Evidence for Microfilament Involvement in Norethandrolone-Induced Intrahepatic Cholestasis

M. James Phillips, MD, Masaya Oda, MD, and Kazuo Funatsu, MD

An experimental study of norethandrolone (NED)-induced intrahepatic cholestasis was made. NED was infused via a portal vein catheter into rat liver *in vivo*, and measurements were made of bile flow. Liver specimens were taken at intervals for light microscopy and for transmission and scanning electron microscopy. Bile-canalicular-rich membrane fractions were prepared. The effects of NED were also examined in isolated hepatocytes in suspension culture. NED infusion induced total cholestasis by 3 hours. Canalicular alterations commonly associated with cholestasis were found in *in vivo* infused liver and in isolated hepatocytes. Pericanalicular microfilament changes were also noted in both, with loss of filament structure and replacement by a granular zone. In isolated canalicular membrane fractions prepared from NED-treated animals, the normal investment of pericanalicular filaments was no longer present. Loss of the bile canalicular ruthenium red surface coat was also noted. In view of the identical findings in isolated hepatocytes and in *in vivo* liver, obstruction and mechanical factors can be excluded as possible causes. The results raise the possibility that the mechanism of NED-induced cholestasis may be related to disaggregation and/or detachment of microfilaments from the canalicular membranes. (Am J Pathol 93:729–744, 1978)

THE ADMINISTRATION of certain clinically used drugs such as the 17-alkyl-substituted steroids can result in intrahepatic cholestasis.[1,2] The mechanism of this type of drug-induced cholestasis is not known. Hypofunction of the cytochrome P450-dependent microsomal biotransformation system causing impairment of bile acid hydroxylation and resulting in altered micelle formation has been proposed [3,4] but does not directly explain the possible reduction in bile-acid–independent bile flow [5] or the functional [6] and morphologic [7] alterations of bile canalicular membranes observed in this form of intrahepatic cholestasis. There is increasing evidence that the bile canalicular membranes are specialized for bile secretion [8–12] and that enzymatic and structural changes in the canalicular membranes could be primary in certain forms of intrahepatic cholestasis.[13–16]

Norethandrolone (NED, 17α-ethyl-19-nortestosterone), one of the 17α-alkylated anabolic steroids, causes impairment of hepatic sulfobromophthalein (BSP) clearance and of maximal bilirubin excretion in patients receiving the drug [17,18] and in healthy human volunteers.[19,20] Similar

From the Department of Pathology, University of Toronto, Toronto, Ontario.

Supported by Grant-in-Aid MT-785 from the Medical Research Council of Canada.

Accepted for publication August 4, 1978.

Address reprint requests to M. James Phillips, MD, Department of Pathology, University of Toronto, Banting Institute, 100 College Street, Toronto, Ontario, Canada M5G 1L5.

0002-9440/78/1211-0729$01.00

changes in biliary excretion of bilirubin or of exogenous dyes and a reduction of bile flow following administration of NED can be produced in certain species of laboratory animals,[21-25] providing a useful experimental model of intrahepatic cholestasis. Electron microscopic studies of the NED-induced cholestatic liver in humans [19,20,26] and in the rat [26,27] revealed dilatation of bile canaliculi with decrease or loss of microvilli and no significant alterations of other liver cell organelles, suggesting that NED might primarily affect the bile canaliculi. These findings are consistent with the bilirubin and exogenous dye transport studies [21-23] that point to a problem in biliary excretion. In the present study, the cholestatic effects of NED were studied in rat liver *in vivo* and in liver cells *in vitro*.

Materials and Methods

Two experimental models were used as follows:

Isolated Liver Cells

Isolated hepatocytes were prepared from Wistar strain rat liver and maintained in Ham's F10 medium containing heat-inactivated horse serum according to the method described previously.[28] The norethandrolone (NED) (a generous gift of G.D. Searle & Co., Chicago) used in this study was shown to be chromatographically pure. NED dissolved in propylene glycol (PG) (J.T. Baker Chemical Co., NJ) was added to 150 ml of the suspension culture medium containing approximately 1.5 g (wet weight) of liver cells at the final concentration of 65 μg of NED per milliliter and 1.5% PG. In the controls, only PG was added to the culture medium at the same concentration. Samples for light and electron microscopy were obtained from each group at 1, 3, and 6 hours cultivation after addition of NED plus PG or PG alone. Five NED-treated and five control cultures were examined.

In Vivo Infused Liver

Female Wistar strain rats (High Oak Ranch, Toronto) weighing from 250 to 320 g were used throughout the study. All were maintained on a standard diet (Rockland mouse and rat diet) and tap water. The NED solution was prepared as follows: NED was dissolved in PG at the concentration of 40 mg/ml and was then added to phosphate-buffered saline (pH 7.4) containing 10% bovine serum albumin (Sigma Chemical Co., St. Louis) at a concentration of 1.2 mg of NED per milliliter. The solution was completely clear. The animals were anesthetized by intraperitoneal injection of sodium pentobarbital (Veterinary Nembutal, Abbott) in a dose of 30 mg/kg of the rat body weight, and the common bile duct was cannulated with a polyethylene tubing (PE 50) for bile collection throughout the experiment. The rate of basal bile flow was determined by weighing the hourly bile collected in a preweighed tube. Two to three hours after cannulation of the bile duct, the liver was infused via a mesenteric vein catheter (PE 10) with the NED solution described above. The infusion was continued at a constant rate of 39 μg of NED/min/100 g of body weight for up to 3 hours using a Harvard infusion pump. In the controls, the liver was infused with the same solution without NED at the infusion rate equivalent to that in the NED-infused groups. Liver tissue samples were taken from each group for light, transmission, and scanning electron microscopy at 2 or 3 hours after the beginning of portal infusion. Ten NED-treated and ten control infused rat livers were examined.

Procedures for Light, Transmission, and Scanning Electron Microscopy

In cultured hepatocytes, an aliquot of the suspension cultures containing separated hepatocytes was fixed for 15 minutes with an equal volume of 2.5% glutaraldehyde (Electron Microscopy Sciences, Fort Washington, Pa) buffered with 0.1 M cacodylate buffer (pH 7.4) and was then centrifuged to form a pellet, as described elsewhere.[29] The pellet obtained was cut into small blocks and was further fixed in 1.25% buffered glutaraldehyde for 45 minutes at 0 C. In the *in vivo* experiment, liver tissue was fixed for electron microscopy by a perfusion technique [30] using fixative composed of 4% formaldehyde freshly prepared from paraformaldehyde (Fisher Scientific Co., New Jersey) and 1.25% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4). The well-perfused areas were cut into small blocks and were further fixed in the same fixative for 15 minutes at 0 C. After being rinsed with the buffer, the small tissue blocks were postfixed with 2% osmic acid in 0.1 M cacodylate buffer (7.4) for 2 hours at 0 C and were processed in the usual manner for routine electron microscopic preparations. Other samples were also subjected to the ruthenium red (RR) stain and the uranyl acetate en bloc stain (UA) technique, as described previously.[47] After dehydration, the tissue blocks were embedded in an Epon–Araldite mixture. Ultrathin sections, stained with lead citrate, were examined in a Philips 300 electron microscope with 60 kV acceleration voltage. One-micron sections were stained with toluidine blue and were used for light microscopic observation. Liver samples for light microscopy were fixed in 10% formalin, processed routinely, and stained with hematoxylin and eosin.

