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BILIARY EXCRETION OF POLYETHYLENE GLYCOL MOLECULAR WEIGHT 900

EVIDENCE FOR A BILE SALT-STIMULATED VESICULAR TRANSPORT MECHANISM

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Abstract—Polyethylene glycol molecular weight 900 (PEG-900) has been used as a marker of vectorial water transport into bile canaliculus. However, the mechanisms by which this compound is excreted have not been clarified. To gain more information on this process, we studied the biliary excretion of [^{3}H]PEG-900 in rats during choleresis induced by canalicular choleretics. In addition, the effects of the microtubule inhibitors colchicine and vinblastine, and of the acidotropic agent chloroquine, on PEG-900 excretion were studied to determine whether a vesicular pathway is involved. Continuous i.v. infusion of either dehydrocholate (DHC, a non-micelle forming bile salt choleretic) or 4-methylumbelliferone (4-MU, a non-bile salt canalicular choleretic) at stepwise-increasing rates [0.7, 1.0 and 1.2 μmol·min^{-1}·(100 g body wt)$^{-1}$] induced a gradual increment in bile flow, whereas a transient increment of [^{3}H]PEG-900 excretion was observed only during DHC-induced choleresis. Furthermore, studies in which two consecutive i.v. injections of DHC (10 μmol/100 g body wt) were administered showed that [^{3}H]PEG-900 excretion induced by a second administration of DHC was 54% lower than that induced by the first one, despite a similar excretion in bile flow. Finally, colchicine (0.5 μmol/100 g body wt), vinblastine (0.5 μmol/100 g body wt) and chloroquine (50 mg/kg body wt) pretreatments inhibited the DHC-induced increment in biliary [^{3}H]PEG-900 output, while DHC-induced choleresis was almost unaffected. Conversely, excretion of [^{14}C]sucrose, when coadministered with [^{3}H]PEG-900, was not impaired by the treatments. These results suggest that, unlike sucrose, PEG-900 excretion is not associated with canalicular water movements. Instead, it may be related to a vesicular transport process followed by a bile acid-stimulated discharge of secretory vesicles into bile through the lysosomal compartment.

Biliary clearance of inert solutes has long been used in studies of hepatic bile formation [1]. Thus, erythritol and mannitol, which are thought to cross the canalicular membrane freely via an aqueous pathway, have been proposed as suitable solutes for the estimation of canalicular bile formation [1–3]. Other relatively large hydrophilic solutes, such as sucrose or inulin, present a pore-size restricted leakage through the transjunctional shunt pathway. Thus, their biliary clearances would provide a measure of paracellular pathway permeability [1, 3, 4].

Recent studies, however, have questioned the validity of using such markers. The major criticisms arise from the following facts: (1) reabsorption of small hydrophilic solutes following water reflux from the canaliculus may occur, so that its biliary clearance would measure net, but not total, canalicular bile flow [5–7], (2) inert solutes may cross bile ducts [8], and (3) these markers can be partially internalized by hepatocytes and excreted into bile involving a vesicular transport mechanism [9–11].

In an attempt to provide a suitable marker of canalicular flow, it was proposed that polyethylene glycol molecular weight 900 (PEG-900)† would be excreted into bile by a mechanism similar to that of erythritol or mannitol but, once secreted, it might self-associate or associate with other constituents of bile, thus preventing its back-diffusion [5–7]. However, other investigators have proposed a transjunctional restricted pathway for PEG-900 [12–14], so that its clearance would provide a relative measure of paracellular permeability [15].

The aim of this study was to clarify the mechanism by which PEG-900 reaches the bile. For this purpose, we assessed PEG-900 excretion under choleresis induced by different canalicular choleretics in the rat. Further, to discern whether vesicular transport is involved, the effects of inhibitors of the microtubular system and acidic intracellular compartments on PEG-900 excretion were also studied. The results suggest that in the rat PEG-900 is transported through hepatocytes by a vesicular mechanism and finally reaches the bile via the lysosomal compartment.

