CHANGES IN HEPATIC STRUCTURE IN RATS PRODUCED BY BREATHING PURE OXYGEN

FENTON SCHAFFNER and PHILIP FELIG

With the Technical Assistance of Esther Trachtenberg

From the Department of Pathology of The Mount Sinai Hospital, New York, and the 6570 Aerospace Medical Research Laboratories, Wright-Patterson Air Force Base, Ohio

ABSTRACT

The livers of rats exposed to pure oxygen were examined electron microscopically to study toxic effects of oxygen in a metabolically sensitive organ. Pressures of 1/3 (258 mm Hg), 1 (760 mm Hg), and 3 (2280 mm Hg) atmospheres were used, with exposures up to 90 days with the lowest pressures. The first changes in the hepatocytes were loss of glycogen and enlargement of mitochondria with development of mitochondria with bizarre shapes which were seen after 3 days at 258 mm, 1 day at 760 mm, and 3 hours at 2280 mm. These changes were followed by formation of increased numbers of cristae, membranes surrounding mitochondria, autophagic vacuoles, and polyribosome clusters. After 2 weeks at 258 mm, which is the pressure of the atmosphere of space cabins, numerous mitochondrial myelin figures appeared but the mitochondrial enlargement had begun to regress. After 90 days at 258 mm, the liver cells appeared almost normal except that many pigment granules had accumulated in the pericanalicular zones. The changes were non-specific and seemed to parallel biochemical alterations recorded elsewhere. They are not considered the result of toxicity but rather of adaptation. These atmospheres, which are used in clinical medicine and in space travel, appear to have no permanent deleterious effects on the liver in rats under the conditions of this experiment.

The toxic effects of oxygen have been appreciated for almost 100 years, yet only the recent introduction of hyperbaric oxygenation in medical therapy and of pure oxygen atmospheres for space exploration has provided sufficient stimulation to investigate the mechanisms of this toxicity (1–5). Many organs are affected by hyperoxia but clinical symptoms arise primarily because of functional alterations in the central nervous system, lungs, hematopoietic system, and perhaps the liver. Because details of hepatic structure have been described under many experimental conditions, and because the liver seems sensitive to metabolic changes, this organ was chosen for studying, by ultrastructural parameters, the effects of breathing pure oxygen for prolonged periods. Concurrent biochemical studies reported elsewhere (5, 6), using a succinic dehydrogenase system on dispersed cells, have shown an over-all increase in oxygen consumption, probably as a result of some uncoupling of oxidative phosphorylation.

MATERIAL AND METHODS

Sprague-Dawley rats weighing 150 to 200 gm were exposed to essentially pure oxygen at 258 or 760 mm Hg in a closed-system environmental chamber in which the temperature, humidity, and carbon dioxide content were closely regulated (Table I). The exposure facility employed, along with its en-

TABLE I

Experimental Environmental Conditions

Parameter	Mean ± S.E.
Oxygen concentration (per cent)	98.3 ± 0.1
CO_2 partial pressure (mm Hg)	0.56 ± 0.03
Temperature (°C)	23.6 ± 0.4
Relative humidity (per cent)	47.4 ± 0.5
Total pressure (mm Hg)	258, 760, 2280

TABLE II

Number of Rats Exposed to Oxygen at Different Pressures for Various Times from Which Liver Tissue Was Taken for Examination

Atmospheres O_2	Days exposed	Number of animals
1/3	3	11
1/3	7	21
1/3	14	14
1/3	90	8
1	1	11
3	1/8	3
Controls	1-90	20

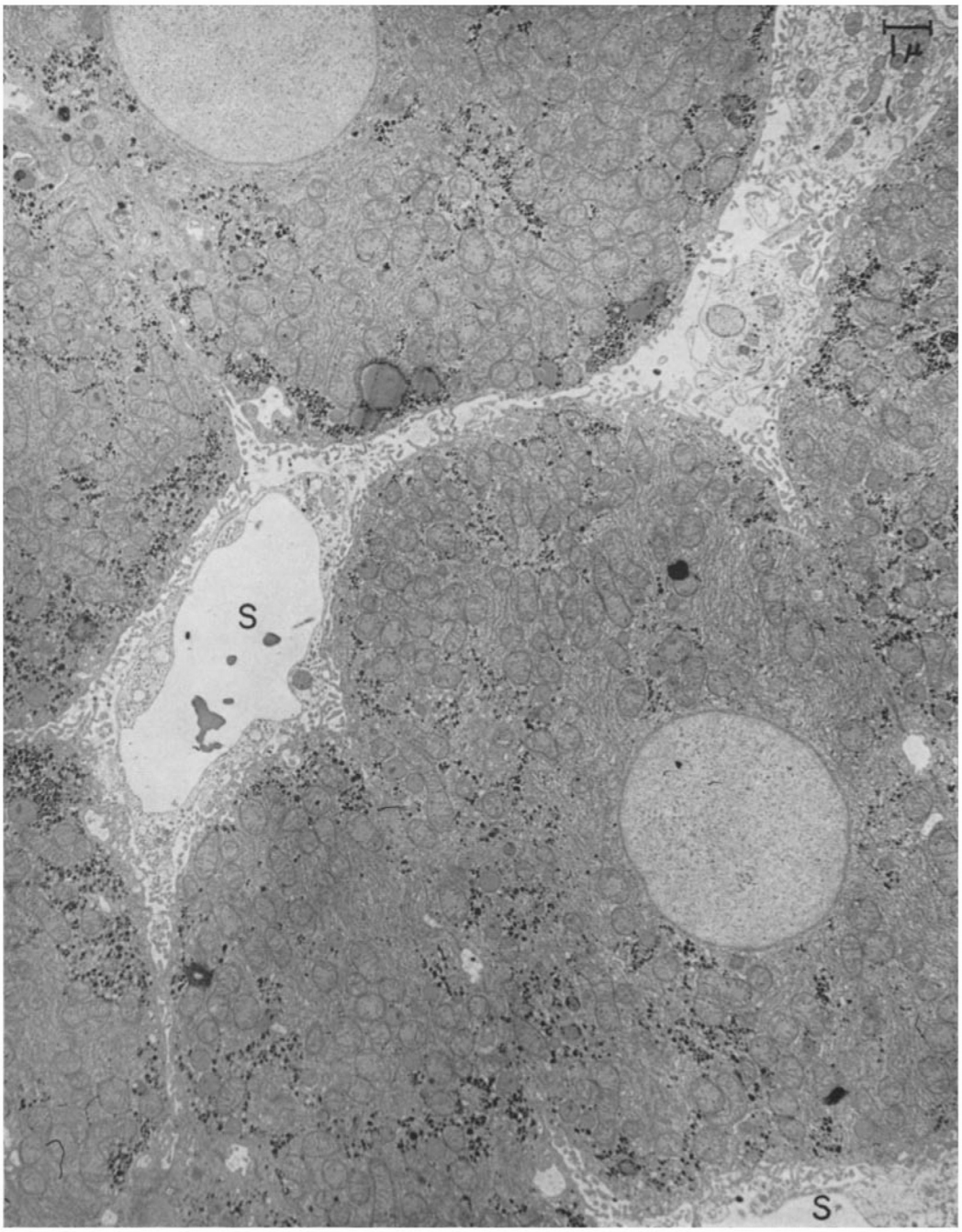
vironmental control system, is described elsewhere (6). In the experiments conducted at 258 mm Hg (⅓ atmosphere), groups of animals were continuously exposed for 3, 7, 14, and 90 days. This pressure was used for the more extensive studies because this is the cabin atmosphere used in American space flights. The rats breathing oxygen at 760 mm Hg were removed after 24 hours. Many would develop convulsions and die if kept much longer than 36 hours under these circumstances. Control animals were simultaneously maintained in identical cages in room air. All animals were allowed food and water *ad libitum*. A few pilot studies were also performed in animals placed in a hyperbaric chamber and exposed to 99 per cent oxygen at three atmospheres absolute pressure for 3 hours. The total number of animals in each group is listed in Table II.

