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REDISTRIBUTION OF 5'-NUCLEOTIDASE IN CHOLESTATIC LIVER

SAN-DUO CHEN

Department of Veterinary Medicine, National Chung-Hsing University, Taichung, Taiwan, 40227 Republic of China

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The mechanism behind the elevation of 5'-nucleotidase (5'-N) activity in cholestatic serum was studied by immunocytochemistry. By using immunolabelling, it was observed that 5'-N was transported from the bile canalicular membrane to the sinusoidal membrane in bile duct ligated liver. The process was carried out by canalicular internalization, vesiculation, intracellular vesicle migration, and fusing with the sinusoidalmembrane. There was no evidence of misaddressing to the lateral membrane, and only very few cases showed a rupture of the tight junction releasing bile into serum from the canalicular lumen. In addition, some hepatic cells were necrosed after bile duct ligation. It is suggested that the reverse transcytosis is the main route for bile regurgitation, although the leakage of the tight junction and the necrosis of hepatocyte can not be ruled out. The elevation of 5'-N activity in cholestatic serum is most likely caused by the release of 5'-N into the blood stream from the sinusoidal membrane which had been enriched with 5'-N from the canalicular membrane following bile duct ligation.

Key words: 5'-nucleotidase, Immunogold labelling, Reverse transcytosis.

5'-nucleotidase is an ectoenzyme which is widely distributed in animal cells (Trams, 1974; De Pierre and Karnovsky, 1974; Newby *et al.*, 1975; Farquhar *et al.*, 1974; Stanley *et al.*, 1980, 1983). It spans the plasma membrane bilayers (Zachowski *et al.*, 1981), and its active site is expressed on the external face of a cell (De Pierre and Karnovski, 1974; Newby *et al.*, 1975). Its stability and ease of assay has made it a convenient and popular marker of the cytoplasmic membrane (Evans, 1976).

The distribution of 5'-nucleotidase in rat liver has been studied in detail. From cell fraction studies, it was found that 5'-nucleotidase exhibits a dual distribution-peaking in both the nuclear and microsomal fractions (Beaufay *et al.*, 1974; Draye *et al.*, 1987). It has also been reported that 5'-nucleotidase is mostly concentrated in the bile canalicular membrane, as compared with the basolateral membrane (Meier *et al.*, 1984; Sztul *et al.*, 1987).

Via cytochemistry (Essner *et al*, 1958; Desmet *et al.*, 1968; Farquhar *et al.*, 1974) and immunolabelling techniques (Stanley *et al.*, 1983; Geuze *et al.*, 1984; Matsuura *et al.*, 1984; Chen, 1990), it has been concluded that 5'-nucleotidase is distributed not only in the bile canalicular and sinusoidal membranes but also in the Golgi complex and endoplastic reticulum. However, Geuze *et al.* (1984) localized this enzyme by immunogold labelling of ultracryosections, and reported that 5'-nucleotidase was not distributed on the lateral membranes of hepatocytes. Chen (1990) also reported that 5'-nu-

cleotidase was more concentrated at bile canalicular membranes but less at sinusoidal membranes and absent from the lateral membranes of hepatocytes.

Several investigators have found that 5'-nucleotidase levels prominently increase in the sera of cholestatic patients (Brocklehurst *et al.*, 1976; Chuang *et al.*, 1984) or bile duct-ligated rats (Kryszewski *et al.*, 1983; Kaplan and Righetti, 1970; Bel *et al.*, 1973; Simons and Arias, 1973; Mullock *et al.*, 1977; Chen *et al.*, 1990). In bile duct-ligated rats, levels rapidly increased in less than one day (Chen *et al.*, 1990). It has also been suggested that it is a useful indicator of cholestasis when increased levels of 5'-nucleotidase activity are present in a patient's sera (Brocklehurst *et al.*, 1976).

Quantitative changes in 5'-nucleotidase activity in rat liver after experimentally-induced cholestasis was reported by Frederiks *et al.*, (1990). Using cytophotometry, they found that total 5'-nucleotidase activity in liver parenchyma increased significantly after four or more weeks of cholestasis, and that 5'-nucleotidase was redistributed over all three plasma membranes surfaces.

