CYTOCHEMICAL AND ELECTRON MICROSCOPIC STUDIES OF RAT LIVER WITH REDUCED CAPACITY TO TRANSPORT CONJUGATED BILIRUBIN

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Plates 45 to 50

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Although significant progress has been made in the understanding of bilirubin metabolism the hepatocellular structures and mechanisms which are concerned with the uptake, conjugation, and excretion of bilirubin remain largely unknown. Available cytochemical and electron microscopic methods may permit detection of morphological alterations that are not detectable in routine microscopic preparations. Functional as well as morphological information about the liver cell membrane and the lysosomes (1) may be obtained by cytochemical staining reactions for phosphatase activities (2).

It has recently been shown (3) that a marked reduction of the maximum capacity of normal rats to excrete conjugated bilirubin into the bile follows the administration of icterogenin, an alkaloid derived from *Lippia rehmanni*, or 17-ethyl, 19-nortestosterone (norethandrolone), an analogue of testosterone. This reduction in the excretory maximum of conjugated bilirubin has been demonstrated during the constant intravenous infusion of conjugated or unconjugated bilirubin. The administration of these agents to homozygous Gunn rats, genetically deficient in glucuronyl transferase activity, results in a markedly reduced capacity to excrete infused conjugated bilirubin. Hepatic uridine diphosphate glucose dehydrogenase and glucuronyl transferase activities are unaffected by either icterogenin or 17-ethyl, 19-nortestosterone. These observations suggest that these agents interfere with the transport of bilirubin from the liver cell into the bile (3).

Despite these profound changes in excretory transport, liver sections stained with hematoxylin and eosin appear essentially normal. However, cytochemical and electron microscopic preparations from the same animals reveal changes

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in: (*a*) the levels of alkaline phosphatase and apparent adenosine triphosphatase (ATPase) activities of the cell membrane at the sinusoids and canaliculi, and between adjoining cells, (*b*) the morphology of the bile canaliculi, and (*c*) the number and distribution of lysosomes. Extrahepatic obstruction induces similar changes in the cytochemistry of the liver cell.

Materials and Methods

Icterogenin was injected into six female Wistar rats intraperitoneally, at laparotomy. Four female Wistar rats were injected with 17-ethyl,19-nortestosterone. Doses were those required to produce a significant reduction in the excretion of conjugated bilirubin (3). Extrahepatic bile duct ligation was performed on five female Sprague-Dawley and five male Wistar rats. Control animals were chosen from the same colonies.

After 24 hours the animals were sacrificed by decapitation and liver samples were fixed in cold formol-calcium (4, 5). Following overnight fixation at 4°C, some samples were dehydrated and embedded in paraffin in the usual fashion for hematoxylin-eosin preparations. Others were processed for phosphatase activities in frozen sections and cut at about 10 μ with a Bausch and Lomb freezing microtome. The incubation media of Gomori (6) were used for acid and alkaline phosphatase and that of Wachstein and Meisel (7) for apparent adenosinetriphosphatase (ATPase) activity.[1]

For electron microscopy samples were fixed rapidly in cold 1 per cent osmium tetroxide buffered at pH 7.2 (10). After 1 hour fixation in osmium tetroxide, the tissues were washed, dehydrated, and embedded in a 1:5 mixture of methyl and butyl methacrylate containing 75 mg per 100 ml uranyl nitrate (11). After overnight polymerization at 50°–60°C, sections were cut with either glass or diamond knives and mounted on formvar-coated copper grids. Some sections were stained for 30 minutes with potassium permanganate and covered with a layer of carbon. They were examined with an RCA EMU 3B electron microscope and photographed at magnifications of 3,600 to 14,000.

OBSERVATIONS

A mild degree of cholangitis and periportal inflammatory cell infiltration is present in both the experimental and control animals. In both groups of animals the parenchymal cells show remarkably little change in the hematoxylin-eosin preparations. In contrast, changes are evident when these cells are examined in the phosphatase preparations and in the electron microscope.

Animals treated with icterogenin:

Bile Canaliculi.—In the normal rat the bile canaliculi show weak alkaline phosphatase activity and only in the peripheral portions of the lobule. This is true even when incubated for up to 2 hours at 37°C (Fig. 1). In the icterogenin-treated rats the canaliculi stain throughout the lobule after 30 minutes of incubation, the shortest incubation time tested. In addition the canaliculi are

[1] For discussion of the substrate specificities of this "apparent ATPase" see references 8 and 9. In the case of normal rat liver the bile canalicular and sinusoidal enzymes will hydrolyze the mono-, di-, and triphosphates of adenosine, guanosine, uridine, inosine, and cytidine. Of these the triphosphates are split most extensively in the bile canaliculi, and the monophosphates in the sinusoids.

frequently dilated and fragmented, with irregular margins showing granules and vacuoles (Fig. 2).

With ATP as substrate, all bile canaliculi of the normal rat liver are stained black. Most canaliculi are relatively straight and smooth (Fig. 5). They are wider in the periphery of the hepatic lobule than in the centrolobular region (12). In the experimental animals dilatations, fragmentations, and twisted branchings of the canaliculi are seen throughout the lobule. Numerous small pericanalicular granules and larger vacuoles are also seen. Equally striking is the diminution of canalicular staining seen in periportal areas (Fig. 6).

