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Vesicular Uptake System for the Cation Lucigenin in the Rat Hepatocyte

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SUMMARY

The hepatic transport mechanism for the fluorescent bivalent hydrophilic organic cation lucigenin (LU) was characterized employing kinetic and morphological methods. The extraction of LU by the perfused rat liver was 50% and uptake was saturable. LU did not inhibit the carrier-mediated hepatic uptake of the model organic cationic compounds tributylmethyl ammonium (type 1) and vecuronium (type 2) in isolated hepatocytes, whereas the uptake of LU in the perfused liver was not affected by either type of cation or by the cardiac glycoside cymarin, a potent type 2 inhibitor. The cytoskeleton-disrupting agents cytochalasin B and nocodazole, however, significantly lowered hepatic uptake of LU. In the intact liver, LU did not stimulate fluid phase endocytosis, as indicated by a lack of effect on the internalization of horseradish peroxidase. These kinetic data point to adsorptive endocytosis as the most probable uptake mechanism. This was confirmed by the inhibitory effect of neomycin and the polycation poly(L-lysine) on LU uptake. Fluorescence microscopy revealed that LU accumulated in the hepatocytes in discrete vesicular structures. Partial co-localization of rhodamine-dextran and acid phosphatase with LU indicated that part of the LU fluorescence was present in lysosomes, although not all lysosomes contained LU. Taken together, we conclude that we identified a novel vesicular pathway for uptake of organic cations by hepatocytes.

One of the major hepatic functions is the uptake and excretion of a large variety of compounds supplied to the liver via portal and arterial blood. Among the drugs that enter the hepatic circulation are many organic cations. Lipid-soluble drugs may enter the hepatocyte by passive diffusion, whereas hydrophilic compounds may need translocating machinery to gain access to the cytoplasm. For organic cations, at least two carrier-mediated transport systems are known to operate, one for relatively large, often bivalent (type 2), cations, and a second one for smaller, mostly monovalent (type 1), cationic drugs (1, 2). The role of these systems in hepatic uptake of low molecular weight compounds in general is not yet completely defined. Kinetic experiments and photoaffinity labeling studies indicate that the presently identified uptake systems may transport substrates with a large variety in structure and charge (1, 2), refuting the traditional charge-specific uptake mechanisms that have been postulated before (2). Type 1 and type 2 cations are known to accumulate in lysosomes (3–5) or mitochondria (6, 7) of hepatocytes by partitioning from the cytoplasm, which always leaves a considerable portion of the drug in the cytoplasmic compartment (8, 9).

The fluorescent dye LU (bis-*N*-methylacridinium nitrate) is a large bivalent cation that, considering its structural features, might enter the cell via the type 2 carrier protein used by drugs like vecuronium and *d*-tubocurarine (10). In the present study, we examined the mechanism of hepatic uptake of LU in combination with its intracellular localization.

Experimental Procedures

Materials. Male Wistar rats of 280–320 g were used in the experiments. They were fasted overnight before the liver perfusion and fed *ad libitum* before hepatocyte isolation. LU was obtained from Molecular Probes (Eugene, OR). Fatty acids, Triton X-100, tyloxapol, staining reagents for cytochemistry, collagenase, rhodamine 123, rhodaminated dextran, neomycin sulfate, cytochalasin B, and nocodazole were from Sigma Chemical Co. (St Louis, MO). HRP and poly(L-lysine) (molecular weight 3800) were obtained from Fluka AG (Buchs SG, Switzerland). [*methyl*-^{14}C]vecuronium bromide was synthesized by Dr. F. Kaspersen (Organon Drug Metabolism Research Labs, Oss, The Netherlands) and [^{3}H]tributylmethylammonium iodide was synthesized by Dr. W. E. M. Mol. All other chemicals were from sources described in recent previous papers from this laboratory (10, 11).