Bile-canaliculus–rich fractions (BC fractions) of liver cell plasma membranes were isolated from NED-infused livers and from control livers by the method described below and were processed for electron microscopy according to the UA and the RR staining procedures.[29]

For scanning electron microscopy, the well-fixed portions of liver tissue subjected to the perfusion fixation technique were cut into strips (approximately 1 × 1 × 3 cu mm). After osmification, the tissue pieces were dehydrated with a graded series of ethanol. The tissue was then frozen under liquid nitrogen and fractured using a precooled razor blade. The fractured pieces were transferred to fresh absolute ethanol, and the ethanol-infiltrated tissues were dried with a Sorvall critical point drying apparatus using CO_2 according to the method described by Humphreys et al.[31] The dried tissue pieces were mounted on an aluminium stud and were coated with gold using an Edwards vacuum coating unit. The specimens were observed in a Cambridge Stereoscan electron microscope with a 20 kV acceleration voltage.

Morphometric Procedures

Morphometric analysis for quantitative assessment of the bile canalicular dilatation caused by NED was performed as described below.

In the isolated liver cells, at least 100 paired hepatocytes (normal and/or dilated canaliculi) were counted by light microscopy from each control and NED-treated culture in 1-μ sections stained with toluidine blue. Three to five blocks from each of 5 controls and of 5 NED-treated cultures at each stage (1-, 3,-, and 6-hour cultivation) were available for the estimation. The results were expressed as the percentage of the numbers of dilated bile canaliculi to the total numbers of paired hepatocytes counted.

From *in vivo* infused livers, three to four blocks from the centrilobular zone and the same number of blocks from portal zones from each of 5 controls and of 5 NED-infused animals were examined by transmission electron microscopy. More than 50 randomly selected bile canaliculi were observed from centrilobular and periportal zones from each animal. The bile canaliculi less than 1 μ in largest diameter and largely filled with microvillous projections in lumen were rendered "normal," while those more than 1 μ in diameter and partially or totally devoid of microvilli were "dilated." The numbers of

dilated bile canaliculi were expressed as the percentage of the total numbers of bile canaliculi observed.

Three BC fractions were isolated from NED-infused or control livers for the morphometric study. Three to four blocks from each BC fraction sample were examined by transmission electron microscopy. More than 50 isolated bile canaliculi were observed from each BC fraction. The dilated bile canaliculi devoid of pericanalicular microfilaments except those around the desmosomes and the tight junctions were counted, and the numbers were expressed as the percentage of the total numbers of the isolated bile canaliculi observed.

Preparation of Canaliculus-Enriched Membrane Fractions

The isolated BC membrane fractions of rat liver plasma membranes were prepared as described in detail elsewhere.[8] The liver was perfused via the portal vein with ice cold physiologic saline, pH 7.4, at 15 cm pressure. The liver was removed and placed in 10 ml ice cold 1 mM sodium bicarbonate, pH 7.5, and was homogenized using a Willems Polytron homogenizer at Speed 7 for 5 to 10 seconds. The homogenate was mixed with bicarbonate to a total volume of 100 ml and was filtered twice through four layers of cotton gauze (Texpack Ltd., Brantford, Ontario) and then through eight layers. The filtered homogenate was centrifuged for 5 minutes at 500*g* followed by 10 minutes at 1000*g*. The supernatant was discarded, and the pellet was resuspended and recentrifuged for 10 minutes at 1000*g*.

Following suspension by gentle homogenization, a volume of sucrose solution (density, 1.26) sufficient to provide a final density of 1.22 was added. Fifteen-milliliter aliquots of this solution were overlayered with 9 ml of sucrose (density, 1.18) and then with 3 ml of sucrose (density, 1.16). Following centrifugation for 60 minutes at 66,000*g* (Rav) using a Beckman 30 fixed-angle rotor, the membrane layers were observed at the 1.22–1.18 interface, which represent pure bile canalicular (BC) membranes (a second membrane fraction at the 1.18–1.16 interface contains predominantly noncanalicular plasma membranes). The BC membrane fraction was washed two more times for 10 minutes using bicarbonate solution. The resultant pellet was the BC membrane fraction used.

Results

Isolated Liver Cells

By light microscopy, 20 to 30% of isolated liver cells in each culture were found to be in pairs, allowing observations of bile canaliculi. In control specimens incubated up to 6 hours, no obvious lumen was seen in most of the paired hepatocytes under the light microscope, and the bile canaliculi of these cells were found to be intact electron microscopically (Figure 1). Morphometrically, only 2.8 ± 0.9% (mean ± SE, N=5) of the paired hepatocytes showed slightly dilated canalicular lumina at 1 hour, 3.2 ± 1.8% (N=5) following 3 hours, and 4.3 ± 2.5% (N=5) following 6 hours of PG administration.

In the NED-treated specimens, round spaces were recognized between some paired hepatocytes 1 hour after administration of NED. Electron microscopy revealed that these spaces between cells correspond to dilated bile canalicular lumina (Figure 2). After 1 hour of NED treatment, 63.2 ±

3.8% (N=5) of the paired hepatocytes showed remarkable canalicular dilatation. There was wide dilatation with loss microvilli in 67.4 ± 3.0% (N=5) of the bile canaliculi at 3 hours and in 66.5 ± 1.9% (N=5) at 6 hours after administration of NED. Hence, the bile canalicular dilatation caused by NED in isolated liver cells was statistically significant ($P < 0.001$) compared with the controls. The smooth endoplasmic reticulum appeared increased in many liver cells. Autophagic vacuoles were occasionally increased in the pericanalicular region. The other liver cell organelles were unchanged. Bleb-like cytoplasmic protrusions were observed over the surface of many liver cells. The pericanalicular microfilamentous network detected around normal bile canaliculi by the UA en block stain (Figure 3) appeared nonfilamentous and granular around the dilated bile canaliculi induced by NED (Figure 4). In the RR-stained control preparations, the tight junctions sealing the bile canaliculi in paired hepatocytes and the canalicular membranes consistently stained positively with RR (Figure 1). The RR-positive surface coat was absent from all the dilated bile canalicular membranes of NED-treated liver cells observed (Figure 2).