METHODS

Animals and surgical procedure. Adult male Wistar

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† Abbreviations: PEG-900, polyethylene glycol molecular weight 900; DHC, dehydrocholate; and 4-MU, 4-methylumbelliferone.

rats weighing 300–350 g were used throughout. Before the experiments, animals were maintained on a standard laboratory diet in a constant temperature environment (25°) and under a constant 12-hr light/12-hr dark cycle.

The animals were anesthetized with sodium pentobarbital (50 mg/kg body wt, i.p.) and thus were maintained throughout the experiments. A polyethylene catheter (PE-10, Intramedic, U.S.A.) was inserted into the bile duct nearly to its bifurcation to avoid contamination with pancreatic juice [16]. The femoral vein and artery were also catheterized (PC-40, Rivero y Cia, Argentina). A tracheal cannula was employed systematically to remove bronchial secretion induced by the anesthetic, and the rectal temperature was maintained at 37.5 to 38.0° with a heating lamp to prevent hypothermal alterations of bile flow [17]. Unless otherwise indicated, renal pedicles were ligated in all animals to avoid urinary excretion of administered inert solutes. At the end of the experiments, the animals were killed by exsanguination, and the liver was promptly removed and weighed.

Experimental procedures. [^{3}H]PEG-900 (12.5 μCi; 6.8 mCi/g, New England Nuclear, U.S.A.) was administered as a single i.v. dose in all the experiments and a 30-min equilibration period was established to reach a constant bile-to-plasma ratio. Bile was collected during 10 min (basal period) and then the canalicular choleretics sodium dehydrocholate (DHC) or 4-methylumbelliferone (4-MU) (both from the Sigma Chemical Co., U.S.A.), dissolved in saline, were infused i.v. at stepwise-increasing rates [0.7, 1.0 and 1.2 μmol·min^{-1}·(100 g body wt)$^{-1}$]. Bile samples were collected at 10-min intervals for 60 min and arterial blood samples (100 μL) were obtained at the midpoint of the bile collection periods.

In another experimental group, [^{3}H]PEG-900 was administered as described above and then a single i.v. dose of DHC (10 μmol/100 g body wt), dissolved in 0.3 mL of saline, was administered and flushed with 0.2 mL of solvent. Bile samples were collected at 2-min intervals for 20 min and arterial blood samples (100 μL) were obtained every 4 min at the midpoint of the bile collection periods. Next, a second injection of an equal dose of DHC was administered and plasma and bile samples were also collected.

Separate groups of animals were injected over a 1-min interval with colchicine (L. Light & Co. Ltd., England) or vinblastine sulfate (E. Lilly Argentina S.A., Argentina) as a single i.v. dose of 0.5 μmol/100 g body wt, dissolved in 0.5 mL of saline. At 60 min, this dose produces a marked reduction of microtubules in rat liver [18]. Renal pedicle ligation was omitted in this group because administration of microtubule poisons diminished markedly the DHC-induced choleresis in rats subjected to this surgical procedure. Chloroquine (Sigma Chemical Co., U.S.A.), a compound that inhibits acidic intracellular compartments [19], was administered in another experimental group as a single i.p. dose (50 mg/kg body wt, dissolved in 0.5 mL of saline). The experiments were initiated 60 min after chloroquine injection as was previously indicated [20]. The pretreated animals were injected i.v. with [^{3}H]PEG-900 (12.5 μCi) and [^{14}C]sucrose (2.5 μCi; 360 mCi/mmol, ICN Pharmaceutical, U.S.A.). Thirty minutes later, DHC was injected and bile samples were obtained as stated above.

For each experimental group, a corresponding control group injected with saline alone was used.

Analytical methods. The volume of bile was determined by weight assuming a density of 1.0 g/mL. Bile flow was expressed as microliters per minute per gram liver.

[^{3}H]PEG-900 and [^{14}C]sucrose were measured by liquid scintillation. For this purpose, aliquots of bile or plasma were mixed in 1 M methylbenzethonium (hyamine) in methanol and 10 mL of 0.4% (w/v) 2,5-diphenyloxazole (PPO) and 0.01% (w/v) 1,4-bis-[2-(5-phenyloxazolyl)]-benzene (POPOP) toluene solution. To avoid spurious counts from chemiluminescence, the yellow-green coloration resulting from the addition of bile into the scintillation fluid was reduced substantially by placing the vials in indirect sunlight for 120 min. Afterward, vials were set in the dark for 6 hr and the radioactivity was measured in a liquid scintillation spectrometer (Beckman Instruments, Inc., U.S.A.). An external standard method was used for quenching correction.

