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On the Mechanism of Cuprizone-Induced Formation of Megamitochondria in Mouse Liver

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Abstract

The livers of mice maintained on a diet containing 1% cuprizone for periods up to 18 days formed giant (mega) mitochondria equaling or exceeding the size of the nucleus. The megamitochondria were isolated from the liver and shown to be competent in respect to oxidative phosphorylation, although the P/O ratio and respiratory control index were somewhat reduced. When cuprizone was deleted from the diet, the size and function of the liver mitochondria returned to normal. Electron microscopic studies have made it possible to demonstrate the intermediate steps by which the membranes of paired mitochondria fuse and defuse to form megamitochondria, and from these studies a general mechanism may now be proposed for processes in which one continuous membrane splits into two separate membranes or by which two separated membranes fuse to form one continuous membrane.

Introduction

Suzuki [1, 2] and Tandler and Hoppel [3] have demonstrated that the mitochondria of the liver of mice fed on a diet containing cuprizone (biscyclohexanone oxaldehydrazone) gradually augment in size to the point that their volume can reach or exceed that of the nucleus. Such an augmentation would require stepwise coalescence of some five or more mitochondria. The transition from liver mitochondria of normal size to megamitochondria is relatively slow *in vivo* (days or weeks) on this dietary regimen. The transition, however, is reversible; when cuprizone is eliminated from the diet, the mitochondria return to normal size within several hours. The present article is aimed at exploring more fully some features of megamitochondrial formation—in particular, the normalcy of function of these mitochondria and the ultrastructural mechanism by which mitochondria interact to generate megamitochondria.

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Experimental

Cuprizone Regimen: Male Swiss-Webster mice (4-5 weeks old) were fed a powdered complete diet containing 1% cuprizone (obtained from G. Frederik Smith Chemical Company, Columbus, Ohio). Control animals (sex) c were fed the same diet minus cuprizone. Animals were sacrificed after 13 days (5 animals), 15 days (9 animals) and 18 days (5 animals) on the cuprizone-containing diet. In the recovery experiment, 5 mice were fed the cuprizone-containing diet for 19 days and then a cuprizone-free diet for 12 hours. As reported by others [1-3], a small proportion of the experimental animals on the cuprizone diet became progressively weak and died during the course of the experiments.

Isolation of Mitochondria: The liver of a decapitated mouse was removed and rinsed in a solution which was 0.21 M in mannitol, 0.07 M in sucrose, 0.1 mM in EDTA and 5 mM in Tris-chloride of pH 7.4. The washed liver was cut into small pieces with a scissors and homogenized in a Teflon homogenizer in the medium specified above. The isolation of mitochondria was carried out essentially according to the method of Hogeboom and Schneider [4]. The isolation medium was supplemented with 0.1% bovine serum albumin (Fraction V, fatty acid-free, Miles Laboratory, Kankakee, Illinois).

Analytical Procedures: Oxidative phosphorylation (P/O ratio) and the respiratory control ratio were determined in a medium which was 0.25 M in sucrose, 3 mM in $MgCl_2$, 10 mM in Tris-chloride (pH 7.4), 0.1% in bovine serum albumin, 5 mM in succinate, 5 mM in potassium phosphate (pH 7.4) and 5 mM in ADP. The rate of respiration was measured at 30° in a Clark-type electrode (Beckman Company). The suspending medium (5 ml) contained 1.5-2.0 mg of mitochondria. Esterification of inorganic phosphate was determined by the method of Lindberg and Ernster [5]. In the determination of the P/O ratios, an aliquot was withdrawn from the cell used for oxygen electrode measurements and analyzed for the esterification of inorganic phosphate.

The cytochrome contents of the isolated mitochondria were determined by the method of Williams [6].

Cytochrome oxidase activity was determined with a Clark-type electrode in a medium which was 0.25 M in sucrose, 10 mM in Tris-chloride (pH 7.4), 50 mM in ascorbate, and contained 0.6 mg per ml of cytochrome *c* (Type IV of Sigma) and lysolecithin (1.2 mg per mg protein). Lysolecithin was used as a solubilizing agent for the oxidase preparation and for induction of maximal oxidase activity.

Electron Microscopy: Samples were fixed in 2% glutaraldehyde which was 0.25 M in sucrose and 0.05 M in potassium cacodylate (pH 7.4). After fixation in glutaraldehyde, the samples were post-fixed in 1% osmium tetroxide. In the dehydration procedure, the first exposure was to 25% ethanol containing 1% uranyl acetate and this was followed by

graded increases in the ethanol concentration until 100% was reached. This dehydration was followed by exposure to absolute propylene oxide before embedding in 100% Epon.

