprizone treatment or both is not clear. Morphologic evidence favoring the altered permeability of mitochondrial membranes in this case is the loss of either outer or inner membrane seen in some mitochondria at 14 days. It is interesting to note that amine oxidase has been localized in outer membrane of mitochondria.[36] The loss of membrane, therefore, might be a reflection of biochemical effect of Cuprizone on the outer membrane. Surface adhesiveness and fusion of the mitochondria, as the genesis of megamitochondria, may also play a role, as shown in Fig. 7A, but the role was probably secondary, as judged by the scarcity of these findings.

The second phase of liver cell changes after Cuprizone treatment was the enormous proliferation of SER with shrinkage of mitochondria. Proliferation of SER has been seen after the administration of some chemicals and drugs. Such alteration was most thoroughly studied after the administration of phenobarbital,[37] azo dyes and related compounds,[38,39] and allylisopropylacetamide (AIA).[40] These studies indicated that ER was important in the metabolism of certain exogenous

materials. In case of AIA administration, it has been shown that there was increase of microsomal reduced nicotinamide adenine dinucleotide phosphate-cytochrome C reductase and glucose 6 phosphate-dehydrogenase,[41,42] which were closely associated with drug oxidation and reduction processes.[43] Therefore, the proliferation of SER in Cuprizone-treated liver cells probably is a similar reflection of drug metabolizing activity, which probably is unrelated to Cuprizone's action against mitochondria.

The relation between CNS and hepatic lesions is not clear. It is worth noting, however, that in Cuprizone-treated mice, the lesions in CNS and liver appeared almost simultaneously. From these findings, it appears to us more likely that Cuprizone produced both liver and CNS damage directly, rather than that the liver damage induced the CNS alteration.

In connection with this, it must be remembered that spongy status has been well documented in certain hepatic disorders and was thought to be secondary to the liver disease. There remains, however, a possibility that lesions of both CNS and liver may be produced directly by the same noxious agents.

Summary

Oral administration of Cuprizone (biscyclohexanone oxalyldihydrazone) produced severe status spongious in the CNS, most prominently in the brain stem and cerebellar white matter of mice. Ultrastructural study showed the status spongious was due to the formation of the vacuoles in the myelin sheaths, as seen in triethyltin edema and isonicotinic acid hydrazid-induced encephalopathy, and also due to vacuole formation in the cytoplasm of glial cells. The mice fed with Cuprizone were also found to have giant mitochondria and proliferation of smooth endoplasmic reticulum in the hepatocytes. The possible mechanisms of the CNS lesion and hepatic mitochondrial changes, and their relationship were discussed.

