A LIGHT AND ELECTRON MICROSCOPE STUDY OF THE MORPHOLOGICAL CHANGES INDUCED IN RAT LIVER CELLS BY THE AZO DYE 2-ME-DAB

J. G. LAFONTAINE, Ph.D., and C. ALLARD, Ph.D.

From the Department of Pathology, Laval University Medical School, Quebec, Canada, and the Institute of Cardiology, Maisonneuve Hospital, Montreal, Canada. Dr. Lafontaine's present address is the Department of Biology, Faculty of Sciences, Laval University, Quebec

ABSTRACT

The cytological changes induced in rat liver cells by the aminoazo dye 2-Me-DAB have been examined by light and electron microscopy. It is observed that this non-carcinogenic compound duplicates most of the morphological alterations produced by other hepatotoxins, some of which, such as the closely related aminoazo dye 3'-Me-DAB, are potent carcinogens. These non-specific effects involve both the granular and agranular forms of the endoplasmic reticulum as well as the glycogen content of hepatic cells. The arrays of cisternal profiles of the granular reticulum in normal hepatic cells become disorganized and the dispersed cisternae often appear fragmented and irregular. Large cytoplasmic inclusions, consisting of loosely organized tubules and vesicles, are also observed which result from a hypertrophy of the agranular reticulum. The glycogen in the cells progressively decreases in amount. The most specific effect of 2-Me-DAB is to induce an increase in the number of mitochondria per cell. Many of these organelles are characterized by the presence of a median double membrane continuous with the inner limiting membrane of the mitochondrial envelope. Evidence is presented in favor of the view that this partition is directly related to the phenomenon of mitochondrial division. It was noted also in the course of the experiment that an increasing number of cells appear which stain quite intensely with methylene blue and appear denser than normal under electron microscopy. The significance of these cells is not known.

INTRODUCTION

Price *et al.* (1) and Potter *et al.* (2) have shown that larger amounts of mitochondrial material are obtained from homogenates of the liver of rats fed the aminoazo dye 2-Me-DAB than from homogenates of either normal livers or livers of animals fed the carcinogen 3'-Me-DAB. Allard *et al.* (3) and Striebich *et al.* (4) subsequently demonstrated that 2-Me-DAB actually induces a significant increase in the number of mitochondria per cell. The last group of authors also reported that 2-Me-DAB does not disturb the normal histological architecture of rat liver as does the carcinogen 3'-Me-DAB (5, 6).

The observations of Porter and Bruni (7) on the ultrastructural changes induced in liver cells by 3'-Me-DAB have now been followed by a number of studies of various other hepatic carcinogens (8–15). Concurrently, several reports (16–21) have appeared describing the effects of other toxic but non-carcinogenic substances on the fine structure of the hepatic cells.

Key to Symbols

aer, agranular endoplasmic reticulum
b, body
bc, bile canaliculus
bz, basophilic zone
cy, cytosome
gc, Golgi complex
ger, granular endoplasmic reticulum
gr, granule
gu, glycogen unit
gz, glycogen zone
hi, hyaline inclusion
l, lipid inclusion
ly, lysosome
m, mitochondrion
pt, partition
r, ribosome
v, vacuole
ve, vesicle

FIGURE 1 Normal hepatic cell illustrating the distribution of the mitochondria (*m*) and of the basophilic zones (*bz*). Most mitochondria are either at the periphery of these zones or actually embedded within them. The lighter cytoplasmic areas correspond to zones of glycogen. The section, 0.25 μ thick, was stained with basic fuchsin, counterstained with methylene blue, and photographed under phase contrast. × 2400.

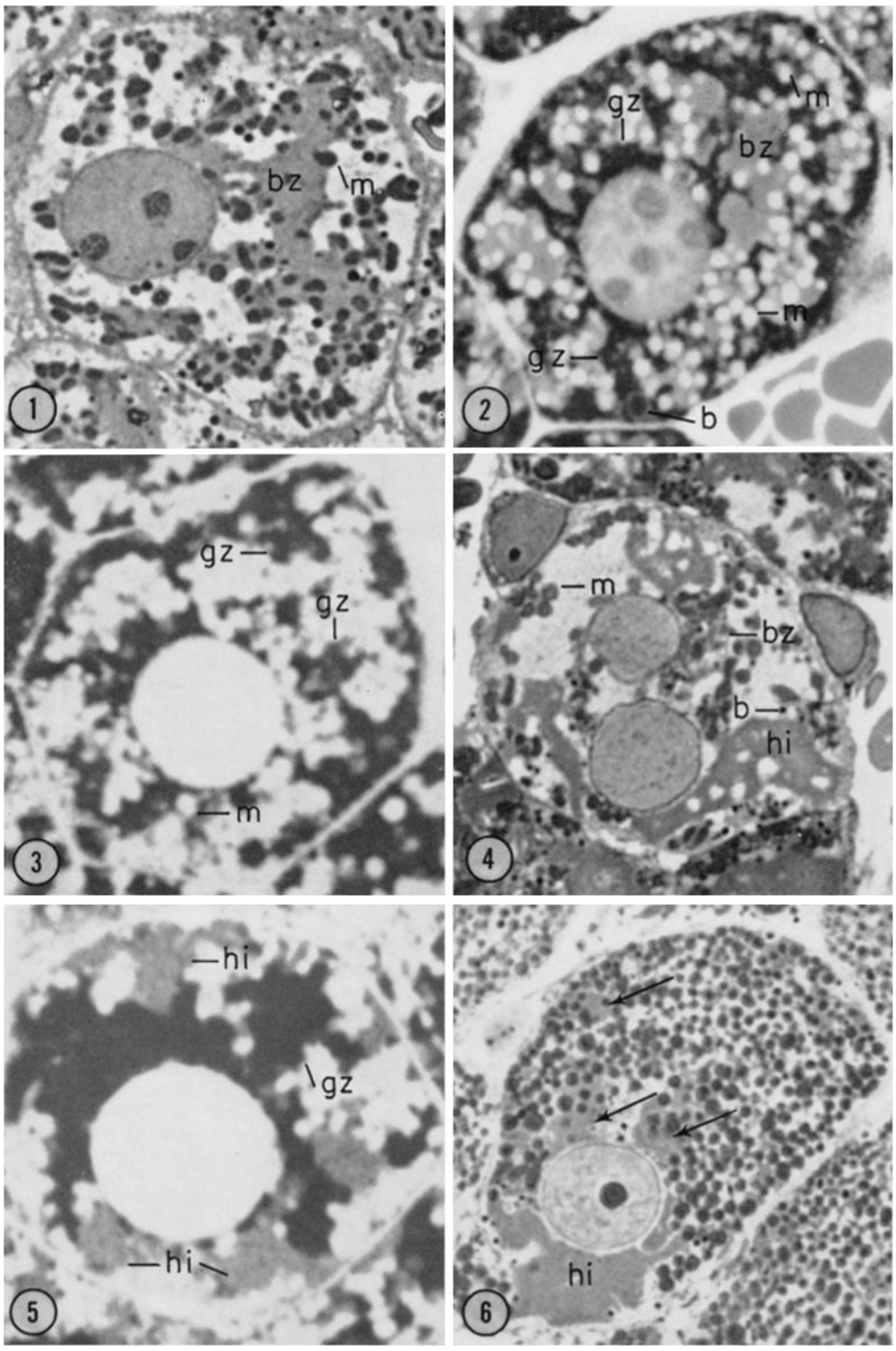
FIGURE 2 Liver cell of control animal; *i.e.*, animal fed a low protein-high carbohydrate basal diet for 10 days. The section, 0.5 μ thick, was stained with the PAS procedure and counterstained with methylene blue. Mitochondria (*m*) appear as negative images after such staining and are seen to be mostly grouped in the immediate neighbourhood of the basophilic zones (*bz*) of the cytoplasm. Glycogen is present in appreciable quantity and permeates all cytoplasmic areas not occupied by formed structures. A roundish refractile body (*b*), probably lipidic in nature, is noted within a zone of glycogen. × 2400.

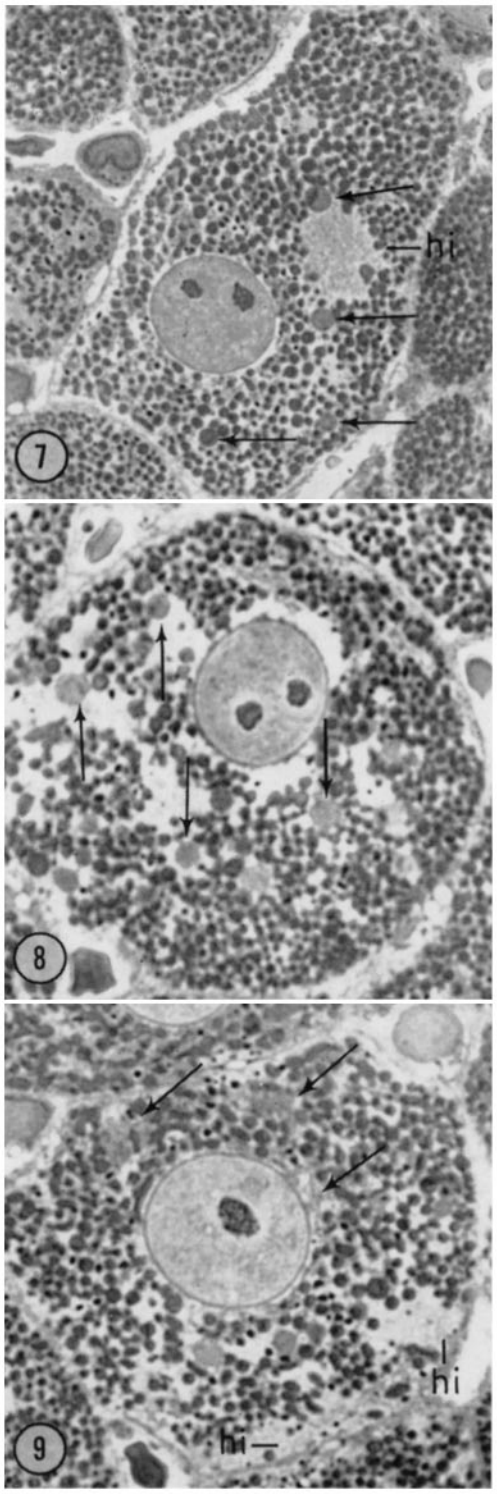
FIGURE 3 Liver cell from the same block that was used for preparation of section illustrated in Fig. 2. The section, 0.5 μ thick, was stained with the PAS technique only. In this preparation, contrary to that shown in Fig. 2, the basophilic areas of the cytoplasm do not show up and, as a result, individual mitochondria are not so clearly delineated. Instead, only groups of mitochondria (*m*) are detected, due to the low concentration or absence of glycogen amongst these organelles. Note that the concentration and distribution of glycogen in this cell corresponds closely to the situation observed in normal hepatic cells (Fig. 1). × 2800.

FIGURE 4 Liver cell of experimental animal; *i.e.*, animal receiving 0.06 per cent 2-Me-DAB for a period of 10 days. The section, 0.25 μ thick, was stained with methylene blue and photographed under phase contrast microscopy. The cell illustrated is of the light type and is surrounded by dark cells which stain much more intensely with methylene blue. Note in this light cell the presence of conspicuous hyaline inclusions (*hi*) which form a coarse network throughout the cytoplasm. Some mitochondria (*m*) are still located within the dispersed basophilic zones (*bz*), but now many are also found scattered within other areas of the cytoplasm. A number of smaller, more refractile bodies (*b*) are also observed within various areas of the cytoplasm. × 2400.

FIGURE 5 Liver cell of experimental animal of the 10-day period. The section, 0.5 μ thick, was stained for glycogen only. Besides the dense cytoplasmic areas corresponding to zones of high glycogen content (*gz*), lighter zones may now also be detected which are pinkish in colour after the PAS procedure. These correspond to the hyaline inclusions (*hi*) illustrated in Fig. 4. Note that at this time glycogen is still present in appreciable amounts. × 3200.

FIGURE 6 Liver cell from a rat that had been fed for 9 weeks on a diet containing 0.06 per cent 2-Me-DAB. The section, 1 μ thick, was stained with methylene blue. The number of mitochondria has increased quite markedly as compared with that observed at the 10-day period (Fig. 4). These organelles now occupy a large proportion of the cytoplasm. Smaller, very dense particles are also observed scattered amongst the mitochondria. Besides the large crescent-shaped hyaline inclusion (*hi*) covering part of the nucleus, a number of similar but much smaller zones may also be observed which in many cytoplasmic areas (arrows) surround the mitochondria. × 1500.


bz
m
1
gz
bz
m
m
gz
b
2
gz
gz
m
3
m
bz
b
hi
4
hi
gz
hi
5
hi
6



The present electron microscope study was initially undertaken with the main purpose of examining in more detail than previously possible (3, 4) the effects of the non-carcinogenic (22, 23) aminoazo dye 2-Me-DAB on the mitochondrial population of the hepatic cell. However, as this work progressed, the realisation that 2-Me-DAB induces a number of changes in other cytoplasmic structures similar to those observed after feeding the potent carcinogen 3′-Me-DAB (7) prompted us to extend the scope of our observations. It was felt that such a study might possibly help to clarify which of the several morphological changes brought about by ingestion of hepatocarcinogens (7–15) are actually specific for the early stages of carcinogenesis.

MATERIAL AND METHODS

Wistar rats averaging 200 to 250 grams were divided into two groups. One group, the control animals, were fed a low protein–high carbohydrate basal diet and water *ad libitum*; the other group received the same diet to which 0.06 per cent 2-Me-DAB was added for periods up to 16 weeks. Every two weeks or so a number of rats were sacrificed by being stunned and decapitated. The liver was rapidly excised and portions of the left lobe fixed for 90 minutes in an ice-cold 1 per cent solution of osmium tetroxide adjusted to pH 7.3 and to which sucrose had been added (24). After dehydration in a graded series of ethanol, the tissue blocks were embedded in a 19:1 mixture of *n*-butyl and methyl methacrylates. More recently, pieces of liver from normal rats were also fixed in a

FIGURE 7 Hepatic cell from the same animal used for Fig. 6. The section, 0.5 μ thick, was stained with methylene blue and photographed under phase contrast microscopy. This figure depicts a low density hyaline zone (*hi*) as well as a number of mitochondria (arrows) larger than normal. Numerous particles smaller and denser than the mitochondria are also found amongst these organelles. × 1400.

FIGURE 8 Hepatic cell from same section that is shown in Fig. 7, illustrating a number of large mitochondria (arrows). This cell, judging from the remaining light cytoplasmic areas, most likely still contains appreciable amounts of glycogen. × 2000.

