LIVER PARENCHYMAL CELL INJURY

III. The Nature of Calcium-Associated Electron-Opaque Masses in Rat Liver Mitochondria Following Poisoning with Carbon Tetrachloride

EDWARD S. REYNOLDS, M.D.

Departments of Pathology, Harvard Medical School and Peter Bent Brigham Hospital, Boston

ABSTRACT

Accumulation of calcium in the mitochondria of rat liver parenchymal cells at 16 and 24 hours after poisoning with carbon tetrachloride is associated with an increase in amount of liver inorganic phosphate, the persistence of mitochondrial adenosine triphosphatase activity, and the formation of electron-opaque intramitochondrial masses in cells with increased calcium contents. These masses, which form within the mitochondrial matrix adjacent to internal mitochondrial membranes, resemble those observed in isolated mitochondria which accumulate calcium and inorganic phosphate; are present in a locus similar to that of electron opacities which result from electron-histochemical determination of mitochondrial ATPase activity; and differ in both appearance and position from matrix granules of normal mitochondria. After poisoning, normal matrix granules disappear from mitochondria prior to their accumulation of calcium. As calcium-associated electron-opaque intramitochondrial masses increase in size, mitochondria degenerate in appearance. At the same time, cytoplasmic membrane systems of mid-zonal and centrilobular cells are disrupted by degranulation of the rough endoplasmic reticulum and the formation of labyrinthine tubular aggregates. The increase in amount of inorganic phosphate in rat liver following poisoning is balanced by a decreased amount of phosphoprotein. These chemical events do not appear to be related, however, as the inorganic phosphate accumulated is derived from serum inorganic phosphate.

Mitochondria isolated from liver and kidney of normal animals sequester up to several hundredfold their normal calcium content when this alkaline earth is presented to them *in vitro* (4, 5). Inorganic phosphate accumulates concurrently, under these conditions, in a metabolic process which requires either adenosine triphosphate (ATP) or an electron transport-linked translocation of inorganic phosphate from the medium to the mitochondrial matrix (2, 6). The continued uptake of calcium and phosphate by mitochondria results in the appearance of electron-opaque masses within the mitochondrial matrix (2, 7).

As a consequence, it has been postulated that mitochondria take part in the translocation of calcium and phosphate in normal cells (4, 5). Indeed, similar granules are observed in osteoclasts of normal bone (8), have been described in calcium phosphate-sequestering tumors (9), and appear in renal tubular epithelium following the administration of parathormone (10). Although calcium uptake by mitochondria has been most

extensively studied, other metals such as barium and strontium are deposited within mitochondria of epithelial cells *in vitro* (7), and iron accumulates intramitochondrially within erythroblasts *in vivo* (11).

The sequestering of calcium by liver parenchymal cells also occurs following poisoning by chemical agents such as carbon tetrachloride (12), thioacetamide (13, 14), dimethylnitrosamine (12), and phosphorus (14). Intracellular calcium accumulation in carbon tetrachloride poisoning is localized to mitochondria (12, 15), is associated with continuing mitochondrial respiration in calcium-sequestering cells (12, 16), and is accompanied by an apparent activation of an alkaline earth–dependent mitochondrial adenosine triphosphatase (ATPase) (17). At the same time, lead-stainable cation-binding sites appear within the cytoplasm of the cells involved (12). Continued accumulation of calcium by mitochondria results in failure of their respiration and ultimate disintegration of this organelle (12, 16).

Electron-opaque calcium-containing masses also form within the matrix of mitochondria of midzonal and centrilobular parenchymal cells as a consequence of carbon tetrachloride poisoning. The present study examines in detail this cytochemical event, which occurs between 8 and 24 hours after the oral administration of hepatotoxin.

METHODS

Healthy young male rats (Charles River Breeding Laboratories, Boston, Massachusetts) weighing between 150 and 250 grams were maintained on a diet

Key to Abbreviations

c, central vein
Ca, intramitochondrial calcium-associated electron-opaque mass
G, Golgi apparatus
LTA, labyrinthine tubular aggregate
Li, lipid droplet
Mb, microbody
MG, mitochondrial matrix granule
Mt, mitochondrion
N, nucleus
p, portal triad
PM, plasma membrane
Rp, polysomes
R_f, ribosomes, free
Rer, granular endoplasmic reticulum
Ser, agranular endoplasmic reticulum.
Vc, vacuole

FIGURES 1 TO 6 Light microscope illustrations of alterations in periportal (Figs. 1 to 3) and centrilobular (Figs. 4 to 6) cells following poisoning with carbon tetrachloride. Osmium tetroxide fixation; Epon-embedded 1-μ sections; staining with toluidine blue O in borax. × 1500.

Fig. 1. Mitochondria in periportal cells of controls appear as short rods 1 μ in smallest diameter.

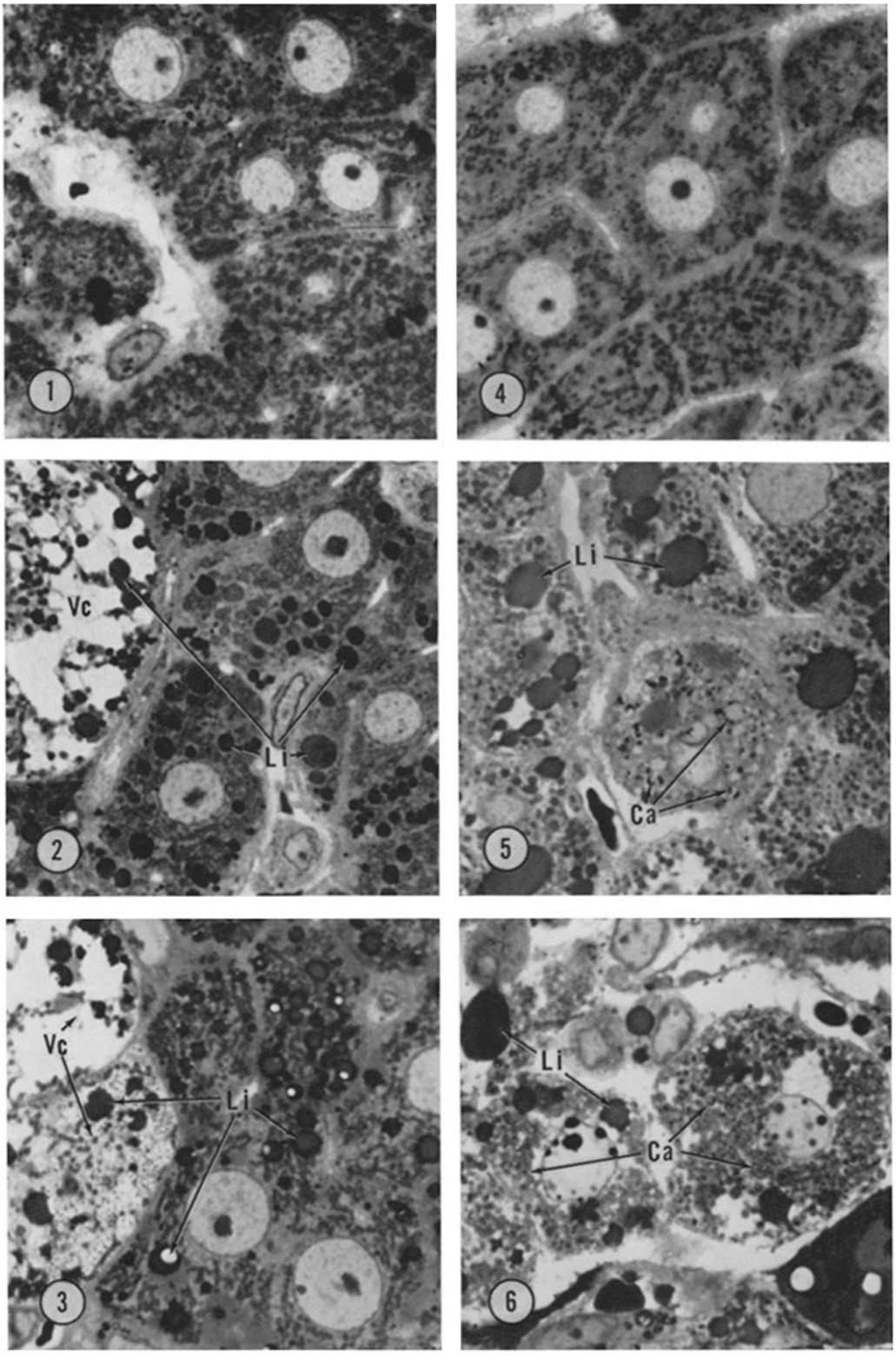
Fig. 2. Sixteen hours after poisoning. Mitochondria of periportal cells are similar to those of controls. Increased amount of cytoplasmic fat is present, however, and the cytoplasm of many periportal cells is diffusely vacuolated.

Fig. 3. Twenty-four hours after poisoning. Mitochondria of periportal cells are elongated and minute osmiophilic droplets appear in previously optically empty vacuoles of diffusely vacuolated cells seen at 16 hours (Fig. 2).

Fig. 4. Mitochondria in centrilobular cells of controls are more filamentous than those of periportal cells. (39 μg Ca/gm liver.)

Fig. 5. Sixteen hours after poisoning. Mitochondria of most centrilobular cells are spherical; nucleoli are absent. Large cytoplasmic droplets of lipid are present. Numerous punctate intensely basophilic deposits are present within mitochondrial remnants of occasional cells which correspond to those staining for increased calcium. (101 μg Ca/gm liver.) (Compare with Fig. 20.)

Fig. 6. Twenty-four hours after poisoning. Plasma membranes of centrilobular cells are pulled away from one another, and mitochondria of many more cells contain punctate basophilic deposits. Nuclear chromatin is reduced to rounded basophilic masses. Macrophages and extravascular erythrocytes are also present. (680 μg Ca/gm liver.)


1
4
Vc
Li
2
Li
Ca
5
Vc
Li
3
Li
Ca
6

of Purina chow and water *ad libitum*. Replicate rats were killed by decapitation and exsanguination 16 and 24 hours after the oral administration by polyethylene stomach tube of a single oral dose of analytical grade carbon tetrachloride, 0.25 ml in an equal volume of mineral oil per 100 grams of animal. Control animals received mineral oil alone and were killed at 24 hours. All animals were fasted for 16 hours before sacrifice.

For morphologic and histochemical studies, the left lateral lobe of the liver was removed immediately and sliced. Some slices were cut into minute cubes measuring less than 2 mm in greatest dimension, and the cubes were fixed in collidine-buffered osmium tetroxide (18) containing 0.125 M sucrose and 0.005 M calcium chloride. Omission of calcium from the standard fixative or use of phosphate-buffered osmium tetroxide (19) failed to preserve the electron-opaque deposits formed within the mitochondrial matrix following poisoning with carbon tetrachloride. In addition, cubes of liver quenched in liquid propane at 90°K were either freeze-dried (220°K) (20) or freeze-substituted (190°K) in acetone, or in acetone containing 1 per cent osmium tetroxide. Upon completion, tissues were warmed to room temperature and embedded in Epon. Unstained and lead citrate–stained sections (21) of Epon-embedded (22) liver were examined in an RCA EMU 3F or 3G microscope. Thick sections (1 micron) from blocks examined in the electron microscope were stained with toluidine blue O (23) for light microscopy.

