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Immunocytochemical localization of γ-glutamyltransferase in induced hyperplastic nodules of rat liver

(bile canaliculi/carcinogenesis/electron microscopy/immunocytochemistry/plasma membrane)

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ABSTRACT **The immunocytochemical localization of γ-glutamyltransferase [(5-glutamyl)-peptide:amino-acid 5-glutamyltransferase, EC 2.3.2.2; γ-GluTase] was demonstrated in hyperplastic liver induced by the carcinogen 2-acetylaminofluorene (2-AAF). The method used a specific antiserum and protein A-horseradish peroxidase and permitted visualization of antigenic sites at both the light and electron microscopic levels. Electron microscopy revealed deposits of 3,3′-diaminobenzidine (DAB) reaction product in the plasma membranes of (*i*) hyperplastic cells, (*ii*) bile canaliculi, (*iii*) endothelial cell membranes, and (*iv*) lymphocytes. The so-called ATPase activity was localized in the plasma membrane in bile canaliculi and in endothelial cells; the hyperplastic cells show marked variability in the levels of this activity.**

Increased activity of γ-glutamyltransferase [(5-glutamyl)-peptide:amino-acid 5-glutamyltransferase, EC 2.3.2.2; γ-GluTase] has been observed frequently in biochemical studies of chemically induced transitions of hepatocytes from normal to hyperplastic and neoplastic states. Elevated activities of this enzyme in rat and mouse liver have been reported by Fiala *et al.* (1, 2) and by Cameron *et al.* (3) after carcinogen feeding; by Laishes *et al.* in cultured rat hepatic cells obtained from induced premalignant and malignant lesions (4); and by Vanderlaan *et al.* in suspensions of liver cells from rats fed the carcinogen 2-acetylaminofluorene (2-AAF) (5). Busachi *et al.* (6) also demonstrated increased γ-GluTase activity in biopsies of human liver affected by alcoholism, cirrhosis, or hepatitis with cholestasis. Increased γ-GluTase activity has been reported also as an indicator of neoplastic change occurring in the human gastrointestinal tract (7) and in elevated serum levels in humans with hepatoma or alcoholic hepatitis (8). However, γ-GluTase has not been associated consistently with induced hepatocarcinogenesis (9, 10), and its role, whether elevated or reduced, has not been defined sufficiently as a reliable indicator of tumorigenesis.

In none of these studies has γ-GluTase localization been demonstrated by electron microscopy.

In the study reported here, we used the immunocytochemical method of Spater *et al.* (11), which is valid for light microscopy and electron microscopy. γ-GluTase-positive cells of 2-AAF-fed rats showed the reaction product in plasma membranes of hyperplastic cells, endothelial cells, and lymphocytes.

MATERIALS AND METHODS

Materials. Protein A and protein A-horseradish peroxidase (protein A-HRP) were purchased from E-Y Laboratories (San Mateo, CA); 3,3′-diaminobenzidine (DAB), from Sigma; and glutaraldehyde, from Ladd Research Industries (Burlington, VT). Other reagents used were of analytical grade.

Animal Preparation. Male Sprague–Dawley rats were fed a regimen of 2-AAF as described by Stout and Becker (12) to induce the development of hyperplastic liver.

Enzyme and Antisera. Purified γ-GluTase was prepared from rat kidney by using the papain digestion method of Inoue *et al.* (13), and enzyme activity was measured by the procedure of Orlowski and Meister (14). Antiserum against purified γ-GluTase was produced in New Zealand White rabbits as described by Horiuchi *et al.* (15). The antibody, seen as a single precipitin line upon double immunodiffusion, inhibited the transferase activity of the purified enzyme by 55%. Control serum was obtained from nonimmunized animals. γ-GluTase localization was demonstrated by the immunocytochemical procedure of Spater *et al.* (11).

