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Ultrastructural and Biochemical Effects of D-Penicillamine on Mouse Hepatocytes

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ABSTRACT The ultrastructural and oxidative metabolic effects of D-penicillamine were examined in hepatocytes of mice fed various concentrations of the drug for periods up to 11 days. In virtually all animals studied, membranous material in the form of whorls, sheaves, and irregular networks was observed in some bile canaliculi. In several mice a few mitochondria were substantially enlarged, with diameters of 4–6 μm. In other animals some mitochondria became quite elongated, measuring 10 μm in length but only 0.5 μm in width. Certain mitochondria had small deposits of dense material in their outer compartment. Lysosomes contained numerous small dense particles; aggregates of these particles were also observed free in the cytosol. Despite these alterations, the vast majority of hepatocytes showed no changes whatsoever. Study of isolated hepatic mitochondria derived from the experimental animals showed no alterations in oxidative metabolism. It may be concluded that, unlike other copper-chelating agents, D-penicillamine has little significant morphological and biochemical effect on mouse hepatocytes, even when its concentration exceeds standard clinical dosages.

Wilson's disease, an inborn error of copper homeostasis, is characterized by the accumulation of excessive amounts of copper in certain organs (Scheinberg and Sternlieb, '65). The copper chelator D-penicillamine is used therapeutically to reduce tissue concentrations of this metal (Sternlieb and Scheinberg, '64). While the ultrastructural effects of prolonged D-penicillamine therapy have been examined in hepatocytes of patients with Wilson's disease (Sternlieb and Feldmann, '76), little is known of the morphological and oxidative metabolic effects of this agent on liver cells of experimental animals. Other copper-chelating drugs, including cuprizone (Suzuki, '69; Hoppel and Tandler, '73; Wakabayashi et al., '75) and diethyldithiocarbamate (DDC) (Asano and Wakabayashi, '74), produce, in a matter of a few days, profound structural and metabolic perturbations in mouse hepatic cells. It was therefore of interest to compare the acute effects of D-penicillamine on mouse liver to those produced by other copper-chelators.

MATERIALS AND METHODS

Male 21-day-old weanling mice (CF1 strain, Charles River) were randomly separated into control and experimental groups. Each experimental group consisted of a minimum of 25 mice. Controls were fed powdered Purina Lab Chow ad libitum; the experimental groups were fed the same diet supplemented with D-penicillamine. Water was freely available.

Electron microscopy

The concentration of D-penicillamine was different for each of the seven experimental groups. Concentrations (on a w/w basis) used included 0.1%, 0.15%, 0.2%, 0.3%, 0.4%, 0.5%, and 0.55%. For each level of drug dosage, two experimental animals were sacrificed at daily intervals starting at day 1 and proceeding to day 11. The experiment was repeated three times for the higher (0.5% and 0.55%) dosages of the drug.

Specimens of liver obtained from decapitated mice were fixed for 2 hours in 2% osmium tetroxide buffered with phosphate (Millonig, '61a). After a brief rinse in distilled water,

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tissue blocks were soaked overnight in cold 0.25% uranyl acetate. Brief rinsing in distilled water was followed by dehydration in ascending concentrations of ethanol, passage through propylene oxide, and embedment either in Maraglas-D.E.R. 732 (Erlandson, '64) or in Epon-Maraglas (Tandler and Walter, '77). Thin sections of specimens from every animal used in this study were stained with methanolic uranyl acetate (Stempak and Ward, '64) followed by lead tartrate (Millonig, 61b) and examined in a Siemens Elmiskop 1A electron microscope.

Biochemistry

The experimental group was fed a 0.5% D-penicillamine-supplemented diet for 6–8 days. Water was freely available. On days 6, 7, and 8 of the diet, five control and 15 experimental animals were sacrificed. Livers from the 15 experimental mice were randomly separated into three groups, each group containing five livers.