With twice the usual dose of icterogenin these changes are outstanding, with vacuolization and dilatation of canaliculi readily apparent in alkaline phosphatase as well as ATPase preparations (Figs. 3 and 7). Electron micrographs of this liver are shown in Figs. 9 and 10. The canaliculi are markedly dilated and their microvilli much reduced in number. Numerous pericanalicular vacuoles corresponding to those seen in Figs. 3 and 7, are present. They may have separated from the canaliculi. The livers of animals receiving smaller doses of icterogenin showed similar but less marked changes when examined with the electron microscope (Fig. 13).

Sinusoidal (Space of Disse) Aspects of the Cell Membrane.—In normal liver the sinusoidal surfaces show no staining for alkaline phosphatase activity after 2 hours of incubation (Fig. 1). They show slight ATPase activity after 30 minutes of incubation, especially in the peripheral parts of the lobule (Fig. 5) (12).

In treated animals, staining for alkaline phosphatase activity is present at the sinusoidal surfaces after only 30 minutes of incubation (Fig. 2). Staining for ATPase activity is also increased, particularly at the periphery of the lobule (Fig. 6).

No morphological alterations are apparent at the sinusoids when examined in the electron microscope (Fig. 9).

Membranes at Adjacent Cell Surfaces.—Generally in the normal rat the cell membranes at adjacent cell surfaces show no staining reaction with any of the phosphate esters used (Figs. 1 and 5). However, in the experimental animals both alkaline phosphatase and ATPase activities are clearly evident, especially in the peripheral areas of the lobule (Figs. 2 and 6). Electron microscopy reveals no alterations in these areas of the plasma membrane.

Lysosomes.—Considerable evidence suggests that the cytoplasmic granules demonstrable in the acid phosphatase preparations and concentrated along the bile canaliculi (Fig. 11) and the pericanalicular dense bodies seen in the electron microscope are the lysosomes of rat liver (see summary in reference 13).

In drug-treated animals the granules are present not only near the bile canaliculi but scattered throughout the cytoplasm of the cell. Frequently the number of granules is markedly increased (Fig. 12).

Electron microscopic observations in icterogenin-treated animals show an increased number of dense bodies (Fig. 13). Large vacuoles are associated with the dense bodies (Fig. 10). Although these vacuoles do not appear to contain electron-dense material they may be related to similar vacuoles seen in dense bodies in human obstructive jaundice (2).

Animals treated with 17-ethyl, 19-nortestosterone (norethandrolone):

Cytochemically these livers display changes that are similar to but less severe than those seen in icterogenin-injected animals. In animals injected with a maximal dose of 17-ethyl, 19-nortestosterone less pronounced branching, fragmentation and dilatation of the canaliculi and staining at the sinusoids is seen in ATPase and alkaline phosphatase preparations (Fig. 15). Acid phosphatase preparations show widespread distribution of the lysosomes rather than the predominantly pericanalicular localization of normal liver. An increase in lysosomes is also frequently seen (Fig. 14).

Electron micrographs of these livers reveal frequent dilatations of bile canaliculi and reduced numbers of microvilli (Fig. 16). Apparently unaltered bile canaliculi are also present.

Extra-hepatic obstruction:

In animals sacrificed 24 hours after bile duct ligation the cytochemical alterations resemble those obtained in animals treated with icterogenin (Figs. 4 and 8). This includes the alterations in bile duct morphology and activity seen in ATPase preparations and the increased alkaline phosphatase activity in the canaliculi, sinusoids, and adjoining cell surfaces. Similarly, the lysosomes are increased in number and are distributed throughout the cell. The changes that we have seen in the alkaline phosphatase preparations are in accord with those reported by Jacoby and Martin (14).

Direct communication between the bile canaliculi and the sinusoids was not apparent in either stained preparations or electron micrographs.

DISCUSSION

The hepatic parenchymal cells of rats with ligated bile ducts and of rats treated with icterogenin and 17-ethyl, 19-nortestosterone show strikingly similar changes in fine structure and enzymatic cytology. These changes include: dilatation, fragmentation, and vacuole formation at the bile canaliculi (excretory surfaces), accompanied by decreased ATPase activity and increased alkaline phosphatase activity, and considerable loss of microvilli; increased staining for ATPase and alkaline phosphatase activities at the sinusoidal surfaces and surfaces between adjacent cells; and an increase in number and more widespread cytoplasmic distribution of lysosomes. The degree of change

in the drug-treated animals appears to vary with the demonstrated ability of these agents to limit hepatocellular transport of conjugated bilirubin (3).

It is tempting to relate the loss in ability to transport conjugated bilirubin and the presence of conjugated bilirubin in the blood to a reduction in apparent ATPase activity at the bile canaliculus and its increase at the sinusoid. Unfortunately, the significance of the morphological and enzymatic properties of the plasma membrane for bilirubin uptake, transport, and excretion is presently unknown (15). Schaffner, Popper, and Perez have described dilatation of bile canaliculi and loss of microvilli in human and rat liver after administration of norethandrolone (16). They suggest that the bile canaliculus is the prime locus of hepatocellular dysfunction in drug-induced cholestasis. In our injected animals aspects of the cell membrane other than the bile canaliculus, while unaltered morphologically, also display changes in enzyme activity. Steiner and Carruthers (17) have recently described the presence of edematous microvilli that frequently occlude the bile canaliculi as well as canalicular dilatation and loss of microvilli, in a variety of intra- and extrahepatic lesions. These authors caution against ascribing a specific form of cholestasis to defects that are apparently non-specific.