Immediately upon the termination of the oxygen exposure, the animals were given light ether anesthesia and liver biopsies from the center of the large right lobe were obtained at laparotomy with a Menghini needle to obtain specimens of uniform size away from the edges of the liver rapidly and with a minimum of trauma. The small pieces of liver tissue were fixed in a cold 1 per cent solution of osmium tetroxide buffered with veronal-acetate (5-ml Michaelis stock solution, in 25-ml final fixative), to which 0.045-gm sucrose was added per ml of fixative, dehydrated, and embedded in Epon 812 (7). They were sectioned on an LKB Ultrotome, stained with lead hydroxide, monoxide, or citrate (8), and examined with an Hitachi HS 7 or HU 11a microscope. Thicker sections from the same blocks were stained with toluidine blue and the periodic acid-Schiff (PAS) reaction (9). Acid phosphatase stains after fixation in formol calcium were done in a few of the animals exposed for 1 week at ⅓ atmosphere, and for 1 day at one atmosphere. Routine sections of formalin-fixed tissue which was embedded in paraffin and stained with hematoxylin and eosin and PAS after diastase digestion were also studied.

RESULTS

The initial changes seen at 72 hours in the hepatocytes in the group of rats maintained at 258-mm Hg were a decrease in the amount of glycogen and an increase in the size of mitochondria (Fig. 1). The density of the mitochondrial matrix appeared the same as in control animals, and the dense intramitochondrial granules were not increased in number despite the larger size of the organelles. A few mitochondria appeared elongated with a constriction in the middle (Fig. 2), or they had double membranes extending across the entire diameter (Fig. 12). The cavities of the endoplasmic reticulum were only slightly dilated. The remainder of the cell appeared normal.

At 1 week the glycogen depletion persisted and mitochondria appeared which were even larger than previously, up to 8.0 microns in length, having bizarre shapes with increased numbers of cristae of increased length (Fig. 4). Some mitochondria seemed split down the middle (which could represent fission or fusion), and many were elongated with constrictions at their middle as though the two ends were pulling apart. Numerous autophagic vacuoles near bile canaliculi contained single mitochondria (Figs. 3, 5, 10). Fewer vacuoles contained only the endoplasmic reticulum. Each cell on cross-section had 3 to 10 such autophagic vacuoles. The Golgi apparatus appeared strikingly enlarged in every cell. The stacked Golgi, cisternae and vesicles usually contained dense granules. These were uniform in size and density and about 500 A in diameter. They were much less numerous although present in control animals. Larger vacuoles, up to 1.0 micron in diameter, associated with the Golgi



Key to Figures

A, autophagic vacuoles	*M*, mitochondria
C, bile canaliculus	*P*, polyribosomes
G, Golgi apparatus	*S*, sinusoids

FIGURE 1 Low-power electron micrograph of liver of rat exposed to pure oxygen at 258 mm Hg for 3 days. Note scanty dark-staining glycogen granules, crowding of the cytoplasm by mitochondria, and normal sinusoids (*S*) and spaces of Disse. × 5,500.

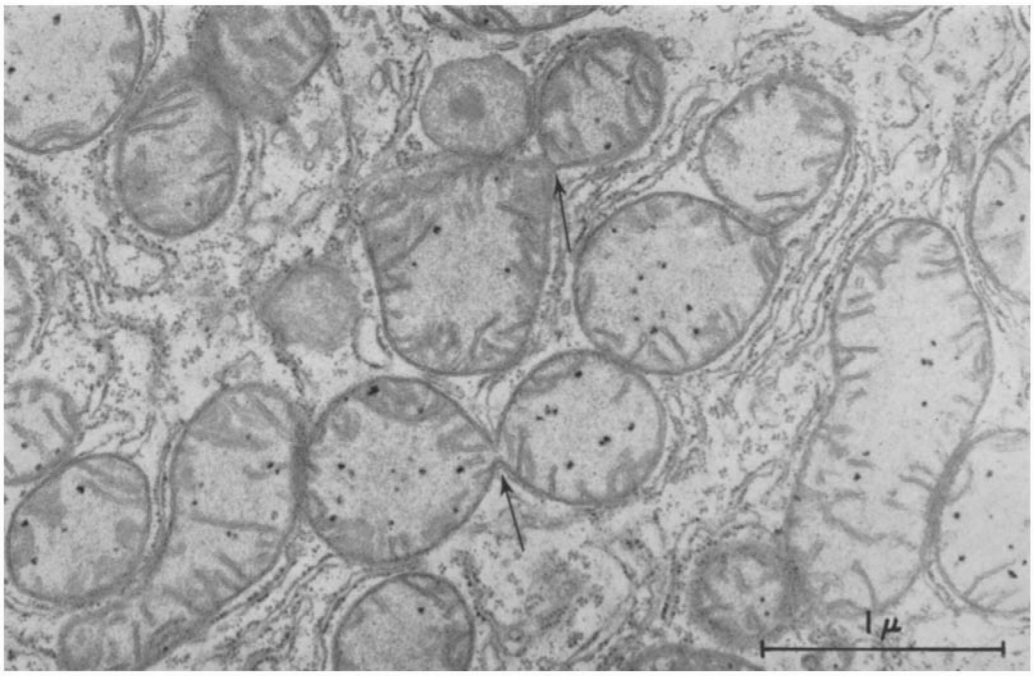


FIGURE 2 Several mitochondria, apparently in the process of budding or dividing by fission (arrows), in rat exposed to oxygen at 258 mm Hg for 3 days. × 31,000.

