Carrier-mediated uptake of lucifer yellow in skate and rat hepatocytes: a fluid-phase marker revisited

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Ballatori, Nazzareno, David N. Hager, Surajit Nundy, David S. Miller, and James L. Boyer. Carrier-mediated uptake of lucifer yellow in skate and rat hepatocytes: a fluid-phase marker revisited. Am. J. Physiol. 277 (Gastrointest. Liver Physiol. 40): G896–G904, 1999.—Uptake of lucifer yellow (LY), a fluorescent disulfonic acid anionic dye, was studied in isolated skate (Raja erinacea) perfused livers and primary hepatocytes to evaluate its utility as a fluid-phase marker in these cells. However, our findings demonstrated that LY is transported across the plasma membrane of skate hepatocytes largely via carrier-mediated mechanisms. Isolated perfused skate livers cleared 50% of the LY from the recirculating perfusate within 1 h of addition of either 22 or 220 µM LY, with only 4.5 and 9% of the LY remaining in the perfusate after 7 h, respectively. Most of the LY was excreted into bile, resulting in high biliary LY concentrations (1 and 10 mM at the two doses, respectively), indicating concentrative transport into bile canalicular lumen. LY uptake by freshly isolated skate hepatocytes was temperature sensitive, exhibited saturation kinetics, and was inhibited by other organic anions. Uptake was mediated by both sodium-dependent (Michaelis-Menten constant (Km), 125 ± 57 µM; maximal velocity (Vmax), 1.5 ± 0.2 pmol·min⁻¹·mg cells⁻¹) and sodium-independent (Km, 207 ± 55 µM; Vmax, 1.7 ± 0.2 pmol·min⁻¹·mg cells⁻¹) mechanisms. Both of these uptake mechanisms were inhibited by various organic anions and transport inhibitors, including furosemide, bumetanide, sulfobromophthalein, rose bengal, probenecid, N-ethylmaleimide, taurocholate, and p-aminoinhippuric acid. Fluorescent imaging techniques showed intracellular vesicular compartmentation of LY in skate hepatocyte cultures. Studies in perfused rat livers also indicated that LY is taken up against a concentration gradient and concentrated in bile. LY uptake in isolated rat hepatocytes was saturable, but only at high concentrations, and demonstrated a Km of 3.7 ± 1.0 mM and a Vmax of 1.75 ± 0.16 nmol·min⁻¹·mg wet wt⁻¹. These results indicate that LY is transported into skate and rat hepatocytes and bile largely by carrier-mediated mechanisms, rather than by fluid-phase endocytosis.

Lucifer yellow (LY) is a fluorescent disulfonic acid (Fig. 1) that has been used extensively as a fluid-phase marker in different cell types (5, 6, 10, 15, 29, 31, 34, 36, 37), including hepatocytes (11, 18, 23, 26) and Hep G2 cells (13, 14). The properties that make LY a useful fluid-phase marker include 1) a high quantum yield, 2) resistance to bleaching, 3) a low acidic dissociation constant so that it is negatively charged at pH values >2, 4) lack of toxicity to cells at concentrations normally employed to measure fluid-phase endocytosis, and 5) absence of cellular metabolism.

However, the physicochemical properties of this compound would also appear to make it a virtually ideal substrate for organic anion transport systems that are expressed at high levels in liver and other transporting epithelia (8, 21, 24). A major hepatic function is the clearance of a multitude of structurally unrelated metabolite intermediates, drugs, and other foreign chemicals from blood and the excretion of these compounds and their metabolites into bile. To accomplish these functions, hepatocytes are equipped with specialized transport proteins on both their sinusoidal (basolateral) and canalicular (apical) membrane domains, including the organic anion transporter protein (Oatp) family of sinusoidal membrane transporters (19, 21, 25) and the canalicular multidrug resistance-associated protein 2 (Mrp2) transport pump (27). Each of these transport proteins exhibit a broad substrate specificity, although they prefer negatively charged amphiphilic compounds with a molecular mass >250 Da (24). The molecular mass of LY (443 Da), its relative hydrophobicity, two sulfonic acid groups, and net negative charge (Fig. 1) make it a candidate substrate for these organic anion transporters, although this has not been directly examined.