It is not unlikely that the changes in canalicular fine structure and enzyme activity that are reported here are manifestations of a more basic disturbance in the excretory mechanism of the liver cell. The presence of numerous pericanalicular vesicles and the widespread distribution of the lysosomes throughout the cytoplasm may also reflect such disturbances. Steiner and Carruthers (17) have also described a markedly increased number of pericanalicular bodies (referred to as "dense, lipid, and lipofuscin bodies") near the pathologically altered bile canaliculi. The identity of similar dense bodies and lipofuscin granules with lysosomes, on the basis of their acid phosphatase activity, has been described by Essner and Novikoff (18). The intimate spatial relationship between lysosomes and secretory granules in a wide variety of cells has been described (19–20). Their relationship in liver is currently under investigation.

It is evident that hepatocellular elements that appear unaltered in present electron microscopic and in cytochemical preparations may have undergone important alterations in function.

The direct communications between the bile canaliculi and the sinusoids described by Rouiller (21) in extrahepatic obstruction have not been seen in these investigations and those of others (16, 22, 23). The studies of Hampton (22) suggest the absence of such communications. Popper and Schaffner (23) and Steiner and Carruthers (16) have been unable to fine any evidence of such channels in cholestatic and extrahepatic obstructions. The absence of continuities between the bile canaliculi and sinusoids and the similarity of the hepatocellular changes in animals with ligated bile ducts to those treated with exogenous agents lend support to the view that altered functional properties

and polarity of the parenchymal cell are responsible for the altered bilirubin transport seen in obstructive jaundice. The similarity of changes in enzymatic cytology in the two groups of animals suggests that the changes seen with extrahepatic obstruction are due to the hepatotoxic nature of some substances normally excreted in the bile.

SUMMARY

Although the livers of rats treated with agents known to suppress bilirubin transport appeared relatively normal on routine histological sections, cytochemical and electron microscopic preparations revealed marked changes in: (*a*) levels of alkaline phosphatase and apparent ATPase activities of the cell membrane at the sinusoids, bile canaliculi, and between adjacent cells; (*b*) morphology of the bile canaliculi such as dilatation, fragmentation, vesiculation and reduced numbers of microvilli; (*c*) the number of lysosomes and their distribution within the cell. Similar changes in the cytochemistry of the liver cell were induced by extrahepatic obstruction.

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Addendum.—Since this manuscript was submitted we have studied the liver of icterogenin-treated rats and rats in which the bile ducts were ligated for enzymatic activities that serve as markers for two other cytoplasmic organelles, the endoplasmic reticulum and Golgi apparatus (9, 24). The observations support the view that the experimental procedures employed produce widespread alteration of the parenchymal cells.

The endoplasmic reticulum shows changes in nucleoside diphosphatase activity following both procedures. The extent to which this is correlated with morphologic alteration of the organelle has not been determined.

The Golgi apparatus becomes much more prominent following these experimental procedures. In most liver parenchymal cells of untreated animals the Golgi apparatus is not visualized by the nucleoside diphosphatase procedure (thiamine pyrophosphate as substrate) and, when seen, it is relatively small and confined to pericanalicular regions. In the experimental animals, the lamellae of the Golgi apparatus are stained in essentially all cells, and they extend from the pericanalicular regions deep into the cytoplasm, sometimes approaching close to the nucleus (Fig. 15).