FIGURE 9 Hepatic cell from same section that is shown in Figs. 7 and 8. This figure illustrates several small groups of cisternae of the granular endoplasmic reticulum (arrows), two light hyaline zones (*hi*), as well as many dense particles of various sizes. Phase contrast. × 1800.

1 per cent solution of osmium tetroxide in phosphate buffer (25) and embedded in Epon (26). This latter material was examined under light microscopy only.

In the course of this study we have depended heavily on the light microscopic examination of 0.25 to 1 μ sections prepared from the same blocks that were used for electron microscopy. Although such sections were usually only 1 mm^2 in area, they nevertheless greatly facilitated the interpretation of our electron micrographs and permitted a much more adequate sampling of the material than would have been possible with the electron microscope. These sections were generally stained with either the periodic acid–Schiff reaction (PAS) (27), 1 per cent borated methylene blue, or 1 per cent basic fuchsin. Combinations of these stains were frequently used also. Where more contrast was needed, the thinnest sections were examined under phase contrast microscopy.

For electron microscopy the sections were stained with lead hydroxide (28) and sandwiched between thin films of Formvar and carbon (29). These preparations were examined in a Siemens Elmiskop I using the double condenser, 80 kv, and 50 μ objective apertures.

OBSERVATIONS

Our description of the morphology of hepatic cells from experimental animals will be mostly limited to the major differences in these cells as compared with hepatocytes of control animals. No attempt will be made, therefore, to cover the numerous modulations in structural details which may be recorded from cell to cell with the electron microscope.

In order to facilitate comparison of the early effects on hepatic cells of the non-carcinogen 2-Me-DAB and the closely related but highly potent substance 3′-Me-DAB, our findings at the end of the 10-day period are reported in some detail. The period extending from the 3rd to 16th week is then discussed and, wherever possible, the sequence of the morphological alterations observed is described.

Basophilic Zones and the Granular Endoplasmic Reticulum

Control Animals

The zones which in the normal hepatic cell (Fig. 1) correspond to arrays of closely and regularly disposed cisternae of the granular endoplasmic reticulum are also observed in 0.3 to 0.7 μ sections of cells from control animals. A number of such basophilic zones remain quite conspicuous during the period investigated but others become partially disorganized. Indeed, examination of 0.5 μ sections under phase contrast reveals amongst the mitochondria the presence of narrow linear structures which most likely correspond to small groups of cisternae of the endoplasmic reticulum.

The most common situation recorded under electron microscopy, especially during the later weeks of the experiment, is that of sinuous cisternal profiles dispersed individually (Fig. 10) or in small groups within the cytoplasm (Figs. 10 and 11).

Experimental Animals

Under light microscopy, the basophilic zones are less easily detected in most hepatic cells of animals fed 2-Me-DAB for a period of 10 days than in cells of normal or control animals. The electron microscope shows that these cells still contain cisternal profiles of the granular reticulum (Figs. 12 and 13) but the impression is definitely gained, however, that on the whole many cells contain fewer such structures. At the end of the 10-day period there already has occurred some degree of fragmentation (Fig. 12) and redistribution of these cisternae in most cells. Profiles of these are now often found scattered individually (Fig. 12) or in small groups amongst the mitochondria or in parts of the cytoplasm not occupied by zones of either glycogen or tubules of smooth endoplasmic reticulum. These cisternal profiles of granular reticulum are also now sinuous in contour, forming loops and bends and sometimes curling partly around mitochondria (Fig. 13). Many ribosomes are found free among these profiles.

Fragmentation (Fig. 12) of the cisternal profiles and disorganization (Fig. 13) of their arrays, processes that are already quite evident in the early stages of the experiment, become more pronounced during the following weeks. By the 6th week, and even more so during the 12th to 16th week period, many cells show only short, sinuous, individual profiles (Figs. 14 and 15) mostly located amongst the mitochondria. Occasionally during these last weeks, cells are encountered which still show arrays of long, membranous profiles (Fig. 17) of granular reticulum similar to the irregular groups of cisternae observed at the end of the 10 day period (Fig. 13).

The appearance of the granular endoplasmic reticulum in the "dark" cells will be described in a later section.

Hyaline Inclusions and the Agranular Reticulum

The liver cells of control animals show no evidence of hyaline inclusions during the period studied.

Such inclusions, however, represent the most striking cytological change observed in the liver of animals fed for 10 days on a diet supplemented with 0.06 per cent 2-Me-DAB. They are generally sinuous in outline and form coarse networks extending throughout portions of the cytoplasm (Fig. 4). After PAS staining, all cells show irregular dark-red zones corresponding to those observed in normal hepatic cells. However, in most cells other zones appear which are pinkish or lighter red in color (Fig. 5) and are adjacent and usually continuous with the typical glycogen areas just referred to. These pinkish zones are often heterogeneous in staining intensity and are, then, characterized by small dark-red spots of glycogen scattered within their mass. In size and distribution, these pinkish areas appear to correspond to the hyaline inclusions seen after methylene blue staining (Fig. 4). Evidence in support of this conclusion will be presented later in this paper.

During the 3 to 16 week period, the distribution and importance of the hyaline inclusions vary markedly from cell to cell. Large, often crescent-shaped inclusions are found at one side of the nucleus (Fig. 6), and other smaller inclusions are scattered amongst the mitochondria. Due to the increasing mitochondrial population, the numerous narrow and sinuous hyaline zones become progressively more difficult to detect under light microscopy.

Around the 9th week period many cells show, besides or instead of the dense hyaline zones just described, cytoplasmic areas which appear distinctly different in their internal structure. They are characterized by a loose organization (Fig. 7), the nature of which cannot be conjectured under light microscopy.

The hyaline inclusions (Figs. 4 and 5) are seen under electron microscopy to consist of a network of membranous elements (Fig. 12) which, at high magnification, appear as vesicles and sinuous tubules of varying diameter. These tubules may be loosely or closely packed and thus give rise to inclusions which in different cells show correspondingly either a loose or a dense organization. The continuity of many of these membranous elements with cisternal profiles of the granular endoplasmic reticulum indicates that the inclusions originate as a result of hypertrophy in the smooth reticulum.

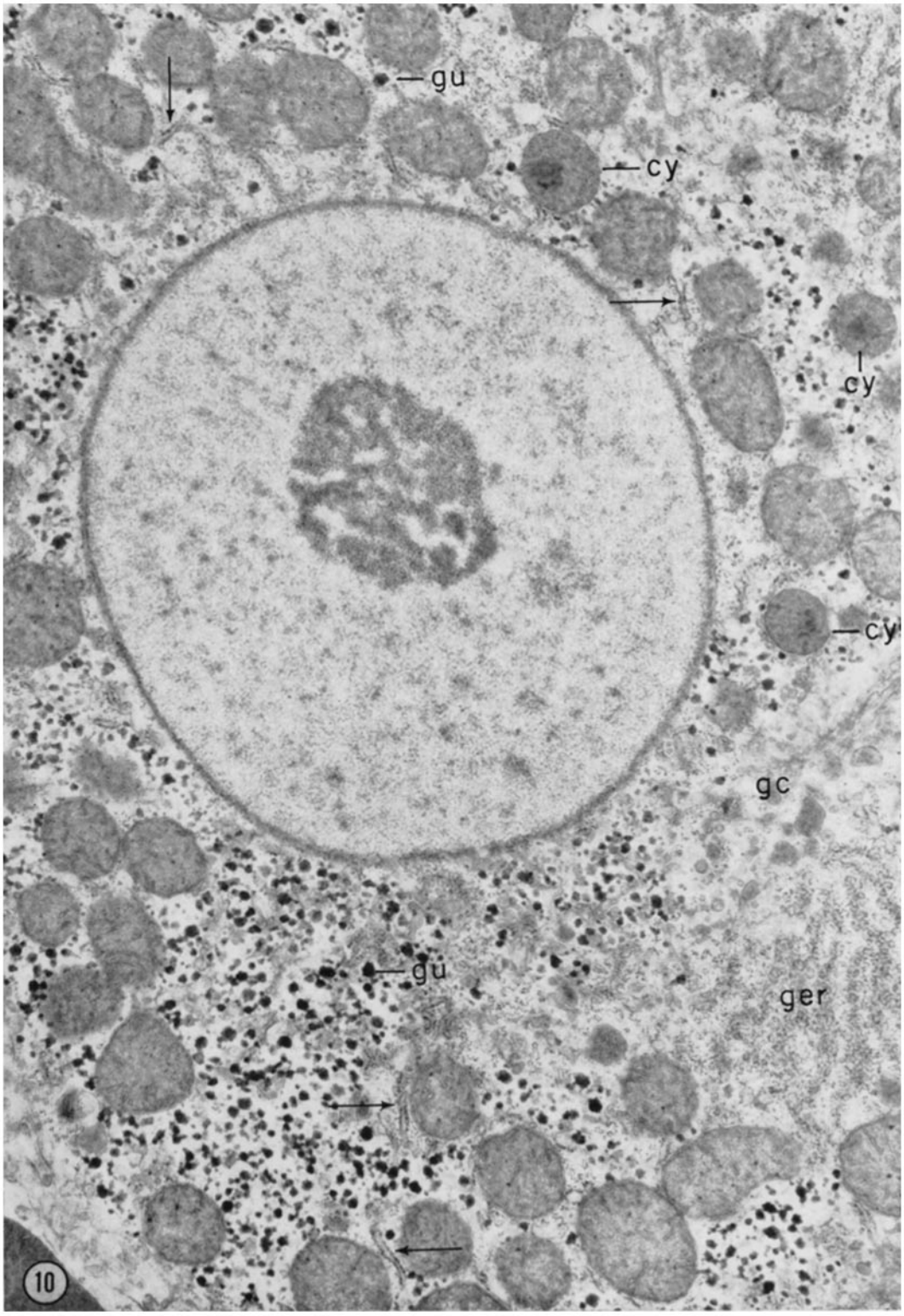
Electron microscopy also sheds light on the presence of both dark-red and pink cytoplasmic areas observed after PAS staining. Our electron micrographs reveal that glycogen units are grouped into large zones (Figs. 12 and 13), containing also a few scattered mitochondria and elements of both the granular and agranular reticulum, and because these zones have a high content of glycogen units they stain densely with the PAS procedure (Fig. 5). The zones which contain fewer glycogen units scattered amongst a large amount of vesicles and tubules of the agranular reticulum, however, stain a pinkish color (Fig. 5). The fact that zones of both types are always contiguous (Fig. 12) accounts for the light micrographic observation that red and pink areas are generally adjacent (Fig. 5).

Glycogen

Control Animals

After PAS staining, no detectable difference in the amount and distribution of glycogen in hepatic cells could be noted between control animals of the 10-day period (Fig. 3) and normal animals. During the following period, up to the 16th week, large uniform zones of glycogen become progressively less frequent and the glycogen is found dis-

FIGURE 10 Electron micrograph of portion of hepatic cell from control animal of the 9-week period. The mitochondria show short cristae as in normal hepatic cells. Three cytosomes (*cy*) with dense nucleoids are also recognized amongst the mitochondria. Part of a Golgi complex (*gc*) is noted close to the nucleus. The granular endoplasmic reticulum (*ger*) in this cell consists of a fairly regular array of cisternae cut obliquely, as well as of a number of short, sinuous double membranes (arrows) found predominantly amongst the mitochondria and often curling partly around some of them. The glycogen units (*gu*) are located mostly within one large cytoplasmic zone but may also be found in small groups among the mitochondria. A number of small, smooth vesicles may be detected within these various glycogen zones. × 17,000.


gu
cy
cy
cy
gc
gu
ger
10

tributed in the form of a network throughout the cytoplasm. This phenomenon is especially striking during the 9th to 16th week period and may result partly from the concomitant dispersion of the mitochondria.

Experimental Animals

As already noted, the liver cells of experimental animals of the 10-day period show large, PAS-positive zones corresponding closely to those observed in normal cells. During the following weeks the glycogen in the hepatic cells of these animals gradually disappears. Observation at low magnification of sections 1 mm^2 in area reveals a patchy distribution of glycogen. Scanning of the same preparations under oil immersion reveals that glycogen is absent from many cells, some of which are adjacent, accounting for its uneven distribution. The decrease in the amount of glycogen is still more noticeable by the 5th week, and by the 9th week most hepatic cells show only small, scattered areas of glycogen or none at all. Occasionally, however, one sizeable zone of glycogen may still be observed within a cell. As a result of dispersion and eventual disappearance of the glycogen in these cells, the contours of the cell, as well as those of the nucleus and mitochondria, which were previously delineated by PAS-staining material, are no longer visible in such preparations.

In the course of our electron microscopic observations it has become apparent that some relationship exists between the evolution of the zones of hypertrophied agranular reticulum and the glycogen content of hepatic cells. The hyaline inclusions of the 10-day period consist of rather loosely packed tubular and vesicular elements (Fig. 12). A number of glycogen units are found within the membranous network thus formed, but most of the glycogen is still aggregated within large cytoplasmic areas adjacent to the zones of hypertrophied agranular reticulum. The electron microscope reveals that during the period of progressive glycogen depletion that occurs during the following weeks the glycogen units become restricted almost exclusively to zones of membranes of the agranular reticulum. Moreover, the organization and glycogen content of these zones vary considerably from cell to cell. Cells which show a loosely organized network of tubular elements invariably still contain glycogen units within such zones (Figs. 14 and 15). A number of the glycogen units stain as densely with lead hydroxyde as they do at the end of the 10 day period, but a large proportion is now much lighter (Figs. 14 and 14) and, therefore, appears as ghosts. The lighter units are usually somewhat larger than the denser particles. On the other hand, cells which are characterized by zones of tightly packed tubular elements show only a few (Fig. 16) very small glycogen units amongst these tubules or none at all.

