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Ultrastructural and Biochemical Effects of Prolonged Oral Arsenic Exposure on Liver Mitochondria of Rats

by Bruce A. Fowler,* James S. Woods,* and Carol M. Schiller*

This investigation was undertaken to further delineate the subcellular manifestations of arsenic toxicity following chronic exposure using combined ultrastructural and biochemical techniques. Male rats were given access to deionized drinking water solutions containing 0, 20, 40, or 85 arsenic as arsenate (As^{+5}) for 6 weeks. *In situ* swelling of liver mitochondria was the most prominent ultrastructural change observed. Mitochondrial respiration studies indicated decreased state 3 respiration and respiratory control ratios (RCR) for pyruvate/malate but not succinate mediated respiration. Specific activity of monoamine oxidase which is localized on the outer mitochondrial membrane showed increases of up to 150% of control and cytochrome-C oxidase which is localized on the inner mitochondrial membrane showed increases in specific activity of 150–200%. Activity of malate dehydrogenase which is localized in the mitochondrial matrix was unchanged at any dose level. These studies indicate that decreased mitochondrial respiration is only one aspect of arsenic toxicity to this organelle. Marked arsenic-mediated perturbation of important enzyme systems localized in mitochondria which participate in the control of respiration and other normal mitochondrial functions are also important manifestations of cellular dysfunction.

Introduction

The liver is known to concentrate arsenic following exposure (*1, 2*) and to play an important role in the *in vivo* metabolism of this element. Humans exposed to dietary arsenic for prolonged periods (*3, 4*) have been observed to develop pronounced liver damage, thus identifying this organ as a target for arsenic toxicity. The exact cellular mechanisms by which arsenic produces hepatotoxicity *in vivo* are unknown but probably reflect differential damage to a number of cellular organelle systems and their attendant biochemical functions. *In vitro* studies (*5–9*) have previously demonstrated that mitochondria are highly sensitive to damage from arsenicals. These organelles which have a number of cellular functions (Fig. 1) are actively known to accumulate arsenic (*10*) and to possess an arsenylated binding component (*11*) which is thought to be associated with the electron transport chain.

The present study was undertaken to evaluate the effects of ingested arsenate (As^{+5}) on liver mitochondria of rats using a combination of ultrastructural and biochemical techniques. *In situ* morphological findings were related to changes in respiratory function and the activities of marker enzymes localized in different mitochondrial compartments. Other studies dealing with the inhibitory effects of arsenate exposure on pyruvate dehydrogenase activity (*12*) and heme biosynthetic pathway (*13*) are presented in other papers.

Materials and Methods

In this experiment, 72 male Charles River CD rats were divided into four groups of 18 each and fed a casein-based purified diet (*14*). These groups were given access to deionized drinking water containing 0, 20, 40, or 85 ppm arsenic as sodium arsenate (As^{+5}) respectively for 6 weeks. At the end of this period, the animals were killed by decapitation

*Environmental Toxicology Branch, National Institute of Environmental Health Sciences, P. O. Box 12233, Research Triangle Park, North Carolina 27709.