Biliary [^{3}H]PEG-900 concentration was expressed as disintegrations per minute per microliter. Biliary output was calculated as the product of bile flow and bile concentration values corrected by the delay suffered due to the transit time through the biliary tree [21]. Since this method estimates the canalicular concentration interpolating the biliary concentration curves at a later time (interval time plus biliary tree transit time), the biliary output corresponding to the last period of bile collection cannot be calculated.

Statistics. All data are presented as mean values ± SEM. The paired Student's *t*-test was used for comparison within groups, and the unpaired Student's *t*-test for comparison between groups; P values of 0.05 or less were considered statistically significant.

RESULTS

Effects of canalicular choleretics on biliary [^{3}H]*-PEG-900 excretion.* As shown in Fig. 1, bile flow in control rats remained constant throughout the experiments, whereas [^{3}H]PEG-900 output declined gradually. Infusion of DHC gradually increased bile flow, whereas [^{3}H]PEG-900 output rose to a peak reached during the first collection period and decreased thereafter. Unlike DHC, 4-MU infusion did not increase [^{3}H]PEG-900 excretion despite the significant increment in bile flow induced by the choleretic.

Effect of consecutive i.v. injections of DHC on biliary [^{3}H]*PEG-900 excretion.* As shown in Fig. 2, acute administration of DHC induced a fast increment in bile flow, which reached its maximum during the third 2-min period of bile collection followed by a progressive decline. This pattern was reproduced after administration of a second identical dose of the choleretic. Although an initial rapid increment in [^{3}H]PEG-900 excretion was also observed, the maximum value was reached during