Previous studies that utilized LY as a fluid-phase marker in rat hepatocytes provided only a cursory examination of its transport properties and largely assumed that transport was similar to that observed in nonepithelial cells (11, 18, 23, 26). One of the earliest studies with rat hepatocytes reported that LY uptake was not saturable at concentrations up to 1.09 mM and thus was a useful fluid-phase marker (26). However, the data presented in Fig. 1 of that study suggest the opposite result, particularly when early time points are considered. These data show that the initial rate of LY uptake increased in a nonlinear fashion as concentrations were increased from 0.05 to 0.8 mM, a result consistent with mediated transport (26). Camacho and...
co-workers (11) also reported that LY uptake by rat hepatocytes was not saturable but only up to a concentration of 0.2 mM, leaving open the possibility that uptake occurred by a carrier-mediated mechanism with a Michaelis-Menten constant (K_m) of >0.2 mM. Once internalized, LY is accumulated within intracellular compartments of hepatocytes (26). More recent studies in plant vacuolar membranes indicate that LY is transported into this intracellular organelle by an ATP-dependent organic anion transport system, rather than by fluid-phase endocytosis (17, 30).

The initial goal of our studies was to use LY as a fluid-phase marker in skate hepatocytes, but we quickly realized that the characteristics of transport in this cell type were not consistent with fluid-phase endocytosis. Transport of LY in skate hepatocytes exhibited characteristics of carrier-mediated organic anion transport rather than fluid-phase endocytosis. These observations in the skate were confirmed in rat liver, which also concentrated this organic anion in bile. LY transport in isolated rat hepatocytes also occurred by a saturable, carrier-mediated mechanism. The apparent affinities (K_m) of the uptake systems in skate and rat hepatocytes are relatively low, a factor that may explain the lack of saturability noted in some previous studies of LY transport.

**EXPERIMENTAL PROCEDURES**

**Materials and animals.** Male skates (Raja erinacea; 0.7-1.2 kg body wt) were obtained by trawl from waters in the Gulf of Maine and transported to the Mount Desert Island Biological Laboratory by boat. At the laboratory, skates were maintained in large tanks filled with aerated flowing seawater for up to 4 days before use. The lithium salt of LY-CH, collagenase type I, and deoxyribonuclease II were obtained from Sigma (St. Louis, MO). All other chemicals were obtained from either J. T. Baker (Phillipsburg, NJ) or Sigma. Male Sprague-Dawley rats were obtained from Charles River Laboratories (Kingston, NY) and were fed ad libitum until time of experimentation.

**Isolation of skate hepatocytes.** Skate hepatocytes were isolated as previously described (35). Immediately after isolation, cells were resuspended in elasmobranch Ringer solution (containing in mM: 270 NaCl, 4 KCl, 2.5 CaCl_2, 3 MgCl_2, 0.5 Na_2SO_4, 1 KH_2PO_4, 8 NaHCO_3, 350 urea, 5 d-glucose, and 5 HEPES-Tris, pH 7.5) or in a sodium-free medium (see below) and centrifuged at 250 g. The washed hepatocytes were resuspended at a concentration of 30–50 mg wet weight/ml (3 × 10^7–5 × 10^8 cells/ml), in either normal elasmobranch Ringer or sodium-free medium. The sodium-free medium was prepared by isosmotic substitution of NaCl and NaHCO_3 with either the respective choline or N-methyl-D-glucamine salts and by replacing Na_2SO_4 with MgSO_4 (MgCl_2 was decreased to adjust the Mg^2+ concentration). The cells were preincubated at 15°C in 50-ml polypropylene tubes for 20–30 min before the start of each experiment.