apparatus were filled with an amorphous and less dense material. The endoplasmic reticulum showed a slight increase in the number of smooth vesicles. In areas in which the enlarged mitochondria appeared tightly packed, multiple layers of reticulum membranes were wrapped about them without forming myelin figures (Fig. 4). Many polysomes, often several hundred in number, were clustered mostly in the perinuclear zone, on or very near the membranes of the endoplasmic reticulum (Fig. 5). These were usually spirals or rosettes, with 16 or 18 ribosomes aligned along the fine thread of presumably messenger RNA, which occasionally could be visualized at magnifications over 100,000 diameters (Fig. 6). In some areas very light and very dense hepatocytes were seen adjacent to one another. Some cells on cross-section also had one to five large, empty-appearing vacuoles up to 5.0 microns in diameter which did not have the appearance of fat droplets from which the fat had fallen out. The bile canaliculi and pericanalicular ectoplasm, the microbodies, and the sinusoidal microvilli were normal, as were the sinusoidal lining cells and the Kupffer cells.

At the end of the 2nd week, the mitochondria were less large and less bizarre in shape. The number and length of the cristae were reduced. However, small myelin figures, 0.2 to 0.3 micron in diameter, were attached to or within mitochondria (Fig. 7). These were found in 10 of the 14 animals examined and, when present, were in each block studied but not always in each cell. The remainder of the cell appeared quite normal except for persistence of the pericanalicular autophagic vacuoles and the large Golgi apparatus previously noted (Fig. 8). Kupffer cells appeared larger and had larger phagosomes than previously.

At 90 days the liver cells appeared to have returned almost entirely to normal. Occasional light and dense cells were seen. A few large mitochondria of bizarre shape, a few autophagic vacuoles, enlarged Golgi apparatus, and glycogen depletion were found in some cells, but this was not uniform and the changes in any single cell were not so severe as at 1 to 2 weeks. In addition, dense bodies with the appearance of small lipofuscin granules (lysosomes), less than 1.0 micron in diameter, were clustered about the bile canaliculi (Fig. 9). These were greatly increased in number when compared to control rats or to rats

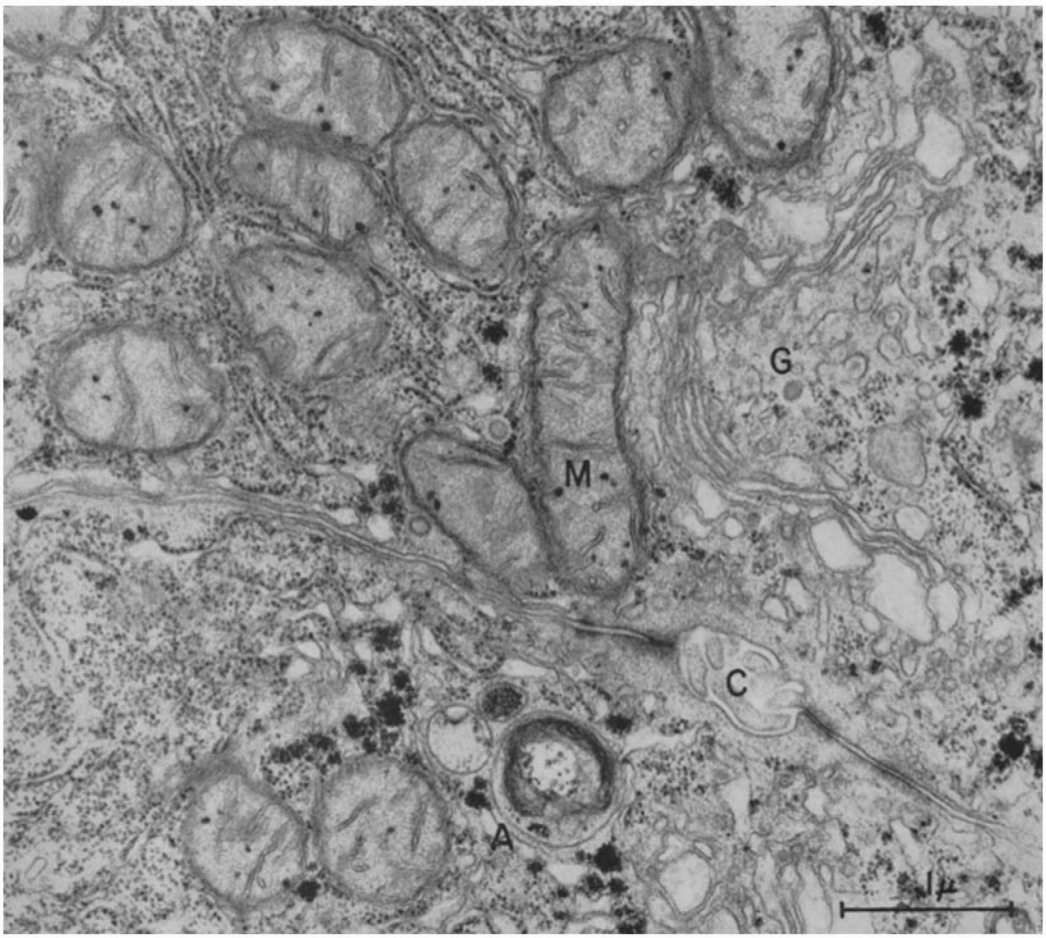


FIGURE 3 In lower cell is an autophagic vacuole (*A*). Immediately above, in an adjacent cell are two mitochondria (*M*) with membranes darker than those of adjacent mitochondria. Rat exposed to oxygen at 258 mm Hg for 1 week. Note the large Golgi apparatus (*G*) on the right in the upper cell and below it a bile canaliculus (*C*). × 22,000.

exposed for shorter times. They could not be seen in routine paraffin sections stained with hematoxylin and eosin. The enlarged Kupffer cells containing numerous phagosomes were still present at 90 days.

