DIVISION OF GIANT MITOCHONDRIA DURING RECOVERY FROM CUPRIZONE INTOXICATION

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INTRODUCTION

Giant mitochondria may be induced in mouse hepatocytes either by riboflavin deficiency (30) or by addition of the copper-chelating agent, cuprizone (27, 28), to the diet. In both cases, the mitochondria attain enormous size, often exceeding the cell nuclei in diameter. It has recently been shown that the megamitochondria resulting from riboflavin deficiency could be restored to normal dimensions within a relatively short period of time by injection of vitamin B_2 into the deficient animals (29). Mitochondrial normalization was brought about by division of the enlarged organelles. This process involved the formation of a membranous partition, which separated the mitochondrion into two distinct chambers. Progressive constriction of the organelle at the level of the partition ultimately resulted in formation of two daughter mitochondria.

In the present study, it was found that cuprizone-induced giant mitochondria could be returned to normal size in an astonishingly short time simply by removing the noxious agent from the diet. Mitochondrial recovery involved division, but proceeded by a totally different route than in recovery from riboflavin deficiency.

MATERIALS AND METHODS

Weanling male CFW Webster mice (Carworth Div. of Becton, Dickerson and Co., New City, N. Y.) were placed on a powdered complete diet containing 0.5% cuprizone (biscyclohexanone oxaldihydrazone) (G. Frederick Smith Chemical Co., Columbus, Ohio). After 9–14 days, depending on the clinical appearance of the animals, the cuprizone-containing diet was replaced by Purina lab chow (Ralston Purina Co., St. Louis, Mo.). Animals were sacrificed by decapitation at various intervals after the normal diet was instituted (Table I).

Specimens of liver were fixed in 2% OsO_4 buffered with phosphate (20) or collidine (5) After rinsing in saline, the tissue blocks were soaked in 0.5% uranyl acetate made up in saline. The blocks were again rinsed in saline, dehydrated in graded ethanol, and embedded in Maraglas-DER 732 (Polysciences, Inc, Rydal, Pa.) (8). Thin sections were stained with uranyl acetate (26) and lead tartrate (21), and examined in a Siemens Elmiskop Ia electron microscope.

RESULTS

Hepatic cells in mice exposed to cuprizone were similar to those described by Suzuki and Kikkawa (28). The cells were characterized by the presence of giant mitochondria as well as mitochondria of normal size, and by a paucity of granular endoplasmic reticulum. Inspection of 1 μm thick sections in the light microscope revealed that enlarged mitochondria were evenly distributed throughout the liver lobules. At the ultrastructural level, the megamitochondria had a characteristic morphology (Fig. 1). They were predominantly spherical to ovoid, with few surface irregularities. Irrespective of organelle shape, the cristae were not increased in length when compared to those in normal mitochondria, and were restricted entirely to the periphery of the organelle. The inner compartment contained a vastly augmented matrix, which in its central regions was devoid of cristae. In any given hepatocyte, the density of the enlarged mitochondria was identical to that of normal sized organelles. The biochemical effects of cuprizone intoxication on mitochondria are reported elsewhere (14).

Animals fed cuprizone for varying lengths of time were placed on a cuprizone-free diet, and their livers were examined at various time points during recovery. For the first 2 h after the normal

TABLE I

Animals Fed Cuprizone for Varying Lengths of Time were Placed on Cuprizone-Free Diet and Recovery Time was Recorded

No. of animals	Days on cuprizone	Recovery time (h on recovery [normal] diet)
7	9	0
5	11	0
2	14	0
2	9	1
2	9	2
3	11	2
1	9	3
3	11	3
1	14	3.5
4	9	4
3	11	4
1	14	4
1	14	4.5
3	9	5
1	14	5
1	9	5 5
3	9	6
1	9	8
1	9	12
2	9	24
2	9	48
2	9	72

diet was initiated, no changes were noted After 3 h, some of the enlarged mitochondria were quite pleomorphic. A few of these organelles were greatly elongated, and were branched or *Y* shaped. Other giant mitochondria were cup shaped.