Results and Discussion

We isolated mitochondria from the livers of male Swiss-Webster mice administered a diet containing 1% cuprizone for 18 days and showed that in respect to rates of respiration, efficiency of oxidative phosphorylation as measured by the P/O ratio, and the respiratory control index, preparations containing a high proportion of megamitochondria as determined by electron microscopy (see Fig. 1) were by no means incompetent (Table I). These mitochondria appeared to be somewhat less efficient than normal mitochondria in respect to oxidative phosphorylation. On removal of cuprizone from the diet, within a few hours the size of the mitochondria returned to normal and the P/O ratios of the isolated mitochondria approximated more closely the P/O ratios of normal mitochondria. We have found that the isolated megamitochondrial suspension showed normal cytochrome oxidase activity (the specific activity was 1.25 times higher than that of normal mitochondria) and a copper content which was 86% of the normal value, 42.1 μatoms per mg protein versus 48.9). Moreover, the yield by isolation of liver mitochondria in the cuprizone-treated animals was about 66% of normal.

Two lines of evidence attest to the relative normalcy of megamitochondria—the evidence here presented that isolated megamithochondria are capable of oxidative phosphorylation and respiratory control, and the evidence of Tandler and Happel [3] confirmed by us, of the reversibility of megamitochondrial formation by

TABLE I. Coupling efficiency of liver mitochondria isolated from mice maintained on a normal or cuprizone-containing diet.

Source of Liver Mitochondria	Addition to Medium	Respiratory Control Ratio	P/O
Control mice	0	3.4	1.74
Control mice	cuprizone (0.16 mM)	3.7	1.77
Cuprizone-treated mice (18 days)	0	2.3	1.20
Cuprizone-treated mice (19 days) maintained 12 hours on a cuprizone-free diet	0	2.5	1.49

The medium and methods used were described in the Methods Section.

