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Contribution of the Fluid Phase Endocytosis to Bile Flow in Cholestasis and Choleresis in Rats (42790)

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Abstract. Using a single iv injection of 1 mg Evans blue dye/100 g body wt in rats, the contribution of the fluid phase endocytosis to bile flow was estimated. In control rats, this pathway contributed 2–4% of the bile flow or 10–20 μl/hr/100 g body wt. This contribution was not affected by cholestasis induced by taurolithocholic acid or bile duct obstruction or by choleresis induced by dehydrocholic acid. It is concluded that fluid phase endocytosis does not significantly influence bile formation but, rather, may be implicated in the remodeling of liver cell plasma membranes.

All eucaryotic cells are continually ingesting bits of their plasma membrane in the form of pinocytotic vesicles. Trapped in these vesicles are extracellular fluid and many substances dissolved in it (1). Although some endocytotic vesicles could fuse with lysosomes which degrade their content, and transport the breakdown products to the cytosol where it could be used by the cells, most endocytotic vesicles escape degradation, are retrieved by unknown mechanism(s), and are returned to plasma membranes. Thus, cell surface area and volume remain unchanged during this process. Some endocytotic vesicles, however, traverse the cytoplasm and release their content by exocytosis at another surface and thus can shuttle fluid and material from one surface of the cell to another (2, 3). Recently, Lake *et al.* (4) and Scharschmidt *et al.* (5), utilizing isolated perfused rat liver and cultured rat hepatocytes, characterized the fluid phase endocytosis in hepatocytes. They demonstrated that each hour the hepatocyte can endocytose the equivalent of 20% of its cell volume and five times its plasma membrane surface area. Of the total amount of inulin (fluid phase marker) endocytosed, 80% is regurgitated back into plasma, 18% is transported in sequestration compartments, and 2% is secreted in bile. They further showed that this pathway does not require vesicle acidification and is unaffected by taurocholate. However, intact microtubules were necessary for the transport of endocytosed substrates to bile or to a sequestration compartment.

Evans blue (T-1824) is a dye used as a marker of extracellular fluid. It binds to albumin after injection and mixes completely within the plasma after about 10 min of iv injection and, thus, was used for measurement of blood volume (6–8). In this study, dye injection has been used to evaluate the contribution of the fluid phase endocytotic pathway to bile flow in normal and abnormal conditions.

Material and Methods. *Animal experiments.* Male Wistar rats weighing 200–250 g (High Oak Ranch, Montreal, Québec) were maintained in an environment of constant temperature (22°C) and lighting (7 PM to 7 AM darkness) with free access to food (Purina Lab Chow) and water prior to experimental use. They were anesthetized with pentobarbital (48 mg/kg/body wt, ip) and the common bile duct was cannulated with a PE-10 catheter. Body temperature was maintained throughout the experiment at 37°C using a rectal probe and a thermostatically controlled heat lamp. Bile was collected for 60 min, after which the animals were divided into the following groups.

(A) Control group: (n = 18) the animals were injected in the right jugular vein, with 0.5, 1.0, or 2 mg Evans blue dye T-1824/100 g body wt (six animals/each dose). Evans blue dye dissolved in 2 ml of 4% albumin and 0.9% sodium chloride solution.

(B) Cholestatic group: two cholestatic models were employed (n = 5 each). In the first, 6 μmole of taurolithocholic acid/100 g body wt was injected with 1 mg Evans blue after 1 hr of initial bile collection. At this dose taurolithocholic acid induced a 50% reduction in bile flow within 30 min after in-

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jection (9). In the second model (n = 5) the common bile ducts were obstructed for 20 hr, after which, the bile duct was cannulated above the obstruction point and bile was collected for 60 min. The animals were then injected with 1 mg/100 g body wt Evans blue dye. This protocol induces cholestasis associated with increased permeability of the tight junction of the hepatocytes (10).

(C) Choleretic group: (n = 5) the animals were injected with 1 mg Evans blue and 30 μmole dehydrocholic acid/100 g body wt, 1 hr after bile duct cannulation. Dehydrocholic acid injection at this dose induced a 100% increase in bile flow (choleresis) (11).

