Adenosinetriphosphatase and 5-Nucleotidase Activities in the Plasma Membrane of Liver Cells as Revealed by Electron Microscopy*,‡

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ABSTRACT

The sites of reaction product resulting from ATPase and 5-nucleotidase activities remaining in parenchymatous cells of osmium-fixed rat liver were studied by electron microscopy of thin sections. These indicate that both ATPase and 5-nucleotidase activities are localized in the plasma membrane where it folds to form the microvilli of the bile canaliculus, and that 5-nucleotidase activity is also present in the microvilli at the sinusoidal aspects of the cells.

It is suggested that these enzymes, particularly ATPase, may play a role in molecular transport or in some kind of membrane activity at the cell surface. Of special interest is the apparent differential localization of these enzymes at the absorptive and secretory regions of the plasma membrane of the cell. It may be of interest to study changes in these enzyme localizations in pathologic states, as a sign of changed cell function.

Some of the difficulties in the interpretation of enzyme reaction products seen in electron micrographs are discussed.

Cytochemical staining studies in our laboratory have indicated that adenosinetriphosphatase (ATPase) is localized in certain areas of the plasma membrane of some cells (8, 19), suggesting that the enzyme may be involved in the active transport of molecules across the cell surface or in cell membrane movements such as occur in pinocytosis and cytopempsis (5). Earlier studies had shown that high levels of ATPase activity are localized in the bile canaliculi of rat liver while 5-nucleotidase is present both in the bile canaliculi and blood sinusoids (7, 20, 10). Even with oil immersion, however, it is not possible to determine the precise sites of reaction product in these areas. That such reaction products might be seen in electron micrographs of thin sections of osmium-fixed tissue was indicated by the studies of Sheldon *et al.* (18) and of Brandes *et al.* (2). These workers found that in intestinal epithelium sufficient alkaline and acid phosphatase activities survived brief osmium tetroxide fixation to produce electron-opaque deposits of reaction products.

In the present study of parenchymatous cells of rat liver, the reaction product of ATPase activity is shown to be localized directly in the plasma membrane where it folds to form the microvilli of the bile canaliculi (13, 15, 4). The reaction product of 5-nucleotidase activity is also shown to be localized there; in addition it is present in the microvilli exposed to the blood sinusoid (space of Disse).

Material and Methods

Adult Sprague-Dawley rats, usually males, were decapitated and bled in the cold room. Thin slices of liver were cut from areas near the periphery of the median lobe and placed immediately into cold, 1 per cent osmium tetroxide (OsO_4) buffered at pH 7.2 (13). The slices were cut into smaller blocks while in fixative. After a total of 7 minutes fixation, the blocks were

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washed several times in veronal-acetate buffer to remove the OsO_4.

Tissue blocks were incubated at 37°C. (15 to 30′) in the ATPase medium of Wachstein and Meisel which contains lead and is buffered at pH 7.2 (20). Other samples were incubated at 37°C. (30′) in the same medium containing 5-adenylic acid instead of ATP. Because the accumulated precipitate of lead phosphate is opaque in the electron beam, conversion to lead sulfide was usually omitted. Samples incubated in the same medium without substrate served as controls. After incubation, all tissue samples were washed in cold veronal-acetate buffer and stored overnight in the cold in 1.6 per cent formaldehyde-0.28 M-veronal-0.28 M acetate buffer, pH 7.2 (21). Unincubated control tissue was fixed for 15′ in cold buffered 1 per cent OsO_4, washed, and stored in buffered formalin overnight. After rapid dehydration in alcohols, the tissue samples were embedded in a 1:5 mixture of methyl-butyl methacrylate according to the method of Ward (21). Sections (silver colored) were cut on a Porter-Blum (Servall) microtome, using glass knives, and were mounted on formvar-coated copper grids. Micrographs were taken with an RCA-EMU 3B electron microscope at original magnifications of from 2600 to 8400.

Preliminary experiments were done to determine whether fine structure would be adequately preserved when incubation was performed *prior* to osmium fixation. Unfixed tissue was incubated as a block and formol-calcium–fixed tissue was incubated as 10 μ sections cut on a routine Bausch and Lomb freezing microtome. Following incubation, the tissue was rinsed and fixed in buffered osmium tetroxide, to minimize damage in the electron beam (6, 12).

The effect of osmium fixation on the levels of ATP and 5-adenylic acid dephosphorylation was studied. Small pieces of liver were fixed in cold, buffered 1 per cent OsO_4 for 7 minutes, rinsed several times in buffer and in sucrose, and homogenized in 0.25 M sucrose. Assays were carried out as described previously for formol-calcium–fixed tissue (10), using the Wachstein-Meisel medium containing 4.1×10^{-3} M ATP or 5-adenylic acid and 1×10^{-2} M $MgSO_4$. All trichloroacetic acid extracts obtained after incubation were treated with norit A prior to the determination of their orthophosphate content. This was necessary because the reduced osmium, or some other substance in the extracts of the osmium-fixed tissue, interfered with the color reaction for orthophosphate.

RESULTS

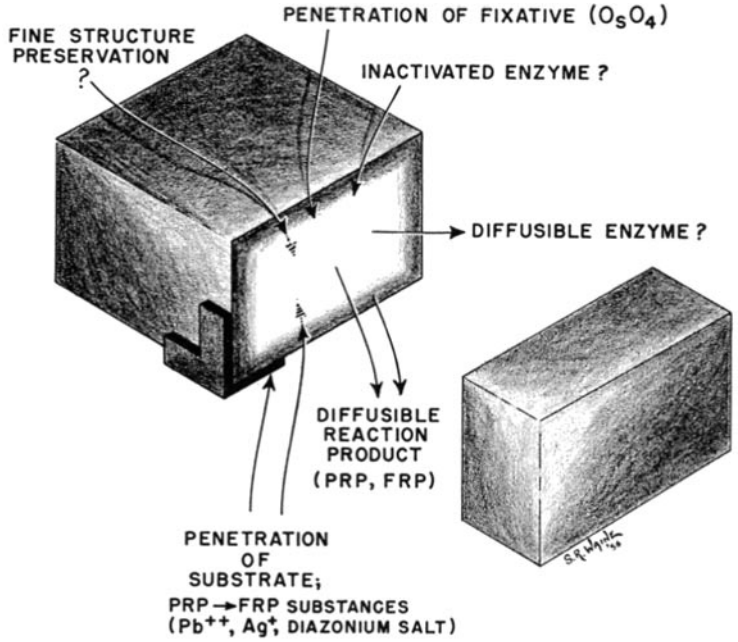
Attempts to use unfixed tissue blocks or frozen sections of formol-calcium–fixed tissue for enzyme incubation were unsuccessful. Damage to fine structure during incubation was too extensive for valid interpretation of the electron micrographs. Much better preservation resulted when osmium-fixed tissue blocks were used.