Isolation of Mitochondria. 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 200 mM mannitol, 70 mM sucrose, 5 mM MOPS (morpholinopropane sulfonic acid), 2 mM EDTA, pH 7.4. The mitochondrial fractions were isolated by differential centrifugation (Hoppel and Tandler, '75) and were washed twice in the same medium as that used for homogenization, but without EDTA.

Oxidation Experiments. A Clark oxygen electrode was used to monitor oxygen uptake in 1 ml of incubation medium containing 80 mM KCl, 50 mM MOPS, 5 mM P_i, 1 mM EDTA [ethyleneglycol bis (β-amino ethyl ester)-N,N^1-tetra-acetic acid], 1 mg of defatted bovine serum albumin, and 2 mg mitochondrial protein/ml. The final pH was 7.0 and the temperature was 30°C. Endogenous substrates were depleted by addition of 150 nmoles of ADP, and the desired substrate was then added (concentrations given in Table 2). Respiration was initiated by further additions of 150 nmoles of ADP. States 3 (ADP-stimulated) and 4 (ADP-limited) rates of oxygen consumption were measured according to Chance and Williams ('55). Respiratory control ratios (the ratio of state 3 to state 4 respiration) and ADP/O ratios were determined by the method of Estabrook ('67).

Chemicals. D-penicillamine was obtained from Sigma. ADP (P-L Biochemicals, Inc., Milwaukee, Wisc.) was standardized by the method of Chappel ('64). Defatted bovine serum albumin (Pentex, Kankakee, Ill.) was prepared according to the method of Chen ('67) and dialyzed as outlined by Hanson and Ballard ('68). Other chemicals were of reagent quality and obtained commercially or prepared as described previously (Hoppel and Tandler, '75).

RESULTS

Gross observations

Irrespective of drug concentration, mice fed a diet containing D-penicillamine gained weight at normal rates (Table 1). The size of the liver was unaffected by the experimental diet (Table 1).

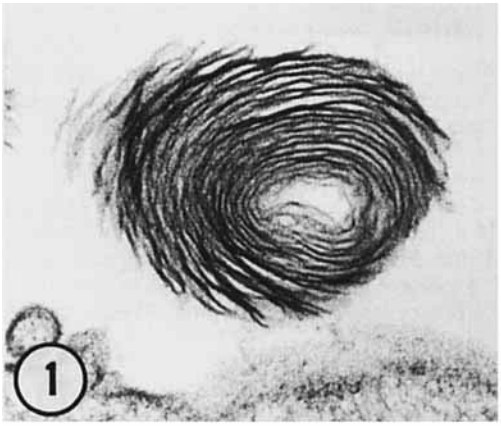
Microscopic observations

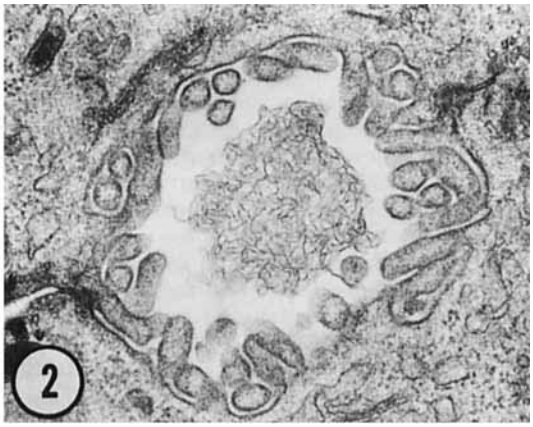
Regardless of dosage levels, relatively few cells in any liver showed obvious morphological alteration. Some of these effects appeared only at low dosages, while others became apparent only at higher levels of the drug. Certain effects that were observed only after 8–10 days in the mice receiving low dosages showed up at earlier time points with higher doses. Despite this observation, no general trends in morphology could be discerned that could be directly related to drug concentration.

