LOCALIZATION OF ACID PHOSPHATASE
ACTIVITY IN HEPATIC LYSOSOMES BY MEANS
OF ELECTRON MICROSCOPY

E. ESSNER, Ph.D., and ALEX B. NOVIKOFF, Ph.D.

From the Department of Pathology, Albert Einstein College of Medicine, Yeshiva University, New York

ABSTRACT

Samples of liver from untreated rats, from rats infused with unconjugated bilirubin, and from biopsies of human liver were fixed overnight in cold formol–calcium. Frozen sections were stained for acid phosphatase activity by the Gomori lead–glycerophosphate procedure. Small *blocks* of fixed tissue were also incubated in this medium. These were then treated briefly with osmium tetroxide, dehydrated, and embedded in methacrylate. Thin sections were studied by electron microscopy. The sites of reaction product of acid phosphatase activity as visualized in electron micrographs are consistent with those seen in frozen sections studied by light microscopy. They indicate that the pericanalicular bodies of parenchymatous cells, the large spherical bodies of Kupffer cells, the microbodies appearing after bilirubin infusion and lipofuscin granules belong to the class of cytoplasmic organelles called lysosomes by de Duve.

de Duve (1, 2) has recently reviewed the biochemical concept of lysosomes. Activities of acid hydrolases, originally five in number, show the same sedimentation characteristics and are released in similar fashion by a variety of experimental procedures, including treatment by lecithinase and proteolytic enzymes. From this it is deduced that these enzymes are contained in particles with a mean diameter of about 0.4 μ and with an average density of approximately 1.15, and that the particles are surrounded by semipermeable lipoprotein membranes. The number of acid hydrolases known to be concentrated in these particles is now ten (2).

In 1956, lysosome-rich fractions isolated from rat liver were examined in the electron microscope and found to contain numerous distinctive particles seldom seen in fractions poor in acid phosphatase activity, the enzyme marker used for lysosomes in these experiments (3). The particles were characterized by "single" outer membranes, the presence of many electron-opaque grains resembling ferritin molecules and, occasionally, internal cavities. Attention was directed to their resemblance to the "dense bodies" seen along the bile canaliculi *in situ*. However, the identification of lysosomes with pericanalicular dense bodies could only be provisional since the fractions also contained many mitochondria. Support for the presence of ferritin in hepatic lysosomes came from studies of Beaufay *et al.* (4) indicating that lysosome-rich fractions contain more easily detachable iron than do the other particulate fractions.

The present study provides direct evidence for the link between hepatic lysosomes and pericanalicular dense bodies. It gives support to the view that acid phosphatase activity of lysosomes can be demonstrated reliably by the Gomori acid phosphatase procedure (5) applied to tissue or cells fixed in cold formol–calcium (6–9). It strengthens the conclusion that the lipofuscin granules of human liver are altered lysosomes

in which acid phosphatase activity is restricted to the periphery (10). Finally, it suggests that the microbodies which appear in rat liver following experimental treatment (11) are also lysosomes.

MATERIAL AND METHODS

The materials studied included normal rat liver (Sprague-Dawley strain, male and female, weighing 150–200 gm.), rats (Wistar strain, 150 gm.) infused with unconjugated bilirubin (7.2 mg. in a total of 6 ml. saline, infused during 10 minutes), and surgical biopsy specimens of human liver. Samples were fixed rapidly in cold 1 per cent osmium tetroxide buffered at pH 7.2 (12) and in cold formol-calcium (13).

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 (14). After overnight polymerization at 50–60°C., sections were cut with either glass or diamond knives and mounted on formvar-coated copper grids. They were examined with an RCA EMU 3B electron microscope and photographed at magnifications of 3,600 to 14,000.

The formol–calcium-fixed tissues were used for demonstrating the localization of acid phosphatase activity. This was done in two ways: (1) for light microscopy, frozen sections, 10 μ thick, were cut on the Bausch and Lomb freezing microtome. They were rinsed briefly in water, and incubated in the lead–glycerophosphate medium of Gomori (5) for 15–45 min. at 37°C. After the rinsing, they were treated with dilute ammonium sulfide, rinsed and mounted in glycerogel. (2) For electron microscopy, the tissue was diced into small blocks, rinsed briefly, incubated for 30 or 50 minutes at 37°C. in the Gomori medium containing 15–25 per cent sucrose, rinsed, treated for 1 hour with buffered osmium tetroxide, and processed for electron microscopy in the manner described above. Sometimes, the small tissue blocks were passed through ammonium sulfide to permit preliminary examination of sites of reaction product in thick (2 μ) sections by light microscopy. Accumulated lead phosphate and lead sulfide are both opaque to electrons.

RESULTS AND DISCUSSION

Normal Rat Liver

Fig. 1 shows a light micrograph of an acid phosphatase preparation. In the parenchymatous cells the acid phosphatase-containing particles show the typical pericanalicular arrangement. A few may be found near the sinusoidal surfaces and elsewhere in the cell, but most are clustered

Explanation of Figures

KEY TO ABBREVIATIONS

BC,	bile canaliculus	LY,	lysosome
ER,	endoplasmic reticulum	M,	mitochondria
EX,	extraneous precipitate	MI,	microvilli
K,	Kupffer cell	N,	nucleus
L,	lipofuscin	U,	unidentified body
LI,	lipid	V,	vacuole

FIGURE 1

Light micrograph of rat liver, incubated 30 minutes for acid phosphatase activity. × 470.