Mitochondria

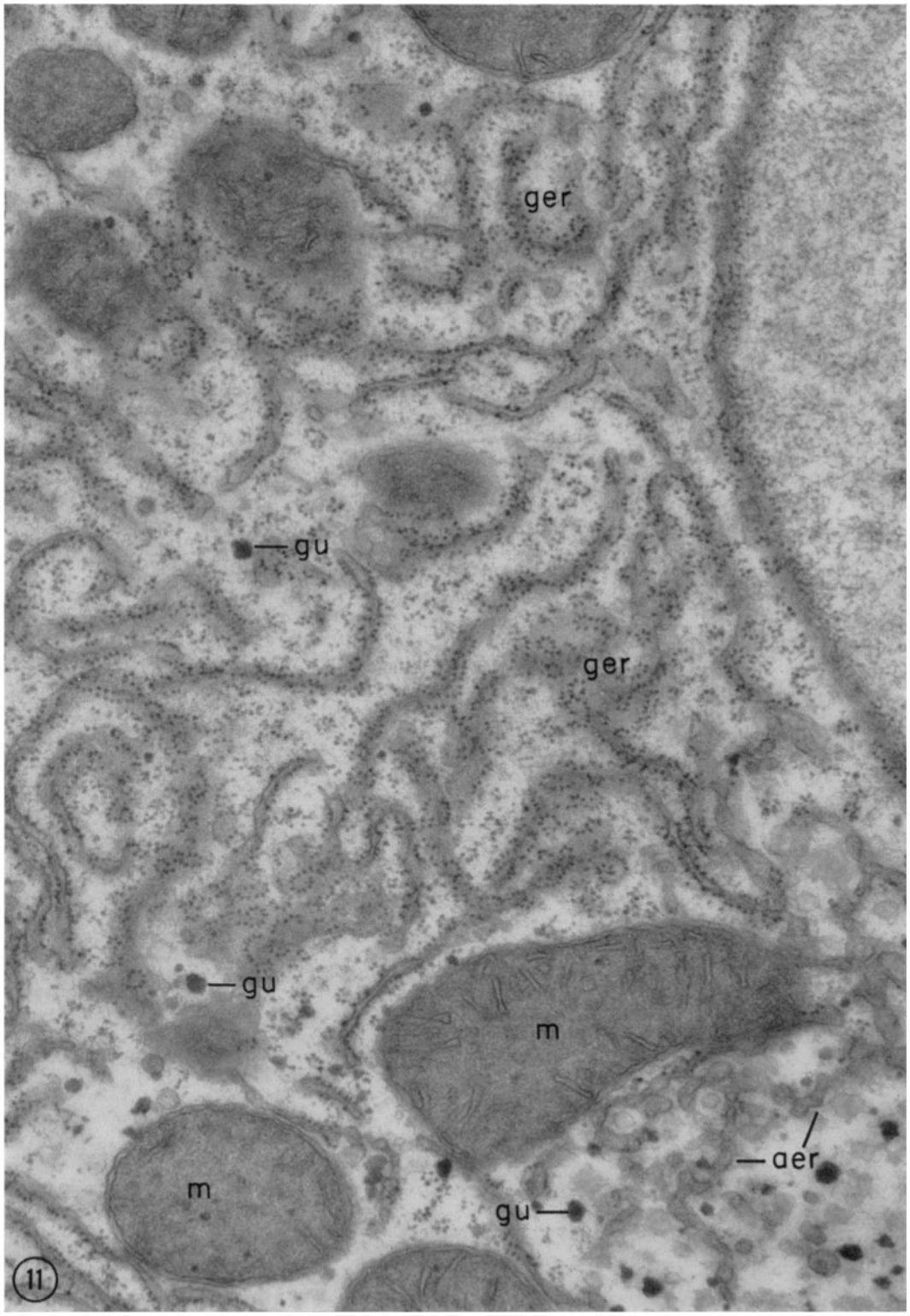
Control Animals

At the end of the 10-day period, the mitochondria are as numerous as in normal hepatic cells and likewise mostly grouped within the basophilic areas of the cytoplasm (Fig. 2). During the following weeks the mitochondria remain in normal number, but in the lighter cells a certain proportion of them are dispersed in non-basophilic zones of the cytoplasm. The impression is gained, however, that in the dark cells the mitochondria are more consistently restricted to the basophilic zones.

Experimental Animals

Starting with the 3rd to 4th week period, it is observed that the number of mitochondria per cell gradually increases, and that a few weeks later the cytoplasm is crowded with them (Figs. 6 and 7). A number of cells are then characterized by large, lightly stained mitochondria, the diameter of which is up to three times that of the remaining mitochondria (Figs. 7, 8, and 9). Many such mitochondria show under light microscopy a series of dense

FIGURE 11 Micrograph of portion of hepatic cell from same period as Fig. 10 but depicting in more detail the ultrastructure of the mitochondria (*m*) and organization of the granular endoplasmic reticulum (*ger*). The cisternal profiles of the granular reticulum are short, fragmented and very irregularly disposed. Also, a fairly large number of free ribosomes are found among these profiles. Tubular and vesicular elements of the agranular reticulum (*aer*) may be recognized at the lower right together with a few glycogen units (*gu*). × 40,000.


ger
gu
ger
gu
m
aer
m
gu
11

streaks which, as will become evident later, correspond to arrays of closely packed cristae.

Besides this gradual increase in the number of mitochondria per cell which is noted in 0.5 μ sections under the light microscope, the electron microscope reveals concomitant changes in their internal structure. It is observed that more and more mitochondria show arrays of long double membranes (Figs. 14, 17 to 21, and 24). These membranes are usually rather straight and extend only partly within the mitochondrial body (Figs. 14, 18, 21, and 24), but in a number of mitochondria they are variously curved (Fig. 17) and in some cases may be concentric with the mitochondrial envelope. In most of our electron micrographs, the origin of these arrays of double membranes is not easily assessed, presumably due to the plane of sectioning. In a number of cases, nevertheless, at least one, and sometimes two or three, out of a group of seven to ten double membranes, are continuous with the inner leaflet of the mitochondrial envelope (Fig. 18). No attempt was made to determine by means of serial sectioning whether all the membranes of such arrays show a similar continuity or whether repeated folding of some of them is involved instead.

A second and certainly more significant type of morphological change observed in many mitochondria during later stages of the experiment is the occurrence of a double membrane structure extending across their body and fusing with the internal membrane of their envelope (Figs. 19 and 20). In the most convincing instances, this structure is located about halfway along the length of the mitochondrial body which is usually noticeably longer than the neighboring mitochondria. Such mitochondria also often show evidence of pinching at the level of this transverse double membrane. This membrane is usually quite regular and straight (Fig. 19) and is characterized by an interleaflet space corresponding in width with the space between the two membranes of the mitochondrial envelope. Occasionally, the transverse double membrane is corrugated (Figs. 20 and 21), so that the interleaflet space becomes irregular and in places (Fig. 21) several times wider than it usually is. A further characteristic of such partitioned mitochondria is the invariable presence of several long cristae frequently organized into arrays (Figs. 20 and 21). Such arrays are more often observed only on one side of the median double membrane but they may also be present on both sides.

Finally, a large percentage of the mitochondria in both light and dark cells from livers of experimental animals and, occasionally, also from control animals, is characterized by the presence of cristae which in cross-section appear triangular or sometimes quadrangular (Figs. 14, 17, 22, and 24).

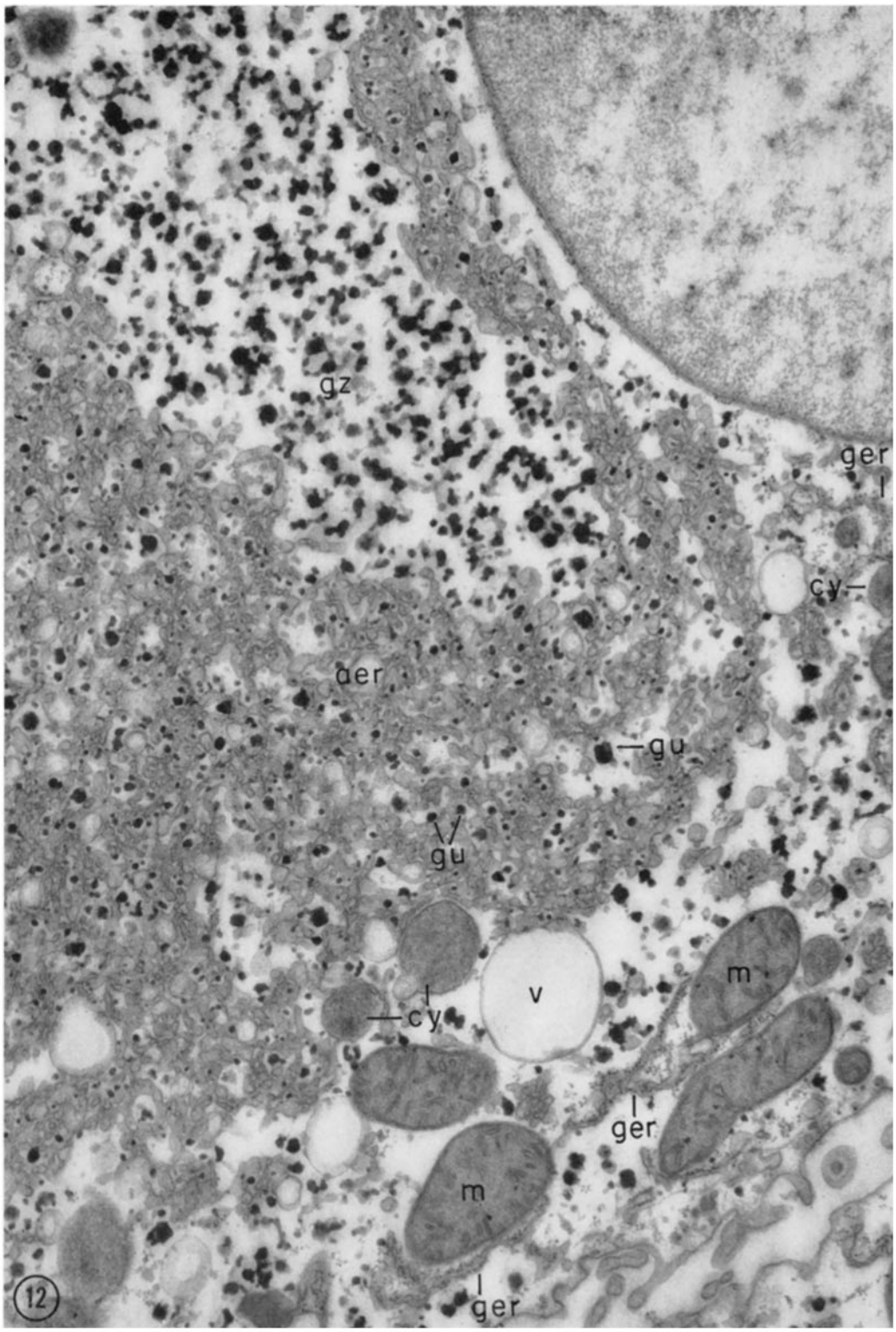
Large mitochondrion-like cytoplasmic particles (Figs. 7 to 9), which are observed sometimes in liver cells of the 9 to 16 week period, possess the ultrastructural features of mitochondria (Fig. 23). They are limited by a double-layered envelope and show a number of cristae some of which are continuous with the internal membrane of the envelope. Besides these cristae, the total width of which is normal, other membranous elements have invariably been noted in the large mitochondria. These elements appear as isolated vesicles or networks of flattened and irregular sac-like structures. The matrix of such mitochondria is often lighter than that of the smaller neighbouring organelles but may almost match their density in other instances.

Lipid Inclusions, Lysosomes, Cytosomes and the Golgi Complex

Phase contrast observation of 0.25 to 0.5 μ sections of liver from experimental animals reveals

FIGURE 12 Micrograph of a cell from animal fed for a period of 10 days on a diet containing 0.06 per cent 2-Me-DAB. This figure illustrates the fine structure of a zone of agranular endoplasmic reticulum (*aer*). The cytoplasm in this area consists of a loose network of irregular membranous tubules and vesicles. A number of glycogen units of various sizes are located within the mesh of this network. Similar units are grouped into a large cytoplasmic area which is adjacent to the mass of tubules and corresponds to a glycogen zone (*gz*).

Isolated cisternal profiles of granular endoplasmic reticulum (*ger*) are found amongst the mitochondria. One of these (arrow) cisternae seems to bifurcate. The mitochondria (*m*) appear unaffected at this time. The cell also contains a number of cytosomes (*cy*) and vacuole-like (*v*) structures which appear to represent swollen vesicles of the agranular reticulum. × 25,000.


gz
ger
l
cy
aer
gu
gu
v
m
cy
ger
m
ger
12

that the cytoplasm of the hepatic cells contains, besides mitochondria of various sizes and density, an appreciable number of roundish, darkly stained, and highly refractile particles (Figs. 7 to 9). Many such particles are only slightly smaller than mitochondria, but the majority are noticeably so. No clear-cut preferential distribution of these particles could be noted in our preparations: most are found intermingled with mitochondria, but some are located at the periphery of both the dense and light hyaline inclusions or sometimes within the latter.

Under electron microscopy it is observed that different types of bodies fit the above description. The first group of such bodies is observed mostly during the 9th to 16th week period. They are amorphous in texture, show no limiting membrane, and have irregular, serrated contours. These lipid-like inclusions (Figs. 15 and 16) are found amongst the mitochondria as well as at the periphery of (Fig. 15) or within (Fig. 16) the zones of tubules and vesicles of the hypertrophied agranular reticulum.

The second type of body has the characteristic features of lysosomes (30). These lysosomes are variously distributed within the cytoplasm, but the majority are found at the cell periphery (Figs. 14 and 17). In some instances groups of up to twenty of these have been found near bile canaliculi.

Most if not all hepatic cells also show a number of roundish, membrane-bounded bodies of the type identified as microbodies (31) or cytosomes (7, 32) (Figs. 12, 17, and 23). These organelles contain a dense nucleoid which at high magnification shows evidence of a latticed substructure. Microbodies are most frequently found amongst the mitochondria and, like the latter, may sometimes be embedded within zones of agranular reticulum.