The difficulties encountered in this tissue block technique have been discussed earlier (9) and are summarized in Text-fig. 1. Because osmium tetroxide penetrates the tissue very slowly, only the outer layer of one or two cells is relatively well fixed in the brief (7 minute) fixation period. Thus, it is from the periphery of the block that thin sections suitable for electron microscopy must be prepared. It is in this layer that extensive loss of enzyme activity is to be anticipated. Enzyme in the interior may not be fixed at all and may be free to diffuse into the incubation medium. Primary and secondary reaction products may diffuse from sites of formation. In addition, the use of tissue blocks rather than sections restricts the penetration of substrate or other reagents into the tissue. It is thus to be expected that the tissue block technique will have but limited applicability.

An estimate of the residual enzyme activities which remain in the tissue blocks exposed to osmium tetroxide for 7 minutes is given in Table I. When assayed in the Wachstein-Meisel medium (*cf.* (10)) there is about 15 per cent survival of the ATPase activity and about 40 per cent of the 5-nucleotidase activity. Clearly, these are maximum figures; they may be considerably lower in the well fixed periphery of the tissue block. There is, however, no question but that some enzyme survives even in areas to which the fixative has complete access. This is shown by two observations we have made: (1) in paraffin sections of liver blocks fixed for 7 minutes in buffered osmium tetroxide, ATPase activity was clearly evident in the canaliculi of the outermost cell layer; (2) canaliculi and sinusoids showed 5-nucleotidase activity throughout the section when 5 μ frozen sections were covered with cold buffered osmium tetroxide for 10 minutes and rinsed, prior to incubation.

Fig. 1 illustrates a typical section through a bile canaliculus from control, unincubated liver. Because of the plane of sectioning, the microvilli appear in cross-, oblique, or longitudinal section. For the same reason, some areas of the plasma membrane may appear not to be folded into microvilli.

The appearance of such tissue after incubation in the Wachstein-Meisel medium without substrate is shown in Fig. 2. Even brief periods of incubation (15′, 37°C.) result in extensive cytoplasmic vacuolization and damage to the endoplasmic reticulum. The mitochondria are better preserved. The microvilli in the bile canaliculi, like those in the Disse space, suffer damage but areas with minimal



TEXT-FIG. 1. Schematic diagram to illustrate some of the problems involved in the use of the tissue block technique. The darkened periphery of the block indicates the limited depth to which osmium tetroxide may penetrate during brief (*i.e.*, 7 minute) periods of fixation. Possible diffusion of unfixed enzyme, or primary and final reaction products (*PRP*, *FRP*) are indicated, as is the possible difficulty of penetration of substrates and reagents into the tissue, due to intrinsic tissue character or to accumulated reaction product (shown in black).

change are readily found. In general, there is very little electron opacity which might be ascribed to lead binding; this is even less common in the cytoplasm than in the nuclei.

Fig. 3 illustrates the general appearance of a bile canaliculus, in longitudinal section, after 30 minutes of incubation in the *ATPase* medium. The irregular pattern caused by unequal deposition of the precipitated lead salt is characteristic of many stained canaliculi. All of this precipitate, however, is deposited *within* the canaliculus. A light precipitate often forms on the nuclei of parenchymatous cells, but it is not present in Fig. 3.

Wide variation in staining intensity occurs within a single block of tissue and, indeed, often in adjacent areas of the same thin section. Within a single bile canaliculus the accumulated precipitate may also be distributed unevenly. It is in the areas with light deposits of lead phosphate that the deposit can be localized in the plasma membrane. Fig. 4, for example, shows light deposits in the plasma membrane in the lower region of the canaliculus. In Fig. 5, deposits in the plasma membrane and at the tips of several microvilli are apparent. The plasma membrane outside of the canaliculus (seen extending above and below it) shows no deposit of reaction product. Although the moderately stained bile canaliculus seen in Fig. 6 is partly disrupted, well-stained microvilli can readily be seen. The plasma membrane, which forms the border of these microvilli, is also stained. In contrast to the less intensely stained areas, regions of the closely opposed cell membranes of adjacent liver cells, which extend from the canaliculus, also contain some precipitate. No significant amount of precipitate is deposited within the lumen. In other instances of heavily stained canaliculi, however, such luminal deposits are present. The light precipitate in the vicinity of the canaliculi may represent lead-binding or, more likely, diffusion of enzyme or reaction product from the reactive sites.

The lead phosphate resulting from *5-nucleotidase* activity is readily identified in the bile canaliculi and blood sinusoids. The former are generally well stained; the deposition and quantity of precipitate appear essentially similar to that of ATPase. Sinusoidal staining is typically much lighter, although it should be noted that it is difficult to assess differences in intensity of staining in electron micro-

TABLE I

Effect of Osmium Tetroxide Fixation upon the Levels of ATP and 5-Adenylic Acid Dephosphorylation by Rat Liver

As controls, small blocks of fresh liver tissue (unfixed) were homogenized in 0.25 M sucrose. Similar tissue blocks were fixed for 7 minutes in buffered 1 per cent osmium tetroxide, washed several times in veronal buffer and in sucrose, and finally homogenized in 0.25 M sucrose. Activity is expressed in μg. P released in 15 minutes by 1 mg. (wet weight) of original tissue. The results are averages of two experiments which were in close agreement.