The only change that was consistently present in the livers of mice receiving D-penicillamine at all concentrations and at virtually all time points involved the contents of the bile canaliculi. Normally empty of formed elements, the canaliculi of treated animals frequently contained membranous masses in the form of myelin figures or tangled skeins (Fig. 1–3). These membranes, which had a trilaminar structure, measured about 6 nm in thickness.

Hepatic mitochondria displayed morphological alterations, but these varied according to concentration and duration of the drug. Mice fed a diet containing 0.1% D-penicillamine had occasional hepatocytes with somewhat enlarged or elongated mitochondria. Adjacent enlarged mitochondria were sometimes linked by a myelin figure.

The increase in mitochondrial enlargement reached its maximum in mice receiving 0.15% D-penicillamine. In these animals occasional cells possessed quite large, more or less spherical mitochondria that measured on the average about 4 μm, with some organelles attaining diameters greater than 6 μm (Fig. 4). In most of the large mitochondria the cristae were restricted to the periphery, with the





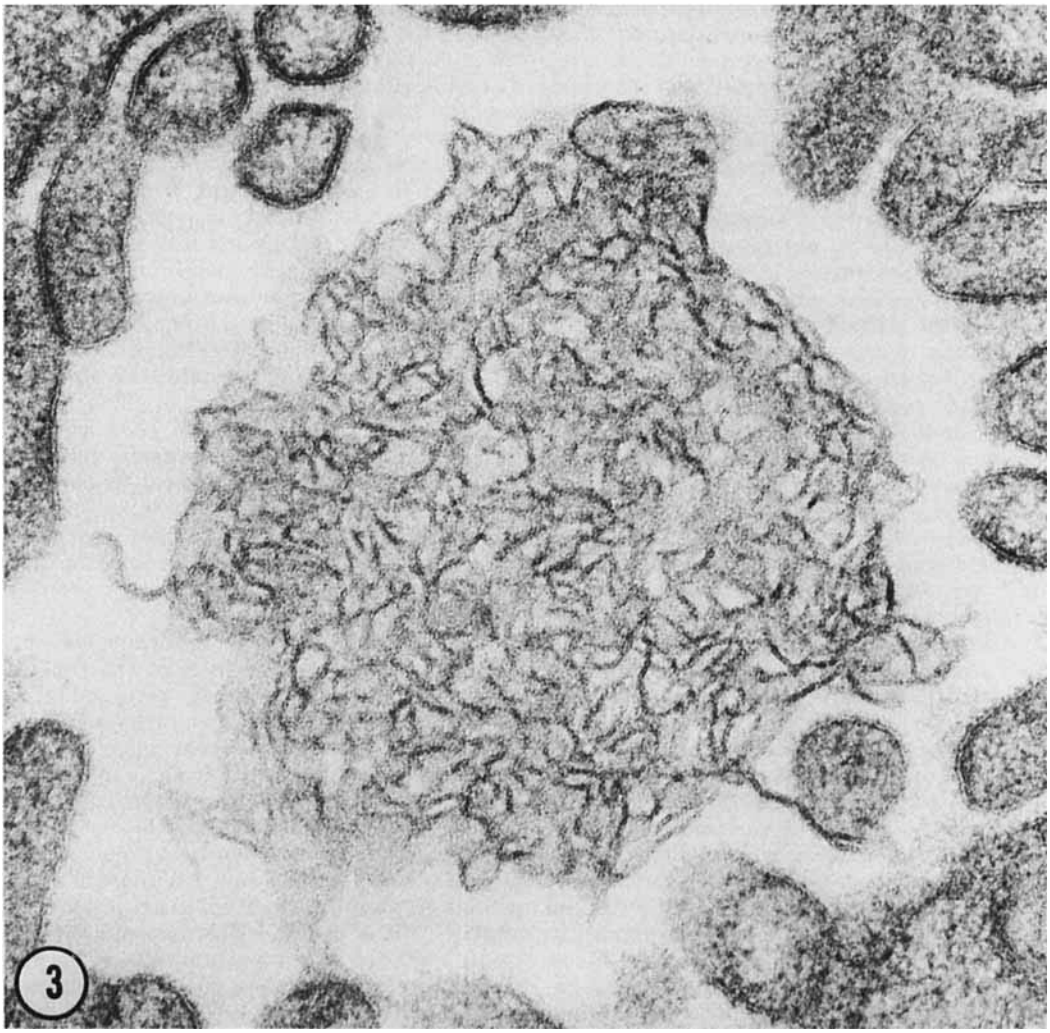


Fig. 1. A laminated myelin figure in a bile canaliculus of a mouse fed 0.55% D-penicillamine for 9 days. × 46,000.

Fig. 2. A bile canaliculus in a mouse fed 0.55% D-penicillamine for 9 days. A tangled mass of membrane-like structures is present. × 34,000.

Fig. 3. A portion of the preceding micrograph at a higher magnification. The tangled threadlike structures within the canalicular lumen have a trilaminar substructure. × 97,000.

center of the matrix compartment free of membranes, but some enlarged organelles had cristae that were uniformly distributed throughout the inner compartment. Some large mitochondria were in the process of division, signaled by the presence of a bisecting membranous partition accompanied by constriction of the organelle at the level of the partition. Other mitochondrial changes were noted in cells that lacked enlarged mitochondria. These included mitochondrial elongation and the presence in a few normal-sized organelles of one or more deposits of extremely dense material in the outer compartment (Fig. 5). Such densities were also present in the group of mice fed 0.2% D-penicillamine.

