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BIOCHEMICAL EFFECTS OF CUPRIZONE ON MOUSE LIVER AND HEART MITOCHONDRIA

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Abstract—Mitochondria isolated from livers of mice fed the copper-chelating agent, cuprizone, showed a decrease in state 3 respiration, respiratory control ratios and ADP/O or P/O ratios. This inhibition of state 3 respiration was not relieved by the addition of dinitrophenol (DNP). These inhibitory effects apparently involved all three sites of mitochondrial-coupled oxidative phosphorylation as well as possibly having an effect on substrate-level oxidative phosphorylation. Cuprizone produced a greater degree of inhibition of phosphorylation than of oxidation and therefore has the characteristics of an inhibitor uncoupler. Liver mitochondria isolated from control mice or from normal rats were unaffected by treatment *in vitro* with cuprizone. In contrast to liver mitochondria, mitochondria isolated from the hearts of mice fed cuprizone show no defects in oxidative phosphorylation.

CUPRIZONE (biscyclohexanone oxalyldihydrazone) is a potent copper-chelating agent.[1] Added to the diet, it produces profound morphological and clinical alterations in the central nervous system of mice, rats and guinea pigs,[2,3] mimicking status spongiosus.[4] In addition, ultrastructural changes have been noted in hepatic[4–6] and cardiac[7] cells of mice after administration of this compound. These effects were more dramatic in hepatocytes where many mitochondria attained enormous size, up to 15 μm dia. In such organelles, the cristae were short and confined to the periphery, the central portion of the mitochondria being occupied by a greatly augmented matrix of normal electron-density. Although each hepatic cell contained giant mitochondria, organelles of normal and of intermediate size were also present. Unlike the liver cells, cardiac mitochondria did not increase in size under the influence of cuprizone. Instead, in some but not all hearts the number of mitochondria was substantially increased.[7]

Despite these striking morphological effects on mitochondria, little is known of the manner in which cuprizone affects mitochondrial biochemistry. This report describes the changes in oxidative metabolism of mitochondria isolated from both liver and heart of mice treated with cuprizone.

MATERIALS AND METHODS

Animals. Weanling mice of the CFW strain (Carworth Farms) weighing 8–10 g were used in all experiments. The control animals were fed pelleted mouse food *ad lib.* The experimental animals were maintained on a 0·5% (w/w) cuprizone-supplemented powdered mouse diet *ad lib.* for 8–12 days. Water was freely available.

Livers from both control and experimental mice were homogenized using a Potter–Elvehjem homogenizer with a loose-fitting Teflon pestle. Mitochondria were isolated from the homogenates in 0·3 M sucrose, 0·01 M MOPS (morpholinopropane sulfonic

acid), 0·002 M EDTA, pH 7·2, at 450–7000 *g* and were washed twice in 0·3 M sucrose, 0·01 M MOPS, pH 7·2.[8] The nuclear fraction was isolated from the liver homogenates at 450 *g* and washed twice in 0·3 M sucrose, 0·01 M MOPS, pH 7·2. Ten livers were pooled for each experiment.

Heart mitochondria were prepared using the bacterial protease, Nagarse, by the method of Tomec and Hoppel.[9] In these experiments, the first low speed fraction, which contained nuclei and cell debris, was rehomogenized in isolation medium, recentrifuged, and the supernatant pooled with the supernatant of the first low speed centrifugation. Mitochondria were isolated from this pooled supernatant from the hearts of 40–50 mice in each experiment.

Oxidation experiments. A Clark oxygen electrode was used to monitor oxygen uptake in 1 ml of incubation medium containing 80 mM KCl, 50 mM MPOS, 5 mM P_i, 1 mM EGTA [ethyleneglycol bis(β-aminoethyl ester)-*N*,*N*′-tetra-acetic acid], 1 mg of defatted bovine serum albumin, and between 1·8 and 2·2 mg of mitochondrial protein/ml. Substrate concentrations are given in Tables 2–5. The final pH was 7·0 and the temperature was 30°. State 3 respiration (ADP-stimulated) and state 4 respiration (ADP-lacking) were measured and respiratory control ratios (RCR) [the ratio of state 3 to state 4 respiration] and ADP/O ratios were calculated as described by Chance and Williams[10] and by Estabrook.[11]

To study uncoupled respiration, 0·1 mM dinitrophenol (DNP) was added in some experiments to both control and cuprizone-liver mitochondria (CLM).