	Fresh tissue (unfixed)	Fixed 7′ in 1 per cent OsO_4	Survival
			per cent
Wachstein-Meisel medium, with 4.1×10^{-3} M ATP....	6.86	1.15	17
Wachstein-Meisel medium, with 4.1×10^{-3} M 5-adenylic acid.....	3.44	1.37	40

graphs. This lighter staining of the sinusoids and the more intense staining of canaliculi, when 5-adenylic acid is used as substrate, correlates well with the appearance in the light microscope of frozen unfixed sections or paraffin sections of acetone-fixed tissue.

Although much damage to fine structure, presumably during incubation, has occurred in the tissue shown in Fig. 7, precipitate is clearly localized on the microvilli in the space of Disse. The stained nucleus of a leucocyte may be seen in the sinusoidal space. Similarly, nuclei of Kupffer and endothelial cells are generally well stained.

DISCUSSION

The attempt to localize on the level of fine structure the precise sites of enzymatic activity is at best a compromise between the conditions required for visualization of the reaction product in the staining procedure and those required for electron microscopy. In some tissues, for example, even brief periods of incubation may result in considerable damage to fine structure. Heart tissue is apparently more resistant to such alterations than is liver (1). With liver, even after brief fixation in osmium tetroxide, incubation of small blocks for 15 to 30′ in the ATPase medium results in vacuolization of the cytoplasm and extensive damage to the endoplasmic reticulum. The nuclei, nucleoli, and mitochondria are better preserved. Fortunately, the membranes of the bile canaliculi and the microvilli in the space of Disse also remain sufficiently well preserved to permit identification of the precipitation loci. Any interpretation of enzyme localization from electron microscopy, however, must carefully weigh the degree of fine structure preservation.

Interpretation of enzyme localization from electron micrographs must also consider the loss of enzyme activity resulting from fixation. Osmium fixation, presently the method of choice for the preservation of fine structure, leads to very extensive loss of both ATPase and 5-nucleotidase activities. Like formol-calcium (10) it eliminates completely the ATPase activity of mitochondria (11). However, in the microvilli of the bile canaliculi sufficient enzyme activities survive for abundant production of lead phosphate. The same applies to 5-nucleotidase activity in the microvilli of the Disse space.

The evidence, although not unequivocal, suggests that in our experiments the substrate-specific phosphatases rather than non-specific alkaline phosphatase are being visualized. From the distributions among subcellular fractions isolated from liver by differential centrifugation, and from staining results, Novikoff *et al.* (10) have concluded that the enzyme visualized by the Wachstein-Meisel technique is most likely a specific ATPase, rather than non-specific phosphatase. Further evidence has recently been obtained in our laboratory. Three weeks after feeding the carcinogen, 3′-methyl,4-dimethyl-aminoazobenzene, to rats the bile canaliculi have lost virtually all their staining in the ATPase and 5-nucleotidase procedures. However, there is a marked *increase* in alkaline phosphatase activity, using glycerophosphate as substrate.

When 5-adenylic acid is used as substrate at neutral pH the staining pattern, observed by light microscopy, differs from that with either ATP at neutral pH or glycerophosphate at alkaline pH. The bile canaliculi near the central veins are more deeply stained than those in the periportal area. With ATP the reverse is true, as is the case with glycerophosphate at alkaline pH in which only the periportal canaliculi are stained. In addition, with 5-adenylic acid, but not with ATP or glycerophosphate, small periportal granules give a positive reaction.

It should be noted that the absence of stain cannot be taken to mean absence of enzyme. Thus, the enzymes under study may be present in the cell membrane outside the canaliculus and sinusoid areas (*i.e.*, where it is not folded into microvilli) but in concentrations too low to be visualized by the staining technique employed, which involves aqueous fixation at relatively high temperature (0–2°C.) and incubation with enzyme-inhibiting lead ions (10).

Finally, we should consider the possible significance of the enzyme localizations in the cell membrane. As already indicated, high ATPase activity has been found in the cell membranes of other tissues. Novikoff (8) described it in the cell membranes which line the "extracellular channels" coursing through a transplantable rat liver tumor. Spater *et al.* (19) have shown high ATPase activity in the deeply infolded membranes at the base of renal tubule cells in a variety of mammals. These membranes are in intimate relationship with the ATP-generating mitochondria (16, 14, 17).

How this enzyme functions in cells remains to be elucidated. It seems safe to expect that it plays a significant role in "active transport" across the cell membrane, and in cell movements such as pinocytosis and cytopempsis (5).

Of particular interest is the apparent enzymic differentiation of the absorptive and secretory regions of the plasma membrane in the parenchymatous cells of liver. In the former region, exposed to the blood plasma (Disse space), ATPase activity is not demonstrable but the level of 5-nucleotidase activity is high. In the latter region, exposed to the bile canaliculi, the level of ATPase activity is very high; appreciable 5-nucleotidase activity is also present. The alkaline phosphatase activity of the bile canaliculi is also likely to be in the plasma membrane microvilli.

The presence of other enzymes in cell membranes should be investigated. In the liver cell, it may be of interest to study changes in the apparent enzymic differentiation of the plasma membrane during pathologic states (*e.g.*, those leading to jaundice) as a sign of changed cell polarity and cell function.

