CARRIER MEDIATED UPTAKE OF LUCIFER YELLOW IN LIVER OF RAJA ERINACEA, THE LITTLE SKATE: A FLUID PHASE MARKER REVISITED

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Lucifer Yellow (LY) is a 443 Dalton fluorescent disulfonic acid organic anionic dye which has been used as a fluid phase marker in a number of different cell types, including hepatocytes (Camacho et al. Hepatology 17:661-667, 1993; Oka et al. J. Biol. Chem. 264:12016-12024, 1989). To verify its usefulness as a fluid phase marker we examined its transport properties in isolated skate hepatocytes and perfused liver preparations. Our findings indicate that transport of LY by skate liver is characteristic of carrier mediated organic anion transport rather then a fluid phase marker.

Isolated hepatocyte suspensions were obtained from skate liver by collagenase perfusion as previously described from this laboratory (Smith et al. J. Exp. Zool. 241:291-296, 1987). LY (20-500 uM) was added to hepatocytes suspended in elasmobranch Ringers (15-45mg cells/ml) maintained at 15° C. To measure uptake of LY, 0.5-1.0 ml aliquots were removed at timed intervals and centrifuged in a Beckman microfuge for 5 sec. The supernatant was discarded and packed cell pellets carefully dried, weighed and disrupted in 4% salicylic acid. The resulting suspension was centrifuged for 20 sec. The supernatant was then assayed for LY content in a Perkin Elmer spectrofluorometer (excitation at 430 nm; emission 540 nm). To correct for the residual LY in extracellular space of the packed cell pellets, LY uptake was also measured at 4° at short time intervals (5 sec) and this value subtracted from all measurements. LY (22 and 220 uM) was also added to isolated perfused skate liver preparations to assess its biliary excretion. Finally isolated hepatocyte clusters were also examined to visualize the intracellular compartmentalization of LY.

Hepatic uptake of LY was a linear function of time at all LY concentrations for up to 30 min, and reached stable levels in the cells at about 2 hr. When cells were preloaded with LY, the time course of LY efflux was similar to that of uptake. However when cells were incubated with LY at 4°C, uptake was markedly inhibited (69%, n=6 experiments). When sodium was replaced by choline or N-methyl D-glucamine, hepatic uptake of LY at 60 min was inhibited significantly in 5 separate experiments from 40 to 56%.

To determine whether the hepatic uptake of LY was saturable, initial uptake rates

were determined at 15 min at increasing LY concentrations from 20, 50, 80, 100, 200, 300, 400 and 500 uM in the presence and absence of sodium. The difference in uptake between sodium-containing and sodium-free media was considered the sodium-dependent component. LY uptake was saturable both in the presence and absence of sodium. Sodium dependent LY uptake demonstrated a Km of 138 ± 60 uM and a Vmax of 1.5 ± 0.22 pmol min^{-1} mg^{-1}. Sodium independent LY uptake demonstrated a Km of 207 ± 55 uM and a Vmax of 1.71 ± 0.19 pmol min^{-1} mg^{-1} (mean ± S.E. determined by a curve fitting Enzfitter program, n = 3 experiments).

Substrate inhibition studies of LY uptake were performed by addition of various organic anions, $HgCl_2$, and N-ethylmaleimide (NEM), in 4 separate experiments performed both in the presence and absence of sodium. Substrates that inhibited sodium dependent LY uptake included 1mM para-aminohippuric acid (19.3%), 100 uM taurocholate (36.3%), 100 uM probenecid (57.7%), 25 uM Rose Bengal (59.3%), 50 uM bromosulphathalein (71.7%), 100 uM bumetanide (76.3%), and 100 uM furosemide (85.1%). NEM (1mM) also inhibited LY uptake (64.3%) in contrast to 25 uM $HgCl_2$ which had no inhibitory effect. Similar inhibitory effects of these compounds were noted for sodium independent uptake of Lucifer Yellow.

To confirm that LY was transported into skate hepatocytes by carrier mediated mechanisms rather than fluid phase endocytosis, LY was added to the perfusate of isolated perfused skate liver preparations to examine its hepatobiliary transport. Approximately 50% of the original dose of LY (22 and 220 uM) was removed by 1 hour and only 9% and 4.5% of the 220 uM and 22 uM dose respectively remained in the perfusate by 7 hours. LY appeared in bile within two hours, the time that is needed to clear the biliary dead space. Bile LY concentrations reached maximum concentrations of 2.75 mM by 3-5 hours following the 22 uM dose, approximately 100 times the maximum perfusate concentration. Biliary concentrations of LY following the 220 uM dose, continued to increase over 7 hours reaching a mean concentration in bile of 13.5 mM at the end of the experiment.

To determine if LY was compartmentalized within the hepatocyte, clusters of hepatocytes were examined by a fluorescent imaging system after exposure to LY. LY accumulated within intracellular vesicular compartments as previously described with rat hepatocytes (Oka et al. J. Biol Chem. 264:12016-12024, '89).

Contrary to previously published studies, the present findings indicate that LY enters the hepatocyte via saturable sodium dependent and sodium independent mechanisms characteristic of organic anion transport systems in this and other species rather than by fluid phase endocytosis. The major evidence for this conclusion is the saturability of the uptake mechanism. For a fluid phase marker, uptake rates should vary linearly with external marker concentrations over a broad range. The ranges of LY concentration used in this study were comparable to those previously reported, although it is not certain that initial rates of hepatic uptake were obtained over linear portions of the uptake curves in previous reports. Initial uptake rates in skate liver are significantly slower than in mammalian liver allowing for measurements of linear uptake at later time points. It is also possible that species differences exist. However, previous studies clearly indicate that

organic anion transport systems analogous to similar systems in mammalian liver have evolved in these primitive elasmobranchs. Furthermore, the chemical structure with two sulfonic acid groups and its molecular size suggest that LY should be a substrate for hepatic organic anion transport. The substrate inhibition studies support this conclusion. The ability of the perfused liver to accumulate LY in bile at bile:plasma concentration ratios of > 100:1 also strongly suggests that this solute is actively transported into bile as observed with many other organic anions. Experiments carried out in the presence and absence of sodium suggest that there are at least two different transport systems of similar affinity and capacity for LY uptake, similar to findings for other organic substrates such as bumetanide (Blumrich et al. Amer. J. Physiol. 28:G926-G933, '93). Preliminary findings also suggest that LY uptake is chloride dependent.

Finally, the apparent accumulation of LY within intracellular compartments observed with fluorescence imaging techniques suggest that transport systems for LY may be present in intrahepatic vesicular compartments. (Supported by National Institutes of Health Grants ES 03828, DK 340989 and 25636 and a Burroughs Wellcome Fund Grant).