Note (1) the pericanalicular arrangement of the acid phosphatase-containing lysosomes in the parenchymatous cells; and (2) Kupffer cells (arrows).

FIGURE 2

Electron micrograph of rat liver, incubated 30 minutes for acid phosphatase activity. ×28,000.

Two pericanalicular bodies (LY) are seen in a parenchymatous cell. Enzyme reaction product completely fills one such particle and is deposited in a thick peripheral zone around the other; it may be forming in a third particle (unmarked arrow).

The bile canaliculus (BC), microvilli (MI), endoplasmic reticulum (ER), are visible. Large vacuoles (V), probably artifacts due to incubation, and extraneous precipitate (EX) are also seen. Endoplasmic reticulum (ER) and mitochondria (ER) are visible, but show no significant deposits of reaction product.
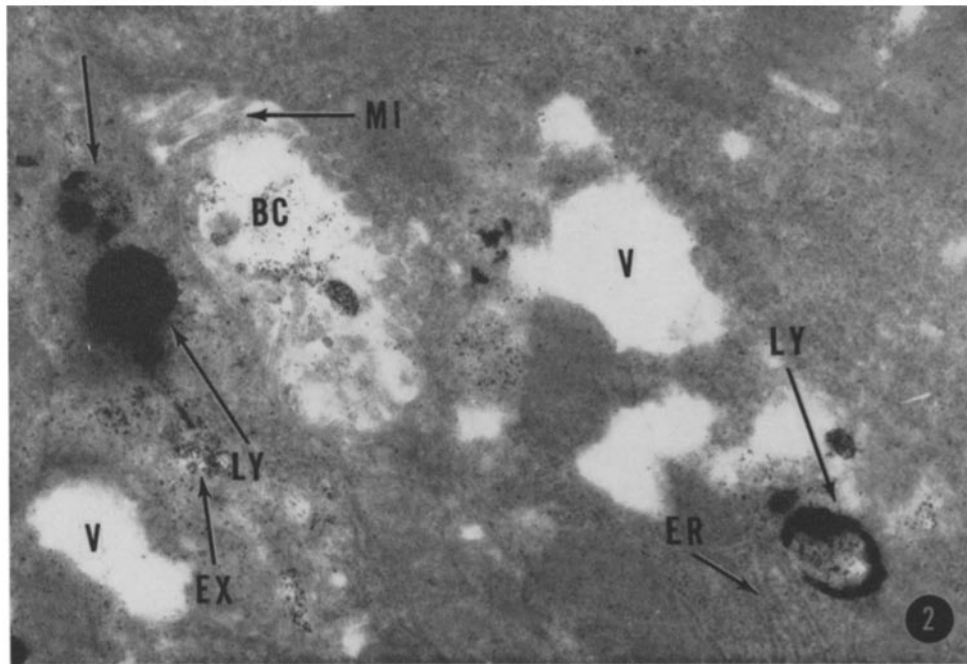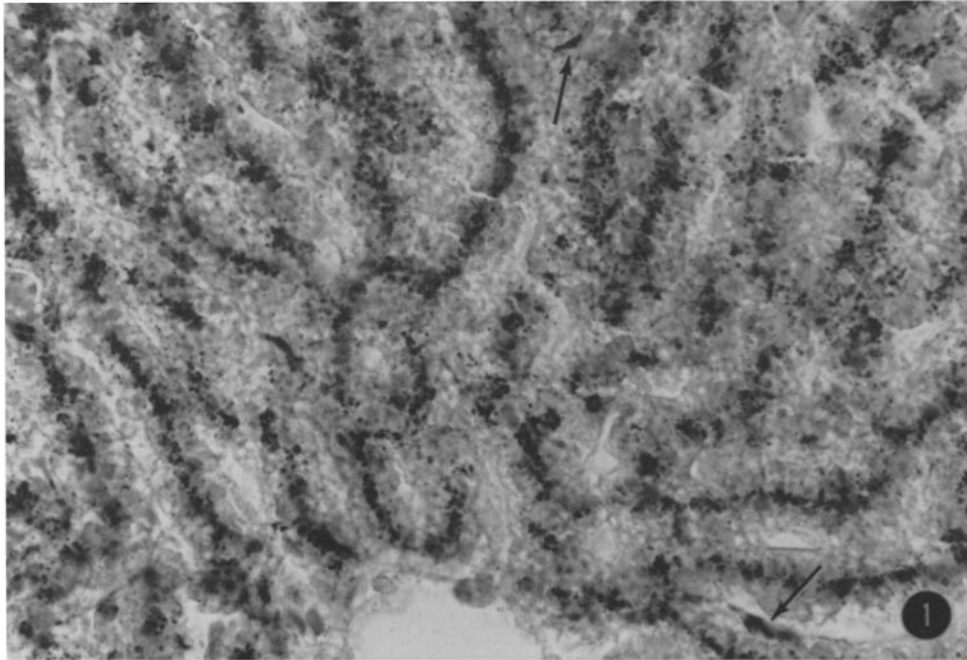
around the bile canaliculi. The Kupffer cells, particularly in the periportal areas, may contain large spherical bodies rich in acid phosphatase activity. It is of interest that in both cell types these bodies, presumably lysosomes, contain a material which gives a positive reaction in the periodic acid–Schiff procedure (15).