At no time did the animals appear sick; they continued to eat and gain weight as did the controls. Light microscope study of thin sections did not yield any additional information but confirmed the glycogen depletion and variation in cell hydration. Acid phosphatase activity at 1 week of 1/3 atmosphere and at 1 day of one atmosphere was normal. The ultrastructural changes after exposure to oxygen at three atmospheres for 3 hours or at one atmosphere for 24 hours were comparable to those seen at 1 week of 1/3 atmosphere, the mitochondria being enlarged and of odd shapes and showing apparent fission, and membranes transversely dividing the mitochondria (Figs. 10, 12), although the numbers of autophagic vacuoles were less (Fig. 11).

DISCUSSION

All of the ultrastructural cellular changes in liver described following prolonged breathing of pure oxygen even at reduced pressure have been seen under other experimental circumstances, including ischemia (10–12), regeneration (13), partial starvation (14), or chemical intoxication (15). In fact, some of them, such as small myelin

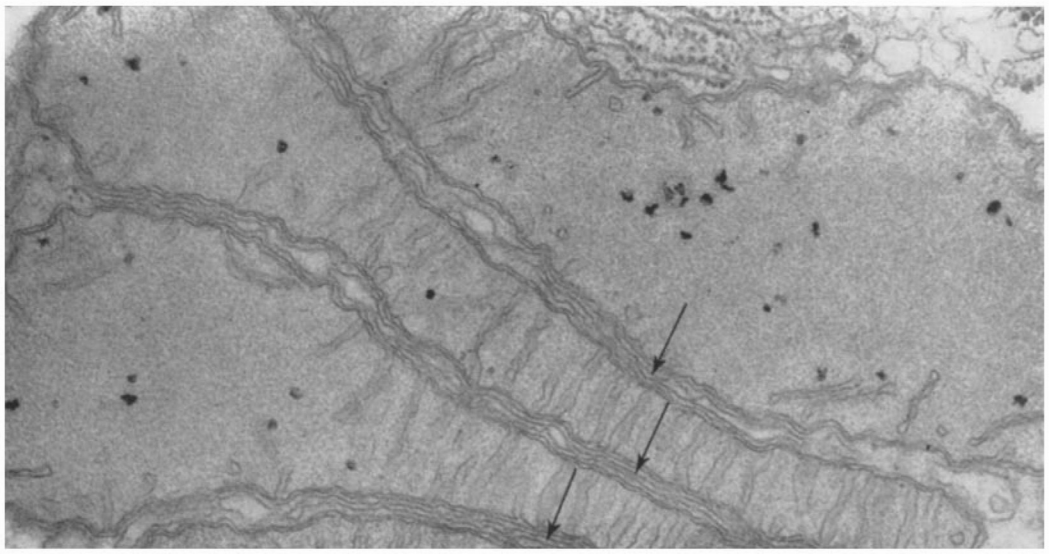


FIGURE 4 Cluster of enlarged mitochondria with increased numbers of cristae, many traversing the entire width of the mitochondria, and extra membranes surrounding the mitochondria (arrows). Rat exposed for 1 week to oxygen at 258 mm Hg. × 72,000.

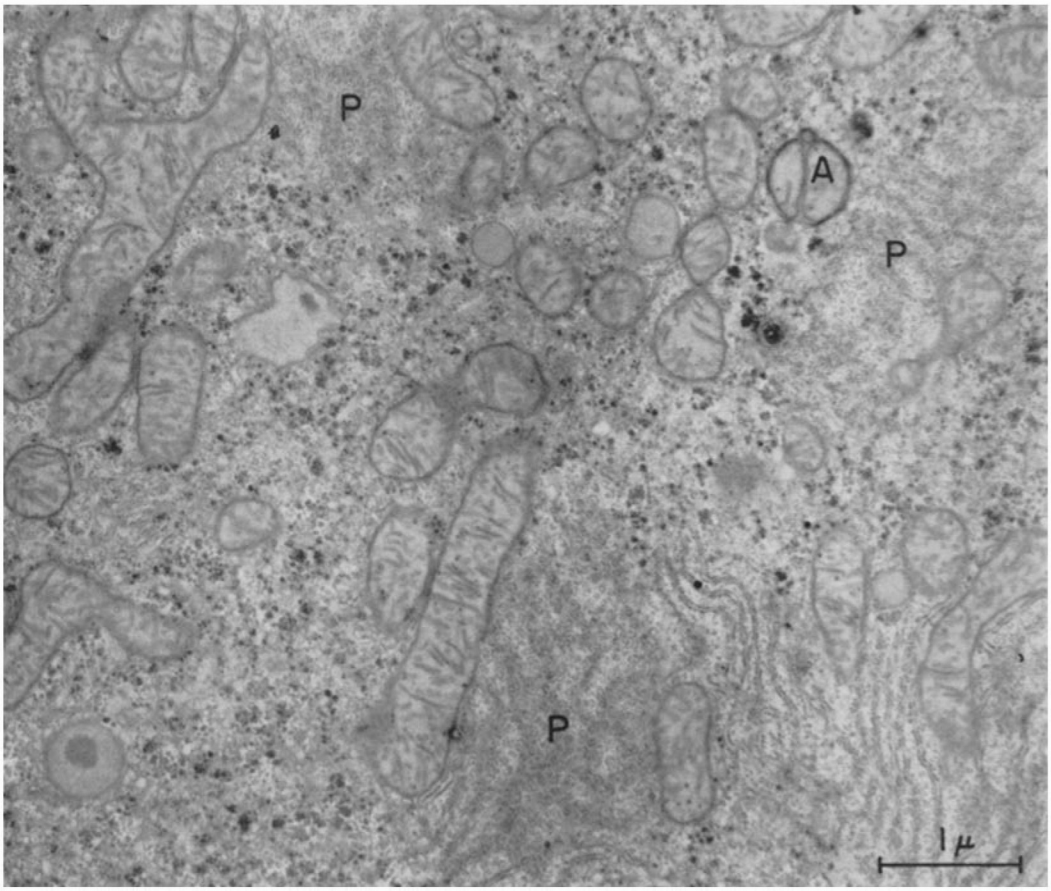


FIGURE 5 Aggregation of many polyribosomes (*P*) in clusters usually near the endoplasmic reticulum, in animal exposed for 1 week to oxygen at 258 mm Hg. A large, irregularly shaped mitochondrion is seen at the upper left while an autophagic vacuole containing a degenerating mitochondrion (*A*) is seen at the right. × 19,000.