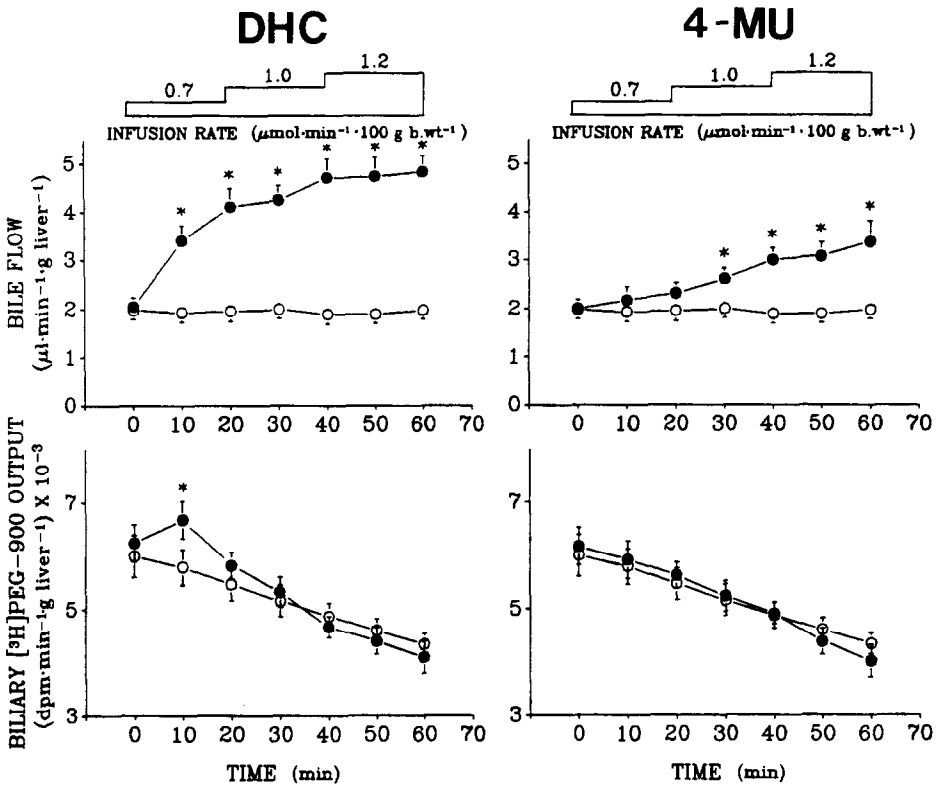

Fig. 1. Bile flow and biliary output of [^{3}H]PEG-900 in rats infused at step-wise increasing rates with DHC or 4-MU. Key: (●—●) DHC or 4-MU infusion (N = 5); (○—○) saline infusion (N = 5). Values are means ± SEM. * Significantly different from control values ($P < 0.05$).

the second 2-min period, thus preceding that of bile flow. On the other hand, the second DHC administration induced a lower excreted amount of the labeled compound in spite of a similar excreted bile volume (Table 1).

Effect of organelle inhibitors on biliary [^{3}H]*PEG-900 and* [^{14}C]*sucrose excretion.* During the basal period, [^{3}H]PEG-900 excretion was not affected by microtubular inhibitors. Conversely, colchicine and vinblastine blocked the DHC-induced excretion of this compound while modifying both bile flow and [^{14}C]sucrose excretion only slightly (Fig. 3).

As shown in Fig. 4, chloroquine treatment significantly diminished [^{3}H]PEG-900 excretion during both basal and DHC-induced choleresis periods, whereas excretion of [^{14}C]sucrose was unaffected.

Plasma disappearance of [^{3}H]*PEG-900.* After [^{3}H]PEG-900 was allowed to equilibrate, its plasma concentration levels dropped following first-order kinetics in all experimental groups. As shown in Table 2, the apparent fractional removal rate was not significantly different in any group when compared to its corresponding control group. The higher plasma removal rate recorded in the groups not subjected to renal pedicle ligature reflects preferential urinary loss of this compound [22].

Since biliary concentration of [^{3}H]PEG-900 declined in parallel with plasma concentration, a constant bile : plasma ratio was observed under basal conditions for each experimental group (Table 2). The bile : plasma ratios were not modified by the treatments, except for the group pretreated with chloroquine. In this group, the lower value obtained was fully accounted for by a lower basal biliary concentration of this compound.

DISCUSSION

Little is known about the mechanism of biliary excretion of PEG-900, and the reports are controversial. It has been proposed that this inert solute, like erythritol or mannitol, may enter the canaliculus by nonrestricted osmotic filtration across the hepatocyte membranes [6–8]. Other authors, however, have postulated a paracellular restricted movement through the tight junctions for this compound [16–19].

The aim of this study was, therefore, to elucidate the mechanism by which PEG-900 reaches the bile. For this purpose, PEG-900 excretion during choleresis induced by canalicular choleretics was analyzed. In addition, to discern whether a vesicular pathway is involved, inhibitors of microtubules or acidic compartments were utilized.

DHC is a synthetic triketo bile salt possessing high choleretic efficiency and a very weak cytotoxic effect [23]. It was selected because its choleretic properties are not affected significantly by microtubule blocking agents [24, 25]. Besides, DHC stimulates the release