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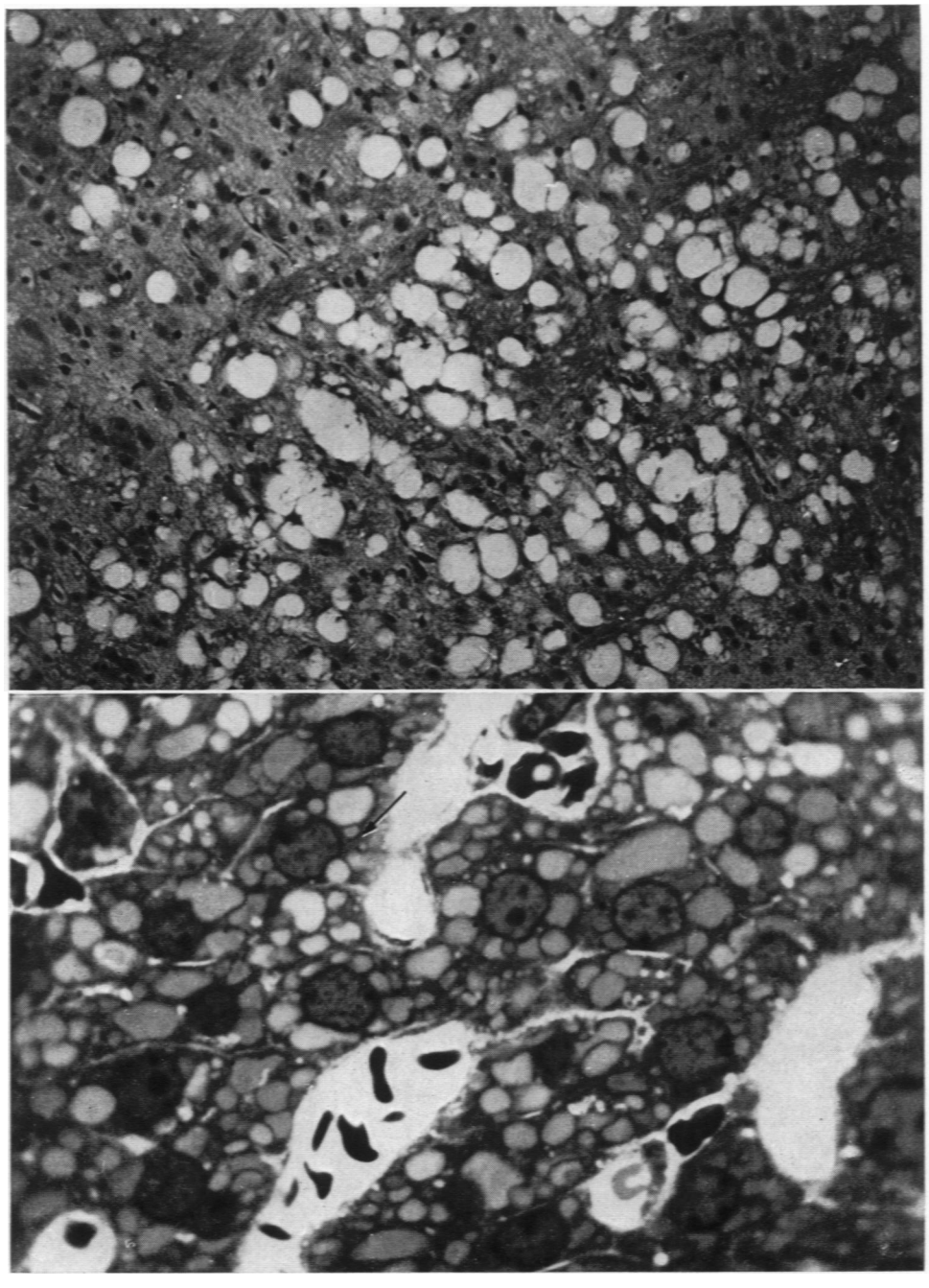
[*Illustrations follow*]

Legends for Figures

Fig. 1. Midbrain from mouse fed with Cuprizone for 10 days. Note diffuse status spongiosus. Hematoxylin and eosin stain. × 160.

Fig. 2. Liver from mouse fed with Cuprizone for 8 days, embedded in Epon. Many vacuoles of variable size occupy cytoplasm of hepatocytes. Indentation of nuclei by vacuoles is sometimes seen *(arrow)*. Cresyl violet and azure II stain. × 1600.