Although the elevation of plasma 5'-nucleotidase activity in the sera of cholestatic patients or bile duct-ligated rats has been reported, the elevation mechanism is still obscure. The purpose of this study was to investigate the mechanism behind the increase of 5'-nucleotidase activity in cholestatic serum by comparing its morphological distribution in normal and bile duct-ligated livers.

MATERIALS AND METHODS

Animals

Male Wistar rats weighing between 150 and 250g were fed ad libitum, then fasted overnight before sacrifice. Seven groups of six rats each were used. The bile ducts of the experimental rats in six of the groups were ligated, and sham-operations were performed on control group rats as described by Vaerman *et al.*, (1988). The animals were sacrificed at 0, 1, 2, 4, 6 hours, 1 day, and 7 days following each operation. Livers were fixed by perfusion with 5% paraformaldehyde supplemented with 0.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) for 15 min, then removed and minced into tiny pieces and refixed by immersion with 5% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) on ice for 1 hour.

Antibody and colloidal gold preparation

Anti-5'-nucleotidase antiserum was obtained by immunizing rabbits against rat liver 5-nucleotidase as described by Widnell (1974); it was subsequently used at various dilutions. Gold particles were prepared via the reduction of chloroauric acid with citrate in the presence of tannic acid, as described by Slot and Geuze (1985). Protein A (purified from the Gowas strain of Staphylococcus aureus and purchased from Pharmacia Fine Chemicals Company of Uppsala, Sweden) was added to the gold particles to produce protein A-gold, as described by De Roe *et al.*, (1987).

Sectioning and labelling

All fixed liver tissues were cut by routine histologic sectioning, ultrathin sectioning, and ultrathin frozen sectioning. Histologic sections were prepared by dehydration of the tissue with graded alcohol, then embedded in paraffin, sectioned at a thickness of 6μ, and stained with Hematoxylin and Eosin.

As described by Spurr (1969), routine ultrathin section was prepared by dehydration with alcohol and embedded in the mixture. Approximately 75nm sections were cut and stained with lead citrate and uranium acetate.

Ultrathin frozen sections were prepared in a Reichert-jung Cryoultramicrotome; thickness was set between 80nm and 100nm, and the cryochamber temperature was adjusted to between –80°C and –110°C. Sections were removed with a tungsten-coated glass knife as described by Geuze *et al.*, (1983), retrieved with a loop containing one drop of 2.3 M sucrose in PBS, transferred by onto the surface of 100 mesh hexagonal grids which had been covered with Formvar film and coated with a layer of carbon, and moved to drops of PBS on parafilm paper.

Immunogold labelling was applied as described by Geuze *et al.*, (1983). Ultrathin frozen sections were labelled with anti-5'-nucleotidase antiserum, incubated with Protein-A gold, stained with uranyl acetate, and mounted with methyl cellulose (Sigma Chemical Company, St. Louis, MO.,USA).

Both ultrathin sections and ultracryosections were observed with a Philips 301 electron microscope.

RESULTS

Clinical signs after bile duct ligation

Signs, indicating abdominal pain (i.e., back arching, hair erection, and loss of appatite) appeared one hour after bile duct ligation. After six hours these signs became more prominent (abdominal cavities became distended, and heads were hidden between forelimbs). After one day, the rats lay prostrate in one corner of their cage and refused to move; hairs surrounding the urethral orifice were contaminated with yellowish urine residue. By the end of seven days, the rats had lost body weight and vigor.

Gross pathologic findings

From two to four hours following bile duct ligation, abdominal cavity vessels were congested and frequent yellowish ascites (about 3ml) were observed. Extravasated bile had also collected in the interstitial tissues of the pancreatic region (i.e. close to the connection of the bile duct with the intestine). After one to seven days, plasma color became greenish, with gingivae and subcutaneous tissue stained with yellowish pigment. Livers were enlarged and dark red. The proximal portion of the ligated bile ducts had dilated considerably and were filled with dark bile. Kidneys were also swollen and greenish, and urinary bladders were filled with brownish urine.