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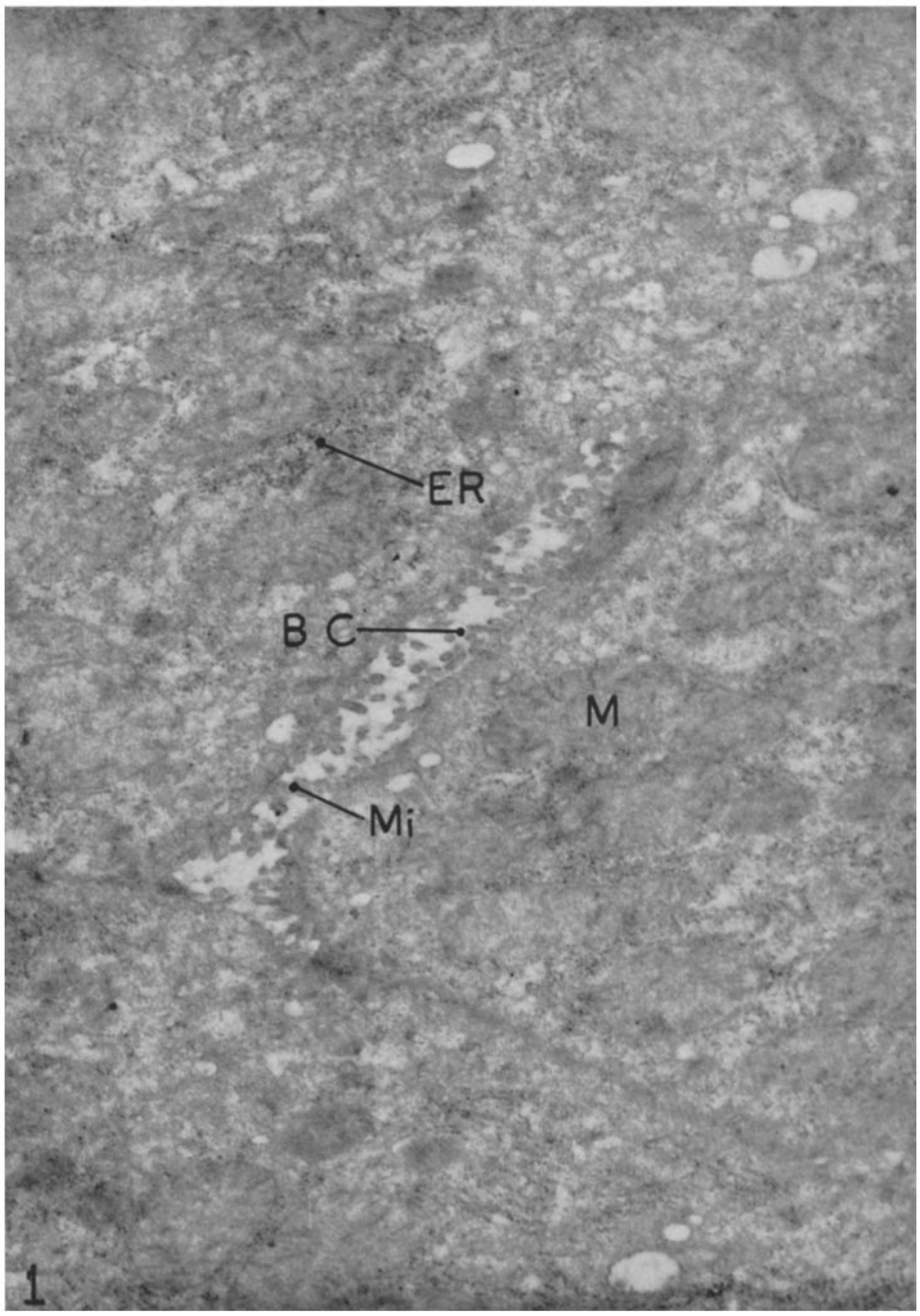
EXPLANATION OF PLATES

PLATE 354

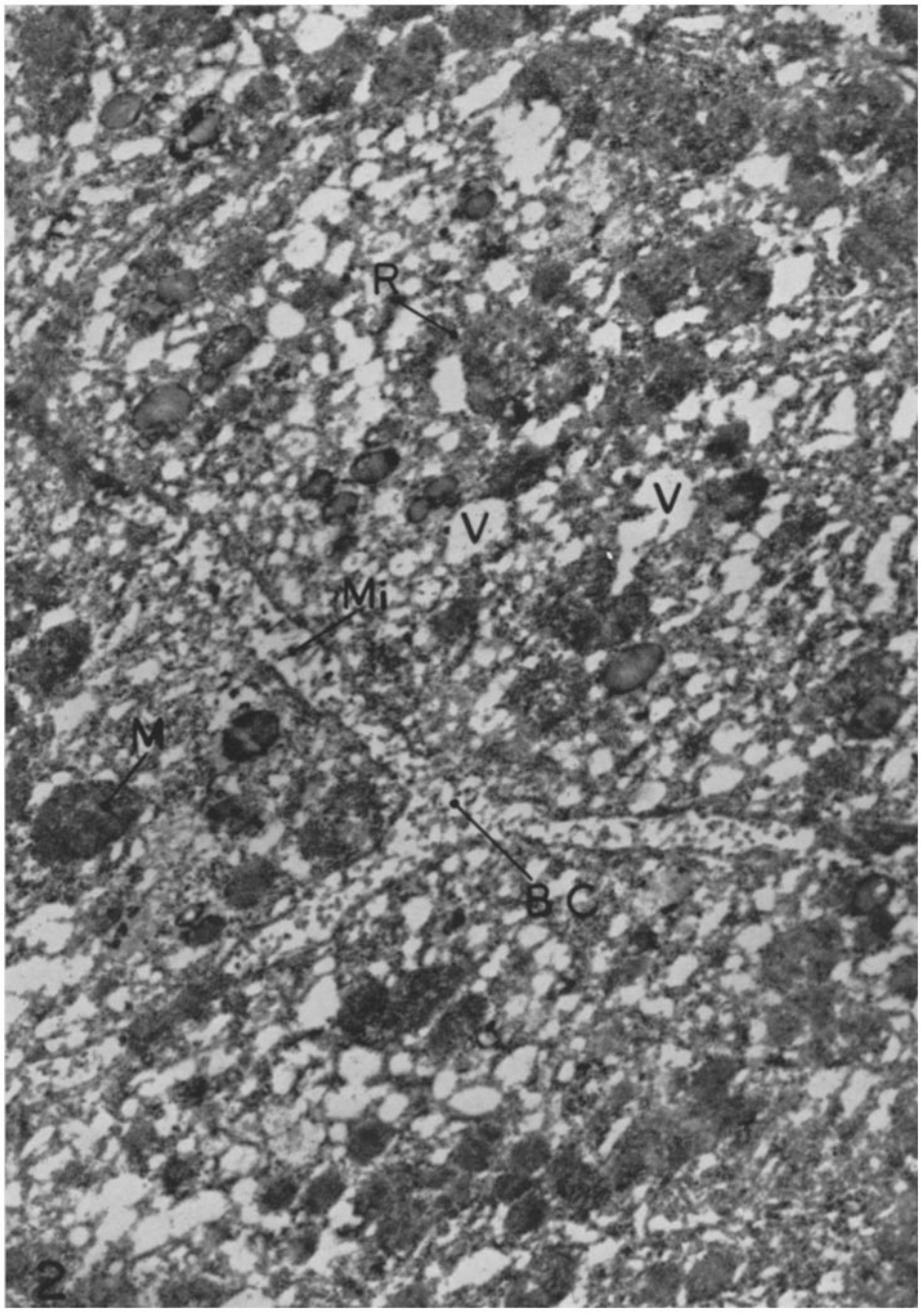
FIG. 1. Control, unincubated liver, fixed 15 minutes in buffered 1 per cent OsO_4 and left overnight in buffered formalin. The bile canaliculus (*BC*) shows numerous microvilli (*Mi*) projecting into the lumen. Because of the plane of sectioning some of the microvilli are seen in cross or oblique section and some areas of the plasma membrane appear free of microvilli. Mitochondria (*M*) and ergastoplasm (*ER*) are also evident. × 22,000.



