LIVER PARENCHYMAL CELL INJURY

I. Initial Alterations of the Cell Following Poisoning with Carbon Tetrachloride

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ABSTRACT

The structure of the endoplasmic reticulum, plasma membrane, mitochondria, and Golgi apparatus of the liver parenchymal cell is strikingly altered within 1 hour following the administration of a single oral dose of carbon tetrachloride to rats. Progressive loss of glucose-6-phosphatase activity accompanies dispersal of the ergastoplasm. Electron microscopy reveals that these changes are associated with vacuolization of the cisternae of the granular endoplasmic reticulum, degranulation of its membranes, and the appearance of increased number of free ribosomes in the adjacent cytoplasmic matrix. Concomitantly, calcium enters the liver parenchymal cell and is sequestered by mitochondria. First increased at 30 minutes, calcium content is maximal at 1 hour and returns to normal at 2 hours. Although succinic and glutamic dehydrogenase activity patterns within the liver lobule are unaffected, liver cell mitochondria enlarge and some appear to fuse or assume cup-like configurations. Microvilli lining the space of Disse become irregularly indistinct and increasingly pleomorphic by 30 minutes when the plasma membrane becomes increasingly permeable to calcium. Golgi vesicles swell and discharge their granules during the period of poisoning studied. Although all the changes observed may be the result of direct interaction of carbon tetrachloride with the membranes of the cytoplasmic constituents of the liver parenchymal cell, the possibility that the irreversible changes observed in the granular endoplasmic reticulum may be due to the chemical interaction between the poison and this system is discussed.

INTRODUCTION

Carbon tetrachloride reaches maximum concentrations in liver parenchyma—approximately 1 mg CCl_4/gm liver—within 2 hours following its oral administration (2). Should this hepatotoxin, a relatively inert, sparingly water-soluble, non-polar lipid solvent (3), act directly on parenchymal cells of livers of intact rats, all lipid-containing structural components of the cell might well be affected by the presence of the solvent at these times. Thus, compositional, functional, and morphologic changes in all cytoplasmic membrane systems of the liver cell would be expected to occur during times of concentration of the lipid solvent by the liver.

Recent studies of the mechanism of action of carbon tetrachloride have demonstrated striking alterations in the endoplasmic reticulum of rat liver parenchymal cells within minutes following poisoning (4). These changes, which consist of dispersal of the ergastoplasm seen by light microscopy, and vacuolization of the cisternae of the granular endoplasmic reticulum, its degranulation, and the appearance of increased numbers of free ribosomes in the adjacent cytoplasmic matrix shown by electron microscopy (4, 5), coincide with the times of concentration of carbon tetrachloride by the liver (2) and are accompanied by altered enzymatic activities of the component parts of the endoplasmic reticulum at 2 hours (5–7). In contrast, swollen mitochondria have been reported to be infrequent (4, 5, 8) and the oxidative function of mitochondria unaffected during this initial 2-hour period (5, 6, 9). Alterations in other cytoplasmic components of the liver parenchymal cell have not been reported during this period (4, 5), although liver parenchymal cells swell and circulation of the blood is impaired (10).

The following studies demonstrate that the morphology of the plasma membrane, mitochondria, and Golgi apparatus of the liver parenchymal cell are, indeed, altered during the first 2 hours following the oral administration of a single dose

Key to Abbreviations

Bc'	Bile canaliculus	*Mt,*	Mitochondria
c,	Central vein	*mv,*	Microvillus
G,	Golgi apparatus	*N,*	Nucleus
Gly,	Glycogen	*p,*	Portal triad
Ku,	Kupffer cell cytoplasm	*Pm*	Plasma membrane
Li,	Lipid droplet	*Rbc,*	Red blood cell
Lym,	Lymphocyte	*Rer,*	Granular endoplasmic reticulum
Mb,	Microbody	*Ser,*	Agranular endoplasmic reticulum

FIGURES 1 to 3 Time course of dispersion of the ergastoplasm in parenchymal cells of liver following administration of CCl_4. Centrilobular liver parenchyma, frozen-dried, methacrylate embedded, 1-μ-thick sections, toluidine blue 0. × 650. (AFIP Neg. 63-179-1.)

FIGURE 1 Liver of normal fasted rat. The ergastoplasm is present as dense angular cytoplasmic masses (arrows).

FIGURE 2 Liver of rat 15 minutes following poisoning. The ergastoplasm is less distinctly clumped than that of the control.

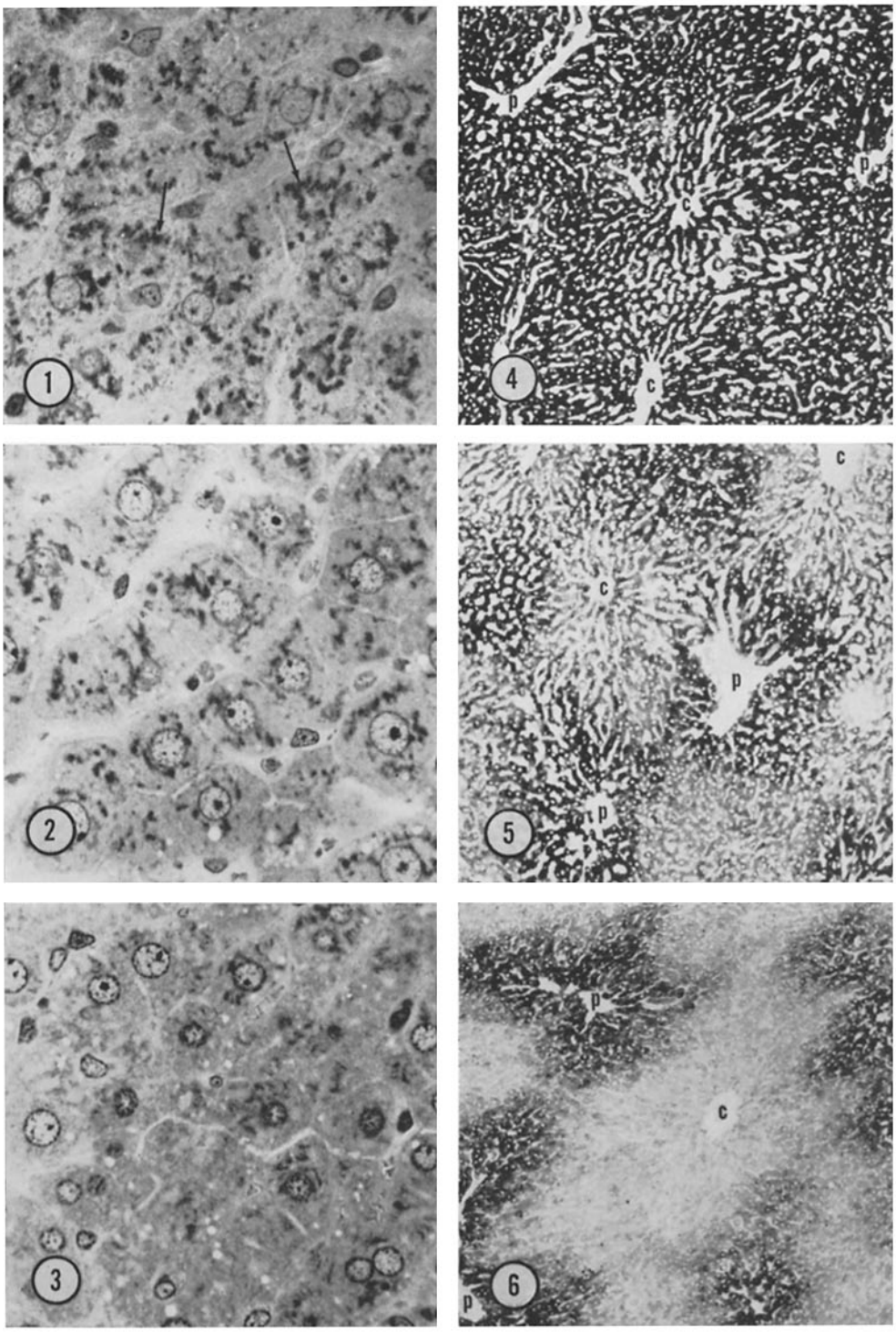
FIGURE 3 Liver of rat 60 minutes following poisoning. The ergastoplasm is no longer present as defined cytoplasmic masses. Basophilic material in the cytoplasm is now present as a faint webwork.

FIGURES 4 to 6 Depression of glucose-6-phosphatase in the liver lobule following administration of CCl_4. Fresh-frozen, cryostat sections, 6 μ thick, method of Wachstein and Meisel (23). × 65. (AFIP Neg. 63-179-1.)

FIGURE 4 Liver of control rat which received mineral oil 60 minutes prior to sacrifice Glucose-6-phosphatase activity of liver parenchymal cells (black areas) is uniformly distributed throughout the liver lobule as in normal fasted animals. Sinusoids and nuclei appear as white areas.

FIGURE 5 Liver of rat 15 minutes following poisoning. Glucose-6-phosphatase activity is moderately depressed centrilobularly.

FIGURE 6 Liver of rat 60 minutes following poisoning. Glucose-6-phosphatase activity remains only in the periportal zone parenchymal cells. Sinusoids are decreased in width at this time.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of carbon tetrachloride to the animal. In addition, these changes are associated with altered properties of the plasma membrane, such that it permits entrance of calcium into the cell, and alterations of the membranes of the endoplasmic reticulum resulting in loss of a membrane-associated (7) enzymatic activity—glucose-6-phosphatase. Succinic and glutamic dehydrogenase activities are unaltered during this period, in spite of the presence of carbon tetrachloride within the cell and sequestration of increased amounts of calcium by mitochondria. Oral administration of the poison was employed, so that these findings could be directly compared with previous findings (9, 11–14) and analytic data concerning the presence of the solvent in the liver (2).

