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A Characterization of Cuprizone-Induced Giant Mouse Liver Mitochondria

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Abstract

Cuprizone affects the liver of treated mice in a random manner, causing no appreciable change in some cases and inducing the formation of megamitochondria with altered properties in others. Lack of a full appreciation of this variability may be at the origin of some discrepancies in published work dealing with the properties of cuprizone mouse liver mitochondria (CMLM).[2] CMLM from fully affected livers were remarkably labile and difficult to isolate in a coupled state by homogenization and centrifugation techniques. The integral respiratory chain proteins of CMLM were functionally normal, with the exception of succinic dehydrogenase which showed considerable inhibition. Coupled morphological and functional analysis provided evidence that these properties were independent of CMLM size, a matter which had remained doubtful thus far and bears on the validity of literature reports.

Key Words: Megamitochondria; cuprizone; redox chain; mouse liver mitochondria.

Introduction

While "giant" mitochondria occur naturally in some cells (Munn, 1974), the considerable interest they have generated (Tandler and Hoppel, 1986) has been mainly due to their association with a variety of pathological conditions. The size attained by the organelles varies from one condition to the other, and ranges in all cases from near normal to a maximum. Most of the published studies concern morphological or ethiological aspects. While the

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[2]Abbreviations: MLM, mouse liver mitochondria; CMLM, cuprizone mouse liver mitochondria; PMS, phenazine methosulfate; Mops, 4-morphoinopropanesulfonic acid; TMPD, tetramethyl-*p*-phenylenediamine; AA, Antimycin A; RCR, respiratory control ratio; BSA, bovine serum albumin.

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precise mechanism of formation of gigamitochondria remains uncertain, the view that they arise by fusion of normal-sized organelles is supported by ultrastructural observations (Wakabayashi *et al.*, 1975a, 1977). As pointed out by Tandler and Hoppel (1986), the studies directed at the biochemical characterization of giant mitochondria have in fact often utilized preparations of near-normal size organelles, the reason presumably being the greater ease of isolation and purification. Whether the results obtained with material of this type can be extended to larger organelles might be questioned.

In a different research perspective, oversize mitochondria have found utilization in experiments involving the micromanipulation and observation of single organelles, difficult to carry out on normal-size mitochondria. Tedeschi and colleagues impaled giant mitochondria with microelectrodes in order to measure their transmembrane voltage and pH gradients and the electrical properties of the inner membrane (Tupper and Tedeschi, 1969a,b; Maloff *et al.*, 1978a,b; Campo *et al.*, 1984; Campo and Tedeschi, 1984). Others have taken advantage of their large surface area to study the lateral mobility of proteins by the FRAP (fluorescence recovery after photobleaching) technique (Hochman *et al.*, 1982, 1985; Gupte *et al.*, 1985).

Cuprizone-induced giant liver mitochondria (Suzuki, 1969; Tandler and Hoppel, 1986) have been used in most of the studies mentioned, as well as in recent applications of the patch-clamp technique to the study of the conductance properties of the outer (Tedeschi *et al.*, 1987; Tedeschi and Kinnally, 1987; Kinnally *et al.*, 1989a) and inner (Sorgato *et al.*, 1987, 1989; Kinnally *et al.*, 1989b) mitochondrial membrane. It is therefore important to characterize isolated cuprizone mouse liver mitochondria (CMLM) as thoroughly as possible, and to ascertain whether they are good substitutes for normal liver mitochondria. Only a few studies have been devoted to this goal, and contradictory reports have been published.