Slices of liver adjacent to those taken for electron microscopy were frozen and sectioned with a hollow-ground steel knife in a Master-Bilt cryostat. Serial fresh-frozen sections, 6 to 10 microns thick, were picked off the knife on warm clean coverslips and stained immediately, or placed in appropriate incubation media.

Cryostat and plastic sections of liver were stained with alizarin red S for calcium (20, 24) and with lead nitrate for inorganic phosphate (25).

Mitochondrial and bile canalicular ATPase activity distribution within the liver lobule was determined in fresh-frozen sections at pH 7.4 by the method of Wachstein and Meisel (26). Under these conditions, ATPase activity of mitochondria of normal livers is dependent upon magnesium (10^{-2} M), and stimulated by the presence of 2,4-dinitrophenol (DNP) (5×10^{-4} M) (27). Addition of exogenous succinate (10^{-2} M) does not affect the histochemical determination of ATPase activity in sections of either control or experimental animals. Cyanide (10^{-2} M) abolishes both bile canalicular and mitochondrial ATPase activities.

Glucose-6-phosphatase activity was stained by the method of Wachstein and Meisel (28) in cryostat sections of liver. Acid and alkaline phosphatase staining patterns were determined by the methods of Gomori (25). Thiamine pyrophosphatase was stained by the method of Eranko and Hasan (29) and inorganic pyrophosphatase (pH 7.3) by the method of Kurata and Maeda (30).

Acid-soluble phosphates, lipid phosphate, nucleic acid phosphate, and protein phosphate were separated by a Schmidt-Thannhauser procedure (31) modified as follows: All steps were carried out at 0–4°C unless otherwise indicated. Immediately after sacrifice of the animal, the liver was removed and pulped in a hand press, and samples of liver pulp weighing less than 1 gram were placed directly in 0.3 M perchloric acid. The supernatant, containing inorganic phosphate and acid-soluble organophosphates, was decanted after centrifugation and the pellet washed twice with fresh aliquots of 0.3 M perchloric acid. After extraction of lipids from the pellet with ethanol and 3:1 ethanol:diethylether, nucleic acids were solubilized from the pellet with hot (100°C) neutral 10 per cent sodium chloride (32). The remaining protein residue was dried to constant weight (at 104°C) and dissolved in 10 per cent potassium hydroxide. Recoveries of liver phosphoprotein were reduced when there were delays between the death of the animal and the perchloric acid precipitation step.

Inorganic phosphate in the acid-soluble fraction of the liver pulp and blood serum was determined directly by the method of Fiske and SubbaRow (33). Total liver phosphate and liver acid-soluble phosphate, lipid phosphate, nucleic acid phosphate, and protein phosphate were determined as inorganic phosphate after fusion with ethanolic magnesium nitrate of aliquots containing less than 1 mg nitrogen (34).

Uptake of inorganic P^{32} by inorganic and organic

FIGURE 7 Part of cytoplasm of two adjacent midzonal liver parenchymal cells of a normal fasted animal. In one, on the left, parallel strands of granular endoplasmic reticulum form ergastoplasm. Numerous interconnections with smooth endoplasmic reticulum are present in both cells. Mitochondria, short rods in the plane of section, are slightly denser than adjacent cytoplasm and contain numerous (3 to 15) freely dispersed, small (300 A), irregular electron-opaque granules within their matrix. × 16,000.

Inset. Mitochondrial matrix granules consist of clusters of electron-transparent subunits (≅ 75 A in diameter) embedded within an electron-opaque mass. × 115,000.

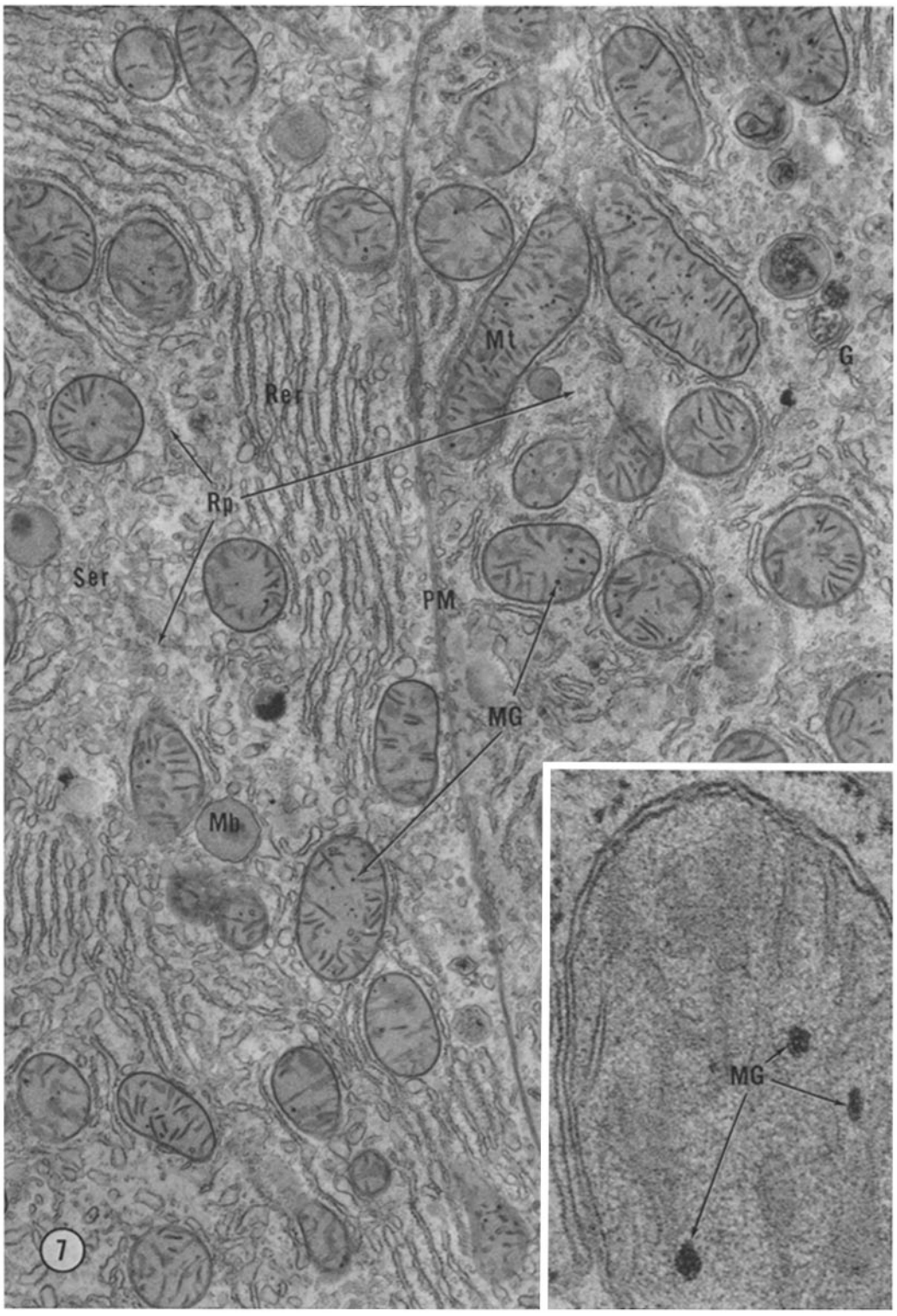

Mt
G
Rer
Rp
Ser
PM
MG
Mb
MG
7

TABLE I

Calcium and Phosphate Content of Livers of Rats Following Poisoning with Carbon Tetrachloride

Interval after poisoning	Calcium	Phosphate					
		Acid soluble					
		Inorganic	Total	Protein	Lipid	Nucleic acid	Total
				μmole/mg N			
Control (7)	0.030	0.32	0.84	0.43	1.00	0.71	3.23
SEM	±0.001	±0.02	±0.03	±0.06	±0.03	±0.03	±0.08
16 hours (5)	0.20*	0.43*	1.14‡	0.30	1.09	0.63	3.35
SEM	±0.07	±0.05	±0.04	±0.03	±0.04	±0.06	±0.11
24 hours (8)	0.57‡	0.54‡	1.10*	0.22*	0.99	0.62	3.29
SEM	±0.06	±0.06	±0.08	±0.05	±0.05	±0.03	±0.10

Number in parentheses is number of animals examined at each time.
SEM = standard error of the mean.
* P, 0.05.
‡ P, 0.01.

phosphate components of liver during the period between 16 and 24 hours after poisoning, when calcium uptake by the liver is maximal, was determined as follows: Control animals and experimental animals were fasted from the time mineral oil, or the standard dose of carbon tetrachloride in mineral oil, was administered. At 16 hours, animals of both groups were injected intraperitoneally with 1.0 ml of a 0.90 per cent saline solution containing 200 μc carrier-free $Na_2HP^{32}O_4$. Control and experimental animals were decapitated at 24 hours, blood was collected from the neck in a beaker containing 5 mg disodium ethylenediaminetetraacetate (Na_2 EDTA), and the liver was immediately excised and analyzed for phosphate contents—acid soluble, lipid, nucleic acid, and protein. P^{32} contents of these fractions were counted at infinite thickness (0.00085 gm/cm^2) in a gas-flow thin window counter. Liver inorganic phosphate in the acid-soluble fractions and serum inorganic phosphate were isolated by elution from Dowex 1 columns with 0.02 N HCl. Aliquots of eluate fractions were analyzed colorimetrically for inorganic phosphate and counted for P^{32}. As a general rule, at least 95 per cent of the radioactivity and phosphate in total liver phosphate was recovered in the inorganic and organophosphate fractions of control and experimental livers.