LY uptake into isolated skate hepatocytes. LY dissolved in appropriate elasmobranch Ringer solution was added to 4–8 ml of cell suspension (15–45 mg wet wt/ml) to final concentrations of 20–500 µM, and hepatocytes were incubated at either 4°C or 15°C. Aliquots of 0.5–1.0 ml of cell suspensions were removed at timed intervals and centrifuged in a Beckman Microfuge for 4 s. The supernatant was rapidly removed by aspiration, and extracellular fluid on the surface of the cell pellets was removed by adsorption onto filter paper strips. Cell pellets were then weighed, disrupted, and centrifuged at 4°C at 5 s, and this value was subtracted from all measurements.

**Fluorescence microscopy.** Cell clusters in the chamber were mounted on the stage of a Nikon Diaphot inverted microscope equipped with epifluorescence optics, Nikon fluorescent objectives (×20 phase 3, numerical aperture = 0.8; ×40 oil, numerical aperture = 1.4), a 50-W mercury lamp, and a fluorescein filter set (Nikon B-2A). To avoid photobleaching of the dye, neutral density filters that passed only 1 or 10% of the excitation light were kept in the light path and fluorescence measurements were made over periods of about 1–2 s.

Images of fluorescent tissue were collected as described previously (22). Briefly, transmitted light and corresponding epifluorescence images were acquired through the microscope side port by the use of a Hamamatsu 2400 charge-coupled device video camera connected to an 8-bit video image capture card (Scion Video Image LG-3) in an Apple Macintosh IIcx computer. With the use of image capture and analysis software (Image 1.49, NIH), incoming images could be displayed at the video rate on a high-resolution computer monitor (Apple), and frame averages could be stored on a Pioneer Laser Memory optical disk recorder for later analysis.

**Isolated perfused skate liver preparation.** Skate livers were isolated and perfused according to the method of Reed et al. (28), as modified by Simmons et al. (33). The bile duct was cannulated with a 14-cm segment of polyethylene tubing (PE-90; Clay Adams, Parsippany, NJ). Because the proximal gallbladder and cystic duct are intrahepatic and cannot be ligated, the cystic duct was excluded by inserting a plug at the neck of the gallbladder through an incision at the gallbladder apex. Next, the collateral tributaries of the portal vein were ligated, the portal vein was cannulated with a 2- to 3-cm segment of polyethylene tubing (PE-205) attached to an equal length of latex tubing, and the liver was flushed with 40–50 ml of heparinized (0.6 U/ml) elasmobranch Ringer solution. The liver was then excised and perfused at a rate of 30 ml/min with 250 ml of elasmobranch Ringer solution containing 5 mM glucose and 10 mM HEPES-Tris, pH 7.5. The first 150 ml of perfusate drained from the perfusion dish were discarded.
after single passage, and the remaining 100 ml were collected in a perfusate reservoir and recirculated. The recirculating perfusate was continuously filtered through a 60-µm nylon mesh stretched over a small funnel. The temperature of the perfusion dish and solutions was maintained at 15°C.

LY uptake and biliary excretion by isolated perfused skate liver. After a 30- to 60-min stabilization period, LY was added at an initial concentration of either 22 or 220 µM to the perfusate reservoir. Bile was collected into tared, ice-chilled Microfuge tubes at 1-h intervals, and perfusate samples (1 ml) were taken from the perfusate reservoir at 1-h intervals. All samples were analyzed for LY content with a PerkinElmer spectrofluorometer (excitation at 430 nm; emission 540 nm).

Isolated rat liver perfusions. Livers isolated from male Sprague-Dawley rats were perfused as previously described (4). Animals were anesthetized with 55 mg/kg pentobarbital sodium. A midline incision was made along the abdomen, and the bile duct was isolated and then cannulated with PE-10 tubing. The liver was perfused with an oxygenated Krebs-Henseleit solution (37°C) containing heparin. After the thoracic vena cava was cannulated with PE-205 tubing, the liver was excised from the body cavity and placed inside the perfusion chamber. The perfusate solution was then changed to a Krebs-Henseleit buffer without heparin. Livers were perfused with 100 ml of an oxygenated Krebs-Henseleit buffer containing 5 mM glucose. Liver temperature was maintained at 37–37.5°C. After a 20-min control period, LY was added to the recirculating perfusate at an initial concentration of 100 µM. Perfusate samples were collected from the perfusate reservoir at regular intervals, and bile was collected into tared, ice-chilled Microfuge tubes. Bile flow rates were determined gravimetrically, assuming a density of 1. Liver viability was monitored by perfusion pressure, bile flow rates, gross appearance, and the release of lactate dehydrogenase into perfusate.