Representative electron micrographs of tissue incubated for acid phosphatase activity are shown in Figs. 2 and 4. Despite improvement resulting from the addition of sucrose to the incubation medium, considerable damage to the fine structure occurs and vacuole-like areas are formed. However, most structures are readily identifiable: bile canaliculi with their microvilli, sinusoidal aspects, mitochondria with their typical cristae and ergastoplasm. The location, size and number of bodies with heavy deposits of reaction product (Fig. 2) correspond to those of dense bodies: they are generally adjacent to bile canaliculi; most are smaller than mitochondria but a few approach mitochondrial size; and two or three are frequently seen adjacent to a canaliculus. Since the deposits of reaction product are too extensive to permit identification of their fine structure, the identification of these structures as dense bodies cannot be definitive.

One of the large Kupffer cell lysosomes is seen in Fig. 3. Its heterogeneous nature is evident, as is its external membrane. Fig. 4 shows the extensive reaction product that accumulates in such lysosomes when liver is incubated in the acid phosphatase medium. The nuclei of Kupffer cells also show dense deposits of reaction product.

Frequently, a fine sprinkling of reaction product is seen in the thin sections, apparently haphazardly distributed. This can readily be distinguished from the heavy deposits of reaction product which form the subject of this report. The fine "extraneous precipitate" is interpreted as resulting from diffusion of either reaction product or enzyme, and therefore no significance is attached to it. It accumulates in moderate amounts in the nuclei of parenchymatous cells. The accumulations on the Kupffer cell nuclei (Fig. 4) are sufficiently more dense to warrant further investigation before they, too, can be attributed to the adsorption of diffusing enzyme or reaction product.

When tissue is incubated in control medium containing lead but not glycerophosphate, only a light, scattered precipitate is seen in some areas. Unlike treatment of thin sections with alkaline lead solutions (16), incubation of tissue blocks in the lead-containing control medium produces little increase in contrast of cell structures.

Liver of Bilirubin-Infused Rats

After infusion of unconjugated bilirubin, there is a dramatic increase in the number of acid phosphatase-rich particles, mostly but not exclusively near the bile canaliculi (Fig. 5). This is paralleled by the appearance, in the same location, of numerous "microbodies" resembling those described by Rouiller and Bernhard (11). Micrographs demonstrating their fine structure (inner core of electron-opaque material arranged in geometric arrays) have already been published (17).

Accumulations of reaction product of acid phosphatase activity are readily seen in electron micrographs (Fig. 6). It can be concluded that most, if not all, of these bodies with reaction product


FIGURE 3

Electron micrograph of rat liver. × 25,000.

A Kupffer cell (K), lining the blood sinusoid (S), contains a large lysosome (LY) with heterogeneous structure and outer membrane (unlabelled arrow). Some of the small grains resemble ferritin. Also visible are the microvilli (MI), mitochondria (M) and lipid (LI) of adjacent parenchymatous cells.

FIGURE 4

Electron micrograph of rat liver incubated 30 minutes for acid phosphatase activity. × 14,000.

Two Kupffer cell nuclei (N) show heavy deposits of reaction product. Between them is a lysosome (LY) with heavy deposit of reaction product (compare with Fig. 3).