(D) Colchicine treatment: (n = 6), the animals were injected with 1 mg Evans blue/100 g body wt after 60 min of initial bile collection. Thirty minutes after Evans blue injection, 0.2 μmole colchicine/100 g body wt in 0.5 saline was injected in the jugular vein. This dose of colchicine was found to cause a significant inhibition of biliary secretion of IgA after 90 min and, therefore, was taken as evidence that colchicine significantly inhibited microtubular function (12). In all experiments the bile ducts were cannulated with a PE-10 catheter i.d. 0.3 mm and 500 mm length and bile was collected for 2 hr after Evans blue injection, in 30 min aliquots. To avoid the problem of deadspace the first 30 min of bile collection was discarded. At the end of the experiment, the animals were sacrificed and plasma samples were prepared for each animal. To determine the disappearance rate of Evans blue from the plasma during the duration of experiments (2 hr), five rats were injected with 1 mg/100 g body wt. Evans blue dye and blood samples were taken every 30 min for the determination of Evans blue concentration in plasma.

Bile volume was determined gravimetrically, assuming 1 ml of bile equivalent to 1 g (density 1.00). The Evans blue concentration in plasma and bile was measured by the Zweens and Frankena method (8), in which one part of polyethylene glycol (average molecular mass 4000) is added to one part of plasma or bile. The tubes were allowed to stand for 10 min to precipitate the nonalbumin fraction of the plasma or bile protein (at this concentration the albumin fraction remains dissolved). The tubes were then centrifuged at 7000g for 10 min at 4°C. The absorbance of the supernatant was read against a similarly treated plasma or bile blank at a wavelength absorbance of 580 nm. Scanning bile samples and plasma at wavelength absorbances from 400 to 760 nm gave a single peak for Evans blue with maximum absorbance at 580 nm.

The concentrations were determined by the aid of a calibration line made by mixing different amounts of Evans blue solution with plasma or bile. At the wavelength used, the absorbance of Evans blue obeys Lambert-Beer's law from 0.5 to 10 μg/ml (the concentration tested) with an r = 0.9999 and there was no differences between the standard curves made with plasma or bile. The amount of water that reached the bile by the Evans blue route and which may represent the fluid phase endocytosis was calculated as follows:

$$\mu\text{l/hr/100 g body wt} = \frac{\mu\text{g Evans blue secreted/hr/100 g body wt}}{\mu\text{g Evans blue/}\mu\text{l plasma}}.$$

In vitro studies. Binding of Evans blue dye to albumin. Five concentrations of Evans blue (60, 120, 180, 240, and 300 μg/ml) were dissolved in 4% albumin–0.9% saline. One milliliter of this solution was dialyzed against 10 ml of 0.9% saline by equilibration dialysis. The dialysis was carried out for 1, 2, and 24 hr at room temperature or at 4°C and the amount of Evans blue present in the dialysate (saline) was measured. At least four experiments were performed with each concentration of Evans blue.

Binding of Evans blue to liver cell plasma membranes. Liver cell plasma membrane fractions consisting of contiguous areas of hepatocytic plasma membrane with attached canalicular structures or containing vesicles and sheets of the sinusoidal cell surfaces were isolated from 200-g male rats as described elsewhere (13). The purity of the fractions was satisfactory as assessed by measuring glucose-6-phosphatase (microsomal enzyme marker), 5′-nucleotidase (liver cell plasma membrane marker), and leucine aminopeptidase (canalicular membrane marker) as outlined earlier (14). The membranes were suspended in 0.075 M NaCl/0.075 M KCl,

pH 7.4, at a concentration of 1.0 mg protein/ml.

The binding of Evans blue to the membranes was determined by incubating 0.5 mg of membrane protein with 0.5 ml of Evans blue dissolved in 4% albumin–0.9% NaCl. The Evans blue concentration in final incubation mixture was 120 μg/ml. The incubation was carried out at 37°C for 5, 15, and 30 min. At the end of the incubation the membranes were centrifuged at 5000*g* for 30 min and the concentration of Evans blue was determined in the supernatant. The specificity of Evans blue binding to cell membranes was tested by incubating Evans blue as described above with cell membranes boiled for 10 min after which nonspecific binding of Evans blue to cell membranes was determined.

Chemicals. Evans blue and colchicine were obtained from Sigma Chemical Co., St. Louis, Missouri. Taurolithocholic acid and dehydrocholic acid were obtained from Calbiochem, San Diego, California. Other chemicals used were obtained from various Scientific Laboratory Supplies in Canada.

Statistical analysis was carried out using one-way or two-way analysis of variance. A level of $P < 0.05$ was considered to be significant.

