0026-895X/89/040532-05$02.00/0

MOLECULAR PHARMACOLOGY, 36:532-536

Zonal Distribution of the Cation Lucigenin in Rat Liver: Influence of Taurocholate

INEKE BRAAKMAN,[1] OSCAR VEREST, TJAARD PIJNING, DIRK K. F. MEIJER, and GENY M. M. GROOTHUIS
Department of Pharmacology and Therapeutics, Groningen University, Groningen, The Netherlands
Received March 6, 1989; Accepted July 17, 1989

SUMMARY

The yellow fluorescent cation lucigenin (LU) was used as a model compound to study acinar heterogeneity in transport of hydrophilic cations that enter the liver by adsorptive endocytosis. Hepatic uptake was fast and saturable. The extraction was about 50% in a cyclically perfused rat liver preparation in which endogenous bile salts were replaced by the infusion of taurocholate (TC). Fluorescence microscopy on 8-μm liver sections revealed a striking distribution pattern. LU appeared to be concentrated in micro- and macrovesicular structures in the cell. At the same time, LU skipped the first cells of the acinus, zone 1 in an antegrade and zone 3 in a retrograde perfusion. A downstream localization of the dye was the result. Single-pass perfusions with TC concentrations ranging from 0 to 180 μM showed that hepatic clearance of LU negatively correlated with the TC concentration ($p < 0.005$). Clearance fell from 1.99 ± 0.06 ml/min-g of liver(mean ± SD) without TC to 1.61 ± 0.21 with 45 μM TC and 1.65 ± 0.12 with 180 μM TC. Moreover, in the absence of TC we observed a homogeneous distribution of LU. TC induced in the acinus a nonfluorescent upstream area that expanded with increasing TC concentration. We concluded that TC inhibited uptake of LU; a high medium concentration of TC in zone 1 (antegrade) was accompanied by a low uptake of LU in this zone, resulting in a downstream increasing acinar gradient. Hepatic uptake and acinar distribution of certain cationic drugs *in vivo* may, therefore, vary with the variable input of bile salts in the portal circulation and, hence, with nutritional status and the time of day.

At least four different pathways have been shown to be involved in the uptake of organic cations (1), two carrier-mediated mechanisms, diffusion, and adsorptive endocytosis. The bivalent organic cation LU enters the hepatocyte via the latter route and, therefore, may be used as a model compound to study acinar heterogeneity of this transport system. LU is the first low molecular weight compound (molecular weight 510) for which endocytosis is recognized as the mechanism of transport into liver parenchymal cells. Other cationic compounds with similar molecular weights, like *d*-tubocurarin (molecular weight 686) and vecuronium (molecular weight 611) are taken up into hepatocytes via a carrier-mediated mechanism (hepatic extractions are 4.3 and 58%, respectively) (2).[2] Hence, possible zonal differences in this process have not been studied yet.

Although many papers have been published on acinar heterogeneity of metabolic functions and enzyme distributions (3–8), little is known about zonal differences in transport functions. Upon injection of a compound *in vivo* or in an isolated perfused liver preparation, tissue concentration gradients may arise, depending on both the relative and the absolute rates of uptake into the cells of the acinar zones (9–11). Examination of the intrahepatic localization of a drug is needed in combination with kinetic experiments to detect intrinsic zonal differences (10).

In the present study, we used ante- and retrograde perfused rat livers to determine acinar heterogeneity in hepatic transport of LU. The results displayed a tissue concentration gradient increasing in the direction of the flow, which was caused by an external factor in the medium and not by intrinsic differences between hepatocytes. Single-pass perfusions with variable TC concentrations revealed that this bile salt was the external factor responsible for the observed phenomenon.

Experimental Procedures

Materials. Male Wistar rats having free access to water were fasted overnight before being used as liver donors. They weighed 180–220 g for the single-pass experiments and 280–320 g for the recirculating perfusions. TLC plates (silica gel 60 F_{254} silanized) were from Merck (Darmstadt, F.R. Germany). All other reagents and materials were from sources described in previous papers (1, 9, 10).