Mitochondria (M) in neighboring parenchymatous cells, as usual, have no reaction product. The space (S) is probably a disrupted portion of the sinusoid.
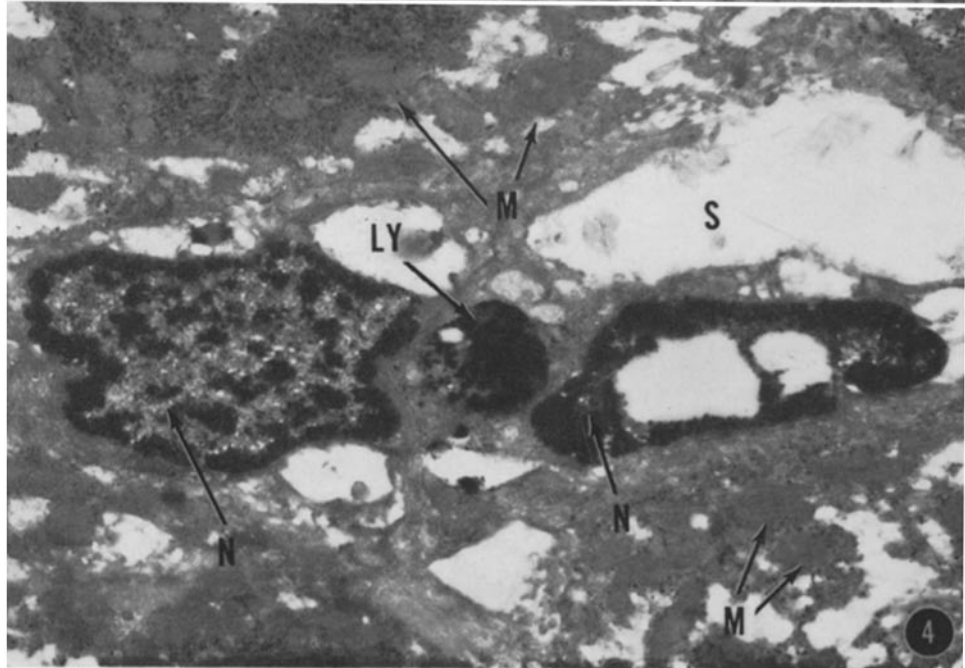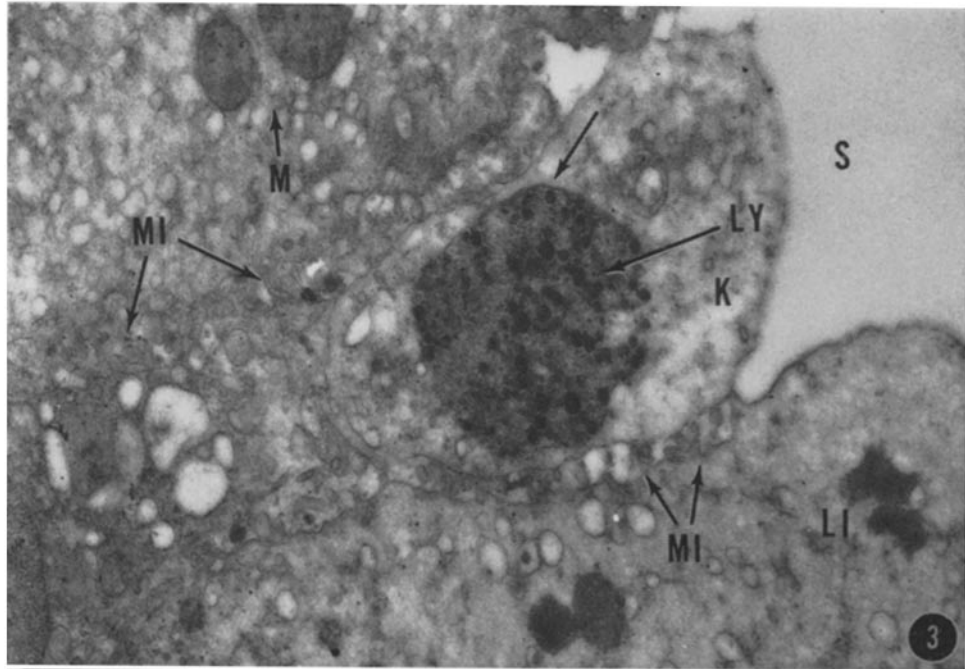
are microbodies, since only these bodies become so numerous following bilirubin infusion.

Rouiller and Bernhard (11) reported the appearance of microbodies following reversal of several experimental procedures that produced hepatocellular damage. They proposed that the microbodies were precursors of the mitochondria. Micrographs were shown of bodies presumed to be in transition from microbody to mitochondrion. These were, however, not unequivocal and, in view of our findings, more compelling evidence is required to suggest so drastic a biochemical transformation in these bodies—the loss of acid hydrolases and the acquisition of oxidative enzymes. Although we have thoroughly searched our material for them, transitional forms have not been encountered.

de Duve et al. (18) have recently reported that when the lysosome-containing mitochondrial fraction is centrifuged in a glycogen gradient in 0.5 M sucrose, biochemically distinctive particles can be separated. They contain uricase, D-amino acid oxidase and catalase, but little or no acid hydrolase activities. From biochemical and electron microscopic studies, de Duve (19) suggests that the microbodies of untreated liver contain uricase and related enzymes but not acid hydrolases. Resolution of the apparent discrepancy between this suggestion and our observations may be achieved when de Duve and colleagues will have analyzed the microbodies of bilirubin-infused liver in the same manner that they have studied those from untreated liver.

Human Liver: Lipofuscin Granules

On the basis of: (*1*) electron micrographs in which transitional stages appeared to be present between pericanalicular dense bodies and "mature" lipofuscin granules, and (*2*) the acid phosphatase activity present in lipofuscin granules, we have suggested (10) that lipofuscin granules are altered lysosomes. Our earlier report was limited to light microscopic examination of the reaction product of acid phosphatase activity. Thus we could suggest only tentatively that the acid phosphatase activity was restricted to the peripheral part of the lipofuscin granule (Fig. 7; *see also* Fig. 2 in Essner and Novikoff (10)) as is the ferritin-like material in the original dense body (Fig. 8). This suggestion is now confirmed by direct electron microscopic examination of reaction product, illustrated by Figs. 9 and 10. When the section passes through a granule in a favorable plane, it is evident that the reaction product is restricted to the periphery of the granule. The inner, moderately opaque material, presumed to be the lipofuscin, shows no reaction product of acid phosphatase activity. As in the case of rat liver (Figs. 2, 4, 6), reaction product is not found in mitochondria (Fig. 9).

It should be noted that Figs. 9 and 10 are printed with reduced contrast so as to show a maximum of structural detail. Since this is not done for Fig. 8, the contrast difference between sites of reaction product and structures of high intrinsic contrast in control sections are minimized.

FIGURE 5

Light micrograph of liver of rat infused with unconjugated bilirubin. × 640. Incubated 10 minutes for acid phosphatase activity, followed by 7 minutes for apparent ATPase activity in the medium of Wachstein and Meisel (40).