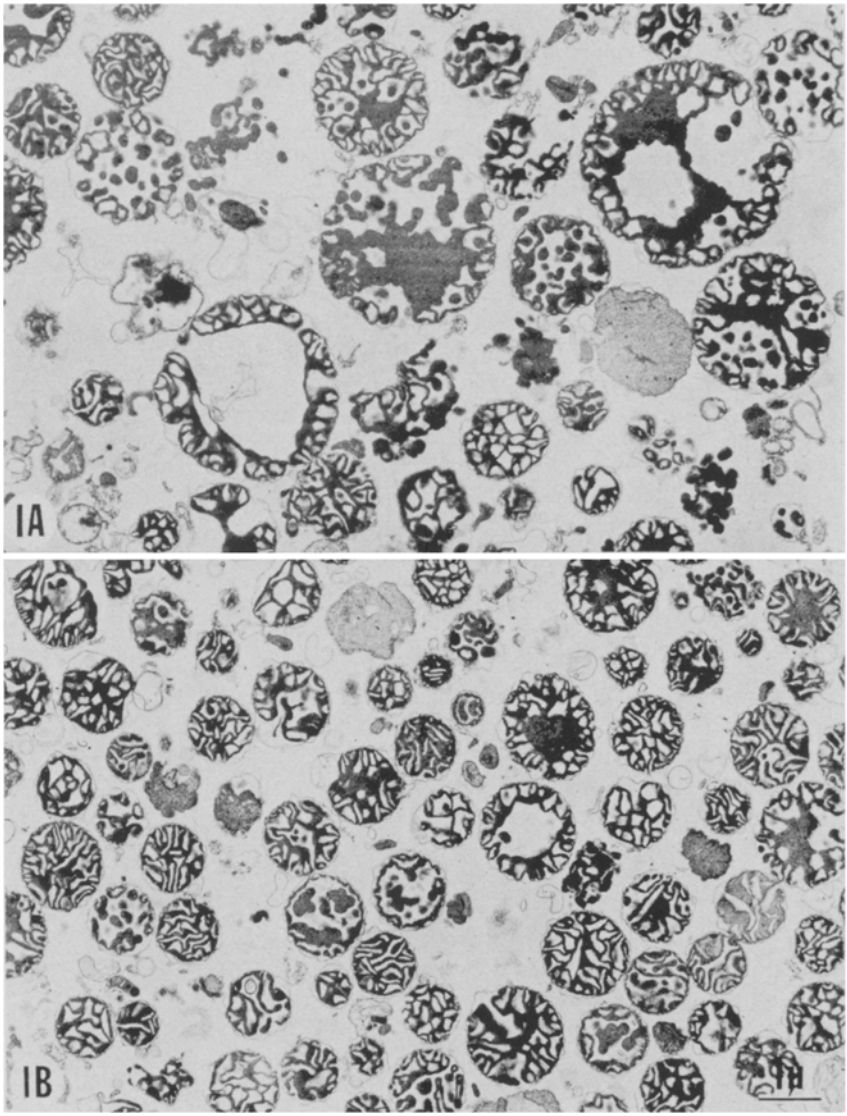

Figure 1. Mitochondrial suspension isolated from mouse liver. (A) Cuprizone-treated animals; (B) Normal animals. ×14,100

elimination of cuprizone from the diet. The point to be emphasized is that megamitochondrial formation is not a pathological process. As we shall discuss later, there are physiological counterparts to the cuprizone-induced generation of megamitochondria.

The normal distribution of mitochondrial size in cells in which mitochondria have translational freedom represents an equilibrium between two processes which take place continually—fusion, or coalescence of two mitochondria, and fragmentation of mitochondrion into two daughter units. In a given cell, mitochondria within a certain size range will be most stable and therefore, the dynamic equilibrium of fusion and fragmentation will tend to generate mitochondria of the most stable size. When this equilibrium is perturbed by a reagent such as cuprizone, which accumulates in the liver, the new equilibrium established for liver mitochondria will tend to favor the formation of megamitochondria (see Fig. 2). When cuprizone is eliminated from the diet and its concentration in the liver is correspondingly reduced, the normal equilibrium for the two competing processes is re-established and the mitochondria gradually revert to normal size. Such an interpretation is fully compatible with three observations. First, during spermatogenesis, mitochondria undergo stepwise fusion to one giant mitochondrion that is twisted around the central core of the sperm cell [7]. Second, time-lapse motion picture studies of fibroblasts show that mitochondria are continuously undergoing both coalescence to larger units and fragmentation to smaller units [8]. Finally, there is evidence that other perturbations can lead to megamitochondria. In rats maintained on a flavin-deficient diet, megamitochondria are found in the liver and these disappear when the dietary deficiency is corrected [9].

The question could be raised why megamitochondria are found only in the liver of cuprizone-treated mice. This selectivity is undoubtedly related to the degree of concentration of cuprizone. In the liver, the concentration achieved is sufficient to modulate the ionic composition of the mitochondrion and thus perturb the control mechanism which determines mitochondrial size. In other organs, the concentration of cuprizone is insufficient to affect this equilibrium. Much the same discrimination is found in cadmium poisoning of the rat [10]. It is only the mitochondria of liver which show deranged function and the necessary degree of accumulation of cadmium. In all other organs of cadmium-poisoned rats, the mitochondria are perfectly normal.

Finally, a comment about the nature of the influence exerted by cuprizone on mitochondrial function. This reagent which is used in analytical chemistry for determination of copper actually forms complexes with both Mg^{++} and Ca^{++} [11]—the two divalent metals which have been shown to influence profoundly the fusion of mitochondrial membranes [12]. It is reasonable, therefore, to postulate that cuprizone will perturb the normal Mg^{++}/Ca^{++} ratio in mitochondria