Preparation of Tissue. Male Sprague–Dawley rats were prepared in the manner described by Stout and Becker (12) and weighed between 400 and 500 g at the time the livers were perfused for this study. The animals were anesthetized with ether, and the livers were perfused through the portal vein in a two-step process with (*i*) 0.9% saline at 20°C for 2.5 min and (*ii*) 0.5% glutaraldehyde in PS buffer (0.1 M phosphate buffer, pH 7.4/5% sucrose) at 20°C for 15 min. Both solutions were administered at the rate of 7.5 ml/min. The livers were removed and transferred to PS buffer at 5°C and cut into 2- to 3-mm slices. Areas were selected for sectioning at 60–80 μm on an Oxford Vibratome (Ted Pella, Tustin, CA) into cold PS buffer. All sections were rinsed twice (15 min each rinse) in buffer and stored overnight at 4°C. This removes residual glutaraldehyde.

Immunocytochemical Procedure. γ-GluTase localization was demonstrated by the immunocytochemical procedure of Spater *et al.* (11) with slight modification. After overnight storage in buffer, all sections were immersed in PS buffer containing 10 mg of ovalbumin and 10 mg of L-lysine per ml and were agitated gently every 10 min for 1.5 hr at 50°C. This was followed by two 10-min rinses in PS buffer at 20°C. All subsequent procedures (except for the overnight incubations in specific antiserum at 4°C) were carried out at 20°C.

The sections were divided into five groups and treated as summarized (see Table 1).

After the outlined procedure, all the groups of sections were rinsed twice (10 min each time) in PS buffer, transferred to Tris·HCl buffer (0.1 M Tris·HCl, pH 7.6/5% sucrose) at 20°C for two additional 10-min rinses, and transferred to DAB-containing medium. The DAB incubation procedure was performed as described (11).

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Abbreviations: 2-AAF, 2-acetylaminofluorene; DAB, 3,3′-diaminobenzidine; γ-GluTase, γ-glutamyltransferase; protein A-HRP, protein A-horseradish peroxidase.