At the end of the 4th h of recovery, the degree of mitochondrial pleomorphism was considerably more pronounced, involving most of the enlarged organelles (Figs 2–6). Many of the largest mitochondria were cup shaped (Fig 3), and often contained a cytoplasmic organelle, usually a peroxisome or another, smaller mitochondrion within their concavities Other megamitochondria with attenuated midregions were shaped like dumbbells, rather than being cotyliform (Fig. 4). At their thinnest point, they measured as little as 0.05 μm (Fig. 5). Based on serial-section analysis of ostensibly similar mitochondria in normal rat liver, Stempak (25) concluded that in reality such organelles were biconcave disks. We have also examined our material in serial sections and are convinced that at least in this case the dumbbell-shaped giant mitochondria have an hourglass configuration in three dimensions A few enlarged mitochondria were observed that lacked an elongated, attenuated midregion, but were deeply and sharply notched or incised, giving them a segmented appearance (Fig 6)

By the end of 5–8 h of recovery, virtually all mitochondria were normal in size and appearance (Fig 7). Despite extensive search, partitioned mitochondria were not observed at any stage of recovery.

In addition to mitochondria, other hepatic organelles underwent progressive changes during recovery. At 3–4 h, the hepatocytes contained greatly increased numbers of pericanalicular dense bodies (lysosomes) (Fig. 2). At the same time, the number of peroxisomes began to rise, continuing in this manner until a peak was reached at 24 h During this interval, peroxisome-desmosome complexes were abundant (31).

DISCUSSION

Mitochondria with attenuated or constricted midregions, as well as mitochondria with surface protrusions or "buds" have been described previously in protists (7, 13, 19, 33), in liverworts (6) and in higher plants (2), in cardiac muscle (9), in brain (24), and in both normal and pathologic liver (3, 4, 10, 15, 16). Such configurations were interpreted as representing stages in mitochondrial fission. It was not possible, however, to determine unequivocally if these mitochondria actually were dividing, were merely displaying normal mitochondrial plasticity, or were in the process of fusion. Interpretation of the significance of the mitochondrial morphology with respect to scission is facilitated by examination of certain lower forms in the logarithmic phase of growth. In *Neurospora crassa*, morphometric analysis (11) and biochemical determination of mitochondrial mass coupled with electron microscopy (17, 18) have been used to follow increases in the mitochondrial population. In the former study (11), cup-shaped mitochondria were abundant during the concomitant doubling of the mitochondrial and mycelial masses. In the latter study (18), when cultures of *N. crassa* maintained on a low-choline medium were exposed to a choline-rich medium, the mitochondria became constricted at some points, producing a lobated profile. Such organelles were rapidly succeeded by considerably smaller mitochondria, suggesting that "pinching off" had occurred (18). In synchronous cultures of the