MATERIALS AND METHODS

Healthy young male rats (Sprague-Dawley derived, Walter Reed Army Institute of Research) weighing between 150 and 250 gm were maintained on a diet of Purina chow and water *ad libitum*. Replicate rats were sacrificed at 0, 15, 30, 60, 90, and 120 minutes following the oral administration by polyethylene stomach tube of a single dose of analytic-grade carbon tetrachloride, 0.25 ml, in an equal volume of mineral oil per 100 gm of animal. Controls received 0.25 ml of mineral oil per 100 gm of animal and were sacrificed at 60 and 120 minutes. All animals were fasted for 16 hours prior to sacrifice by decapitation and exsanguination.

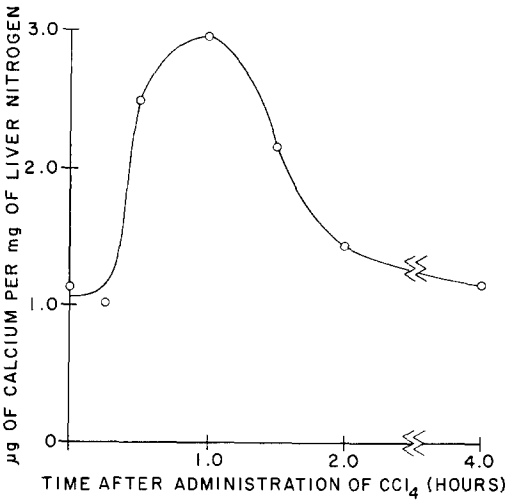


FIGURE 7 Changes in calcium content of liver following administration of CCl_4. Each point is an average of data from 3 or more animals.

The left lateral lobe of the liver was removed immediately and sliced. Some slices were cut into cubes measuring less than 2 mm at greatest dimension and the cubes used for electron microscope and freeze-drying studies.

For electron microscopy, liver cubes were immersed

FIGURES 8 to 11 Accumulation of calcium in parenchymal cells of liver following administration of CCl_4. Fresh-frozen cryostat section 6 μ thick, alizarin red S. Calcium content of each liver was determined analytically as under Methods. × 200. (AFIP Neg. 63-179-2.)

FIGURE 8 Liver of control rat which received mineral oil 60 minutes prior to sacrifice. Calcium content 25 μg Ca/gm liver.

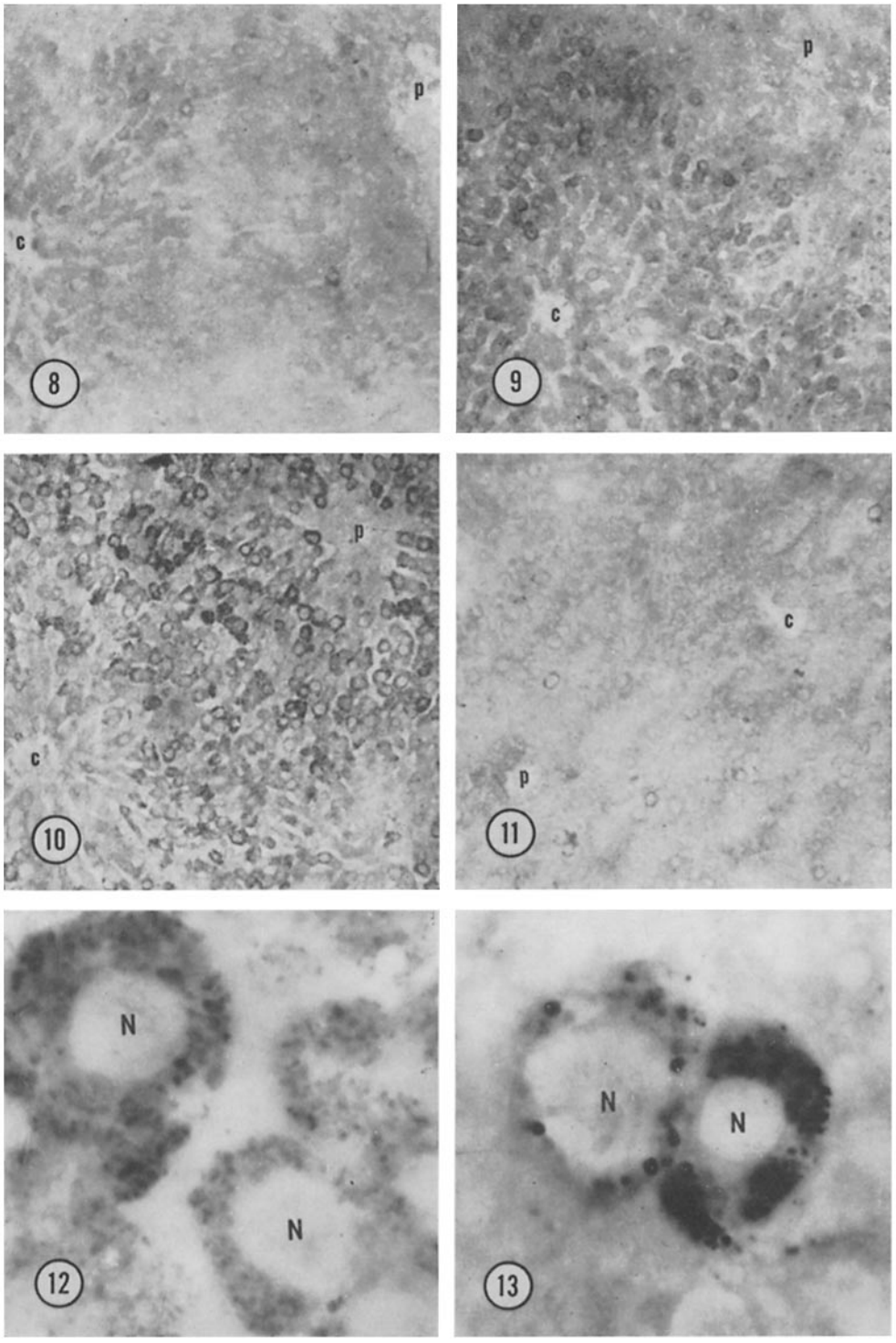
FIGURE 9 Liver of animal 30 minutes following poisoning. Calcium content 53 μg Ca/gm liver. Note increase in staining in midzone of liver lobule.

FIGURE 10 Liver of animal 60 minutes following poisoning. Calcium content 105 μg Ca/gm liver. Only that liver parenchyma immediately adjoining the central veins and portal areas shows no increase in calcium.

FIGURE 11 Liver of animal 120 minutes following poisoning. Calcium content 22 μg Ca/gm liver. Calcium present at 60 minutes has been removed almost completely.

FIGURE 12 Liver of animal 60 minutes following administration of CCl_4. Increased calcium contents are present as small discrete rods and granules in the cytoplasm of liver parenchymal cells. Same liver and preparation as Fig. 10. × 2,400. (AFIP Neg. 63-179-2.)

FIGURE 13 Liver of animal 120 minutes following poisoning with CCl_4. Increased calcium is present as dense droplets of larger size than at 60 minutes (Fig. 12). Calcium is no longer stainable in adjacent cells. Same liver and preparation as Fig. 11. × 2,400. (AFIP Neg. 63-179-2.)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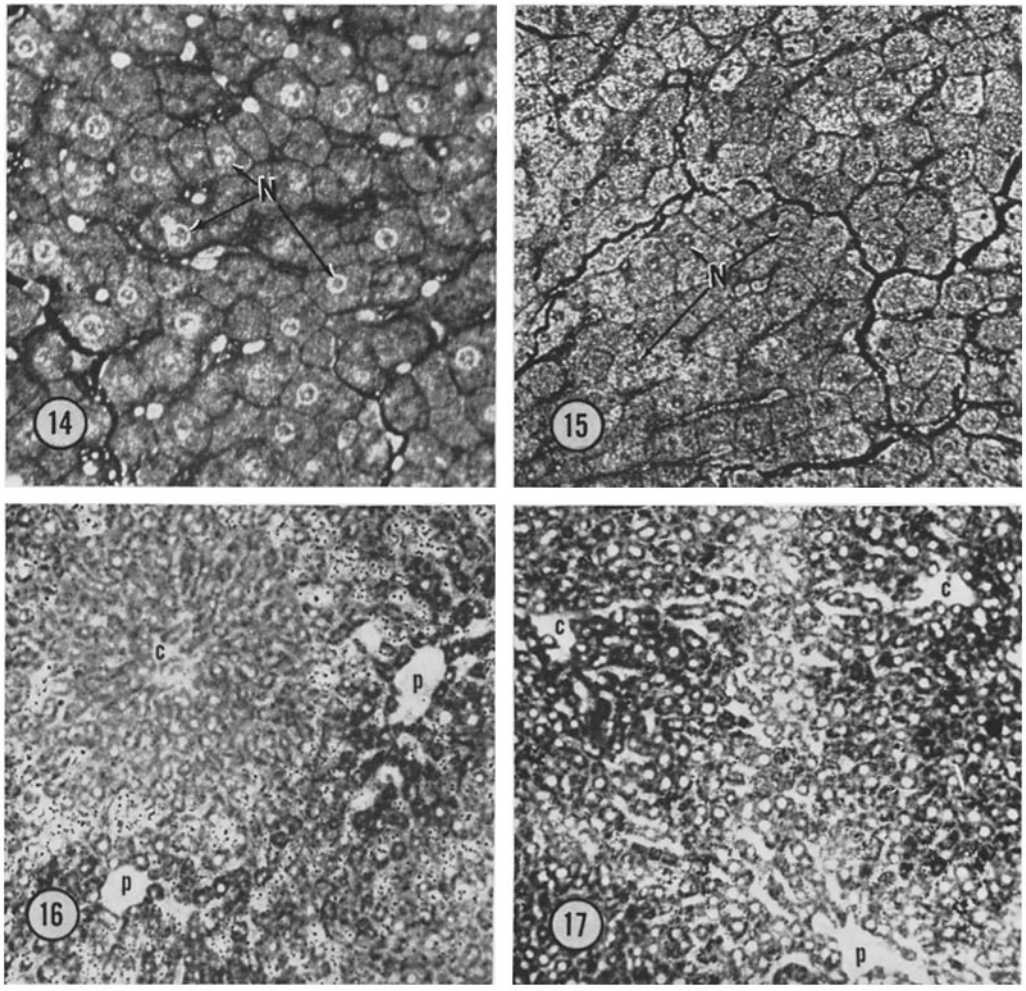


FIGURE 14 Microincineration of liver of control rat. Same liver as Fig. 8. Midzone, frozen-dried, methacrylate-embedded, 2 μ thick. Inorganic ash deposits are light areas against the dark background. Ash patterns of nuclei are clearly seen (*N*). Darkfield microscopy. × 430. (AFIP Neg. 63-179-3.)