Lipophilicity and protein binding of LU. The octanol-water partitioning behavior of LU was determined (12) using Krebs-Henseleit solution (118 mM NaCl, 5 mM KCl, 1.1 mM $MgSO_4$, 2.5 mM $CaCl_2$, 1.2 mM KH_2PO_4, 25 mM $NaHCO_3$) with or without one of the following

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ABBREVIATIONS: LU, lucigenin; BSA, bovine serum albumin; EGTA, [ethylenebis(oxyethylenenitrilo)]tetraacetic acid; HBS, Hanks' balanced salt solution; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; HRP, horseradish peroxidase; KHB, Krebs-Henseleit bicarbonate buffer.

components: 1% Triton X-100, 1% tyloxapol, 0.5 mM taurocholic acid, 0.5 mM NaI, 0.5 mM stearic acid, 0.5 mM undecylenic acid, and 0.5 mM EDTA. LU fluorescence was measured on a SLM-Aminco SPF 125C (excitation 369 nm, bandpass 2.5 nm; emission 510 nm; bandpass 5 nm).

Binding of 10 μM LU to 150 μM BSA was determined at 22° in KHB (118 mM NaCl, 5 mM KCl, 1.1 mM $MgSO_4$, 2.5 mM $CaCl_2$, 1.2 mM KH_2PO_4, 25 mM $NaHCO_3$, saturated with 95% O_2/5% CO_2, pH 7.40) containing 10 mM HEPES, 10 mM glucose, and 150 μM BSA, as used in the hepatocyte preparation. Assays were done in triplicate at 22° by ultrafiltration, in a Micro Partition system equipped with YMT membranes (Amicon, Oosterhout, The Netherlands). LU fluorescence was measured upon dilution with sodium phosphate buffer (330 mM, pH 5.40).

Isolated perfused rat liver. The surgical technique and design of the perfusion apparatus have been described in detail elsewhere (13). The livers were perfused in an antegrade recirculating fashion, as described (14). In short, 100 ml of KHB, containing 10 mM glucose and 1% (w/v) BSA, was used as perfusion medium. The flow rate was established at 3.5 ml/min/g of liver, at a hydrostatic pressure of 10 cm. Oxygenation was effected by gassing with 95% O_2/5% CO_2 and pH was maintained between 7.35 and 7.45. Bile flow was stimulated by continuous infusion of taurocholate to a steady state medium concentration of 10 μM. The liver was allowed to stabilize for 30 min before a compound was injected. Viability was monitored during the experiment by checking bile flow, medium flow, pH, and gross appearance of the liver.

Hepatocyte preparation. To isolate hepatocytes, a modification of the procedure of Berry and Friend (15) was used. Nonfasted rats (290–320 g) were anesthetized with sodium pentobarbital (60 mg/kg, intraperitoneally). Upon cannulation of the portal vein, the liver was perfused with a nonrecirculating HBS (112 mM NaCl, 5.4 mM KCl, 0.88 mM KH_2PO_4, 0.66 mM Na_2HPO_4, 25 mM $NaHCO_3$, 10 mM glucose, saturated with 95% O_2/5% CO_2, pH 7.45), without Ca^{2+} or Mg^{2+} and with 0.5 mM EGTA, for 10 min at 37°. During perfusion the liver was isolated. A 2-min perfusion with HBS with 2 mM Ca^{2+} then removed EGTA from the liver. Collagenase (0.03%) was added to 120 ml of HBS, 2 mM Ca^{2+}, and was recirculated for about 10 min. During the whole procedure, flow was maintained at 30 ml/min and liver entry pressure did not exceed 9 cm H_2O. Isolated cells were incubated for 7 min in a shaker-water bath (37°) under a carbogen atmosphere. After filtration through nylon gauzes (100- and 50-μm mesh, respectively) and cooling on ice, the cells were washed three times with 100 ml of KHB containing 10 mM HEPES, 10 mM glucose, and 150 μM BSA. Cells were collected by centrifugation at 40 × g for 2 min. The final pellet was resuspended in the wash buffer to a concentration of 2.5 × 10^6 cells/ml and kept on ice until use. Viability was routinely tested. Cells were considered suitable for an experiment when more than 90% excluded 0.04% trypan blue.

