RIBOFLAVIN AND MOUSE HEPATIC CELL STRUCTURE AND FUNCTION

II. Division of Mitochondria during Recovery from Simple Deficiency

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ABSTRACT

Mice which had been on a riboflavin-free diet for 6–8 wk were given daily intraperitoneal injections of riboflavin. The hepatic mitochondria, which in the deficient animals were greatly enlarged, were restored to normal dimensions within 3 days. Normalization of the mitochondrial population was brought about by division of the giant organelles. Dividing mitochondria were characterized by a membranous partition separating the inner compartment into two distinct chambers. Such organelles showed varying degrees of pinching at the level of the partition. The most common site of partition formation was at the base of a small mitochondrial bud. During the 1st day of recovery, dividing mitochondria were so common that they could be easily found in mitochondrial pellets. Injection of riboflavin into normally nourished mice also produced an apparent increase in the frequency of dividing mitochondria in the liver cells.

INTRODUCTION

The effects of a riboflavin-free diet on the ultrastructure of mouse hepatic cells were described in a previous report (44). It was found that after 7 wk of deficiency the hepatic mitochondria had undergone striking changes in size. At least a few mitochondria in almost every cell attained gigantic proportions, some being larger than the cell nuclei. Except for this increase in size, the megamitochondria, for the most part, were normal in appearance. A few of the enlarged mitochondria, however, possessed numerous, elongated cristae, which displayed a marked tendency toward alignment in centrally located stacks. Many paired mitochondria were observed, the configuration of which suggested that fusion was occurring. It was postulated that the production of the megamitochondria was in large measure due to fusion of smaller organelles and, to a lesser extent, due to mitochondrial growth.

It is well known that pronounced symptoms of

nutritional deficiency may be relieved by the adminstration of the appropriate trophic substances. The present study was undertaken to determine if the subcellular effects of riboflavin deficiency were reversible. It was found that within 3 days after recovery was initiated by injection of riboflavin the hepatic cells were almost normal in appearance. This article describes the manner in which the mitochondrial normalization takes place.

MATERIALS AND METHODS

The experimental animals were randomly bred Swiss ICR (Millerton Farms, Millerton, N.Y.) female mice. Sixty animals were used in each experiment, the entire experiment being repeated five times. 3-wk-old mice were made riboflavin-deficient by feeding a diet identical with that described in the previous report (44). Control animals were maintained on the deficient diet, which was supplemented with riboflavin in the amount of 2 g per 100 lb. of diet.

After 6–8 wk on the deficient diet, those animals weighing less than 12 g were selected for further observation. (Such animals represented approximately one-third of the original number of mice.) In turn, half of these mice were randomly selected and immediately sacrificed, while each of the remaining mice received a single daily intraperitoneal injection of 0.35 mg of riboflavin[1]. Two or three recovering animals were sacrificed at 2.5, 3.5, 4.5, and 6.5 hr after receiving the initial injection of riboflavin. Additional animals were sacrificed at 1, 2, 3, 7, and 14 days after commencement of daily administration of the vitamin. As a further control, 10-wk-old mice that had been maintained on an *ad libitum* diet of Purina lab chow were injected intraperitoneally with 0.35 mg of riboflavin, and were sacrificed 3.5 hr later.

Samples of liver were prepared for electron microscopy by the procedures described in the previous report (44).

In a separate set of experiments, mitochondria were isolated from livers of control mice and from livers of recovering animals, which were sacrificed 3.5–4.5 hr after injection of riboflavin. The mice were sacrificed by cervical dislocation and the livers were removed and placed in cold sucrose solution (0.25 sucrose, 10 mM Tris-Cl pH 7.5, 1 mM Tris-succinate, and 0.2 mM EDTA). After the livers had cooled to 4°C, they were dried with cheesecloth and weighed. The livers were homogenized in 10 volumes of the sucrose solution (w/v) by using either a loose fitting Dounce homogenizer (Blaessig Glass Specialties, Rochester, N.Y.) with six passes of the pestle or a glass Teflon motor-driven homogenizer with four passes of the pestle. All subsequent operations were carried out at 4°C. The homogenate was centrifuged at 800 *g* for 10 min and the supernatant suspension was decanted off through two layers of cheesecloth and then centrifuged at 6,000 *g* (Spinco rotor No. 30, 8,000 rpm) for 15 min. The supernatant suspension was discarded and the tan mitochondrial pellet was resuspended by homogenization in five volumes of the sucrose solution. The mitochondria were reisolated by centrifugation at 6,000 *g* for 15 min. The mitochondrial pellets were then homogenized in the sucrose solution and the protein concentration was adjusted to 20 mg/ml. Protein was determined by the method of Gornall, Bardawill, and David (15).

The isolated mitochondria were purified by centrifugation in a discontinuous sucrose gradient with the Spinco 25SW or the 39SW rotors. The gradient in the 25SW tube was made up of 5 ml each of 1.6, 1.5, 1.4, and 1.3 M sucrose containing 10 mM Tris Cl, pH 7.5, while the gradient in the 39SW tube consisted of 1 ml each of 1.6, 1.5, 1.4, and 1.3 M sucrose. The mitochondrial suspension (1 ml) was layered on the gradient and centrifuged at 22,500 rpm for 60 min. Each of the sucrose layers was carefully removed with a Pasteur pipette and diluted 1:1 with distilled water, and the mitochondria were recovered by centrifugation at 6,000 *g* as described above.

For electron microscopic examination of the isolated mitochondria, 1–2 mg protein of mitochondria was suspended in 5 ml of the sucrose solution and centrifuged at 26,000 *g* for 10 min. The supernatant solution was removed by aspiration, and the mitochondrial pellets were processed for electron microscopy in the same manner as the solid blocks of liver, except that fixation was carried out at 4°C rather than at room temperature.

OBSERVATIONS

Control Animals The histological and cytological appearance of the hepatocytes was normal in every respect.

Deficient Animals without Riboflavin Alterations in the mitochondrial population of the hepatocytes were evident in all of the mice chosen at random from the 12 g group. At least some mitochondria in almost every cell were greatly enlarged (Fig. 1). These large mitochondria closely conformed in size and appearance to those produced under identical nutritional conditions as reported previously (44).

Deficient Animals with Riboflavin During the first 3 days of recovery, numerous examples of division of giant mitochondria were observed and appeared to be randomly distributed throughout the lobule. A few dividing mitochondria were apparent as

[1] Obtained as Riboderm from Key Pharmacal Co., Memphis, Tennessee.