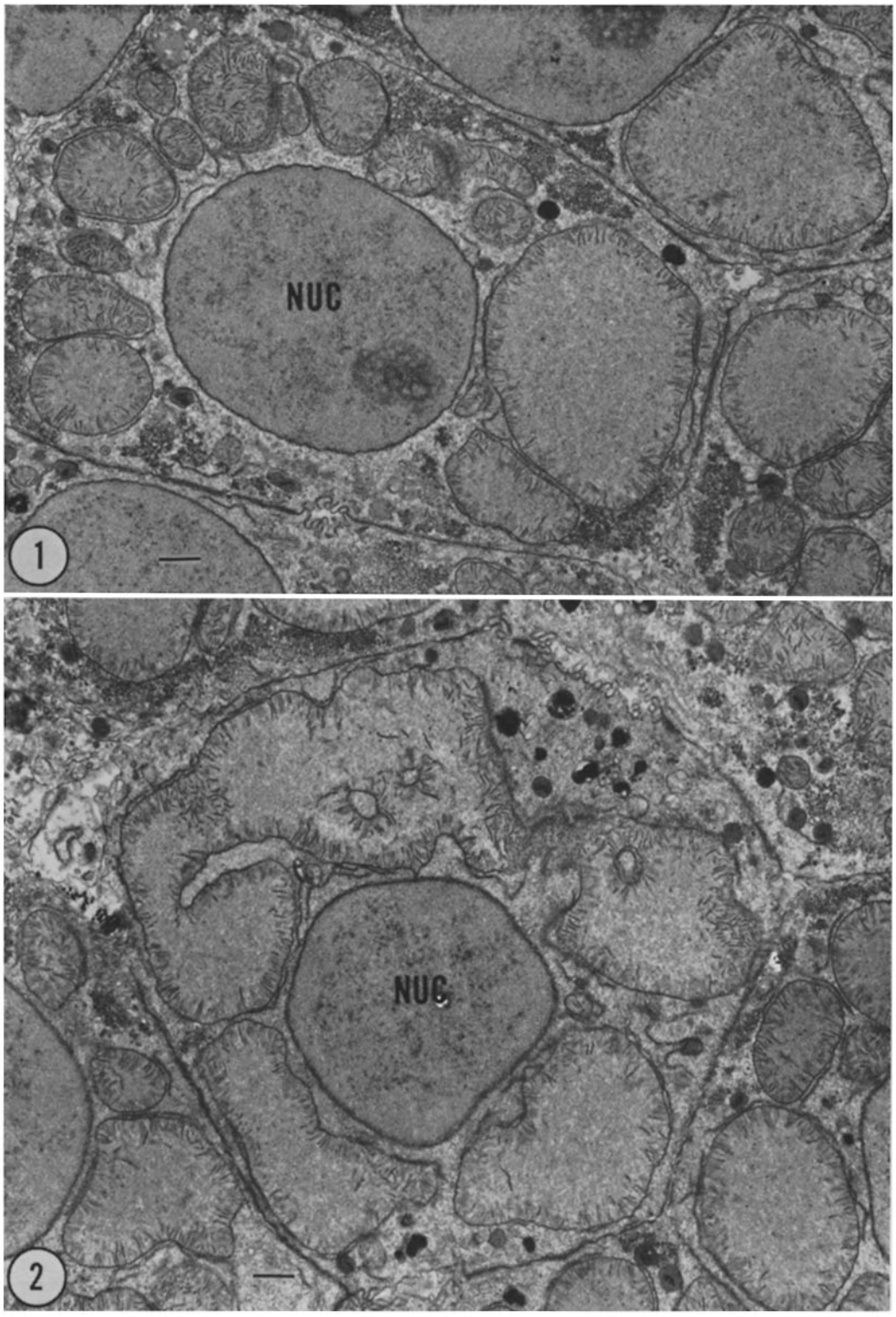

FIGURE 1 Survey electron micrograph of several hepatocytes from a mouse receiving cuprizone for 9 days. Note the large mitochondria with peripheral cristae. *NUC* = nucleus. The scale line on this and all succeeding figures = 1 μm. × 5800.

FIGURE 2 A huge, extremely irregular mitochondrion looms over the nucleus (*NUC*). Cuprizone 11 days, 4 h recovery. × 6200.