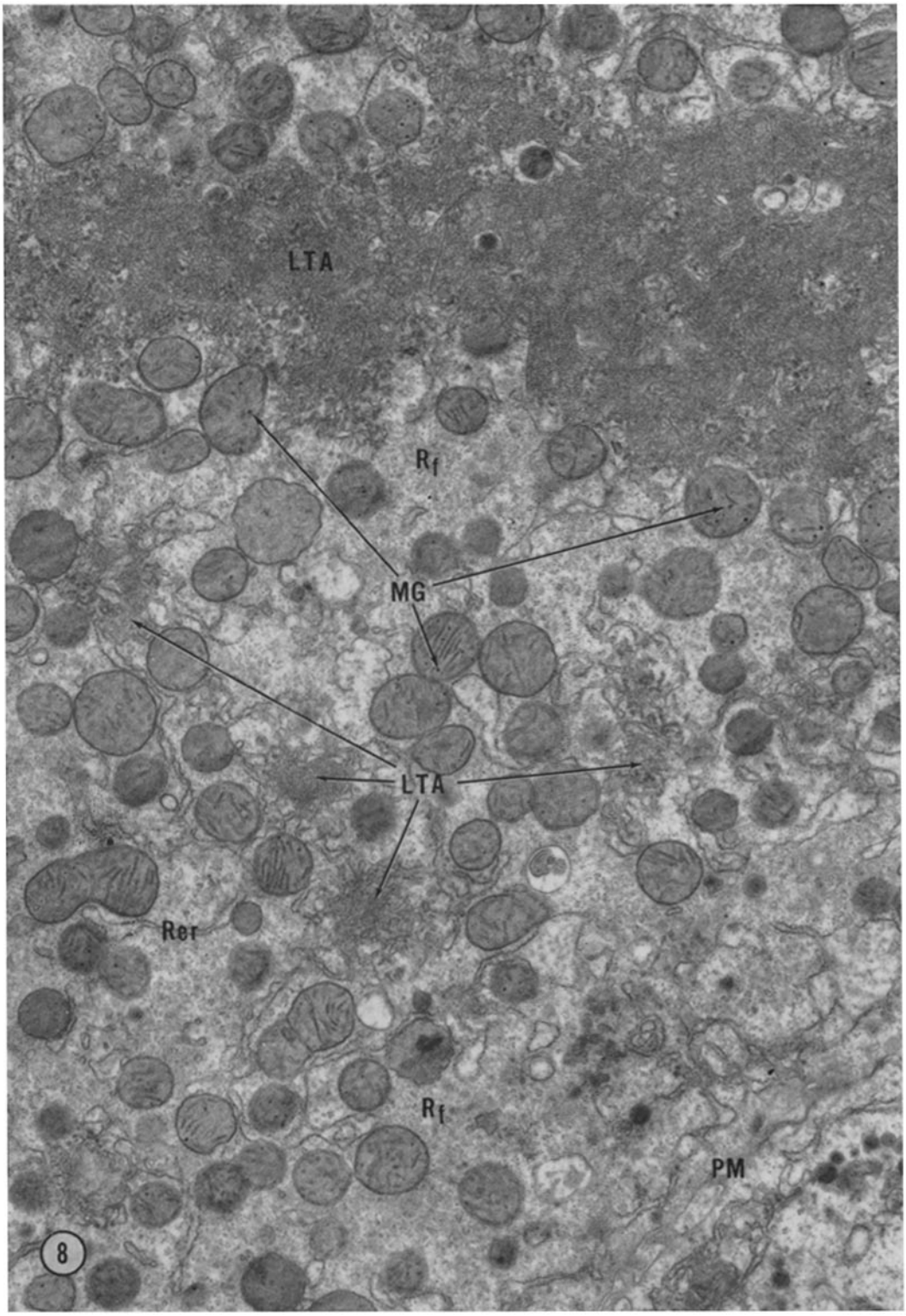
Aliquots of liver pulp were also analyzed for calcium (35) and nitrogen (36). Coefficients of variation for the colorimetric determinations of calcium, nitrogen, and phosphate were 3 per cent.

RESULTS

Livers of control rats (Figs. 1, 4, and 7) do not differ substantially from normal rat livers previously described (24, 37, 38). The uniform depletion of glycogen and the presence of abundant smooth endoplasmic reticulum in control animals which were given only mineral oil can be attributed to their forced fast of 16 hours before sacrifice (37). Microbodies, consistently present in parenchymal cells of control animals, do not increase in number or alter their appearance during the period of poisoning studied.

Striking structural, functional, and compositional alterations of the mitochondria and endoplasmic reticulum of liver parenchymal cells occur in the first 24 hours after the administration of a single oral dose of carbon tetrachloride. These

FIGURE 8 Sixteen hours after poisoning with carbon tetrachloride. Cytoplasmic membrane systems of centrilobular liver parenchymal cells are severely disrupted and form large labyrinthine tubular aggregates. Numerous non-membrane-attached ribosomes are present in the cytoplasm. Although mitochondria are of normal density and size, matrix granules are either absent or reduced in number in the plane of section. × 11,000.


LTA
Rf
MG
LTA
Rer
Rf
PM
8

changes which accompany the massive influx of calcium into liver parenchymal cells (Table I) (12, 15, 16) are described in the order of their light and electron microscopic appearances and associated altered histochemical and compositional parameters.

Light Microscopy

Generalized cytoplasmic staining of centrilobular liver parenchymal cells with toluidine blue O progressively decreases during the first 24 hours after poisoning with carbon tetrachloride (Figs. 5 and 6) and that of periportal parenchyma increases in intensity (Figs. 2 and 3) (12). Concomitantly, an increased amount of lipid accumulates within the cytoplasm of both periportal and centrilobular parenchyma (Figs 2, 3, 5, and 6). In spite of decreased cytoplasmic staining, nuclei of centrilobular cells appear normal at 16 hours and membranes of adjacent cells are adherent (Fig. 5). At 24 hours, however, nuclear chromatin of cells in this zone is aggregated into discrete intensely basophilic masses, and plasma membranes of adjacent cells are separated (Fig. 6). Highly vacuolated cells containing many optically empty vacuoles in the periportal zones 16 hours after poisoning (Fig. 2) often contain punctate lipid droplets within these vacuoles at 24 hours (Fig. 3).

The accumulation of calcium in rat livers during the period from 8 to 24 hours after the oral administration of carbon tetrachloride (Table I) (12, 15, 16) is associated with the appearance of granular cytoplasmic deposits in midzonal and centrilobular liver parenchymal cells which stain intensively for calcium (Fig. 21) (12). These deposits, preserved by the methods of fixation and embedding for electron microscopy employed in this study,[1] also stain as punctate intramitochondrial deposits (Figs. 5 and 6) with toluidine blue O, a cationic dye with a known affinity for calcium-binding sites in tissues (40). Initially, when liver calcium content is increased only severalfold. intramitochondrial deposits staining intensely with alizarin red S (12) and toluidine blue O (Fig. 5) are localized in a few scattered midzonal parenchymal cells. With time and progressive increases in liver calcium content, granules in increasing numbers of midzonal and centrilobular cells stain both positively for this alkaline earth and with toluidine blue O until at 24 hours, when liver calcium content is increased twentyfold (Table I), all cells in the centrilobular half of the liver lobule stain intensely for this metal (Fig. 21) and its binding sites (Fig. 6).

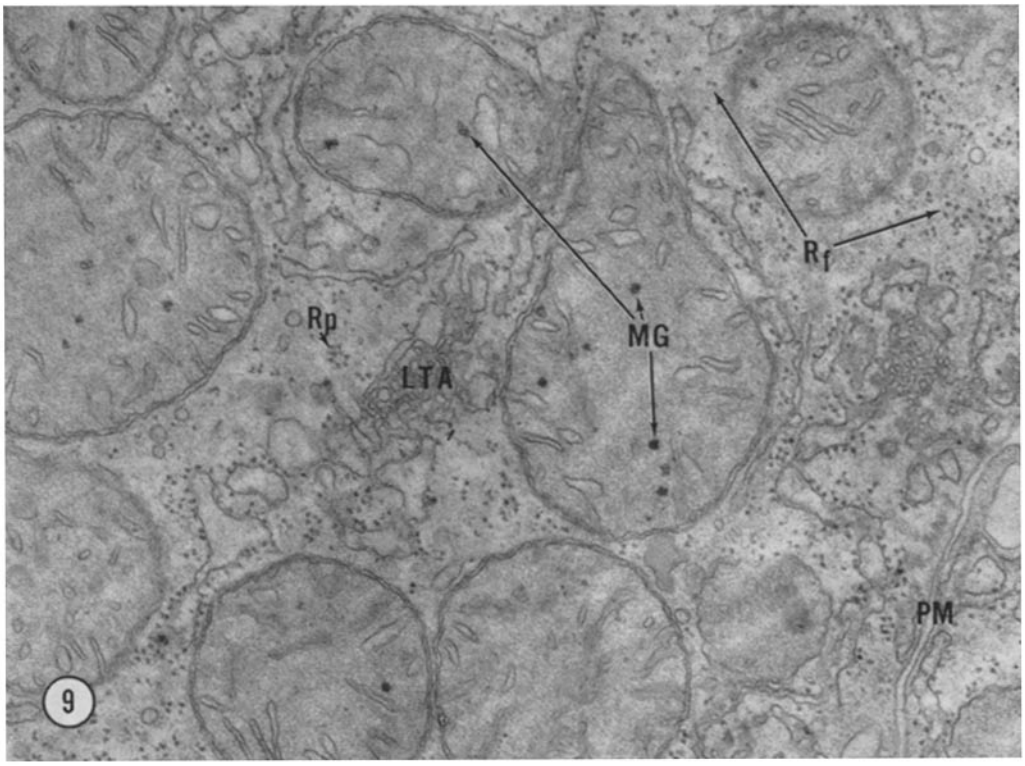
Electron Microscopy

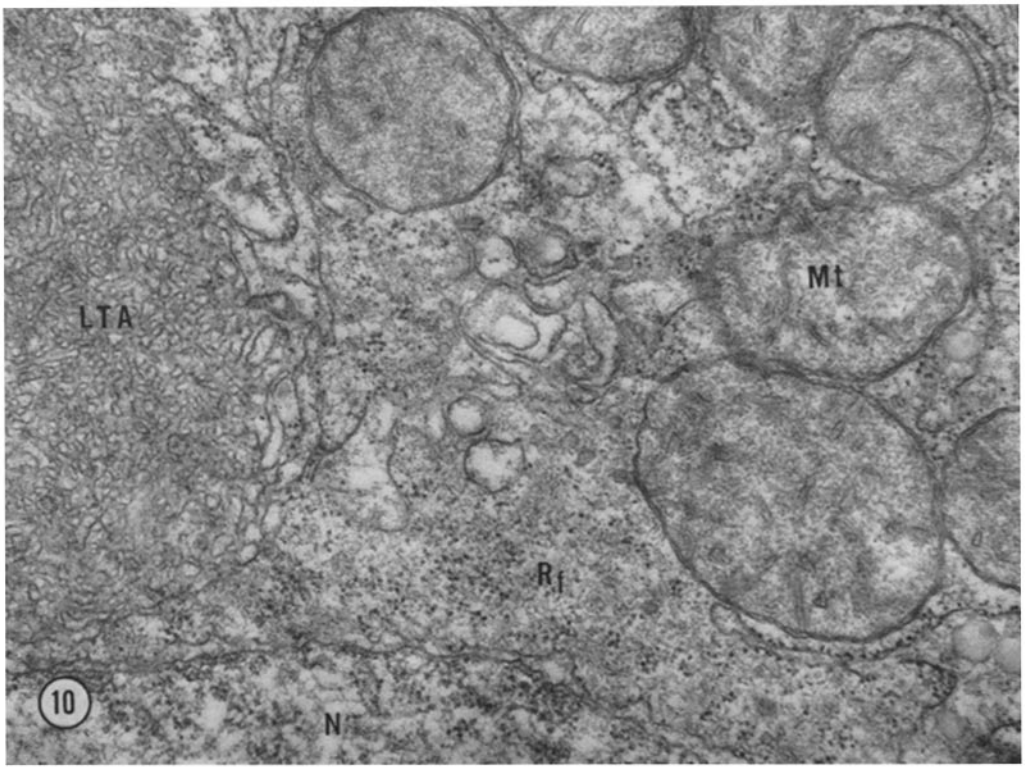
Mitochondria of the parenchymal cells of liver in control animals are slightly more electron opaque than adjacent cytoplasmic matrix and contain up to ten irregular electron-opaque matrix granules (300 to 400 A in diameter) per cross-section of mitochondrion (Fig. 7). These granules, which are normally found within mitochondria, are usually not associated with cristae mitochondriales (Fig. 7) in the plane of section and consist of aggregates of round or cylindrical electron-

[1] The intensity of staining of intramitochondrial masses with alizarin red S after fixation with aqueous osmium tetroxide is reduced as compared with results with frozen-dried or frozen-substituted material (12). Decreased staining with alizarin red S is due to displacement of calcium from its binding site within the mitochondrion and extraction of calcium from the tissue during aqueous fixation (39). Increasing the concentration of calcium in the fixative tends to increase the intensity of alizarin red S staining of intramitochondrial masses toward that found in comparable frozen-dried or frozen-substituted material. Lead staining of intramitochondrial masses, diminished after aqueous fixation, is also better preserved in fixatives with high calcium contents (39).