Results. *Animals experiments.* Measurements of plasma concentration of Evans blue within 2 hr after the injection indicated that the dye distributes evenly in plasma and was in steady state for the duration of the experiment. (The dye concentration was reduced at the end of the 2 hr by 8.0 ± 4.0% (means ± SD) from the initial concentration measured at 30 min.)

Table I shows that the injection of the dye did not significantly influence bile flow ($P > 0.05$). However, plasma and bile concentrations (means ± SD) of Evans blue were dose-dependent (62.6 ± 4.4, 128.8 ± 6.4, and 269.2 ± 10.6 μg/ml for plasma and 1.22 ± 0.54, 2.4 ± 0.39, and 3.18 ± 0.92 μg/ml of bile when 0.5, 1.0, and 2.0 mg/100 g body wt were injected respectively ($P < 0.05$) between varying concentrations in plasma and in bile). When the amount of Evans blue-associated fluid phase was calculated as previously described under Materials and Methods, means and SD obtained were 10.48 ± 0.36, 10.50 ± 1.49, and 5.67 ± 1.59 μl/hr/100 g body wt, when 0.5, 1.0, and 2.0 mg Evans blue/100 g body wt, respectively, were injected. Thus, the contribution of this pathway represents 1.98 ± 0.98, 2.05 ± 0.72, and 1.24 ± 0.52% of bile water when 0.5, 1.0, and 2.0 mg Evans blue/100 g body wt were injected, respectively. The constant ratio of plasma to bile for the 0.5 and 1.0 mg/100 g body wt doses suggests that either of these doses could be used for the studies described here.

Table II shows the effect of cholestasis induced by taurolithocholic acid and bile duct obstruction, choleresis induced by dehydrocholic acid and the effect of colchicine treatment on bile flow, and the contribution of the fluid phase endocytosis. In both taurolithocholic and bile duct obstruction, bile flow was significantly reduced ($P < 0.05$) from control values (cf. Table I). Dehydrocholic acid significantly increased bile flow ($P < 0.05$), and colchicine did not influence bile flow.

The contribution of the fluid phase endocytosis to bile flow was constant and not significantly different from control ($P > 0.05$) during cholestasis or choleresis but was sig-

TABLE I. BILE FLOW, PLASMA CONCENTRATION OF EVANS BLUE, TOTAL EVANS BLUE SECRETED IN BILE, AND CONTRIBUTION OF THE FLUID PHASE ENDOCYTOSIS TO BILE AFTER EVANS BLUE INJECTION IN CONTROL RATS

Amount of Evans blue injected (mg/100 g body wt)	Bile flow (ml/hr/100 g body wt)	Plasma concentration of Evans blue (μg/ml)	Evans blue secreted in bile (μg/hr/100 g body wt)	Contribution of the fluid phase endocytosis (μl/hr/100 g body wt)
0.5 ($n = 6$)	0.55 ± 0.08	62.62 ± 4.36	0.67 ± 0.32	10.48 ± 0.36
1.0 ($n = 6$)	0.55 ± 0.13	128.80 ± 6.38	1.31 ± 0.21	10.50 ± 1.49
2.0 ($n = 6$)	0.48 ± 0.07	269.20 ± 10.60	1.53 ± 0.44	5.67 ± 1.59

Note. Values obtained between 60 and 120 min after the Evans blue injection (means ± SD), and n = number of rats per group.

TABLE II. BILE FLOW, PLASMA CONCENTRATION OF EVANS BLUE, TOTAL EVANS BLUE, TOTAL EVANS BLUE SECRETED IN BILE, AND CONTRIBUTION OF THE FLUID PHASE ENDOCYTOSIS TO BILE FLOW WHEN 1 mg EVANS BLUE/100 g body wt WAS INJECTED IN RATS GIVEN TAUROLITHOCHOLIC ACID, DEHYDROCHOLIC ACID AND COLCHICINE, OR AFTER BILE DUCT LIGATION

Treatment	Bile flow (ml/hr/100 g body wt)	Plasma concentration of Evans blue (μg/ml)	Total amount of Evans blue secreted in bile (μg/hr/100 g body wt)	Contribution of the fluid phase endocytosis (μl/hr/100 g body wt)
Taurolithocholic acid (n = 5)	0.22 ± 0.06	121.80 ± 4.76	1.20 ± 0.21	9.86 ± 1.98
Bile duct ligation (n = 5)	0.34 ± 0.15	125.71 ± 4.95	1.03 ± 0.35	8.46 ± 2.20
Dehydrocholic acid (n = 5)	0.93 ± 0.21	122.22 ± 2.75	1.24 ± 0.50	10.08 ± 3.98
Colchicine (n = 5)	0.56 ± 0.11	126.29 ± 2.89	0.76 ± 0.21	6.14 ± 1.08

Note. Values obtained between 60 and 120 min after Evans blue injection except for colchicine values are between 90 and 120 min after injection (means ± SD). n = number of rats per group.

nificantly ($P < 0.05$) reduced by colchicine treatment.