Kinetic interactions. The effect of 2 μmol of procainamide ethobromide, tributylmethyl ammonium iodide, vecuronium, cymarine, verapamil, and rhodamine B, of 2–5 μmol of neomycin, and of 0.08–0.30 mmol of poly(L-lysine) on the kinetics of 0.5 μmol of LU were established in the isolated perfused liver preparation. The putative inhibitors were injected 2 min before LU. Medium samples were taken at 2, 4, 6, 8, 10, 15, 20, 25, and 30 min after LU addition. Bile fractions of 5 min were collected throughout the experimental period. The microfilament-disrupting agent cytochalasin B (1–4 μmol) and the microtubule-depolymerizing drug nocodazole (5 μmol) were added 30 min before LU.

To examine the effect of LU on fluid phase endocytosis, perfusions were carried out with 50 units of HRP in the presence or absence of 5 μmol of LU. HRP activity in medium samples was measured according to the method of Pütter (16).

The influence of 60 μM LU on the uptake of 15 μM [^{3}H]tributylmethylammonium iodide and [^{14}C]vecuronium bromide was determined in isolated rat hepatocytes. Three milliliters of cell suspension (2.5 × 10^6 cells/ml) were incubated for 30 min at 37° under continuous shaking and gassing with 95% O_2/5% CO_2. Then, substrate was added and samples of 100 μl were taken at 30, 60, 90, 120, 150, 180, and 210 sec. Cells were separated from medium by pipeting the sample onto the center of a Whatman GF/C filter (24 mm). Filtration was performed under a constant vacuum of 600 mbar. The cells were washed three times with 1 ml of ice-cold Krebs-Henseleit solution. Washing with more solution did not change the amount of substrate remaining on the filter. The filters were placed in glass scintillation vials, mixed with 5 ml of Plasmasol (Packard, Groningen, The Netherlands), and counted in a Beckman LS 1800 liquid scintillation counter. Adsorption of label to the filters always stayed below the detection limit. Inhibition by LU was studied near the K_m of the substrate, being 15 μM for both tributylmethylammonium iodide and vecuronium. LU was added 10 sec before the substrate in a 4-fold higher concentration (60 μM), compared with vecuronium and tributylmethylammonium iodide.

Fluorescent labeling of the liver. Rhodamine-dextran was dissolved in 20 mM sodium phosphate buffer, 150 mM NaCl, pH 7.4, and purified by gel filtration on a Sephadex G-25 column (2.5 × 50 cm) to remove low molecular weight components (17). The stock solution was further dialyzed and concentrated by ultrafiltration in a Micro Partition System equipped with YMT membranes (Amicon), at 21°, until the ultrafiltrate was free from rhodamine fluorescence. LU was dissolved in water and rhodamine 123 in dimethyl sulfoxide.

The medium of a cyclically perfused rat liver was injected with fluorescent probes after 30-min stabilization, as described above. LU was added to a concentration of 5 μM and rhodamine 123 and rhodamine-dextran to 10 μM, alone or simultaneously with LU. Thirty minutes after injection, pieces of liver were immediately frozen in liquid Freon (−96°). Liver sections of 8 μm were cut in a cryostat (−20°) and air-dried.

Fluorescence microscopy. Seven microliters of each hepatocyte suspension were placed on a microscope slide and covered with a cover slip. Liver sections were observed unmounted. A Leitz Wetzlar Orthoplux microscope with the Leitz I2/3 filter set (450–490 nm excitation, 515 nm barrier) was used for specific observation of yellow (LU) fluorescence, and the Leitz N2 filterset (530–560 nm excitation, 580 nm barrier) for observation of red (rhodamine) fluorescence. Photographs were taken using Kodak Ektachrome II film at 400 ASA, with an exposure time between 1 and 4 min.