Isolation of rat hepatocytes. The isolation protocol was adapted from the method of Seglen (32). Male Sprague-Dawley rats (200–300 g) were fed ad libitum until time of experimentation. Rats were anesthetized with 55 mg/kg ip sodium pentobarbital. The liver was perfused with calcium- and magnesium-free Krebs-Henseleit buffer containing 0.5 mM EGTA, 5 mM HEPES-Tris (pH 7.5), and 2 U/ml heparin. This perfusate was passed through the liver once at a flow rate of 30–35 ml/min. The liver was then perfused with a recirculating collagenase solution containing 2 mg/ml collagenase (Sigma, Type IV) in a magnesium-free Krebs-Henseleit buffer containing 5 mM CaCl₂ and 20 mM HEPES-Tris, pH 7.5. This buffer was recirculated for 8–12 min, the liver was excised and placed in Krebs-Henseleit buffer, the capsule was removed, and cells were filtered through a 60-µm nylon mesh. The cells were then washed with Krebs-Henseleit buffer to remove collagenase. Cell viability was assessed at this point by trypan blue dye exclusion and was found to be >85%. LY uptake was measured in cells (2 × 10⁶ cells/ml) incubated in 37°C Krebs-Henseleit buffer supplemented with 5 mM HEPES-Tris, pH 7.5, and 5 mM D-glucose.

Statistical analysis. Kinetic data from experiments measuring LY uptake into hepatocytes were fit to the Michaelis-Menten equation by nonlinear least-squares regression analysis using SigmaPlot 4.16. Comparison of data measuring initial rates of LY uptake in the presence and absence of inhibitors was performed by unpaired Student's t-test and correlated to P < 0.05.

RESULTS

Efficient hepatic uptake and concentrative biliary excretion of LY in perfused skate liver. When LY was added to recirculating skate liver perfusate at initial concentrations of 22 and 220 µM, –50% of the LY was removed from perfusate in 1 h, and only 4.5 and 9% of the 22 and 220 µM doses, respectively, remained in the perfusate by 7 h (Fig. 2A). LY appeared in bile within 2 h (Fig. 2B), the time that is needed to clear the biliary dead space in the skate liver (28, 32). Biliary LY concentrations reached maximum mean values of 2.75 mM following the 22 µM dose, or >100 times the
maximum perfusate concentration. Because of the rapid clearance of LY from perfusate, the actual bile-to-plasma ratio was probably closer to 1,000:1, indicating active, concentrative hepatobiliary transport. At the 220-µM dose, biliary LY concentrations continued to increase over 7 h, reaching a mean concentration in bile of 13.5 mM at the end of the experiment (Fig. 2B).

Carrier-mediated uptake of LY by isolated skate hepatocytes. The data from perfused skate livers showed rapid clearance of LY from the perfusate and concentration of the dye in bile, two results that were inconsistent with simple diffusion or fluid-phase endocytosis. To begin to characterize the mechanisms of LY transport, we incubated isolated skate hepatocytes in medium with LY and measured cellular accumulation. LY uptake by isolated hepatocytes incubated at 15°C increased with time of incubation and approached steady state after ~2 h (Fig. 3). Uptake was diminished when the temperature was decreased to 4°C (Fig. 4A) or when sodium was replaced with either choline or N-methyl-D-glucamine (Fig. 4B), indicating the presence of temperature-sensitive and sodium-dependent and -independent components of uptake.