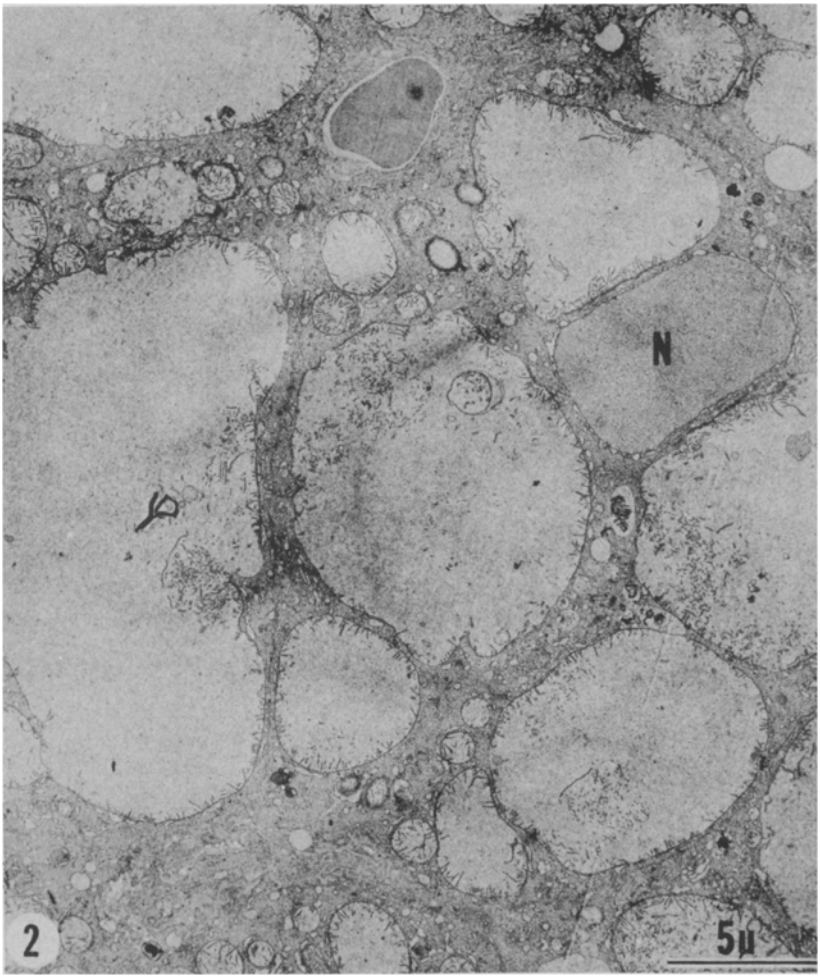

Figure 2. Megamitochondria *in situ* in cuprizone-treated mouse liver. A nucleus is surrounded by a set of six megamitochondria, all of which are larger than the nucleus. Some normal-size mitochondria can be seen interspersed between the megamitochondria. ×6,900

in the liver of mice exposed for a considerable period to a critical level of the reagent and this perturbation will then affect the equilibrium which is the determinant of mitochondrial size.

The central question and the one to which this article is primarily addressed is the mechanism by which two mitochondria can fuse to form a single larger unit with no trace of the septum which originally joined

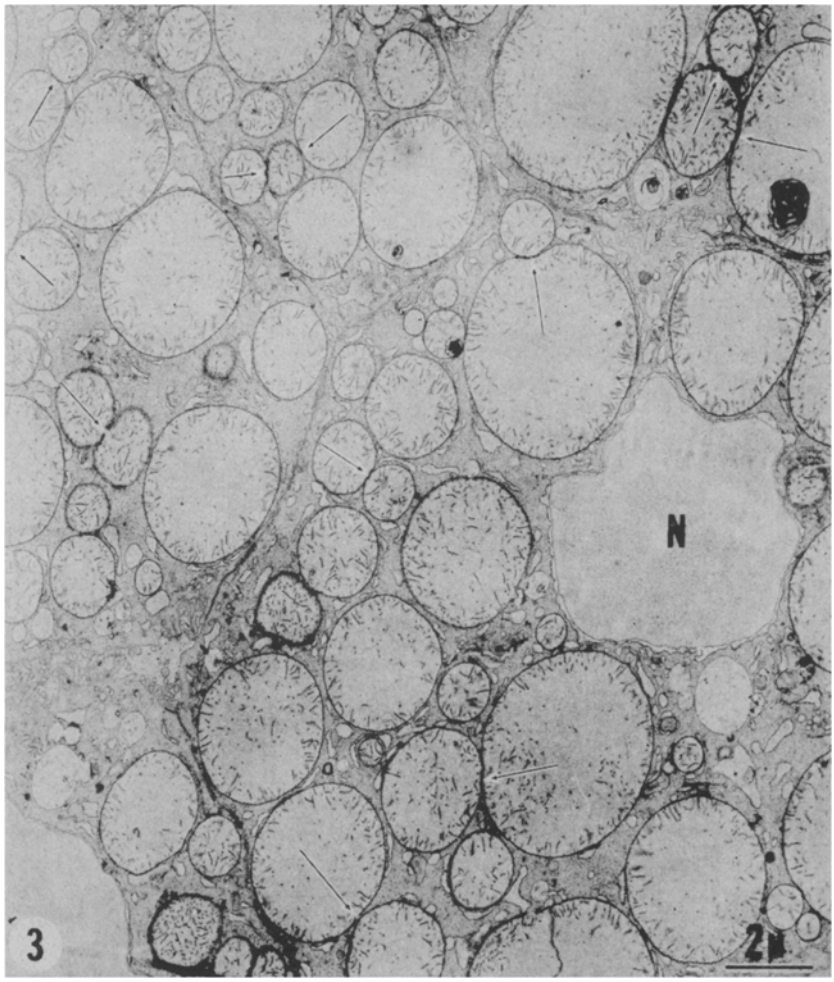

Figure 3. A set of megamitochondria from mouse liver in various stages of the progression leading to megamitochondria. The arrows denote areas of fusion and perforation; nucleus (N) is in the center. x9,900