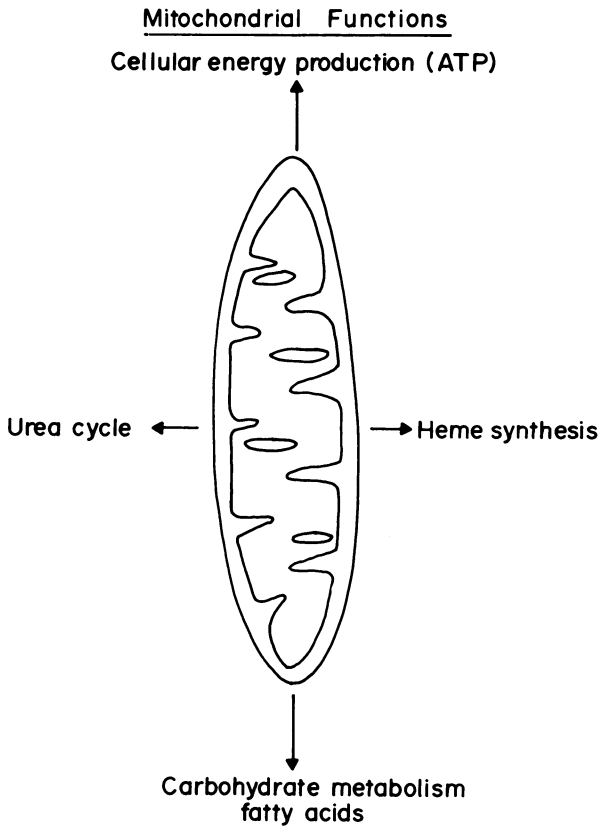

FIGURE 1. Diagrammatic representation of a mitochondrion showing some of its various cellular functions.

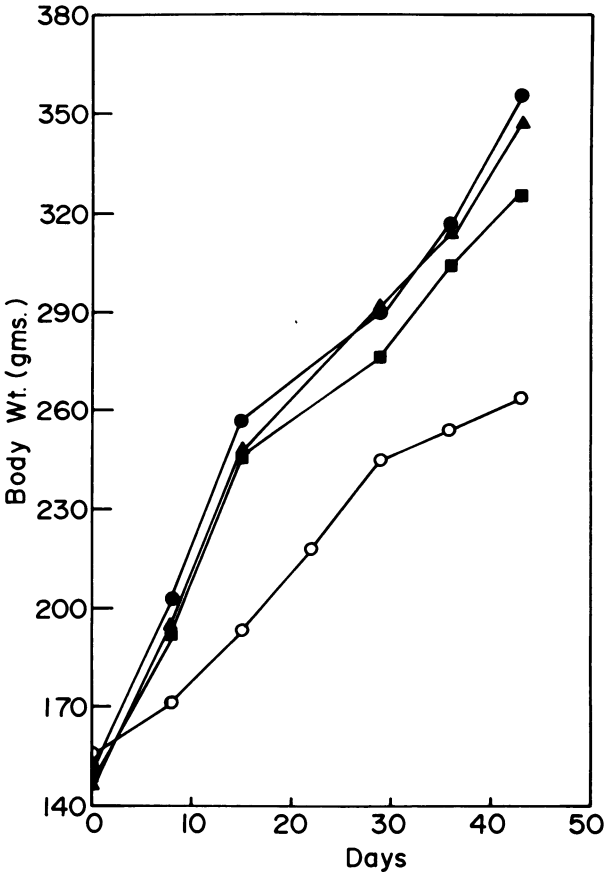

FIGURE 2. Growth curves for rats given a purified diet and arsenate (As^{+5}) in (•) control; (▲) 20 mg As/1.; (■) 40 mg As/l.; (o) 85 mg As/l. drinking water: Significant ($p < 0.01$) depression of growth occurred at only the 85 ppm dose level.

in groups of three each and the livers removed, washed, and pooled. Washed liver mitochondria were isolated from 4.5 g of pooled tissue at each dose level in a series of six replicates. The exact mitochondrial isolation procedure has been extensively described elsewhere (*15*). Mitochondrial respiration studies were conducted using a Clark-type oxygen electrode and the reaction mixture described by Nelson (*16*) with a mitochondrial protein concentration of 1 mg/ml in a final volume of 1 ml. State 4 respiration was measured using both succinate and pyruvate/malate substrates at 30°C. State 3 respiration was initiated by addition of 1.2μM ADP. Respiratory control ratios (RCR) were assessed by comparing the state 3 rate with the state 4 rate following complete utilization of the injected ADP. The mitochondrial marker enzymes, monoamine oxidase (MAO), cytochrome oxidase (CO), and malate dehydrogenase (MDH), which are localized in the outer mitochondrial membrane, inner mitochondrial membrane, and matrix, respectively, were assayed according to the methods of Schnaitman et al. (*17*). Mitochondrial protein concentrations were measured according to the method of Lowry et al. (*18*). Statistical intergroup comparisons were performed by using the Mann-Whitney U test (*19*) and dose-response relationships evaluated by using Jonckheere's test (*20*).