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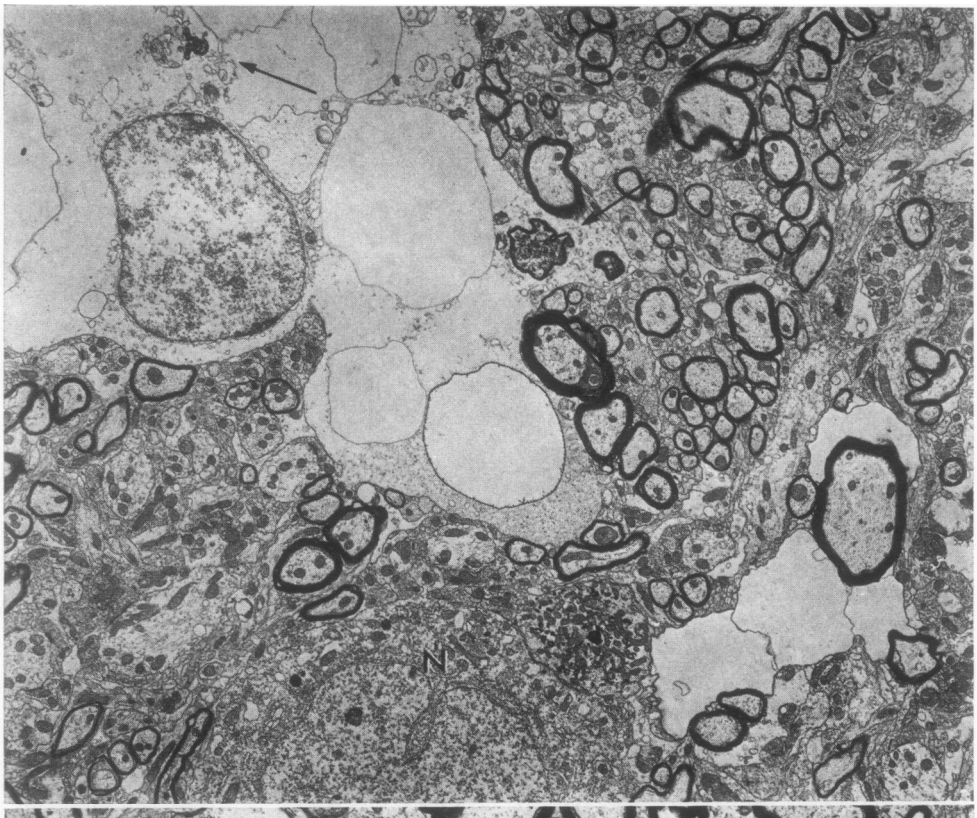

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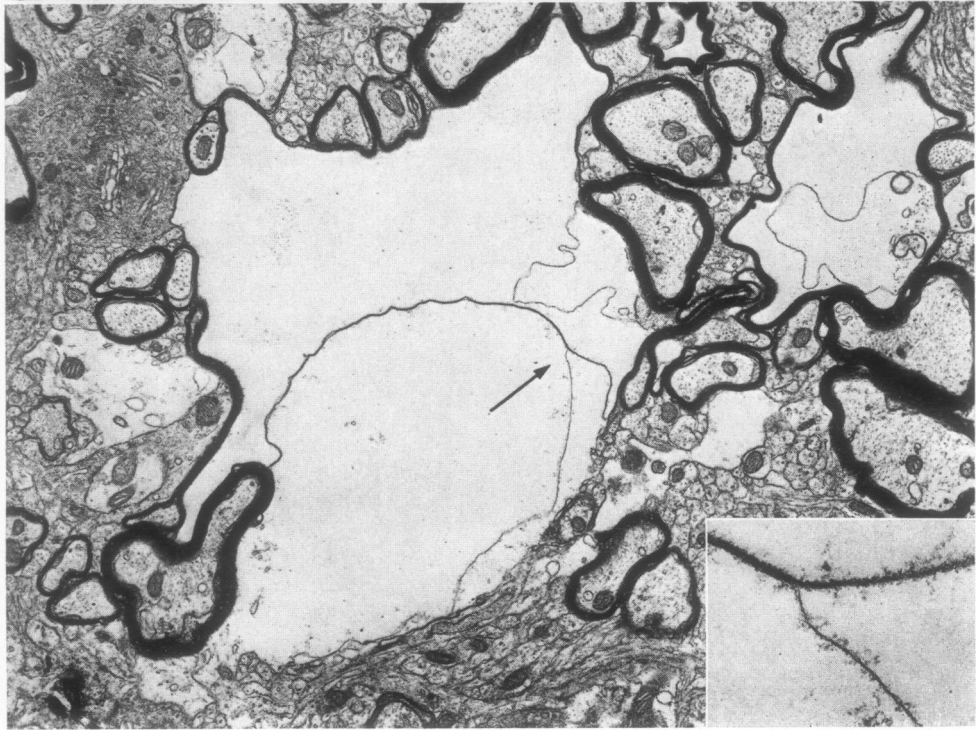