the two mitochondria. There are a variety of physiological processes in which two membranes separated in space become continuous or in which one continuous membrane gives rise to two membranes separated in space. Cell division [13], nuclear pore formation [14], exocytosis [15], endocytosis [16], leucocytosis [17], viral invagination and evagination [18] are among the well-known examples of this fusion-defusion

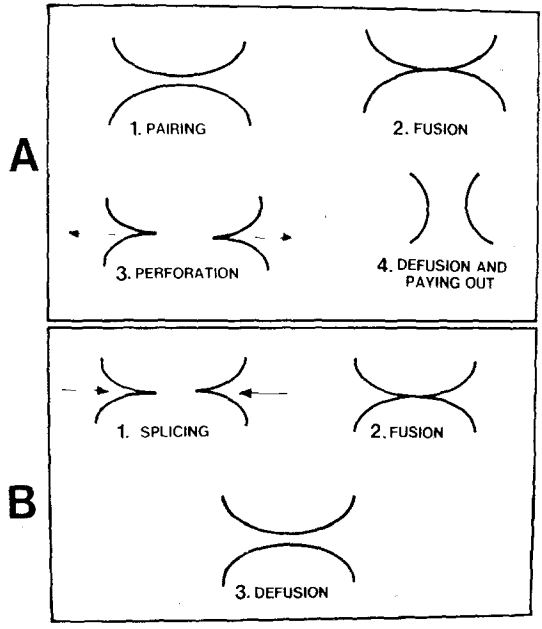

Figure 4. Fragmentation of two fused membranes at some point in the region of fusion, and splicing of the open ends of two fused membranes to form one continuous membrane.

phenomenon. The critical changes in the state of the membrane(s) are usually so short-lived that it has been extremely difficult to document the precise ultrastructural events which underlie the fusion-defusion cycle. The gradualness of megamitochondrion formation in effect provides a slow-motion picture of the critical events. In a large population of mitochondria, there will always be a few in the act of fusion-defusion during the active phase of megamitochondrion formation (see Fig. 3).

We have developed elsewhere the thesis [19] that membranes generally have the capacity to undergo fusion. We shall define a fusion membrane as a hybrid membrane arising from the coalescence of two previously separated membranes. This coalescence in mitochondria usually requires the presence of divalent metals such as Mg^{++} or basic proteins such as protamine and cytochrome c [15]. By virtue of this interaction, the fusion membrane has new properties which are not shown by the component membranes singly. Among the new properties is one that is crucial for the fusion-defusion cycle. Fusion membranes can undergo fragmentation at any point in the area of fusion without loss of the internal contents within each vesicular membrane (see Fig. 4). Moreover,