Histopathologic findings

Up to one hour following operation, no differences were detected between control and ligated rat livers. However, four hours after ligation, small necrotic foci were frequently observed, mostly in the middle zones of the liver lobule. Various stages of necrosis, from early pyknosis to complete karyolysis and cytolysis were recognizable. Necrotic areas were often filled with erythrocytes, or infiltrated by polymorphonuclear neutrophils (Fig. 1). In the portal triads, all blood vessels exhibited dilatation, and connective tissues were often infiltrated by leucocytes. Bile ducts were markedly dilated, and the linings of epithelial cells were flattened.

Ultrastructural findings

The most prominent early changes induced by bile duct ligation were the dilation, distortion, and fragmentation of bile canaliculi. The canaliculi frequently appeared enlarged and tortuous. More than one independent canaliculus was often found between adjacent hepatocytes. Sections of microvilli were usually projected at a shortened distance into the lumen, giving an empty appearance to the dilated canaliculi. The microvilli were swollen or blunted; some of them disappeared from the bile canali-cular membrane, and only a few microvilli re-

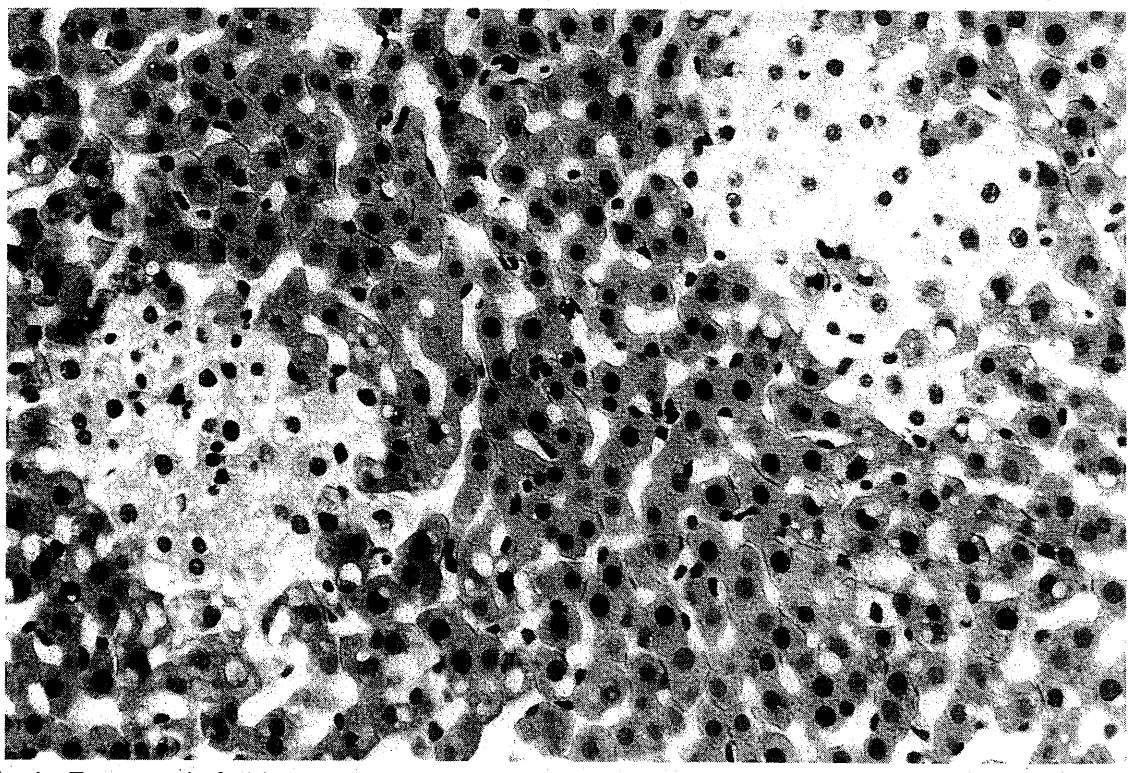

Fig. 1. Two necrotic foci in the parenchyma of bile duct ligated liver. (H & E; 400 X)

mained in the vicinity of the tight junctions. Some small vesicles were occasionally found in the canalicular lumen, in the space of Disse and sinusoids. The canalicular wall bulged into the cytoplasm to form a large, spherical or other irregularly-shaped diverticulum. Discharges of luminal content into the diverticula were observed. The pericanalicular diverticula had apparently pinched off and emigrated into the cytoplasm (vesiculation). Some of the cytoplasmic vesicles still accompanied microvilli-like structures (Fig. 2).