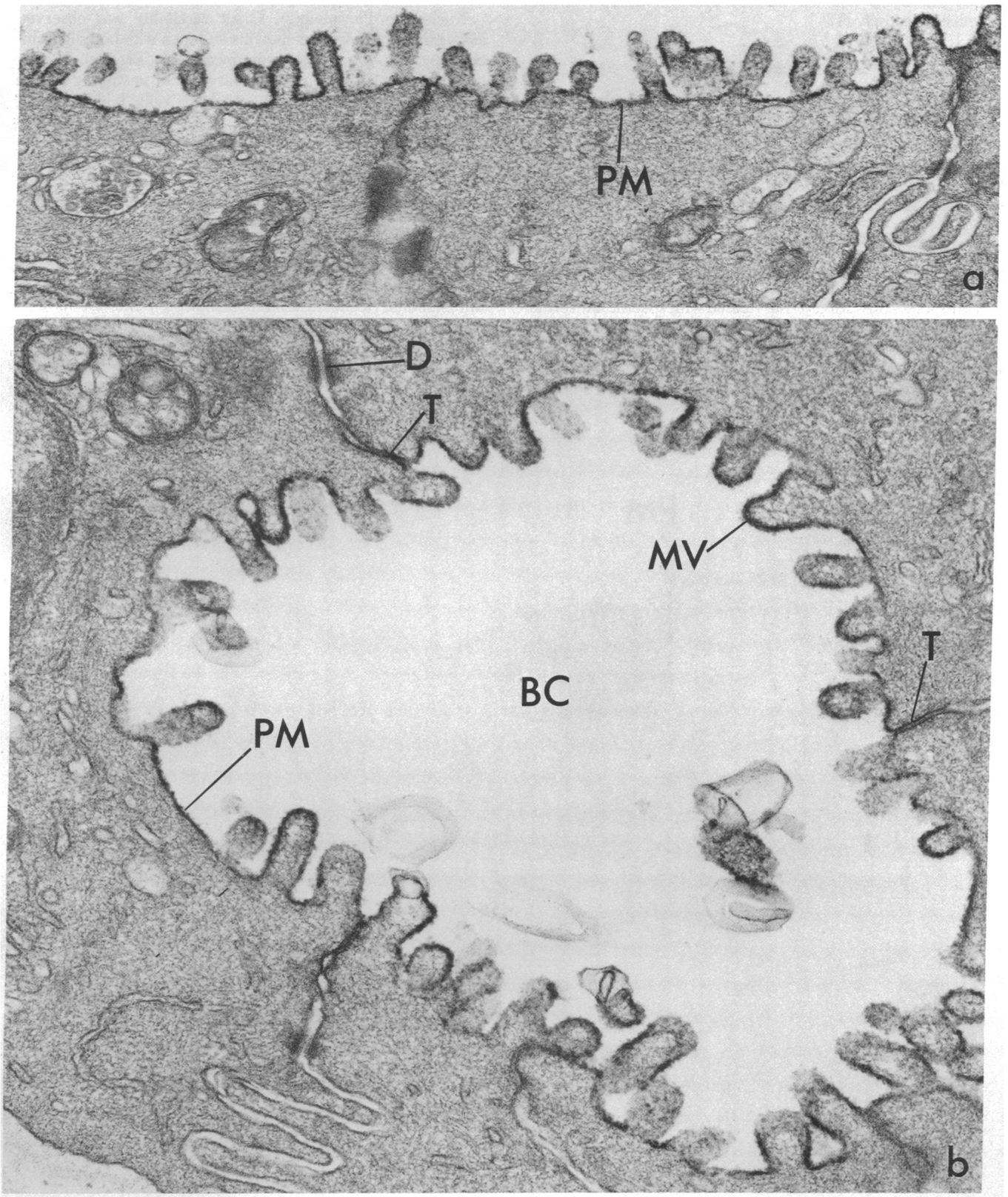

FIG. 1. Electron micrographs. Vibratome sections treated with protein A-HRP after incubation with antiserum specific for γ-GluTase. Thin sections were stained with lead ions. (*a*) Portion of a hyperplastic cell showing reaction product in the plasma membrane (PM). (×42,500.) (*b*) Area shows a bile canaliculus (BC) with reaction product in the plasma membrane (PM) of the microvilli (MV). Desmosomes (D) are negative. (×25,500.) See *Discussion* for explanation of terms.

Determination of "ATPase" Activity. The method of Wachstein and Meisel (16) was used to demonstrate the so-called ATPase activity; in fact, the method demonstrates nucleoside phosphatase activity (17).

RESULTS

γ-GluTase. Antigenic sites were observed best with the 1:10 dilution of specific antiserum. DAB reaction product was seen as electron-dense deposits coating the entire surface of the plasma membranes of hyperplastic cells (Fig. 1*a*), bile canaliculi (Fig. 1*b*), and endothelial cells (Fig. 2*a*); lymphocytes also showed reaction product on the plasma membrane (not illustrated; see ref. 18).

No other organelles showed any reaction product (Figs. 1*a* and *b*, 2*a*, and 3*a*). No evidence of diffusion of reaction product (11) was seen.

Control sections (Table 1) not treated with specific antiserum showed no reaction product. Other control sections, treated with specific antiserum but followed by protein A treatment prior to exposure to protein A-HRP, also had no reaction product. Finally, controls initially exposed to protein A to block spurious staining of endogenous IgG (11) prior to incubation with specific antiserum showed no loss of reaction product due to specific antibody binding.

Nucleoside Phosphatase. Great variability was observed among the hyperplastic cells. This was seen with both light microscopy (Fig. 3*b*) and electron microscopy (Fig. 3*a*). The so-called ATPase reaction product was present in varying degree in the plasma membrane of hyperplastic cells, bile canaliculi, and endothelial cells.

DISCUSSION

The method of Spater *et al.* (11), which was initiated with the proximal convolution of the renal tubule of the rat, has been extended significantly by this report. We studied hyperplastic nodules induced in rat liver by the carcinogen 2-AAF. In both the proximal convolution cells and hyperplastic hepatic cells, the immunocytochemical method showed γ-GluTase to be localized to the plasma membranes. The same localization was observed in the hepatic endothelial cells and in lymphocytes.