Elongated mitochondria were prominent in the 0.4% and 0.55% D-penicillamine mice, especially in the latter group. These were long, rodlike structures, sometimes branched, that measured up to 10 μm in length (Fig. 6). Enlarged spherical mitochondria were not observed at these higher concentrations of the drug.

At all dosage levels of D-penicillamine there was an increase in autophagy compared to controls. Degenerating mitochondria within autophagic vacuoles were frequently observed. Many of the vacuoles contained a variety of cytoplasmic debris displaying a spectrum of electron-lucency. At high magnification most autophagic vacuoles were seen to contain numerous small dense particles measuring about 5–6 nm; clusters of similar particles often were present in the cytosol (Fig. 7). Pericanalicular lysosomes were abundant; several of these enclosed a lucent rectangle, which probably represented the site of a crystalloid that had been extracted during tissue processing for microscopy.

Endoplasmic reticulum, both rough-surfaced and smooth, was not materially affected by D-penicillamine treatment. In a few cells, however, the RER showed some fragmentation. At low concentrations of the drug there appeared to be an increase in the number of centrioles per hepatocyte and it was not uncommon to find four or five of these organelles grouped in the same cell in a single section. There was no apparent change in the number of peroxisomes. Nuclear inclusions with and without bounding membranes were present in appreciable numbers in mice fed 0.15% or 0.2% D-penicillamine. Such inclusions consisted of single or multiple droplets of lipid suspended in matrix substance of moderate density.

Mitochondrial oxidative metabolism

There were no significant differences in mitochondrial yield between normal and experimental animals (Table 1). Mitochondrial pellets from both sources were identical and showed typical organelle morphology. For the five substrates tested, there were no significant differences between hepatic mitochondria isolated from normal and experimental mice with respect to states 3 and 4 respiration, respiratory control ratios, and ADP/O ratios (Table 2).