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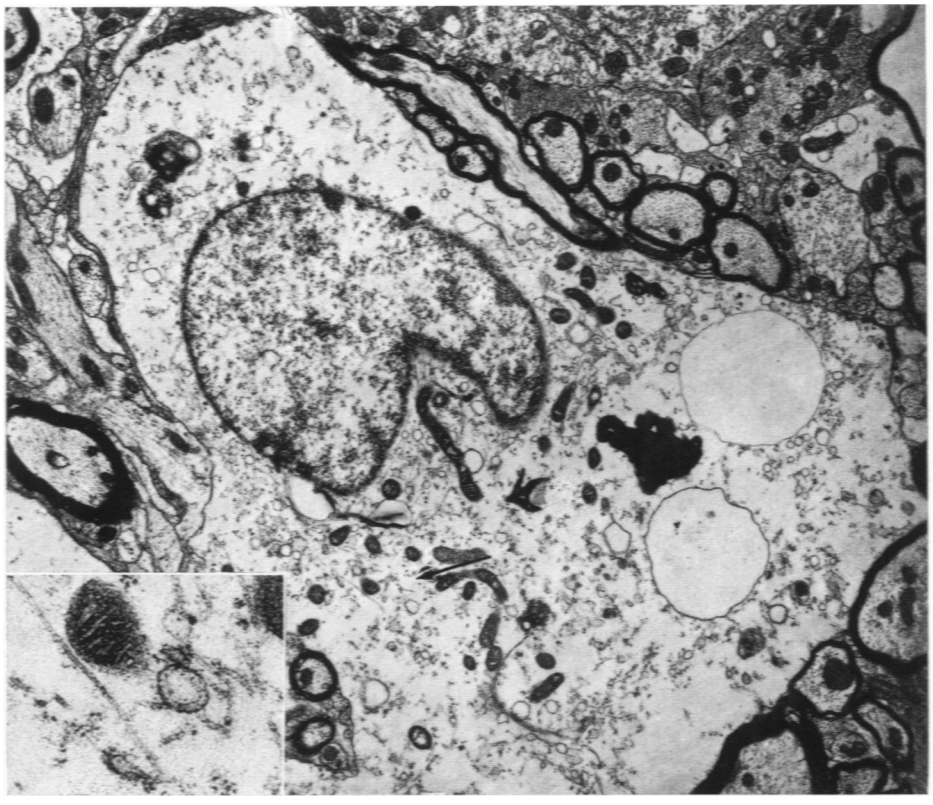