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EXPLANATION OF PLATES

PLATE 45

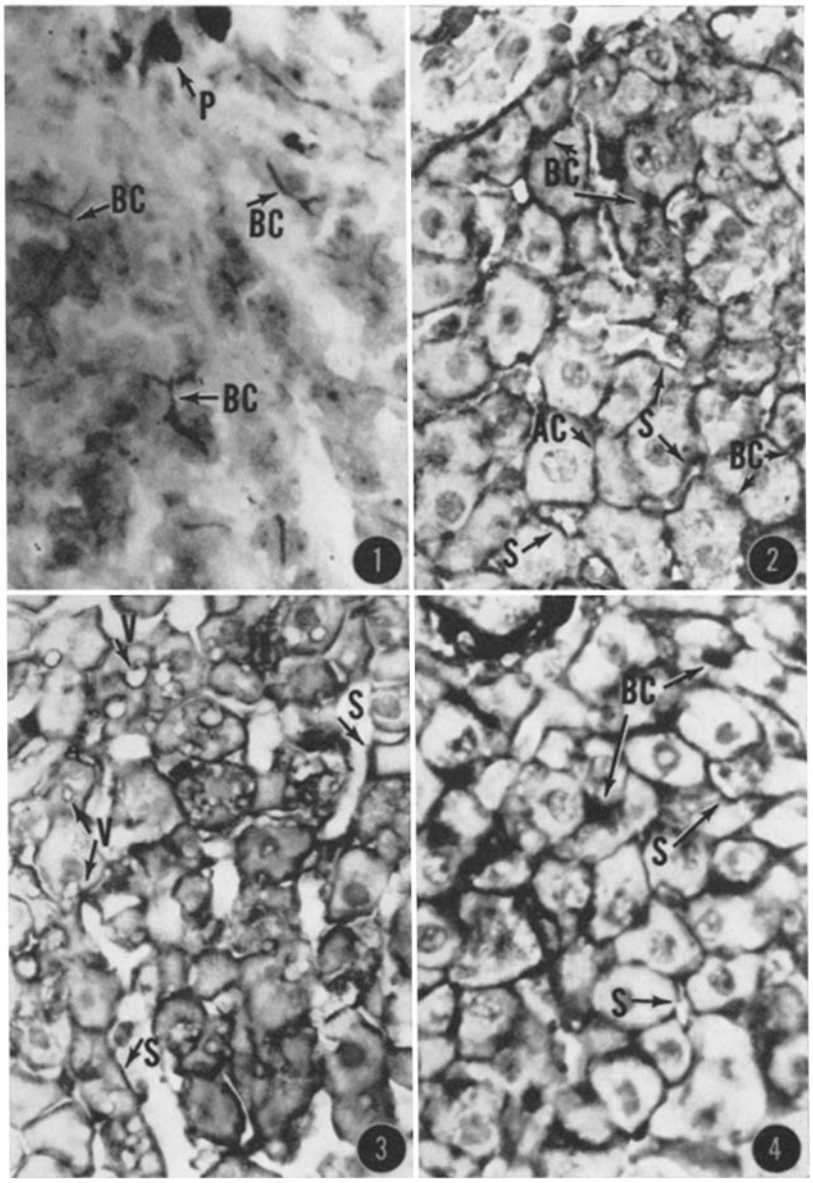
FIGS. 1 to 4. Rat liver stained for alkaline phosphatase activity. × 600.

FIG. 1. Normal rat liver. 2 hours' incubation. Only segments of the bile canaliculi (*BC*) show enzyme activity. Vessels and connective tissue in the portal triad stain black (*P*).

FIG. 2. 24 hours after administration of icterogenin (50 mg/kg). 30 minutes' incubation. There is staining activity in all aspects of the cell membrane: canalicular (*BC*), sinusoidal (*S*) and between adjacent cells (*AC*).

FIG. 3. 24 hours after administration of a maximal dose (100 mg/kg) of icterogenin. 45 minutes' incubation. Numerous vacuoles (*V*) are seen in the region of the bile canaliculi, and frequently the latter are not visible. Marked sinusoidal staining is evident. The fine structure of the canaliculi from the same animal is shown in Figs. 9 and 10.

FIG. 4. 24 hours after bile duct ligation. 1 hour incubation. The staining pattern is similar to that seen in Fig. 2. There is staining of all aspects of the cell membrane, so that the parenchymal cells are clearly outlined.



(Goldfischer *et al.*: Liver and transport of conjugated bilirubin)

PLATE 46

FIGS. 5 to 8. Rat liver stained for apparent ATPase activity. 30 minutes incubation. × 400.

FIG. 5. Normal rat liver. The bile canaliculi (*BC*) are clearly seen. The canaliculi are fairly straight with smooth margins and few branchings. The sinusoids (*S*) show slight activity.

FIG. 6. 24 hours after administration of icterogenin (50 mg/kg). Note the dilated, irregular canaliculi (*BC*) with numerous granules (*G*) at their margins. Increased staining activity is evident at the sinusoids (*S*) and between adjacent cells. In some areas canalicular activity is no longer seen.

FIG. 7. 24 hours after administration of icterogenin (100 mg/kg). Changes in the bile canaliculi (*BC*) are apparent. They show marked dilatation. Numerous vacuoles (*V*) are seen at the sites of the bile canaliculi in many areas.

FIG. 8. 24 hours after bile duct ligation. The alterations in canalicular structure and enzyme activity are similar to those seen in Fig. 6. Numerous granules are seen near the canaliculi. This liver was not congested and therefore the sinusoids are not dilated.