We have used the term "hyperplastic cell" with the understanding that firm evidence is lacking that the cells develop

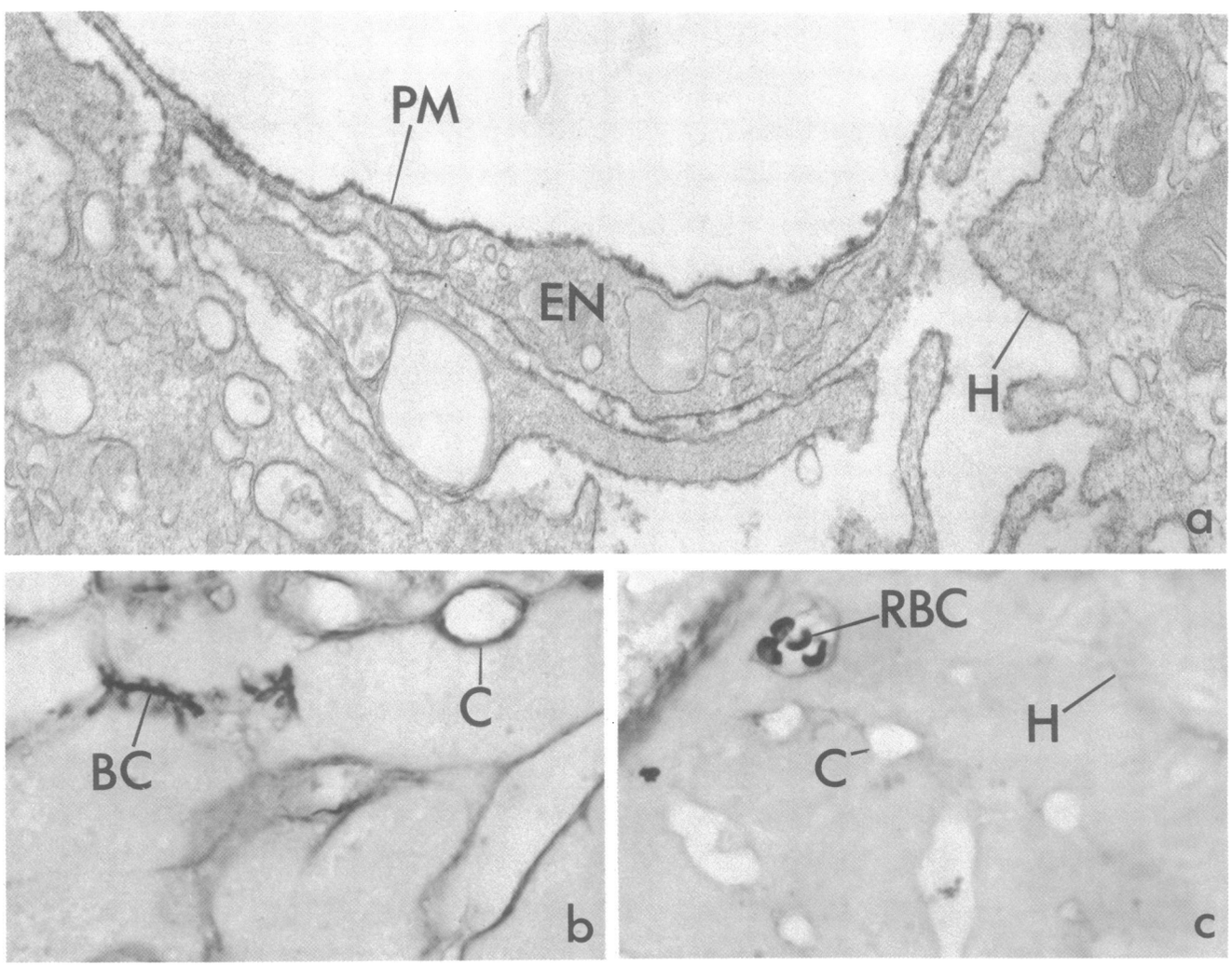

FIG. 2. (*a*) Area of an endothelial cell (EN) with a heavy deposit of reaction product on the luminal surface of the plasma membrane. Less reaction product is seen on its contraluminal membrane and on the underlying membrane of a hyperplastic cell (H). (×36,500.) (*b*) Light micrograph of an Epon-embedded 1-μm-thick Vibratome section treated with protein A-HRP after incubation with antiserum specific for γ-GluTase. Reaction product is seen in the plasma membranes of a capillary (C), a hyperplastic cell (H), and in bile canaliculi (BC). (×1,000.) (*c*) Light micrograph of an Epon-embedded 1-μm thick section of a control treated with protein A-HRP after incubation with nonimmune serum. Only erythrocytes (RBC) show reaction product (from endogenous hemoglobin). All the other structures, positive in Fig. 2*c*, lack reaction product in control. (×1,000.)