FIGURE 15 Microincineration of liver of animal 60 minutes following administration of CCl_4. Same liver as Fig. 10. Cytoplasm now appears to contain as much inorganic ash (light material) per unit area as nuclei. × 430. (AFIP Neg. 63-179-3.)

FIGURES 16 and 17 Dehydrogenase activities in liver of animal 60 minutes following administration of CCl_4. Normal patterns of nitrotetrazolium blue reduction were retained for each substrate. Same liver as Fig. 10. Fresh-frozen cryostat sections. × 140. (AFIP Neg. 63-179-3.)

FIGURE 16 Succinate employed as substrate.

FIGURE 17 Glutamate employed as substrate.

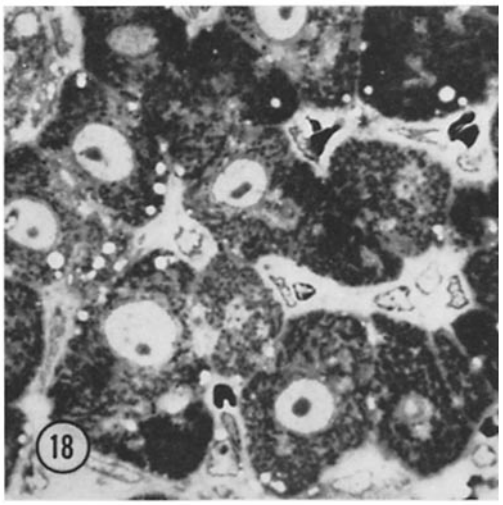
immediately in cold (0–4°C) 1.33 per cent aqueous osmium tetroxide buffered with 0.07 M collidine (15) and containing 0.005 M $CaCl_2$ (16). After 2 hours' fixation, the tissue was dehydrated in ethanol and embedded in Epon 812 mixtures (17). Sections showing silver-to-gold interference colors were cut with glass knives on a Porter-Blum microtome, mounted on copper electromesh grids, and stained

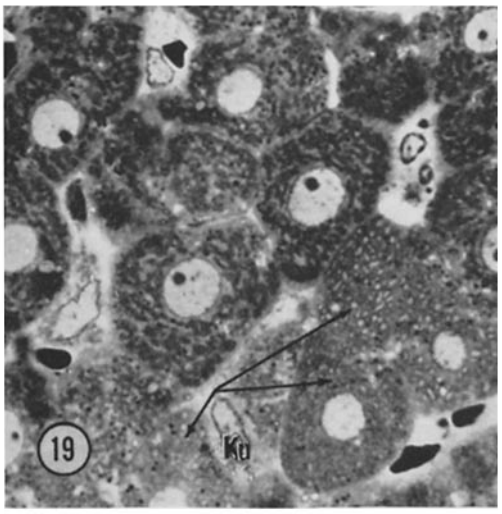
with lead citrate (18). Grids were examined in an RCA EMU 3E or F electron microscope.

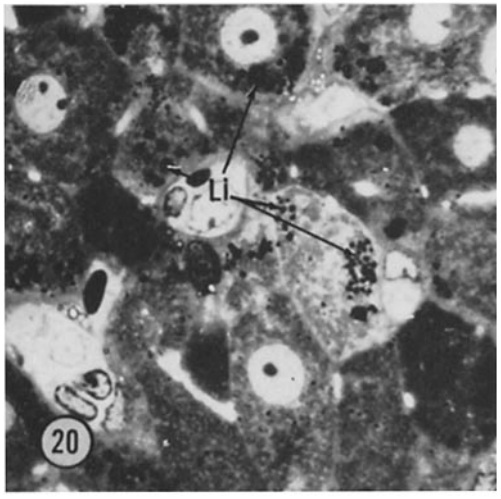
For inorganic ash patterns and staining of the ergastoplasm, liver cubes were frozen-dried by first quenching in liquid propane cooled to 90°K with liquid nitrogen (19). After several minutes at this temperature tissues were transferred to a freeze-drying apparatus (20), previously cooled to 190°K with dry ice, and dried *in vacuo* for 1 week at temperatures not exceeding 220°K. The specimen chamber was then allowed to come to room temperature, the vacuum released, and the dried liver cubes transferred immediately to butyl:methyl methacrylate (5:1) containing 1 per cent benzoyl peroxide. After several changes of monomer, blocks were transferred to prepolymerized methacrylate and polymerization completed at 60°C. Sections 1 μ thick were cut with glass knives on a Porter-Blum microtome without water or water:organic solvent solutions behind the cutting edge of the knife. Sections were then transferred to a small drop of 70 per cent acetone on a glass slide and flattened by heating.

Sections of frozen-dried methacrylate-embedded liver were ashed by placing the mounted section on a quartz mantle in a cold muffle furnace and increasing the temperature to 650°C over a period of 26 minutes. After cooling, the inorganic ash patterns of liver cells were examined by darkfield microscopy.

Ergastoplasm was stained with 1 per cent toluidine blue O in 1 per cent borax in sections of frozen-dried, methacrylate-embedded liver following removal of the plastic with xylene. Mitochondria were stained with 1 per cent toluidine blue O in 1 per cent borax in sections of osmium tetroxide–fixed, Epon-embedded liver, without removal of the plastic (21).







FIGURES 18 to 20 Mitochondrial enlargement in liver parenchymal cells following administration of CCl_4. All figures are of periportal parenchyma. Osmium tetroxide fixation, Epon embedded, 1 μ thick, toluidine blue O in borax. × 1,100. (AFIP Neg. 63-179-4.)

FIGURE 18 Liver of control rat which received mineral oil 120 minutes prior to sacrifice.

FIGURE 19 Liver of rat 60 minutes following poisoning. Mitochondria of all cells are perceptibly larger than those of controls. Those in some cells are enlarged somewhat more than others (arrows).

FIGURE 20 Liver of rat 120 minutes following poisoning. Mitochondria of all cells now appear more uniformly enlarged than at 60 minutes. Note lipid droplets in those aspects of liver parenchymal cells adjacent to sinusoids.

Slices of liver adjacent to those taken for electron microscopy were frozen and sectioned with a hollow-ground steel knife in a cryostat. Sections 2 to 5 μ thick were picked off the knife on warm clean coverslips and stained immediately, or placed in appropriate incubation media.

Cryostat sections were stained for calcium with alizarin red S (Eastman Organic Chemicals, Rochester, New York) by the method of McGee-Russell (22), with the single exception that staining times were reduced to less than 5 seconds to minimize diffusion of calcium alizarinate from its initial precipitation loci in the section.

The distribution of glucose-6-phosphatase activity in the liver lobule was determined in cryostat sections of liver by the method of Wachstein and Meisel (23). This method was considered relatively specific for glucose-6-phosphatase activity as under the condition of the assay β-glycerophosphate was not hydrolyzed and prefixation with 4 per cent formaldehyde (w/v) abolished all activity (24).

Inorganic and organic phosphates were stained by direct incubation of cryostat sections with lead acetate at pH 6.7 and conversion of the precipitated lead to lead sulfide (23).

Succinic and glutamic dehydrogenase activities were determined by incubation of cryostat sections of liver with 1 mg nitrotetrazolium blue (Dajac Laboratories, Philadelphia) (25) in a medium containing either 100 μmoles succinate or glutamate and 600 μmoles glycylglycine buffer, pH 7.5, 100 μmoles sodium phosphate, pH 7.5, 30 μmoles KCN, 15 μmoles $MgCl_2$, 4 μmoles adenosinetriphosphate (Pabst Laboratories, Milwaukee), and sufficient 1 M sucrose to render the medium 0.30 osmolar. Six micromoles diphosphopyridine nucleotide (Pabst Laboratories) was added to the glutamate-containing medium. Controls contained all reagents but substrate. Total volume of liquid present in each vessel was 6.0 ml. Sections were incubated at 0 to 4°C for 16 hours and the reaction stopped by immersion of the section in 1 per cent acetic acid followed by fixation in 4 per cent formaldehyde (w/v).

The remainder of the liver was perfused with cold (0 to 4°C) metal-free 0.25 M sucrose via the hepatic vein (11), pulped, and aliquots were analyzed for calcium (26) and nitrogen (27). Coefficients of variation for the colorimetric determinations of calcium and nitrogen were 3 per cent.