Results

No mortality occurred among any of the treatment groups during the course of the experiment and significant depression of growth rate occurred among only those animals exposed to the 85 ppm As^{+5} dose level (Fig. 2).

The ultrastructural appearance of a hepatocyte from a control rat is given in Figure 3. Hepatocytes from animals given the 20 ppm As^{+5} dose level had a similar architecture and but occasional cells containing swollen mitochondria were observed. Mitochondria in hepatocytes from animals given the 40 and 85 ppm As^{+5} dose levels showed extensive swelling and matrix rarification. This phenomenon appeared to be most pronounced in those cells near the periphery of liver lobules (Fig. 4). At the 85 ppm dose level, large lipidic vacuoles

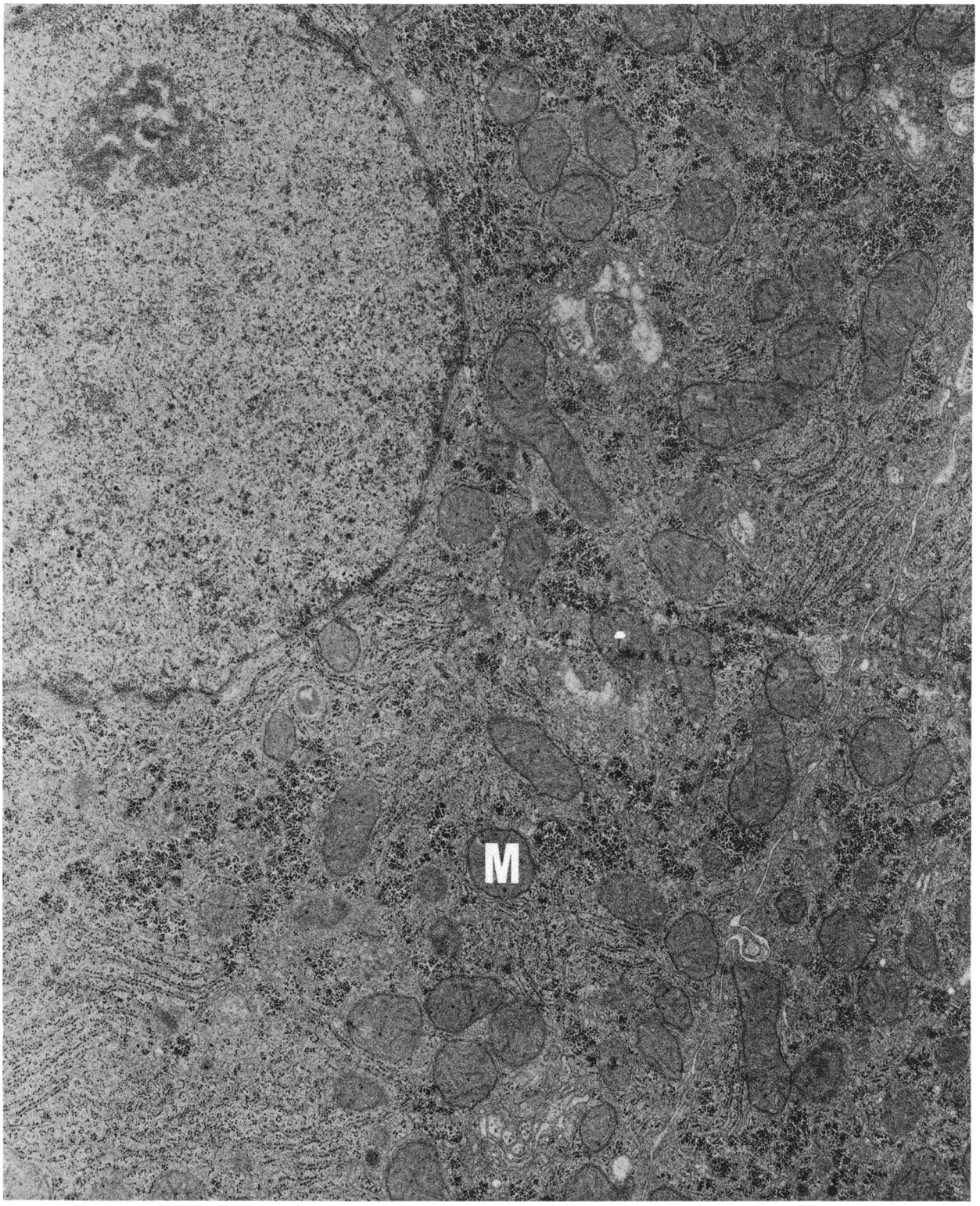

FIGURE 3. Ultrastructural appearance of a hepatocyte from a control illustrating normal cellular architecture. Numerous mitochondria (M) are present. × 16,758.