In Vivo Infused Liver

The livers of the controls showed no significant alterations by light and electron microscopy. By light microscopy, livers of the NED-treated group also appeared normal in the hematoxylin-and-eosin–stained sections except for canalicular dilatation and some vacuolization in the hepatocytes of the centrilobular zone. Bile ducts and ductules were also unchanged. Electron microscopically, marked bile canalicular ectasia accompanied by reduction or complete loss of canalicular microvilli was noted in the NED-infused livers (Figure 5). Normal bile canaliculi were also occasionally seen near the dilated canaliculi (Figure 5). In the NED-treated group, morphometric analyses showed 73.8 ± 2.8% (mean ± SE, N=5) of the bile canaliculi observed in the centrilobular zone were dilated with partial or total loss of microvilli, and 30.6 ± 1.9% (N=5) canaliculi were similarly affected in the periportal zone. In the controls, only 1.2 ± 0.4% (N=5) canaliculi in the periportal zone were slightly dilated with reduction of microvilli. Diverticula formed from the dilated canaliculi appeared irregularly deformed. Although most of the widened canalicular lumina appeared empty, a few contained fine fibrillar or vesicular material. The junctional complexes remained intact. Autophagic vacuoles including electron-dense amorphous or fibrillar materials as well as smooth-surfaced small vesicles were occasionally notable around the dilated bile and canaliculi. Proliferation or redistribution of the smooth endoplasmic

reticulum was inconsistently found. Mitochondria and other liver cell organelles appeared normal. Cytoplasmic protrusions of various sizes were noted on the sinusoidal liver cell plasma membranes, with concomitant decrease of normal surface microvilli over the protrusions (Figure 6). These protrusions were pinched off from the liver plasma membranes, forming round ball-like structures in the space of Disse and in the sinusoids. They were occasionally seen in gaps between sinusoidal endothelial lining cells.

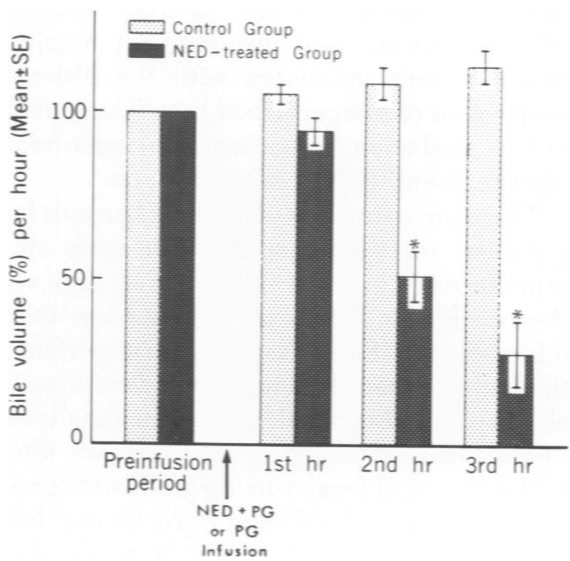
In the UA-stained preparations of the control livers, the microfilamentous network surrounding the bile canaliculi was visualized. Microfilaments also extended into the cores of canalicular microvilli and were attached to the canalicular membranes. In the NED-infused liver preparations, these pericanalicular microfilaments were rarely found around dilated bile canaliculi, and the pericanalicular ectoplasm appeared thickened, electron-dense, and granular. Subplasmalemmal microfilaments appeared condensed at the bases of the protrusions noted on the sinusoidal liver cell surfaces in animals that received NED. Inside the protrusions, the microfilamentous structures were scanty, but electron-dense, granular substances were prominent. By scanning electron microscopy, the bile canaliculi were seen as a chicken-wire–like anastomosing network on the fractured surfaces of the control livers. The bile canalicular channels were straight and almost uniform in their diameter. The canalicular lumens were filled with regular microvillous projections as seen on transmission electron microscopy (Figure 7). In the NED-infused liver preparations, a large part of the bile canalicular system was irregularly dilated, especially in the centrilobular zone, compared with the controls (Figure 8). Canalicular microvilli were lost in the canalicular wall except for those at the marginal ridges, where shortened microvilli were found. In the sinusoids, there was a large number of ball-like structures which originated from the sinusoidal liver cell surface as shown by transmission electron microscopy. In the RR-stained preparations of isolated bile canaliculi (BC fractions) obtained from the NED-infused livers, approximately 50% of the isolated bile canaliculi counted were dilated and devoid of microvilli (Figure 9). No microfilaments were recognized around the canalicular membranes, indicating that they were dissociated from the membranes, except for those attached to the tight junctions and desmosomes. On the other hand, the microfilamentous network was normally attached to the bile canaliculi isolated from the control livers (Figure 10). No dilated bile canaliculi devoid of pericanalicular microfilaments were detected in the control BC fractions.

Bile Flow

Bile flow was significantly affected by the portal infusion of NED compared with controls, as summarized in Text-figure 1. Two and three hours after the beginning of the portal infusion of NED, bile flow (μl/hr/100 g body weight) was significantly reduced by 51.86 ± 6.02% (mean of reduction percentages from 19 determinations [N=19] ± SE) or by 72.40 ± 10.30% (N=7), respectively ($P < 0.001$), compared with that observed in the preinfusion period. In the controls, bile flow rate was not significantly reduced during the portal infusion without NED ($P < 0.001$ [N=10]) and was even increased at 3 hours ($P < 0.01$ [N=5]).

Discussion

The results of this study suggest that the subcellular lesion in the intrahepatic cholestasis produced by NED may be located in the canalicular membrane and its associated microfilaments. Knowledge of cell membranes suggests that certain actin filaments are closely associated with plasma membranes, and a wide variety of contractile functions have been ascribed to these filaments in various nonmuscle cells.[32-35] There is also substantial evidence that certain hepatocellular pericanalicular microfilaments contain actin.[15,29,36-38] It has been speculated that in the liver they provide a motive force for the movement of materials within the hepatocytes.[38] We have suggested that the contractile force exerted by



TEXT-FIGURE 1—Effects of norethandrolone (NED) infusion on bile flow of rats. The volume of bile secreted per hour (μl/hr/100 g body weight) was measured. The graph shows the percentage change of the pre- and post-infusion values. The *asterisk* denotes $P < 0.001$. The NED-treated group received 1.2 mg NED per milliliter plus 3% propylene glycol (PG) in phosphate-buffered saline (pH 7.4) containing 10% bovine serum albumin. Infusion rate, 39 μg NED/min/100 g body weight. Controls received the same infusate as did the NED-treated group, but without NED.

pericanalicular filaments might facilitate bile flow in the canalicular system of the liver.[15,29] Support for this hypothesis was found in experiments using cytochalasin B (CB), an agent which alters actin filament structure and function in many cell types.[39-46] In the liver, CB produced intrahepatic cholestasis which correlated with structural changes in pericanalicular microfilaments.[47,48]

With NED, the earliest changes observed are canalicular ectasia, loss of microvilli, and loss of identifiable microfilaments with their replacement by a granular zone presumed to be derived from altered microfilaments. The finding of almost identical canalicular structural changes in isolated hepatocytes *in vitro* excludes the possibility that obstruction or strictly mechanical factors are involved. The observation that isolated bile canaliculi prepared from animals pretreated with NED are devoid of their normal investment of pericanalicular microfilaments suggests that the attachment of the filaments to the canalicular membrane is weakened by the NED treatment or that the filaments are dissociated and become detached *in vitro* by NED prior to the membrane isolation procedure. The loss of RR staining over the canalicular membranes of both isolated hepatocytes and *in vivo* liver is of interest in view of proposed transmembrane linkages between cytoskeletal elements (microfilaments and microtubules) and surface components.[35] Altered microfilament function is also suggested by the cytoplasmic protrusions noted on the sinusoidal border of the hepatocytes. The process of formation of this type of protrusion has been termed "zeiosis" and is explained by Godman et al [49] on the basis of focal displacement or disruption of subplasmalemmal microfilaments associated with the plasma cell membranes and tonic contraction of nonperturbed microfilaments attached to the plasma membrane, producing herniation of cytoplasmic contents through the weakened segment of the cell membrane.