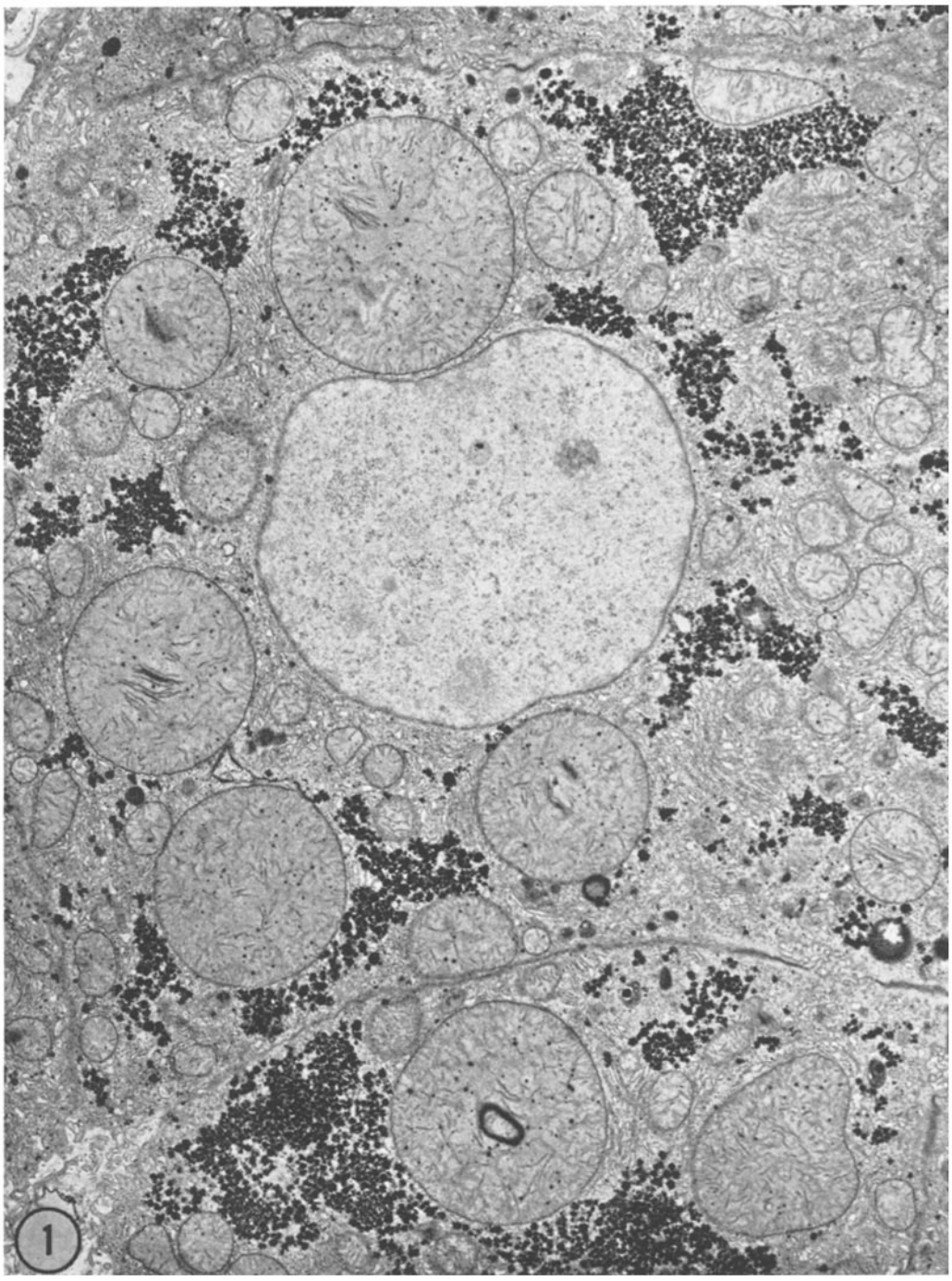


FIGURE 1 Survey micrograph of hepatic cells from a mouse on a riboflavin-free diet for 6 wk. Note the enormous size of many of the mitochondria. Lead citrate. × 6,600.

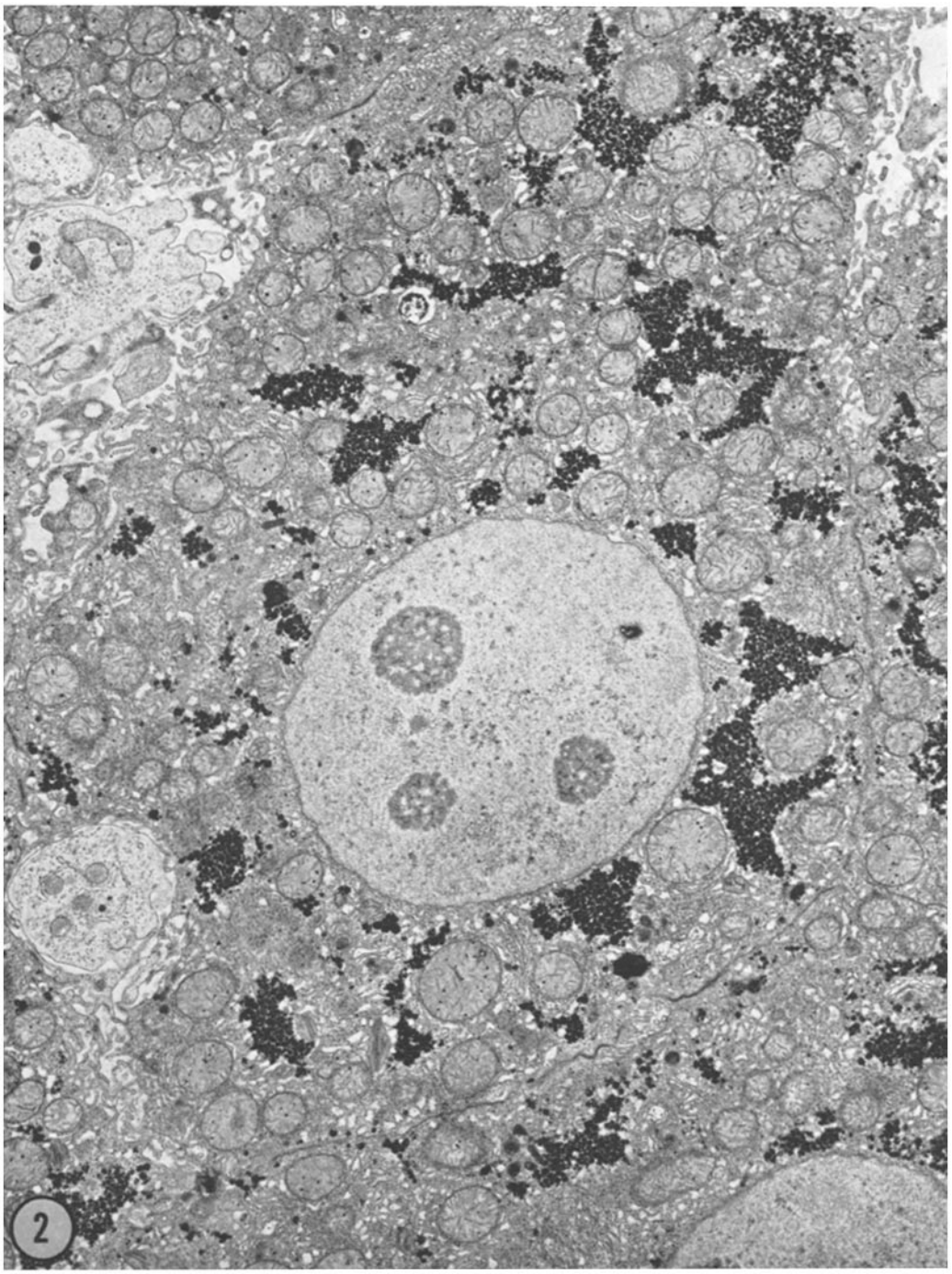


FIGURE 2 Survey micrograph of hepatic cells from a mouse on a riboflavin-free diet for 6 wk, 3 days after initiation of riboflavin injections. The mitochondria are nearly normal in size, but are more spherical than usual. Lead citrate. × 6,600.