Although bile canaliculi often became tortuous, their junctional complexes always remained intact. In all ultrathin plastic section materials examined, only one case of a rupture of the junctional complex was found; a direct communication (fistula) was therefore opened between the canaliculus and sinusoid (Fig. 3). In this case, the luminal content of the bile canaliculus appeared to have been discharged into the sinusoidal lumen via the fistula.

Ultrastructural changes in bile canaliculi of hepatocytes appeared as early as two hours after bile duct ligation; most bile canaliculi were involved during the six hours following an operation, but lesion severity did not constantly increase afterwards.

Distribution of 5'-nucleotidase in bile duct-ligated liver

Figure 4 shows that the 5'-nucleotidase remained mostly associated with the canalicular membrane; it did not cross the tight junctions. In the bile canalicular membrane, even though the gold particles were present on the flattened membrane domains, they appeared to be more concentrated on the microvilli. In addition, gold particles were also found on the membranes of vesicular profiles (in continuity with the canalicular membrane), and in the large cytoplasmic

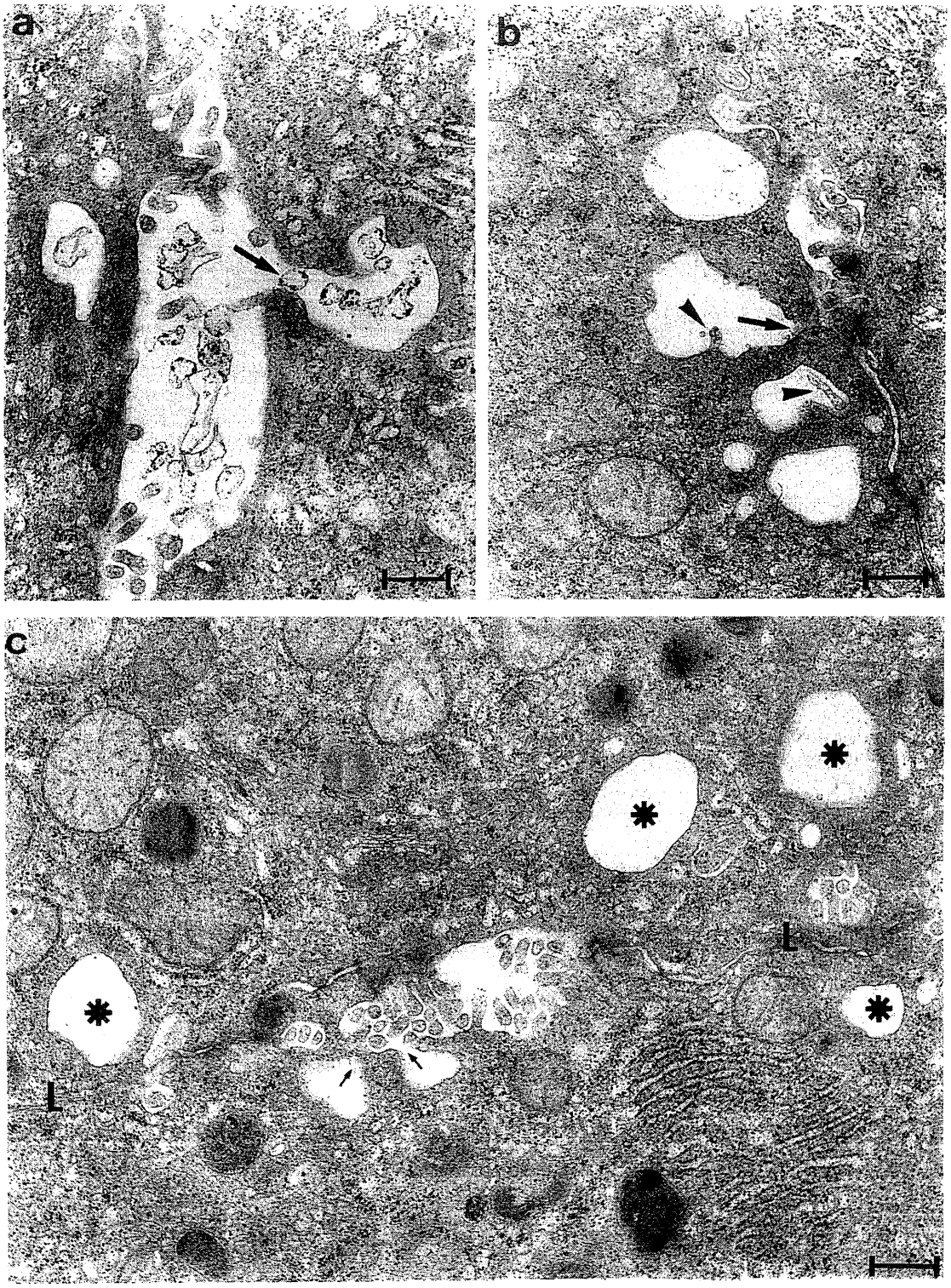

Fig. 2. Ultrastructural changes of vesiculation. a) Bile constituent was discharged into the diverticulum (arrow). b) One vesicle (arrow) was still connected with the bile canaliculus. Two vesicles (arrowhead) still contained microvilli-like structures. c) Four large vesicles (asterisks) in the vicinity of lateral surface (L); two diverticula (small arrows) were being generated from a canaliculus. Bars are 0.25 um.