There are many postulated mechanisms for intrahepatic cholestasis (for a review, see Reference 50). Bile acids are of particular importance in some forms,[51] but it is unlikely that they are important with NED since the canalicular lesions are unlike those found with cholestatic bile acid infusions. Na^+,K^+ATPase may be important in other forms of cholestasis; these are of special interest since functional alterations can occur without electron microscopic alterations in canaliculi in this type of cholestasis.[52] "Pump failure" resulting from primary alterations in canalicular microvilli with loss of local osmotic gradients necessary for bile flow [53] is another mechanism and could be of importance. However, the findings with NED are so similar to those found with cytochalasin B [15,54] that it is possible that both drugs may act in a similar way. The abnormality seems to be located

either in the microfilaments themselves or in the attachment sites in the membrane.[54] Attachment of actin filaments to membranes is implicit in all considerations of filament-mediated membrane movement since a point of insertion is necessary for the contractile filaments to exert a force. If, as we have suggested, the pericanalicular filaments are concerned not only with the structural integrity of the canaliculus but also functionally with maintaining tone and facilitating bile flow in the canalicular system, their altered structure and function could provide a satisfactory explanation for the decrease of flow, dilatation of the canaliculi, and cholestasis observed with NED. Detachment of microfilaments from the BC membrane would result in loss of mechanical cytoskeletal support for this membrane, while loss of contractile function would result in flaccid unsupported canaliculi. Such effects in the widespread canalicular capillary network would not be expected to promote the flow of bile and could explain the reduction and eventual cessation of bile flow as canaliculi became hyperdistended. The loss of the ruthenium red surface coat may itself be contributory to the reduction in bile flow also, since surface glycoproteins also play an important role in cellular secretory processes.[55,56]

References

1. Sherlock S: Biliary secretory failure in man: The problem of cholestasis. Ann Intern Med 65:397–408, 1966
2. Popper H: Cholestasis. Ann Rev Med 19:39–56, 1968
3. Schaffner F, Popper H: Cholestasis is the result of hypoactive hypertrophic smooth endoplasmic reticulum in the hepatocyte. Lancet 2:355–359, 1969
4. Schaffner F: Some observations concerning the molecular biology of cholestasis. Helv Med Acta 37:183–192, 1973
5. Wheeler HO: Secretion of bile. Diseases of the Liver, Fourth edition. Edited by L Schiff. Philadelphia, J. B. Lippincott Co., 1975, pp 87–110
6. Simon FR, Arias IM: Alterations in liver plasma membranes and their possible role in cholestasis. Gastroenterology 62:342–345, 1972
7. Popper H, Schaffner F: Pathophysiology of cholestasis. Hum Pathol 1:1–24, 1970
8. Fisher MM, Bloxam DL, Oda M, Phillips MJ, Yousef IM: Characterization of rat liver cell plasma membranes. Proc Soc Exp Biol Med 150:177–184, 1975
9. Gregory DH, Vlahcevic ZR, Schatzki P, Swell L: Mechanism of secretion of biliary lipids. I. Role of bile canalicular and microsomal membranes in synthesis and transport of biliary lecithin and cholesterol. J Clin Invest 55:105–114, 1975
10. Boyer JL, Reno D: Properties of $Na^{++}K^{+}$-activated ATPase in rat liver plasma membranes enriched with bile canaliculi. Biochim Biophys Acta 401:59–72, 1975
11. Yousef IM, Bloxam DL, Phillips MJ, Fisher MM: Liver cell plasma membrane lipids and the origin of biliary phospholipid. Can J Biochem 53:989–997, 1975
12. Oda M, Yousef IM, Phillips MJ: Cytochemistry of bile canalicular membranes: Effects of digestive enzymes and EDTA on the canalicular surface coat, microfilaments and membrane-bound enzymes. Gastroenterology 69:A-50/850, 1975
13. Simon FR, Arias IM: Alteration of bile canalicular enzymes in cholestasis: A possible cause of bile secretory failure. J Clin Invest 52:765–775, 1973
14. Ronchi G, Desmet VJ: Histochemical study of gamma glutamyl transpeptidase