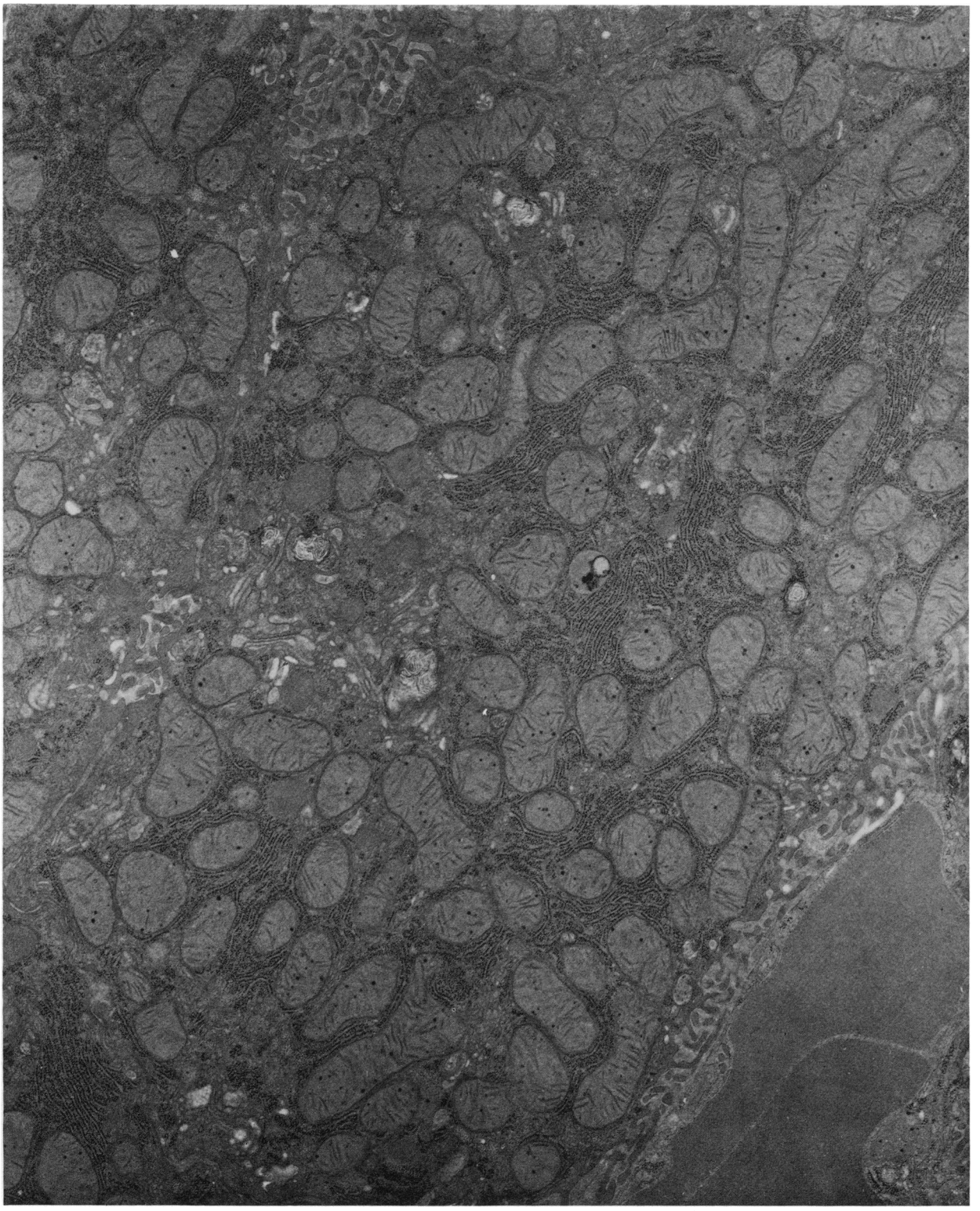

FIGURE 4. Peripheral hepatocyte from a rat exposed to the 40 ppm As^{+5} dose level for 6 weeks. Note swollen mitochondria with rarified matrices. × 16,758.