Staining reactions. Demonstration of acid phosphatase activity in liver sections was carried out as described by Hardonk *et al.* (18).

Fat droplets were visualized after fixation for 10 min in 4% formaldehyde. Upon rinsing with distilled water and a short immersion in 60% 2-propanol, the sections were stained for 10 min in 11 mM Oil Red in 98% 2-propanol (filtered). Upon another short immersion in 60% 2-propanol, counterstaining was done with Mayer's hematoxylin. Sections were mounted in glycerine-gelatine.

Statistical methods. Comparison between two means was made with Student's *t* test, after checking equality of variances with a *F* test. A dose range in the kinetic experiments was considered to be one dose, because variability appeared to be independent of the dose. Microphotographs are representatives of three to five experiments each.

Results

Protein binding and lipophilicity of LU. Protein binding of 10 μM LU to 150 μM BSA was nearly absent; only 5% (0.5 μM) LU was bound, despite the 15-fold molar excess of BSA.

The lipophilicity of LU was extremely low; the compound could not be detected in the octanol phase, and a possible decrease in LU concentration in the aqueous phase due to partitioning into octanol was not observed. Addition of the putative complexing anions to neutralize the positive charge did not change partition behavior and neither did the solubilizing activity of Triton X-100 or tyloxapol.

Disposition of LU by the perfused rat liver. Hepatic clearance of 5 μM LU by the perfused rat liver was 14.4 ml/

min, corresponding to an extraction of 46% at a flow rate of 31 ml/min (Fig. 1). About 30 to 40 min after injection, the medium concentration fell below the detection level. Until that time, LU disappeared with apparent monophasic first-order kinetics, indicating that no significant sinusoidal efflux was present. At a 10 times higher LU concentration (50 μM), saturation of uptake was obvious. The first part of the disappearance curve (above 20 μM LU) displayed zero-order kinetics (not shown).

Fluorescence microscopy of liver sections (Fig. 2A) revealed a punctate pattern, with large and small fluorescent spots. Especially at a higher magnification (Fig. 2B), the difference in distribution of the large and small spots can be seen. The larger ones were located at the edge of the hepatocyte, mostly at the sinusoidal side, whereas the small dots were seen all over the cell. Electron microscopy showed that the large spots were large vacuoles and not clusters of small spots that could not be resolved by fluorescence microscopy.

Intracellular localization of LU. The perfused liver was employed to identify the organelles that were involved in the intracellular accumulation of LU. The nucleus and cytoplasm were not stained at all. The staining pattern of rhodamine-dextran (Fig. 3A) was canalicular, with more intense spots scattered over the tissue. Fig. 3B shows the same tissue at another wavelength; fluorescence of LU is seen in addition to a faint fluorescence of rhodamine-dextran (cf., the fluorescence pattern in Fig. 2A). The overlap in localization of LU and rhodamine-dextran is obvious (Fig. 3, A and B, *arrows*), although no complete congruence exists (Figs. 2A, and 3, A and B). LU was not present in the brighter spots in Fig. 3A, whereas rhodamine-dextran did not label the many smaller dots seen in Figs. 2A and 3B. The staining pattern of the lysosomal enzyme acid phosphatase (Fig. 3C) also showed a partial overlap with LU localization.

The mitochondrial marker rhodamine 123 stained the tissue rather homogeneously (not shown). Staining was less intense in the canalicular areas where the lysosomes are concentrated.