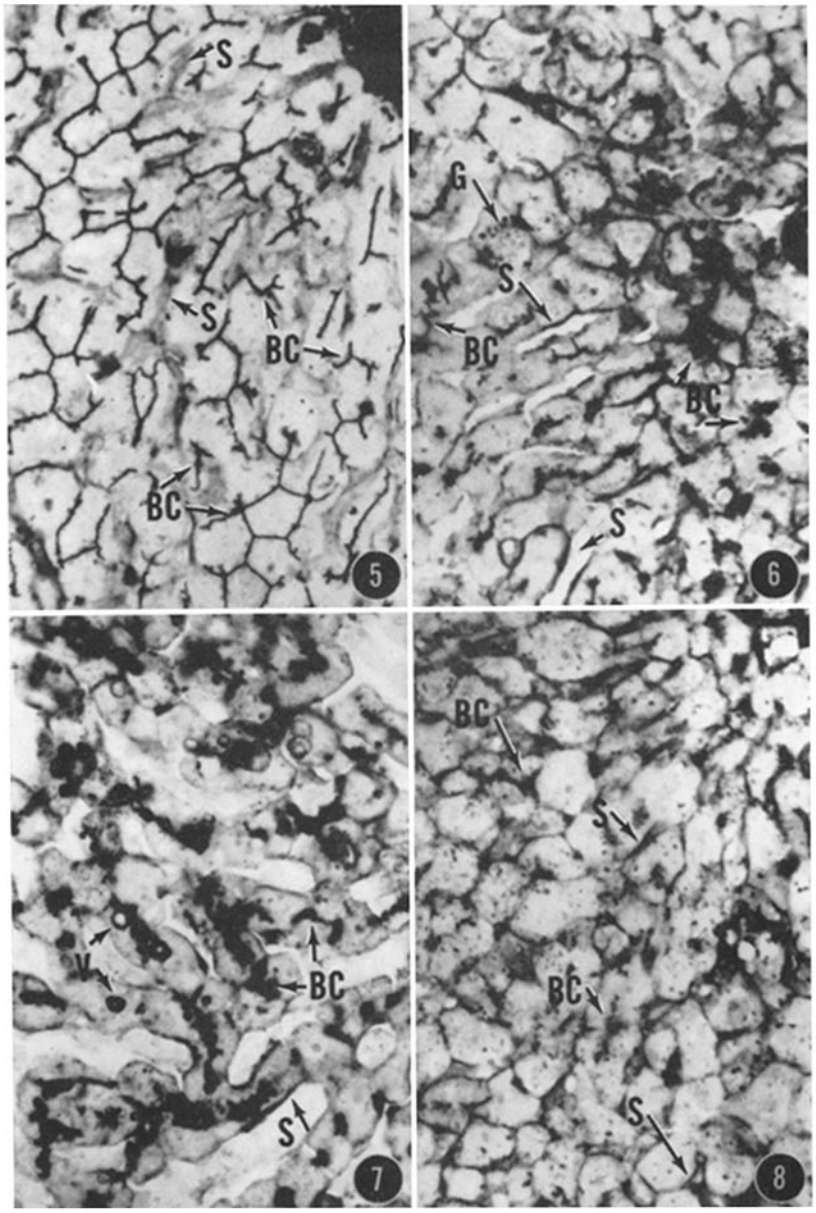


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PLATE 47

FIG. 9. Electron micrograph of rat liver following injection of icterogenin, (100 mg/kg). The section passes through several sinusoids (*S*) and bile canaliculi (*BC*). The bile canaliculi are dilated and tortuous and show reduced numbers of microvilli projecting into the lumina. Satellite small and large vacuoles (arrows) which may have separated from the bile canaliculi are shown in the vicinity. The nuclei (*N*), nucleoli (*NU*), mitochondria (*M*) and ergastoplasm (*ER*) in the parenchymatous cells are indicated. × 5600.

FIG. 10. Electron micrograph of rat liver following injection of icterogenin, (100 mg/kg). A dilated bile canaliculus (*BC*) with reduced numbers of microvilli (*MV*) is shown. The lysosomes (*L*) appear as dense bodies. They are delimited by single membranes. They possess electron-dense matrices with numerous small particles and small or large vacuoles (*V*). Numerous mitochondria (*M*) are seen. × 21,000.