Tedeschi's group obtained single giant CMLM capable of producing ATP and of taking up calcium ions (Maloff *et al.*, 1978b; Campo *et al.*, 1984). Suchy and Cooper (1974) showed that individual, giant CMLM, picked out from disrupted cells with a microcapillary, exhibited good respiratory control. Inhibition of succinate-based respiration in CMLM was reported by Hoppel and Tandler (1973, 1975), who also found low respiratory control ratios and a marked decrease of State 3 respiration with a variety of Site I substrates. Other authors found instead normal succinate-based respiration, cytochrome oxidase activity, and cytochrome aa_3 levels (Hochman *et al.*, 1982, 1985; Wagner and Rafael, 1977; Wakabayashi *et al.*, 1975b, 1987; Flatmark *et al.*, 1980). Wagner and Rafael (1977) reported that megamitochondria could be obtained in a coupled state, despite their intrinsic fragility, and Wakabayashi *et al.* (1975b) reported only slightly lowered respiratory control ratios. Tandler mentioned instead that "small mitochondria obtained

from livers with megamitochondria were poorly coupled" (Tandler and Hoppel, 1986).

This work addresses mainly two points: whether the membrane properties of CMLM are dependent or not on their size, and whether the respiratory chain of CMLM is "normal" or not. Reliable answers to these questions would be of value both to workers investigating ethiological and clinical aspects of megamitochondria formation, and to those using these giant organelles as convenient tools for studies on mitochondrial properties.

Materials and Methods

Production of CMLM

Seventeen-day-old Balb C mice were weaned and maintained on a powdered standard diet supplemented with 0.9% (w/w) cuprizone (Aldrich) recrystallized from ethanol. Six to twenty animals were sacrificed by cervical dislocation after 10–12 days. Each liver was homogenized separately by two strokes of the pestel of a large-clearance tissue grinder (Kontes K-8853301), each homogenate was individually checked by microscopic observation, and only the homogenates subjectively judged to contain many large mitochondria were pooled and utilized for the isolation of the CMLM. Preparations of CMLM were then obtained by a variety of centrifugation protocols, designed to yield two or more fractions with different size distributions. Often the procedure of Hochman *et al.* (1985) was used, with the following modifications: after the second centrifugation the interphase between the two layers was resuspended in the 0.5 M sucrose layer, and the smaller mitochondria contained in the upper layer were harvested separately by centrifugation at 1000 or 6000 *g*. The final resuspension was in 200 mM mannitol, 70 mM sucrose, 0.5 mg/ml BSA and 2 mM Hepes/Tris, pH 7.4.

Assay Procedures

Mitochondrial protein concentrations were determined by the Lowry method. Rates of oxygen consumption by mitochondria incubated at 25°C in a closed, thermostatted and stirred vessel were determined using a Clark electrode (Yellow Springs). When comparison with normal mitochondria was desired, both controls and CMLM were subjected to osmotic shock in order to minimize differences due to the damaged state of the CMLM. All the respiration and ATP hydrolysis rate data presented in Table I and II were obtained using the following procedure: 10–50 μl of mitochondrial suspension were diluted into 1 ml of water, and after 2 min 1 ml of doubly concentrated medium was added to give a suspension in 200 mM sucrose, 20 mM Tris/Mops,

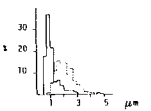
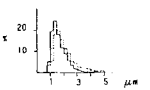
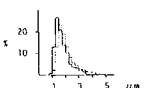
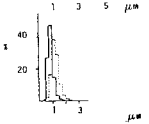
5 mM phosphate, and 1 mM EGTA, pH 7.2. 2 μM Rotenone, or 2 μM Rotenone plus 10 μM cytochrome *c* and excess antimycin A were respectively added when succinate- or TMPD/ascorbate-based respiration were to be measured. Substrate concentrations used were: NADH, 0.5 mM; succinate, 10 mM; TMPD, 1 mM; ascorbate, 5 mM. Rates of NADH and TMPD oxidation were corrected for the low rotenone- or cyanide-insensitive respiration, respectively.