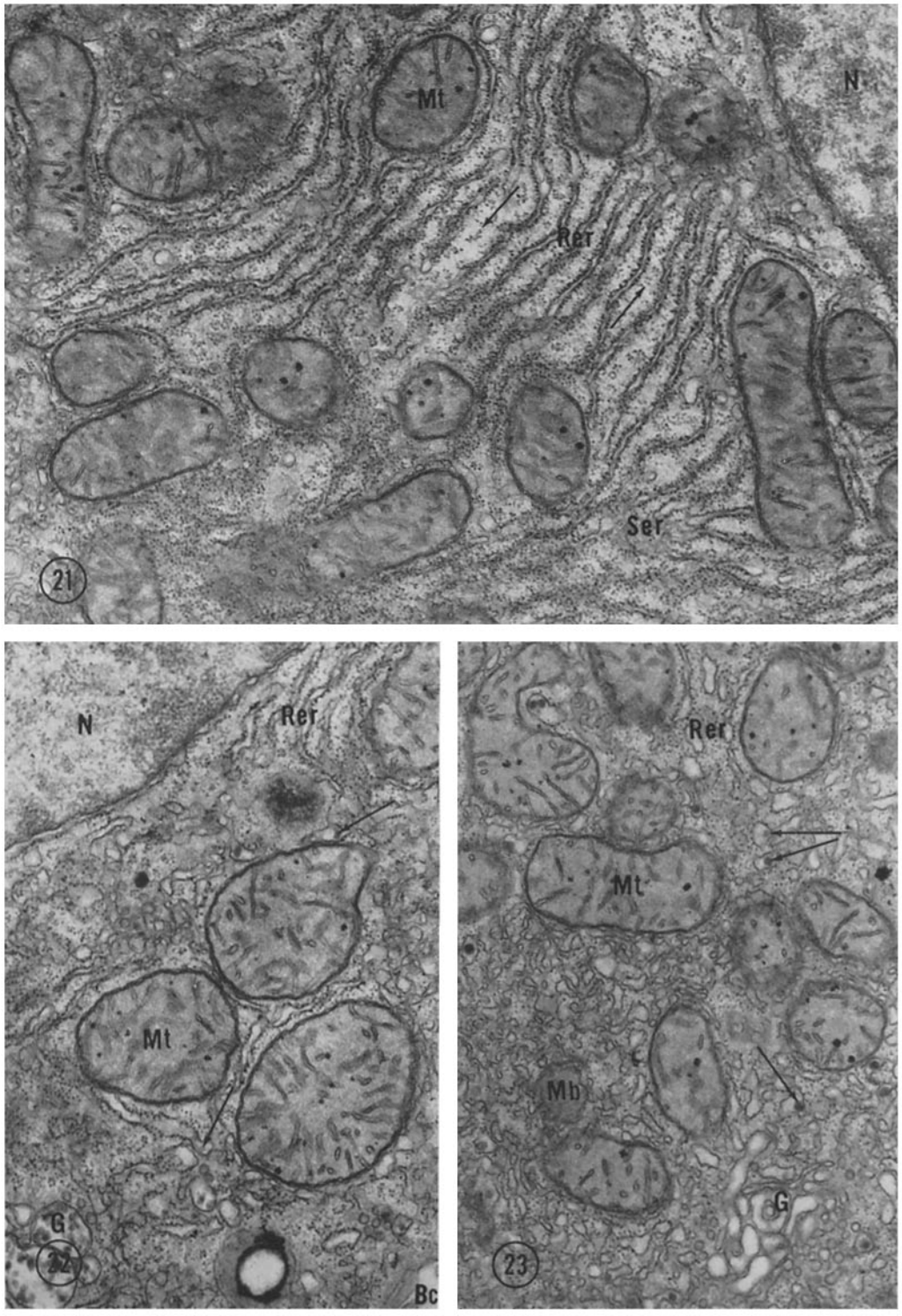
RESULTS

Light and electron microscopic observations of livers of control rats (Figs. 4, 8, 14, and 18) do not substantially differ from those previously described for the livers of normal rats (28, 29), or from those on livers of normal fasted rats examined during the course of this study (Figs. 1, 21, and 25). Uniform depletion of glycogen and the presence of abundant smooth endoplasmic reticulum in normal fasted, control, and experimental animals (Figs. 21 to 24) can be attributed to their forced fast of 16 to 18 hours prior to poisoning and sacrifice (29). Microbodies (30) (Figs. 18 to 20, 23, 24, 26 and 31), con-

FIGURE 21 Portion of cytoplasm and nucleus of centrilobular liver parenchymal cell of normal fasted animal. Parallel strands of granular endoplasmic reticulum form discrete ergastoplasm and are, in places, continuous with smooth endoplasmic reticulum. Ribosomes unassociated with membranes in the plane of section are present in the cytoplasmic matrix between the regularly granulated membranes of the endoplasmic reticulum. In a tangential section of a mitochondrion triangular cristae are seen (lower left) × 25,000. (AFIP Neg. 63-179-5.)

FIGURE 22 Portion of cytoplasm and nucleus of a periportal liver parenchymal cell 30 minutes following administration of CCl_4 to an animal. Cytoplasmic organelles appear normal. Golgi apparatus consists of clusters of small round vacuoles adjacent to larger more irregular vacuoles which contain irregular dense granules (*ca* 400 A). Similar granules are also occasionally seen within granular endoplasmic reticulum (arrows). × 27,000. (AFIP Neg. 63-179-5.)

FIGURE 23 Portion of cytoplasm of a centrilobular liver parenchymal cell 30 minutes following administration of CCl_4 to an animal. Ergastoplasm is dispersed, the cisternae of the granular endoplasmic reticulum are moderately dilated, and increased numbers of free ribosomes are seen in the adjacent cytoplasmic matrix. Membranes of the granular endoplasmic reticulum are sparsely and irregularly lined by ribosomes. Components of the Golgi apparatus are irregularly dilated and devoid of the coarse granules found in the control. Increased numbers of coarse granules (arrows) are seen in the dilated cisternae of the granular endoplasmic reticulum. × 25,000. (AFIP Neg. 63-179-5.)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sistently present in parenchymal cells of livers of control and normal fasted animals, do not appear to increase in number, or alter their appearance during the period of poisoning studied.

Striking alterations are observed in some of the cytoplasmic components of liver parenchymal cells following oral administration of carbon tetrachloride to rats. These changes are described in the order of their light and electron microscopic appearance, respectively.

Light Microscopy

The ergastoplasm, coarse angular masses of basophilic material present in the cytoplasm of normal liver cells (31) (Fig. 1), is less distinctly clumped in centrilobular parenchymal cells within 15 minutes following the oral administration of carbon tetrachloride (Fig. 2). The ergastoplasm undergoes progressive dissolution and by 1 hour it is completely dispersed throughout the cytoplasm in all liver parenchymal cells, coming to lie as faint basophilic strands between mitochondria (Fig. 3).

Depression of glucose-6-phosphatase activity accompanies the progressive dispersion of the ergastoplasm. This enzymatic activity, a property of the cell localized to the membranes of the endoplasmic reticulum (7)—a component of the ergastoplasm—and which is uniformly distributed throughout the liver lobule of normal rats under the assay conditions used (Fig. 4), is depressed centrilobularly within 15 minutes (Fig. 5). Glucose-6-phosphatase activity is progressively depressed, and by 1 hour it is observed only in that parenchyma immediately about the portal areas (Fig. 6). This enzymatic property of the endoplasmic reticulum remains suppressed until approximately 48 hours elapse from the time of administration of the poison.

Calcium enters the liver cell soon after carbon tetrachloride is administered, but after the onset of the dispersal of the ergastoplasm and the depression of glucose-6-phosphatase activity (Figs. 1 to 6). Liver calcium content, normal at 15 minutes, more than doubles within 30 minutes following poisoning (Fig. 7) and continues to increase to a maximum value at 1 hour. At this time, the liver calcium content in poisoned animals may rise from a normal of 24 μg Ca to as high as 105 μg Ca/gm liver. Subsequently, liver calcium content decreases, returning to near normal levels by 2 hours.