(Essner *et al.*: ATPase in plasma membrane)

PLATE 355

FIG. 2. Control liver fixed in buffered 1 per cent OsO_4, incubated 15 minutes at 37°C., Wachstein-Meisel medium without substrate, and left overnight in buffered formalin. The bile canaliculus (*BC*) shows numerous microvilli (*Mi*). The cytoplasm is filled with vacuoles (*V*). Mitochondria (*M*) are present. Although the endoplasmic reticulum is disrupted, clusters of ribonucleoprotein granules (*R*) are still visible. × 16,000.

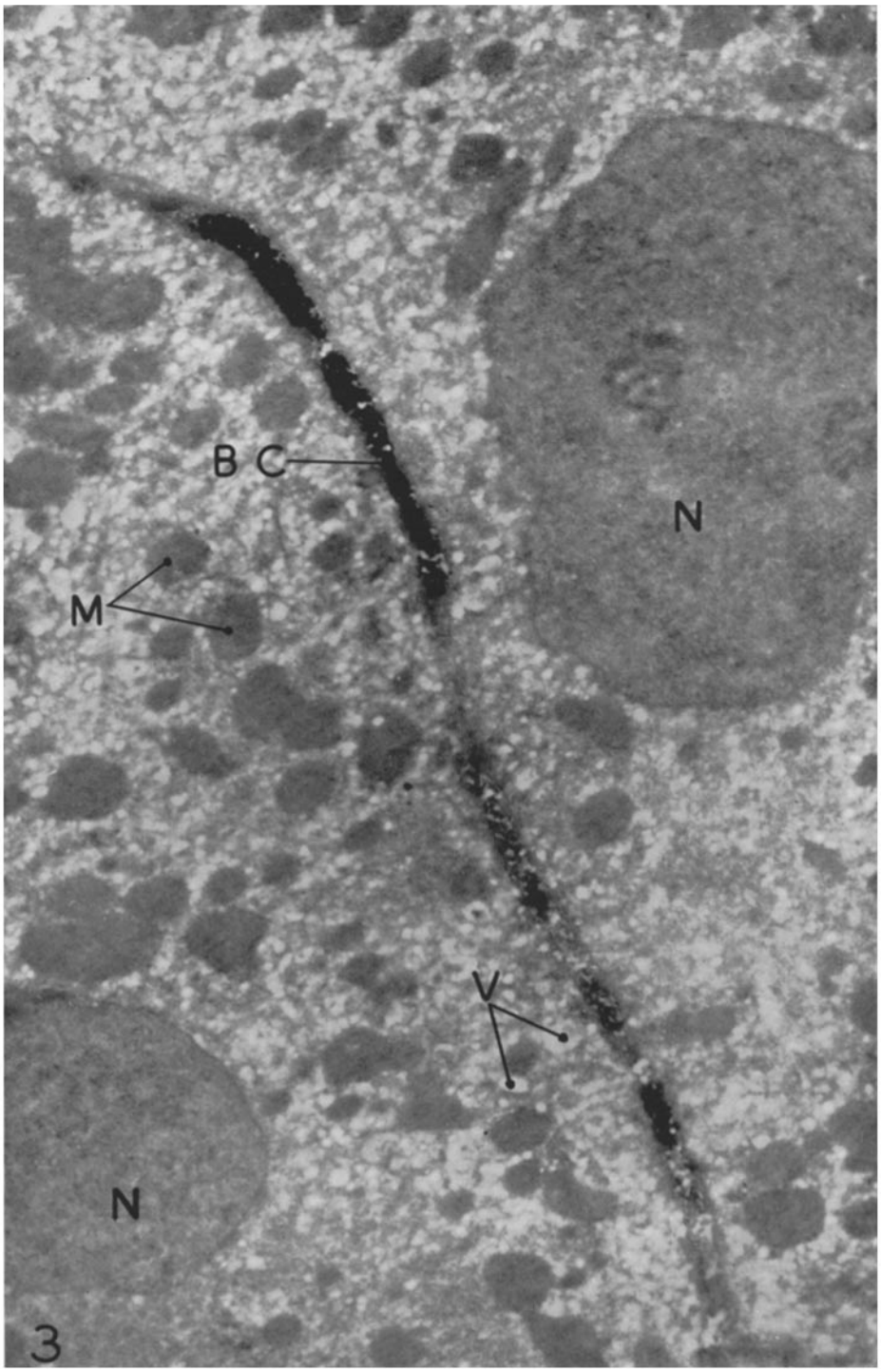


(Essner *et al.*: ATPase in plasma membrane)

PLATE 356

FIGS. 3 to 7. Thin sections of rat liver from tissue blocks fixed 7 minutes in buffered 1 per cent OsO_4, pH 7.2, and incubated 30 minutes in the Wachstein-Meisel medium for ATPase or 5-nucleotidase.