Saturability of LY transport by isolated skate hepatocytes was assessed by measuring initial rates of uptake with increasing LY concentrations (20–500 µM) in normal elasmobranch Ringer and in medium in which sodium was replaced with choline (Fig. 5). The difference in uptake values was considered the sodium-dependent component. LY uptake in the presence and absence of sodium was a nonlinear function of concentration (Fig. 5). When these data were fit to the Michaelis-Menten equation, it provided apparent $K_m$ values of $125 \pm 57$ and $207 \pm 55$ µM and $V_{max}$ values of $1.5 \pm 0.2$ and $1.7 \pm 0.2$ pmol·min$^{-1}$·mg cells$^{-1}$ for the sodium-dependent and sodium-independent components, respectively.

The effects of transport inhibitors on initial rates of LY uptake by skate hepatocytes was also measured in the presence and absence of sodium (Fig. 6). Inhibition of the sodium-dependent component of LY uptake was seen with 1 mM p-aminohippuric acid (19% inhibition), 100 µM DIDS (17%), 100 µM taurocholate (36%), 100 µM probenecid (58%), 25 µM rose bengal (59%), 50 µM sulfobromophthalein (72%), 100 µM bumetanide (76%), and 100 µM furosemide (85%). N-ethylmaleimide (1 mM) also inhibited LY uptake (64%), whereas mercuric chloride (25 µM) had no effect. Roughly comparable inhibitory effects of these compounds were noted for sodium-independent component of LY uptake (Fig. 6B).

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Fig. 3. Time dependence of LY uptake into isolated skate hepatocytes. LY was added at either 20, 100, or 500 µM, and hepatocytes were incubated at 15°C in elasmobranch Ringer for up to 3 h. Values are means ± SD of 4 experiments, each performed in duplicate.

Fig. 4. Effects of temperature (A) and sodium substitutions (B) on LY uptake into isolated skate hepatocytes. Uptake of 100 µM LY was measured at 15°C in normal elasmobranch Ringer or in Ringer in which the sodium was replaced with either choline or N-methyl-D-glucamine (NMDG). Values are means ± SD of 5 experiments, each performed in duplicate.
One advantage of fluorescent substrates to study transport in skate hepatocyte clusters is that a significant fraction of the clusters retain a patent bile canalicular space (22). Indeed, we previously used video microscopy to demonstrate uphill secretion of a fluorescent taurocholate derivative into that space (22). To determine the distribution of LY within hepatocytes and hepatocyte clusters, we imaged cells that had been incubated in medium with 1 µM LY. These hepatocytes showed accumulation of the fluorescent dye within the cells, and the intracellular distribution of dye was nonuniform, suggesting vesicular accumulation (Fig. 7A). When 100 µM taurocholate was also in the medium, overall cellular fluorescence was greatly reduced (Fig. 7D). Some control clusters also showed accumulation of LY within the canalicular space (Fig. 8), and in those clusters the fluorescence intensity of the canalicular space clearly exceeded that of the cells, supporting the results obtained in the perfused skate liver model.

Carrier-mediated uptake of LY by rat hepatocytes. To examine whether the findings in the skate could be extended to mammalian hepatocytes, additional studies measured hepatobiliary excretion of LY in the isolated perfused rat liver and LY uptake in isolated rat hepatocytes. As in the skate, the isolated rat liver rapidly cleared LY from recirculating perfusate (100 µM initial concentration) and excreted this organic anion into bile in high concentrations (Fig. 9). Biliary LY concentrations were about 6 mM, indicating concentrative transport into bile (Fig. 9). Nearly 70% of the dose was recovered in bile over an 80-min collection interval.

Fig. 5. Kinetics of LY uptake into isolated skate hepatocytes. A: sodium-dependent uptake (i.e., total uptake minus uptake seen in choline medium). B: uptake in choline-containing medium (sodium-independent uptake). Initial uptake rates (15 min) were measured in the presence of 20, 50, 80, 100, 200, 300, 400, and 500 µM LY at 15°C in either normal elasmobranch Ringer or in Ringer in which the sodium was replaced with choline. The difference was considered the sodium-dependent component (A). Values are means ± SD of 4 experiments, each performed in duplicate.