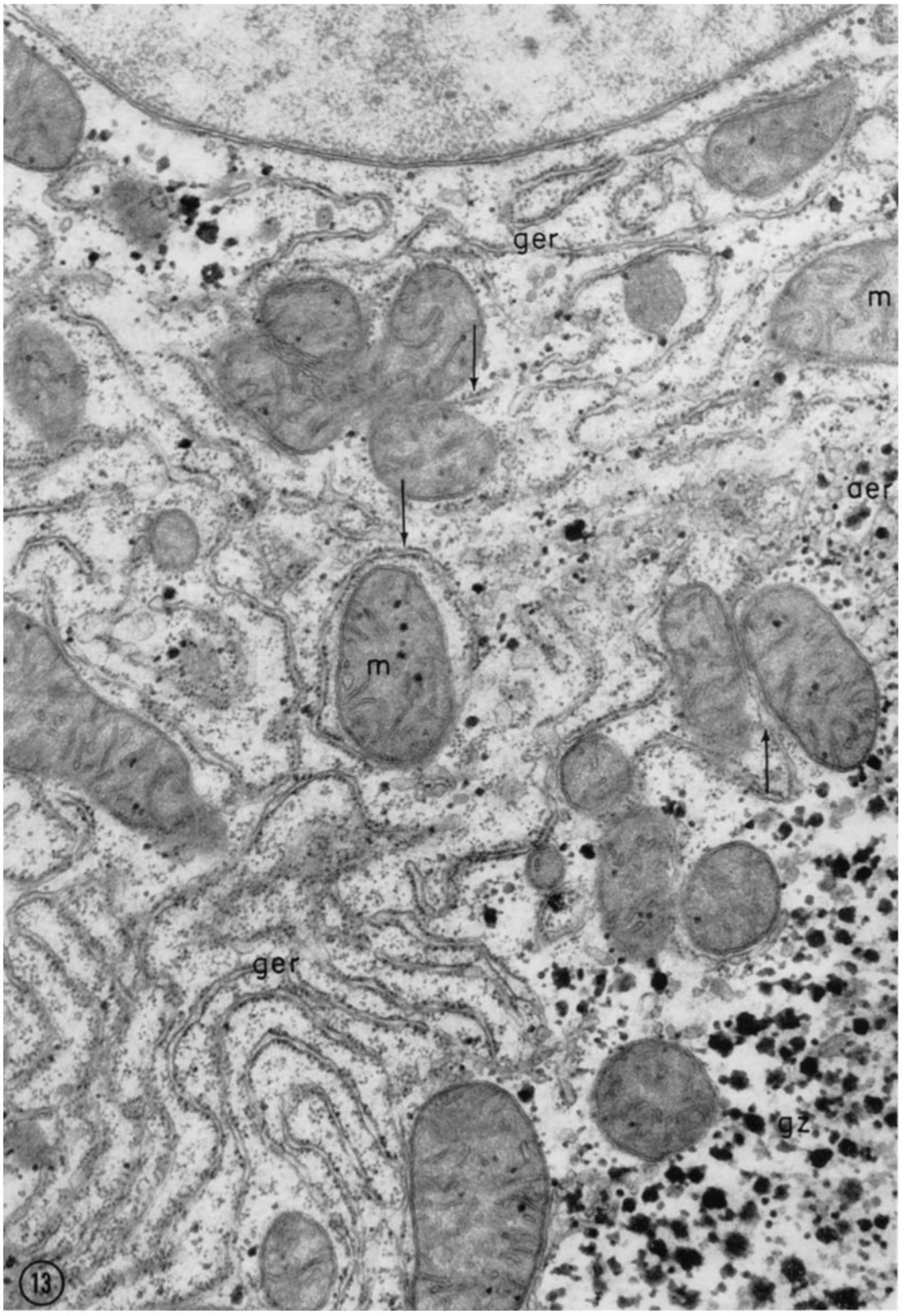
A third type of cytoplasmic body is found typically within or immediately near the Golgi complex. It should be noted at this point that two, three, and up to five Golgi zones are frequently present in hepatic cells during the later weeks of the experiment. In most micrographs these Golgi complexes appear hypertrophied and characteristically contain a number of vesicles of various sizes and shapes (Figs. 24 and 25). Many of these vesicles are located at the extremities of crescent-shaped arrays of smooth double membranes from which conceivably, they are being pinched off. After lead staining, all of these vesicles show a dense matrix, and many contain still denser granules (Figs. 24 and 25) similar to those noted earlier by Karrer (33). Most of the dense vesicles are only 0.1 μ in diameter or even smaller. However, some of them are several times that size and may possibly account for a small proportion of the tiniest cytoplasmic particles observed under phase contrast microscopy.

Dark Cells

Control Animals

Although under light microscopy the hepatic cells from control animals do not vary appreciably in their morphology during the course of the experiment, two groups of cells may nevertheless be distinguished during the last ten weeks or so, on the basis of the intensity of their staining with methylene blue. During this later period an increasing number of cells stain much more intensely with this dye, so that sections of tissue now look like mosaics of varying staining intensity. Since both the nucleus and cytoplasm of these dark cells stain quite densely with methylene blue, it was necessary to examine 0.25 μ sections under phase contrast to detect the mitochondria and other cytoplasmic structures. Examination of such preparations has failed to indicate clearly how these dark cells differ morphologically from the lighter ones, except to show that in the latter cells there is some degree of dispersion of the mitochondria. However, by the 12th week, and

FIGURE 13 Portion of cell from experimental animal of the 10-day period showing a cytoplasmic area occupied by a group of convoluted cisternal profiles of granular endoplasmic reticulum (*ger*). Some of these are partly curled (arrows) around mitochondria or are squeezed (arrows) between them. Many free ribosomes are noted within this zone. At the lower right is part of a glycogen zone (*gz*). Glycogen units, some of them quite small, are also scattered within the zone of agranular reticulum (*aer*) as well as amongst the particle-studded membranes. × 32,000.


ger
m
ger
m
ger
gz
13

more so later on, a large number of cells are of the dark type.

Experimental Animals

In sections of liver tissue from experimental animals of the 10-day period most of the cells stain lightly with methylene blue but a number of others already stain much more intensely. These dark cells do not seem to have any particular distribution: they are found here and there, singly or in groups of two or more. Their density is such that mitochondria are visualized with difficulty, even in 0.5 μ sections examined under phase contrast microscopy.

In the ensuing weeks a larger proportion of the cells is of the dark type. These cells, as well as the lighter ones, now show an appreciable increase in their mitochondrial population. Under electron microscopy, both the nucleus and cytoplasm of the dark cells are noticeably denser than those of the light ones. Our micrographs furnish little clue to the origin of this electron opaqueness. It would appear that some amorphous substance permeates these cells and gives rise to their observed density.

Arrays of cisternal profiles of the granular endoplasmic reticulum are seldom encountered in the dark hepatic cells, as is also the case for the light cells of the same period. Whenever present, they consist of irregular membranes which fold back on themselves and form very sinuous patterns. In those cells where no such agglomeration of particle-studded membranes is present, the granular reticulum takes the form of short profiles most of which are squeezed between the mitochondria and usually curl partly around them. Because these short profiles are frequently closely associated with the surface of the mitochondria, many of them are inconspicuous at first sight.

In accord with the light and patchy PAS staining of these dark cells, very little glycogen is detected in them under electron microscopy (Fig. 25). Areas of the cell which consist predominantly of smooth tubular or vesicular elements are seen to contain isolated glycogen units or small groups of units. The very small size of the glycogen zones together with their scattered distribution accounts for the patchy PAS staining of these cells.

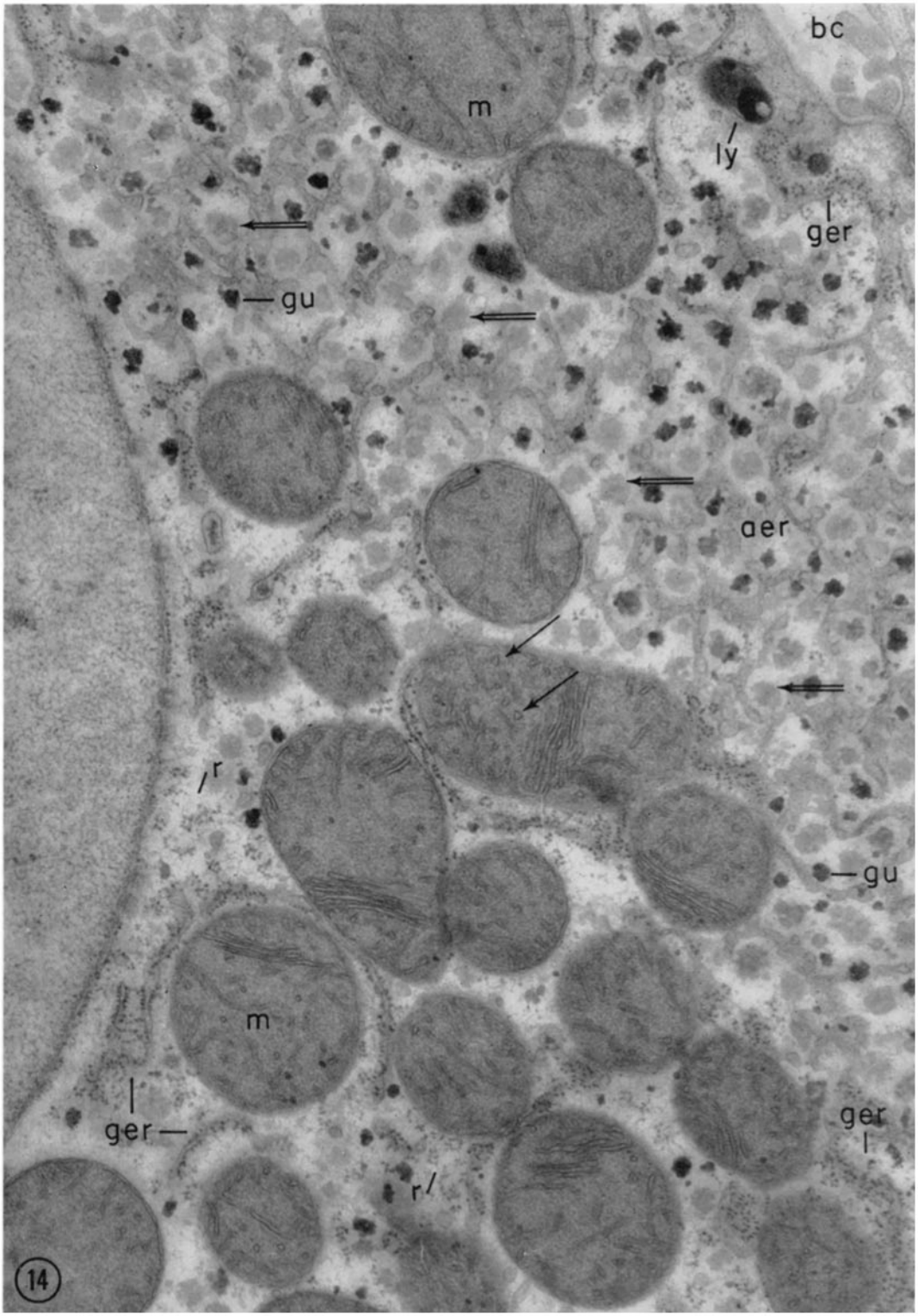
The most significant feature of the dark cells is undoubtedly the large percentage of their numerous mitochondria which shows a double membrane partition of the type described earlier (Figs. 16 and 19) as well as groups of long cristae in parallel disposition. Cells are often encountered in which a quarter to a third of the mitochondria have a transverse partition and in which at least two out of three of these organelles contain arrays of closely disposed double membranes or cristae.

DISCUSSION

The aminoazo dye 2-Me-DAB initially provoked the interest of a number of workers because of its apparent specific action on the mitochondria of hepatic cells. The observation that rats given a diet containing this compound did not develop liver tumors (22, 23) and that the general histological architecture of this organ was maintained (4) has commonly led to the conclusion that this compound, unlike a closely related aminoazo dye such as 3′-Me-DAB (34), is not carcinogenic. From the present study it is apparent, however, that 2-Me-DAB duplicates most of the morphological changes induced in hepatic cells by highly carcinogenic substances (7–15) as well as by a number of other substances which are toxic but non-carcinogenic (16–21). Since, among cytoplasmic components, the endoplasmic reticulum, mitochondria, and glycogen show a pronounced response to the action of 2-Me-DAB, the discussion

Figure 14 Portion of a liver cell from animal fed for a period of 9 weeks on a diet containing 0.06 per cent 2-Me-DAB. A number of mitochondria (*m*) are characterized by long lamellae sometimes organized into arrays. Some of the cross-sections of cristae are triangular (single arrows). The cisternal profiles of the granular reticulum are fragmented and scattered mostly amongst the mitochondria. Several free ribosomes (*r*) are also found near these cisternae.

The agranular reticulum (*aer*) is hypertrophied in this cell and consists of a very loose network of irregular tubular elements. Glycogen units (*gu*), some stained intensely, others only faintly (double arrows) are located within the mesh of this network. Most of these glycogen units are constituted of a number of smaller elements. The great majority of the lightly stained ghost-like units (double arrows) are somewhat larger than the denser ones. A lysosome(*ly*) is located close to a bile canaliculus (*bc*). × 30,000.


bc
m
ly
ger
gu
aer
r
gu
m
ger
ger
r
14

that follows will be limited mainly to these components.

The Granular Reticulum

Our light microscope observations on relatively thin sections of normal hepatic cells show that cytoplasmic areas can be recognized which, on account of their distribution and size, presumably correspond to arrays of cisternae of the granular endoplasmic reticulum. One of the earliest effects of 2-Me-DAB feeding noted under light microscopy is the disappearance of such conspicuous zones in many cells. This phenomenon was shown to be the result of a more or less pronounced dispersal of the component cisternae of these zones amongst the mitochondria and other areas of the cytoplasm. The cisternae in question may remain closely associated in a certain proportion of the cells, but in these instances they appear fragmented and irregularly organized.

These observations are in accord with those of a number of workers who have studied the morphology of hepatic cells under different experimental conditions. Berg (35) and Lagerstedt (36) noted that fasting, for instance, affects the cytoplasmic basophilia of the hepatic cells, and Bernhard *et al.* (37) reported similar observations with the electron microscope. Fawcett (38), in a more detailed study, first related this decreased basophilia with a dispersal of the membranes of the granular endoplasmic reticulum. Later investigations of more acute morphological disturbances in hepatic cells brought about by non-carcinogenic but highly toxic substances, such as carbon tetrachloride (16), phosphorus (18), and α-naphthylisothiocyanate (21), have led to similar conclusions. Oberling and Rouiller (16) report that during the early stages of carbon tetrachloride poisoning the decreased basophilia of the centrolobular cells is accompanied by a corresponding progressive diminution in the number of cisternal profiles of the granular reticulum and their eventual disappearance. According to Jézéquel (18), Smuckler *et al.* (20), and Steiner and Baglio (21), the disorganization of these membranes also leads to a dispersal in the ground cytoplasm of some of the ribosomes.

A similar phenomenon occurs after feeding the potent hepatic carcinogen 3′-Me-DAB (7) and, apparently to some extent at least, also after ingestion of dimethylnitrosamine (8) and thioacetamide (13, 14). In their well documented study, Porter and Bruni (7) show that already at the 2-day period of feeding 3′-Me-DAB the arrays of parallel cisternal profiles of the granular endoplasmic reticulum become disorganized and that individual double-membraned elements are scattered amongst the mitochondria. They estimate, moreover, that a diminution of RNP particles takes place at that time. These authors report that both types of morphological disturbances of the granular reticulum are accentuated during the two following weeks.