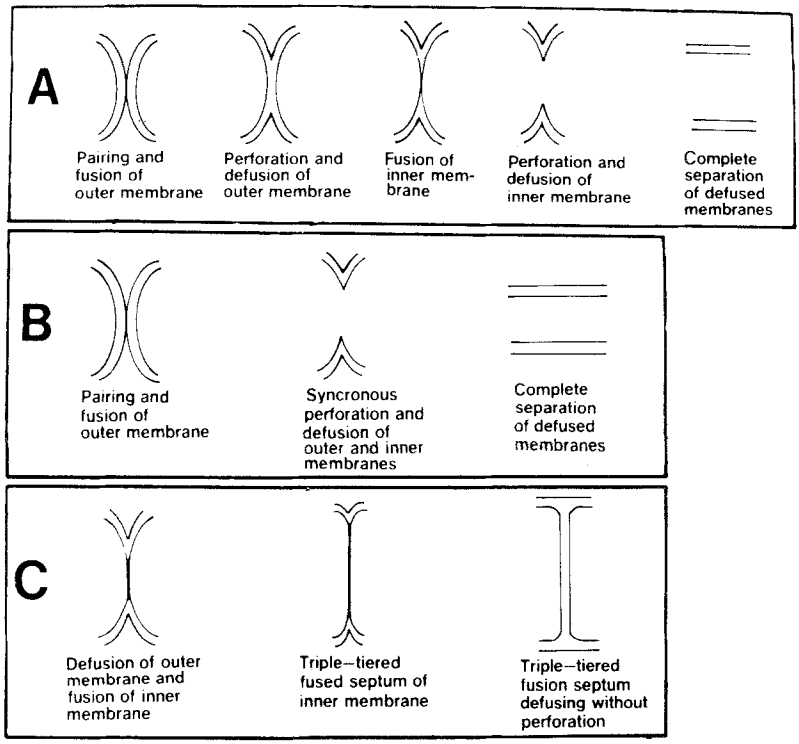

Figure 5. Diagrammatic representation of the progression of events leading to megamitochondrion formation. (A) Fusion of outer membranes, perforation at a point in the fusion area, and defusion of the fused membranes, followed by a similar progression for the inner membranes. (B) Synchronous fusion perforation and defusion of outer and inner membranes. (C) Fusion, perforation and defusion of the outer membrane with a block in the defusion of the inner membrane.

two fusion membranes can splice together to form one continuous fusion membrane. Note that membrane fusion, followed by fragmentation, can lead to exposed ends; unfused membranes are continuous and thus never have exposed ends. Given these properties of fusion membranes and given the reversibility of membrane fusion, it is possible to rationalize many of the physiological processes involving coalescence of previously separated membranes or fragmentation of a continuous membrane into two separated membranes. The application of this thesis to megamitochondrion formation is shown diagrammatically in Fig. 5. The first step is always the fusion of the outer membranes of the two paired mitochondria. The fusion membrane undergoes perforation and the two now separated fusion membranes defuse and pay back into the continuous membrane surrounding the now enlarged mitochondrion.

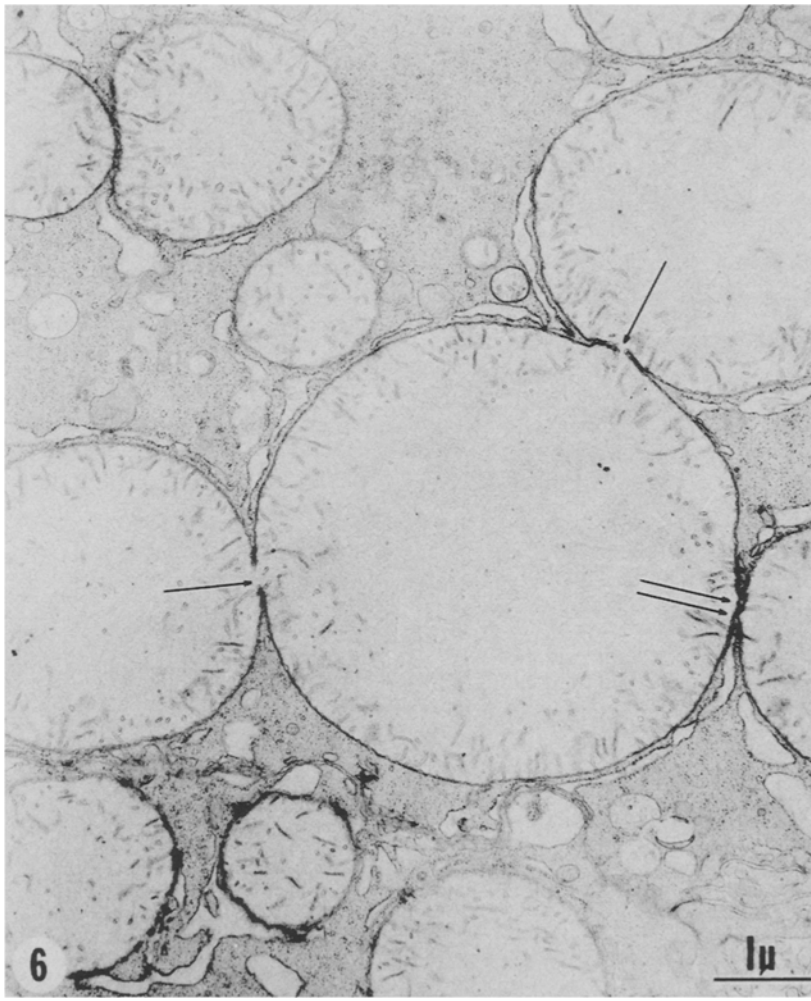

Figure 6. Fusion of the two outer membranes of opposed mitochondria during megamitochondrion formation induced by cuprizone (two arrows) and perforation at some point in the area of fusion (one arrow). ×22,500

The second step is the fusion of the two inner membranes, followed by perforation and defusion and paying back. When the paying back defusion process is completed for the two membranes, the septum linking the two mitochondria disappears and no trace is now left of the original fusion process. There is a second alternative to this progression.