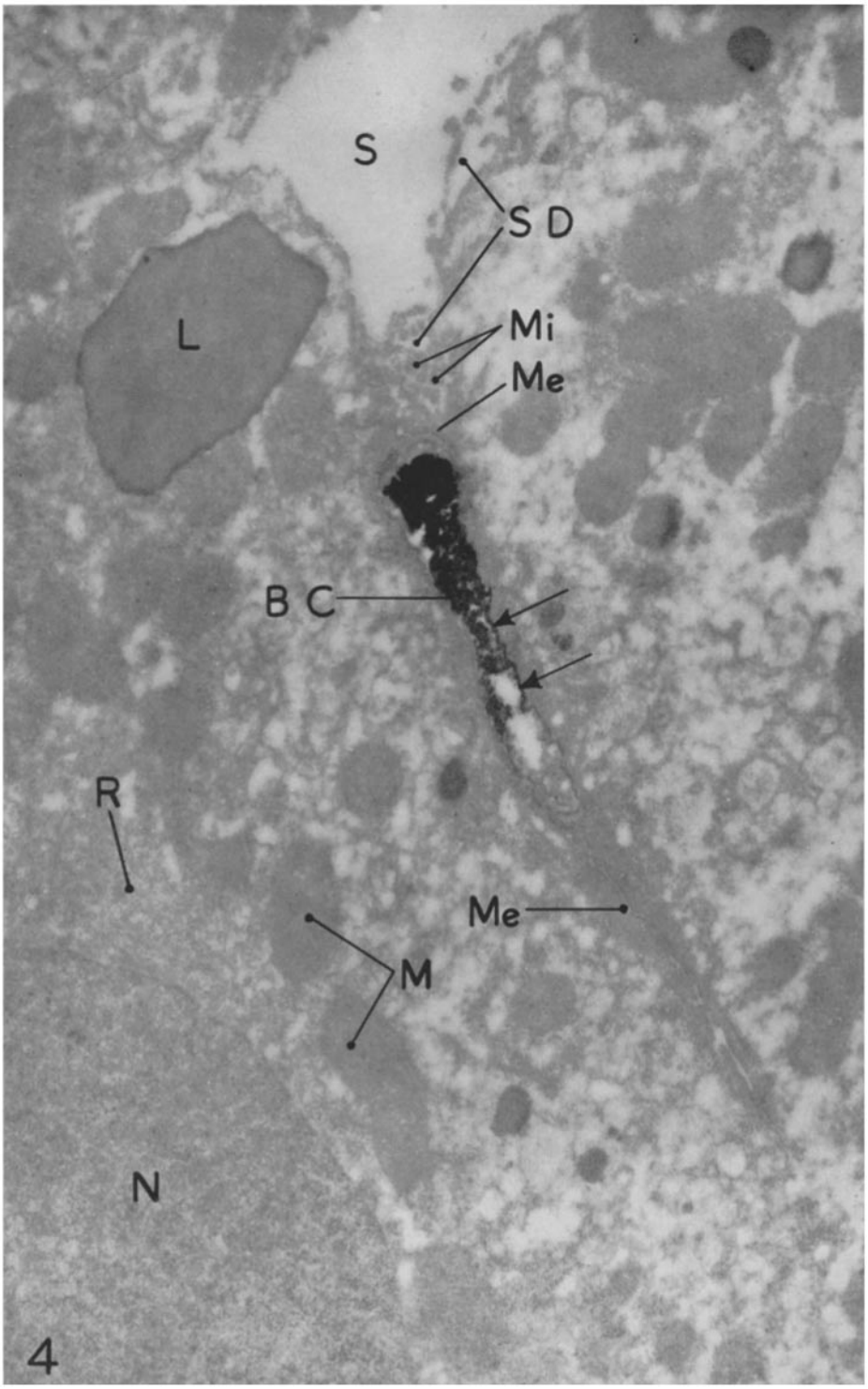
FIG. 3. ATPase. Longitudinal section through a bile canaliculus (*BC*), showing irregular deposition of lead phosphate. On either side are nuclei (*N*) of two liver cells. Mitochondria (*M*) are present in a greatly vacuolated (*V*) cytoplasm. × 11,000.



(Essner *et al.*: ATPase in plasma membrane)