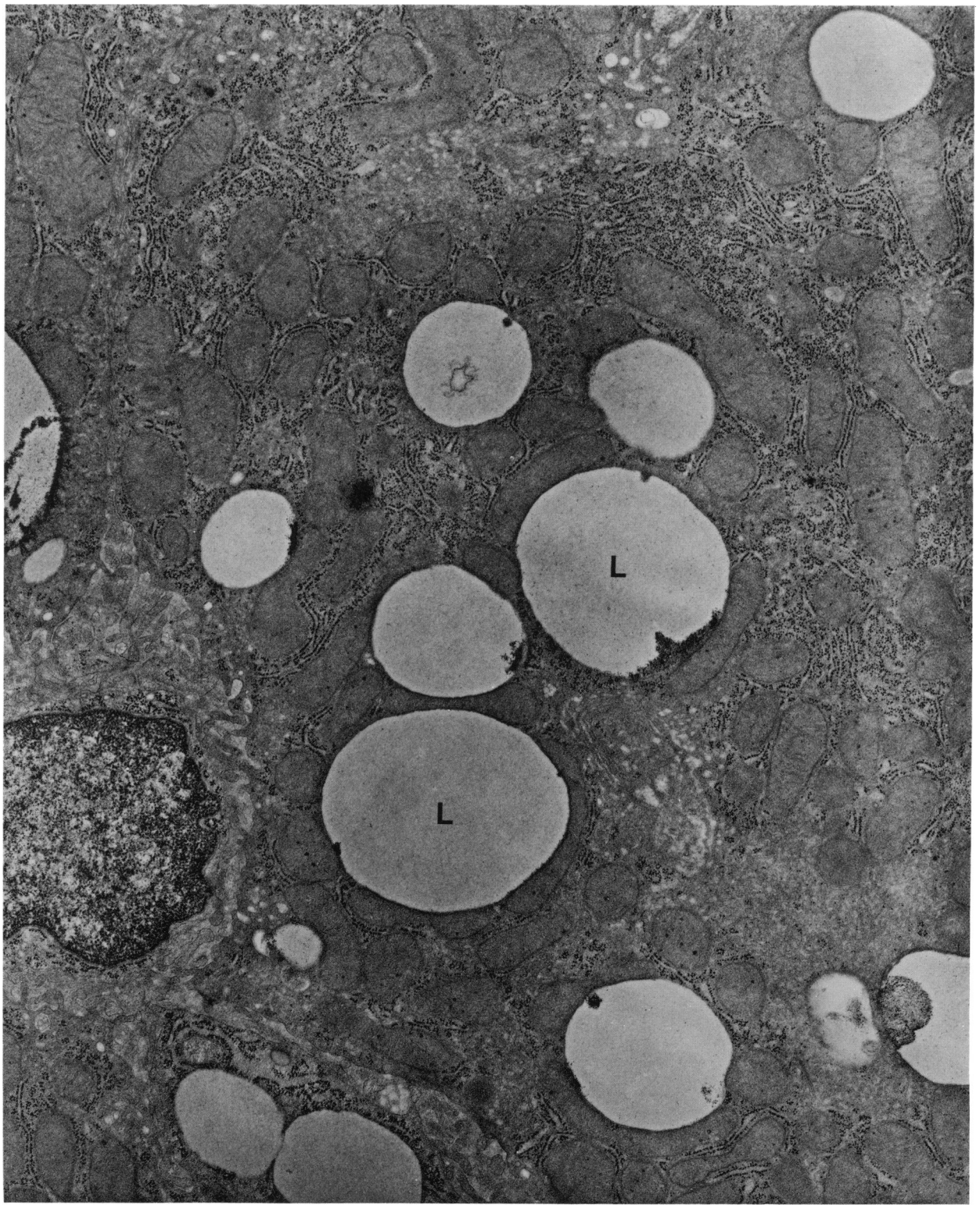

FIGURE 5. Liver parenchymal cell from a rat exposed to the 85 ppm As^{+5} dose level for 6 weeks. Large lipidic vacuoles (L) are prominent. × 16,758.