Oxidative phosphorylation experiments. The basic incubation medium described for the oxidation experiments was used with the addition of 25 mM glucose, 3·8 U hexokinase, and 5·0 mM ADP and 10 mM P_i (containing 10^6 cpm ^{32}P) instead of 5 mM P_i. Oxygen consumption was monitored, and when approximately 60 per cent of the oxygen in the medium had been consumed 0·5-ml aliquots were removed and added to tubes containing 0·05 ml of 65% trichloroacetic acid (TCA); these were mixed and placed on ice. After centrifugation, aliquots of the TCA supernatant were removed and phosphate uptake was determined as described previously.[12] The organic phosphate samples were counted in a Nuclear Chicago liquid scintillation spectrometer.

Electron microscopy. Both mitochondrial and nuclear pellets were fixed for 1 hr in cold (0–4°) phosphate-buffered 2% osmium tetroxide.[13] After dehydration in graded ethanol, the pellets were embedded in Maraglas–D.E.R. 732.[14] Thin sections were stained with methanolic uranyl acetate[15] followed by lead citrate,[16] and examined in a Siemens Elmiskop la electron microscope.

Chemicals. Cuprizone was a product of G. Frederick Smith Company, Columbus Ohio. All other material was purchased or prepared as described previously.[8]

RESULTS

Mice fed a cuprizone-supplemented diet failed to gain significant weight ($P < 0·001$) during the experimental period (Table 1). The ratio of liver wet weight per 100 g body weight was not significantly decreased in the experimental mice. It was noted that the experimental livers had a rather soft consistency, and required extremely gentle handling in order to be removed intact from the animals. Despite this alteration, the yield of mitochondria per gram wet weight of liver was not significantly

TABLE 1. EFFECTS OF CUPRIZONE ON BODY AND ORGAN WEIGHT*

	Control	Cuprizone-treated mice
Average body wt	18·78 ± 0·50 (4)	7·69 ± 1·24 (5)†
Liver wt (g/100 g body wt)	5·22 ± 0·12 (4)	4·77 ± 0·48 (5)
Mitochondrial protein (mg/g wet wt liver)	17·31 ± 3·11 (4)	17·61 ± 3·00 (5)
Average body wt	19·65 ± 0·52 (4)	9·11 ± 0·55 (5)†
Heart wt (g/100 g body wt)	0·525 ± 0·020 (4)	0·523 ± 0·036 (5)
Mitochondrial protein (mg/g wet wt heart)	23·28 ± 2·61 (4)	24·12 ± 3·99 (5)

* Results are given as mean ± S.D., with the number of experiments in parentheses.

† $P < 0·001$ when compared to the control.

different. As reported previously,[6] the hepatic mitochondria in cuprizone intoxication varied in size from normal to giant. Liver mitochondria isolated between 450 and 7000 *g* were used in our experiments and an electron micrograph of the mitochondrial pellet is shown in Fig. 1. The megamitochondria sedimented in the nuclear fraction, as shown in Fig. 2.

The rates of oxidation in isolated liver mitochondria from control mice are shown in Table 2. As judged by RCR and ADP/O ratio, these mitochondria were normal. These values are somewhat lower than those usually obtained in our laboratory for normal liver mitochondria. This is most probably because the livers were deliberately extirpated at a relatively leisurely rate in order to match the time required to dissect out cuprizone livers.

With CLM, the state 3 respiratory rates were inhibited with all substrates tested. Inhibition varied from 89 per cent with hexanoate to 68 per cent with β-hydroxybutyrate (Table 2). The decrease in the RCR was produced by depression of state 3 respiration. The inhibited state 3 respiration was not altered by the addition of DNP to the incubation medium, indicating that inhibition occurred in both coupled and uncoupled CLM.