The number of acid phosphatase-rich particles adjacent to the ATPase-rich bile canaliculi is so large that individual bodies are difficult to see in the photograph. The particles elsewhere in the cell (*unmarked arrows*) are also increased in number (*see* reference 17).

Crystals of bilirubin (*B*) are seen in some areas of the sinusoids.

FIGURE 6

Electron micrograph of liver of rat, infused with unconjugated bilirubin; incubated 30 minutes for acid phosphatase activity. × 16,000.

Unlabelled arrows point to a few of the particles in which enzyme reaction product is deposited.

Endoplasmic reticulum (*ER*), and a portion of a bile canaliculus (*BC*) are also seen. There is much extraneous precipitate.
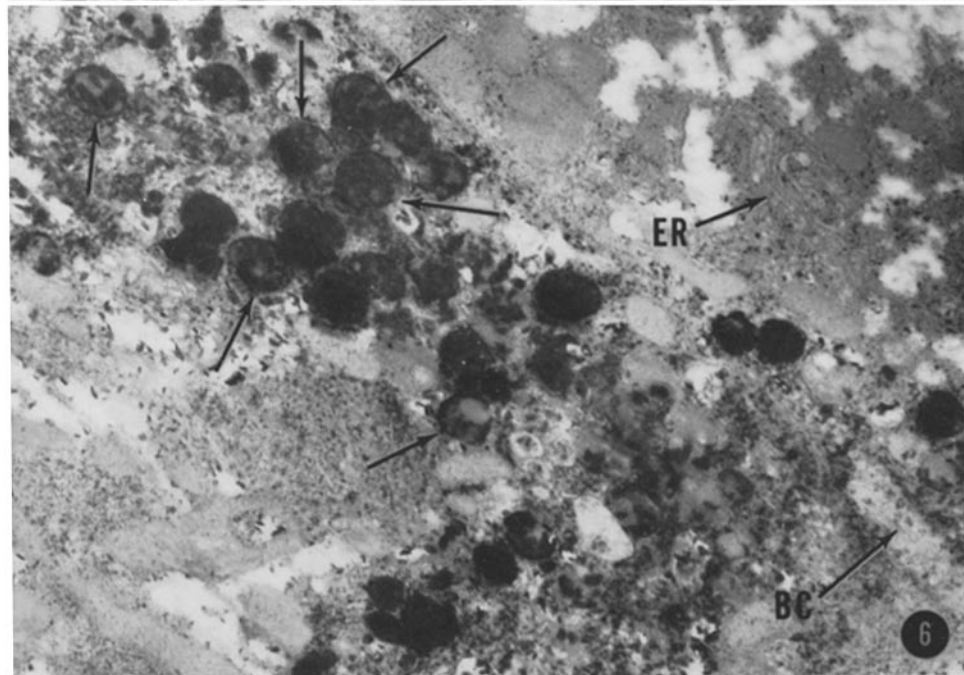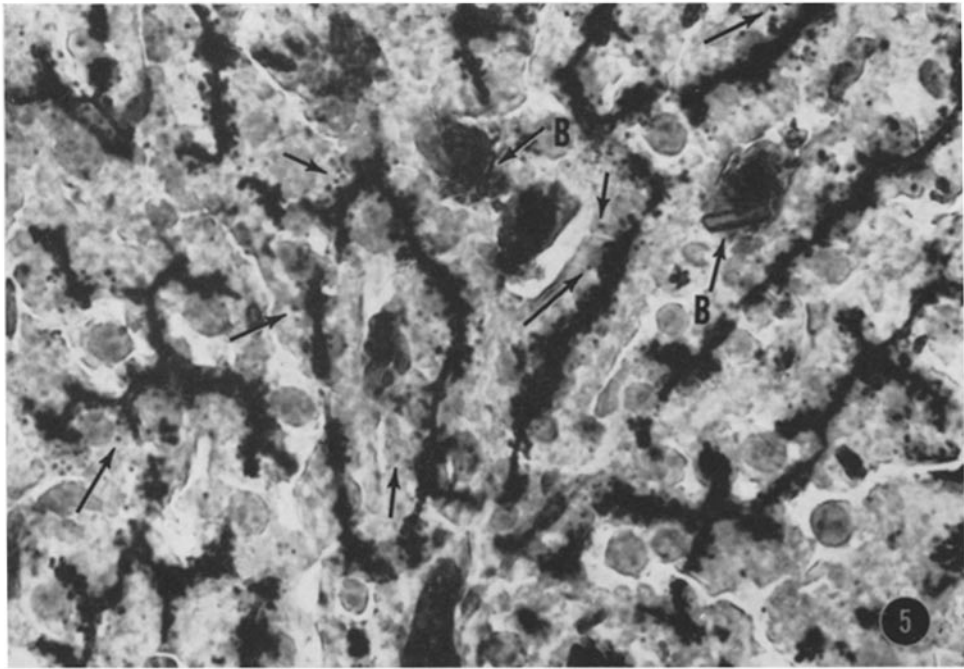
However, the far greater opacity of the reaction product is readily perceived in the electron microscope.