DISCUSSION

In general, the morphological effects of dietary D-penicillamine on mouse liver cells were, with one exception, inconsistent, with no pronounced trends evident. The only consistent hepatic feature resulting from the drug was the presence of membranous aggregates in many of the bile canaliculi, regardless of dosage. Similar canalicular material has been observed in mice recovering from cuprizone intoxication (B. Tandler, unpublished observations). These formations are considerably more complex than the membranous whorls that are occasionally present in bile canaliculi as a result of experimental biliary obstruction (Cooper et al., '74) or of treatment with such drugs as allylisopropylacetamide (Biempica et al., '67), pyrazole (Lieber et al., '70), or 2-deoxy-D-galactose (Kepler and Hübner, '73). The composition of this membranous material is unknown, but its morphological appearance and dimensions indicate that it consists of phospholipid or lipoprotein. Since D-penicillamine has no obvious effect on hepatic lipid metabolism, the source of the canalicular membranes is unclear.

Although some sporadic mitochondrial enlargement was observed in mice fed D-penicillamine, megamitochondria were produced in only a few animals. The rarity of giant hepatic mitochondria in these mice sharply contrasts with the condition of liver cells in mice fed the copper-chelating agents cuprizone and DDC. The addition of either of these agents to the diet engenders enormous mitochondria with volumes several orders of magnitude greater than normal sized organelles (Suzuki, '69; Asano and Wakabayashi, '74). In many instances these megamitochondria are substantially larger than the hepatocyte nuclei (Tandler and Hoppel, '73). Such megamitochondria are characterized by short, peripherally disposed cristae and by an augmented