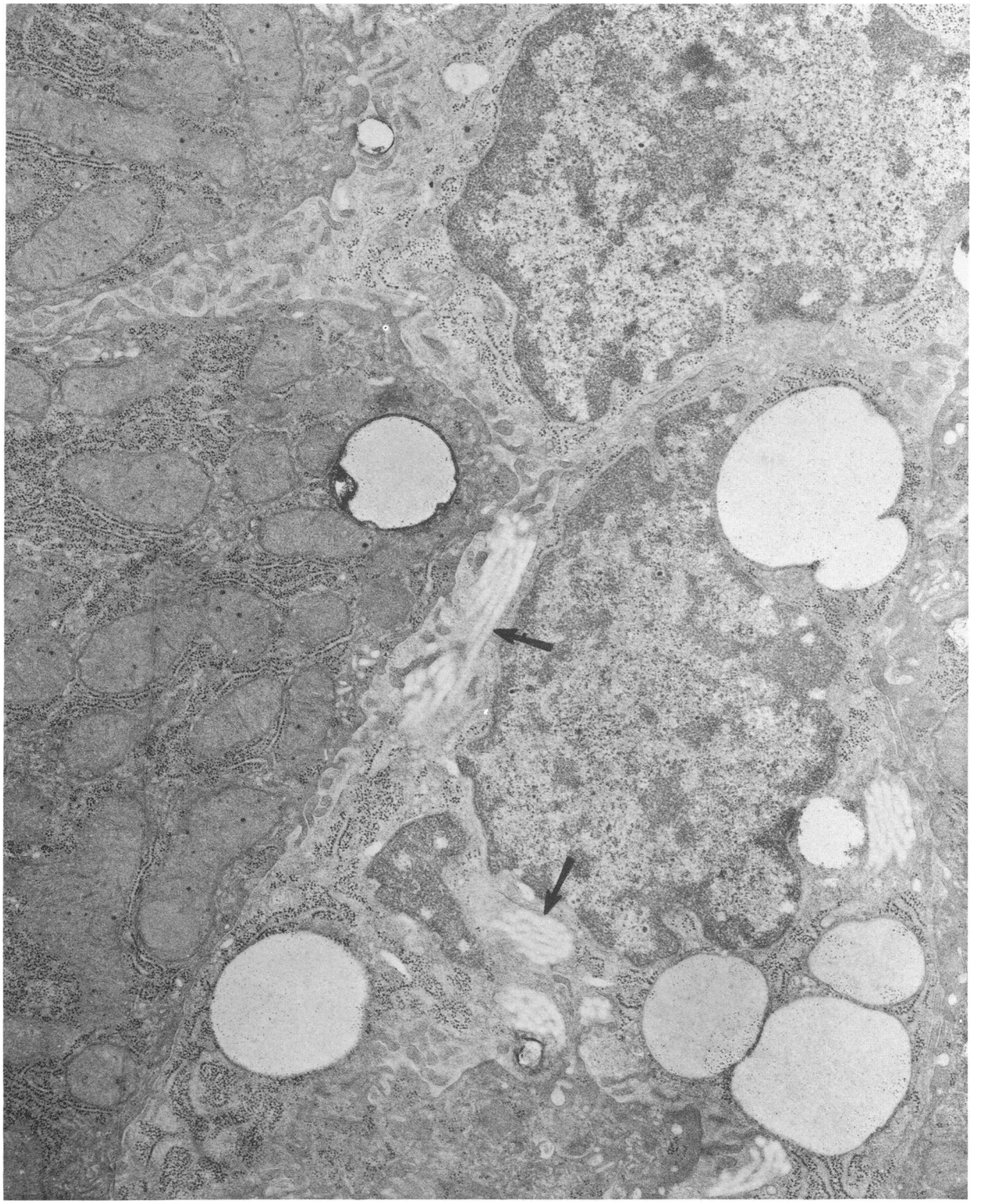

FIGURE 6. Large bundles of connective tissue fibers (arrows) were frequently observed among hepatocytes from rats given the 85 ppm As^{+5} dose level. × 16,758.

were frequently observed in these cells (Fig. 5). Large bundles of interstitial connective tissue were also present in hepatocytes from these rats (Fig. 6).

Mitochondrial respiratory function was assessed in these same animals (Fig. 7). Decreased respiratory control ratios (RCR) and depressed state 3 respiration were observed for pyruvate/malate but not succinate. These effects were most pronounced in animals given the 40 and 85 ppm As^{+5} dose levels. P/O ratios were measurably depressed for both succinate and pyruvate/malate substrates at only the 85 ppm dose level.