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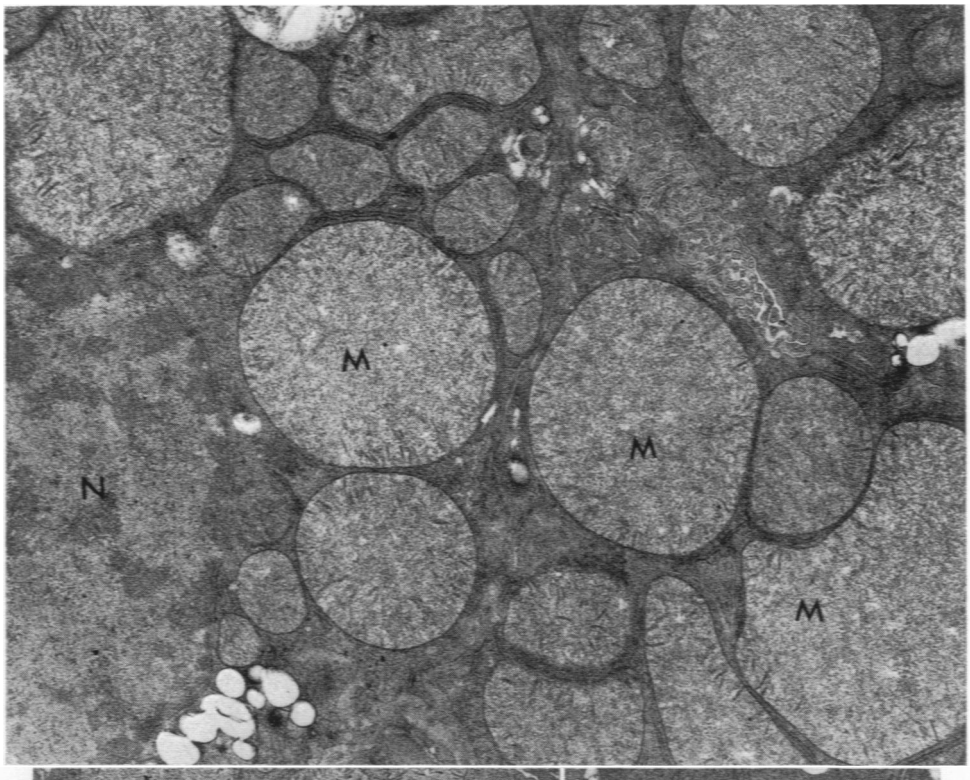
Fig. 3. Cytoplasm of clear glial cell contains many single membrane-bound vacuoles. Phagocytized myelin is seen in cytoplasm *(arrows)*. Formation of intramyelinic vacuoles is seen at lower right. Neuron *(N)*. × 5800.

Fig. 4. Vacuoles are seen between myelin sheaths. × 7500. **Inset** *(arrow)*. Myelin splitting occurs always at intraperiod line. × 48,000.

Fig. 5. Clear glial cell contains membrane bound vacuoles and myelin figures in cytoplasm. × 5800. **Inset** *(arrow)*. Many microtubules are also scattered in cytoplasm. × 28,000.

Fig. 6. Enlarged mitochondria *(M)* in hepatocyte after 7 days of Cuprizone feeding. Cristae are located at periphery. Note smooth mitochondrial contour and lack of mitochondrial granules. Nucleus *(N)* is located at left. × 8000.

Fig. 7. Giant mitochondria in hepatocyte. **A.** Lobulation. **B.** Irregular arrangement of cristae. Note irregular arrangement of granular ER and increase of free ribosomes. **A,** × 5500; **B,** × 9000.



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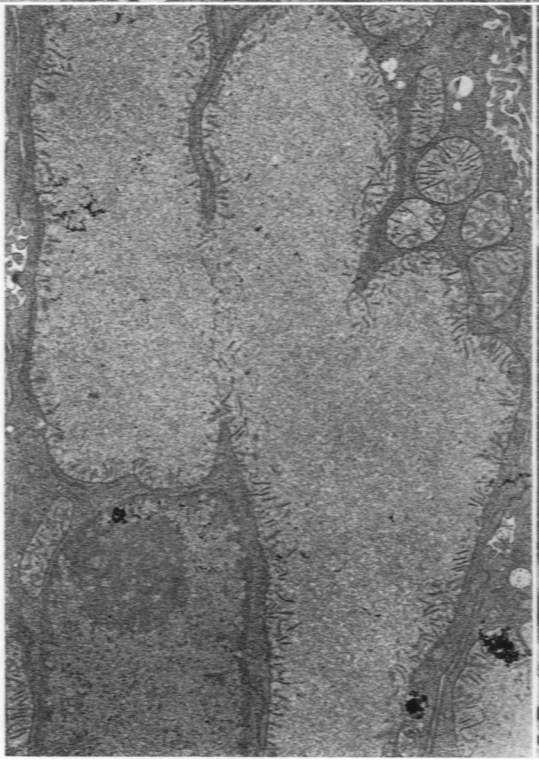