In vitro experiments. Binding of Evans blue to albumin. At all concentrations used (60, 120, 180, 240, and 300 μg Evans blue/ml), 99% of the dye was bound to albumin. The constant ratio of bound to free concentration suggests that the free concentration (1%) was a contaminant which does not bind to albumin.

Binding of Evans blue to liver cell plasma membranes. When liver cell plasma membrane fractions were incubated with Evans blue at a concentration of 120 μg Evans blue/ml, there was no difference between the total and nonspecific binding (4.8 ± 0.4 vs 4.9 ± 0.5% of the total Evans blue, respectively) suggesting that at this concentration, specific binding of Evans blue to the cell membrane fractions was undetectable.

Discussion. The data obtained in these studies showed that the disappearance of Evans blue dye, 2 hr after injection, was only 8%. This suggests that Evans blue distributes evenly in plasma during the duration of the experiment. Furthermore, the dye binds completely to albumin and there was no evidence of specific binding to the sinusoidal- or canalicular-enriched preparations of liver cell plasma membranes. In addition there was no transformation of Evans blue during its passage to bile (unpublished data). These characteristics make this dye an ideal fluid phase marker for studies of the fluid phase endocytosis in the hepatocyte. The binding to albumin suggests that biliary secretion of the dye via the paracellular pathway (15) is unlikely since albumin has not been shown to penetrate the hepatocellular tight junctions (16). Additional support for this view is given by the result that bile duct ligation which alters the tight junction permeability does not influence Evans blue secretion in bile. The absence of specific receptors or binding proteins on the sinusoidal or canalicular membrane fractions suggests that the uptake of the dye was mediated by the fluid phase endocytotic pathway and that secretion was mediated by exocytosis. It may be also suggested that a vesicular cellular transport was involved since a significant reduction of the biliary secretion of the dye occurred when the animals were pretreated with colchicine. Colchicine was shown to influence microtubules and vesicular transport of some substrates (13). In addition Scharschmidt *et al.* showed that colchicine treatment reduced vesicle transport of inulin endocytosed by the liver (5).

Having demonstrated the suitability of the Evans blue as fluid phase marker and that it was probably secreted in bile by transcellular-vesicle pathway, it is possible to calculate the contribution of this fluid phase endocytosis to bile flow during cholestatic or choleretic conditions.

However, two main assumptions must be made since they cannot be ascertained from these studies. The first is that there is no concentration or dilution of the dye during its

transit across the hepatocyte, and the second is that since the dye binds completely to albumin, albumin was not excluded from the intrahepatic interstitial space. The first assumption may be true, since the secretion of Evans blue was similar in cholestasis and choleresis. The second assumption may not be true, since albumin was reported to be excluded from as much as 50% of the intrahepatic interstitial space (17). Thus the results obtained in this study would underestimate this contribution to the fluid phase endocytosis by 50%. Nevertheless, this underestimation will be applicable to basal and cholestatic or choleretic situations.

Table I demonstrates that the contribution of the fluid phase endocytosis to bile flow is minimal and represents about 2%. If these data are corrected for albumin distribution, then the maximum contribution of this pathway to basal bile flow should be an average of 4%, a value which is similar to that obtained by Lorenzini *et al.* (18) using inulin as fluid phase marker. It should be noted that the contribution of the fluid phase endocytosis when 2 mg of Evans blue was used was lower than that obtained when 0.5 or 1 mg of Evans blue was used. Since the dye was bound to albumin at all doses, it could be assumed that there was extra hepatic uptake of the dye at the 2 mg concentration, thus influencing the correlation between bile–plasma concentration. The data obtained in Table II showed that the contribution of the fluid phase endocytosis was not influenced by cholestasis or choleresis.

The low contribution of the fluid phase endocytosis to bile formation throws doubt on its direct importance in bile secretion. Thus this pathway may only have implication on remodeling of plasma liver cell membranes and, therefore, can indirectly influence liver function.

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