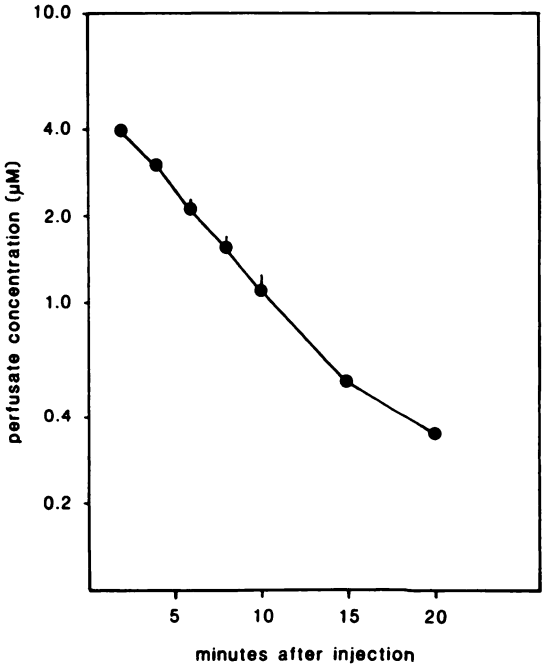

Fig. 1. Disappearance of 500 nmol of LU from the medium of a perfused rat liver.

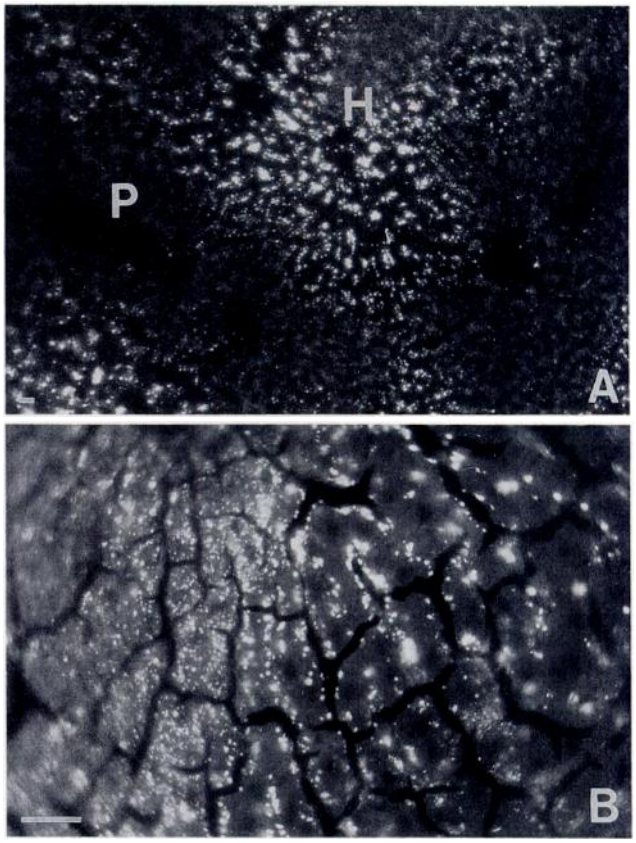

Fig. 2. Intrahepatic localization of LU upon 15-min antegrade recirculating perfusion. Both small and large fluorescent dots are visible. *P*, Terminal portal venule; *H*, terminal hepatic venule. *Bar*, 25 μm.

A subcellular structure that resembles the LU-containing vesicles is the lipid droplet. Although LU was not lipid soluble at all, we definitively excluded this option by comparison with an Oil Red O staining (Fig. 3D). Fat was present only in zone 1 and the pattern did not agree with the hepatic distribution of LU.

Transport mechanism for LU. Although extensive external binding of LU to isolated hepatocytes occurred instantaneously, no appreciable increase in intracellular fluorescence was seen during 30 min of incubation. In fact, the amount of LU taken up into the cells was too small to measure against the high background of external binding.

The characteristics of LU uptake, therefore, were examined further in an intact liver. We added several cations and inhibitors of carrier-mediated cation uptake to the perfusion medium, but no effect was seen from any of them except rhodamine B, which significantly diminished LU uptake to 82% (Table 1). Inversely, a 4-fold excess of LU had no effect on the uptake of tributylmethyl ammonium and vecuronium in isolated cells.