Fig. 6. Effects of transport inhibitors on the sodium-dependent (A) and sodium-independent (B) components of LY uptake into isolated skate hepatocytes. Initial uptake rates of 100 µM LY were measured at 15°C in normal elasmobranch Ringer or in Ringer in which the sodium was replaced with choline. Inhibitors were added at the following concentrations (µM): taurocholate 100, sulfobromophthalein (BSP) 50, bumetanide 100, p-aminohippuric acid (PAH) 1,000, DIDS 100, mercuric chloride 25, N-ethylmaleimide (NEM) 1,000, furosemide 100, probenecid 100, and rose bengal 25. Values are means ± SD of 4 experiments, each performed in duplicate.
LY uptake was also measured in isolated rat hepatocytes, and the data were consistent with transport via a low-affinity carrier-mediated mechanism. The initial 1-min uptake of LY in rat hepatocytes was a nonlinear function of extracellular LY concentrations (Fig. 10). When the data were fit to the Michaelis-Menten equation, they provided an apparent $K_m$ of $3.7 \pm 1.0$ mM and a $V_{\text{max}}$ of $1.75 \pm 0.16$ nmol·min$^{-1}$·mg wet wt$^{-1}$. In contrast to skate hepatocytes, LY uptake into rat hepatocytes was not affected by substituting choline for sodium in the incubation medium (data not shown), indicating that uptake is mediated largely by a sodium-independent mechanism.

**DISCUSSION**

The ideal marker for fluid-phase endocytosis would be a compound that does not enter cells by any carrier-mediated process and that has a low intrinsic membrane passive permeability. In many cells, particularly nonpolarized cells, LY fulfills these criteria (5, 6, 10, 15, 29, 31, 34, 36, 37). However, because LY is negatively charged at physiological pH, it is a candidate substrate for transport systems that are specifically designed to carry organic anions across cell membranes. Such systems are generally present in transporting epithelia, and, consistent with this, recent experiments indicate that LY is a substrate for transporters in renal proximal tubule (20). Liver cells also contain several multispecific organic anion transporters (the Oatp and Mrp families), which are particularly well suited to the transport of compounds that share the physicochemical properties of LY (8, 21, 24). Thus, although LY undoubtedly enters and leaves hepatocytes by fluid-phase
mechanisms, this mode of transport might not be the major route of entry or exit.

The first indication that fluid-phase endocytosis plus simple diffusion could not account for the transport of LY in hepatocytes came from our experiments with isolated perfused skate livers (Fig. 2). LY was rapidly removed from the perfusate and excreted into bile at extraordinarily high concentrations relative to plasma. Both the appearance time of LY in skate bile and the high bile-to-plasma ratios seen are comparable with published data for the biliary excretion of specifically transported organic anions (e.g., sulfobromophthalein and taurocholate) and very different from those of compounds not specifically transported (e.g., sucrose and mannitol) (9).

Several aspects of the transport of LY in isolated skate hepatocytes strongly argue for specific, carrier-mediated transport. Perhaps most importantly, uptake is saturable (Fig. 5) and cellular accumulation is concentrative (Fig. 3). After 3 h of incubation in medium with 100 µM LY, cellular accumulation averaged about 50 pmol/mg wet wt (Fig. 3). This corresponds to an estimated cell-to-medium concentration ratio of about 0.5. Although at first glance a ratio of less than unity may not appear to indicate concentrative transport, it is important to consider that LY is negatively charged and that the membrane electrical potential significantly restricts entry. Indeed, a ratio of 0.5 is much higher than calculated from the Nernst equation for a divalent organic anion passively distributed across a membrane, supporting an electrical potential of ~60 mV (i.e., ~0.01).

Our observations in the skate were supported by studies in rat liver. LY was rapidly removed from the sinusoidal circulation of rat liver and actively concentrated in bile. LY uptake in isolated rat hepatocytes was saturable, with an apparent $K_m$ of $3.7 \pm 1.0$ mM and a $V_{max}$ of $1.75 \pm 0.16$ nmol·min$^{-1}$·mg wet wt$^{-1}$ (Fig. 10), indicating a relatively low affinity but high transport capacity. In contrast to skate liver, the rat liver uptake mechanism displayed no sodium dependence, indicating transport by one of the sodium-independent organic anion transporters.