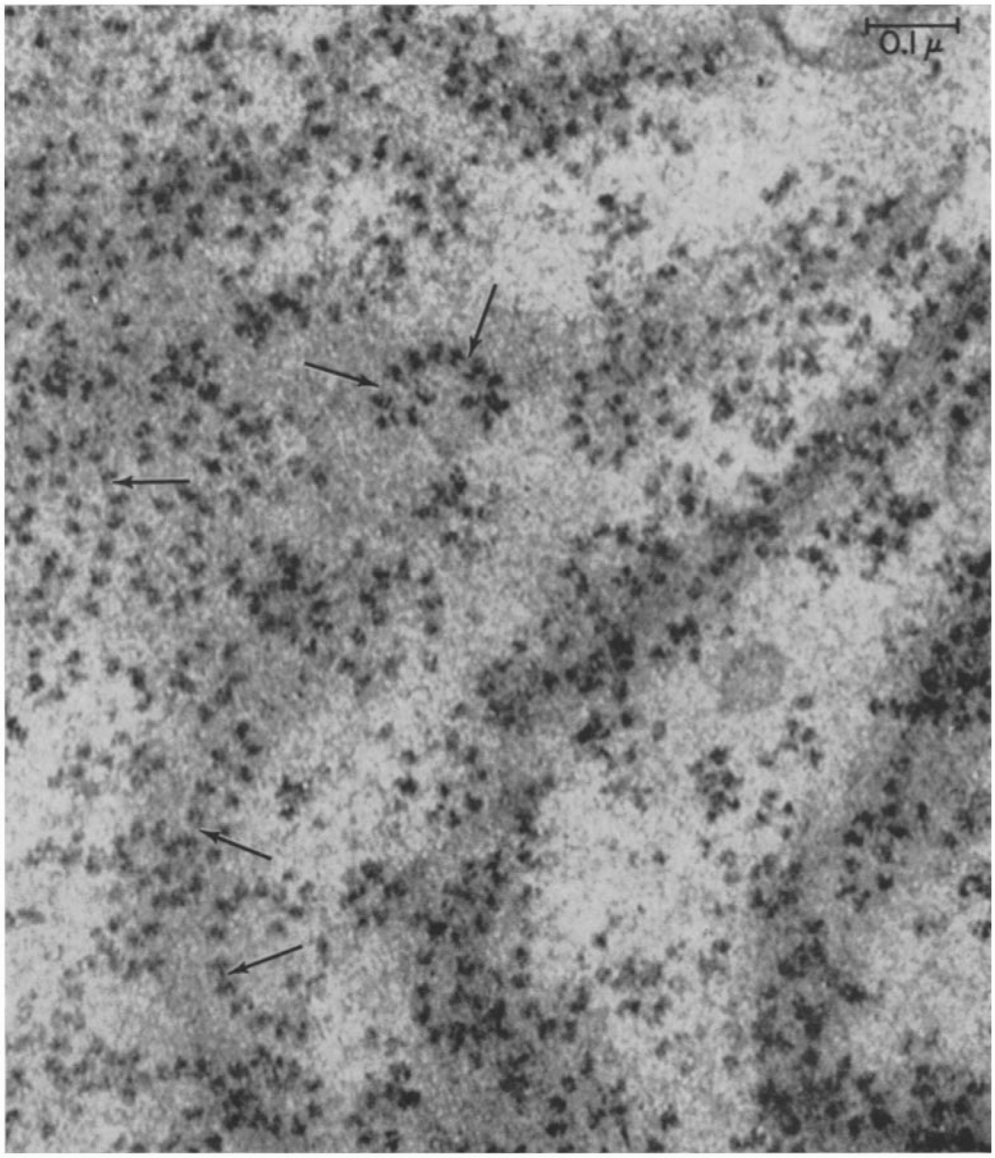


FIGURE 6 Higher magnification of portion of Fig. 5, showing polyribosomes most commonly in a spiral form on or very near profiles of endoplasmic reticulum with fine thread (arrows) sometimes appearing as dense line and other times as lighter line connecting individual ribosomes. × 115,000.

figures in mitochondria, can be produced by freezing and thawing of the liver after it has been excised (16). Therefore, none of the changes can be regarded as specific. Indeed, it is impossible to separate hyperoxic liver, as seen in this study, from the hypoxic one (12). Yet under hyperoxic conditions, the blood oxygen is elevated (17), and the hepatic oxygen consumption *in vitro* is increased above normal (5). Chemically, there is evidence to suggest a change in the NADH/NAD ratio with formation of excess NAD, a situation which is the reverse of that found in hypoxia (18). Studies of rats breathing oxygen at 258 mm Hg for 2 weeks have demonstrated no significant alterations in serum bilirubin, alkaline phosphatase activity, or glutamic-oxaloacetic trans-