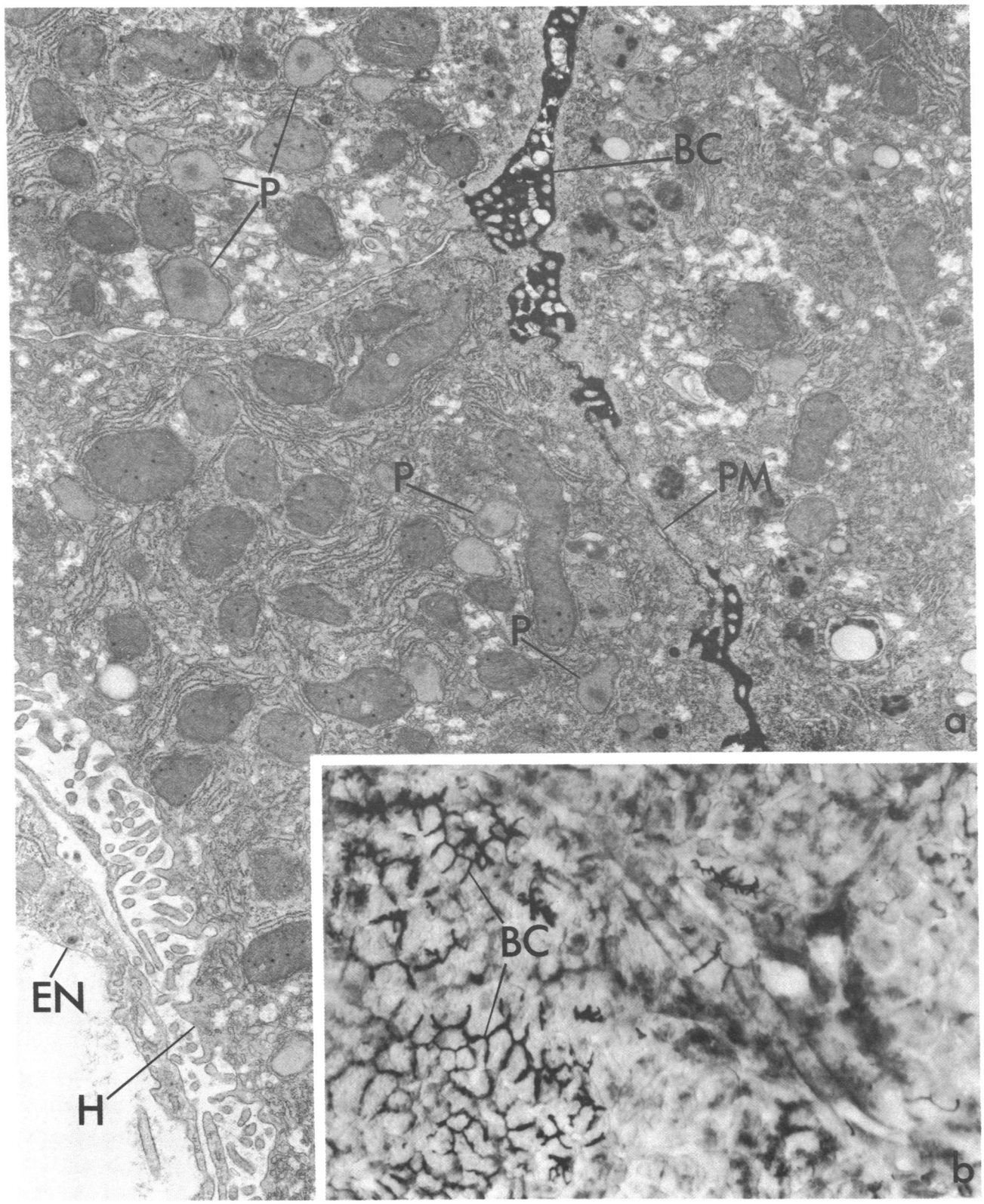

FIG. 3. (*a*) Electron micrograph of a vibratome section incubated for so-called ATPase activity. Reaction product is seen as dark Pb deposits on the plasma membranes of the bile canaliculi (BC). Several peroxisomes with nucleoids are visible (P). Plasma membranes of a hyperplastic cell (H) and an endothelial cell (En) are without reaction product. (×11,700.) (*b*) Light micrograph of a vibratome section incubated for ATPase activity. Note the marked variability in the activity in the tortuous bile canaliculi between the hyperplastic cells. Large areas within the hyperplastic nodule are devoid of reaction product. (×250.)

phenotypically from the original hepatocytes [see reports by Richards *et al.* (10) and Merkow *et al.* (19)]. The same understanding applies to the use of the term "bile canaliculi"; it is not known whether they are phenotypically unaltered from the original bile canaliculi. Recently, Rao *et al.* (9) have shown that the "altered foci," "neoplastic nodules," and hepatocellular carcinoma that develop in rats treated with Wy-14,643 are all γ-GluTase negative. They emphasize the importance of not con-

Table 1. Immunocytochemical incubation procedure

Procedure sequence	Treatment of section groups				
	1	2	3	4	5
Pretreatment	—	—	PrA, 1:100; 20°C, 60 min	—	—
Step 1, at 4°C for 20 hr	Antiserum, undiluted	Antiserum, 1:10	Antiserum, 1:10	Antiserum, 1:10	Normal serum, undiluted
Step 2, at 20°C for 60 min	PrA-HRP, 1:200	PrA-HRP, 1:200	PrA-HRP, 1:200	PrA, 1:100	PrA-HRP, 1:200