Succinate dehydrogenase activity was determined by the PMS method (Veeger *et al.*, 1969) at 38°C, using water-shocked MLM or CMLM (see above). The content of cytochromes aa_3 was determined from reduced-minus-oxidized spectra of solubilized (Triton X-100, 2%) mitochondria at 605–630 nm, using a millimolar extinction coefficient of 26.5 cm^{-1} (Terada and Van Dam, 1975). The concentration of bc_1 complexes was determined by extrapolating antimycin titration curves (Wagner and Rafael, 1977; Kroeger and Klingenberg, 1973). The osmotically shocked mitochondria were incubated with varying concentrations of antimycin for 4 min before addition of succinate and determination of the respiratory activity. Phospholipid content of mitochondrial suspensions was determined by the procedure of Folch *et al.* (1957).

Determination of Morphological Parameters

The information needed to calculate the activities of membrane enzymes per unit of surface area, i.e., the number of mitochondria per suspension unit volume and their size distribution, was obtained using a Kontron-Zeiss IBAS 2000 imaging apparatus. A measured volume of the mitochondrial suspension, normally 0.5 μl, was placed on a glass slide and covered with a coverslip, which caused the droplet to spread over an area of 0.3–0.7 cm^2 (corresponding to optimal depth of the suspension layer). The glass slide was placed on the movable, electrical micromanipulator-controlled stage of the microscope and viewed at a total magnification of 400 or 630 × using phase-contrast optics. The contour of the spot was traced by moving the stage under the objective and note was taken of the automatically displayed coordinates of 50 or more points on the perimeter. The contour was immediately reproduced on graph paper, and a grid of perpendicular, evenly spaced lines was superimposed. The intersection points falling within the contour were then systematically visited by positioning the stage at the appropriate coordinates, and the microscope images, collected by a videocamera, were electronically recorded. Fifty to 100 images, each covering an area of 5900 or 2400 μm^2, were collected for each spot, constituting an unbiased, statistically valid set of images. This procedure was made necessary by the realization that the mitochondria were usually unevenly distributed through the spot, the periphery being poorer in organelles and the central section holding most

Table I. Surface-Normalized Parameters of Mitochondria with Different Size Distributions[a]

Size Distribution[b]	Mean surface[c] (μm^2)	Respiration[d] (g-atoms O $\times$ μm^{-2} min^{-1}) $\times$ 10^{17}			ATP hydrolysis[d] (moles $\times$ μm^{-2} min^{-1}) $\times$ 10^{17}
		NADH	Succinate	TMPD	
 Exp. 1 CMLM	14.8	n.d.	0.74	7.8	n.d
	4.0	n.d	0.77	9.5	n.d
 Exp. 2 CMLM	16.2	1.3	0.76	n.d.	3.7
	9.4	1.0	0.67	n.d.	3.0
 Exp. 3 CMLM	16.2	n.d	0.82	n.d.	7.2
	10.9	n.d	0.79	n.d.	5.5
 Exp. 4 MLM	5.0	1.3	1.73	11.6	n.d.
	2.3	1.2	1.64	11.4	n.d

[a] Representative experiments. For each experiment, two populations are compared having the size distributions shown in the corresponding histograms. The distribution of the suspension with the larger mean mitochondrial surface is given by the dotted line. Both were obtained from the same pooled livers, by slightly different centrifugation protocols. "Equivalent sphere" of a mitochondrion is the sphere the area of whose projection on a plane equals that of the two-dimensional image of the mitochondrion. Number of mitochondrial images used to construct the histograms (larger, smaller): Expt. 1: 527, 369; Expt. 2: 1938, 2274; Expt. 3: 3707, 2473; Expt. 4: 3830, 2818. n.d.: not determined. Bins for $d > 5\,\mu m$ are not shown, since the diameters of only a few of the measured mitochondria fell in that range. They were, however, included in the calculation of mean surface areas.

[b] Histograms giving percent population vs. diameter of the equivalent sphere in bins of $0.25\,\mu m$.

[c] Mean surface of the equivalent sphere.