Because of the technical difficulty in differentiating between states 3 and 4, ADP/O ratios could not be accurately measured in CLM. The data on oxidative phosphorylation in CLM are shown in Table 3. The oxidative rates of these mitochondria were severely inhibited. In addition, the rate of uptake of $^{32}P_i$ was also decreased and the P/O ratios were less than one. This demonstrated that the inhibition of oxidation was accompanied by an even greater inhibition of phosphorylation. The P/O ratio of one with α-ketoglutarate plus malonate was higher than that of the other substrates, and was a reflection of the fact that this substrate undergoes substrate-level phosphorylation in addition to phosphorylation coupled to the electron-transfer chain.

To test for effects *in vitro*, 1 mM cuprizone was added to the incubation medium during measurements of oxidative phosphorylation by normal mouse liver mitochondria. There were no changes in state 3 or state 4 respiration, or in RCR or ADP/O ratios using glutamate or succinate as the substrate. Normal mouse or rat liver mitochondria were also pretreated with 0·5 μM cuprizone/mg of mitochondrial protein

TABLE 2. EFFECTS OF DIETARY CUPRIZONE ON MOUSE LIVER MITOCHONDRIAL OXIDATIVE PHOSPHORYLATION*

Substrates	State 3 respiration (n-atoms O/min/mg)		State 4 respiration (n-atoms O/min/mg)		RCR		ADP/O†
	Control (4)	CLM (4)‡	Control (4)	CLM (4)	Control	CLM	Control (4)
10 mM Glutamate	90·8 ± 12·6	23·8 ± 8·9	21·4 ± 7·6	13·8 ± 4·9	4·6 ± 1·1	1·9 ± 1·1	2·36 ± 0·10
10 mM Pyruvate + 2·5 mM L-malate	58·5 ± 9·5	13·2 ± 3·9	24·0 ± 5·9	13·2 ± 3·9§	2·5 ± 0·2	1·0	2·03 ± 0·07
20 mM D,L-β-Hydroxybutyrate	76·3 ± 13·6	24·3 ± 4·2	22·9 ± 6·4	14·9 ± 4·4	3·4 ± 0·4	1·6 ± 0·4	2·35 ± 0·10
10 mM α-Ketoglutarate + 10 mM malonate	40·5 ± 5·2	6·8 ± 3·7	17·4 ± 4·2	5·4 ± 1·8‖	2·5 ± 0·2	1·2 ± 0·5	2·53 ± 0·26
40 μM Palmityl-L-carnitine + 0·5 mM L-malate	117·9 ± 20·9	19·7 ± 10·1	29·9 ± 6·2	13·4 ± 3·1‖	4·0 ± 0·6	1·3 ± 0·8	2·02 ± 0·05
0·4 mM Hexanoate + 0·5 mM L-malate	104·2 ± 17·7	11·6 ± 5·8	32·0 ± 6·0	11·6 ± 5·8‖	3·4 ± 0·3	1·0	2·08 ± 0·05
10 mM Succinate + 3·75 μM rotenone	187·7 ± 19·1	50·1 ± 17·9	44·9 ± 17·9	41·1 ± 14·4	4·9 ± 2·25	1·3 ± 0·6	1·67 ± 0·31

* Results are given as mean ± S.D. with the number of experiments shown in parentheses in the column heading.
† ADP/O ratios were not calculated for CLM (see text).
‡ $P < 0·001$ when compared to the control.
§ $P < 0·05$ when compared to the control.
‖ $P < 0·005$ when compared to the control.