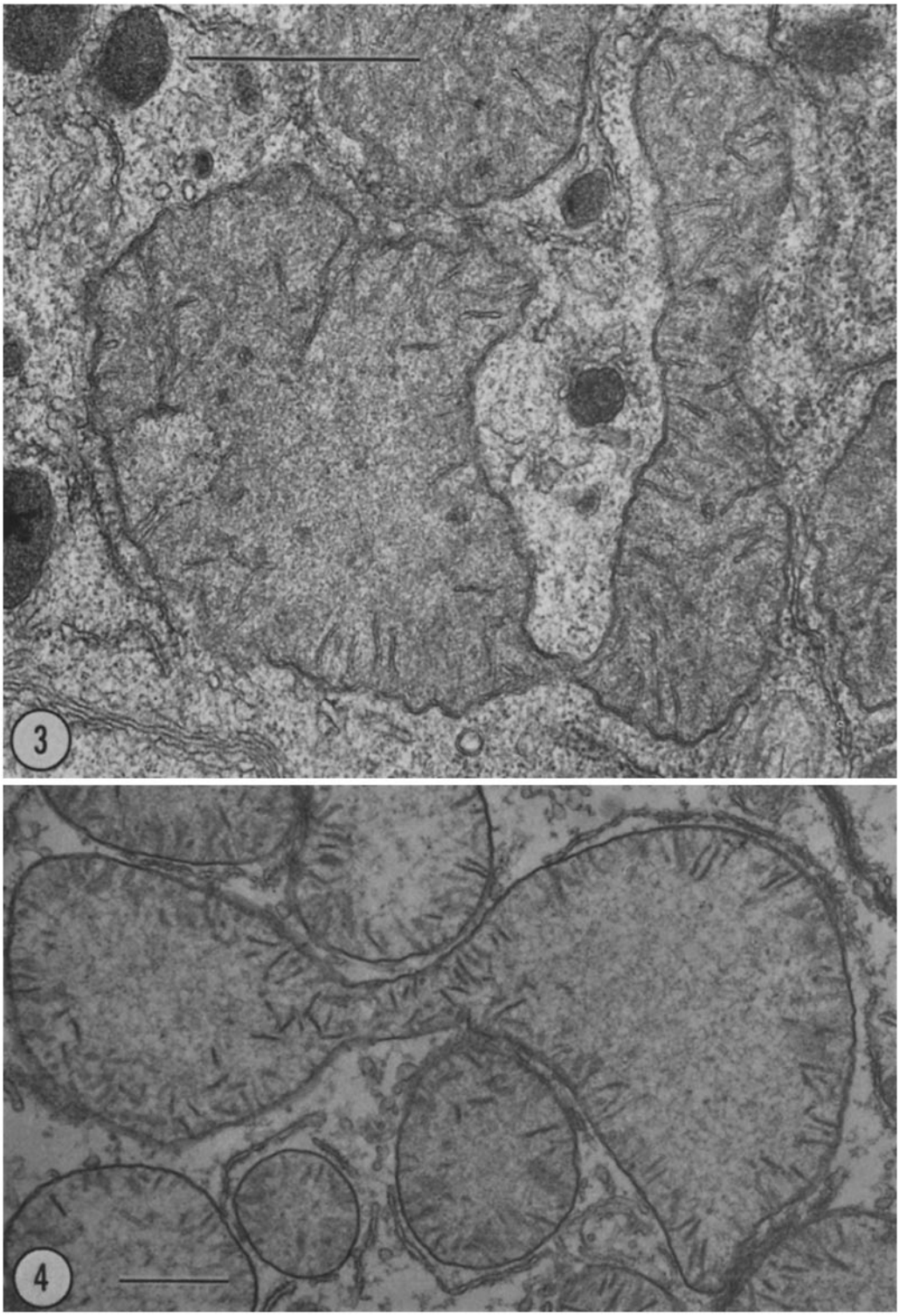

FIGURE 3 A profile view of a cup-shaped megamitochondrion. Cuprizone 9 days, 4 h recovery × 35,000.

FIGURE 4 A dumbbell-shaped giant mitochondrion. Cuprizone 11 days, 4 h recovery. × 16,800.