Two types of transport systems are present on the basolateral membranes of rat and human hepatocytes that function as carriers for organic anions. Sodium-dependent transporters include the bile acid (taurocholate) transporter Ntcp, and sodium-independent transporters include the multispecific organic solute carriers Oatp1 and Oatp2 (21, 25). Analogous transport systems have been described in livers from lower vertebrates, including the dogfish shark and small skate, and a multispecific organic anion transporter has been expressed in Xenopus laevis oocytes injected with skate liver mRNA (16). However, these skate liver transport proteins have not yet been cloned. Probes to the rat and human liver transporters do not give positive signals on Northern blot analysis of skate liver, indicating that these proteins are significantly different from those in this primitive vertebrate species (16). LY uptake by skate hepatocytes was partially sodium dependent. Replacing sodium with choline or N-methyl-D-glucamine reduced uptake by ~50%. Sodium dependence is clearly characteristic of carrier-mediated rather than endocytic transport processes. Sodium-dependent uptake of amino acids, organic anions, and other solutes has previously been measured in skate hepatocytes (1–3, 7, 16, 35), and these processes appear similar to those of mammalian liver.

Both the sodium-dependent and the sodium-independent components of uptake in skate hepatocytes were saturable and had moderate affinities for LY; apparent $K_m$ values were 100–200 µM, whereas in rat hepatocytes this value was higher (3.7 mM). These values are comparable to those observed for many substrates of mammalian Oatp1, Oatp2, and Ntcp. For example, although the $K_m$ for sulfobromophthalein uptake on Oatp1 is only 2 µM, other substrates display a lower affinity, including taurocholate (50 µM) and ouabain (1.7 mM) (21). Thus it is possible that the failure of previous studies to detect a saturable transport system for LY is related to its relatively low affinity for the carrier. Indeed, retrospective examination of published characteristics of LY uptake in rat hepatocytes demonstrates that LY uptake probably was rate limited in one study (26), although the authors reached a different conclusion, whereas another study used LY concentrations that were below the $K_m$ value for rat hepatocytes (11).

The substrate inhibition studies in skate hepatocytes also suggest that LY is transported by both sodium-dependent and -independent multispecific organic anion transport carriers typically found on the sinusoidal membrane of hepatocytes. Although one cannot conclude from cis inhibition studies that the inhibitors are also transported by these carriers, competition from these compounds for LY uptake would not be expected if LY were passively entering these cells as a fluid-phase marker.
Finally, our initial imaging experiments in the skate hepatocyte studies confirm both the perfused liver and isolated hepatocyte studies. First, isolated skate hepatocyte clusters showed a pattern of dye distribution similar to that previously seen with a fluorescent taurocholate derivative (22). This taurocholate derivative was taken up by a sodium-independent, sinusoidal organic anion transporter and secreted into bile on a potential-dependent luminal transporter (22). As with the taurocholate derivative, LY was accumulated by skate hepatocytes and was further concentrated in bile canaliculi. Similar to the uptake studies, LY accumulation measured by fluorescence microscopy was reduced substantially by the inclusion of taurocholate in the culture medium. In addition, the fluorescence images suggest a nonuniform distribution of LY within skate hepatocytes. A similar nonuniform intracellular compartmentment of LY was recently reported for renal proximal tubule (20) and has been described for other fluorescent organic anions in rat hepatocytes (12). The significance of this compartmentment for LY transport remains to be determined.

In summary, the functional properties of hepatic uptake of LY that demonstrate saturation, competition by other substrates, and sodium dependence (in skate hepatocytes), as well as highly concentrative transport into bile, are features typical of carrier-mediated organic anion transport. Caution should therefore be exercised when using this compound as a putative fluid-phase marker in liver and other tissues that express multispecific organic anion transport mechanisms.

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