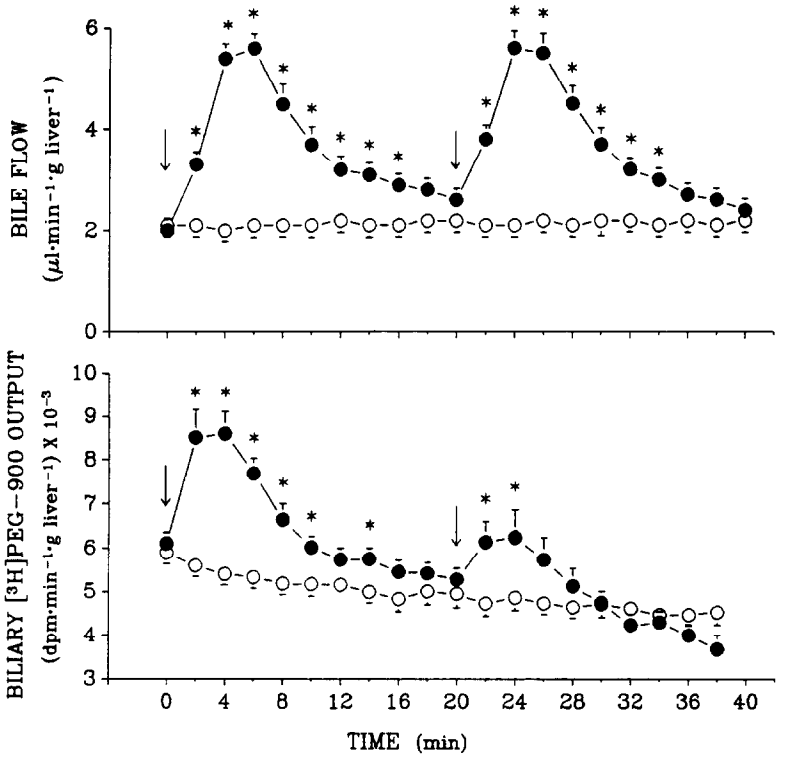

Fig. 2. Effect of DHC on bile flow and biliary output of [^{3}H]PEG-900. Six rats received two consecutive i.v. bolus injections of DHC (10 μmol/100 g body wt) (●). Control values (○) were obtained from six rats that received saline alone. Arrows indicate the administration of DHC or saline. [^{3}H]PEG-900 was administered 30 min before starting the bile collection. Values are means ± SEM. * Significantly different from control values ($P < 0.05$).

Table 1. Cumulative volume of bile and biliary excretion of [^{3}H]PEG-900 after two consecutive administrations of DHC

	Cumulative bile volume (μL/g liver)	Cumulative biliary excretion (dpm/g liver) × 10^{-3}
First injection	35.7 ± 7.0	28.2 ± 4.9
Second injection	29.7 ± 6.5	15.2 ± 5.8*

Two consecutive i.v. doses of DHC (10 μmol/100 g body wt) were administered to six rats. Data correspond to over control values obtained during a 20-min bile collection period after each DHC administration. Values are means ± SEM.

* Significantly different from the first administration value ($P < 0.05$).

of lysosomal content into bile [26, 27], whereas the non-bile salt choleretic 4-MU does not [28]. Thus, both choleretics appear as useful tools to discriminate bile flow associated from vesicular transport mechanisms.

The present results indicated that PEG-900 excretion was not associated with canalicular water movements. The key observations in reaching this conclusion are: (1) 4-MU-induced choleresis did not enhance PEG-900 excretion and only a transient stimulation was observed during DHC-induced choleresis (see Fig. 1), (2) unlike PEG-900, the excretion of erythritol (data not shown) and sucrose (see Figs. 3 and 4), which are transferred into bile mainly by diffusion and solvent drag [1], was closely associated with bile flow, and (3) a second administration of DHC induced a lower PEG-900 excretion than the first administration, whereas bile flow was closely reproduced (see Fig. 2 and Table 1). Thus, taken together, these results suggest that a transport mechanism other than diffusion and solvent drag accounts for PEG-900 excretion.

Conversely, these results may be explained tentatively by a transcytotic vesicular pathway for PEG-900. In fact, a similar behavior following consecutive administrations of bile salt was reported for biliary excretion of the lysosomal enzyme acid phosphatase and attributed to pericanalicular lysosome exhaustion after repeated stimulation [28]. Furthermore, unlike sucrose, PEG-900 excretion was inhibited by colchicine and vinblastine (see Fig. 3). Hence, we can assume that PEG-900 excretion depends on an intact microtubule network.