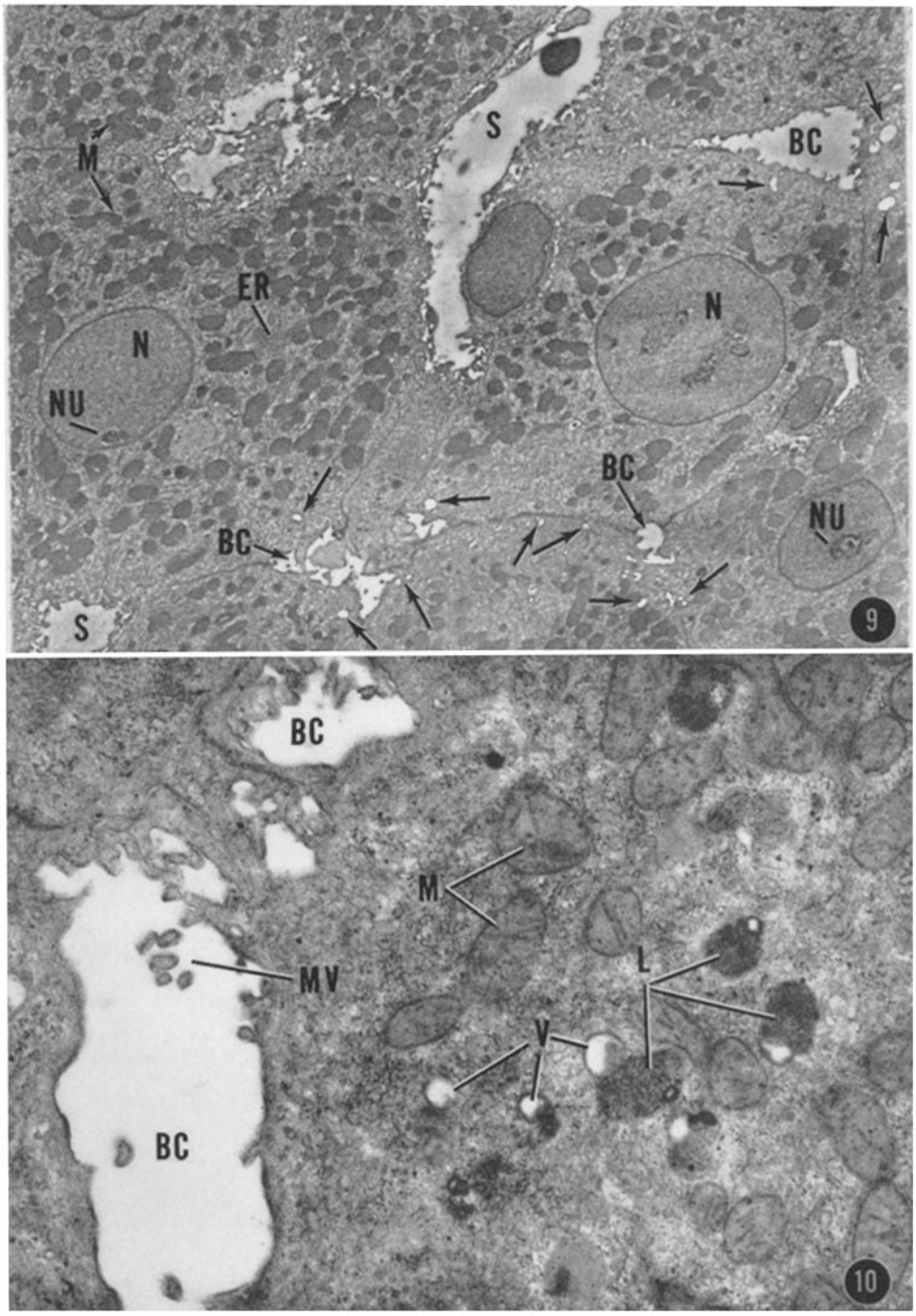

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PLATE 48

FIG. 11. Normal rat liver stained for acid phosphatase activity. 20 minutes' incubation. Numerous granules, presumably lysosomes (*L*), are seen in the cytoplasm. They are predominantly pericanalicular in localization. × 400.

FIG. 12. Rat liver stained for acid phosphatase 24 hours after injection of icterogenin, (50 mg/kg). 20 minutes' incubation. The lysosomes (*L*) are frequently scattered through the cytoplasm, away from their usual pericanalicular position. Although quantitation is difficult, they appear to be increased in number. × 400.

FIG. 13. Electron micrograph of rat liver following injection of icterogenin, (50 mg/kg). Parts of one or more bile canaliculi (*BC*) with microvilli (*MV*) are shown. Several apparently isolated vacuoles (arrows) are shown in the vicinity. These may have separated from the bile canaliculi. Mitochondria (*M*), ergastoplasm (*ER*) and Golgi apparatus (*G*) are also shown. Increased numbers of dense bodies or lysosomes (*L*) are seen in the vicinity of the bile canaliculus, (compare with Fig. 3). × 18,000.