These results provide considerable support for the suggestion, tentatively made in 1956 (3), that the lysosomes of hepatic tissue are the pericanalicular dense bodies. In all three situations—normal rat liver, liver of rats infused with bilirubin, and human liver containing lipofuscin granules—this identification may be suggested on the basis of size and distribution of the granules showing accumulated reaction product of acid phosphatase activity. In the case of the lipofuscin granules (altered dense bodies (10)), the identity of fine structure before and after incubation for enzyme activity provides strong additional evidence. In the bilirubin-infused rats, additional support stems from the great increase, following infusion, in the number of pericanalicular bodies with acid phosphatase activity.

The dense bodies of normal liver, the microbodies of bilirubin-infused rats, the lipofuscin granules, and the large heterogeneous bodies of Kupffer cells are all considered lysosomes since they possess the two criteria we use (6, 10, 20) to characterize lysosomes morphologically: (1) the presence of acid phosphatase activity in stained preparations, and (2) a "single" outer membrane ("unit membrane" of Robertson (21)). Bodies with these two features have also been described in the cells of the proximal convolutions (8) and glomerular epithelium (22, 20) in rat kidney, in rat erythrophagocytes (23), and in other tissues (20). It is readily apparent that lysosomes of different cells show striking differences in fine structure. In these studies, the assumption has been made that all lysosomes possess acid phosphatase activity, although it is recognized that even the biochemical evidence from liver (1, 2) is still not entirely unequivocal on this point. de Duve (2) considers it "reasonable, unless direct evidence to the contrary is obtained, to consider the lysosomes as forming a single group, all of which contain all the acid hydrolases recognized as lysosomal, but in variable proportion." Such evidence may come from continued application of centrifugation procedures, of higher resolution, or from staining procedures for other hydrolases as reliable on the cytological level as that for acid phosphatase. One may expect that, with interest focused on lysosomal enzymes, such high-resolution staining methods may become available before long for β-glucuronidase, glucosaminidase, cathepsins or nucleases. Good staining methods are now available for demonstrating esterase activity, and the first direct comparisons of esterase and acid phosphatase reactions indicate that both are present in the same lysosomes. These

FIGURE 7

Light micrograph of human liver, incubated for acid phosphatase activity. × 1,440.

Lipofuscin granules are present near the bile canaliculus. In one of these (*arrow*) reaction product is seen at the periphery.

FIGURE 8

Electron micrograph of human liver. × 40,000. This figure is printed for high contrast, whereas Figs. 9 and 10 are printed for low contrast.

A "mature" lipofuscin granule occupies the center of the micrograph. The central mass of moderate electron opacity (L) is presumably lipofuscin pigment. The arrow indicates the "single" nature of the limiting membrane. Ferritin-like grains are restricted to the periphery; this is interpreted as the remains of the original pericanalicular dense body (10).

Mitochondria (M), and endoplasmic reticulum (ER) are seen.

FIGURE 9

Electron micrograph of human liver incubated 30 minutes for acid phosphatase activity. × 13,000.

Deposits of enzyme reaction product (contrast minimized by printing) are localized to the periphery of lipofuscin bodies, as can be seen in those cut in favorable planes (*arrow*). The central mass, presumably lipofuscin, is free of reaction product, as are the mitochondria (M) in the surrounding areas.
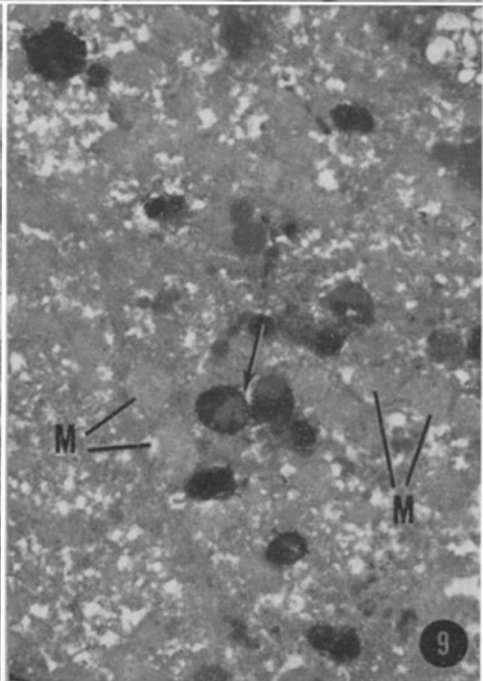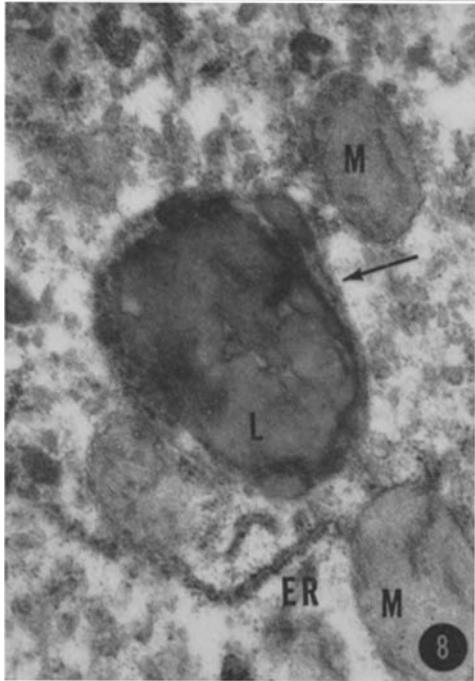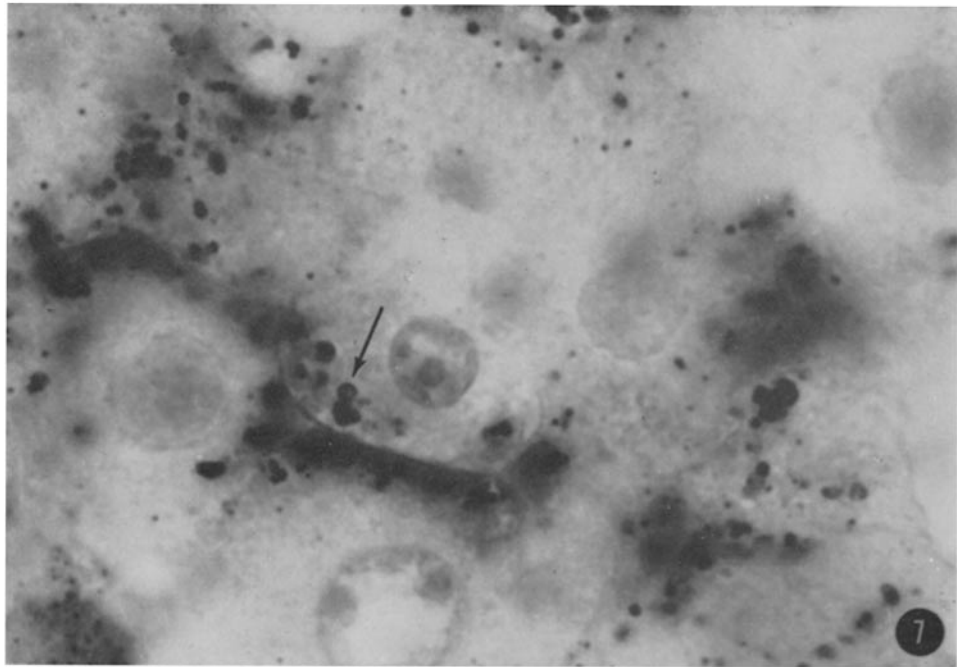
comparisons are discussed elsewhere (20), as is the dilemma posed by the apparent discrepancy in intracellular localizations of esterase activity indicated by staining methods and biochemical analyses of isolated subcellular fractions.