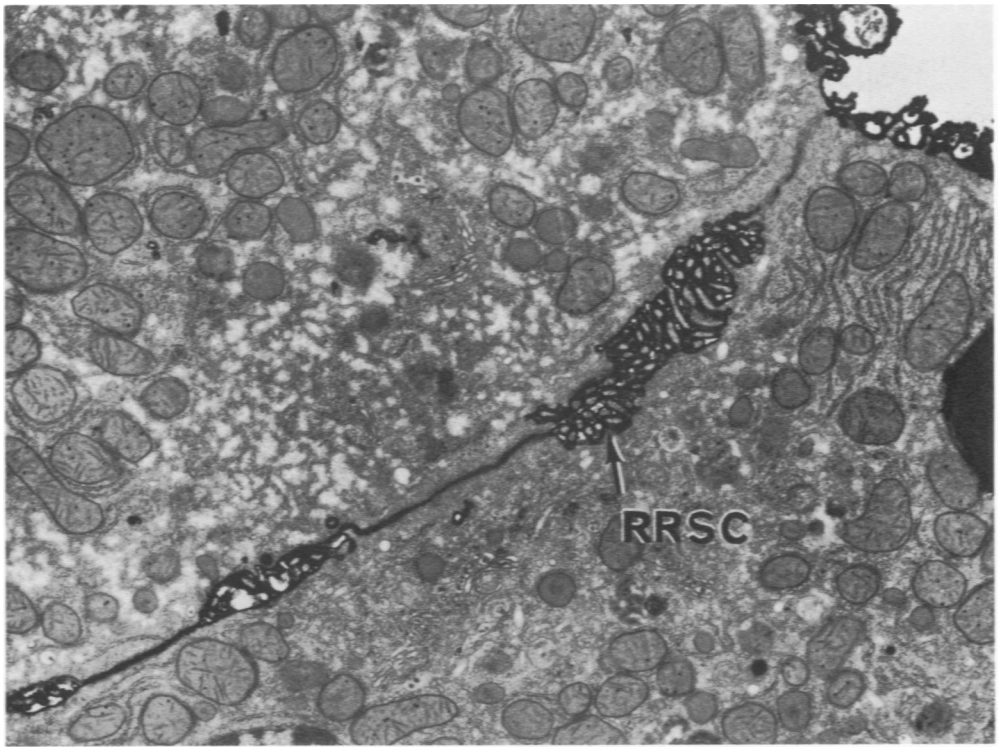
(GGT) in experimental intrahepatic and extrahepatic cholestasis. Beitr Path 150:316–321, 1973
15. Phillips MJ, Oda M, Mak E, Fisher MM: Bile canalicular structure and function. Jaundice. Edited by CA Goresky, MM Fisher. New York, Plenum Press, 1975, pp 367–382
16. Miyai K, Mayr WW, Richardson AL: Acute cholestasis induced by lithocholic acid in the rat: A freeze-fracture replica and thin section study. Lab Invest 32:527–535, 1975
17. Heaney RP, Whedon GD: Impairment of hepatic bromsulphalein clearance by two 17-substituted testosterones. J Lab Clin Med 52:169–175, 1958
18. Dunning MF: Jaundice associated with norethandrolone (Nilevar) administration. JAMA 167:1242–1243, 1958
19. Schaffner F: Effect of anabolic steroids in man. Therapeutic Agents and the Liver. A symposium held on 1 June, 1964 at the Royal Free Hospital, London. Edited by N McIntyre, S Sherlock, Oxford, Blackwell Scientific Publications, 1965, pp 99–117
20. Orlandi F, Jezequel AM: On the pathogenesis of the cholestasis induced by 17 alkylated steroids: Ultrastructural and functional changes of the liver cells during the treatment. Rev Intern Hepatol 16:331–333, 1966
21. Arias IM: Effects of a plant acid (icterogenin) and certain anabolic steroids on the hepatic metabolism of bilirubin and sulfobromophthalein (BSP). Ann NY Acad Sci 104:1014–1025, 1963
22. Hargreaves T, Lathe GH: Inhibitory aspects of bile secretion. Nature 200:1172–1176, 1963
23. Hargreaves T: Cholestatic drugs and bilirubin metabolism. Nature 206:154–156, 1965
24. Lennon HD: Effect of several anabolic steroids on sulfobromophthalein (BSP) retention in rabbits. Steroids 5:361–373, 1965
25. Despopoulos A: Excretion of sulfobromophthalein by perfused rat liver as influenced by steroidal hormones. J Pharmacol Exp Ther 173:37–42, 1970
26. Schaffner F, Popper H, Perez V: Changes in bile canaliculi produced by norethandrolone: Electron microscopic study of human and rat liver. J Lab Clin Med 56:623–628, 1960
27. Goldfischer S, Arias IM, Essner E, Novikoff AB: Cytochemical and electron microscopic studies of rat liver with reduced capacity to transport conjugated bilirubin. J Exp Med 115:467–474, 1962
28. Jeejeebhoy KN, Ho J, Greenberg GR, Phillips MJ, Bruce-Robertson A, Sodtke U: Albumin, fibrinogen and transferrin synthesis in isolated rat hepatocyte suspensions: A model for the study of plasma protein synthesis. Biochem J 146:141–155, 1975
29. Oda M, Price VM, Fisher MM, Phillips MJ: Ultrastructure of bile canaliculi, with special reference to the surface coat and the pericanalicular web. Lab Invest 31:314–323, 1974
30. Fahimi HD: Perfusion and immersion fixation of rat liver with glutaraldehyde. Lab Invest 16:736–750, 1967
31. Humphreys WH, Spurlock BO, Johnson JS: Critical point drying of ethanol-infiltrated, cryofractured biological specimens for scanning electron microscopy. Scanning Electron Microscopy. Part I. Chicago, IIT Research Institute, 1974, pp 275–282
32. Steck TL: The organization of the proteins in the human red blood cell membrane: A review. J Cell Biol 62:1–19, 1974
33. Singer SJ: The molecular organization of membranes. Ann Rev Biochem 43:805–833, 1974
34. Mooseker MS, Tilney LG: Organization of an actin filament-membrane complex: Filament popularity and membrane attachment in the microvilli of intestinal epithelial cells. J Cell Biol 67:725–743, 1975

35. Nicolson GL: Transmembrane control of the receptors on normal and tumor cells. I. Cytoplasmic influence over surface components. Biochim Biophys Acta 457:57–108, 1976
36. Holborow EJ, Trenchev PS, Dorling J, Webb J: Demonstration of smooth muscle contractile protein antigens in liver and epithelial cells. Ann NY Acad Sci 254:489–504, 1975
37. Neifakh SA, Vasilets IM: Actomyosin-like protein in outer membrane of liver cells. Fed Proc 24:T561–T562, 1965
38. French SW, Davies PL: Ultrastructural localization of actin-like filaments in rat hepatocytes. Gastroenterology 68:765–774, 1975
39. Spooner BS, Yamada KM, Wessells NK: Microfilaments and cell locomotion. J Cell Biol 49:595–613, 1971
40. Schroeder TE: The role of "contractile ring" filaments in dividing Arbacia egg. Biol Bull 137:413–414, 1969
41. Malawista SE, Gee JBL, Bensch KG: Cytochalasin B reversibly inhibits phagocytosis: Functional, metabolic, and ultrastructural effects in human blood leukocytes and rabbit alveolar macrophages. Yale J Biol Med 44:286–300, 1971
42. Manasek FJ, Burnside B, Stroman J: The sensitivity of developing cardiac myofibrils to cytochalasin-B. Proc Natl Acad Sci USA 69:308–312, 1972
43. Orci L, Gabbay KH, Malaisse WJ: Pancreatic beta-cell web: Its possible role in insulin secretion. Science 175:1128–1130, 1972
44. Spooner BS, Wessells NK: Effects of cytochalasin B upon microfilaments involved in morphogenesis of salivary epithelium. Proc Natl Acad Sci USA 66:360–364, 1970
45. Wrenn JT, Wessells NK: Cytochalasin B: Effects upon microfilaments involved in morphogenesis of estrogen-induced glands of oviduct. Proc Natl Acad Sci USA 66:904–908, 1970
46. Yamada KM, Spooner BS, Wessells NK: Axon growth: Roles of microfilaments and microtubules. Proc Natl Acad Sci USA 66:1206–1212, 1970
47. Phillips MJ, Oda M, Mak E, Fisher MM, Jeejeephoy KN: Microfilament dysfunction as a possible cause of intrahepatic cholestasis. Gastroenterology 68:48–58, 1975
48. Jahn W: Similarity between the effect of experimental congestion of the isolated perfused rat liver and the action of cytochalasin B. Naunyn-Schmiedeberg's Arch Pharmacol 278:431–434, 1973
49. Godman GC, Miranda AF, Deitch AD, Tananbaum SW: Action of cytochalasin D on cells of established lines. III. Zeiosis and movements at the cell surface. J Cell Biol 64:644–667, 1975
50. Phillips MJ, Oda M, Funatsu K: Ultrastructure of cholestasis. Toxic Injury of the Liver. Edited by E Farber, MM Fisher. New York, M. Dekkar Co., Inc., 1978
51. Boyer JL, Layden TJ, Hruban Z: Mechanisms of cholestasis: Taurolithocholate alters canalicular membrane composition, structure and permeability. Membrane Alterations as Basis of Liver Injury. Edited by H Popper, L Bianchi, W Reutter. Lancaster, England, MTP Press, 1977, p 353
52. Simon FR, Sutherland E, Accatino L, Vial J, Mills D: Studies on drug induced cholestasis: Effect of ethinyl estradiol on hepatic bile acid receptors and (NA^+-K^+)-ATPase. Bile Acid Metabolism in Health and Disease. Edited by G Paumgartner, A Stiehl. Lancaster, England, MTP Press, 1977, p 133
53. Erlinger S: Cholestasis: Pump failure, microvilli defect, or both? Lancet 1:533–534, 1978
54. Oda M, Phillips MJ: Bile canalicular membrane pathology in cytochalasin B-induced cholestasis. Lab Invest 37:350–356, 1977
55. Rennke HG, Cotran RS, Venkatachalam MA: Role of molecular charge in glomerular permeability: Tracer studies with cationized ferritins. J Cell Biol 67:638–646, 1975
56. Brenner BM, Bohner MP, Bayliss C, Deen WM: Editorial review: Determinants of