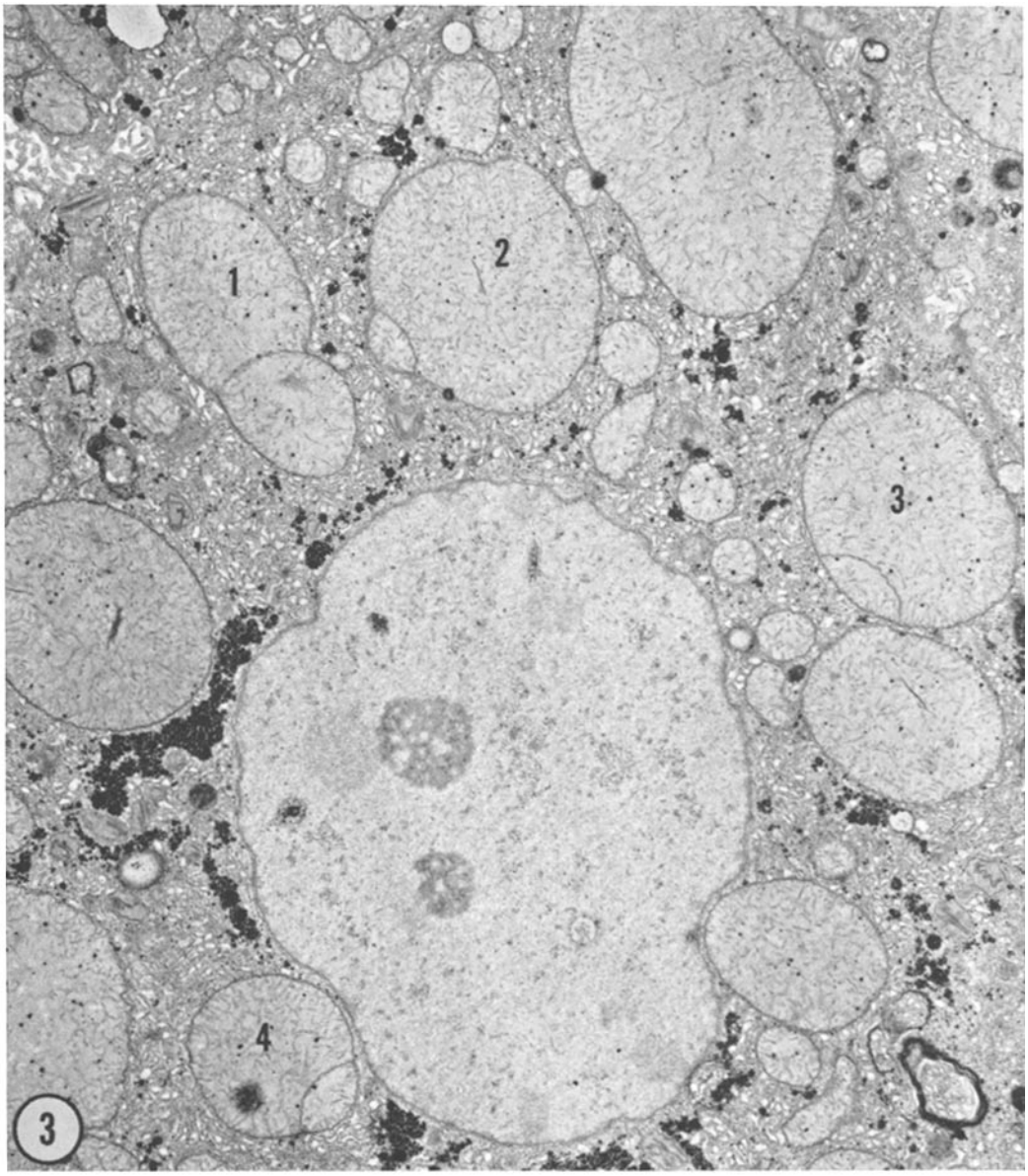


FIGURE 3 Survey micrograph of a liver cell from a 6 wk riboflavin-deficient animal, 24 hr after initial injection of riboflavin. Four dividing mitochondria (1–4) are apparent in this single cell. An autophagic vacuole with unusually dense limiting membranes is in the lower right corner. Lead citrate. × 7,600.

early as 2.5 hr after the first injection of riboflavin. They increased in frequency through the first day to a peak at 24 hr, then quickly declined in number. At the height of mitochondrial division, i.e. 24 hr, a number of cells were seen which contained as many as four or five dividing megamitochondria in the thickness of a single section (Fig. 3). It should be noted that in many other hepatic cells no evidence of mitochondrial division was apparent, although megamitochondria were present. At time points lying on either side of the peak, no more than one dividing organelle was

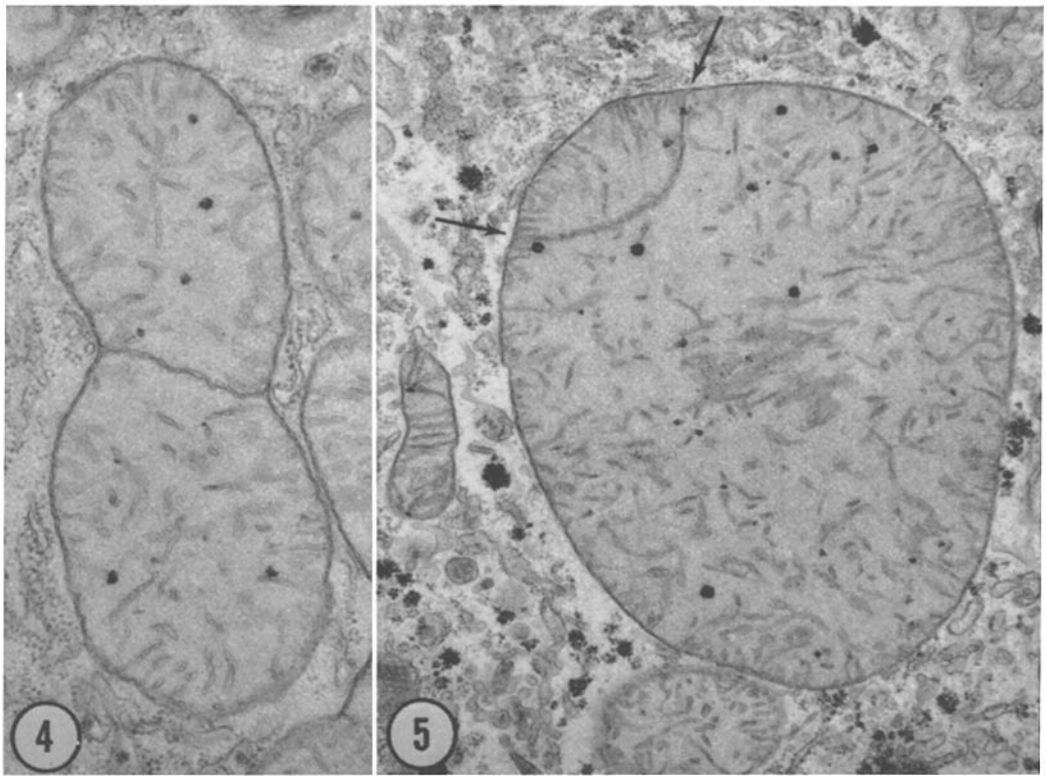


FIGURE 4 A dividing mitochondrion of normal size. This organelle is traversed by a median partition and shows a narrowing at the level of the partition. 6 wk riboflavin-deficient, 6.5 hr recovery. Lead citrate. × 38,000.

FIGURE 5 A dividing megamitochondrion. A partition (arrows) separates a small portion of organelle from the major mitochondrial mass. This sequestered moiety is about equal in size to the normal mitochondrion at the left. 6 wk riboflavin-deficient, 1 day recovery. Lead citrate. × 24,000.

usually present in any cell section, while the majority of cells showed no activity of this nature.

At the end of 3 days, the hepatic mitochondria were nearly normal in size, although they displayed a tendency to be more spherical than usual (Fig. 2). Although a rigorous histometric analysis was not performed, it was obvious that the number of mitochondrial profiles had greatly increased. A few megamitochondria persisted for 1 wk, but none were observed at 2 wk of recovery. At this point, no morphological evidence of cell injury could be detected.