The effects of As^{+5} exposure on the mitochondrial marker enzymes MAO, CO, and MDH are given in Figure 8. Increases in the specific activities of MAO and CO, of up to 150–200% of control were noted. The differences in MAO activity for all As^{+5} treatment groups were significant at $P<.01$ and no dose response was observed. Differences in the specific activity of CO were significant ($p < 0.01$) at the 20 and 85 ppm dose levels and $p < 0.05$ at the 40 ppm dose level. No dose response was obtained. Activity of the matrix marker enzyme MDH was unchanged at any dose level.

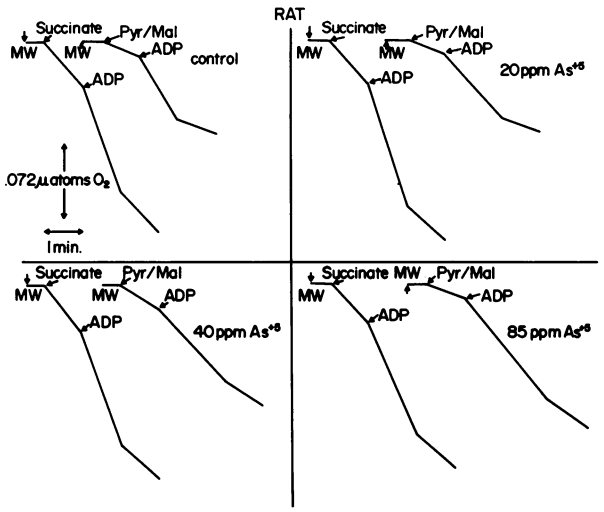

FIGURE 7. Oxygen electrode tracings for washed hepatic mitochondria (MW) from rats in the various dose groups. Note depressed state 3 respiration and RCR values when pyruvate/malate (Pyr/Mal) was used as a substrate in comparison with succinate. P/O ratios were markedly depressed for both substrate categories at only the 85 ppm dose level.