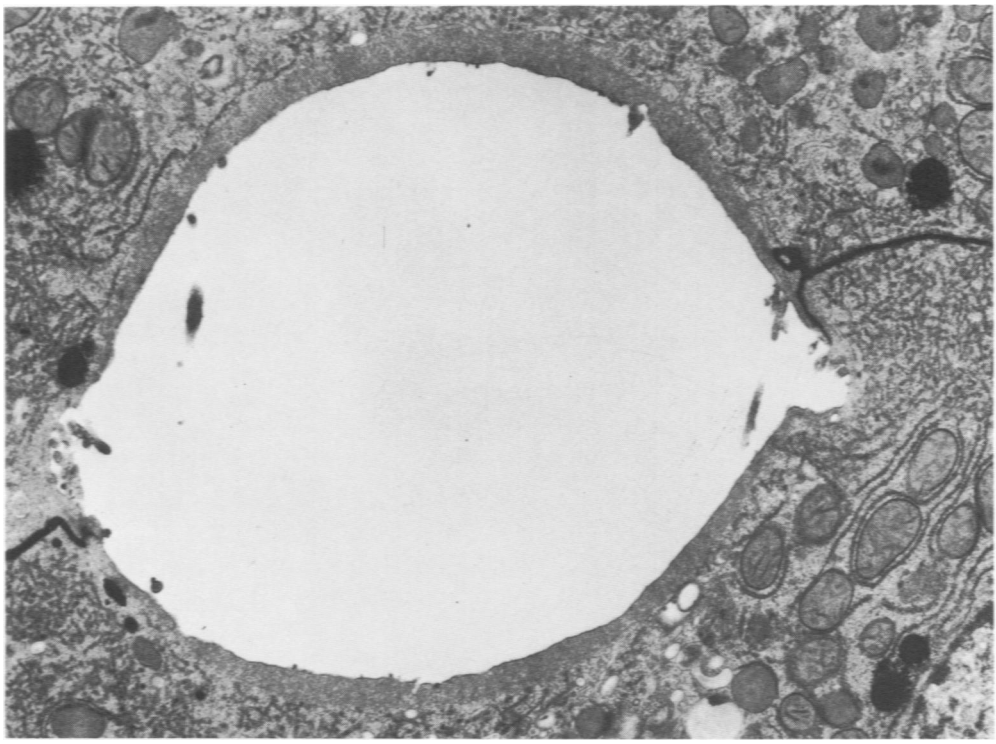
glomerular permselectivity: Insights derived from observations in vivo. Kidney Int 12:229–237, 1977

Acknowledgments

The authors express thanks to Dr. K. N. Jeejeebhoy for providing the isolated hepatocytes and to Drs. M. M. Fisher and I. Yousef for assistance with the liver plasma membrane preparations.

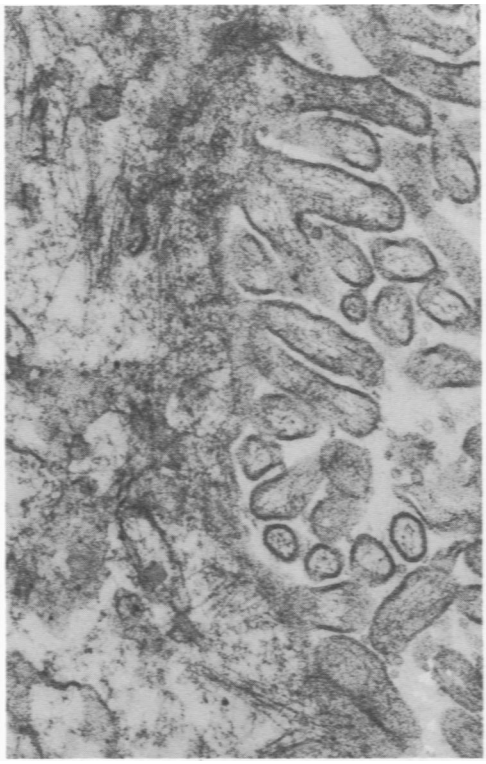


1



2

Figure 1—Isolated hepatocytes from control group following 3 hours of incubation, showing continuous borders of two liver cells. Note the ruthenium-red-positive surface coat (*RRSC*), which appears as a black deposit on the sinusoidal and canalicular membrane surfaces. Note also that the ultrastructural cytology of these cells is normal. (Ruthenium red, × 13,300) **Figure 2**—Isolated hepatocytes from NED-treated group following 3 hours of incubation, showing contiguous borders of two liver cells. There is marked canalicular dilation and loss of microvilli. The RRSC is lost from the canalicular membrane but is still noted over the lateral and sinusoidal plasma membranes. Note also the widened granular-appearing ectoplasmic zone around the canaliculus. This is "morphologic cholestasis *in vitro*." The remainder of the cellular organelles appear normal. (Ruthenium red, × 13,300)