The cytoskeleton-disrupting agents cytochalasin B and nocodazole (Table 1) inhibited LU uptake to 78% and 63% of control values, respectively. In most experiments, cytochalasin B clearly inhibited uptake but sometimes a slight stimulation was observed. Furthermore, the monophasic first-order hepatic uptake of the fluid phase marker HRP ($CL = 0.21 \pm 0.03$ ml/min/g of liver; three experiments) was not at all affected by LU ($CL = 0.24 \pm 0.01$ ml/min/g of liver; three experiments).

Neomycin or poly(L-lysine) were added to the perfusion

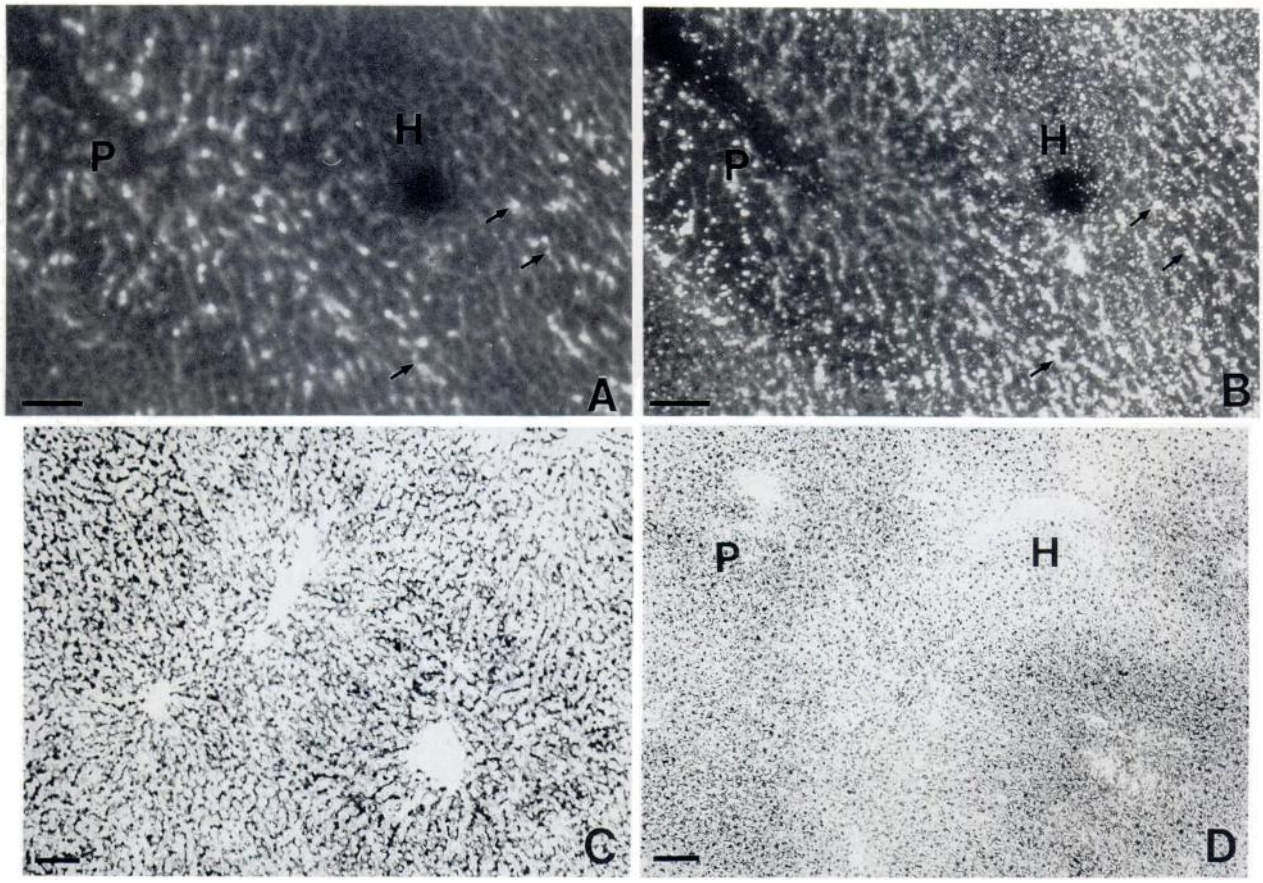

Fig. 3. Partial co-localization (*arrows*) of rhodamine-dextran (A) and LU (B) in intact liver. Livers were perfused for 30 min. Photography was performed with filter sets selective for rhodamine (A) and LU (B). In B, fluorescence of rhodamine is faintly seen next to LU. The acid phosphatase staining pattern (lysosomes) (C) partly resembles that of LU (B), like rhodamine-dextran (A). The staining pattern of fat droplets with Oil Red O (D) does not resemble that of LU (B). *P*, Terminal portal venule; *H*, terminal hepatic venule. *Bar*, 100 μm.

TABLE 1

Influence of various substrates on uptake of 500 nmol of LU in the perfused rat liver

Values are means ± standard deviations. Student's *t* test was used to determine statistical significance.

	Dose range	*n*	Extraction
	μmol		%
Control		4	46.8 ± 3.9
Procainamide ethobromide	2	2	43.8 ± 7.9
Tributylmethyl ammonium	2	2	48.4 ± 3.2
Vecuronium	2	2	46.0 ± 1.9
Rhodamine B	1.3	2	38.2 ± 3.0[a]
Cymarin	2	2	50.0 ± 11.9
Verapamil	2	2	51.4 ± 7.0
Cytochalasin B	1–4	7	36.6 ± 8.4[a]
Nocodazole	5	2	29.3 ± 0.4[b]
Neomycin	2–5	5	37.1 ± 10.5
Poly(L-lysine)	0.08–0.3	4	36.3 ± 3.0[b]

[a] $p < 0.05$.
[b] $p < 0.0025$.

medium to investigate whether adsorptive endocytosis was involved in LU internalization. Neomycin had a clear but not statistically significant effect on LU kinetics, whereas poly(L-lysine) inhibited the uptake of LU significantly (Table 1).