Since interest is currently high in localizing enzyme activities on the electron microscope level, some comments on phosphatases generally may be desirable. It was with alkaline and acid phosphatases that the first electron microscopic studies of enzyme activities were made, by Sheldon et al. (24) and Brandes et al. (25). These workers introduced the block technique of incubation following brief fixation in osmium tetroxide. This procedure was subsequently used by Essner et al. (26) for localizing adenosine triphosphatase (ATPase) and 5-nucleotidase activities in rat liver. Other enzymes, like acid phosphatase, may not survive even brief osmium tetroxide fixation. Yet unfixed tissue cannot be used, since the enzyme would diffuse during incubation and because the liver, unlike tissues such as cardiac muscle (27), undergoes extensive morphological alteration unless fixed. Fortunately, fixation in cold formol–calcium sufficiently prevents both these occurrences, without inhibiting too severely the acid phosphatase activity. However, this block technique cannot be applied to all tissues. Our experience indicated that with rat kidney reagents did not penetrate enough to produce consistent and reliable results. It was for this reason that recourse was had to incubating frozen sections, 25 μ thick, of formol–calcium-fixed tissue (28). As was to be expected, freezing and thawing resulted in extensive damage, yet valuable information was obtained regarding localizations, in renal tubule cells, of acid phosphatase activity (29) and apparent ATPase activity (30, 7, 8). This included the localization of ATPase activity in the cell membranes where adjacent cells interdigitate in the thick limbs of Henle's loops, a localization also seen in frozen sections studied by light microscopy (30). Consistency with such light microscopic observations is lacking in the case of two other

phosphatase activities reported, on the basis of block studies, to be localized in these membranes: 5-nucleotidase ((31); see also Figs. 9–10 in de Robertis et al. (32)) and alkaline phosphatase (33). Neither of these enzyme activities is found in these areas of cell membrane when frozen sections of formol–calcium-fixed tissue are incubated in appropriate media. It may be that, even in the presence of detergent (31), limited penetration of reagents produces misleading deposits of reaction product.

In any case, the present acid phosphatase studies yield results that are consistent with those see in frozen sections. This includes even the restriction of reaction product to the periphery of the lipofuscin granules, as seen with high magnification under the light microscope (Fig. 7). It is unlikely that penetration of materials is restricted in such sections, since they are frozen and thawed slowly.

Shorter incubation periods than we have used, especially where numerous active sites lie close together (Fig. 6), would have been desirable. It would probably reduce the amount of extraneous precipitate, while preserving fine structure more adequately and permitting identification of the initial deposits of reaction product. The presence of sucrose in the incubation medium helps preserve fine structure but it does not eliminate vacuolization and other damage that occurs, even in fixed tissue.