PLATE 357

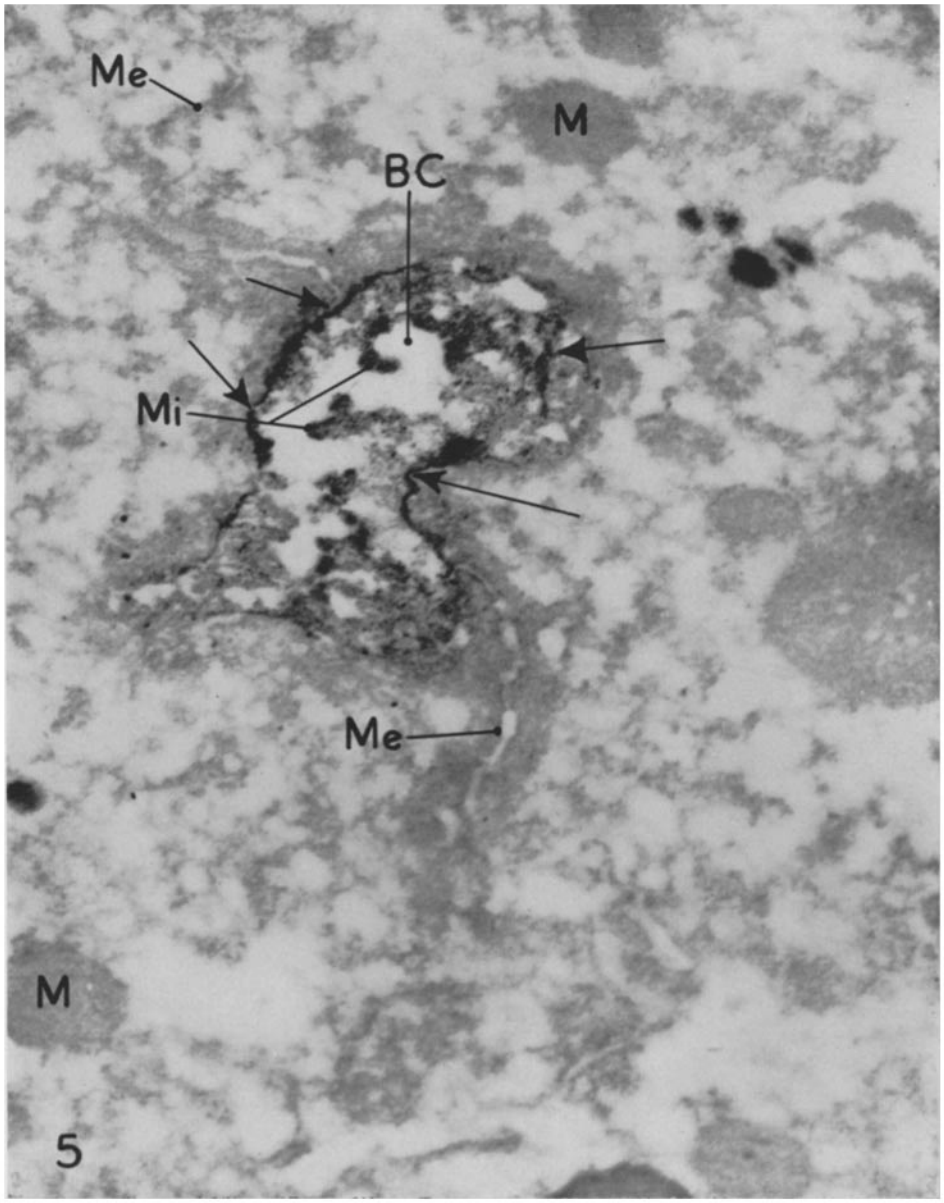
FIG. 4. ATPase. Section through a bile canaliculus (*BC*), showing gradation in deposition of lead phosphate. Arrows indicate stained sections of the plasma membrane. Areas of unstained plasma membrane (*Me*) extend from both sides of the canaliculus. The sinusoid (*S*) shows a part of the space of Disse (*SD*) containing unstained microvilli (*Mi*). Mitochondria (*M*), ribonucleoprotein particles (*R*), a lipide droplet (*L*), and part of a nucleus (*N*) are visible. × 21,000.



(Essner *et al.*: ATPase in plasma membrane)

PLATE 358

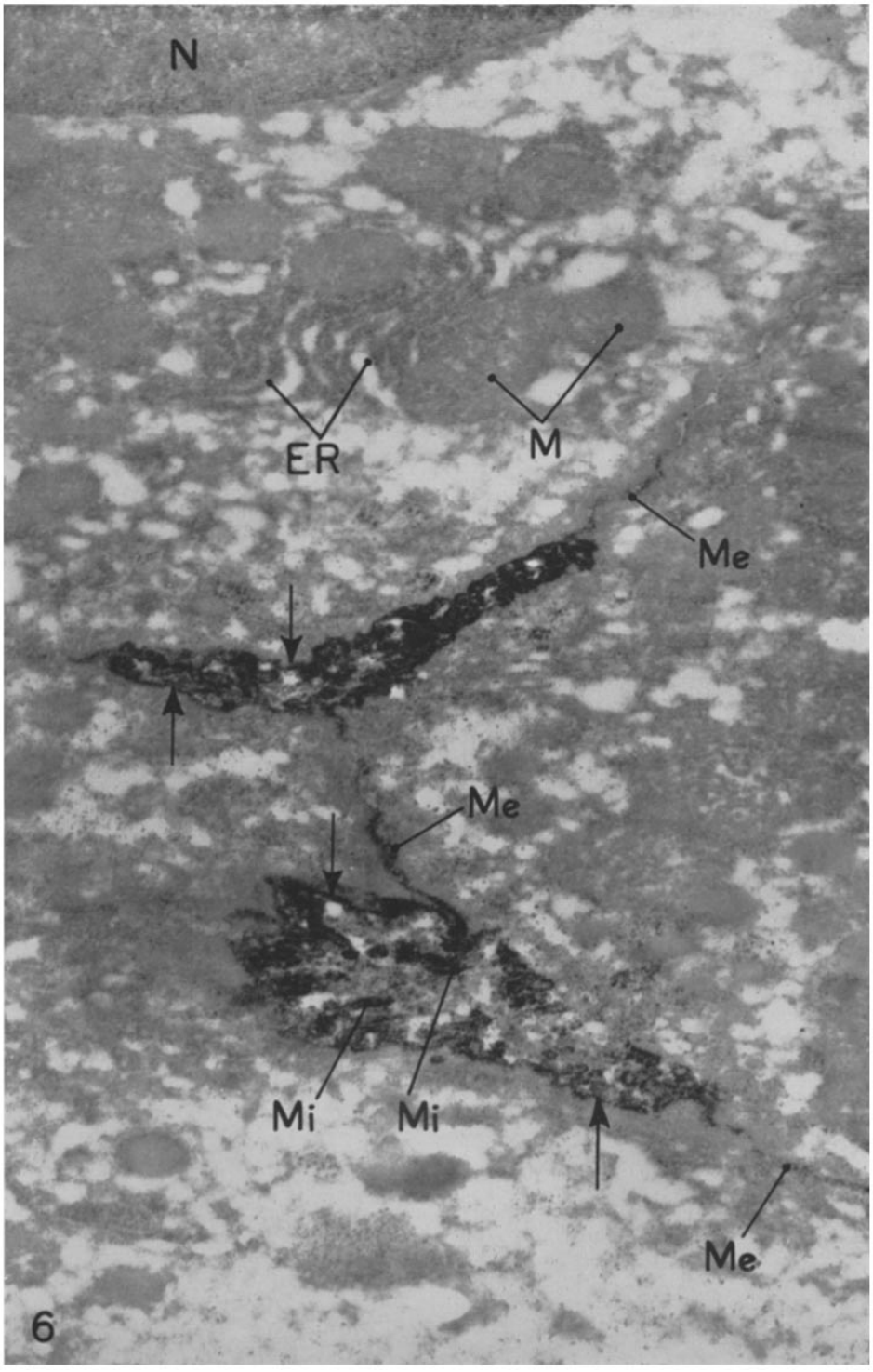
FIG. 5. ATPase. Oblique section through a bile canaliculus (*BC*). Arrows indicate stained areas of the plasma membrane. Unstained membrane (*Me*) outside the canaliculus is evident, as are mitochondria (*M*) and microvilli (*Mi*). The tips of some of the latter appear stained. × 44,000.