Unlike sucrose, PEG-900 excretion was also inhibited by chloroquine (see Fig. 4). This suggested that PEG-900 requires functional integrity of acidic hepatocyte compartments for excretion. Compounds that go through hepatocytes via vesicular transport,

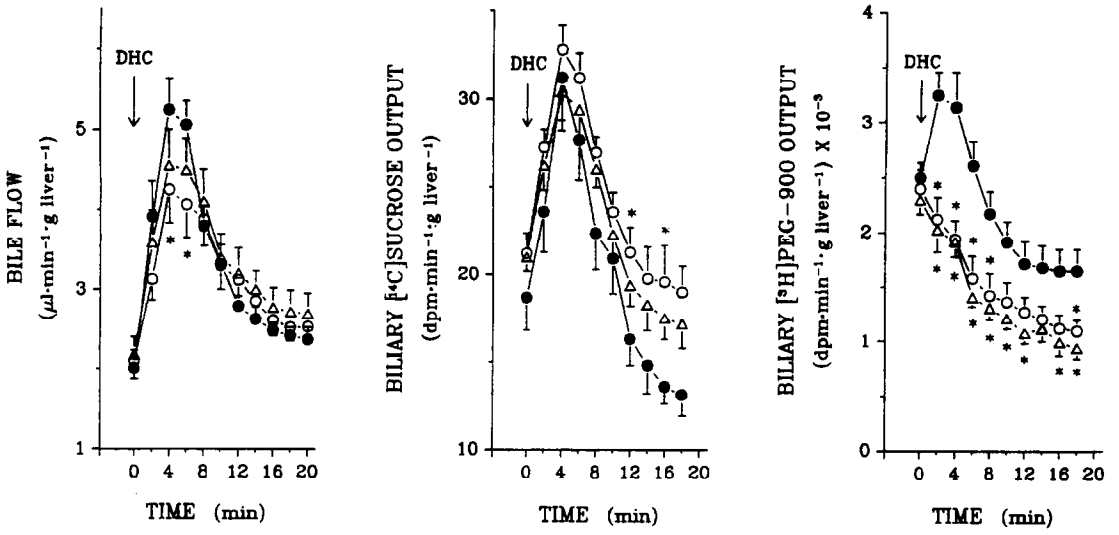

Fig. 3. Bile flow and biliary output of [^{14}C]sucrose and [^{3}H]PEG-900 after i.v. injection of DHC (10 μmol/100 g body wt) in five control rats (●) and four rats pretreated with colchicine (○) or vinblastine (△). The labeled compounds were coadministered 30 min before starting the bile collection. Points represent mean values ± SEM. * Significantly different from control values ($P < 0.05$).

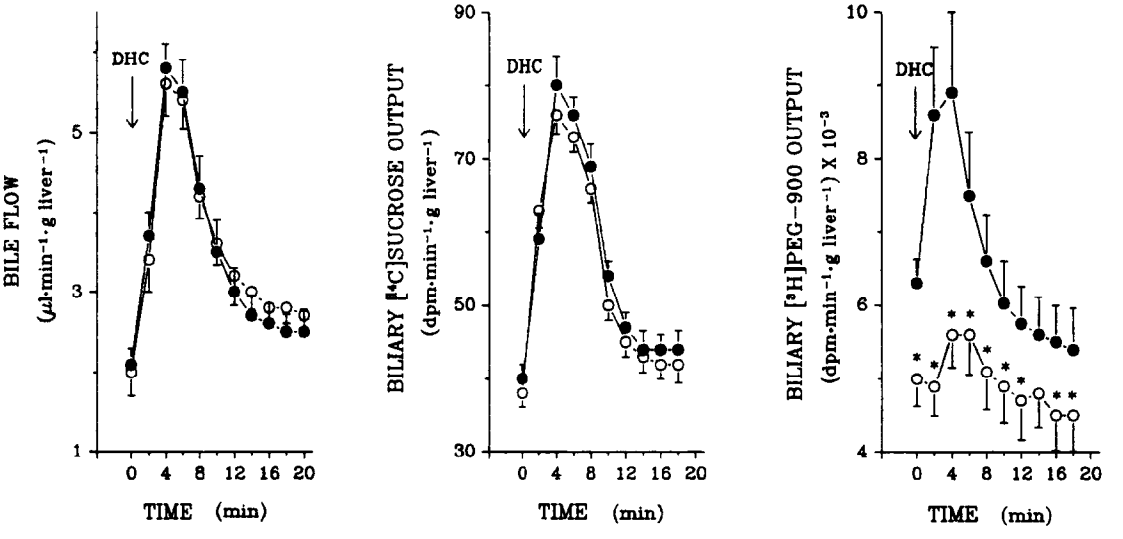

Fig. 4. Bile flow and biliary output of [^{14}C]sucrose and [^{3}H]PEG-900 after i.v. injection of DHC (10 μmol/100 g body wt) in five control rats (●) and in five rats pretreated with chloroquine (○). The labeled compounds were coadministered 30 min before starting the bile collection. Points represent mean values ± SEM. * Significantly different from control values ($P < 0.05$).