Since the deposits of "extraneous precipitate" seen in the electron microscope are below the limit of resolution of the light microscope, the results here reported provide support for the view that the Gomori acid phosphatase procedure, applied to tissue or cells fixed in cold formol–calcium, is a reliable means of demonstrating acid phosphatase–rich lysosomes (6–9, 20). Similar acid phosphatase localizations are obtained by other methods, in which the substrates are indoxyl or naphthyl phosphates (34–36). The absence of acid phosphatase activity from mitochondria is consistent with the biochemical evidence (1).

FIGURE 10

Electron micrograph of human liver, incubated 30 minutes for acid phosphatase activity. × 25,000.

Deposits of reaction product (contrast minimized by printing) are evident in the peripheral areas of lipofuscin granules (arrows). The central areas of moderate electron opacity, presumably lipofuscin, are free of precipitate. Endoplasmic reticulum (ER) is evident; it, too, is free of reaction product.
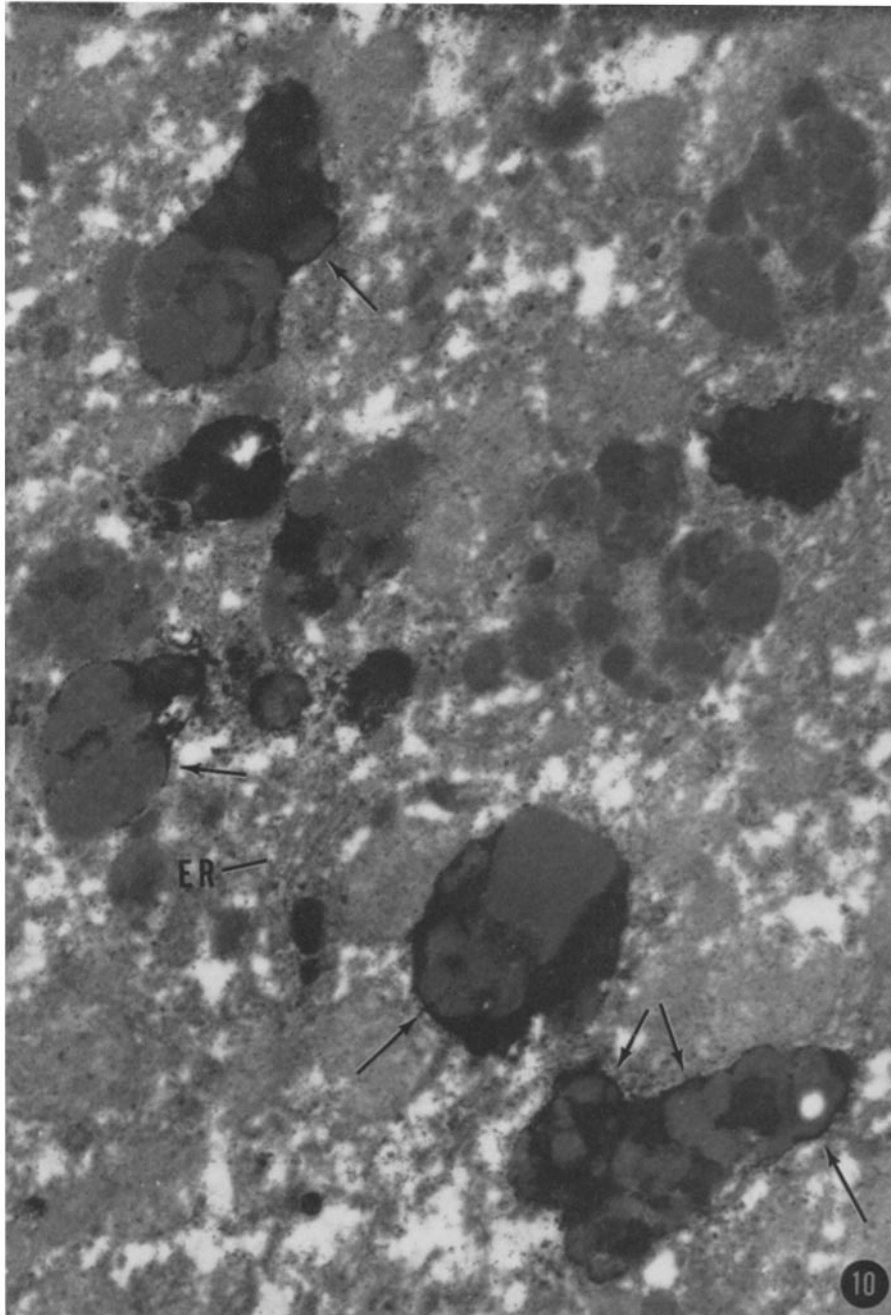
It is a pleasure to acknowledge the generous cooperation of Dr. I. M. Arias, of the Department of Medicine, who performed the bilirubin infusions, Mr. L. J. Walker, who serviced the electron microscope and prepared the photographs, and Mr. W. Y. Shin, who prepared the frozen sections for light microscopy.

ADDENDUM

Following preparation of this manuscript, and its publication in preliminary form (37, 38), a study of

mouse liver was published by de Man *et al.* (39). Repeated intraperitoneal injections of dextran resulted in a marked increase in acid phosphatase–rich granules along the bile canaliculi, where dextran droplets accumulated. These droplets appear as large clear vacuoles in the electron microscope. de Man and associates believe that in the parenchymatous cells, and especially in the Kupffer cells, the dense bodies (presumed to be the sites of acid phosphatase activity) form part of the vacuole wall.

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