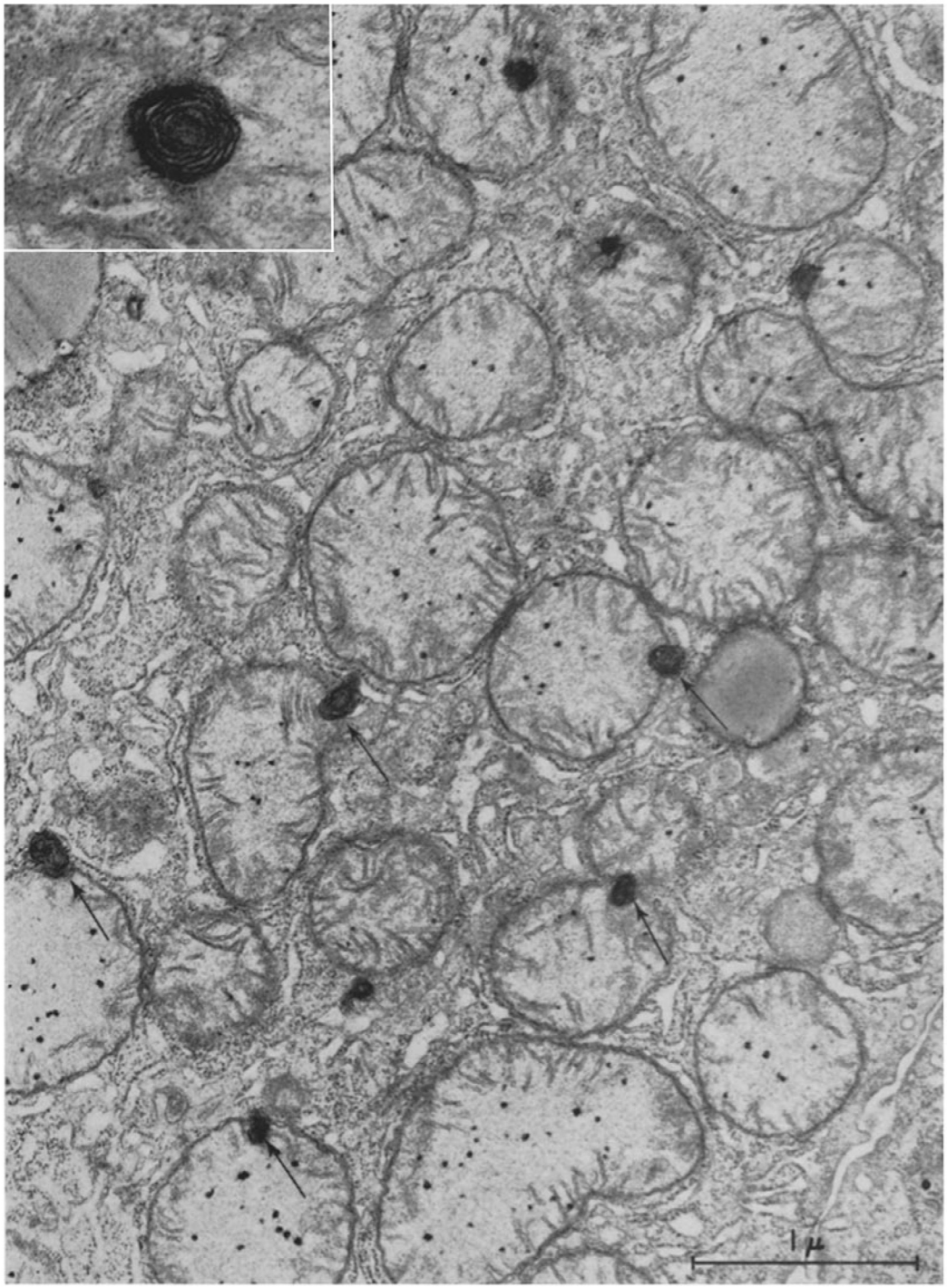


FIGURE 7 Numerous myelin figures (arrows) on edges of mitochondria in rat exposed for 2 weeks to oxygen at 258 mm Hg. Mitochondria with such figures are more normal in size than those without. × 32,000. The details of the myelin figures are seen in the inset on the upper left. Most of these myelin figures were either on the edge of the mitochondrion or between adjacent mitochondria. × 60,000.