Because the effects of cytochalasin B and neomycin were too capricious to find any dose dependence, we statistically analyzed the results by assuming the dose range to be only one dose.

Discussion

The extremely low lipid solubility of LU and the saturability of its uptake system imply that diffusion across the plasma membrane as well as across intracellular membranes is very unlikely to occur. The observation that expected inhibitors of carrier-mediated cation transport had no effect on LU kinetics suggests that a carrier-protein is not involved in LU uptake either. Rhodamine B did inhibit uptake of LU, but this very lipophilic cation mainly enters the cell by diffusion, meanwhile accumulating in the membranes (11).[2] This drug, therefore, might affect any transport pathway across a membrane and the inhibition should not be considered specific. The lack of effect of the inhibitors may be explained in two ways, a different transport system is involved or the affinity of LU for the carrier protein is too high to be effectively diminished. In that case, LU should be able to inhibit the uptake of almost every other cation, but neither uptake of vecuronium nor that of tributylmethyl ammonium was decreased by LU in isolated hepatocytes, which correlates with the undetectable uptake of LU by isolated hepatocytes. These data excluded classical carrier-mediated uptake of LU into the hepatocytes.

We, therefore, tested the hypothesis of vesicular uptake, using some inhibitors of this process. The capricious effect of cytochalasin B on LU kinetics is in line with the contradictory

[2] Unpublished observations.

reports in the literature about effects of the agent in the liver. Cytochalasin B inhibits uptake of immunoglobulin A in cultured hepatocytes (19), whereas a stimulation of endocytosis was reported in the perfused rat liver (20, 21). In the present study, the average effect of cytochalasin B was a statistically significant inhibition of LU uptake, which is compatible with an endocytic transport system.