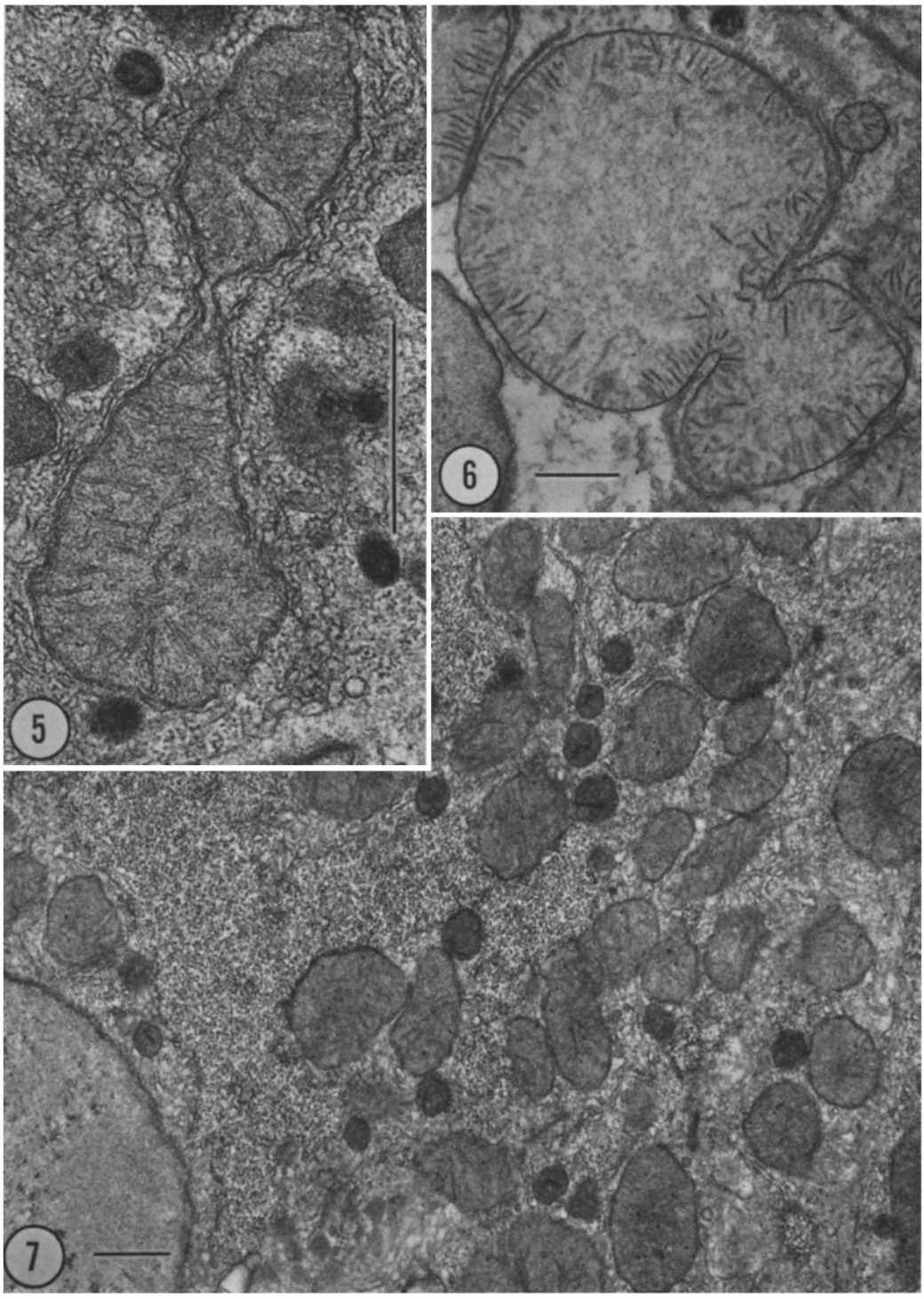

FIGURE 5 A mitochondrion with an extremely attenuated midregion. Cuprizone 9 days, 4 h recovery. × 31,000

FIGURE 6 A large mitochondrion with a deep constriction. Cuprizone 11 days, 4 h recovery. × 12,400.

FIGURE 7 The mitochondria in this cell, and in surrounding hepatocytes as well, have returned to near normal dimensions. The peculiar appearance of the glycogen is due to en bloc staining with uranyl acetate (33). Cuprizone 9 days, 5½ h recovery. × 11,500.

fission yeast *Schizosaccharomyces pombe*, respiratory activity measurements and ultrastructural observations were employed to follow mitochondria through the complete cell cycle (22, 23). It was found that oxygen consumption and the number of mitochondria per cell increased before cell division, during this period, the mitochondria had a dumbbell shape.

A further example of mitochondrial budding may be found in malarial parasites of birds (1). In each trophozoite, there is but a single mitochondrion During schizogony, which is characterized by budding of individual trophozoites to yield many merozoites, the mitochondrion becomes lobated and undergoes fission, so that each forming merozoite receives a portion of the parent organelle, through the agency of cytoplasmic flow It is of interest to note that in another, nonmalarial protozoan (*Boderia*) undergoing schizogony, dividing mitochondria were characterized by partition formation and constriction (12).

In the study referred to earlier, Tandler et al. (29) took advantage of the change in size of hepatic mitochondria during recovery from riboflavin deficiency to establish a temporal sequence in mitochondrial division. Changing dimensions may also be utilized to establish the sequence of events during mitochondrial recovery from cuprizone poisoning The onset of recovery after cuprizone is deleted from the diet appears to be independent, within limits, of the length of time that the mice were receiving the drug In every case, morphological signs of recovery appeared between the 2nd and 3rd h This suggests that cuprizone is either quickly excreted from the body, probably via the kidneys, or is rapidly degraded. The earliest indication of recovery was the appearance of mitochondrial pleomorphism The change in form leads to the produc ion of hourglass- or dumbbell-shaped mitochondria, or organelles which are deeply incised near their equator. Continued constriction at the slenderized region of the organelle must result in a form where the inner limiting membranes from opposite sides of the mitochondrion first touch, then fuse. Ingrowth of the outer limiting membrane completes the process by separating the organelle into two mitochondria. Repetition of this process eventually reduces the mitochondria to normal size, and restores the normal complement of mitochondria to each hepatocyte.

Because the cells in liver are not synchronized, it obviously is difficult to demonstrate solely by electron microscopy that mitochondrial division has in fact taken place. Nevertheless, in the presence of obvious reductions in size and increases in number of mitochondria, and in the absence of redundant mitochondrial membranes or mitochondrial breakdown, an alternative interpretation is not readily apparent

SUMMARY

Mice fed a diet containing the copper chelator, cuprizone, developed greatly enlarged hepatic mitochondria. These enlarged organelles could be restored to normal size within a few hours by removing the drug from the diet. The onset of recovery was signaled by increasing mitochondrial pleomorphism. Mitochondrial normalization was brought about by elongation and attenuation of the midregions of the giant mitochondria, ultimately resulting in separation into two daughter organelles Repetition of this process reduced the mitochondria to normal dimensions. Throughout recovery, *no* partitioned mitochondria were observed.

A brief report of some of this work has appeared (1971 *Anat. Rec.* **169**:442).

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