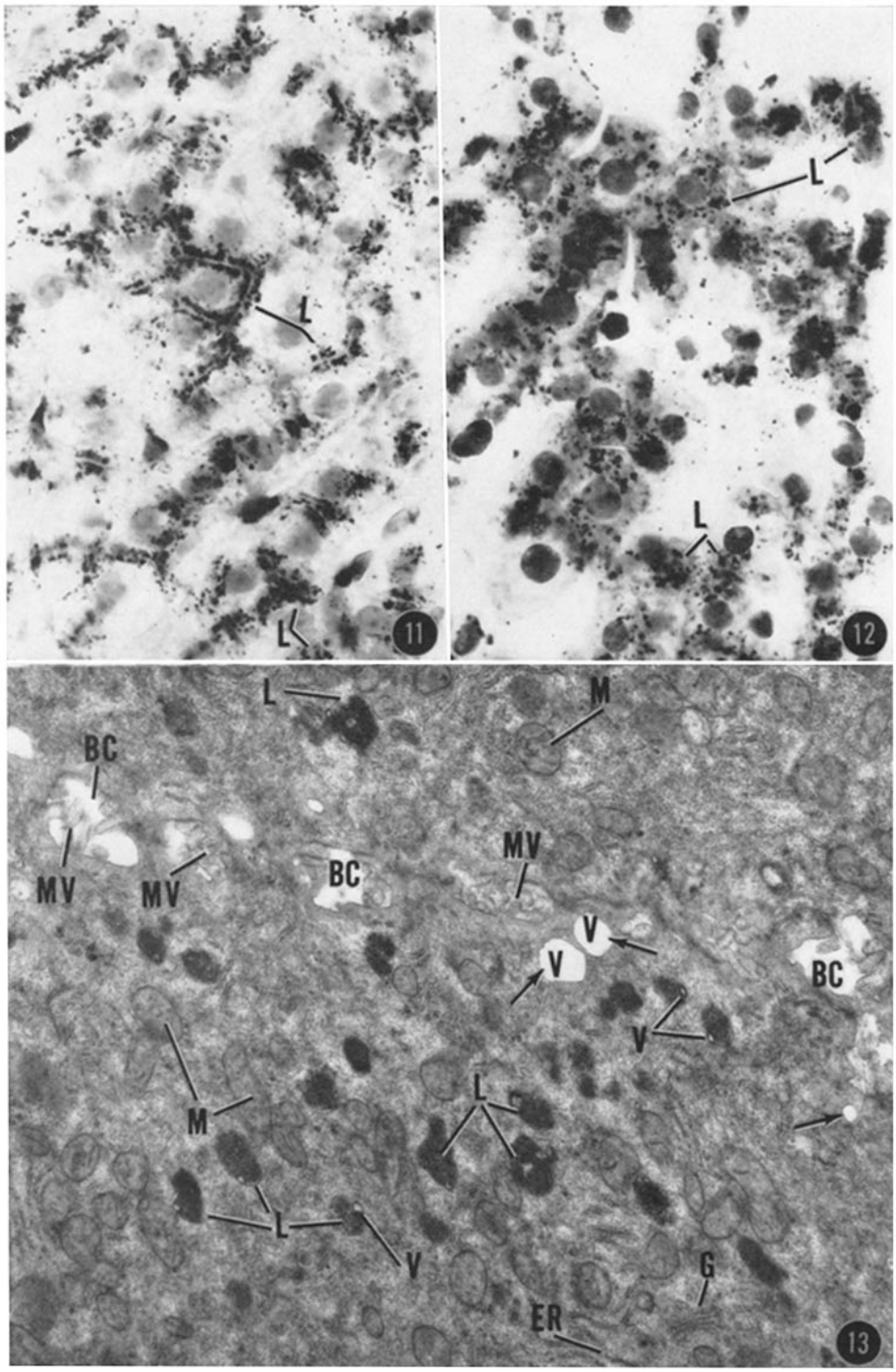

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PLATE 49

FIG. 14. Rat liver stained for acid phosphatase 24 hours after administration of 17-ethyl,19-nortestosterone. Compare with normal rat liver similarly stained in Fig. 11. The lysosomes (*L*) are apparently more numerous and more widely distributed through the cytoplasm. × 700.

FIG. 15. Rat liver stained for apparent ATPase activity 24 hours after administration of 17-ethyl,19-nortestosterone. Compare with normal, Fig. 5. Note the dilated canaliculi (*BC*), pericanalicular granules (*G*), and the sinusoidal staining (*S*). × 700.

FIG. 16. Electron micrograph of rat liver following injection of 17-ethyl,19-nortestosterone. The bile canaliculi (*BC*) are dilated and show reduced numbers of microvilli (*MV*). The nucleus (*N*), nucleolus (*NU*), and mitochondria (*M*) are indicated. Also present are numerous microbodies (*MI*) containing a typical central core of electron of electron-dense material which at higher magnification show crystalline-like structure. × 13,000.