such as proteins, are carried to the acidic endosomal compartment and then vesicles containing proteins reach the bile canaliculus either directly or after fusion with pericanalicular lysosomes [29]. However, acidotropic agents only inhibit the last of these routes [27, 30–32]. Thus, we interpreted the inhibition by chloroquine as indicative of a lysosomal pathway for PEG-900 excretion.

Finally, plasma PEG-900 disappearance was not affected by either choleretic administration or by organelle inhibitors (see Table 2). Thus, availability for uptake of this compound cannot account for the observed changes in bile excretion.

The assumption of a bile salt-stimulated vesicular transport for PEG-900 in the hepatocyte is likely. Indeed, several inert solutes are partially excreted into bile via an endocytotic–exocytotic route, although only a small fraction is transported by this

Table 2. Plasma removal rates and basal bile:plasma ratios of [^{3}H]PEG-900 under choleretic infusion or treatment with organelle inhibitors

Groups	Apparent fractional removal rate* (min^{-1}) × 10^3	Basal bile:plasma ratio
Control (ligated) (5)	5.3 ± 0.4	10.0 ± 0.6
DHC infusion† (5)	5.1 ± 0.4	9.3 ± 0.4
4-MU infusion† (5)	5.2 ± 0.5	9.6 ± 0.6
Chloroquine† (5)	5.4 ± 0.3	7.8 ± 0.5‡
Control (non-ligated) (5)	20.9 ± 2.3	9.9 ± 0.5
Colchicine§ (4)	22.6 ± 3.0	10.2 ± 0.6
Vinblastine§ (4)	21.7 ± 2.6	9.6 ± 0.5

Values are means ± SEM. The number of experiments is given in parentheses.

* Apparent fractional removal rate of [^{3}H]PEG-900 was calculated from the slope of the linear semilogarithmic plot of plasma concentration data vs time once the equilibrium period was reached.

† Groups whose renal pedicles were ligated prior to [^{3}H]PEG-900 administration, which should be compared with the control group referred to as "ligated".

‡ Significantly different from control values ($P < 0.05$).

§ Groups without renal pedicle ligature, which should be compared with the control group referred to as "non-ligated". The higher plasma disappearance compared with the "ligated" group reflects rapid urinary excretion of the compound.

mechanism [9–11]. The capacity of PEG-900 to interact with lipidic membranes, thus mediating membrane fusion [33], may help to explain why a vesicular transport would be its preferential excretion mechanism. Finally, bile salts are known to stimulate transcytotic vesicular transport and discharge of vesicular contents into bile [26–28, 34, 35].

In agreement with previous reports [5–7, 13, 14], the basal bile : plasma ratio for PEG-900 far exceeded one (see Table 2). This is not surprising, since vesicular transport of certain endogenous compounds like IgA has been reported to be largely concentrative [36]. Furthermore, it has been determined that exogenous compounds that are sequestered in hepatocyte lysosomes undergo an efficient biliary excretion [37, 38], and compounds with such characteristics are found to be greatly concentrated in bile [39]. On the other hand, PEG-900 may self-associate or associate with other constituents in bile (e.g. micelles), thus preventing its back-diffusion. Since reabsorption of water was proposed to occur from the biliary lumen [5], this compound might be concentrated further by this mechanism.

In conclusion, our results indicated that plasma-to-bile transport of PEG-900 is not associated with canalicular water movements. Instead, it seems to be related to a vesicular transport process followed by a bile acid-stimulated discharge into bile through the lysosomal compartment. Thus, PEG-900 should not be used as a marker of canalicular water movements in the rat. Alternatively, this compound may be useful for exploring hepatocytic vesicular transport.

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