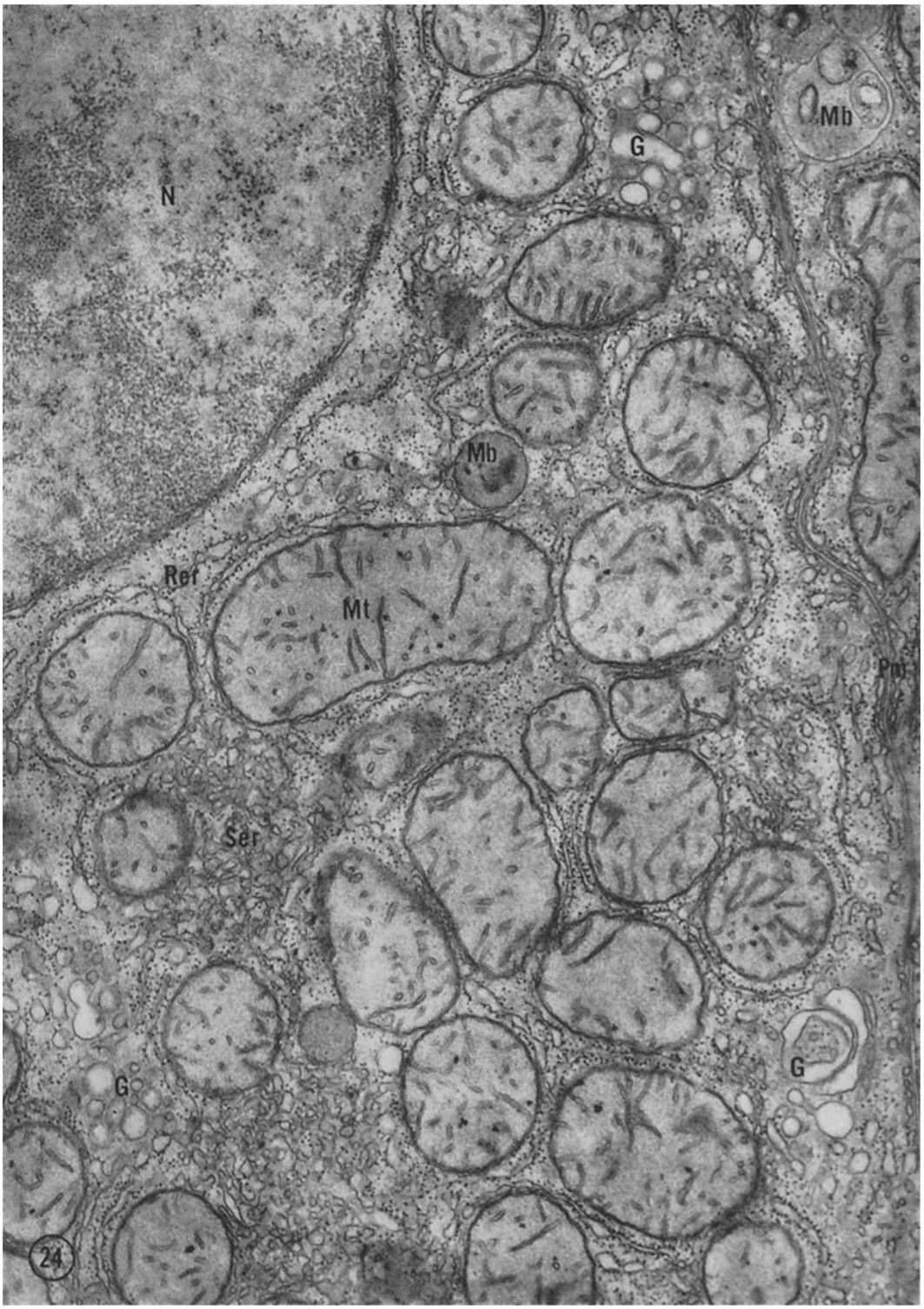
The increased liver calcium at 30, 60, and 90 minutes stains as discrete granules in the cytoplasm of hepatic cells (Figs. 9, 10, 12, and 13), the over-all intensity of staining with alizarin red S corresponding to the quantitative increase in calcium content (Fig. 7). At 30 minutes, calcium deposits are most prominent in pericentral and midzonal liver cells (Fig. 9), whereas at 60 minutes only the cells in the immediate vicinity of portal triads and central veins are spared (Fig. 10). Calcium is cleared from periportal cells last, and by 2 hours remains in only a few liver cells (Fig. 11). In those livers where calcium is maximally increased at 60 minutes following poisoning, increased inorganic ash is detectable upon microincineration (Fig. 15).

Granules giving a staining reaction for calcium at 60 minutes correspond to mitochondria in size and cytoplasmic distribution (Figs. 12 and 19). In those few cells which retain calcium at 2 hours, however, the calcium-containing granules stain more intensely, are increased in size over those present at 1 hour, and are displaced to the periphery of the cell (Fig. 13). Increased calcium at these times is not accompanied by increased organic or inorganic phosphate detectable by histochemical means (23).

Mitochondria of hepatic cells enlarge perceptibly at 1 hour (Fig. 19). This change, observed in all zones of the liver lobule, does not, however, involve mitochondria of all cells equally at this time. Mitochondrial enlargement progresses and by 2 hours it involves almost all liver parenchymal cells (Fig. 20).

Surprisingly enough, respiratory enzyme activity patterns in the liver lobule of poisoned animals do not differ qualitatively from those of

FIGURE 24 Portion of cytoplasm and nucleus of a midzonal liver parenchymal cell 60 minutes following administration of CCl_4 to the animal. Alterations noted previously are present (Fig. 10). Dilation of Golgi vacuoles is more marked than at 30 minutes (Fig. 23). Some mitochondria are moderately enlarged, and increased numbers of *cristae mitochondriales* with irregular "square" rectangular profiles are seen. × 25,000. (AFIP Neg. 63-179-6.)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control animals (Figs. 16 and 17), in spite of the influx of calcium into hepatic cells and its sequestration by mitochondria. At 60 minutes, when calcium is maximally increased in the liver and the carbon tetrachloride is near its peak concentration (2), glutamic dehydrogenase remains most prominent centrally (Fig. 16) and succinic dehydrogenase periportally (Fig. 17). Normal patterns of respiratory enzyme activity persist in the liver lobule until 8 hours after administration of the poison (32).

Increased lipid deposits in liver parenchymal cells are not observed until the end of the initial 2-hour period studied. At this time, increased numbers of osmiophilic droplets are consistently present in the subsinusoidal cytoplasm of periportal cells in livers of all animals studied (Fig. 20).

Electron Microscopy

Ergastoplasm observed in the light microscope (Fig. 1) corresponds to parallel arrays of ribosome-studded or granular endoplasmic reticulum seen in electron micrographs (29, 33) (Fig. 21). The flattened vesicles, or cisternae, of the granular endoplasmic reticulum are continuous with, but distinct from, the tubular agranular endoplasmic reticulum (29). As a rule the granular endoplasmic reticulum does not appear to be intimately associated with mitochondria in liver cells of normal animals (Fig. 21).

Dispersal of the ergastoplasm (Figs. 1 to 3) commencing 15 minutes following the administration of the poison is represented in electron micrographs by disaggregation of the parallel arrays of granular endoplasmic reticulum and the scattering of its individual strands throughout the cytoplasm of the liver cell (Figs. 23 and 24). Concomitantly, cisternae of portions of the granular endoplasmic reticulum dilate and the number of membrane-attached ribosomes diminishes. Unattached ribosomes, present in small numbers in the cytoplasmic matrix of normal hepatic cells (Fig. 21), are found in increased number in the cytoplasmic matrix adjacent to altered granular endoplasmic reticulum of injured cells (Figs. 23, 24, 26, 29 to 33).

Alterations in the morphology of the granular endoplasmic reticulum are accompanied by alterations in the structure of the Golgi complex of liver parenchymal cells (Figs. 23 and 24). The closely approximated parallel arrays of flattened vesicles and small round vesicles which constitute the Golgi complex in normal liver cells (29, 33) (Fig. 21) are transformed, after poisoning, into a cluster of dilated vacuoles with round profiles. This change, first seen in cells of the centrilobular zone within 30 minutes (Fig. 23), is progressive and by 1 hour the swollen Golgi vacuoles (Fig. 24) are observed in the cells of all regions of the lobule. These vacuoles are almost completely devoid of coarse, electron-opaque granules (diameter = 600 to 800 A) present in the Golgi complex in normal liver cells.

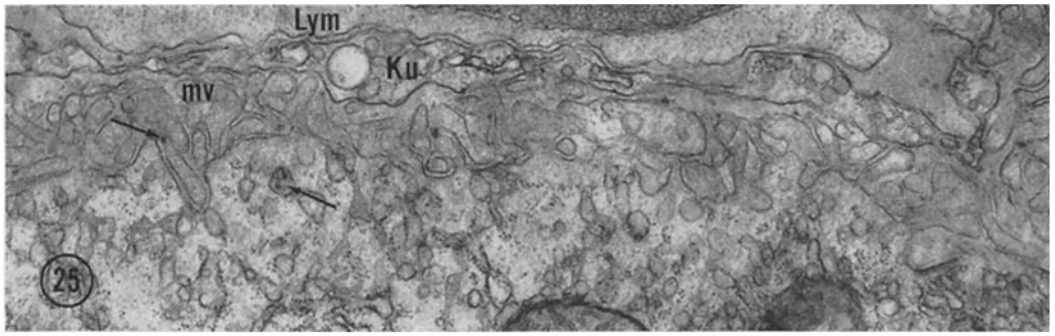
In both normal and experimental animals, coarse dense granules, similar in appearance to those normally found in Golgi vesicles (29, 33) (Fig. 22), are seen within the agranular endoplasmic reticulum immediately subjacent to microvilli which extend into the space of Disse

FIGURE 25 Space of Disse and adjacent structures of a midzonal liver parenchymal cell from a normal fasted animal. A lymphocyte fills the lumen of the sinusoid. Coarse dense granules (arrows) are occasionally seen in the tortuous tubules of the liver parenchymal cell immediately subjacent to the space of Disse and within the space of Disse. × 25,000. (AFIP Neg. 63-179-7.)

FIGURE 26 Space of Disse and adjacent structures of a midzonal liver parenchymal cell 30 minutes following administration of CCl_4. Microvilli often appear to have indistinct margins (compare with Fig. 12). Numerous coarse dense granules (arrows) are observed in tubules subjacent to the villous border of the parenchymal cell and also within the space of Disse. × 25,000. (AFIP Neg. 63-179-7.)

FIGURE 27 Same animal as Fig. 26. Note ballooned microvilli. × 21,000. (AFIP Neg. 63-179-7.)