Dividing mitochondria were characterized by a membranous partition or septum which separated their matrix compartment into two chambers (Figs. 4–8). The partitions consisted of two membranes, each of which was in direct continuity with the inner mitochondrial membrane. By definition, the partitions constituted cristae mitochondriales, albeit of a specialized type. The membranes of the septa usually were parallel, displaying little tendency to separate. In the rare dividing mitochondria of normal size (Fig. 4), the partitions were quite straight and usually had a median location, bisecting the organelle into two approximately equal halves. In contrast, in the much more frequently occurring dividing megamitochondria (Figs. 5, 6), the partitions generally were not in the midline, but rather subtended a small portion of the organelle. These sequestered moieties were lenticular in profile, and usually corresponded in size to normal mitochondria. The most common site of partition formation in the megamitochondria was at the base of a small mitochondrial pro-

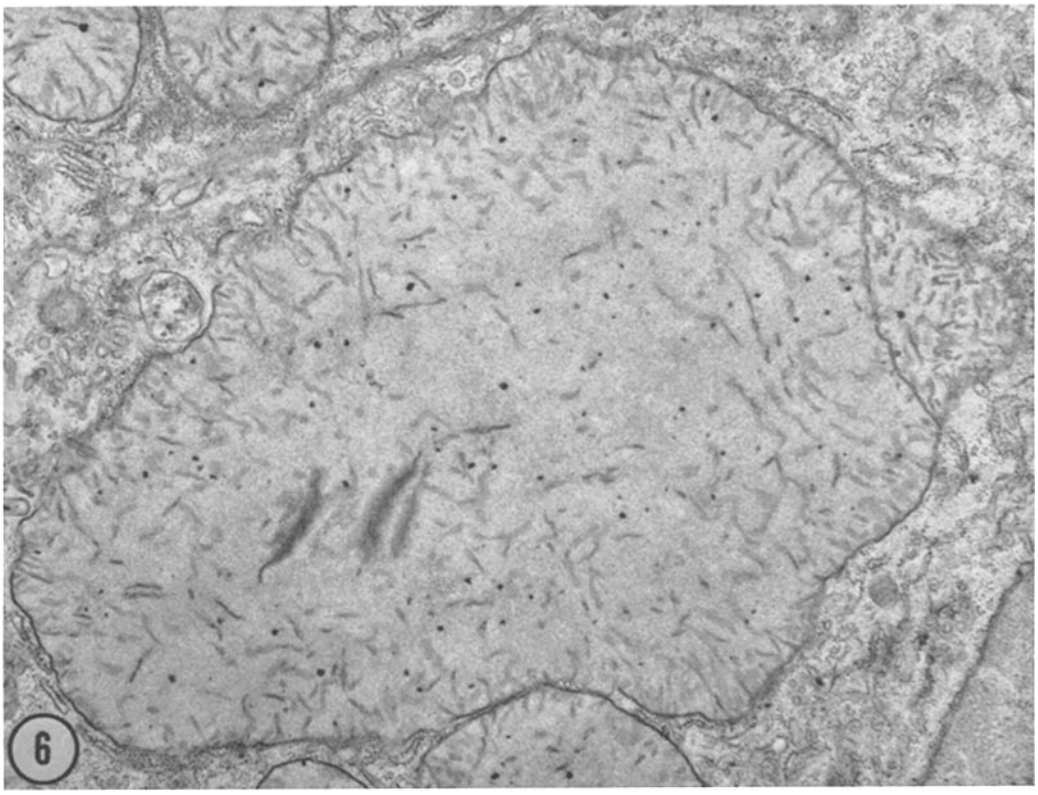


FIGURE 6 A dividing megamitochondrion, showing acentric placement of the partition. 6 wk riboflavin deficient, 6.5 hr recovery. Lead citrate. × 21,000.

trusion or bud (Fig. 7). These buds, which occurred primarily on spherical organelles, were rarely smaller in size than normal mitochondria, but occasionally were appreciably larger. Frequently, the large mitochondria were constricted at the level of the partition (Fig. 8).

In addition to dividing mitochondria, many of the cells contained a few degenerating mitochondria, which invariably were within autophagic vacuoles (Figs. 11, 12). Most of these degenerating organelles were of normal size, although occasionally a degenerating megamitochondrion was observed. Most of the autophagic vacuoles were delimited by a single membrane, as described by Novikoff and Shin (32), but a few were bounded by a congeries of unusually dense membranes resembling a myelin figure (Fig. 12).

Isolated Mitochondria Mitochondria from the normal control animals equilibrated almost entirely in the 1.3 M band in the sucrose gradient. Mitochondria from the riboflavin-deficient mice and from recovering mice were distributed among the 1.3 M, 1.4 M and 1.5 M bands. In both cases, on a protein basis (15) about 50% were in the 1.5 fractions, 10 per cent in the 1.4 M fraction and 40 per cent in the 1.3 M fraction.

Electron microscopic examination of the various fractions revealed that dividing mitochondria were present in appreciable numbers in the 1.5 M fraction obtained from recovering mice (Figs. 14–18). The dividing organelles usually were spheroidal to oblate in form, and generally were larger than the nondividing mitochondria. As in the *in situ* mitochondria, the partitions were usually curvilinear, and segregated a portion of the mitochondrial mass (Fig. 14). That the partitions actually divide the matrix compartment into two distinct chambers was demonstrated by the presence of binary mitochondria with chambers of widely differing electron-opacity (Fig. 15). Apparently during preparation, matrix material was selectively extracted from one chamber, and not