Post-treatment	—	—	—	PrA-HRP, 1:200; 20°C, 60 min	—
Step 3, at 20°C for 5–10 min	DAB	DAB	DAB	DAB	DAB

All of the sections received two 10-min rinses in PS buffer at 20°C between each sequence of the outlined procedure. PrA, protein A.

sidering the appearance of γ-GluTase as a phenotypic marker in hepatocarcinogenesis. Hanigan and Pitot (20) have written, "The isolation of γ-GluTase cells is a first step in obtaining the answer to this and other questions involving the development of hepatocellular carcinoma." It would appear that this is not necessarily the case.

Another observation considered by some to be of importance in hepatocarcinogenesis is the one we have made of the so-called ATPase activity on the cell surface. In the hyperplastic cells induced by 2-AAF, some areas of the cells are without such activity while other areas are positive. In contrast, Karasaki (21) has reported that the entire cell surface shows ATPase activity when hyperbasophilic foci are induced by *N,N'*-dimethylaminoazobenzine.

Our laboratory has described other changes in areas of the hyperplastic cells induced by 2-AAF. One is alkaline phosphatase activity, which may show markedly different levels in adjacent areas; another is the presence of gap junctions of highly variable size, (ref. 22; unpublished data). A final observation to note is the presence of nucleoid-containing peroxisomes characteristic of hepatocytes (23).

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