FIGURE 28 Same animal as Fig. 26. Note obliteration of space of Disse by swollen liver cell cytoplasm. Microvillus-appearing structures (*) are apparently tubules within the cytoplasm of the liver cell. Arrows denote coarse dense granules. × 23,000. (AFIP Neg. 63-179-7.)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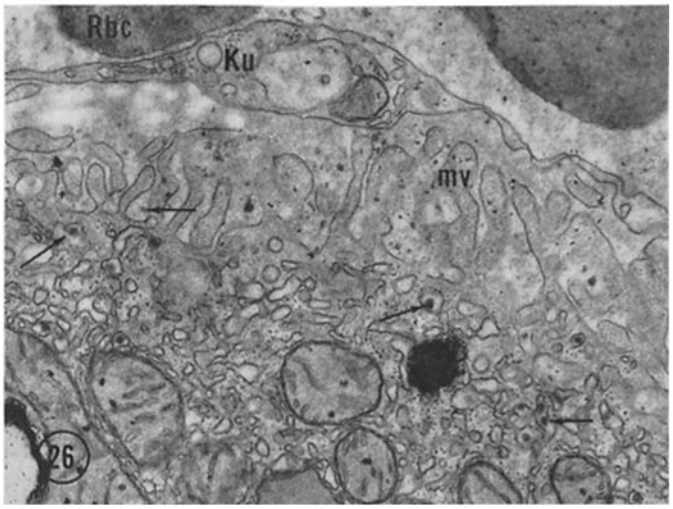
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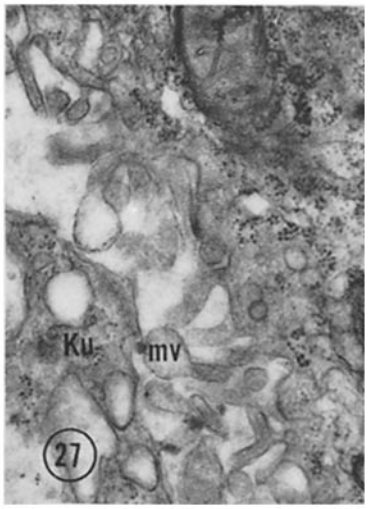
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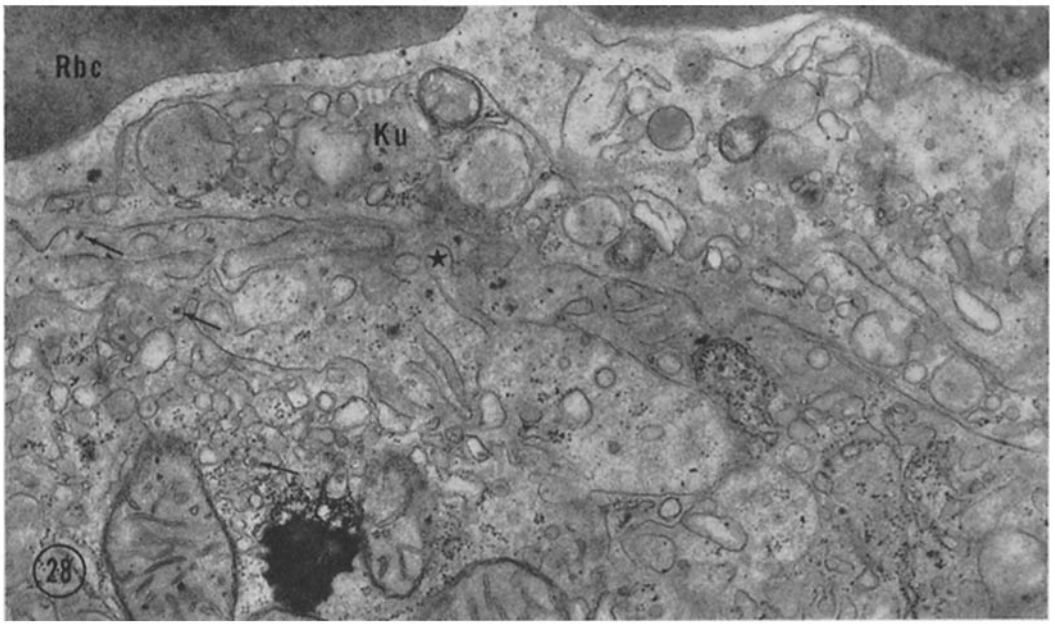
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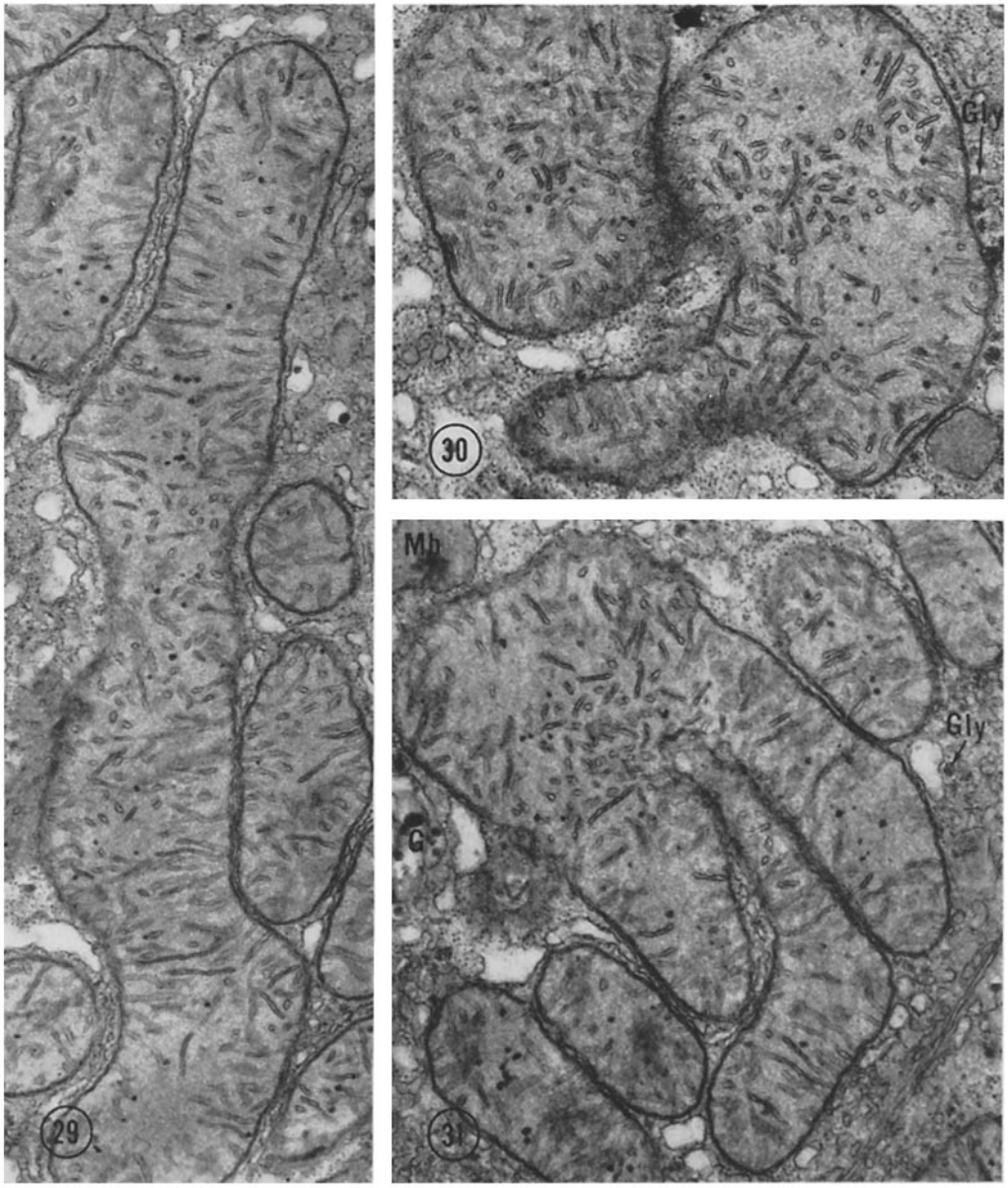
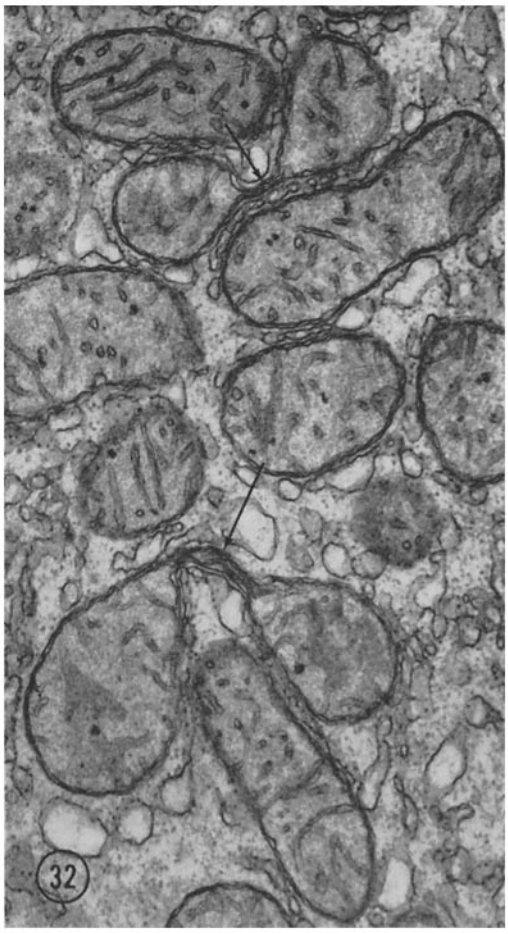
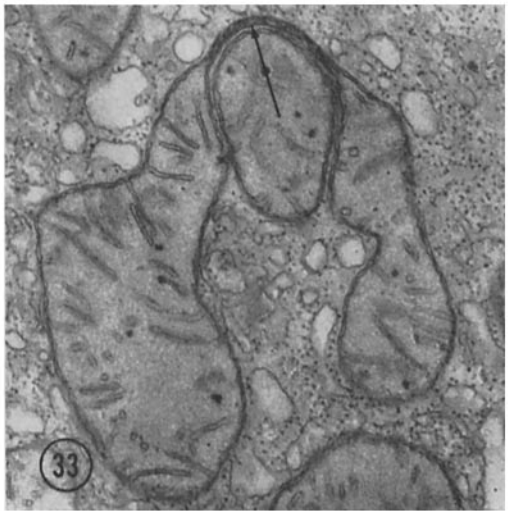


FIGURE 29 Example of the greatly enlarged, or hypertrophied, mitochondria observed in liver parenchymal cells 120 minutes following the administration of CCl_4. This mitochondrion, which measures more than 7.5 μ in length, appears normal with respect to *cristae mitochondriales*, mitochondrial matrix, and the relative ratios of the two. Many cristae are irregularly "square" in cross-section. × 21,000. (AFIP Neg. 63-179-8.)