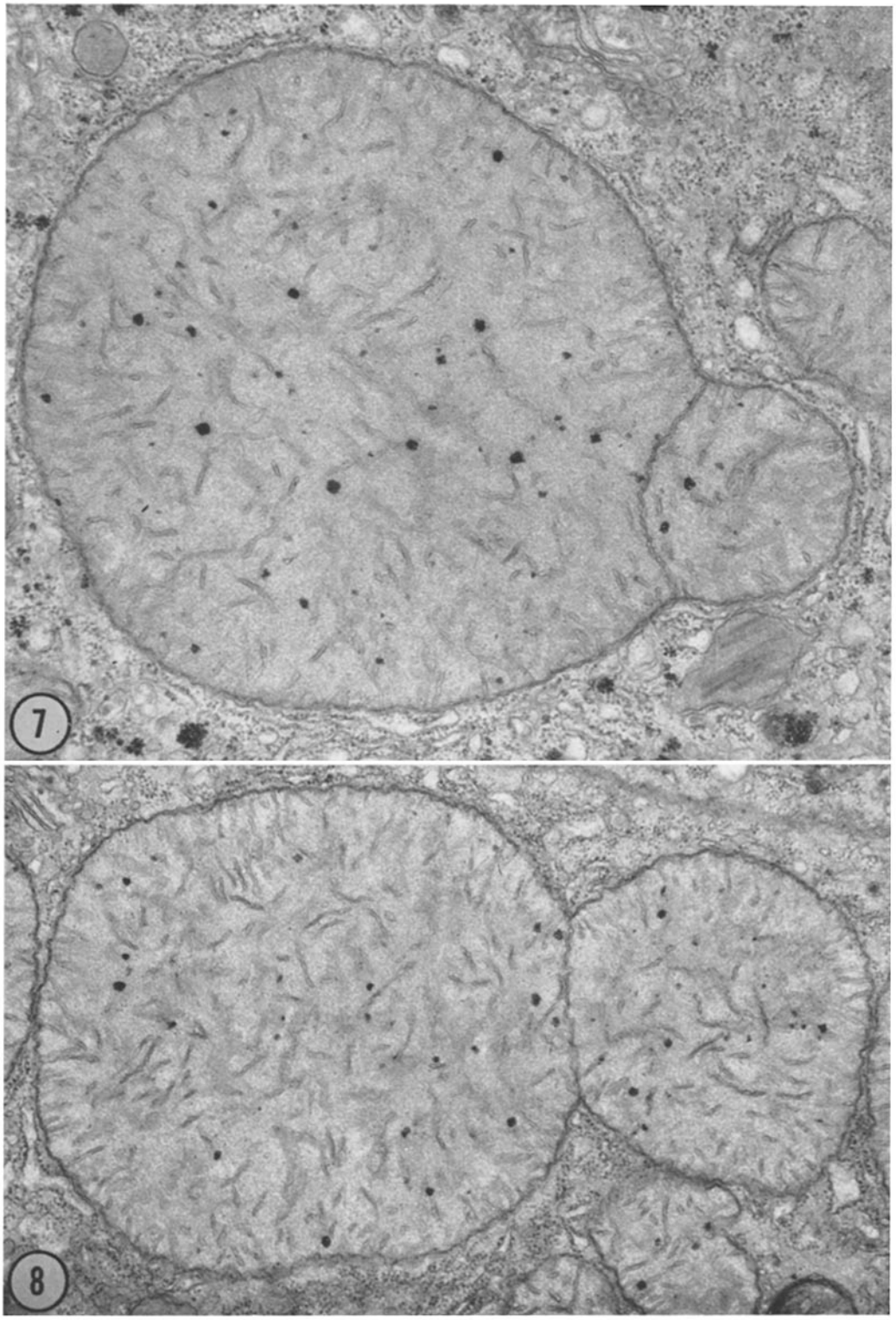


FIGURE 7 A typical dividing megamitochondrion. The partition is at the base of a small bud or protrusion. 6 wk riboflavin-deficient, 1 day recovery. Lead citrate. × 36,000.

FIGURE 8 A dividing mitochondrion with deep furrowing at the level of the partition. 6 wk riboflavin-deficient, 6.5 hr recovery. Lead citrate. × 32,000.

from the other. If the septum were incomplete, the contents of the two chambers would mingle, resulting in a matrix of uniform density.

In a few instances, dividing isolated mitochondria were observed in which the partition, for part of its length, consisted of four parallel membranes (Figs. 16–18). The two outer membranes of the four-ply partition were continuous with the inner limiting mitochondrial membrane, thus corresponding to the partition of *in situ* organelles. In contrast, the two central membranes of the four-ply partition, which were denser than other mitochondrial membranes, appeared to be the walls of an extremely flattened sac. This sac apparently was an extension of a membranous vesicle, a structure which was frequently observed between the inner and outer mitochondrial membranes and continuous with neither. These vesicles consisted of a single membrane or of a complexly arranged series of whorled membranes. In addition to their presence near the septa in dividing mitochondria, the whorls were commonly found in apparently nondividing organelles.

Normal Animals with Riboflavin The only detectable effect on the hepatic cells was manifested by the mitochondria. In a large proportion of liver cells in each animal, the mitochondrial population evinced unusual pleomorphism. Many mitochondria were unusually long, and branched and Y-shaped forms were abundant. In many mitochondria, the cristae were lengthened and often assumed an arcuate configuration, being joined at both ends to the inner mitochondrial membrane. In some of the animals, dividing mitochondria were common (Fig. 19). Such organelles were within the size range of normal hepatic mitochondria. As in the case of the dividing megamitochondria these organelles possessed a partition, occasionally two, and usually showed a furrowing at the level of the septum.

DISCUSSION

Recent studies by Luck (26–29) and by Parsons and Rustad (36), utilizing radioautographic and biochemical techniques, have clearly demonstrated that mitochondria are self-replicating bodies, at least in certain lower organisms. While a similar faculty has been ascribed to mitochondria of higher organisms, the evidence is based primarily on light microscopy or low resolution electron microscopy (see Novikoff (31) and Lehninger (25) for critical reviews). The results of the present study indicate that mammalian mitochondria *do* divide, and that the membranous partitions within the mitochondria play a cardinal role in this process.

Mitochondria with transverse partitions were first described by Fawcett (11). Such configurations, which were relatively rare in normal liver cells, were increased in number by short-term fasting and by refeeding of the experimental animals. Subsequent investigations have revealed the presence of partitioned mitochondria not only in hepatic cells (1, 7, 8, 14, 21–23, 37, 39, 40, 43), but in a variety of other cell types as well. Partitioned mitochondria have been demonstrated in rat hepatoma cells (12), alveolar cells of the rat lung (6), rat cardiac muscle cells (34), rat adrenal gland cells,[2] rat embryonic cells (16), oncocytomas of the human parotid gland (45), ascidian oocytes (20), protozoa (19), caterpillar fat body cells,[3] mushrooms (47, 48), and onion root cells (18).

While the significance of partitioned mitochondria has been the subject of much speculation, in only a few instances have they been linked to mitochondrial fission by experimental evidence (16, 19, 22, 33, 34, 47, 48). In each of these studies, this alleged relationship was based on the fact that partitioned mitochondria were most numerous at a time when the mitochondrial population was increasing in size. A case in point is the work of Lafontaine and Allard (22). These workers fed rats the azo dye 2-Me-DAB over a 16 wk period. In later stages of the experiment, the number of mitochondria per hepatic cell was greatly augmented, and many partitioned mitochondria were present. Such mitochondria usually possessed a median septum consisting of two membranes which were fused with the internal limiting membrane of the organelle. The partitioned mitochondria often showed a slight pinching at the level of the partition, and the space between the two component membranes of the septum often was several times greater than the space within the mitochondrial envelope.

Further evidence that mitochondrial partitions are involved in the fission of these organelles was provided by the work of Onishi (33, 34). In this experiment, Onishi bled rats until the total amount of blood in the animals was reduced to ⅓–½ of normal. The resultant hypoxia led to rapid, acute degeneration of approximately 80% of the cardiac

[2] Friend, D. S. Personal communication.

[3] Larsen, W. Personal communication.