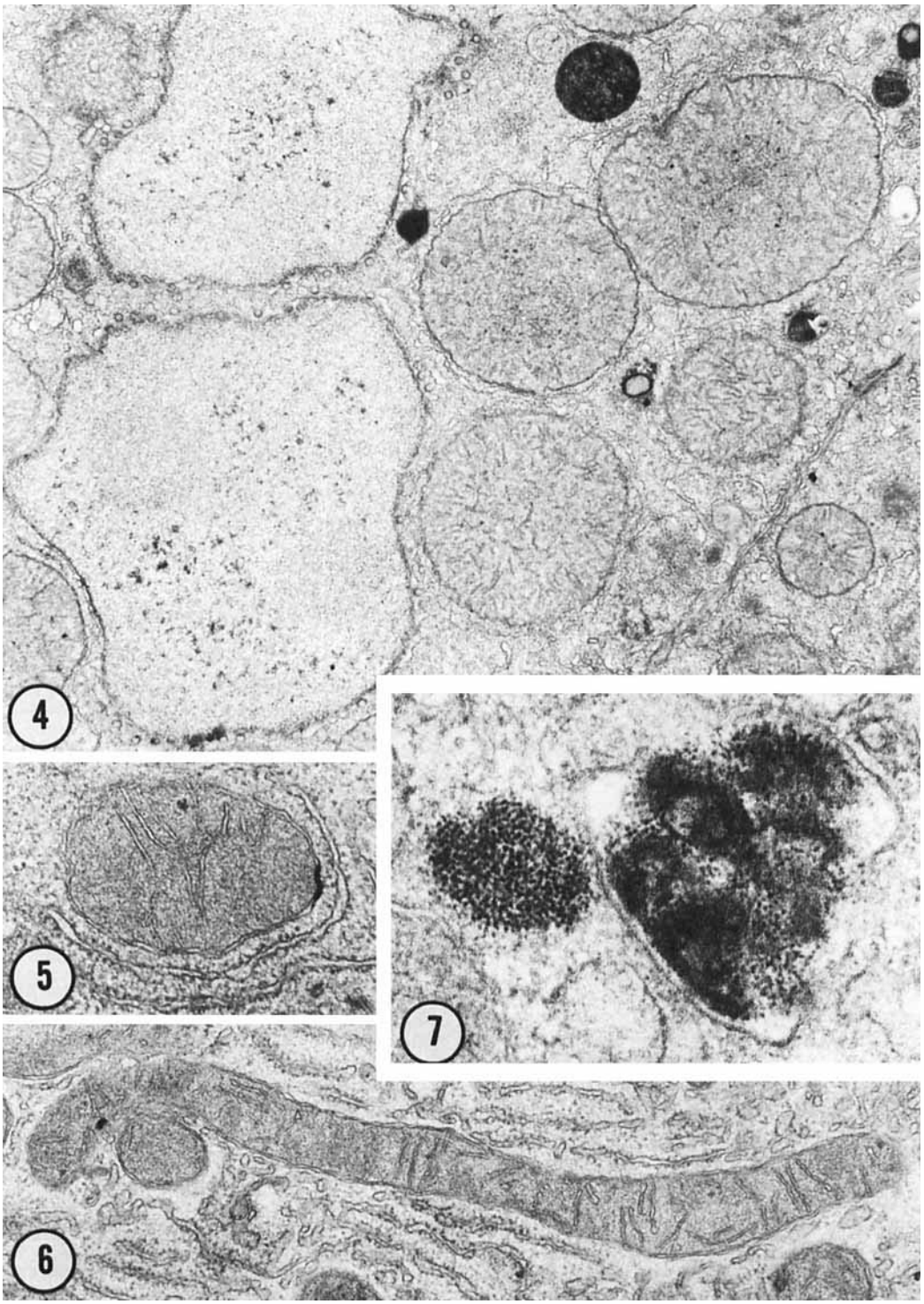


Fig. 4. Hepatocyte with enlarged mitochondria. 0.15% D-penicillamine, day 3. × 10,000.

Fig. 5. A mitochondrion with a dense deposit in its outer compartment. 0.2% D-penicillamine, day 1. × 35,000.

Fig. 6. An elongated mitochondrion. 0.55% D-penicillamine, day 7. × 21,500.

Fig. 7. An autophagic vacuole containing dense material and numerous dense particles. Below it is a cluster of similar particles free in the cytosol. 0.2% D-pencillamine, day 11. × 70,000.

TABLE 1. Macroscopic effects of D-penicillamine

	Control[a]	Experimental[b]
Body weight (g)	17.7 ± 3.7	16.1 ± 2.5
Liver (g wet weight/100g BW)	5.49 ± 0.45	5.01 ± 0.34
Mitochondria (mg protein/g wet weight liver)	15.7 ± 3.9	18.2 ± 2.9

[a] Weanling mice were fed a normal diet for 6–8 days. Data obtained from three experiments involving five animals per experiment. Data are expressed as mean ± S.D.

[b] Weanling mice were fed control diet supplemented with 0.5% D-penicillamine for 6–8 days. Data obtained from nine experimental groups involving five animals per group. Data are expressed as mean ± S.D.

TABLE 2. Oxidative phosphorylation in hepatic mitochondria during D-penicillamine supplementation[a]

Substrate	Animals	Oxygen consumption ngatoms $O_2 \cdot min^{-1} \cdot mg^{-1}$ State 3	Oxygen consumption ngatoms $O_2 \cdot min^{-1} \cdot mg^{-1}$ State 4	Respiratory control ratio	ADP/O
10 mM Glutamate	Control	75.7 ± 19.5	11.0 ± 2.9	7.2 ± 3.0	2.73 ± 0.23
	D-penicillamine	80.2 ± 13.1	10.2 ± 4.2	9.1 ± 3.7	2.78 ± 0.38
40 μM Palmitoylcarnitine	Control	102.9 ± 8.7	14.3 ± 5.3	8.1 ± 3.5	2.39 ± 0.22
+ 5 mM L-malate	D-penicillamine	104.2 ± 8.6	17.8 ± 5.0	6.2 ± 1.4	2.38 ± 0.33
10 mM Pyruvate +	Control	45.2 ± 2.9	15.1 ± 5.1	3.2 ± 1.2	2.58 ± 0.34
5 mM L-malate	D-penicillamine	39.0 ± 4.8	13.0 ± 5.3	3.4 ± 1.1	2.87 ± 0.41
10 mM α-Ketoglutarate	Control	39.0 ± 2.1	11.0 ± 1.1	3.6 ± 0.6	3.60 ± 0.07
+ 10 mM malonate	D-penicillamine	36.4 ± 4.1	11.5 ± 3.1	3.3 ± 0.8	3.75 ± 0.41
10 mM Succinate	Control	179.7 ± 29.6	29.5 ± 14.4	7.0 ± 3.0	1.85 ± 0.37
+ 3.75 μM rotenone	D-penicillamine	179.6 ± 26.5	28.5 ± 10.4	6.9 ± 2.1	1.67 ± 0.24