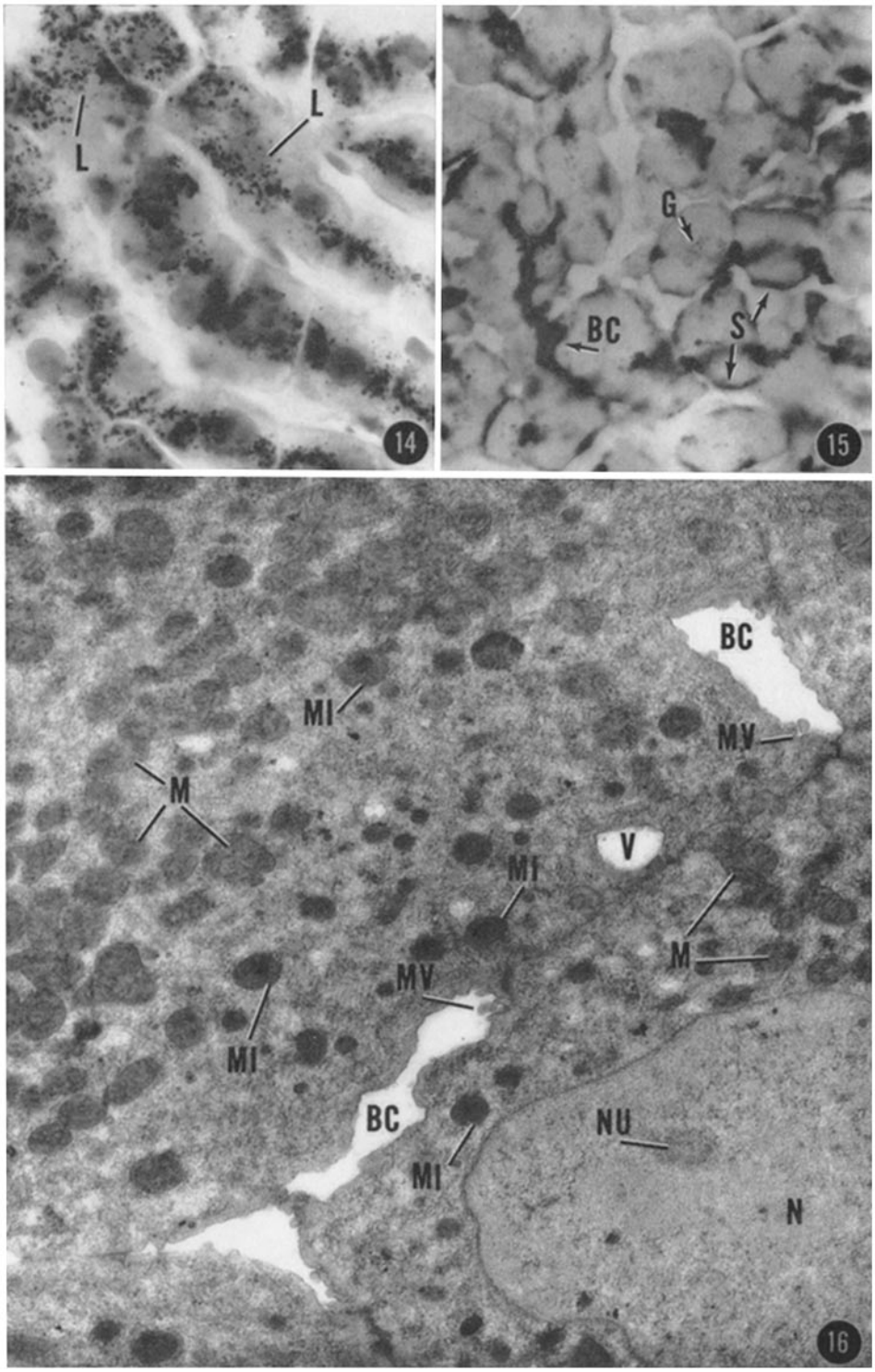

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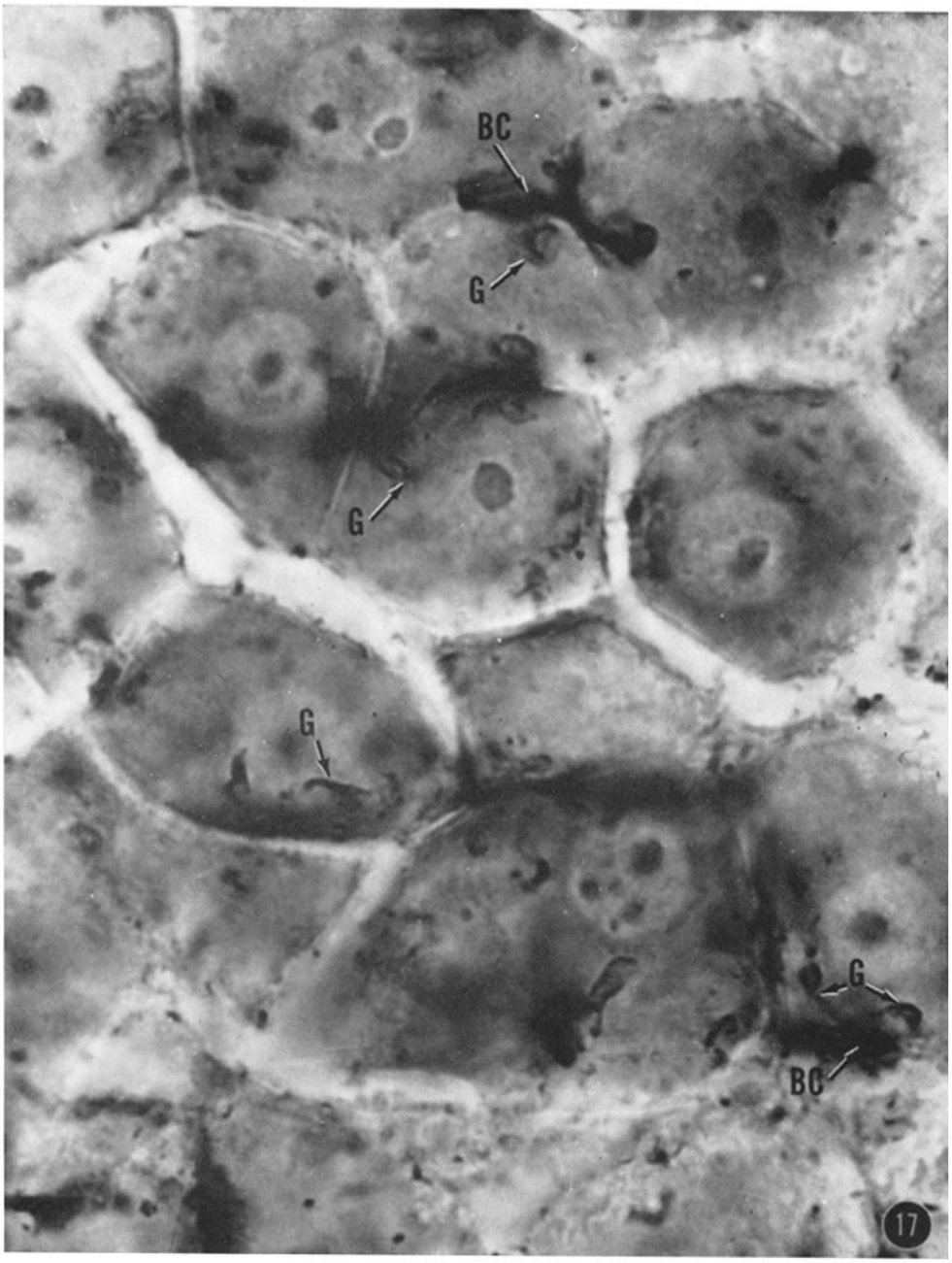
PLATE 50

FIG. 17. Rat liver stained for nucleoside diphosphatase activity 24 hours after injection of icterogenin. Incubated for 30 minutes with thiamine pyrophosphate as substrate. Note staining of the bile canaliculus (*BC*), and the lamellae of the Golgi apparatus (*G*). The Golgi lamellae are much more extensive and more deeply stained than in untreated controls. × 2,000.



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