(Essner *et al.*: ATPase in plasma membrane)

PLATE 359

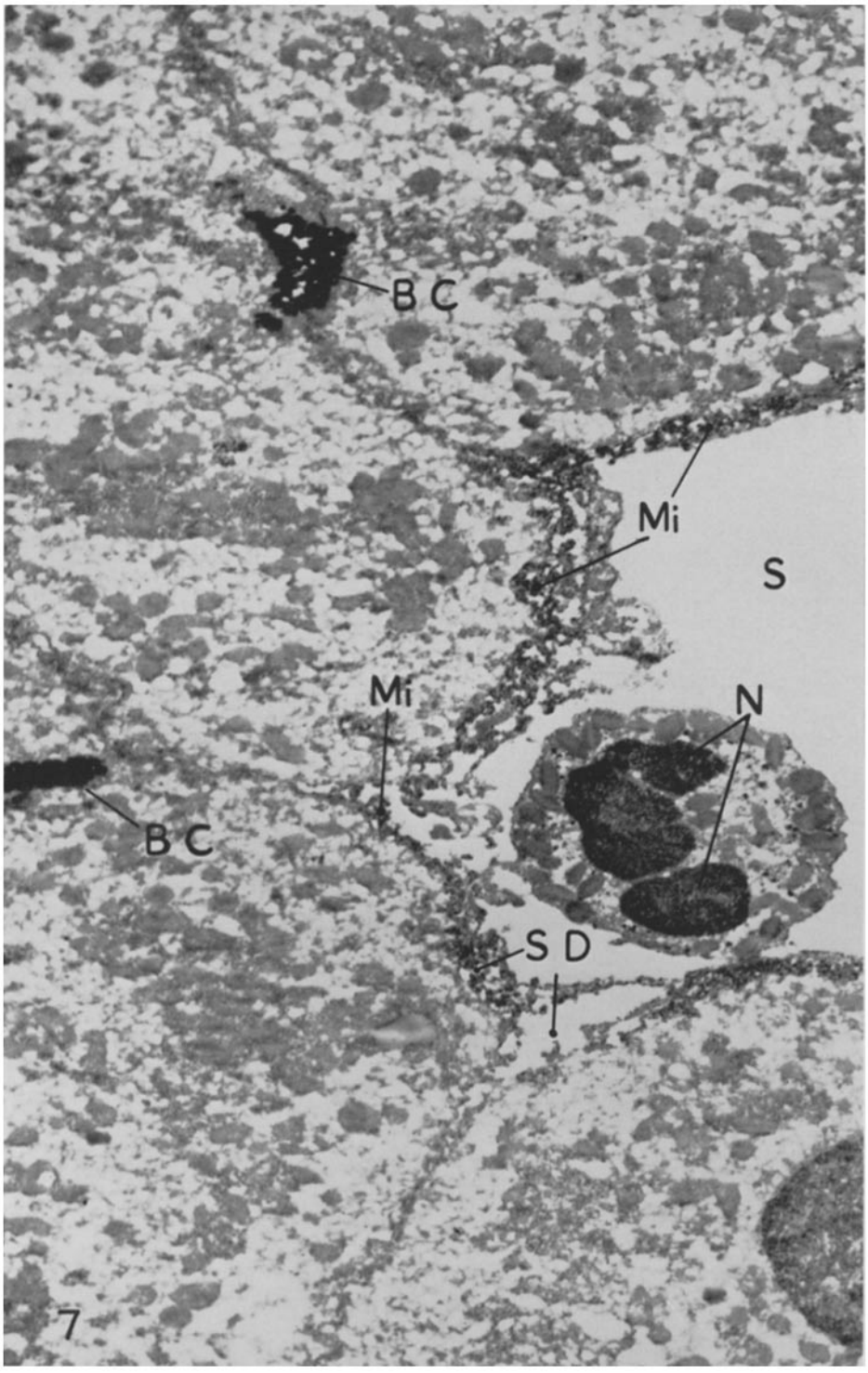
FIG. 6. ATPase. Section through moderately stained bile canaliculus, the lower portion of which is damaged. Arrows indicate the stained plasma membrane in areas with and without microvilli (*Mi*). Some precipitate is also present on adjacent plasma membranes (*Me*) of closely opposed liver cells outside the canaliculus proper. Part of a nucleus (*N*) may be seen at the upper border of the micrograph. Below it, are the ribonucleoprotein granules of the ergastoplasm (*ER*). See text for possible interpretation of the light precipitate in the vicinity of the canaliculus. × 24,000.



(Essner *et al.*: ATPase in plasma membrane)

PLATE 360

FIG. 7. 5-Nucleotidase. Section showing a part of the sinusoid (*S*). Precipitate of lead phosphate is present in the microvilli (*Mi*) in the space of Disse (*SD*). Bile canaliculi (*BC*) are heavily stained. The nucleus of a leucocyte is also stained. × 11,000.



(Essner *et al.*: ATPase in plasma membrane)