FIGURE 9 Cytoplasm of midzonal liver parenchymal cell 16 hours after poisoning. Small labyrinthine tubular aggregates are forming, and free ribosomes are seen in the cytoplasmic matrix. Normal matrix granules are present within mitochondria. × 42,000.

FIGURE 10 Cytoplasm of midzonal liver parenchymal cell 24 hours after poisoning. Part of large labyrinthine aggregate is present at left. Free ribosomes are present in the cytoplasmic matrix. Normal matrix granules are absent from mitochondria. × 42,000.


Rf
Rp
MG
LTA
PM
9


Mt
LTA
Rf
10
N

transparent bodies (≅ 75 A in diameter) within more electron-opaque material (Fig. 7, inset).

Within 16 hours after carbon tetrachloride poisoning (Figs. 8 and 10), normal matrix granules in the mitochondria of mid- and centrilobular parenchymal cells decrease in number and size. Indeed, many mitochondria in centrilobular cells contain no matrix granules at all in the plane of section (Fig. 10). In striking contrast, mitochondrial granules are not decreased in number periportally at this time or at 24 hours (Figs. 9 and 17).

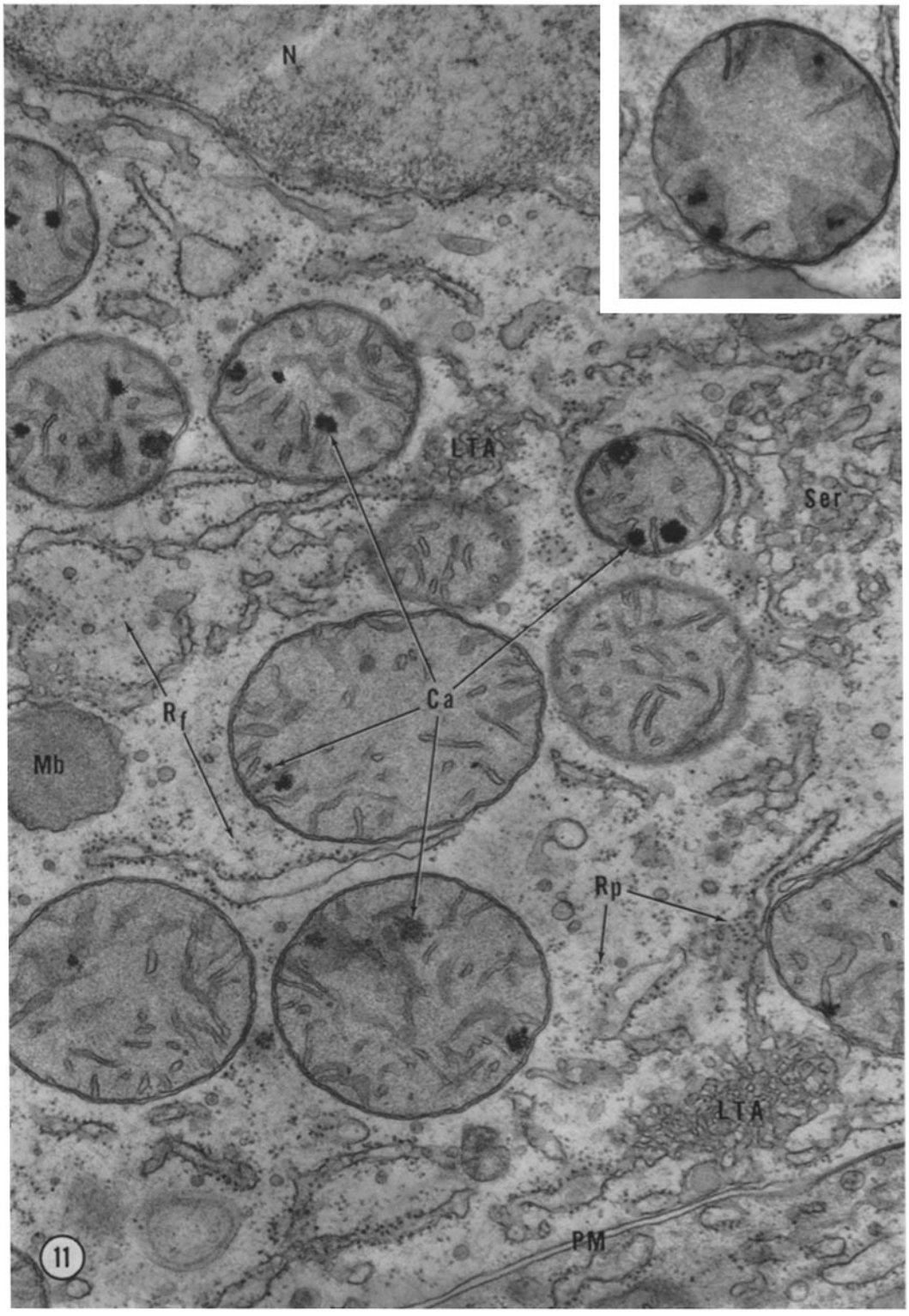
Electron-opaque masses (1000 to 2000 A in diameter) appear within mitochondria (Figs. 11 to 16) of midzonal and centrilobular cells which correspond in number, distribution, and appearance to those parenchymal cells staining positively for calcium and calcium-binding sites at 16 and 24 hours after poisoning (12) (Figs. 5, 6, 21, and 22). These masses, which differ in appearance and position from matrix granules normally present, are first observed as small clusters of electron-opaque granules in the mitochondrial matrix immediately adjacent to (Figs. 11 and 13) or on the surface of cristae mitochondriales (Fig. 11, inset). Granular electron-opaque masses may form in mitochondria which are morphologically indistinguishable from those of normal-appearing cells (Figs. 9 and 17). Between 16 and 24 hours, electron-opaque intramitochondrial masses increase in size and at the latter time may appear confluent (Fig. 12, inset; Fig. 16). Concomitantly, the matrix of the mitochondria involved decreases in density and its normal fine reticular pattern (Fig. 7, inset) is lost (Figs. 12 to 16). Although the amount of intramitochondrial accumulation of calcium phosphate at 16 hours in midzonal and centrilobular cells varies greatly between individual cells, many cells at this time being free of electron-opaque masses, all mitochondria are uniformly involved within any single cell containing these deposits (Figs. 5, 11 and 12). With continued calcium accumulation by the liver (Table II; Fig. 18), however, mitochondria of almost all centrilobular cells increase in size, have highly rarefied matrices, and contain large spherical or tubular shells of paracristal granular electron-opaque masses (Figs. 14 to 16).

The electron opacity of calcium-associated intramitochondrial matrix masses is qualitatively inherent in their native composition and is not attributable to impregnation with osmium tetroxide during fixation, or to staining with lead ion (Figs. 18 to 20). Although the latter treatment may enhance their density, granular matrix masses in unstained osmium tetroxide–fixed tissues (Figs. 18 and 19) are almost as opaque as those in stained tissues, on the one hand (Figs. 11 to 16), and in unstained, unosmicated, freeze-substituted or freeze-dried tissue, on the other (Fig. 20). Selected area electron diffraction of each of the mitochondria shown in Figs. 18 to 20 and other areas in the same sections failed to reveal that these masses are crystalline. The appearance of some of the masses suggests that they may, however, consist of radiating clusters of fine electron-opaque fibrils or spicules (Fig. 11; Fig. 12, inset; Figs. 13, 14, 16, 18, and 20).

The well ordered cytoplasmic membrane systems of the smooth and granular endoplasmic reticulum normally found in centrilobular parenchymal cells (Fig. 7) are also severely disrupted after poisoning (Figs. 8 to 10, 12) (24, 41, 42). This disruption is characterized by degranulation of the rough endoplasmic reticulum and conversion of both smooth and rough endoplasmic reticulum (Figs. 8 to 11) into large labyrinthine aggregates of closely packed smooth-surfaced tubules. Increased amounts of free ribosomes and finely

FIGURE 11 Part of cytoplasm of midzonal parenchymal cells 16 hours after poisoning. Although mitochondria are denser than cytoplasm, normal matrix granules are absent. Small granular electron-opaque deposits of varying size are present within the mitochondrial matrix in close association with internal mitochondrial membranes. Loosely aggregated ribosomal complexes are present within the cytoplasm, some of which are in close association with the cisternae of the endoplasmic reticulum. Non–membrane-bound ribosomes are often smaller and less electron opaque than membrane-bound ribosomes. × 34,000.

Inset. Six electron-opaque deposits are present upon the surfaces of cristae mitochondriales parallel to the plane of section. × 32,000.


N
LTA
Ser
Ca
Rf
Mb
Rp
D
LTA
PM
11

granular material of smaller dimensions are present in intracellular areas depleted of endoplasmic reticulum (Figs. 8, 9 and 11) at 16 hours, and non-membrane-associated multiribosomal complexes within the cytoplasm of these cells are poorly organized (Figs. 8 to 16). In spite of this severe disturbance of membrane systems of all centrilobular cells at this time, mitochondria of most of these cells appear relatively normal (Fig. 8). Conversely, intramitochondrial electron-opaque deposits may be observed in midzonal cells with only slightly damaged granular endoplasmic reticulum (Fig. 11). At 24 hours, however, granular debris is lost from the cytoplasm of centrilobular cells, the cytoplasmic matrix of these cells appears empty (Fig. 16), and calcium-associated electron-opaque masses are uniformly present within their mitochondria (Fig. 6).

In contrast, although the granular endoplasmic reticulum of periportal parenchymal cells is dispersed throughout the cytoplasm, it is otherwise normal in appearance at 16 and 24 hours. In addition, increased numbers of well formed polyribosomal complexes are present within their cytoplasmic matrix (Fig. 17).