Isolated rat liver perfusions. Recirculating liver perfusions in the antegrade and retrograde direction were carried out as described before (9, 10, 12) TC was infused into the medium at a rate of 0.25 μmol/min, corresponding to a steady state medium concentration of 10 μM. Five

[1] Present address: Department of Cell Biology, Yale University School of Medicine, 323 Cedar Street, New Haven, CT 06510.

[2] W. E. M. Mol, F. Rombout, J. E. Paanakker, R. Oosting, A. H. J. Scaf, and D. K. F. Meijer. Pharmacokinetics of steroidal muscle relaxants in isolated perfused rat liver. Submitted for publication.

ABBREVIATIONS: LU, lucigenin; TC, taurocholate; TLC, thin layer chromatography.

hundred nmoles of LU were injected into the medium, which consisted of a Krebs-Henseleit bicarbonate buffer with 1% bovine serum albumin and 0.1% glucose.

To study the influence of TC on the kinetics of LU, livers were perfused in a single-pass mode. The procedure was the same as for the recirculating perfusions, with two modifications. 1) Medium passed the liver only once and was discarded after being drained from the organ or it was collected for determinations of LU concentrations. 2) The medium contained 0.2% bovine serum albumin instead of 1%, 3 μM LU, and differing concentrations of TC, 0, 9, 45, and 180 μM. The medium flow averaged 22 ml/min, corresponding to TC infusion rates into the liver of 0, 0.20, 1, and 4 μmol/min.

TLC. Samples from bile, medium, and liver homogenates were prepared and spotted on a TLC plate, as described before (9). The plates were developed for 1 hr in a chamber saturated with 80% 2-propanol/20% formic acid. Fluorescence was observed in the light of a 366 nm Hg lamp.

Determination of LU concentration and localization. Samples from the liver perfusions were diluted with a 0.33 M sodium phosphate buffer, pH 5.4. Fluorescence was measured on an SLM-Aminco SPF 125C (excitation 369 nm, bandpass 2.5 nm; emission 510 nm, bandpass 5 nm).

Preparation of tissue for fluorescence microscopy and recording of the photographs were as described before (1).

Statistical methods. Comparison between two means was made with Student's t test, after checking equality of variances with an F test (13). Significance of correlation ($r \neq 0$) was tested according to the method of Snedecor and Cochran (13). Fluorescence photographs are representatives of three to five experiments each.

Results

Kinetics and extent of LU uptake. Rat livers were perfused in the recirculating mode with 500 nmol of LU in the antegrade or retrograde direction. LU disappeared from the medium with monophasic first-order kinetics (1). Kinetic data and related parameters are listed in Table 1. All parameters were independent of perfusion direction.

Fig. 1 shows the intrahepatic localization of LU 10 min after injection into the medium. Fluorescence is seen in the downstream area of the acinus, zones 2 and 3 in an antegrade perfusion (Fig. 1A) and zones 1 and 2 in a retrograde perfusion (Fig. 1B). In both liver sections, large and small fluorescent dots are present.

With TLC, no metabolites were detected in bile, medium, or liver homogenates (with the detection limit being 10 pmol of LU, less than 0.1% of the dose was metabolized). The R_f value of LU was 0.44. Many other regular TLC systems did not work because LU was too hydrophilic to be moved quantitatively from the site of spotting.

Influence of TC. Antegrade single-pass perfusions with variable TC concentrations were used to determine the effect of this bile salt on kinetics and acinar localization of LU. Clearance of the dye was negatively correlated with TC concentration (Fig. 2). Maximum inhibition, achieved at about 50 μM TC, was 20%.

Intrahepatic distribution was also influenced by TC (Fig. 3).

TABLE 1

Parameters of LU transport and the perfused liver

Data are means ± standard deviations of four experiments. No statistically significant difference was observed with Student's t test ($p < 0.025$).