Discussion

The results of this study indicate that prolonged oral exposure to As^{+5} produced marked ultrastructural and biochemical changes in hepatocyte mitochondria. *In situ* mitochondrial swelling was most pronounced in rats exposed to the 40 and 85

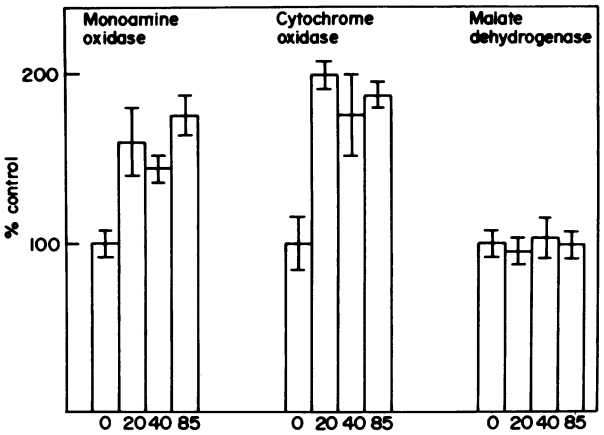

FIGURE 8. Effects of prolonged arsenate administration on mitochondrial marker enzymes expressed as mean ± SEM. Monoamine oxidase (MAO) and cytochrome oxidase (CO), which are localized in the mitochondrial membranes showed marked increases in specific activity, following arsenate exposure. The specific activity of malate dehydrogenase (MDH), which was used as a matrix marker, was unchanged.

ppm As^{+5} dose levels along with lipidic vacuolation and fibrosis. These fibrotic changes are suggestive of cell death and replacement with resultant scarring.

The above morphological changes were associated with decreased state 3 respiration and respiratory control ratios for pyruvate/malate but not succinate supported respiration. This observation is consistent with the early *in vitro* findings of Crane and Lipman (*5*), who found that the respiration of liver mitochondria incubated with varying concentrations of arsenate was increasingly inhibited with time for NAD-linked substrates but not for succinate. These authors suggested that the observed inhibition of NAD-linked respiration was due to the reduction of arsenate to arsenite with the resulting inhibition of the disulfhydryl lipoic acid cofactor. More recent studies (*12*) suggest impaired regulation of the pyruvate dehydrogenase complex as an alternative mechanism for the observed inhibitory effects of arsenate on pyruvate/malate mitochondrial respiration. Depression of the P/O ratios at the higher As^{+5} dose levels for either substrate-type probably results from stimulation of Mg^{+2} ATPase a phenomenon which has also been noted *in vitro* (*6, 8, 9*) and *in vivo* (*21*).

The results of marker enzyme assays performed on liver mitochondria from animals used in the present study, disclosed that mitochondrial swelling was associated with increases in the specific activities of MAO and CO which are localized on the outer and inner mitochondrial membranes, respectively. The enhanced activities of these enzymes suggests direct arsenic perturbation of the mem-

branes leading to increased permeability or conformational change. The absence of change in the activity of MDH indicates that these changes were not due to altered mitochondrial protein content, since all assays were performed on aliquots of the same mitochondrial pellets from each respective dose group. Other studies which relate the ultrastructural damage observed in this report to perturbation of mitochondrial heme biosynthetic pathway enzymes and subsequent porphyrinuria are described in an adjacent paper (*13*). Current research efforts are focussed on relating arsenate-induced changes in mitochondrial membrane structure to ion and substrate transport capability.

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