[d] Rate of respiration or ATP hydrolysis per μm^2 of surface of the equivalent sphere.

of the largest ones. The number of mitochondria in each photograph, the area of their image, and the diameter of the equivalent (same-area) circle were later determined using the IBAS software to construct histograms such as those shown in Table I. Since the mitochondria always appeared as round objects (with diameters up to 12 μm), they were treated as spheres when calculating their outer surface. The area of the recorded images and of the surface occupied by the suspension droplet on the slide was determined on the basis of suitable calibrations. This knowledge permitted the calculation of the number of mitochondria and of the mean and total contour mitochondrial surface areas per unit volume (or per milligram protein) of the original suspension from the number and sizes of the mitochondria in the recorded images.

A similar procedure was used for the determination of the perimeter and area and of the cristal contours within the cross-sections of mitochondria in published electron micrographs. In this case the photographs were viewed through a computer-interfaced videocamera, the contrast of the images was enhanced, and the relevant features measured. Manually, this involved following the lines on the display screen by means of a "mouse."

Results

Formation and Isolation of Megamitochondria

An evident characteristic of the cuprizone method of megamitochondria production was the great variability of effects. In spite of the standardized conditions, the livers of only some of the mice contained a high proportion of large mitochondria, and yielded preparations with the properties described below. Other mice, even belonging to the same litter, had livers containing mitochondria with a near-normal size distribution, with much the same properties as control mitochondria. We also occasionally observed intermediate situations, with somewhat enlarged mitochondria possessing intermediate properties. The occurrence of one or the other of these situations was apparently random. It did not depend on how long the animals had been on the cuprizone diet, or on their age at the beginning of the diet. Possibly the general health state of the mouse or how well it was nourished by the mother may have been of importance. We qualitatively observed a degree of correlation between the development of intoxication symptoms (lack of weight gain, lethargy) by the animals and the abundance of megamitochondria in their livers. Suchy and Cooper (1974) explicitly mentioned similar difficulties in obtaining reproducible effects. Variability is evident as well from literature reports (Hoppel and Tandler, 1975) in the case of mice kept on a riboflavin-deficient diet.

Coupling Properties of the CMLM

We found the CMLM to be extremely fragile. The initial homogenate showed respiratory control ratios of 3–4, but this parameter decreased rapidly during the purification steps. Figure 1 illustrates this for a typical differential centrifugation procedure, and at the same time shows that preparations of CMLM exhibiting normal RCR's and resistance to handling could be obtained from unaffected livers. The isolation procedure also resulted in the ability of the CMLM to oxidize added NADH (at rates 75–95% of those measured after osmotic shock) and in the loss of most of the matrix K^+, indicating that membrane damage was responsible for the fall of the coupling parameters. Application of the isolation protocol of Wagner and Rafael