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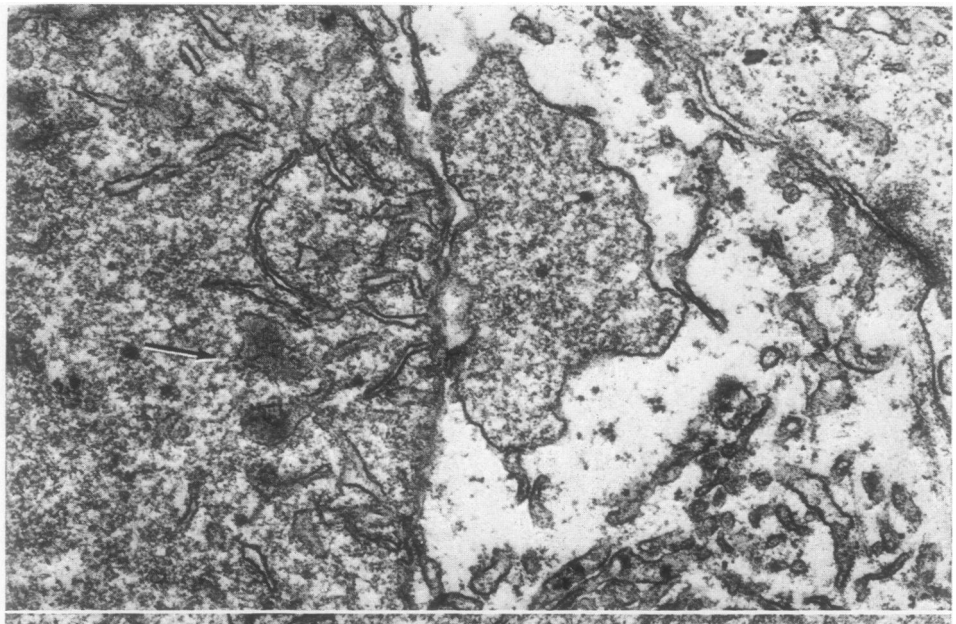

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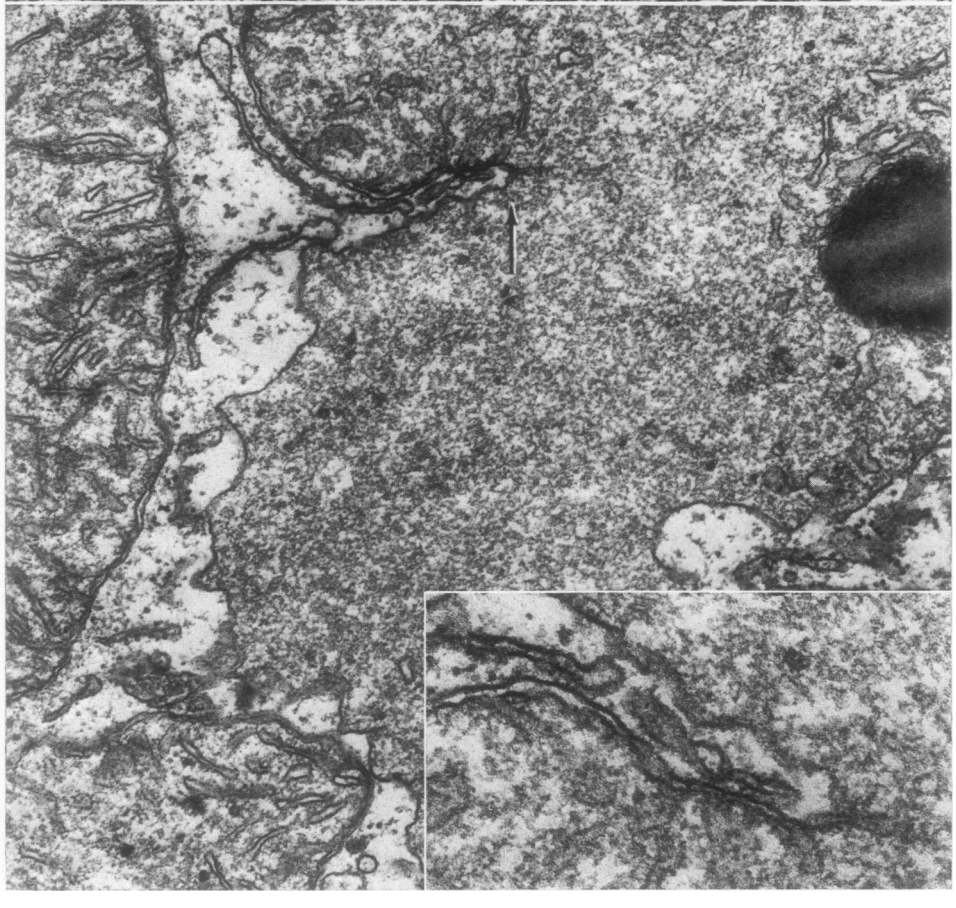