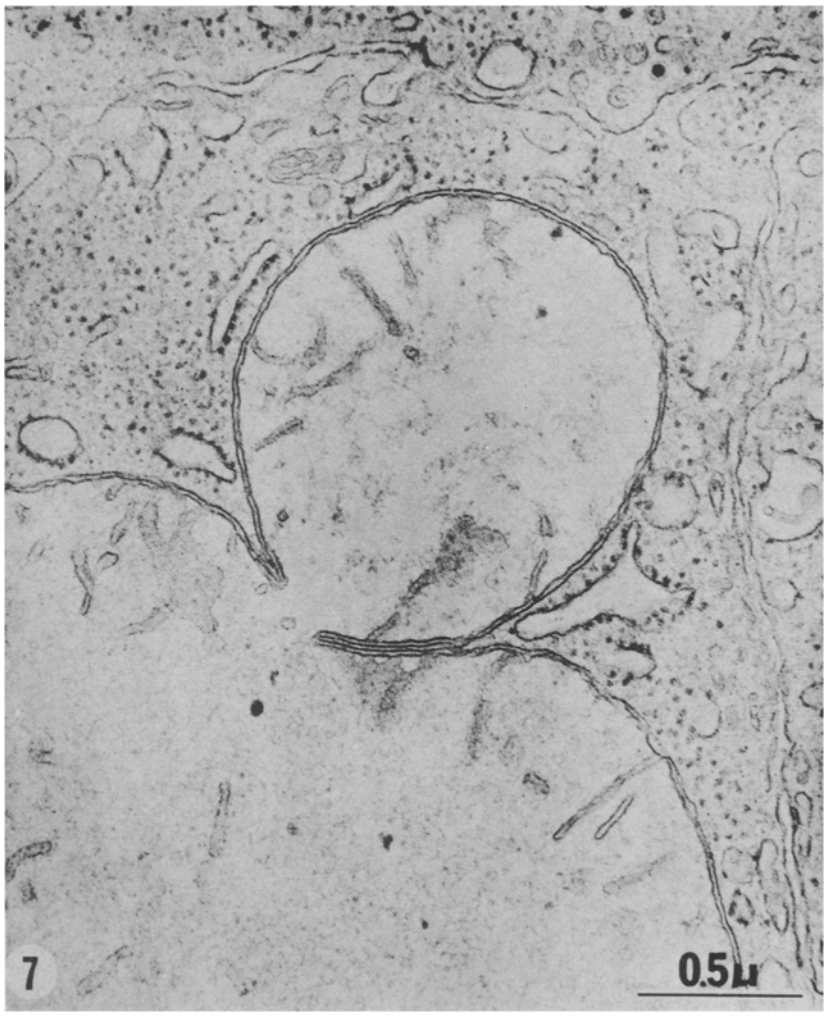

Figure 7. Electron micrograph showing the synchronous perforation, defusion and paying back of the perforated ends of the fused outer and inner membranes. x75,000

The outer membranes will fuse, perforate and separate partially; then the inner membranes will follow suit; finally the two perforated and separated membranes (outer and inner) will pay back in synchrony. In this case it would appear that both inner and outer membranes fuse and defuse simultaneously; fusion involving multiples of two membranes is referred to as laminar fusion. Finally, there is a third alternative to this