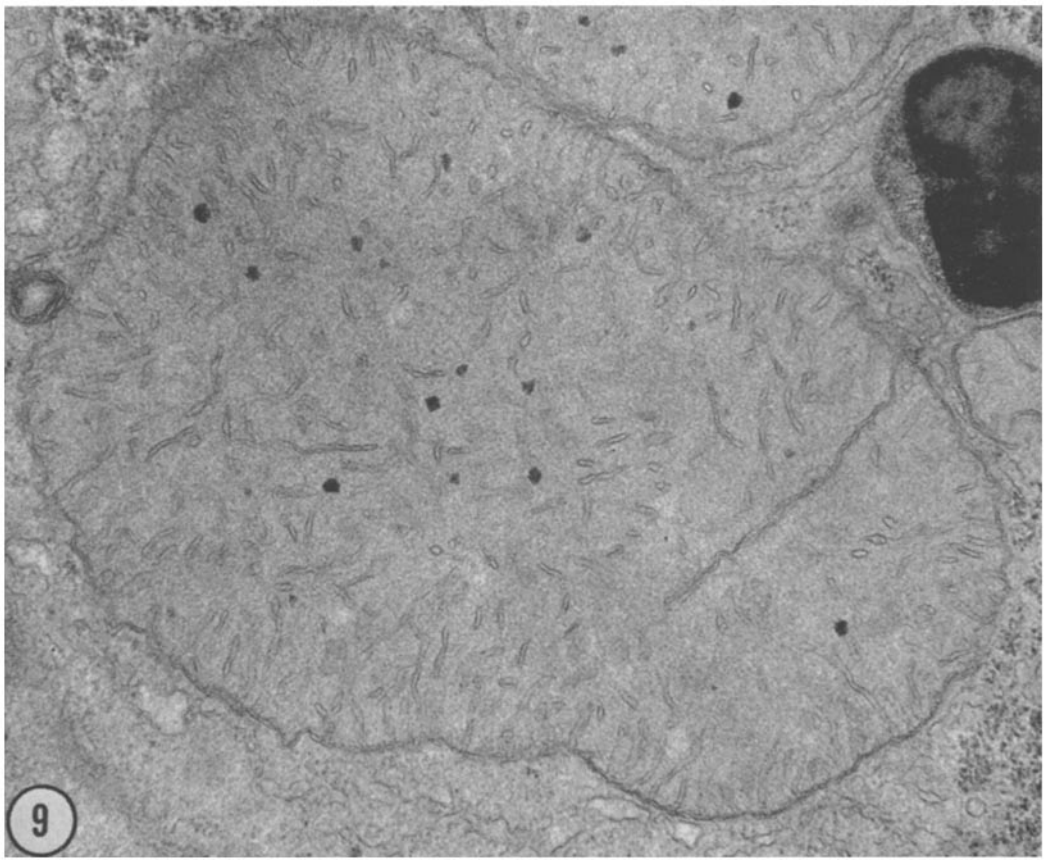


FIGURE 9 A large mitochondrion with a single, elongated, transversely oriented crista. Such cristae probably represent stages in the formation of a partition. 7 wk riboflavin-deficient, 4.5 hr recovery. Lead citrate. × 40,000.

mitochondira. The degenerative changes included swelling, disruption, and complete loss of the cristae, and rarefaction of the matrix. Within 24 hr, however, the remaining unaffected organelles gave evidence of mitochondrial division. As in the hepatic mitochondria described by LaFontaine and Allard (22), partitions were observed which bisected the cardiac mitochondria. The organelles also displayed a pinching-in at the level of the partition. In at least one case, a mitochondrion was described in which the partition for part of its length consisted of two parallel membranes, while for the remainder of its length consisted of four parallel membranes. By 15 days, the number of mitochondria per cell had returned to normal.

In a study of the protozoan *Boderia turneri*, Hedley and Wakefield (19) observed a single case of multiple fission or schizogony in cultures. In contrast to the adult animals, in which the mitochondria were spherical, the rapidly growing schizonts had many mitochondria of highly variable morphology. Approximately 2–4 per cent of these mitochondria were ovoid to elongate in form with a central dissepiment and constriction.

Mitochondria isolated from gill tissue of the common meadow mushroom and maintained in extracellular cultures frequently showed septation (47, 48). In addition to binary forms, many organelles had multiple partitions, forming "clones." Partitions were also described in in vitro cultures of mitochondria derived from rat embryonic tissues (16).

While, in each of the foregoing studies, partitioned mitochondria were implicated in mitochondrial fission, the possibility that such configurations represented stages in fusion of mitochondria

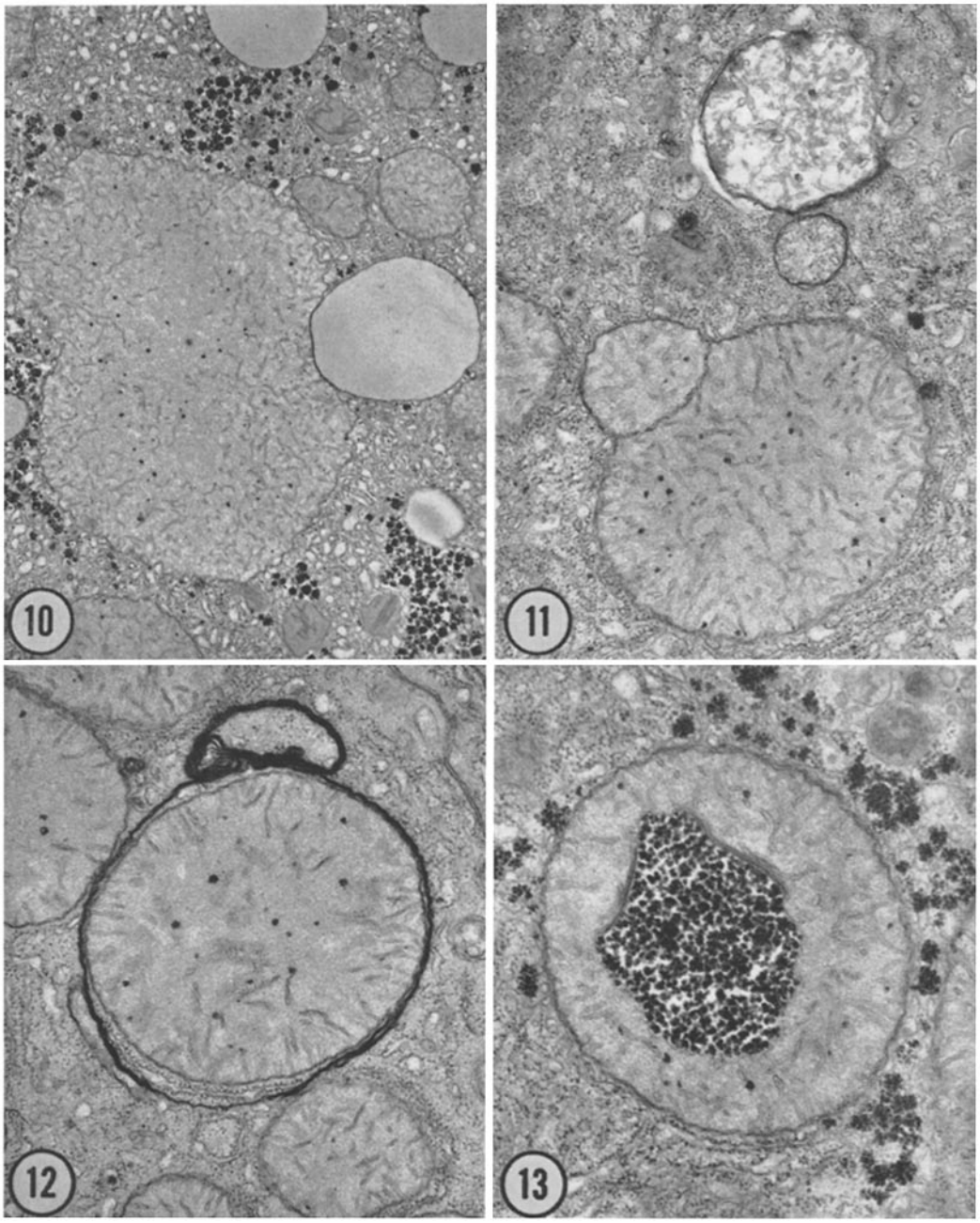


FIGURE 10 A megamitochondrion intimately associated with a lipid droplet. From the 4th wk of riboflavin-deficiency, the capacity of mitochondria to associate with lipid is absent, but this function is rapidly restored by injection of the vitamin. 6 wk deficient, 1 day recovery. Lead citrate. × 11,700.

FIGURE 11 A degenerating mitochondrion adjacent to a dividing organelle. 6 wk deficient, 6.5 hr recovery. Lead citrate. × 24,500.

FIGURE 12 A large mitochondrion within an autophagic vacuole. The limiting membranes of the vacuole are unusually dense. 6 wk deficient, 6.5 hr recovery. Lead citrate. × 26,600.