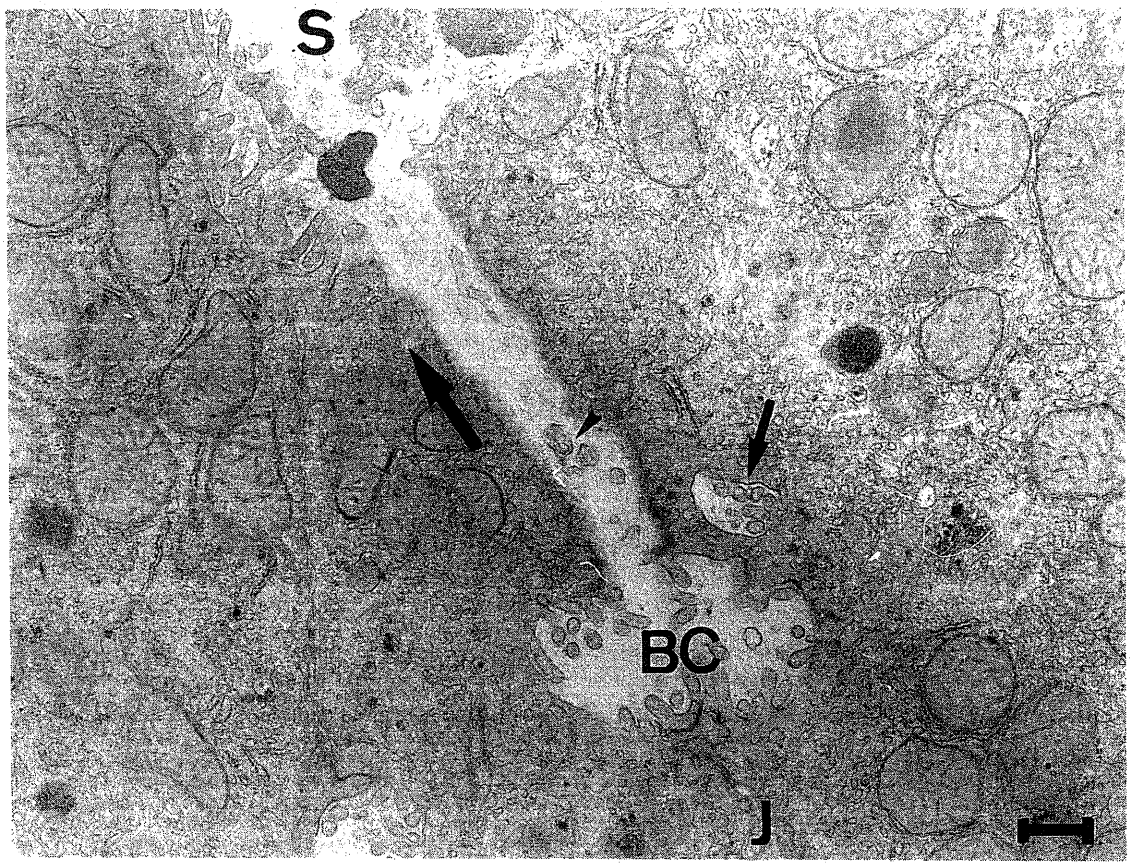

Fig. 3. Rupture of junctional complex. A fistula was formed; between bile the large arrow indicates that the luminal content (arrowhead) was being discharged into the sinusoid. The lower junction (J) is intact. Bar is 0.25 um.

vesicles located some distance from the bile canaliculi and apparently independent from them. Such vesicles were more frequently found along the lateral surfaces, and were also seen in the vicinity of the sinusoidal membrane (Fig. 5). When comparing bile canaliculi size at various ligation times, they obviously increased within two hours, and reached maximum size in six hours; they then maintained dilated state thereafter. In the other hand, the intensity of gold labelling in the bile canalicular membrane increased in two hours and peaked at six hours, but it then decreased gradually until it was equal to the control intensity during the twelve hours following ligation. The intensity of labelling in the sinusoidal membrane appeared to have a similar kinetic pattern as in the bile canalicular membrane-showing a peak at around six hours.

CONCLUSION AND DISCUSSION

According to the data, it may be concluded that elevation in 5'-nucleotidase activity in the plasma of cholestatic patients occurs via three pathways: (1) the reverse transcytosis of bile constituents from bile canaliculi to Disse's space; (2) the rupturing of canalicular tight junctions causing bile leakage into the bloodstream; and (3) the necrosis of hepatocytes. However, the reverse transcytosis is most likely the main pathway; it occurred as early as two to four hours after bile duct ligation, and it may be