FIGURES 30 and 31 Two examples of enlarged, or hypertrophied, mitochondria of liver parenchymal cells 120 minutes following poisoning, the members of one pair of which are closely adjacent, if not confluent (Fig. 30), and those of another pair which are confluent (Fig. 31). Large numbers of tubular *cristae mitochondriales* are observed in these mitochondria. The adjacent cytoplasmic matrix is highly disordered, with dilated granular endoplasmic reticulum and abundant free ribosomes. × 22,500. (AFIP Neg. 63-179-8.)





FIGURES 32-33

(Figs. 25 to 28). Indeed, similar material is also observed in the space of Disse between microvilli (Figs. 26 and 28). Although such granules are not usually observed in the cisternae of the granular endoplasmic reticulum of liver cells of control animals, they become prominent in the dilated elements of this system within 30 minutes following poisoning (Figs. 23, 24, 26, and 29).

The surface of that portion of the parenchymal cell which borders the space of Disse is normally lined by an irregular array of short microvilli (29, 32) (Fig. 25). As a general rule, microvilli are longer and villous borders of cells more prominent centrilobularly. Thirty minutes following poisoning these microvilli become more pleomorphic (Figs. 26 to 28) and often appear to have more indistinct margins than those from livers of control animals (Fig. 26). Ballooning of microvilli (Fig. 27) and partial obliteration of the space of Disse by irregular projections of liver cell cytoplasm (Fig. 28) are not uncommon findings in liver cells of poisoned animals at this time. By 2 hours after administration of carbon tetrachloride, many parenchymal cells have smooth or nonvillous surfaces bordering the space of Disse.

The enlargement of liver cell mitochondria that is apparent by light microscopy within 1 hour following poisoning (Fig. 19) is also evident in electron micrographs (Fig. 24). Indeed, mitochondria of liver parenchymal cells observed in the electron microscope are often seen to be more rounded 30 minutes following poisoning (Figs. 22 and 23). Enlarged mitochondria observed within the 2-hour period immediately following administration of carbon tetrachloride are not simply swollen, inasmuch as their matrix appears as dense as that of normal mitochondria, the normal ratio of cristae to matrix is retained, and the cristae are not displaced peripherally in the mitochondrion (Figs. 24, 29 to 33).

FIGURES 32 and 33 Three examples of mitochondria with attenuated midsections found in liver parenchymal cells 60 minutes following the administration of CCl_4 to the animal. Regions of attenuation are indicated by arrows. In all instances, the inner and outer mitochondrial membranes are intact. In Fig. 33 the attenuated portion of such a mitochondrion partially surrounds an adjacent mitochondrion. As in previous figures, at this and later times the adjacent cytoplasm is disorganized. Fig. 32, × 25,000. Fig. 33, × 30,000. (AFIP Neg. 63-179-9.)

In addition to progressively enlarging following poisoning with carbon tetrachloride (Figs. 24, 29 to 33), mitochondria of liver parenchymal cells assume bizarre configurations. At 1 and 2 hours, mitochondria are observed with attenuated midportions, each end interconnected by parallel strands of inner and outer membranes (Figs. 32 and 33). This attenuated middle portion may, upon occasion, be of sufficient length to surround partially adjacent cytoplasmic organelles (Fig. 33). Greatly enlarged mitochondria present at 1 and 2 hours (Figs. 29 to 33) may also have irregularly rounded contours with "bud-like" protrusions. The tubular cristae of enlarged mitochondria often appear angular in outline (Figs. 22 to 24, 29 to 33).

Although almost all liver parenchymal cells have enlarged mitochondria and a highly disordered endoplasmic reticulum at 2 hours, a small minority of these cells contain a dense cytoplasm with more ordered granular endoplasmic reticulum and swollen mitochondria, *i.e.*, mitochondria with a diluted matrix and a reduced number of cristae which are all peripherally displaced (34). The irregularly concave contrours of these cells and their crenated nuclei suggest their selective dehydration, possibly as a consequence of fixation.

DISCUSSION

Dispersion of the ergastoplasm of liver parenchymal cells (Figs. 2 and 3) and the onset of progressive vacuolization and degranulation of components of the endoplasmic reticulum observed within minutes following the oral administration of carbon tetrachloride (Figs. 23 and 24) (4, 5) are associated with loss of glucose-6-phosphatase activity (Figs. 5 and 6), spontaneously reversible intracellular accumulation of calcium (Figs. 9, 10, 12 and 13), and striking morphologic alterations in the organization of the membranes of various cytoplasmic components of the liver cell (Figs. 23, 24, 26 to 33). These events, all manifest within minutes following the administration of carbon tetrachloride, occur during the time of concentration of the poison by the liver (2).

Carbon tetrachloride directly affects properties of mitochondria and microsomes isolated from livers of normal rats (6, 9). Mitochondria exposed to dilute aqueous solutions of carbon tetrachloride increase in size ("swell"), as determined by changes in optical density of their suspensions (35). Microsomes exposed to carbon tetrachloride *in vitro* suffer loss of glucose-6-phosphatase activity (6). Similar changes observed in the livers of poisoned animals during the times when the lipid solvent is present in the liver cell may represent similar actions of carbon tetrachloride on these components of the liver cell *in vivo*. Although mitochondria may increase in size in the presence of the concentrations of carbon tetrachloride reached in the liver, altered mitochondrial respiratory function would not be expected at these levels of carbon tetrachloride—even in the presence of moderate increases in mitochondrial calcium content (9, 36).

When carbon tetrachloride is administered under conditions and concentrations identical to those employed in this study, it reaches a maximum concentration—1 mg CCl_4/gm liver—90 minutes following its oral administration (4). As a non-polar lipid-soluble substance it is undoubtedly inhomogeneously distributed throughout the cytoplasmic structures of the liver cell. Its solution in the hydrocarbon phases of cell membranes would tend to liquify these structures (37), *i.e.*, transform their constituent bimolecular phospholipid leaflets from crystalline to liquid phase. Such phase shifts in their physical-chemical properties might transform sheet-like membranes with low surface tensions to droplets and vesicles with high surface tension (37). Thus, the breakup and vesiculation of the laminar cisternae of granular endoplasmic reticulum, the dilatation and rounding of Golgi vesicles, the ballooning of microvilli, and the bizarre configurations of mitochondria and their cristae may be manifestations of the physical presence of the lipid solvent in the membranes of these organelles.

Reversible sequestration of calcium by mitochondria of liver parenchymal cells between 30 and 90 minutes following poisoning with carbon tetrachloride may not be a result of direct damage to this organelle by the lipid solvent. Nor can calcium binding by mitochondria following administration of the poison be correlated with increased numbers of histochemically demonstrable calcium binding sites at this time (32). Mitochondria of parenchymal cells of normal liver reversibly bind such concentrations of calcium *in vitro* (9, 36), and contain sufficient binding sites, as judged by phosphate content, to account for the sequestration of far greater amounts of calcium (9, 11).

Altered permeability characteristics of the plasma membrane of the liver parenchymal cell may accompany alterations in its structure (Figs. 26 to 28). Between 15 and 30 minutes following

poisoning these membranes may become increasingly permeable to calcium and may, if increases in cell size can be used as an indicator (10), admit increasing amounts of water and electrolytes to the interior of the cell. Although it is tempting to assume that the uptake of calcium by the cell is due to the direct interaction of the lipid solvent with the plasma membrane, the fact that calcium moves out of the cell between 1 and 2 hours and that liver parenchymal cells are capable of maintaining normal calcium contents when carbon tetrachloride is maximally concentrated in the liver (2) is difficult to explain on the basis of such an hypothesis. In spite of regaining their ability to regulate their internal calcium concentrations, liver cells do not return to their former size—indicating a continuing permeability defect of the plasma membrane of the cell.

Mitochondrial enlargement (Figs. 18 to 20, 24, 29 to 33) during the first few hours of poisoning appears to be unique, so far, in responses of cellular components to cytotoxic agents. Increase in size without associated reduction in number and peripheral displacement of *cristae mitochondriales* and without dilution of the mitochondrial matrix is to be distinguished from mitochondrial swelling (34) seen at later times following poisoning with carbon tetrachloride (4, 8), anoxia (38), and treatment with other agents (39). Although morphologically distinct, the mitochondrial enlargement seen here may not be causally or functionally separate from swelling and may, indeed, represent an early incipient phase of the latter.