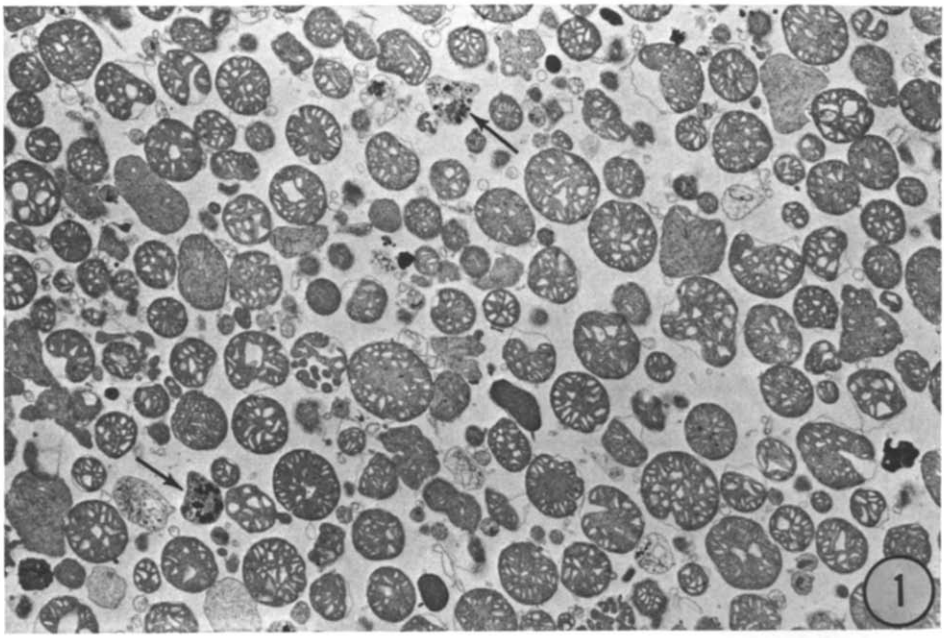

FIG. 1. Mitochondrial pellet from cuprizone-intoxicated mouse livers. Many of the mitochondria show a moderate degree of enlargement, but are otherwise normal in appearance. Some lysosomes (arrows) are present in the pellet (×4000).

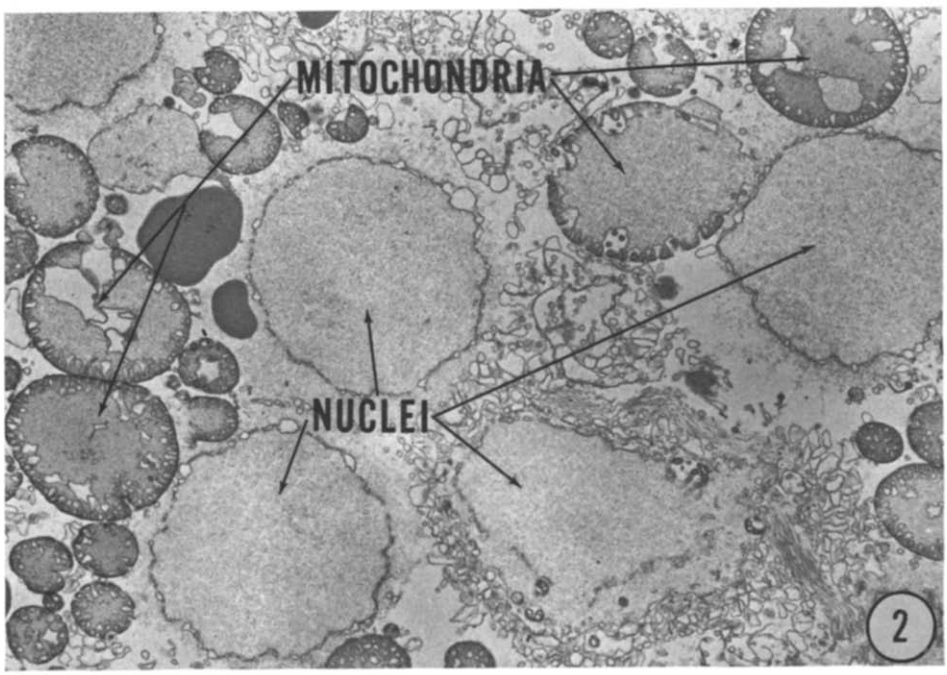

FIG. 2. Nuclear pellet from livers of cuprizone-fed mice. The many giant mitochondria present in this cell fraction show close morphologic correspondence to cuprizone-induced, *in situ* megamitochondria (×3600).