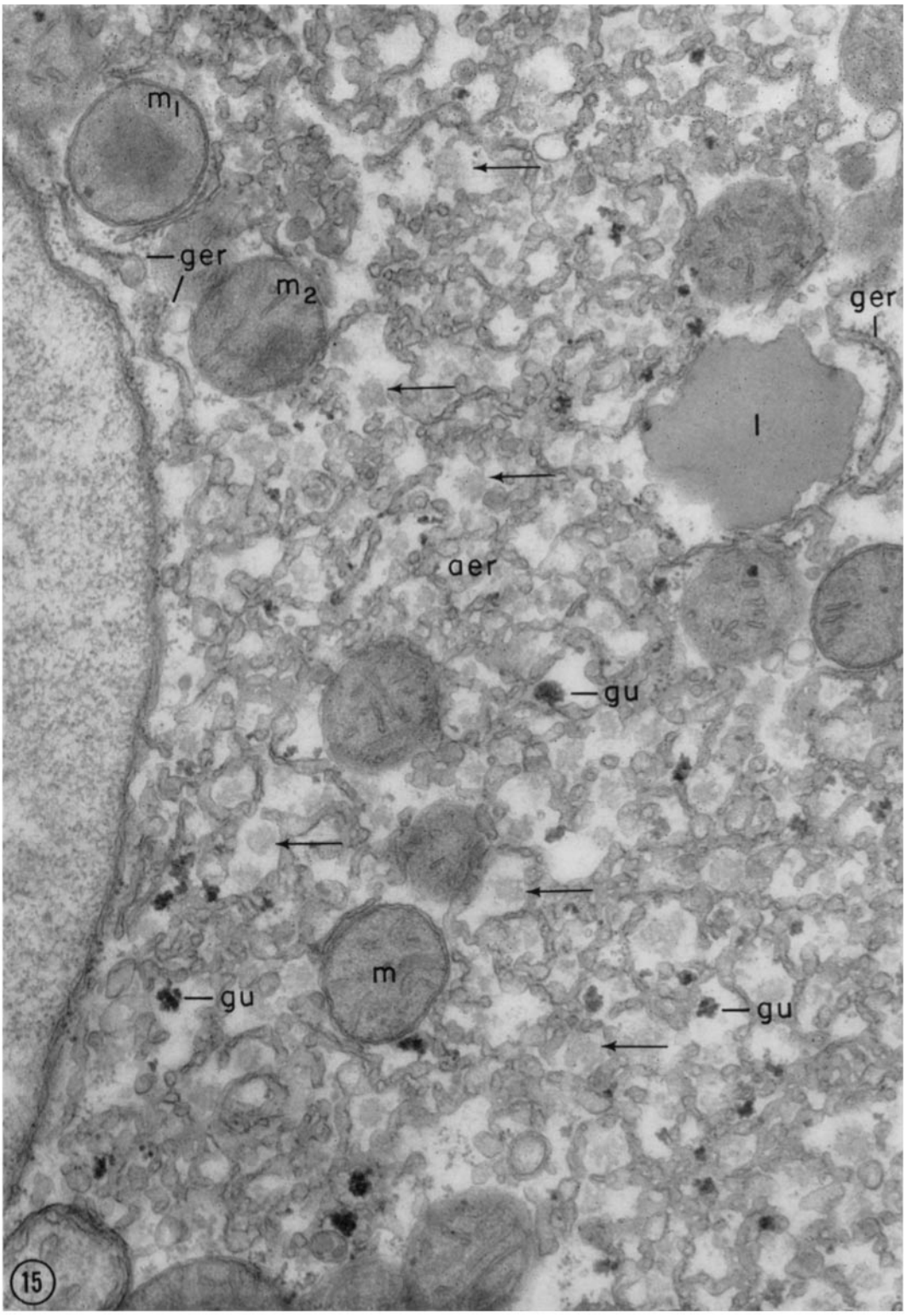
From these studies of various toxic and carcinogenic substances, and from our own studies with 2-Me-DAB, it is clear therefore that the loss of basophilia accompanied by a dispersion of the arrays of linear lamellae of granular endoplasmic reticulum, and in some cases a detachment of their ribosomes, are not morphological changes induced exclusively by carcinogenic substances. A comparison of the effects of the two related azo dyes, 2-Me-DAB and 3′-Me-DAB (7), definitely indicates, however, that the process of fragmentation and dispersion of these cisternae is much more rapid following ingestion of the carcinogen.

The Agranular Reticulum and Glycogen

Since Fawcett's (38) original observation that masses of tubular and vesicular elements appear in liver cells of rats fasted and refed, the apparent relationship of such zones to glycogen has roused much interest and speculation (7, 39–41). It has

FIGURE 15 Electron micrograph of part of a cell from experimental animal of the 11-week period. The area illustrated shows a large zone of tubules of the agranular endoplasmic reticulum (*aer*). This network of tubules is rather loose, as in Fig. 15, but here most of the glycogen units stain very lightly with lead hydroxyde. These ghost-like units (arrows) are all definitely larger than the denser ones (*gu*). Two of the mitochondria (m_1 and m_2) show arrays of lamellae cut tangentially.

A few short profiles of particle-associated cisternae (*ger*) may be seen near some mitochondria. One lipid inclusion (*l*) is illustrated. Its homogenous inner portion is limited by a somewhat denser thin layer. × 36,000.


m1
ger
m2
ger
l
aer
gu
m
gu
gu
15

been shown (7, 42) that in hepatic cells of rats fed a diet containing 3'-Me-DAB these extensive masses of tubules of the agranular reticulum correspond to the hyaline inclusions seen under light microscopy and already well known in a number of pathological conditions. Such membranous zones develop in glycogen areas of the hepatic cell (7). In the course of later studies, Porter and his associates (39, 40) have presented evidence that feeding an animal after prolonged fasting induces an increase in the number of smooth vesicular profiles along with an accumulation of glycogen in the glycogen zones of the hepatic cell. A few hours later, when glycogen storage has reached a maximum these smooth vesicles have more or less completely disappeared. This last observation concurs with the report that in hepatic glycogenosis where cells are literally loaded with glycogen no smooth membranes of the reticulum have been noted (43).

On the basis of the observations mentioned above and others on animals treated with glucagon and epinephrine (40), it has been postulated that the smooth surfaced reticulum of the hepatic cell is somehow involved with synthesis and storage of glycogen. The findings, however, also lend themselves to different interpretations, and some of these have recently been discussed (40). For instance, Fawcett has questioned the role of the agranular reticulum in the synthesis and degradation of glycogen (41) and cited a number of examples of tissues where the presence of large zones of this component of the reticulum is unquestionably related to other cellular functions.

The present study has shown that ingestion of 2-Me-DAB induces in hepatic cells the formation of conspicuous hyaline inclusions which after 10 days are already quite large. At this early stage of the experiment, a noticeable decrease in the size of the zones of glycogen is observed. Electron microscopy has revealed that during the following period up to the 12th week, the remaining glycogen units are restricted to zones of tubules which correspond to the hyaline inclusions seen under light microscopy. Although, on the whole, the feeding of 2-Me-DAB tends to gradually deplete the cells of most of their glycogen, many of the cells still show variable amounts of glycogen within zones of hypertrophied agranular reticulum. These observations are best interpreted at present as indicating some relationship between the disappearance of the glycogen zones and the concurrent hypertrophy of the agranular reticulum. Moreover, our images suggest that the close-meshed networks of tubules characterize cells with a high degree of glycogen depletion whereas zones consisting of less densely packed, irregular tubules and vesicles are found in cells which still contain a certain amount of glycogen.

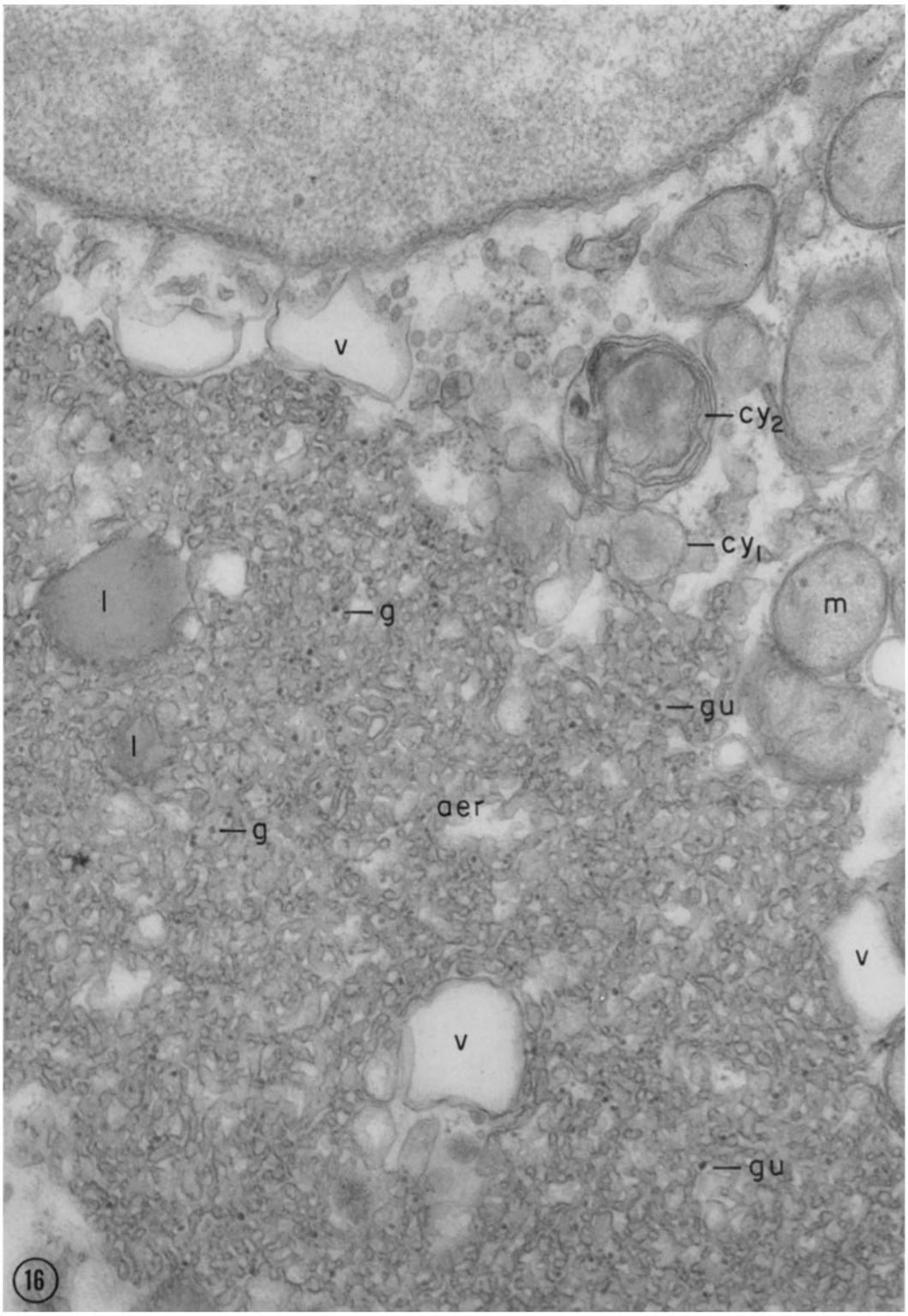
Steiner and Baglio (21) have also noted a close relationship between the hypertrophy of the agranular reticulum and glycogen depletion. They report that this hypertrophy takes place at a time when glycogen is still abundant, as noted in the present study. They further report in accord with our observations, that the masses of tubules of the agranular reticulum are more compact in cells which reach almost complete depletion of their glycogen.

We can offer little explanation for the observation that the glycogen units located within the mesh of loosely organized zones of agranular reticulum do not all stain with equal intensity with lead hydroxyde and that, furthermore, the light, ghost-like units are usually appreciably larger than the dense ones. Since previous studies (7, 21, 39, 40), as well as the present one, clearly suggest a dynamic relationship in hepatic cells between zones of agranular reticulum and glycogen, it is not unreasonable to assume that the observed variation in staining intensity of neighboring glycogen units reflects an evolution in the biochemical or physicochemical state of these various particles.

Mitochondria

Early studies with histological preparations and homogenates (3, 4) have indicated that the

FIGURE 16 Portion of a cell from the same animal used for Fig. 15. Here the zone of agranular reticulum (*aer*) is constituted of closely packed tubules and a few membrane-bounded vesicles (*v*). The residual glycogen material (*gu*) consists of very small dense particles some 200 to 250 A in diameter. The zone illustrated contains two lipid inclusions (*l*). Two cytosomes may also be recognized: both are wrapped by one (cy_1) or several (cy_2) smooth membranes which are most likely related to those forming the neighboring zone of tubules. × 44,000.