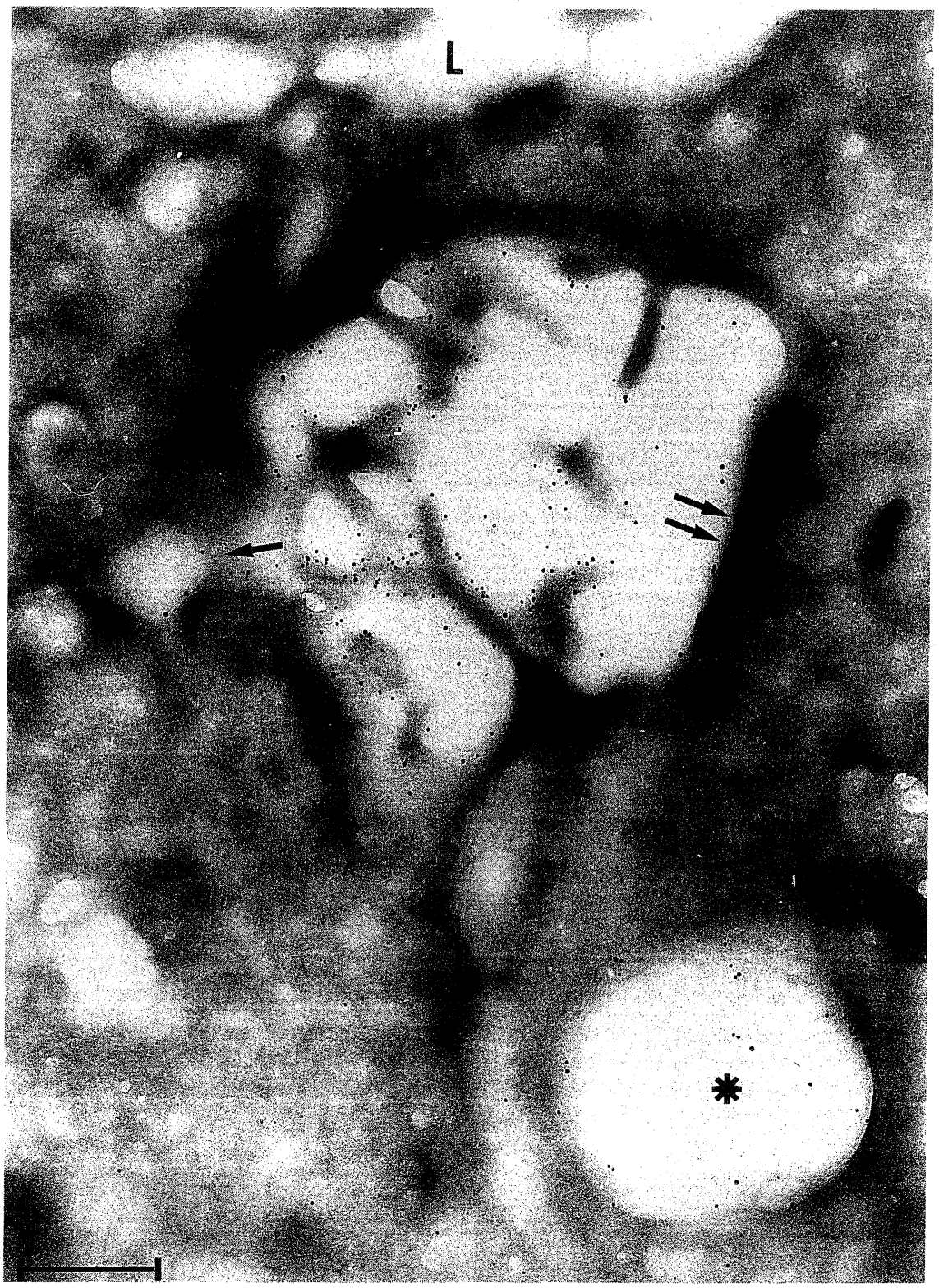

Fig. 4. Immunogold labelling of 5'-nucleotidase. Gold particles were distributed on microvilli and flattened membrane (double arrow), but not at tight junction (arrowhead). A vesicle (arrow) was in the process of being budded from a canaliculus. A large vesicle (asterisk) was heavily labelled, but the lateral surfaces (L) were not labelled. Bar is 0.25 um.