[a] Hepatic mitochondria were isolated as described in the text according to the groupings described in footnotes a and b of Table 1. Data are expressed as mean ± S.D.

structureless matrix compartment. The relatively rare enlarged mitochondria produced as a result of feeding mice D-penicillamine are quite similar in structure to those resulting from cuprizone ingestion.

A number of the enlarged mitochondria observed in mice fed D-penicillamine were bisected by a partition. It was previously demonstrated that megamitochondria resulting from riboflavin deficiency in mice show this configuration during the recovery period when they are returning to normal size (Tandler et al., '69). Partitioned mitochondria were interpreted as being in active division, an interpretation that has subsequently been confirmed in a variety of cell types. However, Wakabayashi et al. ('75) consider partitioned mitochondria to represent a stage in organelle fusion. Arguments against this point of view are presented elsewhere (Tandler and Hoppel, '74).

In addition to occasional enlargement, a second effect was noted in this study in some mitochondria, especially at lower doses of D-penicillamine. Some of these organelles contained small, amorphous, extremely dense deposits in their outer compartment. Similar outer compartment densities have been observed in hepatic mitochondria of rats receiving toxic doses of ferrous sulfate (Ganote and Nahara, '73) and in mitochondria of duodenal absorptive cells of rats after gastric infusion of ferrous chloride (Oki et al., '65). Since D-penicillamine can affect iron levels in tissues (Hourani and Demopoulos, '69), the mitochondrial densities seen in the present study may represent fluxional iron. Given the sporadic occurrence of these densities and the fact that we have been unable to detect them in mitochondrial pellets, the unequivocal delineation of their nature is not feasible at the present time.

In a morphometric study, Riede et al. ('71) investigated the effects of D-penicillamine on rat liver cells. Mitochondrial volume was only slightly increased, but there was a substantial increase in surface area of cristae. In addition, the volume density of microbodies per hepatocyte was nearly double that of controls. While histometric analysis was not carried out in the present study, it is our subjective impression that in the mouse mitochondrial cristae appeared to be of normal length and number in the vast majority of organelles, and that increased numbers of microbodies occurred in only a few animals.

To examine mitochondrial oxidative metabolism in the D-penicillamine-mice, we selected

five substrates yielding reducing equivalents that enter the electron transport chain via several different mechanisms, i.e., at different sites and by different dehydrogenases. These substrates included a glycolytic intermediate (pyruvate), Krebs cycle intermediates (α-ketoglutarate and succinate), an amino acid (glutamate), and a fatty acid (palmitoylcarnitine). Our data demonstrate that state 3 oxidative metabolism was unaffected by dietary D-penicillamine treatment. As judged by respiratory control ratios, the biochemical integrity of the isolated mitochondria was unaffected; as shown by the ADP/O ratios, phosphorylation was similarly unchanged. These findings contrast with the impaired mitochondrial oxidative metabolism produced by cuprizone (Hoppel and Tandler, '73). Mitochondria isolated from livers of weanling mice fed this agent for 8–12 days show a decrease in state 3 respiration, respiratory control ratios, and ADP/O and P/O ratios for seven substrates, including the five substrates used in the present study.

In conclusion, we have demonstrated that in mice D-penicillamine has relatively few acute effects on hepatic morphology and no effect on oxidative phosphorylation, even when the dosages on a body weight basis are double those used therapeutically in patients.

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