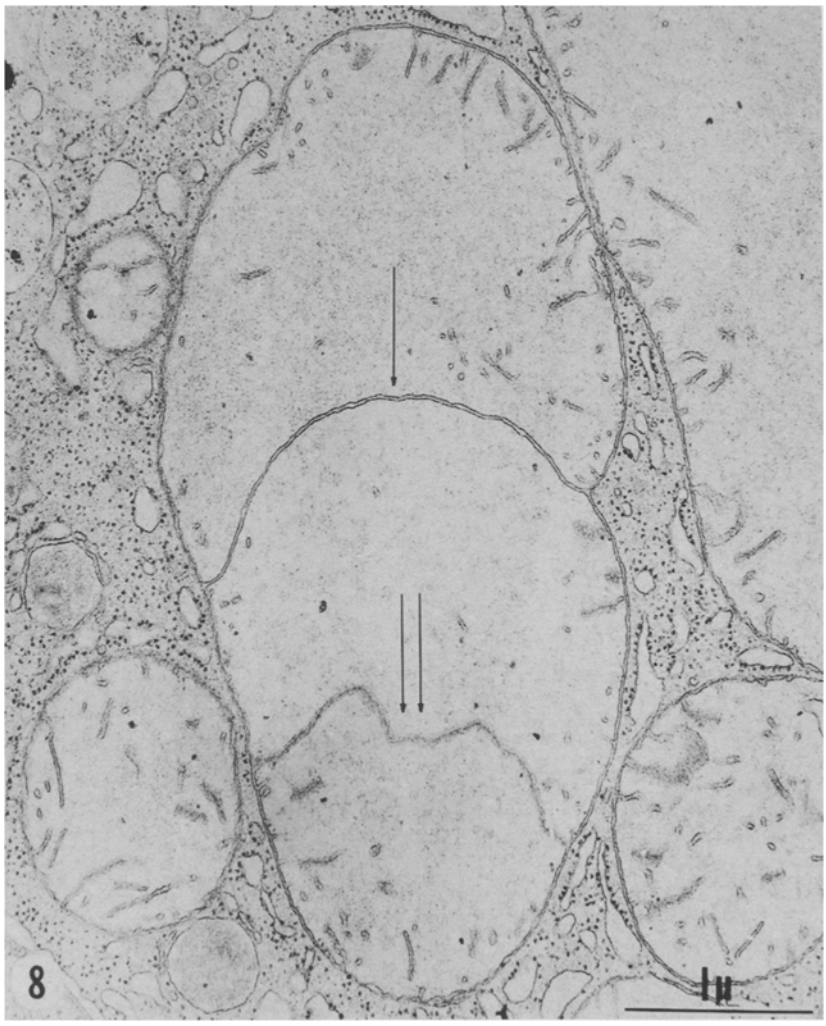

Figure 8. Septa which mark the points of fusion of paired mitochondria during megamitochondrion formation. One arrow points to a septum in which the two inner membranes are separated by a space. Two arrows point to a septum in which the two inner membranes have fused to form a single triple-tiered membrane. x43,000

progression. The outer membranes will fuse, perforate, separate and pay back completely. Then the inner membranes will fuse and remain fused for a considerable period prior to defusion. In such cases, a septum will be found in the middle of a mitochondrion extending from one side to the other, and the two membranes in the septum will be found to be

continuous with the inner membrane envelope. Such a septum can be distinguished from a crista since it is perforated at both ends (cristae are perforated only at one end).

Figure 3 shows a large field of mouse liver megamitochondria *in situ* with multiple instances of membrane fusion. The frequency of fusion is sufficiently high that it is possible to find evidence for all the variations in the progression of fusion events during formation of megamitochondria. In general, the stepwise fusion-defusion of the outer membrane, followed by the fusion-defusion of the inner membrane, is a very rare event. These two fusion-defusion cycles follow one another in quick succession. One can often see the pairing of two mitochondria with a region in which fusion of the two outer membranes has taken place (see Fig. 6), but this is almost immediately followed by the perforation of the fused outer membrane and the fusion-perforation cycle for the inner membrane; then the two pairs of separated membranes pay out in synchrony (see Figs 6 and 7). Finally, in Fig. 8 we see two inner membrane septae still unperforated and yet to be separated. Note that one of the septae appears as a pair of membranes separated by a space, whereas the other appears as a single fused triple-tiered membrane without any internal space. These are in fact two equilibrium forms of the fusion membrane. The two membranes fuse to form a triple-tiered structure (the middle tier being characteristically dark). This fusion membrane can relax back to the original pair of unfused and separated membranes (separated by a space). The triple-tiered fusion membrane is seen only in relatively few cases (Fig. 8 is a fine example of such a fusion membrane). More often it relaxes back to the unfused state. Under other conditions, the reverse is true. In presence of Mg^{++}, e.g., the stable form is the triple-tiered structure and the rare form is the paired membrane separated by a space. That the paired membranes were engaged in the fusion-defusion cycle is readily inferred from the perforations or from the tell-tale geometry. In principle, it is impossible to decide from the electron micrographs whether the transition stages are intermediate to megamitochondrion formation or to the fragmentation of a megamitochondrion. Since megamitochondrion formation took place progressively during the period of feeding cuprizone (1-21 days), it is a reasonable presumption that the intermediate stages were those of megamitochondrion formation and not fragmentation. However, as far as our thesis of the mechanism of megamitochondrion formation is concerned, it would make no difference which the correct direction happened to be, since the process is apparently reversible.

Acknowledgements

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