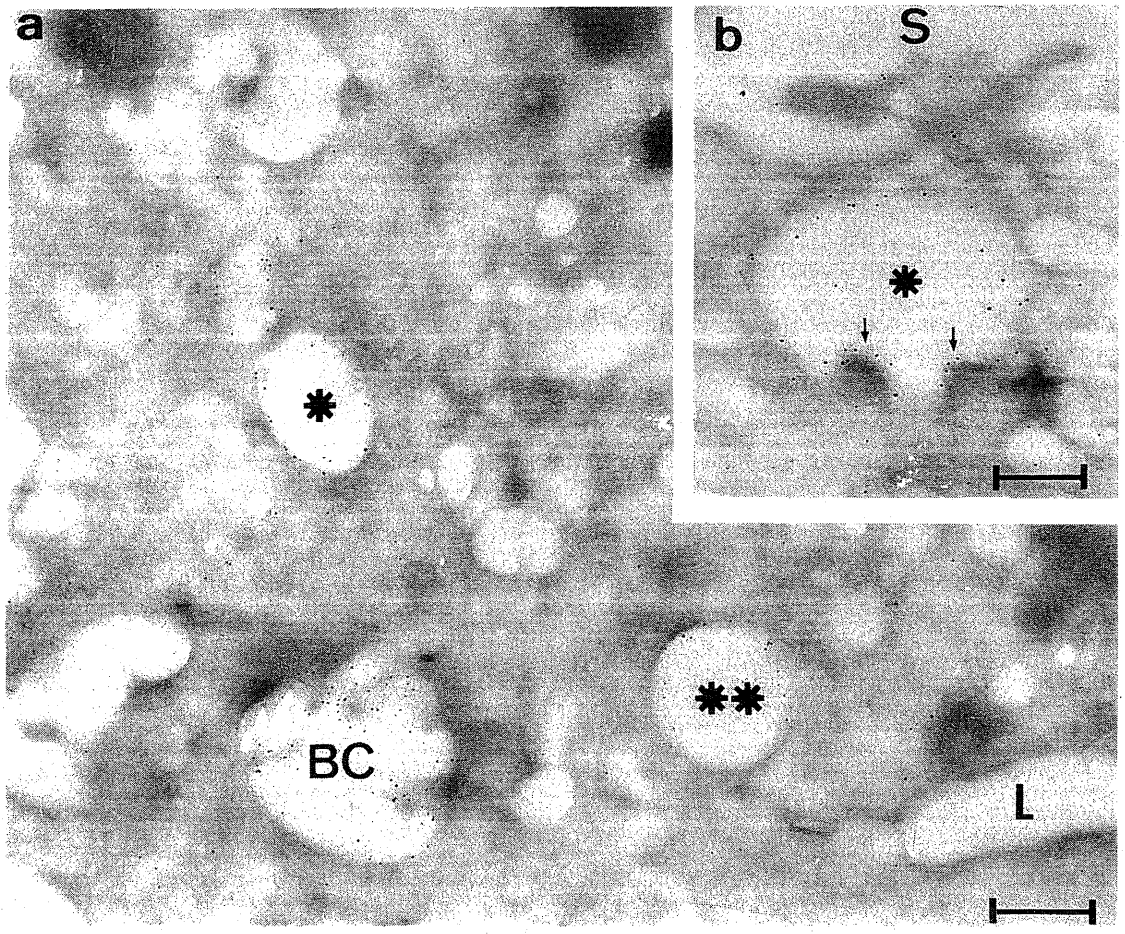

Fig. 5. Evidence of vesicular transport. a) Two vesicles (asterisks) were in the vicinity of canaliculus, one of which was adjacent to the lateral surface (L). b) A vesicle (asterisk) which contained two microvilli (small arrows) was close to sinusoidal membrane. Bars are 0.25 um.

used to explain the rapid and marked elevation of 5'-nucleotidase in cholestatic serum. The data also strongly support the opinion of Bel *et al.*, (1973) that 5'-nucleotidase is the most sensitive indicator of cholestatic patients.

Although the vesiculation of bile canalicular membranes in bile duct-ligated livers was reported by Krstulovic *et al.*, (1968), they did not describe the detailed processes occurring during vesiculation, nor did they uncover the origin of these intracellular vesicles. The data in the present study clearly show serial ultrastructure evidence of vesicle formation, movement, and fusing with the cytoplasmic membrane. In addition, by using immunogold labelling it was strongly suggested that the intracellular vesicles are the transport instruments of reverse transcytosis.

The early appearance of ultrastructural changes during the six hours following ligation is completely compatible with the data reported by Chen *et al.