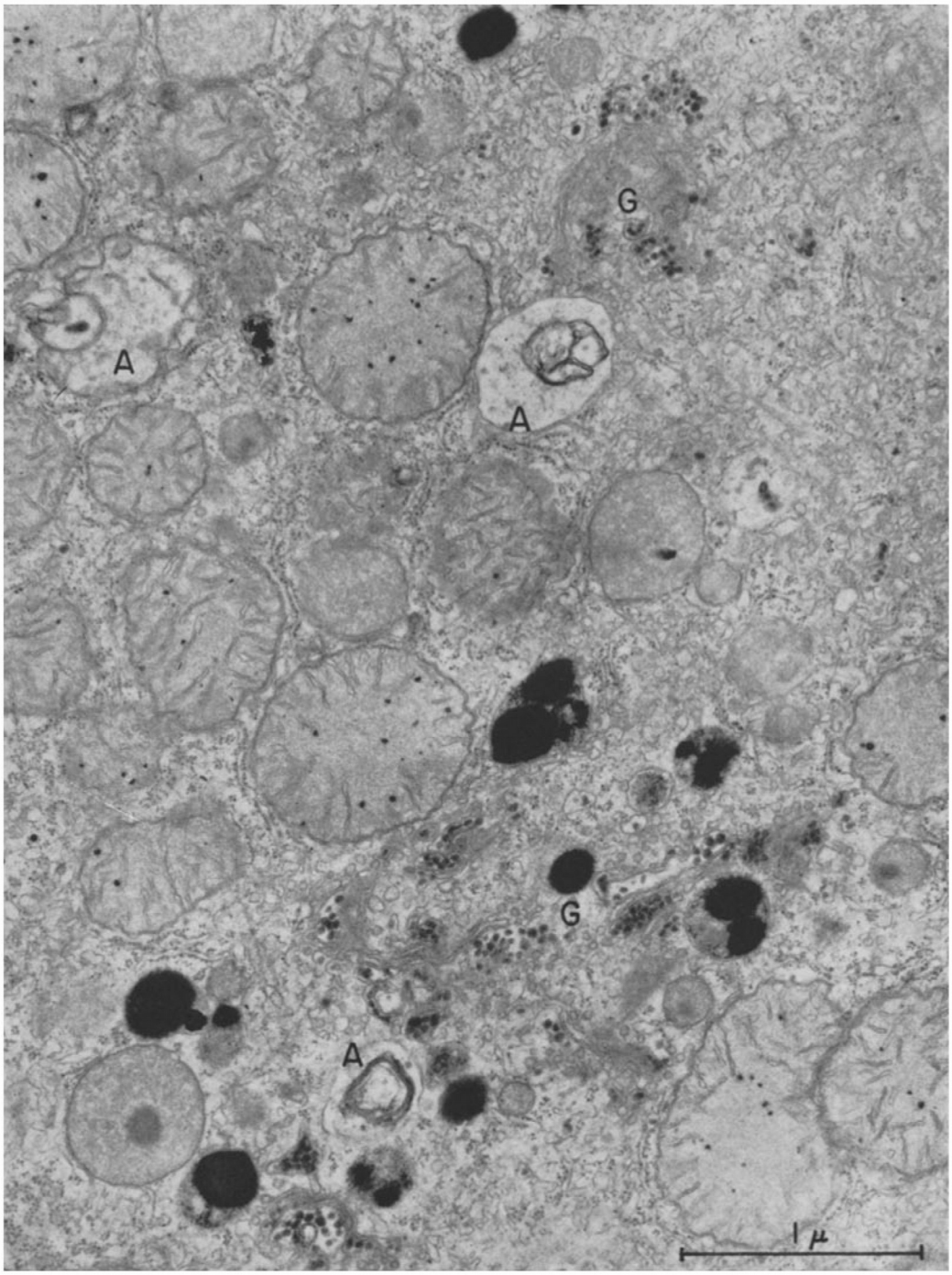


FIGURE 8 Numerous autophagic vacuoles (*A*), some pigment granules, and a large amount of stacked Golgi cisternae and vacuoles (*G*) containing uniform dense granules. Note the crenation of the mitochondrial membranes. Rat exposed to oxygen at 258 mm Hg for 2 weeks. × 34,000.

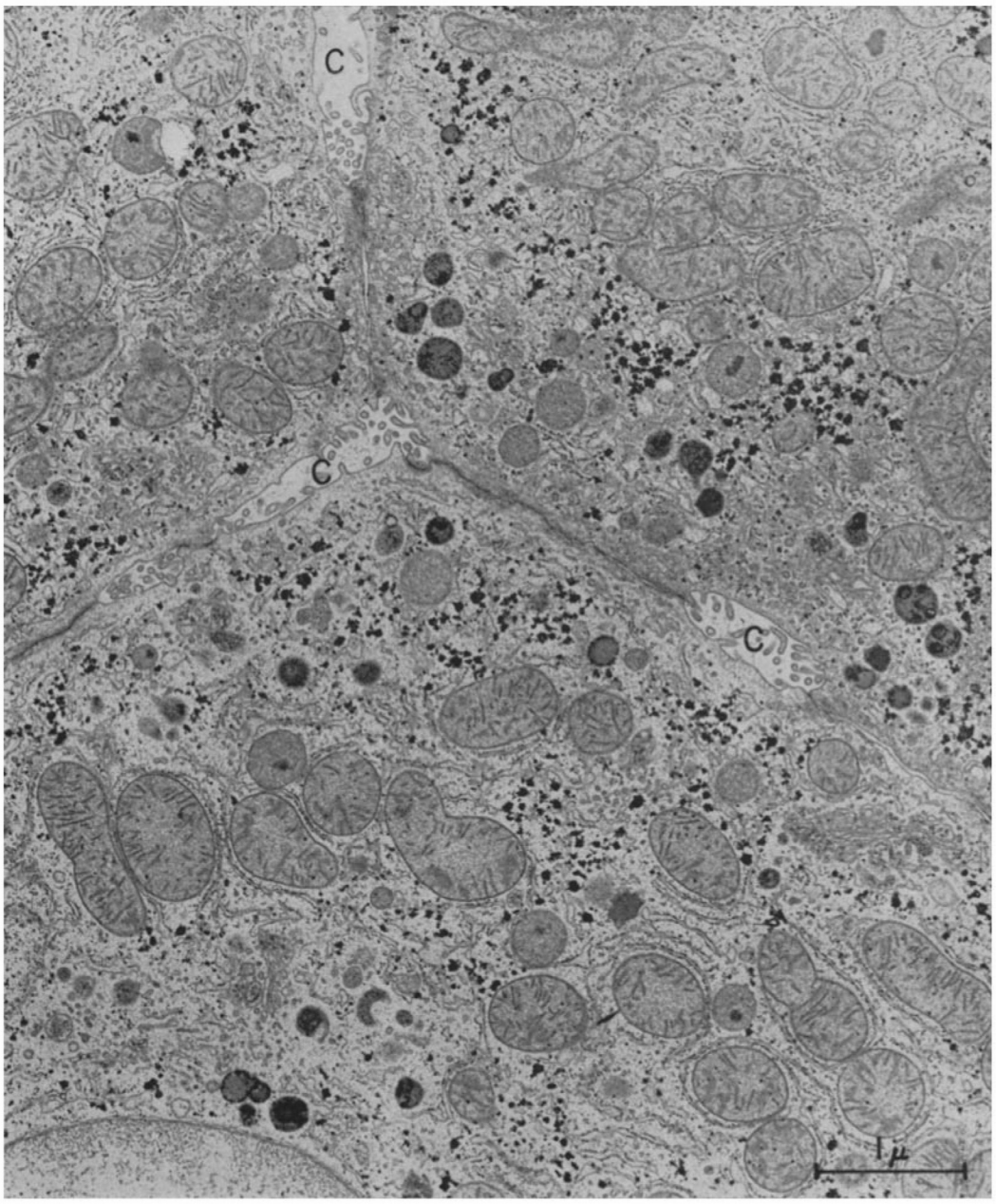


FIGURE 9 Numerous dense lipofuscin granules (lysosomes) near bile canaliculi in rat exposed for 90 days to oxygen at 258 mm Hg. Note the normal size and shape of the mitochondria. × 22,000.