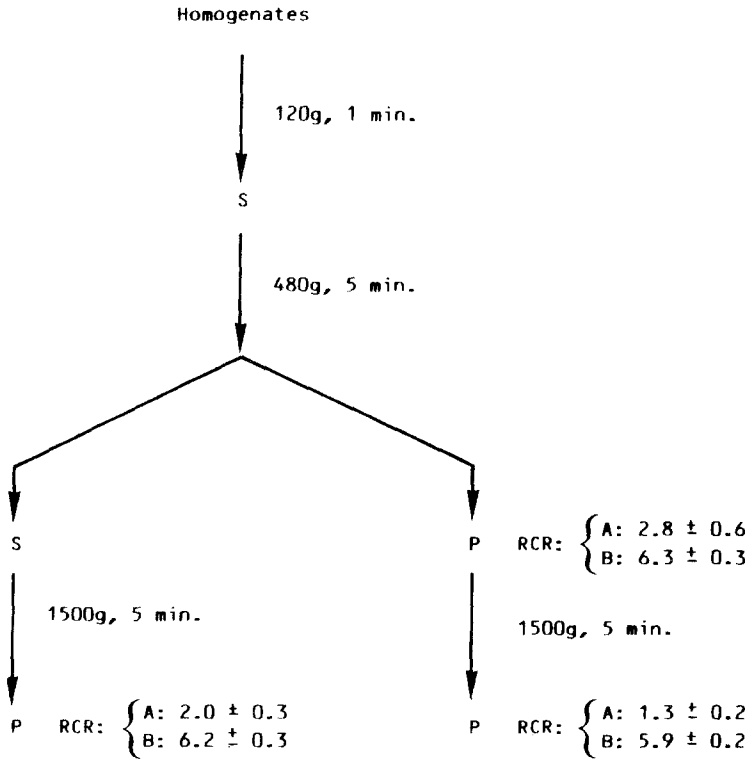


Fig. 1. Decline of the respiratory control ratio during isolation. The centrifugation protocol outlined above was applied in parallel to liver homogenates A and B. Both A and B had been obtained from the livers of mice kept on the cuprizone-containing diet for the same length of time, but in case A the livers had been judged to have been profoundly affected, while in case B the effect had been slight. RCR's were measured with succinate/ADP after gentle resuspension of the pellet in the preparation medium. S: supernatant. P: pellet. Averages with standard deviations (three experiments) are given.

(1975, 1977) and inclusion of up to 0.5% BSA in the isolation medium (Wakabayashi *et al.*, 1975b) in our hands did not result in an improvement.

To test whether this fragility was a consequence of the large size of the CMLM, which might be expected to make them mechanically unstable, we isolated mitochondrial fractions with different size distributions by the simple method of collecting successive pellets from the first supernatant (after elimination of the debris), always spun at the same rate. The various fractions were thus subjected essentially to the same isolation stress, differing only in the length of time spent in the refrigerated centrifuge tubes. Even the smallest CMLM collected in this manner, with size distributions only slightly shifted with respect to MLM, were rapidly uncoupled by procedures such as centrifugation at 1000 × *g* and resuspension by gently swirling (see also Fig. 1). Exposing the purified CMLM to hypoosmotic media did not lead to dramatic morphological changes: in particular, the average diameter barely increased, a further indication of extensive membrane damage.

The Properties of CMLM Do Not Depend on Their Size

Before a meaningful comparison with normal MLM could be carried out, the question had to be answered of whether the characterizing parameters of CMLM depend on their size. Given the variability mentioned above, this could only be achieved by comparing suspensions of mitochondria of different size distributions obtained from the same pool of livers. Since our interest centered on the properties of the inner membrane, the most appropriate comparison seemed to be between membrane respiratory activities, normalized to take into account the different membrane surface areas of the organelles (Table I). Examination of published EM photographs of isolated CMLM (Hochman *et al.*, 1982; Wakabayashi *et al.*, 1974, 1975b) as well as our own (not shown) indicated that directed morphometeric determination of the surface of the inner membrane could not be done with the available means. In Table I the fluxes have therefore been normalized using the mean outer surface area with the underlying assumption that the ratio of the envelope surface to the actual inner membrane area is independent of CMLM size. This would be expected if the megamitochondria arise via fusion of smaller organelles, (Wakabayashi *et al.*, 1977, 1984), and is supported by the observations reported below. Wakabayashi *et al.* (1984) have presented ultrastructural evidence that in their thin sections of livers from cuprizone-fed mice the number of cristae per unit surface area of the mitochondrial section depended on the size of the mitochondrion. This was, however, due to the fact that the cristae extended only a finite and constant depth into the matrix space, and the observation suggested a constant cristae/outer membrane ratio. This was confirmed by the results of measurements