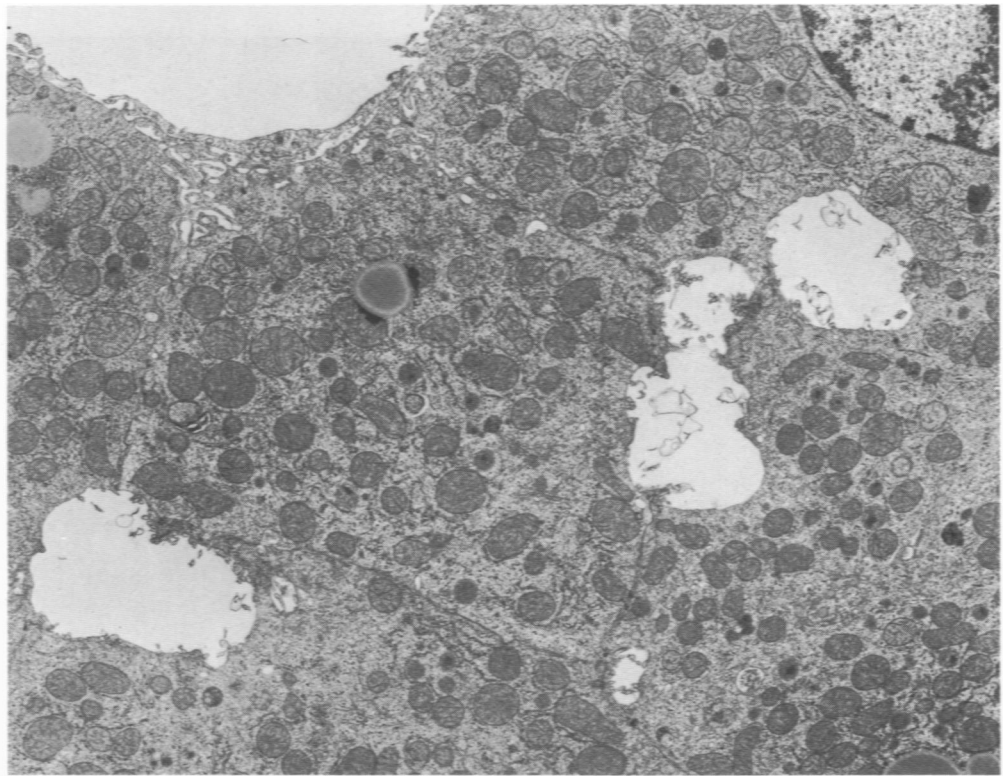
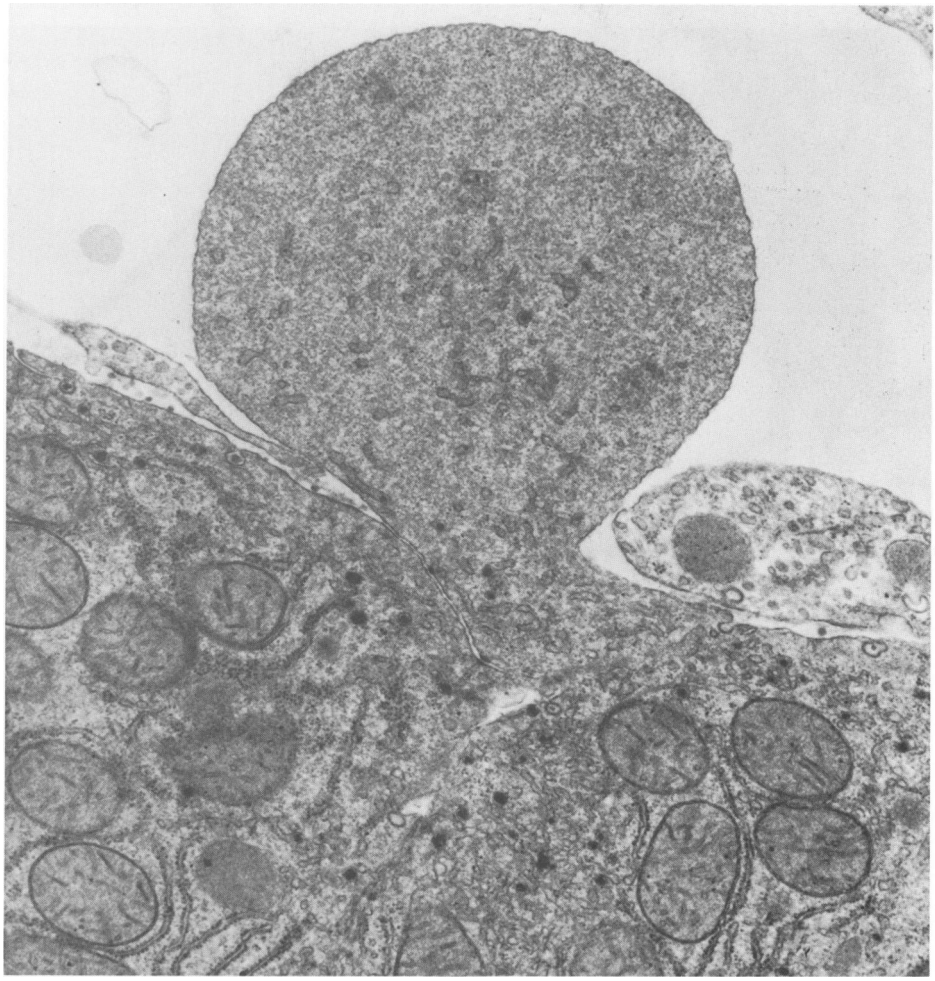
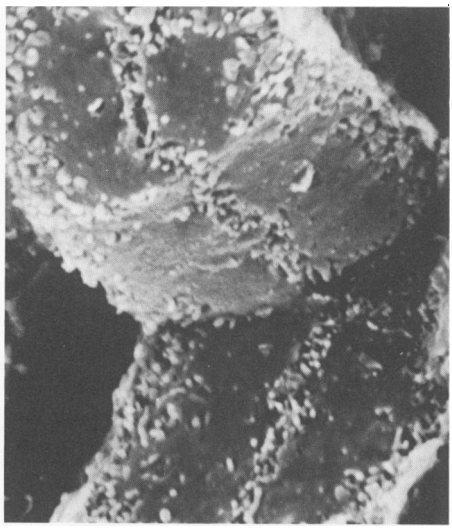


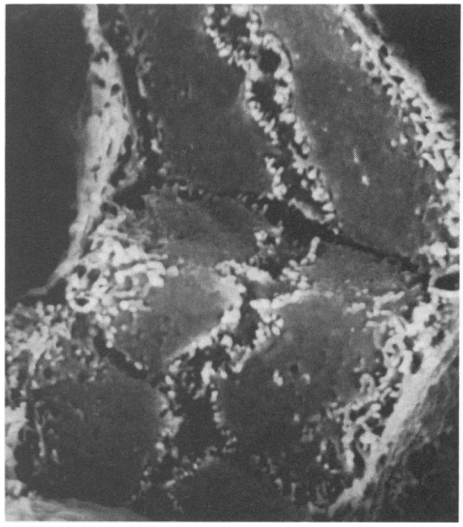
Figure 3—Edge of bile canaliculus from normal control isolated hepatocytes. Many microvilli project into the canalicular lumen which is situated on the *right*. Note the abundance of microfilaments in the cores of microvilli and in the pericanalicular organelle-free ectoplasmic zone of the hepatocyte. (Uranyl acetate, en bloc stain, × 56,000) **Figure 4**—Edge of dilated bile canaliculus from NED-treated isolated hepatocytes. Microvilli are absent. The ectoplasmic zone is widened and filled with small granules, presumably derived from microfilaments. Filaments cannot be identified. (Compared with Figure 3.) (Uranyl acetate, en bloc stain, × 56,000) **Figure 5**—*In vivo* rat liver following 3 hours of NED infusion. Three markedly dilated and one normal bile canaliculus are shown. The dilated canaliculi are nearly devoid of microvilli except for those near the terminal ridges. One diverticulum is shown. The remainder of the ultrastructure of these cells is normal. (Lead citrate, × 6300)