Mitochondria can enlarge by at least three other mechanisms. Firstly, mitochondria can undergo an apparent increase in size by spherical transformation of filamentous, or rodlet forms, as has been described in the liver following feeding with 3-methyldiamino-azobenzene (28). Secondly, adjacent mitochondria can fuse, *e.g.*, as during spermiogenesis (40). Thirdly, mitochondria can grow by synthesis of new mitochondrial material. Indeed, all these events may occur simultaneously during the first few hours following administration of carbon tetrachloride to the animal. The increase in size, however, is far greater than that which would be expected to result from spherical transformation of mitochondria present in the liver cell of the normal animal. Although some adjacent mitochondria may fuse as suggested in Figs. 30 and 31, reduction in the number of mitochondria contained per cell was not qualitatively apparent, in spite of a generalized increase in the volume of liver parenchymal cells (10, 41). Growth by actual synthesis of mitochondrial material cannot be ruled out. Reversible sequestration of calcium by mitochondria does not appear to be directly associated with mitochondrial enlargement, as mitochondria continue to increase in size after the removal of the increased calcium from the liver cell.

Alterations in mitochondrial fine structure, *i.e.*, tubular transformation of *cristae mitochondriales* (Figs. 25 and 29 to 33) and attenuation of portions of mitochondria (Figs. 32 and 33), are concomitants of enlargement and have not been observed in liver parenchymal cells of normal or control animals. The attenuated mitochondria resemble the bizarre, cup-shaped mitochondria which appear in liver parenchymal cells following intrasplenic injections of ammonium carbonate (42) and the profiles of cup-shaped mitochondria normally found in steroid-producing cells of the adrenal cortex (43) and testis (44).

Dispersal of the ergastoplasm, a striking initial cytotoxic event in the liver lesion of carbon tetrachloride poisoning, also is observed following the administration of 3′-methyldiaminoazobenzene (28), phosphorus (39), and dimethylnitrosamine (44), as a concomitant of prolonged fasting (29), and as a sequel to plasmapheresis (45) and partial hepatectomy (45). The fact that such diverse chemical and physiologic stimuli can induce a similar type of alteration in the cytoplasmic structure of this organellar system in the liver cell indicates that this may be a generalized pathophysiological response of the liver cell.

On the other hand, progressive vacuolization of the cisternae of the granular endoplasmic reticulum, degranulation of their membranes, and depression of glucose-6-phosphatase activity are not such widespread cytotoxic phenomena. Similar morphologic alterations have been observed only following poisoning with dimethylnitrosamine (44) and phosphorus (38, 46). However, glucose-6-phosphatase activity is unaffected by dimethylnitrosamine (47), and the fate of this enzymatic activity is unknown in the case of phosphorus poisoning. Other forms of liver intoxication, such as those following feeding of ethionine (48) and 3′-methyldiaminoazobenzene (28), do not result in such alterations in the membranes of the granular endoplasmic reticulum.

The morphologic similarities between the lesion produced by dimethylnitrosamine, an *in situ*

methylating agent (44), and that produced by carbon tetrachloride, and the fact that both agents profoundly and similarly affect protein synthesis by the liver parenchymal cell (5, 44, 46) suggest that, in addition to its lipid solvent actions, this halogenated hydrocarbon may chemically interact with the constituents of the cell. Carbon tetrachloride is known to be metabolized by the liver following its administration (41, 49–51). The conversion of carbon tetrachloride to phosgene or chloroformate following its reduction intracellularly to chloroform (51), and the subsequent interaction of these acid chlorides with cellular components could be expected to cause profound biochemical and possibly morphologic alterations of these structures. Indeed, selective chemical alteration of components of the ergastoplasm by these highly reactive compounds produced within the cell could account for the apparent irreversibility of this cytotoxic lesion in carbon tetrachloride poisoning, in contrast to the spontaneous reversal of calcium retention by the liver parenchymal cell.

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REFERENCES

1. Reynolds, E. S., *Fed. Proc.*, 1962, **21**, 305.
2. Recknagel, R. O., and Litteria, M., *Am. J. Path.*, 1960, **36**, 521.
3. Von Oettingen, W. F., United States Public Health Service Publication No. 414, Washington, D. C., U. S. Government Printing Office, 1955.
4. Oberling, C., and Rouiller, C., *Ann. Anat. Path.*, 1956, **1**, 401.
5. Smuckler, E. A., Iseri, O. A., and Benditt, E. P., *J. Exp. Med.*, 1962, **116**, 55.
6. Recknagel, R. O., and Lombardi, B., *J. Biol. Chem.*, 1961, **236**, 564.
7. Chauveau, J., Moulé, Y., Rouiller, C., and Schneebeli, J., *J. Cell. Biol.*, 1962, **12**, 17.
8. Bassi, M., *Exp. Cell Research*, 1960, **20**, 313.
9. Reynolds, E. S., Thiers, R. E., and Vallee, B. L., *J. Biol. Chem.*, 1962, **237**, 3546.
10. Glynn, L. E., and Himsworth, H. P., *Clin. Sc.*, 1948, **6**, 235.
11. Thiers, R. E., Reynolds, E. S., and Vallee, B. L., *J. Biol. Chem.*, 1960, **235**, 2130.
12. Cristie, G. S., and Judah, J. D., *Proc. Roy. Soc. London, Series B*, 1954, 142, 241.
13. Wilgram, G. F., Smith, S. W., Ingle, D. J., and Kennedy, E. P., *Fed. Proc.*, 1961, **20**, 290.
14. Rees, K. R., Sinha, K. P., and Spector, W. G., *J. Path. Bact.*, 1961, **81**, 107.
15. Bennett, H. S., and Luft, J. H., *J. Biophysic. and Biochem. Cytol.*, 1959, 6, 113.
16. Baker, J. R., *J. Histochem. and Cytochem.*, 1958, **6**, 303.
17. Luft, J. H., *J. Biophysic. and Biochem. Cytol.*, 1961, **9**, 409.
18. Reynolds, E. S., *J. Cell Biol.*, 1963, **17**, 208.
19. Bell, L. G. E., *Internat. Revs. Cytol.*, 1952, **1**, 35.
20. Stowell, R. E., *Stain Techn.*, 1951, **26**, 105.
21. Richardson, K. C., Jarett, L., and Finke, E. H., *Stain Techn.*, 1960, **35**, 313.
22. McGee-Russell, S. M., *J. Histochem. and Cytochem.*, 1958, **6**, 22.
23. Wachstein, M., and Meisel, E., *J. Histochem. and Cytochem.*, 1956, **4**, 592.
24. Chiquoine, A. D., *J. Histochem. and Cytochem.*, 1955, **3**, 471.
25. Nachlas, M. M., Tsou, K., DeSouza, E., Cheng, C., and Seligman, A. M., *J. Histochem. and Cytochem.*, 1957, **5**, 420.
26. Reynolds, E. S., and Linde, R. E., *Anal. Biochem.*, 1963, **5**, 246.
27. Koch, F. C., and McMeekin, T. L., *J. Am. Chem. Soc.*, 1924, **46**, 2066.
28. Porter, K. R., and Bruni, C., *Cancer Research*, 1959, **19**, 997.
29. Fawcett, D. W., *J. Nat. Cancer Inst.*, 1955, **15**, 1475.
30. Ashford, T. P., and Porter, K. R., *J. Cell Biol.*, 1962, **12**, 198.
31. Deane, H. W., *Am. J. Anat.*, 1956, **78**, 227.
32. Reynolds, E. S., *J. Histochem. and Cytochem.*, 1961, **9**, 602.
33. Porter, K. R., *J. Histochem. and Cytochem.*, 1954, **2**, 346.
34. Rouiller, C., *Internat. Revs. Cytol.*, 1960, **9**, 227.
35. Recknagel, R. O., and Malamed, S., *J. Biol. Chem.*, 1958, **232**, 705.
36. Vasington, F. D., and Murphy, J. V., *J. Biol. Chem.*, 1962, **237**, 2670.
37. Schulman, J. H., personal communication.
38. Mölbert, E., and Guerritore, D., *Beitr. path. Anat. u. allg. Path.*, 1956, **117**, 31.

39. JEZEQUEL, A. M., *Ann. Anat. Path.*, 1958, **3,** 512.
40. ANDRÉ, J., *J. Ultrastruct. Research*, 1962, suppl. 3.
41. REYNOLDS, E. S., *Fed. Proc.*, 1963, **22,** 370.
42. DAVID, H., and KETTLER, L. H., *Z. Zellforsch.*, 1961, **53,** 857.
43. YATES, R., unpublished observations.
44. CHRISTENSEN, A. K., and CHAPMAN, G. B., *Exp. Cell Research*, 1959, **18,** 576.
45. GLINOS, A. D., *in* Liver Function, (R. W. Brauer, editor), Washington, D. C., American Institute of Biological Sciences, 1958.
46. BARKER, E., and SMUCKLER, E. A., abstract, 2nd Annual Meeting American Society of Cell Biology, 1962.
47. BROWERS, J. A. J., and EMMELOT, P., *Exp. Cell Research*, 1960, **19,** 467.
48. HERMAN, L., EBER, L., and FITZGERALD, P. J., *in* Electron Microscopy, (S. S. Breeze, Jr., editor), New York, Academic Press, Inc., 1962, 2.
49. MACCOLLISTER, D. D., BEAMER, W. H., ATCHISON, G. J., and SPENCER, H. C., *Fed. Proc.*, 1950, **9,** 300.
50. ROQUE, A. L., and FEDORKO, M. E., *J. Histochem. and Cytochem.*, 1961, **9,** 613.
51. BUTLER, T. C., *J. Pharmacol. and Exp. Therap.*, 1961, **134,** 311.