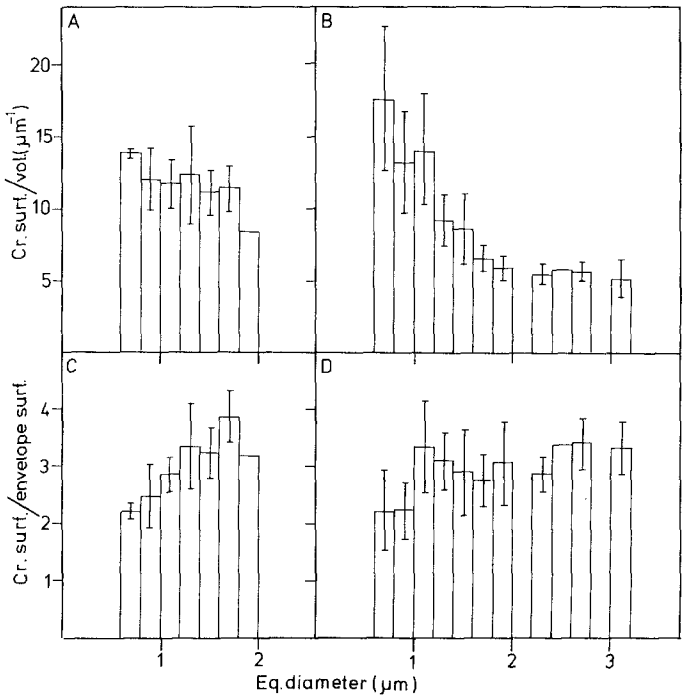


Fig. 2. Ratio of the cristal membrane surface to mitochondrial volume (Panels A and B) or to envelope surface (Panels C and D) in CMLM as a function of size. A and C: "Cristae-enriched" mitochondria (from Wakabayashi *et al.*, 1984, Fig. 1); B and D: "Cristae-poor" mitochondria (*ibid.*, Fig. 4 and Wakabayashi *et al.*, 1975a, Fig. 3a). Bin size: 0.25 μm. A total of 25 (A and C) and 45 (B and D) images of mitochondrial sections was analyzed. Error bars give the standard ($n > 2$) or average deviations around the mean. For methods and rationalization, see text and the experimental section.

carried out with the aid of the imaging system on a few EM photographs published by Wakabayashi *et al.* (1975a, 1984) (Fig. 2). The ratio of the cristal profiles to the section contour is always close to 3. By the postulates of morphometry (Loud *et al.*, 1965), the ratio of the inner membrane surface to the three-dimensional contour or to the outer membrane of CMLM is therefore also constant and close to 4. This value is in excellent agreement with the observations reported by Loud (1968) for normal mitochondria. The amount of phospholipids present per mg protein was measured to be the same, namely 115 nmols/mg protein, in CMLM and control MLM, thus suggesting that the membrane complement is indeed the same in the two cases.

The results presented in Table I indicate that in isolated CMLM respiration proceeds at the same rate per unit of membrane surface irrespective of the dimensions of the organelles, whose size distribution is given in

Table II. Comparison of Mitochondria from Cuprizone-treated Mice (CMLM) and from Controls[a]

	Respiration (ng-atoms O × mg protein^{-1} min^{-1})			Antimycin binding sites (pmol × mg protein^{-1})	Respiration[b]/AA binding sites (ng-atoms O × min^{-1} pmol^{-1})	Surface[c] (μm^2 × mg protein^{-1}) × 10^{-10}
	NADH	Succinate	TMPD			
CMLM	141 ± 23 (7)	95 ± 16 (26)	1293 ± 345 (5)	52 ± 9 (14)	1.9 ± 0.4 (14)	1.07 ± 0.26 (19)
Controls	136 ± 21 (4)	159 ± 18 (12)	1377 ± 345 (4)	52 ± 4 (5)	3.1 ± 0.5 (5)	0.92 ± 0.17 (5)

[a] Values ± standard deviations are given. Numbers in parentheses refer to the number of independent experiments averaged.
[b] Succinate-based.
[c] Mean surface of the equivalent spheres × number of mitochondria per mg protein.

the histograms presented. Data are also provided, to allow comparison, for osmotically shocked mouse liver mitochiondria. It should be emphasized that the higher (dashed line) size distribution histogram in exp. 4 of Table I concerns only a small fraction of the MLM in the preparation. Neither of the two histograms actually reflects the naturally occurring size distribution: both fractions had been enriched in larger organelles in order to facilitate size measurements.