v
—cy2
—cy1
l
—g
m
—gu
l
aer
—g
v
v
—gu
16

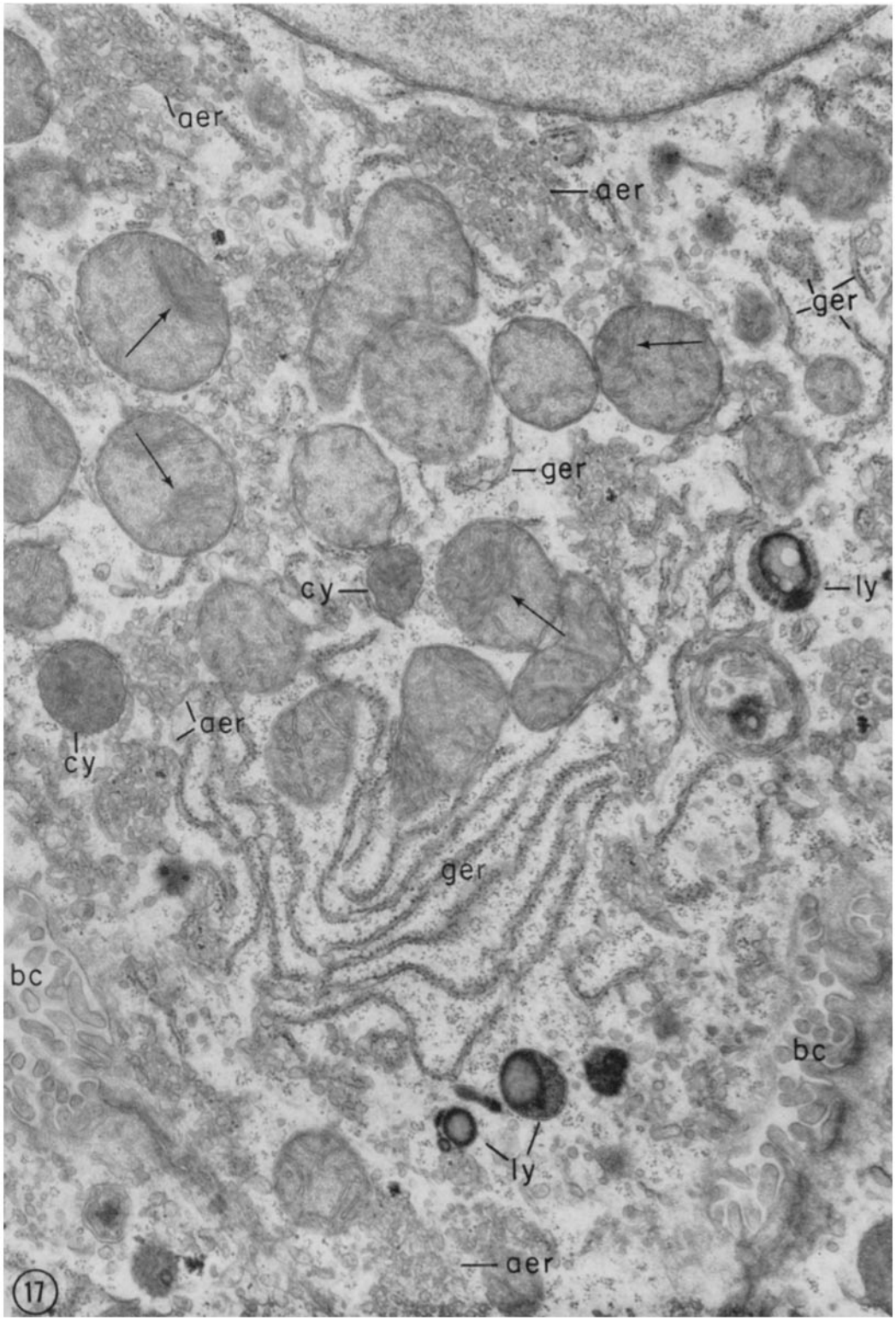
most specific effect of the non-carcinogenic azo dye 2-Me-DAB on the hepatic cell is to induce a remarkable increase in the mitochondrial population. The present observations have wholly confirmed these findings and shown, moreover, that this increase in the number of mitochondria per cell is accompanied by changes in their ultrastructure. Most of these changes are similar to the morphological variations characteristic of mitochondria in liver and other tissues or to variations occurring under experimental conditions other than those prevailing in the present study. Mitochondria with cristae which are angular in cross-section have, for instance, been recorded in a variety of normal tissues (44), and they may also be recognized in hepatic cells of rats subjected to fasting (reference 40, Fig. 5). A second ultrastructural alteration of mitochondria, the appearance of arrays of long crista-like lamellae, is also a frequent feature. Such arrays have been observed in liver epithelioma by Jézéquel (45) who also noted continuity of some of these double membranes with the inner leaflet of the mitochondrial envelope. Similar membranous arrays are present in mitochondria of cells of cholestatic livers (46) and in liver cells following ammonium carbonate poisoning (47), dietary deficiencies (48), and extrahepatic biliary obstruction (49). The many variations in the internal morphology of mitochondria in liver and various other tissues have recently been reviewed (50, 51).

Since the morphological changes discussed above are observed in a variety of situations where no indication exists of a significant increase in the number of mitochondria, it is, therefore, doubtful whether in the present study such ultrastructural alterations of the mitochondria are in any way related to their apparent duplication. A third ultrastructural change, the presence of a transverse double membrane continuous at both ends with the inner layer of the mitochondrial envelope, is more suggestive in this respect, however, and certainly represents the most significant type of modification induced in rat liver mitochondria by the ingestion of 2-Me-DAB. Such transverse membranes were first noted by Fawcett (38) in the mitochondria of hepatic cells of rats during both fasting and refeeding, and the possibility was considered that these mitochondria might be in the process of division. A preliminary report (52) has appeared describing an increased number of mitochondria in hepatic cells of guinea pigs subjected to fasting or to a scorbutic diet. Indications were apparently obtained suggesting that fission of the mitochondria occurs under such experimental conditions, but the internal morphology of these organelles is not described.

Previous observations (3, 4), as well as those reported here, to the effect that 2-Me-DAB induces an appreciable increase in the number of mitochondria per cell raise the question of the origin of these organelles. Are they formed *de novo* or rather do they originate from preexisting bodies by division of these bodies? Our electron micrographs provide evidence strongly suggesting the latter mode of formation. First, no cytoplasmic organelles are observed in our material which would qualify as premitochondrial bodies such as those recently described (53) in both regenerating and embryonic rat livers. We feel, moreover, that the presence of a transverse membrane in many mitochondria is highly indicative that such organelles are in the process of dividing. The objection could be raised that, due to their packing within the cytoplasm, many mitochondria are in close enough contact to fuse, thus, transitionally at least, giving rise to the double partition observed. This line of reasoning, undoubtedly, would find support in the often quoted cinematographic observations of Frederic and Chèvremont (54) that mitochondria of tissue culture cells readily fuse as well as divide. Although this may well be

FIGURE 17 Portion of a cell from experimental animal fed for 9 weeks on a diet containing 0.06 per cent 2-Me-DAB. Many of the mitochondria illustrated are characterized by arrays (arrows) of curved double membranes cut obliquely. Two cytosomes (*cy*) and several lysosomes (*ly*) are shown.

Some of the cisternal profiles of the granular reticulum (*ger*) are grouped into one irregular array in the lower part of the figure but several much shorter particle-studded ones are scattered individually amongst the mitochondria. Small zones of closely packed smooth tubules (*aer*) containing tiny glycogen units are located in various areas of the cytoplasm. A bile canaliculus (*bc*) runs obliquely on each side of the lower portion of the figure. × 14,000.


aer
aer
ger
ger
ly
cy
cy
aer
ger
bc
bc
ly
aer
17

the case, the ultrastructure of such fusing mitochondria, unfortunately, is not known at present. Certainly mitochondria are quite often seen touching one another and sometimes even pushing into each other to the point of deforming their normally regular contours, but in such cases, however, the boundary between them is always four-layered (see Fig. 24).

A point that remains to be discussed is that the transverse double membrane most likely represents a partition between two separate compartments and is not merely a long tubular crista which happens to have extended completely across a mitochondrion. Without serial sectioning it is impossible to prove the first possibility categorically, and therefore the following observations are offered instead in support of it. First, in all sections of such mitochondria longitudinally cut, as established by the double character of their outer envelope, the transverse membrane is likewise clearly double and, moreover, is always located medially or closely so. The former of these two characteristics is suggestive of the presence of a partition orientated perpendicular to the long axis of the mitochondria. Secondly, the transverse membrane always extends across the mitochondrial cavity and fuses at both ends with the inner leaflet of the envelope; moreover, it is always found to be perpendicular to that envelope or closely so. Now it should be recalled that the long cristae, which also characterize such mitochondria are randomly orientated, instead, with respect to the long axis of these organelles. Finally, in all cases recorded these long cristae may be either straight or curved but the spacing between their two limiting membranes is always quite regular and of a constant value. The transverse double membrane, on the other hand, is often wavy, and in such cases the spacing between the two leaflets varies greatly. Significantly enough, a slight to appreciable amount of pinching of the mitochondrion is invariably present at the level of these wavy transverse membranes, but such is not always the case when the transverse membranes are straight.

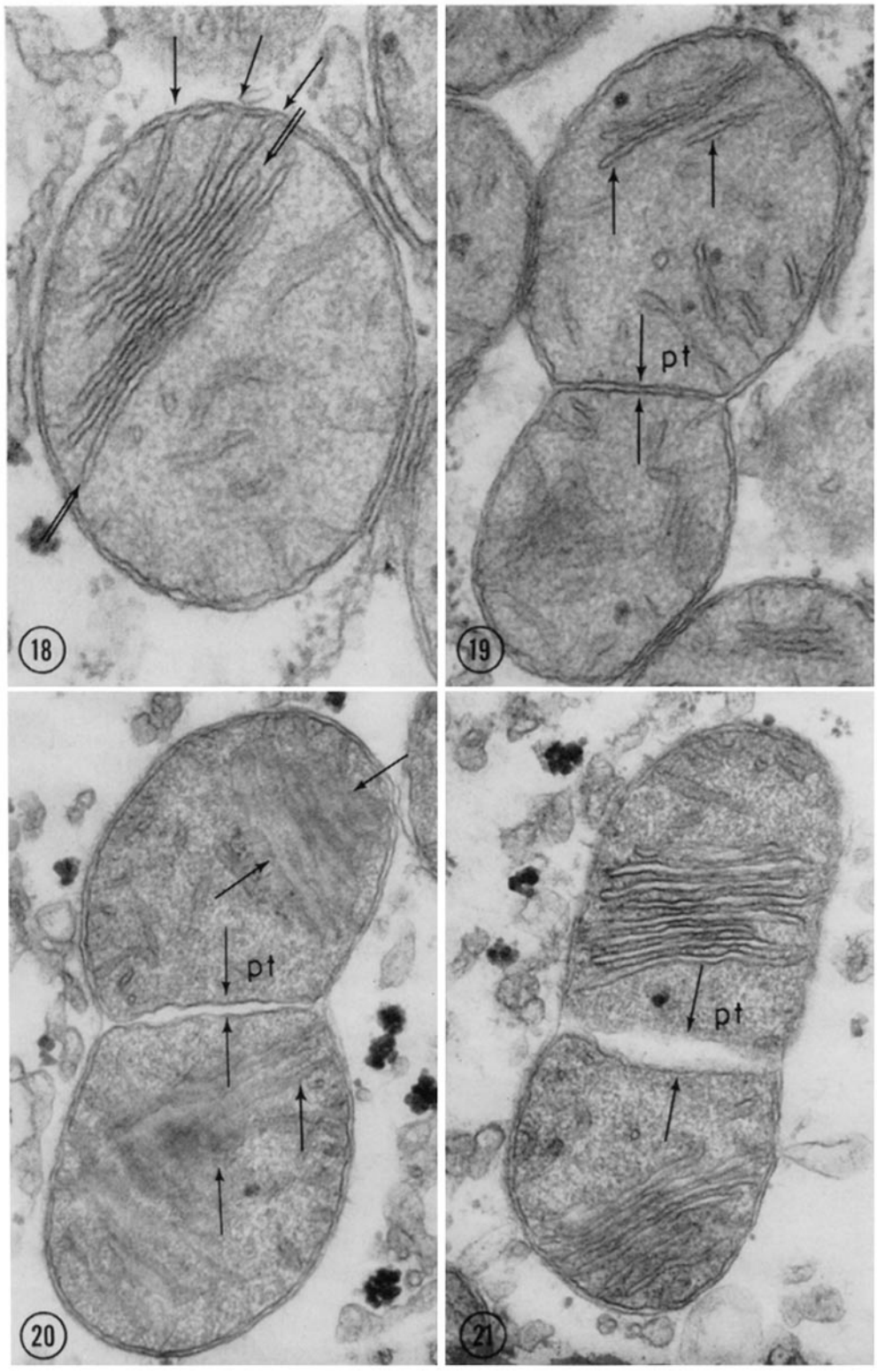
We propose that the group of observations just summarized is best interpreted by assuming that the transverse mitochondrial membrane is quite distinct from the long cristae and arrays of membranes observed after feeding 2-Me-DAB and corresponds, in fact, to a partition. It seems not too unreasonable to postulate, then, that this partition is directly related to the division of the mitochondria. Finally, it is suggested that a slight pinching of the mitochondrial body takes place at the onset of division and that concomitantly

FIGURE 18 Mitochondrion from hepatic cell of experimental animal of the 11-week period. This micrograph depicts an array of long double membranes three of which (single arrows) are continuous with the inner layer of the mitochondrial envelope. Note that some of these double membranes are definitely closed (double arrows) at their extremity. × 80,000.

FIGURE 19 Micrograph of a mitochondrion from hepatic cell of experimental animal of the 11-week period. This mitochondrion is characterized by a number of long, crista-like double membranes (arrows) and by a partition (*pt*) both membranes of which are continuous with the inner leaflet of the mitochondrial envelope. The total thickness of this transverse double membrane corresponds closely to that of the mitochondrial envelope itself. × 75,000.

FIGURE 20 Mitochondrion from hepatic cell of animal fed for 9 weeks on a diet containing 0.06 per cent 2-Me-DAB. Groups of long crista-like double membranes (arrows) have been sectioned obliquely and do not show up clearly. The median partition (*pt*) in this mitochondrion is irregular and its inter-leaflet space is a few times wider than that of the mitochondrial envelope. It should be noted that the outer membrane of this envelope is still intact at the level of the median constriction of the mitochondrion. × 47,500.

FIGURE 21 Mitochondrion from same preparation used for Fig. 20. A group of long double membranes is observed on each side of the median partition (*pt*). The two component membranes of the partition show a wider separation than in Fig. 20. Nevertheless, the two halves of the mitochondrion are still united, at the level of this partition, by the outer membrane of the mitochondrial envelope. × 52,000.