Histochemistry

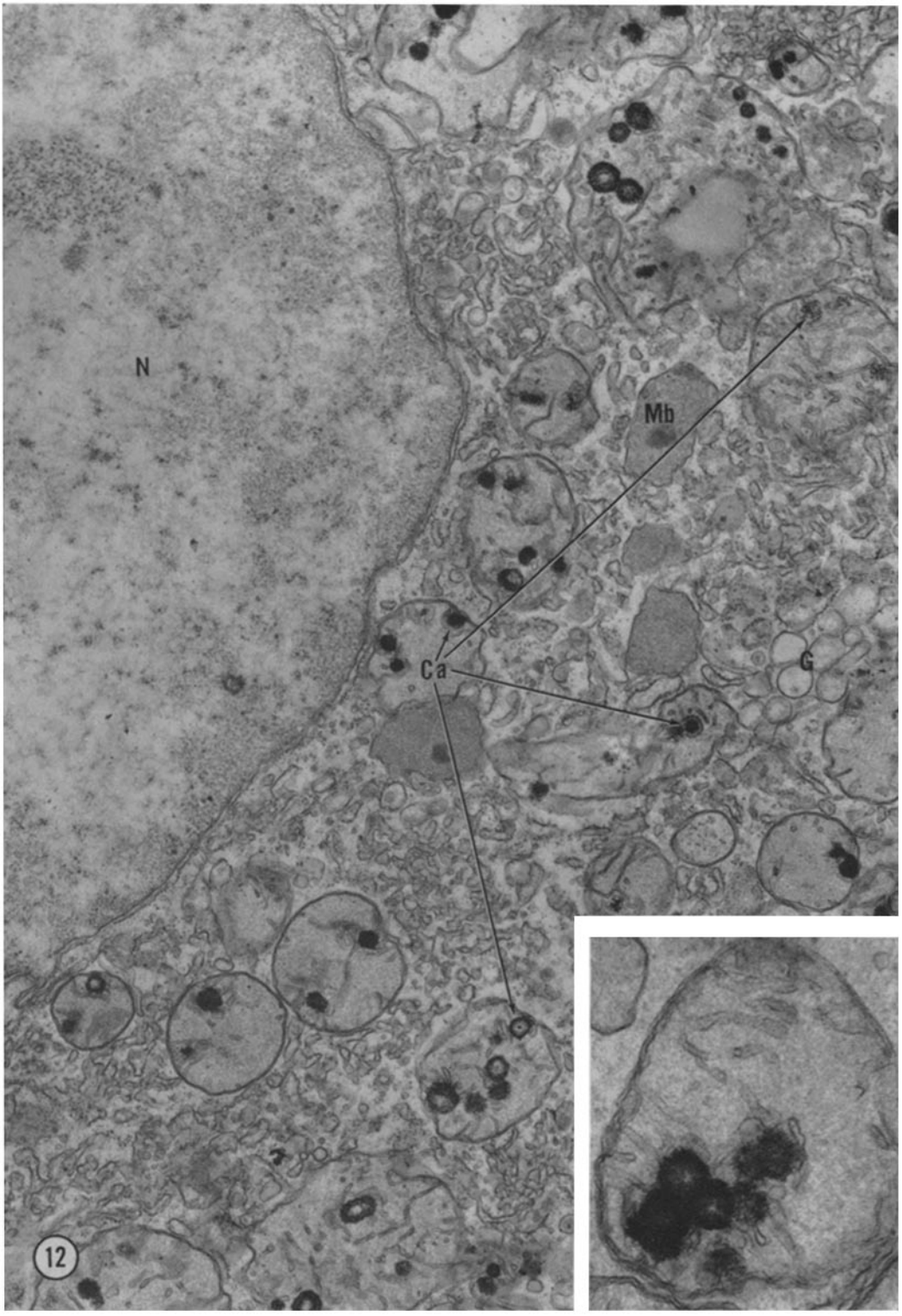
Mitochondrial ATPase activity, *i.e.*, magnesium-dependent ATPase, persists in calcium-rich centrilobular parenchymal cells 24 hours after poisoning (Figs. 21 and 22). Indeed, its activity appears to be more intense than that seen periportally at this time (Fig. 23). This apparent increase in centrilobular ATPase activity is, however, due to the staining of preexisting cation-binding sites with lead ion in mitochondria of calcium-containing cells. This staining, which can be demonstrated by dipping the section momentarily into the incubation medium for mitochondrial ATPase, increases in intensity only when magnesium is present during incubation, *i.e.*, under conditions where mitochondrial ATPase is activated (Figs. 22 and 23). Thus, calcium itself does not appear to activate mitochondrial ATPase, and the increased lead staining which appears during incubation (Fig. 23) is attributable to the persistence of mitochondrial ATPase activity in calcium-containing cells. In contrast to the persistence of mitochondrial ATPase activity, glucose-6-phosphatase (Fig. 24), pyrophosphatase, and acid and alkaline phosphatase (Figs. 25 and 26) activities are suppressed centrilobularly at this time.

Analytical

Increases in liver calcium content following poisoning with carbon tetrachloride are associated with increased inorganic phosphate content (Table I). Normally present in liver in tenfold greater amounts than calcium (Table I), inorganic phosphate almost doubles in amount within 24 hours, when its content is equimolar with that of calcium. This change in inorganic phosphate content is quantitatively balanced by decreased phosphoprotein content (Table I). As lipid phosphate and nucleic acid phosphate contents do not change in livers of poisoned animals, total liver phosphate contents are unchanged from those of controls.

Although it would appear from Table I that increased inorganic phosphate is derived from the splitting of cellular phosphoproteins, tracer studies with inorganic P^{32}-orthophosphate indicate it to be derived from serum inorganic phosphate (Table II). Administered inorganic P^{32}-orthophosphate, which reaches maximal specific activities in the serum within 1 hour (43), is rapidly incorporated into and retained by the liver inorganic phosphate in normal animals (Table II). Its incorporation into lipid phosphate, protein phosphate, and nucleic acid phosphate pools is slower, as indicated by the three-, five-, and twelvefold lower specific activities of these pools, respectively, 8 hours after administration of the label (Table II). Massive influxes of calcium and altered phosphate distribu-

FIGURE 12 Part of nucleus and cytoplasm of midzonal parenchymal cell 16 hours after poisoning. Large spherical electron-opaque masses, 2000 to 4000 A in diameter and consisting of one or more concentric rings of electron-opaque granules, are present within the mitochondrial matrix adjacent to cristae. Density of mitochondria is equal to that of adjacent cytoplasmic matrix. Cytoplasmic membrane systems are disrupted. × 18,000.
Inset. Large electron-opaque masses are closely associated with cristae. × 51,000.


N
Mb
Ca
G
12

tion following poisoning (Table II) do not alter these relationships. Thus the high specific activity of the inorganic phosphate accumulated to balance calcium following poisoning could not have been derived from cellular phosphoproteins with low specific activities.

DISCUSSION

Electron-opaque masses which form within the matrix of liver cell mitochondria following poisoning with carbon tetrachloride are in all probability sites of precipitation of markedly increased amounts of calcium and inorganic phosphate concentrated by mitochondria. These granular masses are morphologically identical with masses of calcium phosphate which form in isolated respiring mitochondria exposed to calcium and inorganic phosphate *in vitro* (2) and are similar in appearance to granular electron-opaque intramitochondrial deposits which are associated with hydroxyapatite masses formed in renal epithelial cells of parathormone-treated rats (9). After poisoning, the site of initial deposition of electron-opaque masses within the matrix adjacent to cristae mitochondriales is in a locus similar to that of electron-opaque deposits which result from electron-histochemical demonstration of mitochondrial ATPase activity (3, 44).

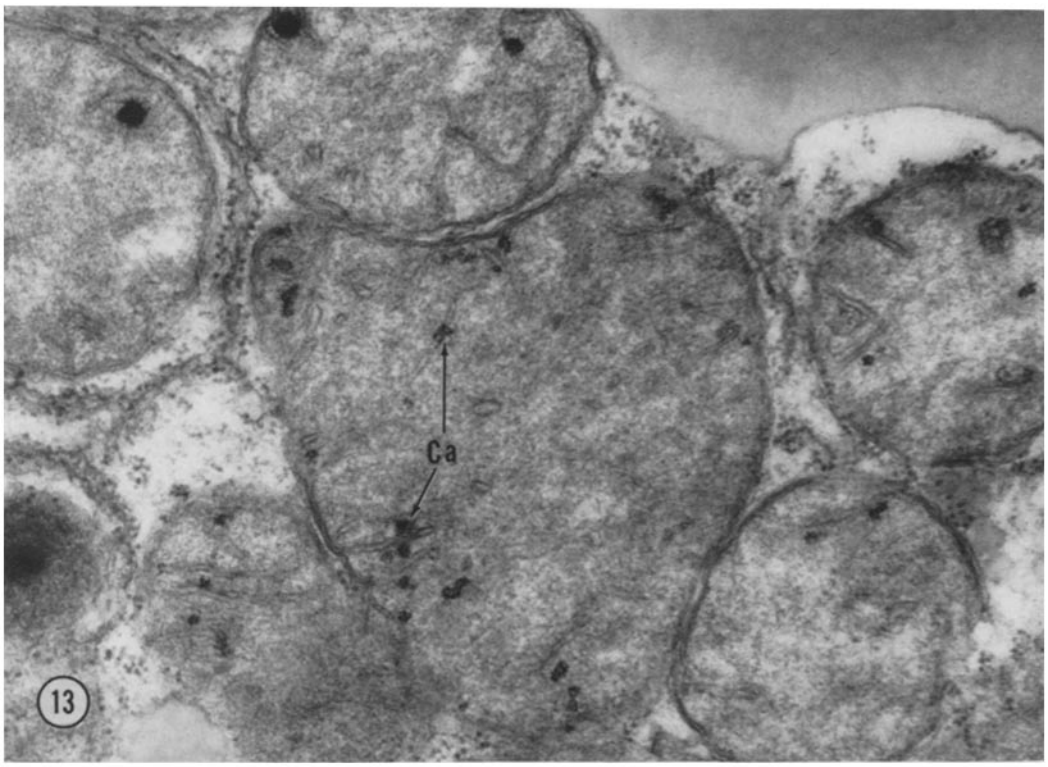
Intramitochondrial deposits of calcium phosphate in livers of carbon tetrachloride–poisoned rats are morphologically and positionally distinct from electron-opaque matrix granules normally found in mitochondria (45). Normal matrix granules consist of small (300 to 400 A), irregular, electron-opaque bodies which for the most part are unrelated to cristae mitochondriales and within which is present an array of 75-A electron-transparent subunits. Normal matrix granules disappear from mitochondria of some normal cell populations during physiological activity (46) and following cellular injury (47, 48). Indeed, in the case of the studies presented here, matrix granules normally present disappear from mitochondria before the appearance of intramitochondrial calcium-associated electron-opaque masses. Thus it does not appear that these latter masses, which arise in close association with the internal surfaces of the cristae mitochondriales following carbon tetrachloride poisoning, arise from randomly distributed matrix granules normally found in mitochondria.