Comparison of CMLM and MLM Respiratory Properties

The relevant data are presented in Table II. The only significant difference among the parameters reported concerns the activity of the succinate/cyctochrome *c* segment of the respiratory chain. While the extent of inhibition varied between 20 and 50% depending on the individual preparations, succinate-based respiration, expressed either per mg protein or per unit of membrane surface, was always lower in CMLM. Expressed as electrons per second per cyt. aa_3, the rates of succinate oxidation were about 29 and 48 for CMLM and MLM respectively, in excellent agreement with the report by Gupte *et al.* (1984). The inhibition was not relieved by preincubation with malonate or succinate, which is known to activate the succinic dehydrogenase of submitochondrial particles (Kimura *et al.*, 1967). In agreement with published results (Wakabayashi *et al.*, 1975a, 1978; Hochman *et al.*, 1982; Wagner and Rafael, 1977; Flatmark *et al.*, 1980), cytochrome aa_3 levels, about 0.11 nmol mg protein^{-1}, and cytochrome *c* oxidase activity were not substantially affected by the cuprizone treatment. These results, as well as the normal rates of NADH and TMPD oxidation, indicate that the terminal segment of the respiratory chain is not to be blamed for the inhibition of succinate oxidation, and confirm that the CMLM have an inner membrane/protein ratio comparable to that of MLM. Our determinations (Table II), as well as the observation that first-site NADH oxidation proceeds at a higher rate than succinate oxidation, rule out a reduced bc_1 content as the reason for the decrease. Furthermore, the bc_1 complex has been reported to exert only a very limited degree of control on mitochondrial respiration (Groen *et al.*, 1982). An indication that something must instead be amiss with succinic dehydrogenase comes from comparisons of the ratio of the rate of succinate oxidation to the number of antimycin-binding sites. The lower ratio found for CMLM indicates that the supply of electrons reaching each bc_1 complex is not sufficient for it to operate at the turnover number prevailing in the case of MLM. Since the mitochondria were completely uncoupled (osmotically shocked) in all these experiments, no thermodynamic forces existed to exert a control, which must therefore be due to the succinic dehydrogenase. This conclusion is borne out by direct measurement of this

emzyme's activity by the PMS method: in CMLM the activity was 78% of that of controls.

With CMLM, irrespective of whether they had been subjected to osmotic shock, respiration with a variety of citric acid cycle substrates was invariably much lower than in MLM, in agreement with Tandler and Hoppel (1975). The normal rates of NADH oxidation (Table II) suggest that this may be due to the loss of matrix components during isolation rather than to modification or loss of membrane proteins. The ATPase activity did not appear to have been greatly altered by the cuprizone treatment. However, we found it to vary so much from one preparation of CMLM to the other as to render a meaningful comparison difficult. Factors such as the use or not of a sucrose gradient and perhaps the degree of damage sustained by the organelles were presumably at the origin of this variability (see Table I). As the ATPase activity was always completely oligomycin-sensitive, detachment and/or loss of the F_1 moiety cannot provide a rationale for the scatter, which might be linked to alterations of the ATPase inhibitor protein activity or content.

Discussion

Two major conclusions may be drawn from the results presented above: (i) the biochemical properties of the CMLM do not depend on their size, and (ii) CMLM resemble normal MLM as far as the activity of much of the redox chain is concerned. The only respiratory chain complex affected by the cuprizone treatment appears to be succinate dehydrogenase, the activity of which is considerably lower in the CMLM membrane. Furthermore, the finding, from image analysis of published EM photographs, that the ratio of CMLM cristal membrane to organelle contour surface is very close to that of normal liver mitochondria and does not depend on the size of the organelles provides some support to the idea that giant mitochondria arise via fusion of smaller ones. We found cuprizone mouse liver mitochondria to be extremely susceptible to damage during isolation. This lability is not a characteristic of the larger organelles only, but affects all CMLM, irrespective of size. Finally, the variability of the effects of cuprizone itself was fully recognized.