18
19
pt
20
pt
21
pt

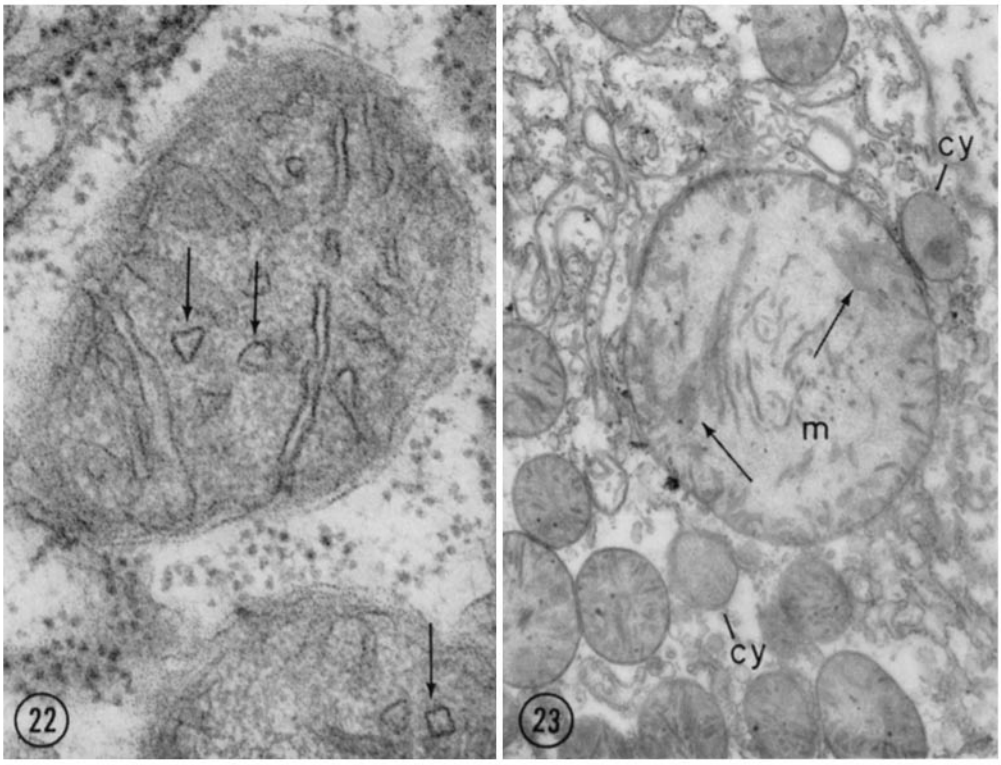


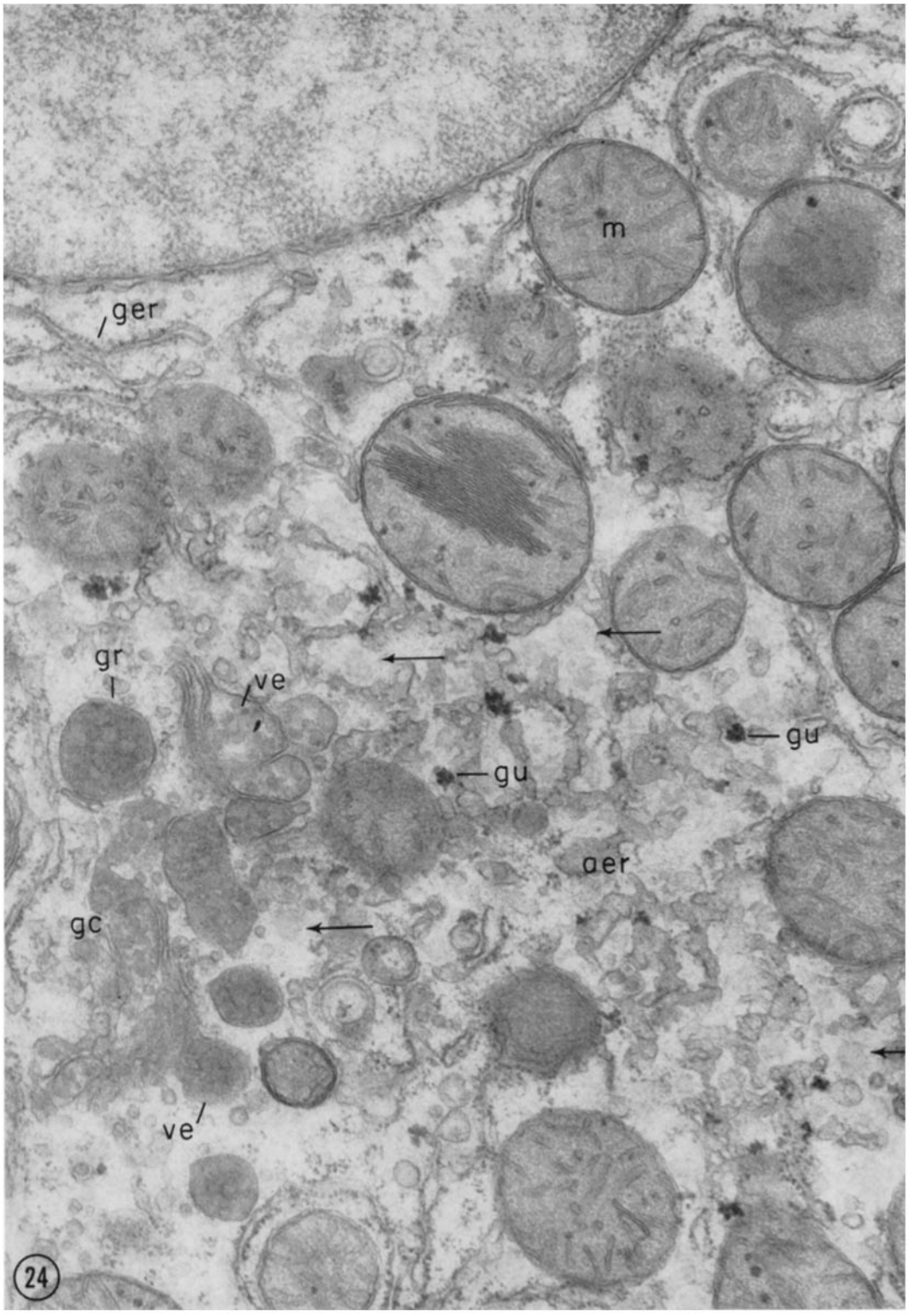
FIGURE 22 Higher magnification micrograph of portion of Fig. 17 illustrating the fine structure of certain mitochondria in experimental animals. This figure shows that in cross-section certain cristae are quite angular (arrows). Some are rather irregular in contour whereas others are nearly triangular or quadrangular. × 90,000.

FIGURE 23 Portion of cytoplasm of hepatic cell from animal of the 11-week period. This micrograph illustrates two cytosomes (*cy*) and several mitochondria. The smaller mitochondria are of normal size and show a number of long cristae. The large mitochondrion (*m*) is limited by a two-layered envelope and is further characterized by cristae of different length. Several sac-like structures (arrows) appear to have been sectioned tangentially. × 19,000.

the two wavy leaflets of the partition begin to separate.

Our observations and conclusions are consistent with Luck's (55) recent autoradiographic study of the repartition of newly incorporated choline in mitochondria during the logarithmic growth of *Neurospora crassa*. His data exclude *de novo* formation of the mitochondria, in this organism at least,

FIGURE 24 Portion of a hepatic cell from animal of the 11-week period. The granular endoplasmic reticulum (*ger*) is represented by a group of long cisternae close to the nucleus as well as by several shorter membranes scattered amongst the mitochondria. A large portion of the area illustrated consists of smooth tubules and vesicles organized into a loose network (*aer*). A few dense glycogen units (*gu*) are clearly seen in the mesh of this network, but several much lighter ones may also be detected (arrows). The Golgi complex (*gc*) contains crescent-shaped membranes together with numerous vesicles (*ve*) of various sizes. Most of the latter show a number of much smaller structures similar to the tiniest vesicles found within the Golgi zone. A dense granule (*gr*), located close to the Golgi complex, is characterized by a heterogeneous matrix. × 40,000.


m
ger
gr
ve
gu
gu
aer
gc
ve
24

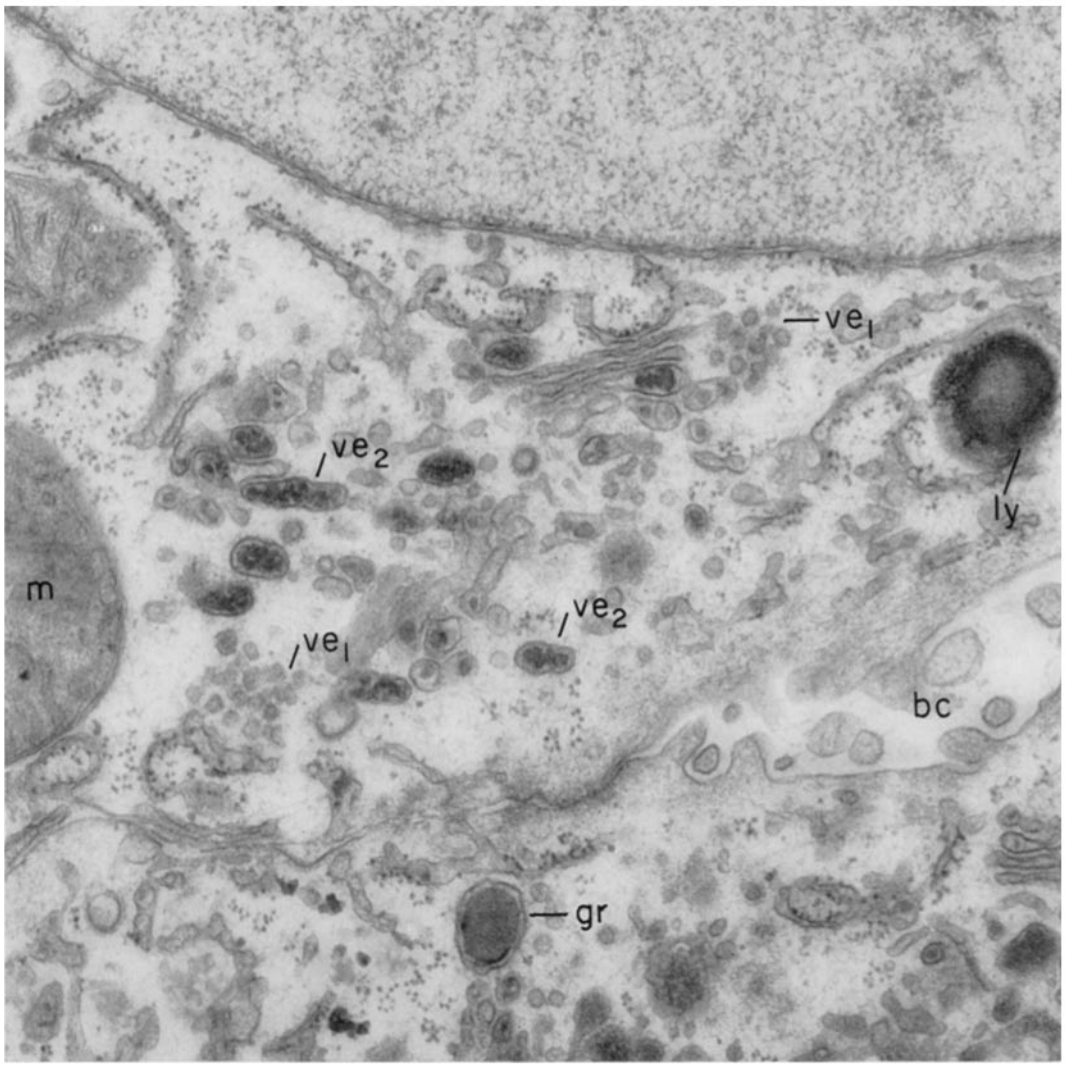


FIGURE 25 Cytoplasmic area of hepatic cell of animal from the 9-week period depicting a Golgi zone. The latter consists of groups of smooth double membranes and vesicles (*ve*) of various sizes. Some of these vesicles (ve_1) are roundish in outline, rather small, and characterized by a light matrix. Other such Golgi vesicles (ve_2) are more irregular in outline, larger, and contain one or more dense granules. The micrograph also shows parts of two mitochondria (*m*), a lysosome (*ly*), and a bile canaliculus (*bc*). A dense, membrane-bounded granule (*gr*) is located in the lower part of the figure. It is quite similar to but somewhat larger than the dense Golgi vesicles just described. × 42,000.

and strongly favour the view that the observed increase in their number results from division of these organelles.

It is evident that the azo dye 2-Me-DAB is well suited to induce duplication of mitochondria in rat hepatic cells. In view of the incompleteness of some of our observations, and of the interest of the problem at hand, the present investigation is being continued to obtain more direct and conclusive evidence in support of our proposed mechanism for the duplication of mitochondria.

Nature of the Dark Cell

The presence of dark and light cells within the normal liver has been known for some time. In a recent preliminary report Aterman (56) dis-

tinguishes these two cell types on the basis of the distribution of their basophilic material. According to this author, cytoplasmic basophilia is a dynamic characteristic of liver cells and follows the variation of their glycogen content. Groups of cells which stain quite intensely with methylene blue may also be observed in 0.5 μ sections of normal liver fixed in phosphate-buffered osmium tetroxide and embedded in Epon (57). To our knowledge, this feature of the normal hepatic cells has not yet been reported at the ultrastructural level. At the present time it is difficult, therefore, to estimate to what extent the presence of dark cells in our preparations of both control and experimental animals reflects uniquely the experimental conditions used in the course of this study. One fact, however, that remains clear is that a larger proportion of dark cells is observed in rat liver when 2-Me-DAB is added to the basal diet than when the basal diet alone is fed to the animals. Moreover, during the later stages of the experiment, the mitochondria in the dark cells are consistently more crowded and a larger proportion is characterized by a transverse double membrane than in the light cells which otherwise also show an increased number of these organelles.

The presence of dark cells has recently been reported in the liver of rats subjected to a number of quite different experimental conditions (10, 11, 21, 58–60). According to Steiner and Baglio (21) the occurrence of these cells is not dependent on alterations of the granular reticulum or diminution in the amount of glycogen. Their observation concerning the greater density of both the nucleoplasm and cytoplasm concurs with ours. They do not state, however, what cellular constituent is responsible for this increased density. We would favour the view that some amorphous substance(s) permeates both the nucleoplasm and cytoplasm and causes a greater opacity of these cells.

This study was initiated while both authors were on the staff of the Montreal Cancer Institute. The financial assistance of the National Cancer Institute of Canada during this early phase as well as during the later phases of our work is gratefully acknowledged.

Received for publication, August 19, 1963.