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TABLE 3. OXIDATIVE PHOSPHORYLATION IN CUPRIZONE LIVER MITOCHONDRIA

Substrate	O (n-atoms/mg/min)*	^{32}P uptake (nmoles/mg/min)	P/O
10 mM Glutamate	10·2	4·0	0·39
10 mM D,L-β-Hydroxybutyrate	14·1	5·3	0·38
10 mM α-Ketoglutarate + 10 mM malonate	2·5	2·4	0·96
40 μM Palmityl-L-carnitine + 0·5 mM L-malate	4·0	2·1	0·53
10 mM Succinate + 3·75 μM rotenone	47·1	8·6	0·18

* Oxygen consumption was allowed to proceed until 60 per cent of the oxygen in the medium was consumed; therefore, unlike those in Table 2, these values are not initial rates.

for 15–60 min at 0°, after which oxidative phosphorylation was measured. As before, no effects were observed (Table 4).

In contrast to liver, the hearts were unaffected grossly by a cuprizone-supplemented diet (Table 5). Although previous studies[7] demonstrated that the number of mitochondria was increased in some hearts, the recovery of mitochondrial protein per g wet weight of heart was not materially enhanced (Table 1). It was found that cuprizone had no effect on oxidative metabolism of cardiac mitochondria (Table 5).

TABLE 4. NORMAL RAT LIVER MITOCHONDRIA ARE UNAFFECTED BY TREATMENT WITH CUPRIZONE *in vitro**

Pretreatment†	Substrate	Oxygen consumption (n-atoms O/min/mg) State 3	State 4	RCR	ADP/O
None	10 mM Glutamate	91·4	12	7·6	2·75
Cuprizone	10 mM Glutamate	94·8	12·2	7·3	2·81
None	10 mM Succinate + 3·75 μM rotenone	119·4	29·3	4·1	1·70
Cuprizone	10 mM Succinate + 3·75 μM rotenone	135·4	27·1	5·0	1·68

* The results are expressed as the mean of three serial additions of ADP.

† Rat liver mitochondria (50 mg of protein/ml) were preincubated for 60 min with 25 mM cuprizone (0·5 μmole of cuprizone/mg of mitochondrial protein) at 4°.

DISCUSSION

The present study was undertaken to determine whether the morphological alterations in hepatic and cardiac mitochondria produced in mice by a cuprizone-supplemented diet were accompanied by alterations in oxidative metabolism. It was found that hepatic mitochondria demonstrated defective oxidative phosphorylation. State 3 respiration was severely depressed and state 4 respiration was depressed with four out of seven substrates, resulting in respiratory control ratios of less than 2. The P/O ratios were also greatly depressed. The depressed state 3 respiration was not altered by the addition of DNP to produce uncoupling. In CLM, the inhibition of phosphorylation

TABLE 5. EFFECTS OF DIETARY CUPRIZONE ON MOUSE HEART MITOCHONDRIAL OXIDATIVE PHOSPHORYLATION*

Substrate	State 3 respiration (n-atoms O/mg/min)		State 4 respiration (n-atoms O/mg/min)		RCR		ADP/O	
	Control (4)	Cuprizone (5)	Control (4)	Cuprizone (5)	Control (4)	Cuprizone (5)	Control (4)	Cuprizone (5)
10 mM Glutamate	159·9 ± 23·9	132·6 ± 19·0	31·4 ± 10·7	32·3 ± 6·5	5·4 ± 1·0	4·3 ± 1·2	2·70 ± 0·39	2·71 ± 0·34
10 mM Pyruvate + 2·5 mM L-malate	265·0 ± 26·7	253·8 ± 23·6	33·4 ± 22·6	34·1 ± 10·9	10·6 ± 5·6	8·1 ± 3·1	2·84 ± 0·18	2·86 ± 0·17
10 mM α-ketoglutarate + 10 mM malonate	183·4 ± 50·5	178·8 ± 34·2	31·0 ± 4·1	23·4 ± 8·7	5·8 ± 1·8	8·4 ± 3·2	3·11 ± 0·47	3·67 ± 0·37
40 μM Palmityl-L-carnitine + 0·5 mM L-malate	315·4 ± 38·8	281·5 ± 44·9	42·4 ± 16·3	48·1 ± 5·4	8·2 ± 3·9	5·8 ± 0·5	2·37 ± 0·18	2·83 ± 0·26
40 μM Palmityl-CoA + 2 mM L-carnitine + 0·5 mM L-malate	257·3 ± 29·5	249·7 ± 21·9	33·7 ± 9·1	38·0 ± 7·4	8·5 ± 3·3	6·8 ± 1·8	2·57 ± 0·26	2·95 ± 0·16
10 mM Succinate + 3·75 μM rotenone	384·8 ± 35·4	418·8 ± 43·8	95·2 ± 43·5	84·8 ± 19·5	5·4 ± 3·0	5·1 ± 0·8	1·66 ± 0·16	1·90 ± 0·30