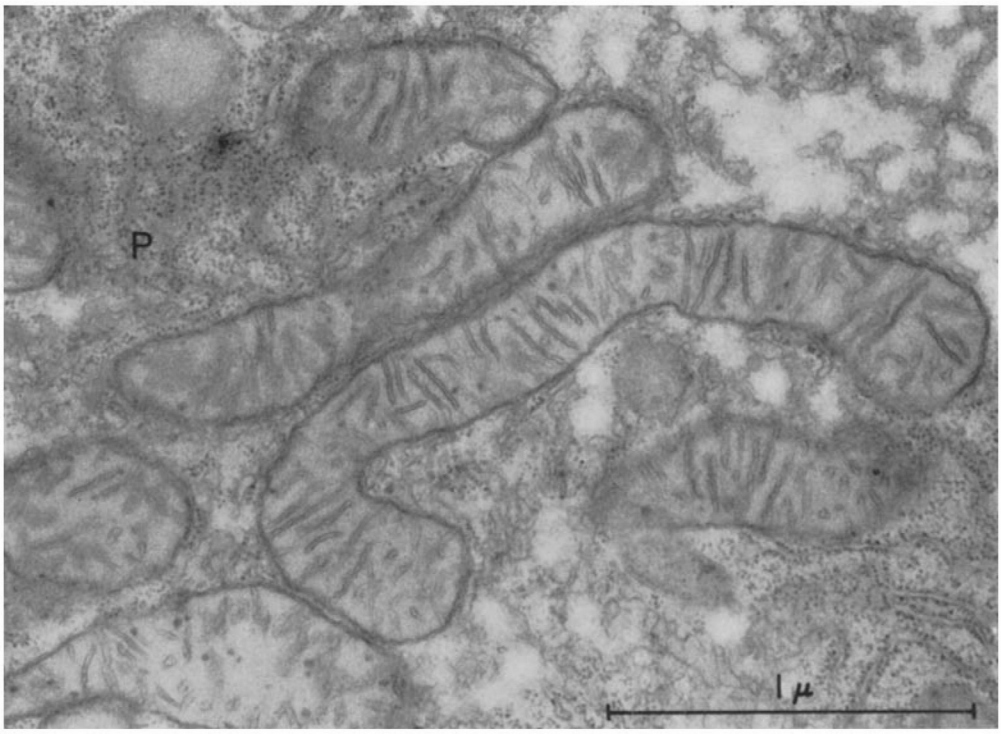


FIGURE 10 Very elongated mitochondria with many cristae traversing almost the entire width of the organelle. Note the polyribosome cluster (*P*) on the upper left. The glycogen failed to stain and is seen as light "holes." Rat exposed to oxygen at 760 mm Hg for 1 day. × 44,000.

aminase activity (6). However, more sensitive indices are required to fully evaluate over-all cellular metabolism.

In hepatocytes, which were selected as a model of metabolically active cells, the chief sites of initial alterations are the mitochondria which increase in size, by either hypertrophy or fusion, and also appear to increase in number. Furthermore, these organelles represent the common locus of subcellular hepatic reactivity to hyperoxia, regardless of the pressure to which inspired oxygen is increased. Similar suggestions have come from the study of lung tissue (19) and kidney tissue (20), as well as from biochemical investigations (1). From the time relationships seen in the groups at 258 mm Hg, this effect would appear to be a direct one. The mitochondria appear to undergo a rapid turnover, as evidenced by the formation of many lysosomes (autophagic vacuoles), with the formation of new mitochondria by fission. This observation has led to the idea that hyperoxia makes the lysosomal membranes more permeable and leads to leakage of hydrolytic enzymes from the lysosomes, resulting in cell damage (21). This sequence of events seems unlikely because damaged organelles are sequestered in the autophagic vacuoles and the remaining cell structures are normal in appearance rather than deteriorated. Therefore, it is more plausible that oxygen exerts a direct effect upon mitochondria. This effect reaches a peak in about 1 week. During adaptation to oxygen exposure, mitochondrial turnover is presumed to be increased during the 1st and 2nd weeks because the autophagic vacuoles containing mitochondria are most numerous at this time. Then as the animal has become adapted after 1 to 3 months, the liver returns to normal with only some lipofuscin pigment granules in lysosomes as a residue. Whether these changes should be classified as toxic or physiologic remains unanswered. The animals, however, continued to gain weight normally.

Several problems must yet be studied before

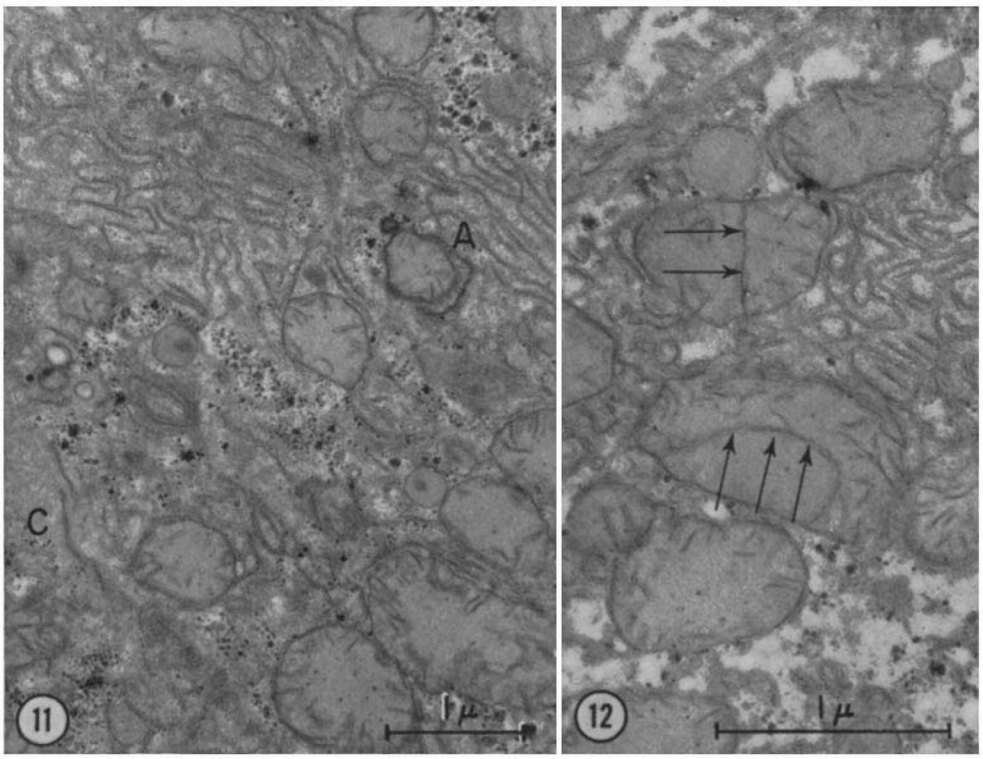


FIGURE 11 Autophagic vacuole (*A*) containing mitochondrion with membranes darker than those of surrounding mitochondria. Rat exposed to oxygen at 760 mm Hg for 1 day. × 18,000.

FIGURE 12 Membranes traversing entire mitochondria (arrows), suggesting either fission or fusion. Rat exposed to oxygen at 760 mm Hg for 1 day. × 30,000.

pure oxygen at reduced pressure can be considered innocuous for men, as far as the liver is concerned. These are: (1) determination of normal function of the liver using sensitive methods; (2) demonstration of a lack of increased vulnerability to toxic substances such as volatile solvents or irradiation; and (3) extension of these observations in rats to primates, particularly man.

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