Nocodazole was used instead of colchicine because the latter substance interfered with the determination of the LU concentration. Colchicine inhibits uptake of indocyanin green (22) and HRP (23) in rat liver, whereas nocodazole was shown to inhibit hepatic uptake of polymeric immunoglobulin A and its subsequent biliary excretion (24). The uptake of LU was lowered significantly by nocodazole (Table 1), supporting the idea of endocytic transport.

Three different endocytic uptake mechanisms have been described. First is nonspecific fluid phase endocytosis, which involves substances like HRP (25, 26), insulin (27, 28), or sucrose (29). This system is not saturable (29) and is 2 orders of magnitude slower than the uptake of LU (27). If LU is taken up by this route, it should stimulate fluid phase endocytosis. Our observation that LU did not stimulate HRP uptake further negated the participation of this pathway in LU uptake.

Other possible systems are adsorptive and receptor-mediated endocytosis. Both processes are in principle saturable, although the number of nonspecific binding sites connected with adsorptive endocytosis may be very large (30). The interaction of a cation like LU with a specific receptor initiating receptor-mediated endocytosis has not been described, either in liver or in any other tissue. In the kidney, the positively charged aminoglycoside antibiotics, like neomycin, enter the cells of the proximal tubule via such a pathway (31, 32). Adsorptive endocytosis of aminoglycosides can be inhibited by the polycation poly(L-lysine) (33). These compounds supposedly interact specifically with anionic sites of the brush border and basolateral membranes. Adsorptive endocytosis, therefore, is a more likely mechanism for LU uptake.

In the present study, LU uptake was shown to be inhibited by poly(L-lysine) and neomycin, although the latter effect was quite variable and not significant. We conclude from our data that LU was taken up into the hepatocytes by means of adsorptive endocytosis, which implies that the small dots may represent endosomes and lysosomes. The only partial inhibition by poly(L-lysine) and neomycin might be explained by a higher accessibility or affinity of LU for the supposed binding sites of the plasma membrane.

In isolated hepatocytes, the uptake of LU was too slow to be detected reliably. This is explained by the fact that endocytosis is retarded in freshly isolated hepatocytes, compared with cells in culture or *in vivo* (34). Yet, an extensive binding to the exterior of the cell was found.

The LU fluorescence was seen in small endocytic vesicles all over the cell next to lysosomes, which is characteristic for a general vesicular transport pathway through the cell. In addition, larger vacuoles were induced. Such vacuoles have been described for a group of cationic amphiphilic drugs in several cell types. A relatively high lipophilicity, however, appeared to be a prerequisite for the induction of such vacuoles (35); in contrast, LU is very hydrophilic. On the other hand, vacuolization may occur during hypoxia, a condition that may exist in downstream areas of the acinus (36). The large granules indeed were seen predominantly in zones 2 and 3 of the hepatic acinus. Therefore, the large vacuoles might be related to a relatively low oxygen concentration in the *in vitro* preparation. This does not imply that these livers were anoxic or not viable; electron microscopy showed intact mitochondria and a normal cellular structure, in spite of the presence of the large vacuoles. Alternatively, LU itself might induce toxic effects and clustering and fusion of endosomes, especially in cells with a suboptimal energy supply. These hypotheses remained to be studied. The presence of LU in such vacuoles can easily be explained by its extensive membrane binding, followed by pinocytosis and fusion of endocytic vesicles.

With a vesicular uptake mechanism for LU, many observations can be explained, 1) the obvious absence of cytoplasmic staining by the dye, 2) its extremely low rate of biliary excretion, 3) the lack of kinetic interaction with other organic cations or related compounds, 4) the probably endosomal and partly lysosomal localization, and 5) the discrepancy in uptake rate between freshly isolated hepatocytes and hepatocytes in intact liver. Taken together, we showed that, in addition to diffusion and carrier-mediated uptake, adsorptive endocytosis may play a role in the clearance of organic cations by hepatocytes. LU is the first compound for which this mechanism is recognized, but it is likely that many other cations use this same pathway next to the other uptake systems.

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