BIBLIOGRAPHY

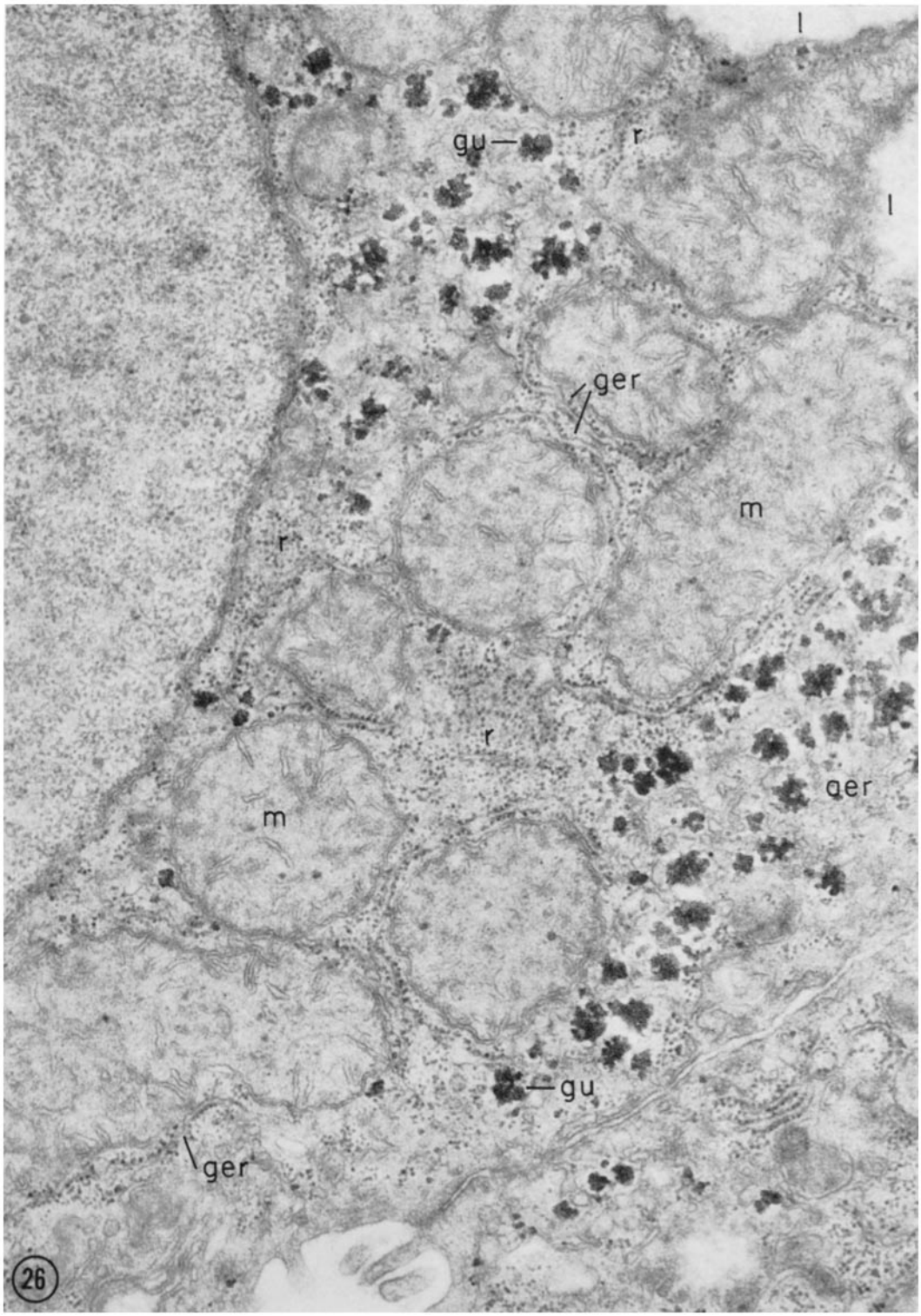
1. Price, J. M., Miller, E. C., Miller, J. A., and Weber, G. M., Studies on the intracellular composition of livers from rats fed various aminoazo dyes. II. 3′-Methyl-, 2′-Methyl-, and 2 - Methyl - 4 - dimethylaminoazobenzene, and 4′-fluoro-4-dimethylaminoazobenzene, *Cancer Research*, 1950, **10**, 18.
2. Potter, V. R., Price, J. M., Miller, E. G., and Miller, J. A., Studies on the intracellular composition of livers from rats fed various aminoazo dyes. III. Effects of succinoxidase and oxalacetic oxidase, *Cancer Research*, 1950, **10**, 28.
3. Allard, C., de Lamirande, G., and Cantero, A., Mitochondrial population in mammalian cells. IV. Preliminary results on the variation in the mitochondrial population of the average rat liver cell during azodyes carcinogenesis, *Canad. J. Med. Sc.*, 1952, **30**, 543.
4. Striebich, M. J., Shelton, E., and Schneider, W. C., Quantitative morphological studies on the livers and liver homogenates of rats fed 2 - methyl - or 3′ - methyl - 4 - dimethylaminoazobenzene, *Cancer Research*, 1953, **13**, 279.
5. Orr, J. W., The histology of the rat's liver during the course of carcinogenesis by butter-yellow (p-dimethylaminoazobenzene), *J. Path. and Bact.*, 1940, **50**, 393.
6. Price, J. M., Harman, J. W., Miller, E. C., and Miller, J. A., Progressive microscopic alterations in the livers of rats fed the hepatic carcinogen 3′ - Methyl - 4 - dimethylaminoazobenzene and 4′-Fluoro-4-dimethylaminoazo-benzene, *Cancer Research*, 1952, **12**, 192.
7. Porter, K. R., and Bruni, C., An electron microscope study of the early effects of 3′-Me-DAB on rat liver cells, *Cancer Research*, 1959, **19**, 997.
8. Emmelot, P., and Benedetti, E. L., Changes in the fine structure of rat liver cells brought about by dimethylnitrosamine, *J. Biophysic. and Biochem. Cytol.*, 1960, **7**, 393.
9. Emmelot, P., and Benedetti, E. L., Some observations on the effects of liver carcinogens on the fine structure and function of the endoplasmic reticulum of rat liver cells, *in* Protein Biosynthesis, (R. J. C. Harris, editor), New York, Academic Press, Inc., 1961, 99.
10. Arakawa, K., An electron microscopic observation on hepatic cells of albino rats after DL-ethionine administration. I. Changes of the rough surfaced elements of endoplasmic reticulum, *J. Electronmicroscopy*, 1960, **8**, 54.
11. Herman, L., Eber, L., and Fitzgerald, P. J., Liver cell degeneration with ethionine administration, *in* Fifth International Congress

for Electron Microscopy, (S. S. Breese, editor), Academic Press Inc., New York, 1962, **2**, VV-6.

12. THOENES, W., and BANNASCH, P., Elektronen und lichtmikroskopische Untersuchungen am Cytoplasma der Leberzellen nach akuter und chronischer Theocetamid-Vergiftung, *Virchows Arch. path. Anat.*, 1962, **335**, 556.
13. SALOMON, J. C., SALOMON, M., and BERNHARD, W., Modifications des cellules du parenchyme hépatique du rat sous l'effet de la thioacétamide. Etude au microscope électronique de lésions précoces au cours d'une intoxication chronique, *Bull. Cancer*, 1962, **49**, 139.
14. SALOMON, J. C., Modifications des cellules du parenchyme hépatique du rat sous l'effet de la thioacétamide. Etude au microscope électronique des lésions observées à la phase tardive d'une intoxication chronique, *J. Ultrastruct. Research*, 1962, **7**, 293.
15. DUPONT, A., and DEMAILLE, A., Electron microscopy of the experimental azoic hepatoma of the rat. II. "Ultrafine" changes in the cytoplasm, *Compt. rend. Soc. biol.*, 1962, **156**, 1648.
16. OBERLING, C., and ROUILLER, C., Les effets de l'intoxication aiguë au tétrachlorure de carbone sur le foie du rat. Etude au microscope électronique, *Ann. Anat. Path.*, 1956, **1**, 401.
17. ROUILLER, C., Contribution de la microscopie électronique à l'étude du foie normal et pathologique, *Ann. Anat. Path.*, 1957, **2**, 548.
18. JÉZÉQUEL, A. M., Les effets de l'intoxication aiguë au phosphore sur le foie de rat. Etude au microscope électronique, *Ann. Anat. Path.*, 1958, **3**, 512.
19. BASSI, M., Electron microscopy of rat liver after carbon tetrachloride poisoning, *Exp. Cell Research*, 1960, **20**, 313.
20. SMUCKLER, E. A., ISERI, O. A., and BENDITT, E. P., An intracellular defect in protein synthesis induced by carbon tetrachloride, *J. Exp. Med.*, 1962, **116**, 55.
21. STEINER, J. W., and BAGLIO, C. M., Electron microscopy of the cytoplasm of parenchymal liver cells in α-naphthylisothiocyanate-induced cirrhosis, *Lab. Inv.*, 1963, **12**, 765.
22. MILLER, J. A., and BAUMANN, C. A., The carcinogenicity of certain azo dyes related to p-dimethylaminoazobenzene, *Cancer Research*, 1945, **5**, 227.
23. MILLER, J. A., and MILLER, E. C., The carcinogenicity of certain derivatives of p-dimethylaminoazobenzene in the rat, *J. Exp. Med.*, 1948, **87**, 139.
24. CAULFIELD, J. B., Effect of varying the vehicle for OsO_4 in tissue fixation, *J. Biophysic. and Biochem. Cytol.*, 1957, **3**, 827.
25. MILLONIG, G., Further observations on a phosphate buffer for osmium solutions, *in* Fifth International Congress for Electron Microscopy, (S. S. Breese, editor), New York, Academic Press, Inc., 1962, **2**, P8.
26. LUFT, J. H., Improvements in epoxy resin embedding methods, *J. Biophysic. and Biochem. Cytol.*, 1961, **9**, 409.
27. MUNGER, B. L., Staining methods applicable to sections of osmium-fixed tissue for light microscopy, *J. Biophysic. and Biochem. Cytol.*, 1961, **11**, 502.
28. MILLONIG, G., A modified procedure for lead staining of thin sections, *J. Biophysic. and Biochem. Cytol.*, 1961, **11**, 736.
29. WATSON, M. L., Reduction of heating artifacts in thin sections examined in the electron microscope, *J. Biophysic. and Biochem. Cytol.*, 1957, **3**, 1017.
30. NOVIKOFF, A. B., Lysosomes and related particles, *in* The Cell, (J. Brachet and A. E. Mirsky, editors), New York, Academic Press, Inc., 1961, **2**, 423.
31. ROUILLER, C., and BERNHARD, W., "Microbodies" and the problem of mitochondrial regeneration in liver cells, *J. Biophysic. and Biochem. Cytol.*, 1956, **2**, No. 4 suppl., 355.
32. SCHULZ, H., Die submikroskopische Pathologie der Cytosomen in den Alveolar makrophagen

FIGURE 26 Portion of a dark hepatic cell from animal fed for 16 weeks on a diet containing 0.06 per cent 2-Me-DAB. The nucleus consists of lighter fibrillar zones intermingled with more compact areas which appear granular in texture.

In the cytoplasm the mitochondria (*m*) show serrated contours and a number of cristae slightly longer, perhaps, than those in normal hepatic cells. The granular reticulum (*ger*) is represented by a few isolated profiles of cisternae mostly squeezed between the mitochondria and often wrapped partly around them. The most characteristic feature of this cell is the opaqueness of the ground cytoplasm. Note also that some ribosomes are attached to membranes and that many other ribosomes are grouped in zones of various sizes amongst the mitochondria. The remaining cytoplasmic areas of this dark cell contain tubules of the agranular reticulum (*aer*) and glycogen units. The figure also illustrates two lipid inclusions (*l*). × 30,000.


l
gu
r
l
ger
m
r
r
m
ger
gu
ger
26

de Lunge. *Beitr. pathol. Anat. u. allg. Path.*, 1958, **119**, 71.

33. KARRER, H. E., Electron-microscopic observations on developing chick embryo liver. The Golgi complex and its possible role in the formation of glycogen. *J. Ultrastruct. Research*, 1960, **4**, 149.
34. MILLER, J. A., and MILLER, E. C., The carcinogenic aminoazo dyes, *Adv. Cancer Research*, 1953, **1**, 339.
35. BERG, W., Uber funktionelle Leberzellstrukturen I, *Arch. mikr. Anat.*, 1920, **94**, 518.
36. LAGERSTEDT, S., Cytological studies on the protein metabolism of the liver in the rat, *Acta Anat.*, 1949, **1**, suppl. 9, 1.
37. BERNHARD, W., HAGUENAU, F., GAUTIER, A., and OBERLING, C., La structure submicroscopique des éléments basophiles cytoplasmiques dans le foie, le pancréas et les glandes salivaires. Etude de coupes ultrafines au microscope électronique, *Z. Zellforsch.*, 1952, **37**, 281.
38. FAWCETT, D. W., Observations on the cytology and electron microscopy of hepatic cells, *J. Nat. Cancer Inst.*, 1955, **15**, 1475.
39. MILLONIG, G., and PORTER, K. R., Structural elements of rat liver cells involved in glycogen metabolism, Proceedings of the European Regional Conference on Electron Microscopy, Delft, 1960, **2**, 655.
40. PORTER, K. R., The endoplasmic reticulum: some current interpretations of its forms and functions, *in* Biological Structure and Function, (T. W. Goodwin and O. Lindberg, editors), New York, Academic Press, Inc., 1961, **1**, 127.
41. FAWCETT, D. W., The membranes of the cytoplasm, *Lab. Inv.*, 1961, **10**, 1162.
42. BRUNI, C., Hyaline degeneration of rat liver cells studied with the electron microscope, *Lab. Inv.*, 1960, **9**, 209.
43. SALOMON, J. C., HABIB, R., and BERNHARD, W., Etude au microscope électronique de la glycogénose hépatique, *Pathol. Biol.*, 1961, **9**, 1251.
44. REVEL, J. P., FAWCETT, D. W., and PHILPOTT, C. W., Observations on mitochondrial structure. Angular configuration of the cristae, *J. Cell. Biol.*, 1963, **16**, 187.
45. JÉZÉQUEL, A. M., Dégénérescence myélinique des mitochondries de foie humain dans un épithélioma du cholédoque et un ictère viral, *J. Ultrastruct. Research*, 1959, **3**, 210.
46. EKHOLM, R., and EDLUNG, Y., The mitochondria in human normal and cholestatic liver, *Proc. Internat. Kongr. Elektronmikroskopie, 4th*, Berlin, 1958, Berlin, Springer, **2**, 273.
47. DAVID, H., and KETTLER, L. H., Degeneration von Lebermitochondrien nach Ammonium Intoxikation, *Z. Zellforsch.*, 1961, **53**, 857.
48. WILSON, J. W., and LEDUC, E. H., Mitochondrial changes in the liver of essential fatty acid-deficient mice, *J. Cell Biol.*, 1963, **16**, 281.
49. CARRUTHERS, J. S., and STEINER, J. W., Experimental extrahepatic biliary obstruction. Fine structural changes of liver cell mitochondria, *Gastroenterology*, 1962, **42**, 419.
50. ROUILLER, C., Physiological and pathological changes in mitochondrial morphology, *Internat. Rev. Cytol.*, 1960, **9**, 227.
51. NOVIKOFF, A. B., Mitochondria (chondriosomes), *in* The Cell, (J. Brachet and A. E. Mirsky, editors), New York, Academic Press, Inc., 1961, **2**, 299.
52. SHERIDAN, M., Fine structural changes seen in the livers of scorbutic and partially starved guinea pigs, *Anat. Rec.*, 1960, **136**, 276.
53. KILARSLI, W. M., Electron microscope observation on early form of mitochondria, Proceedings of the Second Annual Meeting of the American Society for Cell Biology, San Francisco, 1962, 91.
54. FREDERIC, J., Recherches cytologiques sur le chondriome normal ou soumis à l'expérimentation dans des cellules vivantes cultivées in vitro, *Arch. Biol.*, 1958, **69**, 167.
55. LUCK, D. J. L., Formation of mitochondria in *Neurospora crassa*. A quantitative radioautographic study, *J. Cell. Biol.*, 1963, **16**, 483.
56. ATERMAN, K., The "dark" and the "light" cells of the liver, *Anat. Rec.*, 1960, **136**, 157.
57. CHENARD, C., unpublished observations.
58. HÜBNER, G., and BERNHARD, W., Das submikroskopische Bild der Leberzelle nach temporärer Durchblutungssperre, *Beitr. Path. Anat. u. allg. Path.*, 1961, **125**, 1.
59. GANSLER, H. Feinstruktur heller und dunkler Zellen, *in* Fifth International Congress for Electron Microscopy, (S. S. Breese, editor), New York, Academic Press, Inc., 1962, **2**, N-5.
60. DAOUST, R., Cellular populations and nucleic acid metabolism in rat liver parenchyma during azo-dye carcinogenesis, *Proc. Fifth Canad. Cancer Research Conf.*, 1963, **5**, 225.