FIGURE 13 A mitochondrion containing glycogen which is bounded by a single membrane. Such organelles are observed with some regularity during the later stages of riboflavin deficiency, and persist for several days after recovery is initiated. 6 wk deficient, 2 day recovery. Lead citrate. × 30,000.

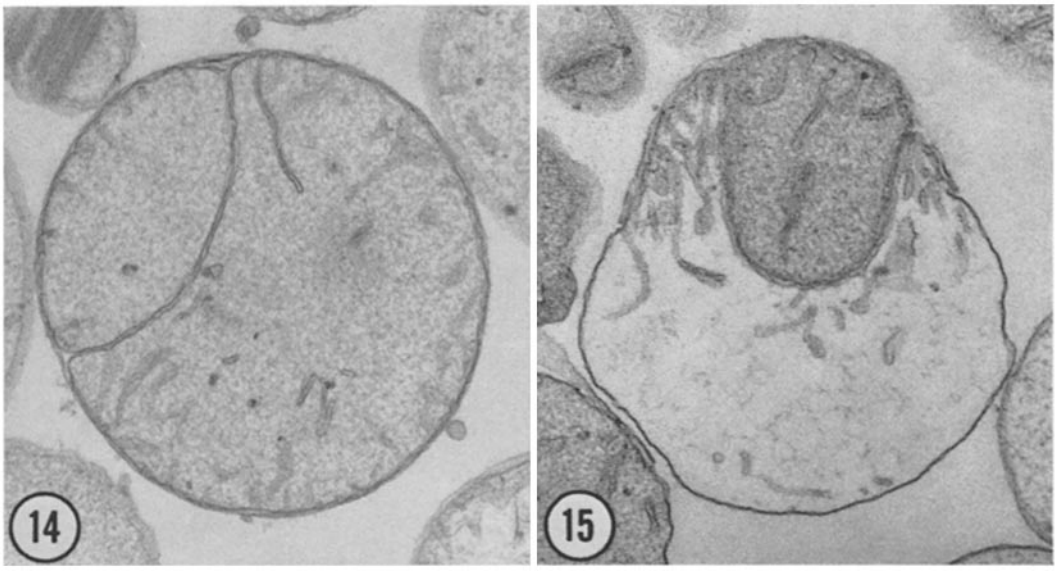
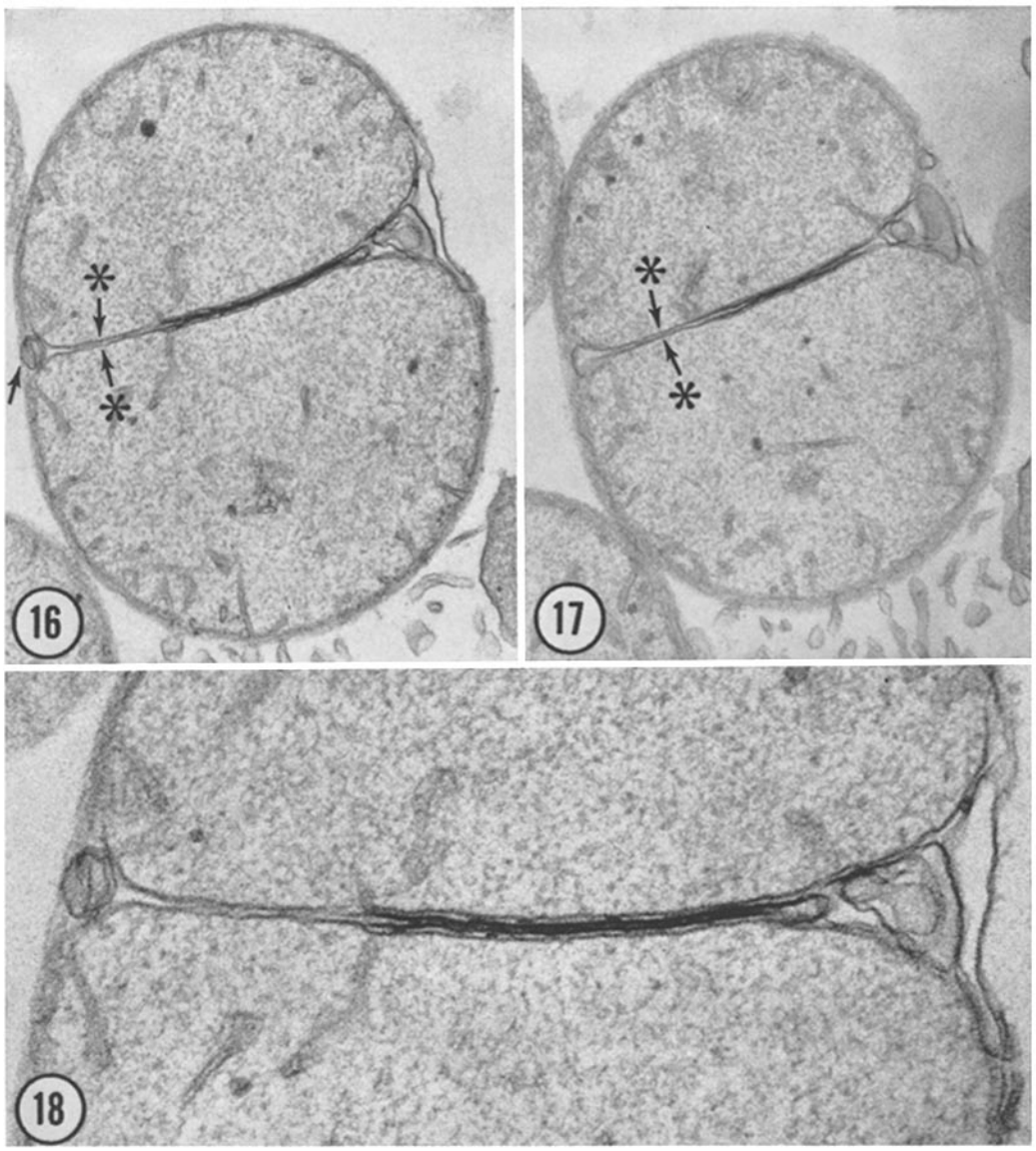


FIGURE 14 An isolated mitochondrion which is dividing. The partition is clearly continuous with the inner mitochondrial membrane. 8 wk riboflavin-deficient, 3.5 hr recovery. Uranyl acetate and lead tartrate. × 47,500.

FIGURE 15 A dividing isolated mitochondrion with two chambers of widely differing density. This difference is due to differential extraction or leakage of matrix material, and demonstrates that the partitions are complete and separate the mitochondrion into distinct segments. 8 wk riboflavin-deficient, 3.5 hr recovery. Uranyl acetate and lead tartrate. × 42,500.

could not be entirely excluded. The present study provides evidence that partitioned mitochondria do in fact represent division figures. The direction of the process is easily recognized, since the giant mitochondria are rapidly succeeded by mitochondria of normal size. If the organelles were fusing, then the obvious reduction in mitochondrial size could not have occurred. Furthermore, in an earlier study (44), we described stages in mitochondrial fusion brought about by riboflavin deficiency, and these stages in no way resembled partitioned mitochondria. In the period of most rapid mitochondrial growth (5–8 wk after initiation of the riboflavin-free diet), *no* partitioned mitochondria were observed in the parenchymal cells; they were seen only after administration of the vitamin. In this connection, it should be added that the frequency of partitioned mitochondria was somewhat enhanced in isocaloric controls, as contrasted to control animals fed *ad libitum*, thus confirming Fawcett's (11) original observation. It is also interesting to note that injection of riboflavin into normally nourished mice produces an increase in the occurrence of mitochondrial division figures. A similar effect was noted by Hayat (18), who found that exposure of newly-formed onion roots to niacin solutions resulted in the rapid, widespread appearance of septa in the mitochondria of the root cells. It may well be that at least some of the B vitamins act as rate-limiting factors in mitochondrial replication.