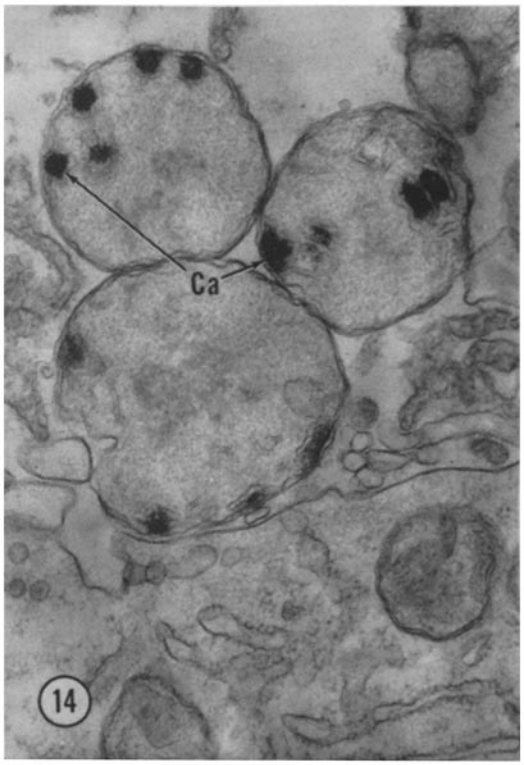
The accumulation of massive amounts of calcium by mitochondria isolated from livers of normal rats is considered to be secondary to both electron transport–linked and the ATPase-linked intramitochondrial accumulation of inorganic phosphate (2, 4, 6). Similar processes may occur within the mitochondria of liver parenchymal cells following poisoning, as mitochondria of calcium-sequestering cells are capable of continuing respiration (12) and retain their ATPase activity (Fig. 23), and the total liver inorganic phosphate (orthophosphate) rises to account for *at least* all increased calcium bound (Ca/P mole ratio = 1.0, Table I). Although inorganic phosphate is actively accumulated metabolically by mitochondria under these conditions (4, 6), the actual mechanism by which calcium accumulates is obscure (16, 49, 50). Normal liver mitochondria sequester up to twentyfold their initial calcium contents under conditions (0–4°C) where metabolic processes are markedly slowed (16). Such binding, which has previously been attributed to calcium sequestration by phospholipid compo-

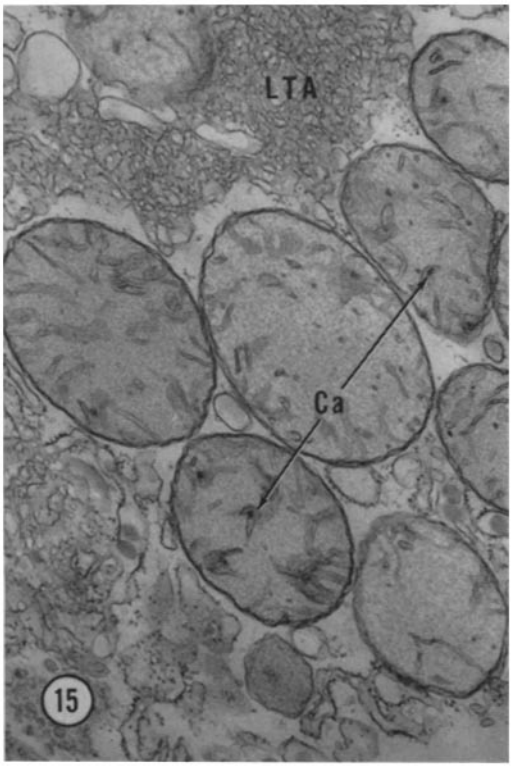
FIGURE 13 Small electron-opaque masses are closely adjacent to cristae of mitochondria in this midzonal liver parenchymal cell 24 hours after poisoning. Larger masses are present in adjacent mitochondria. Polysomes are present in the adjacent cytoplasmic matrix. × 49,000.

FIGURE 14 Some electron-opaque masses present in these mitochondria 24 hours after poisoning are cylindrical and all are enveloped by cristae mitochondriales. Within the central parts of the mitochondrial matrix are irregular areas of slightly increased density. × 37,000.

FIGURE 15 Mitochondrial matrix density is decreased to varying degrees in this cel 24 hours after poisoning. Denser mitochondria contain small electron-opaque masses which are closely associated with the cristae. Adjacent cytoplasm is disorganized, and labyrinthine tubular aggregates are present. × 29,000.


Ca
13


Ca
14


LTA
Ca
15

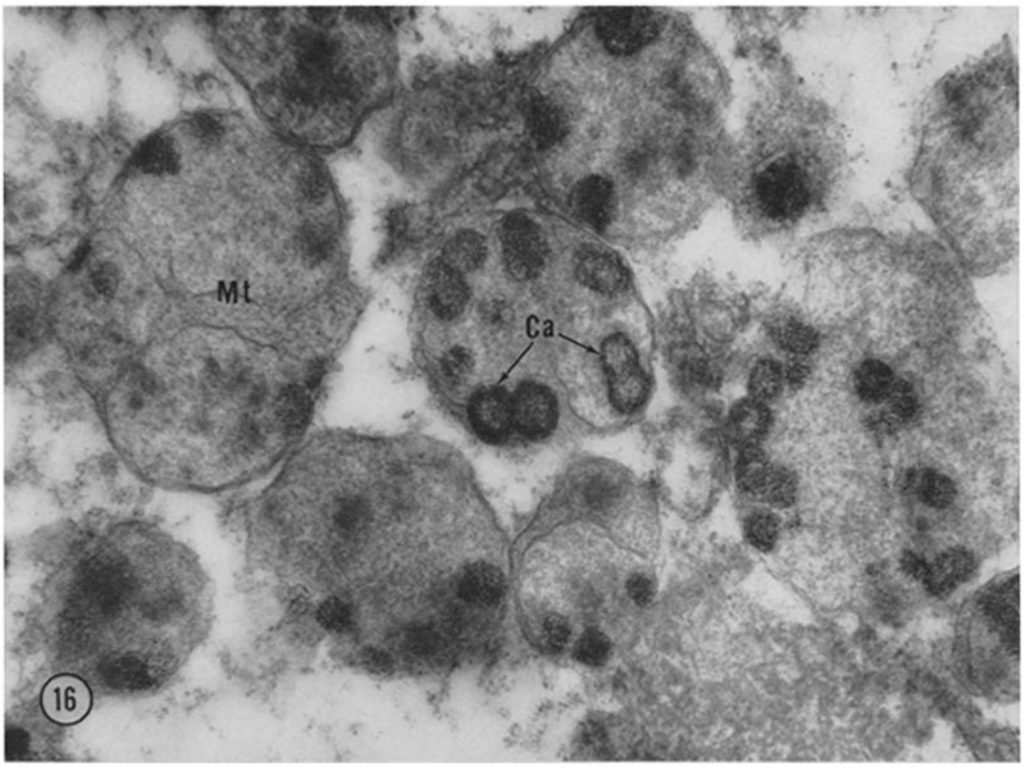
nents of mitochondrial membranes (16, 49), is not known to be respiration linked or energy requiring, although calcium ion in low concentrations and in the absence of added phosphates affects mitochondrial respiration patterns (50). The simultaneous or sequential presentation of calcium and phosphate to respiring mitochondria, or of calcium together with ATP, may result in a condition where calcium, at first passively sequestered by the mitochondrial membranes, is transported into the interior of the mitochondrion and precipitates with metabolically concentrated inorganic phosphate in the mitochondrial matrix (2, 6, 50). Such a mechanism may operate in mitochondria which accumulate up to 500-fold their normal calcium contents *in vivo* (12) and *in vitro* (2, 5, 6).

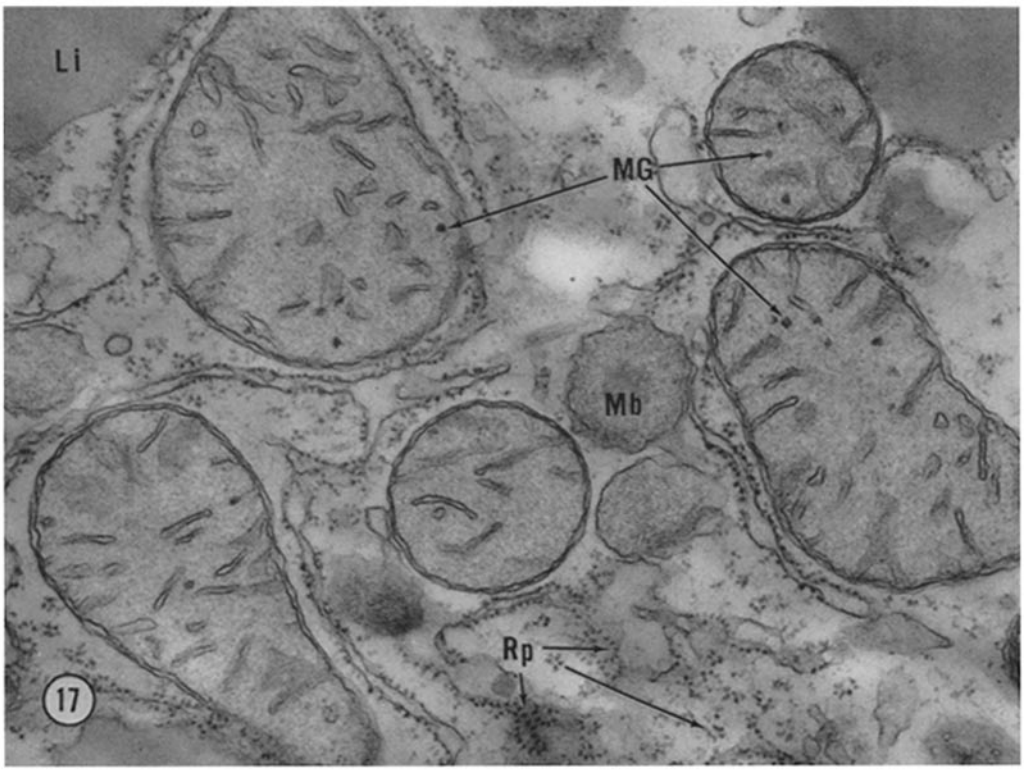
Calcium-associated electron-opaque masses which form in the mitochondria of liver cells of carbon tetrachloride–poisoned rats are most probably precipitates of calcium phosphate, similar in composition to those observed in mitochondria *in vitro* by Rossi, Lehninger, and Greenawalt (2, 6). The fact that crystalline patterns of hydroxyapatite were not elicited upon selected area electron diffraction of the masses formed either *in vivo* (Figs. 18 to 20) or *in vitro* (2) does not necessarily mean that they are not hydroxyapatite crystals, but that they may be present in amounts and concentrations below the limits of detection of the instruments employed. Caulfield (10), who was able to obtain hydroxyapatite electron diffraction patterns on cytoplasmic deposits in renal epithelial cells of parathormone-treated rats, was similarly unable to obtain evidence of crystallinity in the electron-opaque mitochondrial deposits in the same cells (51). In the face of such findings, the possibility that the intramitochondrial deposits are not hydroxyapatite must be considered. Amorphous calcium phosphate deposits do not give a crystalline electron diffraction pattern (52). Nor would one occur if the calcium and phosphate were deposited as an amorphous mineral-organic complex. Indeed, the finding that toluidine blue O, a cationic metachromatic dye with an affinity for the organic matrix of calcifying extracellular systems (40, 53), intensely stains calcium-associated intramitochondrial deposits following poisoning also suggests that these masses may have an organic calcium-binding component.