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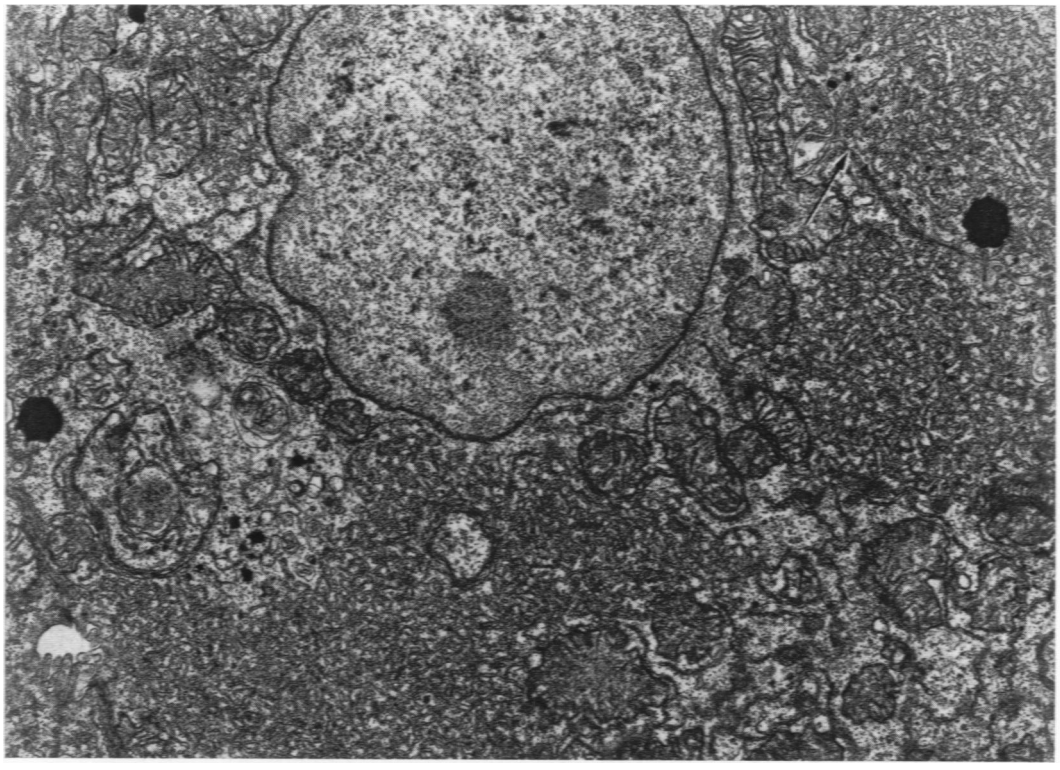

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Fig. 8. Club-shaped dilatation of mitochondrial cristae *(arrow)* in mitochondrion of liver cells after 14 days of feeding. × 28,000.

Fig. 9. Portion of mitochondrion bounded only by a single membrane. Note outward bulging and lack of cristae in this zone. × 29,000. **Inset** *(arrow)*. Junction of double and single membranes. × 66,000.

Fig. 10. Proliferation of smooth endoplasmic reticulum (SER) is noted after 14 days of Cuprizone feeding. Sometimes highly electron-dense, round bodies are seen in cisternae of SER *(arrow)*. Mitochondria are smaller and surface wrinkled. × 7000.