6



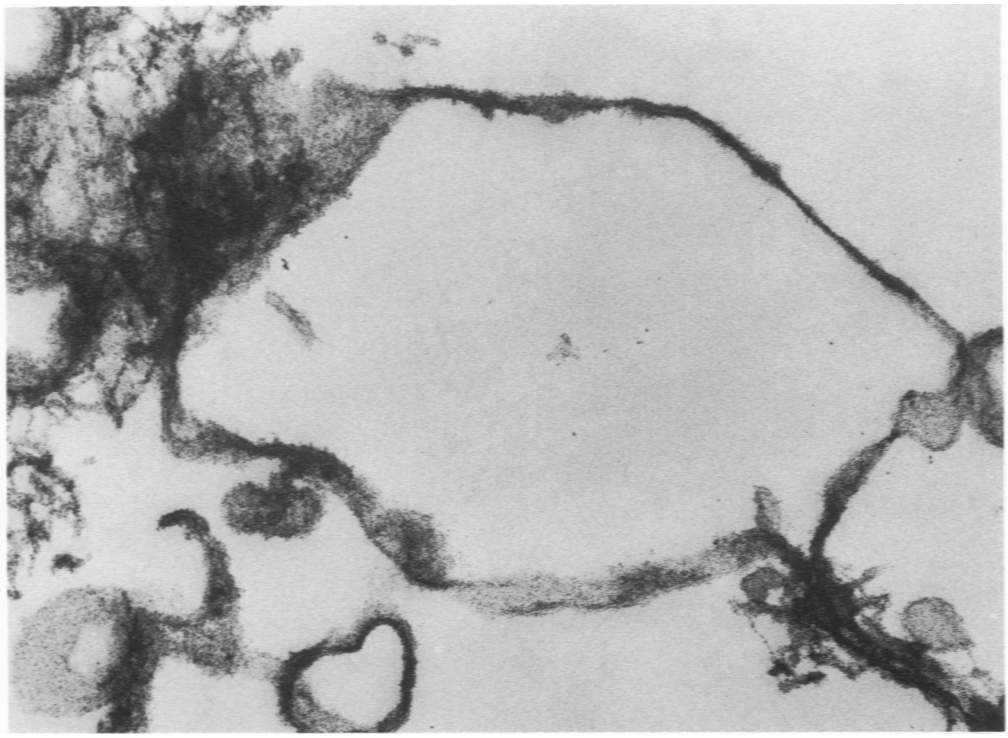
7



8

Figure 6—*In vivo* rat liver following 3 hours of NED infusion. Note the cytoplasmic protrusion which herniates between the Kupffer cells. Microvilli are absent over the protrusion. (Lead citrate, × 10,000) **Figure 7**—*In vivo* rat liver following 3 hours of infusion in control animal. The bile canalicular network is well shown. Microvilli fill the bile canalicular lumina. Scanning electron micrograph. (× 9750) **Figure 8**—*In vivo* rat liver following 3 hours of infusion with NED. The bile canalicular network is dilated and microvilli are reduced. Scanning electron micrograph. (× 9750)

9



10

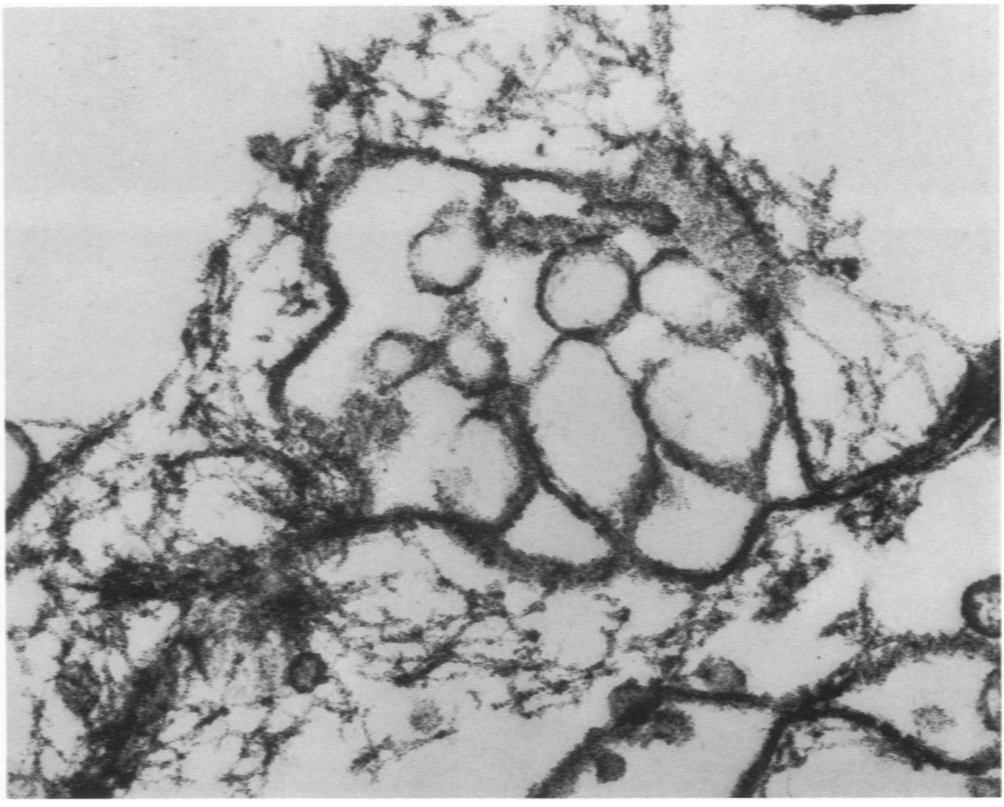


Figure 9—Isolated bile canaliculus from *in vivo* NED-infused rat liver. Note the absence of microfilaments around the entire perimeter of the canaliculus. The only filaments are those in association with portions of the junctional complex. Note also the loss of microvilli and the RRSC. (Ruthenium red, × 67,500) **Figure 10**—Isolated bile canaliculus from *in vivo* infused control rat liver. Note the intact canalicular structure with microvilli, RRSC, and pericanalicular network of microfilaments. (Compare with Figure 9.) (Ruthenium red, × 67,500)