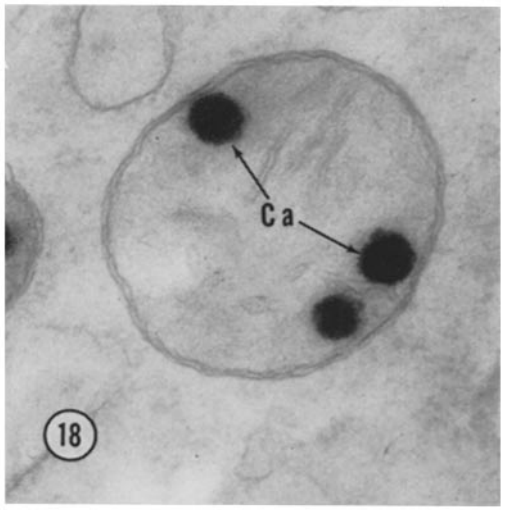
Precipitates of calcium phosphate initially appear in mitochondria which, although they have no normal matrix granules, are otherwise normal in appearance. As the amount of precipitate increases, mitochondria become swollen, or degenerate in appearance. These changes do not appear to be related as to cause and effect, however, as the amount of precipitate found is not directly proportional to the degree of mitochondrial degeneration observed. Relatively normal-appearing mitochondria can contain massive calcium phosphate deposits—particularly at early times after poisoning (Fig. 12)—while obviously swollen mitochondria may contain smaller deposits (Fig. 15). When calcium ion in relatively high concentration ($> 10^{-3}$ molar) is presented to mitochondria in the absence of phosphate, swelling, loss of potassium, and, ultimately, respiratory failure result (16, 49, 54–56). Simultaneous presentation of inorganic phosphate may tend to lessen the direct effect of calcium on mitochondrial structure and functions *in vitro* as calcium phosphate is deposited in the matrix (2, 4–6, 50). Normally calcium is present within the cell together with a large excess of inorganic phosphate (12.3 μmoles/gm) (Table I), and liver calcium content (1.0 μmole/gm) is less than half that of serum calcium (2.5 μmoles/ml). Progressive accumulation of calcium by the cell following poisoning with carbon tetrachloride equalizes intracellular calcium and inorganic phosphate contents (Table II). As a result, intra-

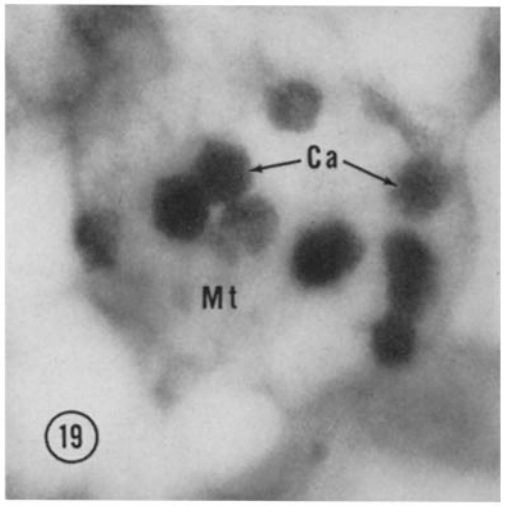
FIGURE 16 Mitochondria of centrilobular parenchymal cells 24 hours after poisoning are degenerate, with poor preservation of cristae and outer membranes. Granular electron-opaque masses within the matrix are large (4000 A) and are closely associated with the mitochondrial membranes. In matrix, irregular amorphous areas of slightly increased density (see also Fig. 13) are present centrally. × 34,000.

FIGURE 17 Part of cytoplasm of periportal parenchymal cell 24 hours after poisoning. Mitochondria appear normal. Normal matrix granules are present. Elements of rough endoplasmic reticulum are well granulated. Numerous polysomes are seen within the cytoplasmic matrix. × 40,000.


Mt
Ca
16


Li
MG
Mb
Rp
17





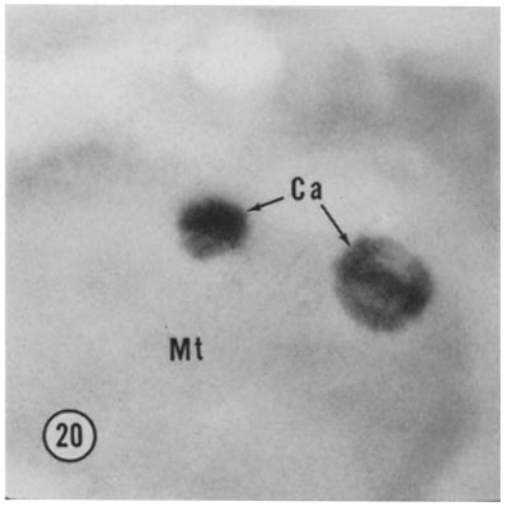
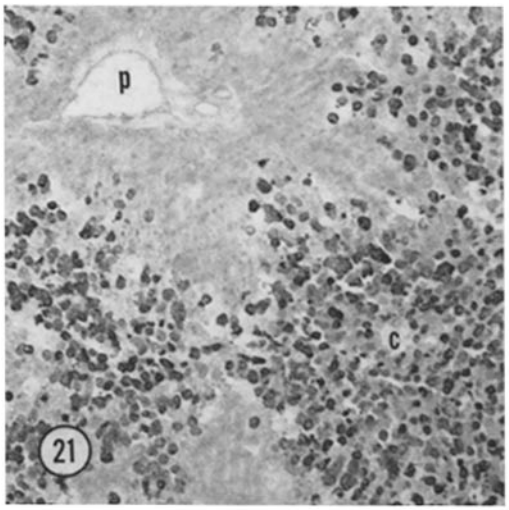
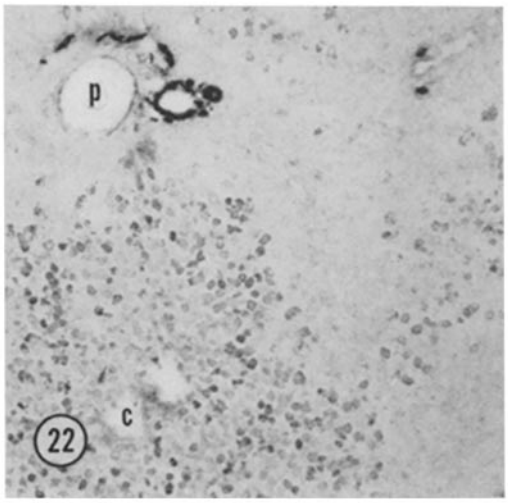


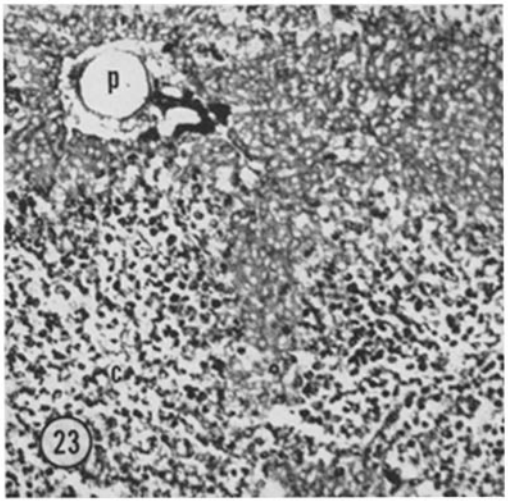
FIGURE 18 Electron-opaque intramitochondrial matrix granules in livers of rats, 24 hours after poisoning, in unstained sections are similar to those in sections stained with lead citrate (Figs. 11, 12, 14 and 16). × 58,000.

FIGURE 19 The appearance of electron-opaque intramitochondrial masses, at 24 hours after poisoning, in tissues freeze-substituted in acetone containing 1 per cent OsO_4. Section is not stained with lead. × 58,000.

FIGURE 20 Electron - opaque intramitochondrial masses, at 24 hours after poisoning, in tissues freeze-substituted in acetone. Section is unstained. A fine pattern of electron-opaque fibrils within the masses can be observed. × 58,000.





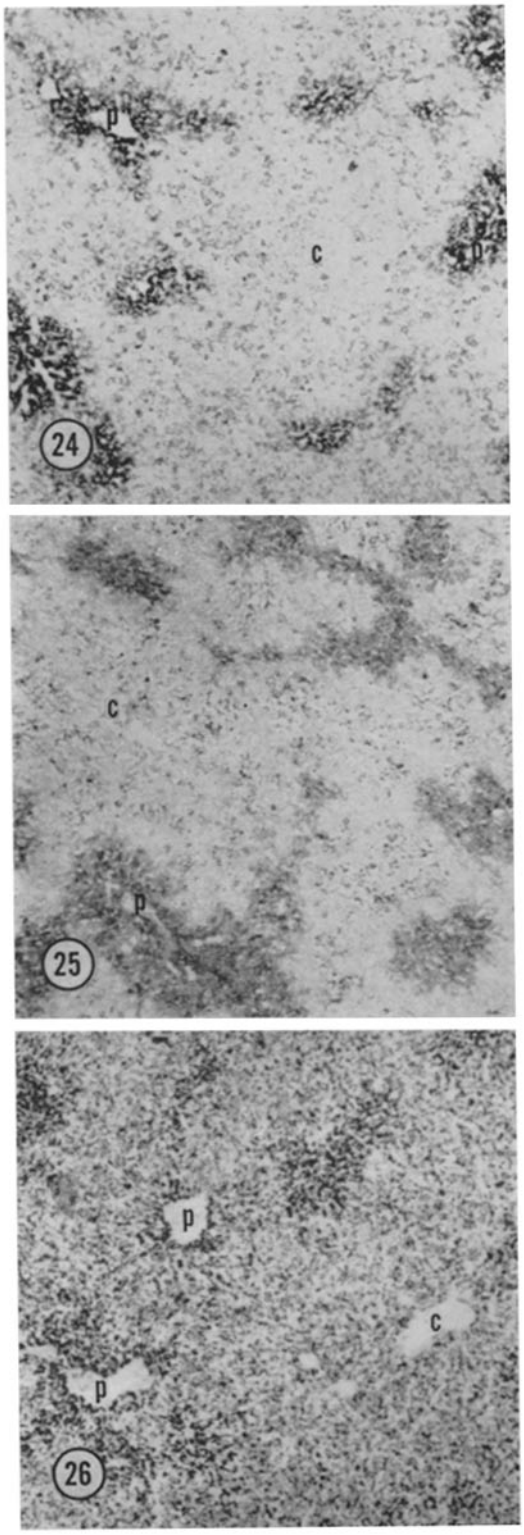


FIGURES 21 TO 23 Liver of rat 24 hours after poisoning. Fresh-frozen serial sections, 8 μ. (680 μg Ca/gm liver.) $\times$ 100.

Fig. 21. Calcium is massively increased in all centrilobular parenchymal cells. Alizarin red S staining.

Fig. 22. Centrilobular "mitochondrial ATPase" activity is due to lead binding—presumably by increased inorganic phosphate accumulated *in vivo*—in cells with increased calcium contents. Similar "mitochondrial ATPase" activities are obtained by momentarily dipping the section in the incubation medium. ATPase (pH 7.4) without additions.

Fig. 23. Magnesium-dependent, 2,4-dinitrophenol–stimulated mitochondrial ATPase is present both periportally and in centrilobular cells with increased calcium contents. Denser centrilobular precipitates of lead phosphate may be due to the superimposition of mitochondrial ATPase activity in cells with already increased inorganic phosphate contents. ATPase (pH 7.4) with 10^{-2} M $MgSO_4$ and 5×10^{-4} M 2,4-DNP added.



FIGURES 24 TO 26 Liver of rat 24 hours after poisoning. Glucose-6-phosphatase (Fig. 24), alkaline phosphatase (Fig. 25), and acid phosphatase (Fig. 26) activities are decreased centrilobularly. Lead staining in centrilobular areas in Figs. 24 and 25 is in mitochondria of calcium-rich cells. Fresh-frozen 8-μ sections. $\times$ 60.

TABLE II

Comparison of Pattern of Inorganic P^{32} Incorporation into Phosphates of Livers of Carbon Tetrachloride–Poisoned Animals with the Pattern in Control Animals

Controls were fed mineral oil, and experimentals were given carbon tetrachloride 24 hours before sacrifice. Sixteen hours after feeding, at a time corresponding to the onset of maximal rates of calcium sequestration by livers of experimental animals (12), 200 μc $Na_2HP^{32}O_4$ was administered intraperitoneally.