*, (1990), in which the plasmatic 5'-nucleotidase activity was detected as early as one hour after bile duct ligation and peaked six hours after bile duct ligation. The intensity of gold labelling decreased and returned to control levels in both the canalicular and sinusoidal membranes twelve hours after ligation; the elevation of 5'-nucleotidase activity in cholestatic serum may be due to the continuous pouring

of this enzyme into the bloodstream via thesinusoidal membrane.

Although Frederiks *et al.*, (1990) reported that 5'-nucleotidase was found on the lateral surfaces of plasma membranes in bile duct-ligated livers, and suggested that this was related to the formation of new sites for bile salt transport out of the hepatocytes, I did not find that gold particle labelling appeared on the lateral membranes of hepatocytes-either in control or bile duct-ligated livers. The reasons for the different results may be either the application of different techniques, or the leakage of bile from the ruptured tight junctions-leading to the previously reported observations of the localization of this enzyme on the lateral membranes of hepatocytes.

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五核甘酸酶在膽汁鬱積肝內之再分佈

陳三多

本實驗之目的乃欲瞭解黃膽血清中五核甘酸酶升高之作用原理。藉著免疫標示法之研究，發現在膽管結紮的鼠肝中，五核甘酸酶可由膽小管膜被運送到血竇膜上。其過程是經膽小管膜之內凹作用，小泡形成作用，小泡細胞內運送，以及與血竇膜融合作用來完成。實驗中沒有發現側膜之錯誤送達作用而且僅有非常少數的例子，其緊密接合破裂使得膽小管之內容物被直接送入血行中。此外，當膽管結紮後，有些肝細胞會發生壞死現象。因此，根據以上結果推論返逆性過細胞現象是黃膽時膽汁進入血行中之主要途徑，而緊密接合的破裂以及肝細胞的壞死則為次要的原因。