The first of these conclusions, strictly speaking, applies to the properties and to the ranges of sizes compared in the experiments (see Table I). We could not extend the comparison to CMLM fractions with more widely different mean surface areas because of the low percentage of truly enormous CMLM in livers and of the difficulty of obtaining them in reasonably uncontaminated form. It seems, however, logical to extrapolate the observations to larger organelles. If this extrapolation is accepted, it removes the doubts as to whether the results of studies conducted on preparations of

CMLM of normal or near-normal size could be considered to apply regardless of size. However, new doubts are introduced by the realization that cuprizone may produce its effects in an uneven manner, sparing some of the experimental animals while affecting others. This may have resulted in preparations containing both cuprizone-affected and practically normal mitochondria, possibly in different proportions in different experiments. The tendency of researchers to use suspensions of relatively small organelles may have increased the proportion of unaffected mitochondria.

Other properties of livers from cuprizone-fed mice have been recognized which may contribute to the variability of preparations. The ultrastructural effects of the cuprizone diet disappear very rapidly upon suspension of the diet: the first signs of reversion to a population of normal-size mitochondria appear within half an hour and after some 6 h an almost normal size distribution prevails (Tandler and Hoppel, 1973; Wakabayashi *et al.*, 1975a). Also, cells in different parts of the liver might develop megamitochondria to different extents and nonhepatocyte cells might not be affected (Wakabayashi *et al.*, 1975a).

Heterogeneity of the preparations might be at the origin of some of the reports of coupled CMLM preparations (Wagner and Rafael, 1977; Wakabayashi *et al.*, 1975b). From livers deeply affected by cuprizone (as subjectively judged by megamitochondria content) we were unable to isolate a purified mitochondrial fraction exhibiting a RCR higher than about 1.3. The reasons for this fragility are unclear. Lipid composition has been reported to be unaltered except for two relatively minor components (Wakabayashi *et al.*, 1978). Megamitochondria *in situ* have been reported to be often connected by narrow junctions (Wakabayashi *et al.*, 1975a), which would be expected to cleave easily during cell disruption and subsequent handling. At times the outer membrane has been observed to be broken, with the inner membrane protruding into the cytoplasm. If such cases occur in the whole liver, it is hardly surprising that isolated mitochondria are easily damaged. Many of the smaller isolated CMLM might actually arise via cleavage, during the isolation, of branched mitochondrial systems. Highly branched mitochondrial networks form in several cell types (Pellegrini, 1980; Amchenkova *et al.*, 1988), and there is some evidence (Wakabayashi *et al.*, 1975a) that what appear to be independent, relatively small mitochondria in liver sections from cuprizone-fed mice might actually be branches of such networks. These would be expected to break, possibly with imperfect resealing, during isolation. Some properties of the isolated CMLM indeed resemble those of osmotically shocked mitochondria. Our own observations and those of others (Maloff *et al.*, 1978b; Suchy and Cooper, 1974) indicate, however, that the organelles are coupled *in situ*. The gentlest possible isolation procedures are obviously necessary.

Apart from permeability characteristics, the only other component of the mitochondrial membrane energy-transducing apparatus definitely altered by the cuprizone diet appears to be succinic dehydrogenase. Our data do not allow us to decide whether the functional enzyme is expressed at a lower density, or whether the activity of the enzyme itself has been modified. Since succinate dehydrogenase contains iron and cuprizone is a metal chelator, the latter case may be considered more likely, but the detailed mechanism of action of cuprizone at this level, as well as at all others (neural disorders, formation if megamitochondria, fragility of mitochondria), remains to be explored.

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