Calcium and inorganic phosphate contents of control and experimental animals	Specific activity of phosphate			
	Liver			Serum inorganic
	Inorganic	Protein	Total	
	μmole/mg N	*cpm/mμmole PO_4*		
Control no. 1 (Ca = 0.030; PO_4 = 0.28)	21.3	3.1	7.1	9.9
Control no. 2 (Ca = 0.035; PO_4 = 0.32)	21.2	2.9	6.3	9.7
Experimental no. 1 (Ca = 0.47; PO_4 = 0.38)	25.0	4.4	6.9	7.8
Experimental no. 2 (Ca = 0.50; PO_4 = 0.44)	22.8	4.7	8.7	8.8

cellular inorganic phosphate contents may become limited. Exhaustion of inorganic phosphate in cells which continue to be permeable to calcium would be expected to result in disruption of mitochondrial structure and function owing to the presence of excess calcium. As liver parenchymal cells which begin to accumulate calcium at different times after poisoning are in different states of cytoplasmic degeneration, such changes may reflect different degrees of loss of cytoplasmic inorganic phosphate within the cell prior to calcium uptake.

In addition to the direct effects of calcium and inorganic phosphate on mitochondria, their uptake by mitochondria within the cell would result in a decreased net synthesis of high energy phosphate compounds (6, 57). ATP contents are markedly decreased in livers of carbon tetrachloride–poisoned rats (58). Failure of mitochondrial production of ATP would be expected to compromise energy-requiring synthetic functions necessary for cell survival, and eventually complete exhaustion of ATP would result in aggravation of calcium- and inorganic phosphate–induced mitochondrial alterations (16, 49, 54–57). Owing to the fact that isolated mitochondria require ATP for the accumulation of calcium and phosphate *in vitro* (6, 57), the exhaustion of intracellular ATP contents may also be a regulatory factor in the eventual size of the mitochondrial electron-opaque masses observed within cells *in vivo*.

Although calcium appears to be primarily responsible for the mitochondrial lesion in carbon tetrachloride poisoning, its entrance into the cell is secondary to altered plasma membrane permeability. Damaged function of this vital structure, either as a result of direct attack of the hepatotoxin (20) or indirectly through destruction of the protein synthetic properties of the granular endoplasmic reticulum (41) or ATP depletion would tend to result in an increased permeability of the membrane to calcium.

Quantitatively balanced decreases in phosphoprotein and increases in inorganic phosphate contents of liver following carbon tetrachloride poisoning do not appear to be directly related. Inorganic phosphate which accumulates in livers of experimental animals is derived from serum inorganic phosphate, as indicated by its relatively high specific activity (Table II). This is in contrast to the low specific activity expected were it derived directly from the splitting of cellular phosphoproteins. Since microsomal and nuclear fractions of normal liver are particularly rich in phosphoproteins (31 and 34 μmoles PO_4 per gm protein, respectively), degranulation of the rough endoplasmic reticulum (41, 42), formation of labyrinthine aggregates of smooth-surfaced tubules

from components of the endoplasmic reticulum (Fig. 8), and nuclear alterations (Fig. 6) may be due, in part, to splitting of phosphoproteins normally present.

The presence of a magnesium-activated, 2,4-DNP–stimulated mitochondrial ATPase activity in normal liver (27) and its persistence following carbon tetrachloride poisoning (Fig. 12) is in direct contrast to the shift from a 2,4-DNP– to a magnesium-activated ATPase observed in mitochondria isolated from livers of poisoned animals (17). Morphologic (59) and compositional (49) alterations resulting from the procedure employed to isolate mitochondria would tend to compound the mitochondrial lesion observed within intact cells and may account for the apparent shift in the properties of mitochondrial ATPase activity. Conversely, the presence of lead ion (3×10^{-3} M) in the medium used for the histochemical determination of mitochondrial ATPase activity may suppress 2,4-DNP–activated ATPase activity in normal mitochondria (60). In any event, magnesium appears to be a specific requirement for activation of mitochondiral ATPase activity determined histochemically, as its activity is apparently unaffected by increased intramitochondrial calcium content following poisoning.

The author would like to thank Dr. S. James Adelstein for the use of his laboratory facilities, in which the chemical studies were carried out, and Dr. James B. Caulfield for instruction in electron diffraction.

This work was supported in part by grants H-1771 and AM-7979 from the National Institutes of Health, United States Public Health Service. A preliminary report of these studies has been presented (1).

The author is a Special Fellow (GSP-7309) of the Division of General Medical Sciences, National Institutes of Health, United States Public Health Service.

Received for publication, August 12, 1964.

REFERENCES

1. Reynolds, E. S., *J. Cell Biol.*, 1963, **19,** 58A (abstract).
2. Greenawalt, J. W., Rossi, C. S., and Lehninger, A. L., *J. Cell Biol.*, 1964, **23,** 21.
3. Ashworth, C. T., Luibel, F. J., and Stewart, S. C., *J. Cell Biol.* 1963, **17,** 1.
4. Engstrom, G. W., and DeLuca, H., *Biochemistry*, 1964, **3,** 379.
5. Vasington, F. D., and Murphy, J. V., *J. Biol. Chem.*, 1962, **237,** 2670.
6. Rossi, C. S., and Lehninger, A. L., *Biochem. Z.*, 1963, **338,** 698.
7. Peachey, L. D., *J. Cell. Biol.*, 1964, **20,** 95.
8. Gonzales, F., and Karnovsky, M. J., *J. Biophysic and Biochem. Cytol.*, 1961, **9,** 299.
9. Lafferty, F., Reynolds, E. S., and Pearson, E. H., *Am. J. Med.*, 1965, **38,** 106.
10. Caulfield, J. B., and Schrag, P. E., *Am. J. Path.*, 1964, **44,** 365.
11. Bessis, M., and Breton-Gorius, J., *J. Biophysic. and Biochem. Cytol.*, 1959, **6,** 231.
12. Reynolds, E. S., *Lab. Invest.*, 1964, **13,** 1457.
13. Gallagher, C. H., Gupta, D. N., Judah, J. B., and Rees, K. R., *J. Path. and Bact.*, 1956, **72,** 193.
14. Kratzing, C. C., Dunstone, J. R., Madsen, N. P., MacDonald, P., and Bell, H., *Biochem. Pharmacol.*, 1960, **3,** 272.
15. Thiers, R. E., Reynolds, E. S., and Vallee, B. L., *J. Biol. Chem.*, 1960, **235,** 2130.
16. Reynolds, E. S., Thiers, R. E., and Vallee, B. L., *J. Biol. Chem.*, 1962, **237,** 3546.
17. Recknagel, R. O., and Anthony, D. D., *J. Biol. Chem.*, 1959, **234,** 1052.
18. Bennett, H. S., and Luft, J. H., *J. Biophysic. and Biochem. Cytol.*, 1959, **6,** 113.
19. Millonig, G., *J. Appl. Phys.*, 1961, **32,** 1637.
20. Reynolds, E. S., *J. Cell Biol.*, 1963, **19,** 139.
21. Reynolds, E. S., *J. Cell Biol.*, 1963, **17,** 208.
22. Luft, J. H., *J. Biophysic. and Biochem. Cytol.*, 1961, **9,** 409.
23. Richardson, K. C., Jarett, L., and Finke, E. H., *Stain Technol.*, 1960, **35,** 313.
24. McGee-Russell, S. M., *J. Histochem. and Cytochem.*, 1958, **6,** 22.
25. Gomori, G., Microscopic Histochemistry, Chicago, Ill., University of Chicago Press, 1952.
26. Wachstein, M., and Meisel, E., *Am. J. Clin. Path.*, 1957, **27,** 13.
27. Padykula, H. A., and Gauthier, G. F., *J. Cell Biol.*, 1963, **18,** 87.
28. Wachstein, M., and Meisel, E., *J. Histochem. and Cytochem.*, 1956, **4,** 592.
29. Eranko, O., and Hasan, J., *Acta Path. et Microbiol. Scand.*, 1954, **35,** 563.
30. Kurata, Y., and Maeda, S., *Stain Technol.*, 1956, **31,** 13.
31. Schmidt, G., and Thannhauser, S. J., *J. Biol. Chem.*, 1945, **161,** 83.
32. Davidson, J. M., and Smellie, R. M., *Biochem. J.*, 1952, **52,** 594.
33. Fiske, C. H., and SubbaRow, Y., *J. Biol. Chem.*, 1925, **66,** 375.

34. AMES, B. N., and DUBIN, D. T., *J. Biol. Chem.*, 1960, **235**, 769.
35. REYNOLDS, E. S., and LINDE, R. E., *Anal. Biochem.*, 1963, **5**, 246.
36. KOCH, F. C., and MCMEEKIN, T. L., *J. Am. Chem. Soc.*, 1924, **46**, 2066.
37. FAWCETT, D. W., *J. Nat. Cancer Inst.*, 1955, **15**, 1475.
38. PORTER, K. R., and BRUNI, C., *Cancer Research*, 1959, **19**, 997.
39. REYNOLDS, E. S., unpublished data.
40. SIMKISS, K., and TYLER, C., *Quart. J. Micr. Sc.*, 1957, **98**, 19.
41. SMUCKLER, E. A., ISERI, O. A., and BENDITT, E. P., *J. Exp. Med.*, 1962, **116**, 55.
42. OBERLING, C., and ROUILLER, C., *Ann. anat. path.*, 1956, **1**, 401.
43. HEVESY, G., *Ann. Rev. Biochem.*, 1940, **9**, 641.
44. SCARPELLI, D. G., and CRAIG, E. L., *J. Cell Biol.*, 1963, **17**, 279.
45. WEISS, J., *J. Exp. Med.*, 1955, **102**, 783.
46. ITO, S., *Abstr. 2nd Ann. Meeting Am. Soc. Cell Biol.*, 1962.
47. ASHFORD, T. P., and PORTER, K. R., *Abstr. Ist Ann. Meeting Am. Soc. Cell Biol.*, 1961.
48. TRUMP, B. F., GOLDBLATT, J. J., and STOWELL, R. E., *Lab. Invest.*, 1962, **11**, 986.
49. SLATER, E. C., and CLELAND, K. W., *Biochem. J.*, 1953, **55**, 566.
50. CHANCE, B., *in* Energy Linked Functions of Mitochondria, New York, Academic Press, Inc., 1963, p. 253.
51. CAULFIELD, J. B., personal communication.
52. WATSON, M. L., and ROBINSON, R. A., *Am. J. Anat.*, 1953, **93**, 25.
53. SOBEL, A. E., and BURGER, M., *Proc. Soc. Exp. Biol. and Med.*, 1954, **87**, 7.
54. HUNTER, F. E., JR., and FORD, L. J., *J. Biol. Chem.*, 1955, **216**, 357.
55. BERGER, M., *Biochim. et Biophysica Acta*, 1957, **23**, 504.
56. ERNSTER, L., and LOW, H., *Exp. Cell Research*, Suppl., 1955, **3**, 133.
57. BRIERLEY, G. P., *in* Energy Linked Functions of Mitochondria, (B. Chance, editor), New York, Academic Press, Inc., 1963, p. 237.
58. DIANZANI, M. U., *Biochem. J.*, 1957, **65**, 116.
59. DESPANDE, P. D., HICKMAN, D. D., and VON KORFF, R. W., *J. Biophysic. and Biochem. Cytol.*, 1961, **11**, 77.
60. PERSIJN, J. P., DAEMS, W. T., DEMAN, J. C. H., and MAIJER, E. A. F. H., *Histochemie*, 1961, **2**, 372.