Riboflavin is rapidly taken up by the liver after intraabdominal injection (50). The initial morphologic event in mitochondrial recovery is the production of a partition. This is accomplished either by fusion of existing cristae, or by elongation of a single cristae, as suggested by Fig. 9. A similar elongated crista in isolated mushroom mitochondria was illustrated by Vogel and Kemper (48), who suggested that it represented a stage in the formation of a partition. The precise placement of the newly formed partition varies, but in the megamitochondria this partition tends to be acentric or at the base of a small bud, usually sequestering a portion of the mitochondrial mass no larger than a normal sized organelle. Since the partitions never consisted of four parallel membranes, at least in the *in situ* organelles, it



FIGURES 16 and 17 Serial sections of a dividing isolated mitochondrion. Fig. 16 is of a section medial to Fig. 17. The vesicle (arrow) in Fig. 16 is lacking in Fig. 17. The components (*) of the partition in Fig. 16, that are continuous with the inner limiting membrane, form a cul-de-sac in Fig. 17. 8 wk riboflavin-deficient, 3.5 hr recovery. Uranyl acetate and lead tartrate. Figs. 16 and 17. × 40,500.

FIGURE 18 A higher magnification of Fig. 16. The partition is seen to consist of four plies at the right, and two plies at the left. The relationship of the components of the partition to the membranous vesicles at either end is not clear. × 84,500.

follows that extension of the outer membrane of the mitochondrial envelope between the component membranes of the septum results in a separation of the two mitochondrial moieties, perhaps due to a repulsive force. Such a separation would account for the figure-8 configuration often seen in the partitioned mitochondria. While the same repulsion may exist in dividing cardiac mitochon-

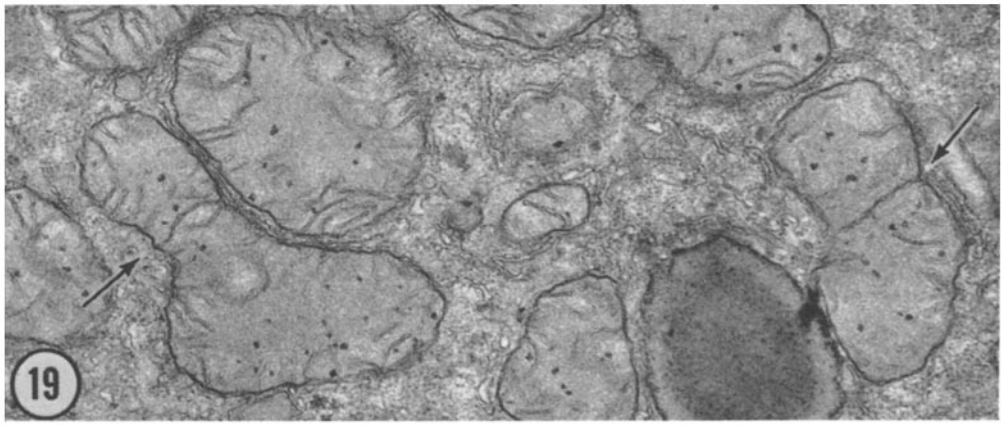


FIGURE 19 A section of a hepatic cell from a normally fed, 10 week-old mouse that was injected with a single dose of riboflavin 3.5 hr prior to sacrifice. Two dividing mitochondria (arrows) are apparent. Lead tartrate. × 29,500.

dria, these organelles are physically constrained by the numerous myofilaments and surrounding mitochondria, thus occasionally permitting visualization of four membranes in the partition, as Onishi (34) has illustrated.

The site of synthesis of the components of the newly-formed outer membrane is undetermined. It has been shown (35, 41) that outer membranes of rat liver mitochondria possess an electron transport system similar or identical to that occurring in membranes of the endoplasmic reticulum, implying a similarity of the two types of membrane. Furthermore, apparent morphologic continuity between ER membranes and the outer mitochondrial membrane has been described (2, 5, 38). However, on the basis of a study of serial sections of hepatic cells, Stempak (42) criticized such presumptions of continuity and suggested that these continuities are spurious associations due to organelle overlap within the thickness of a section. With this caveat in mind, we have searched for instances of continuity between dividing mitochondria and elements of ER. While we have seen several configurations suggestive of continuity between the ER and the outer mitochondrial membranes at the level of the partition, this point could not be unequivocally illustrated.

Examination of mitochondrial pellets derived from livers of mice recovering from riboflavin deficiency introduces a discordant note in any scheme of mitochondrial fission. While partitioned mitochondria were common in such preparations, they displayed an important morphological difference in comparison to the *in situ* organelles; at the level of the partition, these isolated mitochondria usually had a vesicle or whorl of membranous material between the inner and outer limiting membranes. Similar whorls were often present in apparently nondividing mitochondria in the pellets. While membranous whorls were occasionally noted in association with *in situ* mitochondria in the riboflavin-deficient animals, the whorls were always on the outer aspect of the outer membrane, or within the matrix; none were observed in the outer compartment (44). In the isolated mitochondria, it was not possible to discern if the whorls were continuous with either or both of the two limiting membranes. In a few cases, it was determined that the whorls were continuous with the outer membrane component of the newly formed partitions. What relationship these whorls bear to mitochondrial fission is moot, since they do not appear in the *in situ* organelles. It is quite conceivable that they are artifacts of preparation, since the mitochondria were exposed to sucrose gradients of unusually high concentration (up to 1.6 M).

In conclusion, we have demonstrated that partitioned mitochondria actually are dividing. While mitochondria of this configuration have long been recognized and had been implicated in fission, what was previously always absent was a time factor. In the system which we have reported, a temporal sequence has been introduced. In the absence of riboflavin, the hepatic mitochondria grow large. Upon administration of the vitamin, they are restored to normal size by progressive

division, which involves production of partitions. The relative paucity of partitioned mitochondria in normal hepatic cells in no way militates against their involvement in fission. A few simple calculations should suffice to demonstrate this point. There are about 800 mitochondria in a liver cell (25). The mitochondrial population of hepatic cells has a half-life of about 10.5 days (13). Studies by Hawley and Wagner (17) on synchronously dividing mitochondria in *Neurospora* indicate that the mitochondrial mass doubling time is of relatively short duration (6–7 hr). If mitochondrial division in normal hepatic cells is a completely random process, lacking any rhythmicity, and duration of division is short, then only a few organelles in each cell would be in active division at any given time. If the thinness of sections for electron microscopy and the problems of correct orientation are taken into account, it becomes obvious that the observation of a partitioned mitochondrion is of necessity a quite rare event.

This is not to say that partition formation is the sole means by which mitochondria may be replicated. Observations made on other systems suggest that other modalities of mitochondrial division may exist. Thus, Hawley and Wagner (17), in a system where the mitochondria were known to be increasing in number, found only cup-shaped or dumbbell-shaped mitochondria. Such organelles apparently divide by progressive elongation and attentuation of their mid-regions. A similar sequence was described in protists (10, 30, 46, 49) and in liverworts (9). In addition to their occurrence in these lower forms, dumbbell-shaped mitochondria are present in mammalian liver cells (3, 4, 24, 42). In each case, these forms were described as constituting stages in mitochondrial division. While mitochondria of a dumbbell configuration were quite rare in the animals recovering from riboflavin deficiency, the possibility that they play a role in mitochondrial normalization cannot be entirely excluded.

A brief report of some of this work has appeared (1967. *Anat. Rec.* **157**:331).

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