* Results are given as mean ± S.D. with the number of experiments in parentheses in the column heading.

was greater than the corresponding decrease in rate of oxidation; in this respect the alterations produced by cuprizone resemble those produced by inhibitor uncouplers.[17]

In an abstract, Moore *et al.*[18] reported that CLM are uncoupled at sites 2 and 3. In contrast, our results indicate that the defects in oxidative phosphorylation in CLM are not site-specific. Since the experimental design was not included in the report of Moore *et al.*,[18] we are unable to reconcile these disparate findings. Another point of difference concerns the effects of cuprizone *in vitro* on both CLM and normal liver mitochondria. Moore *et al.*[18] reported that when cuprizone is added to CLM, which according to their results are already uncoupled at sites 2 and 3, there is a supervening uncoupling at site 1. Our results show that even without added cuprizone all three sites are already inhibited. They also claim that pretreatment of normal mouse or rat liver mitochondria with 0·5 μM cuprizone/mg of protein for 15 min at 0° resulted in uncoupling at sites 2 and 3. Using identical concentrations of cuprizone with normal mouse or rat liver mitochondria, we were unable to detect any alterations in oxidative phosphorylation, even when pretreatment was prolonged for periods up to 1 hr.

In contrast to hepatic mitochondria, no defects in oxidative phosphorylation were observed in mitochondria isolated from heart muscle. It may be concluded that there is not only a morphological difference between liver and heart cells in their response to cuprizone, but also a biochemical difference. Furthermore, species differences in reaction to cuprizone have been reported.[3] For example, while giant mitochondria are present in hepatocytes of mice fed cuprizone, they are totally lacking in liver cells of rats fed the same diet.*

The mechanism of action of cuprizone is unknown. An obvious possibility is that its effects depend on its chelating properties. Since copper plays an important role *inter alia* in cytochrome oxidase activity,[19] the removal of this cation should interfere with oxidative metabolism. Our evidence, however, shows that the P/O ratio is severely depressed, pointing to a more generalized effect on oxidative phosphorylation than a specific inhibition of cytochrome oxidase. Furthermore, the clinical effects of cuprizone supplementation are not reversed by addition of copper to the diet.†[,2,20] Of course, it is possible that cuprizone is actually chelating other heavy metals, such as Fe^{2+} and Mn^{2+}, but the affinity of cuprizone for these metals has not been determined. Peterson and Bollier[1] have shown that at low concentrations many heavy metals do not affect the determination of serum copper by the cuprizone method. The fact that simple deficiency of metals such as copper,[21] iron[22] or manganese[23] does not produce morphologic or biochemical changes similar to those produced by cuprizone militates against any mechanism that solely involves chelation. It may be speculated that a metabolite of cuprizone is in fact responsible for the effects which we have described. However, it has not been established whether cuprizone undergoes metabolic transformation in the body. The fact that cuprizone does not affect mitochondrial metabolism *in vitro* suggests that mitochondrial perturbations in the intact animal are secondary to changes produced either in other subcellular components of the hepatocyte or in cells of other organs.

* Unpublished observations.

† We have recently found that neither biochemical defects nor morphological alterations of liver mitochondria are prevented by addition of copper sulfate to the cuprizone-supplemented diet.

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