	Antegrade	Retrograde
Liver weight/body weight (%)	3.4 ± 0.1	3.3 ± 0.1
Bile flow/liver weight (μl/min/g)	1.05 ± 0.07	1.13 ± 0.08
Perfusion flow/liver weight (ml/min/g)	3.58 ± 0.06	3.61 ± 0.06
Portal vein pressure (cm H_2O)	10.0 ± 0.7	10.2 ± 0.5
LU excreted in bile in 90 min (%)	1.9 ± 0.2	1.9 ± 0.2
Clearance (ml/min/g)	1.67 ± 0.08	1.45 ± 0.24
Extraction (%)	46.8 ± 3.9	40.4 ± 4.6

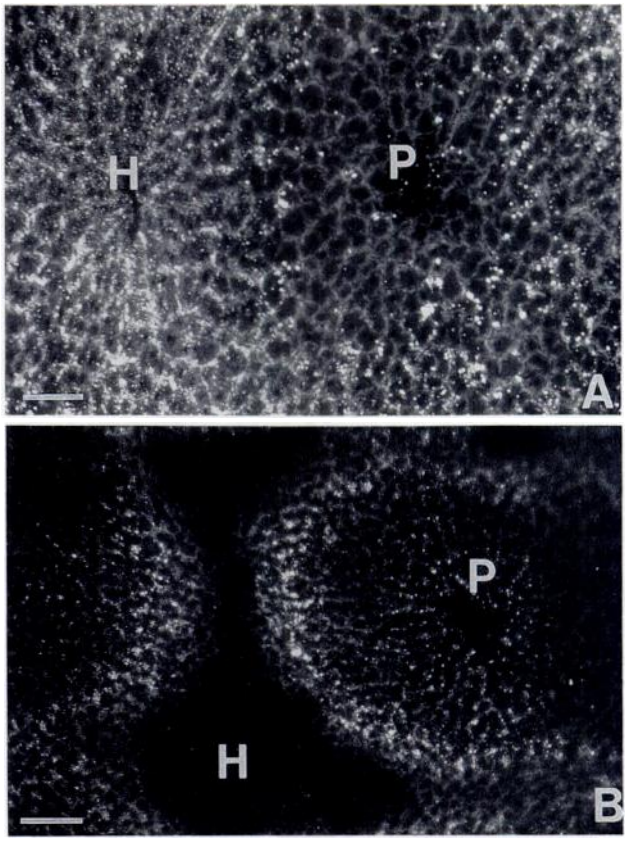

Fig. 1. Localization of LU in the liver upon 10 min of antegrade (A) or retrograde (B) recirculating perfusion after injection of 500 nmol of LU. Intensity differences between photographs are due to photographic artifacts, because clearance and extraction were the same in both perfusions. *P*, Terminal portal venule; *H*, terminal hepatic venule. *Bar*, 100 μm.

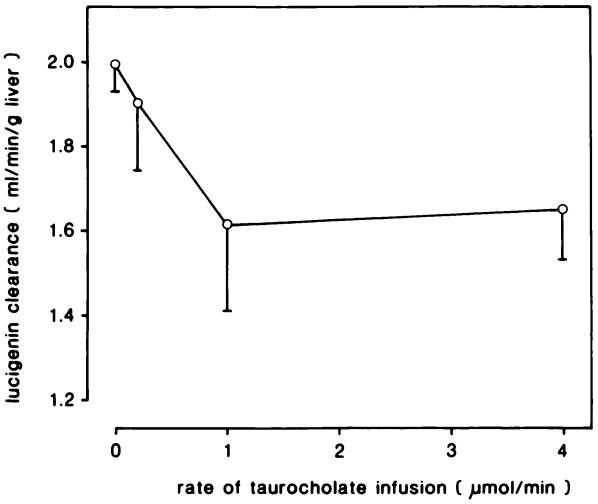

Fig. 2. Effect of TC on hepatic clearance of LU. Values are means ± standard deviations (four to six experiments). The negative correlation between the two parameters was significant ($P < 0.005$). When the value of 4 μmol of TC/min was left out, significance was higher ($P < 0.001$).

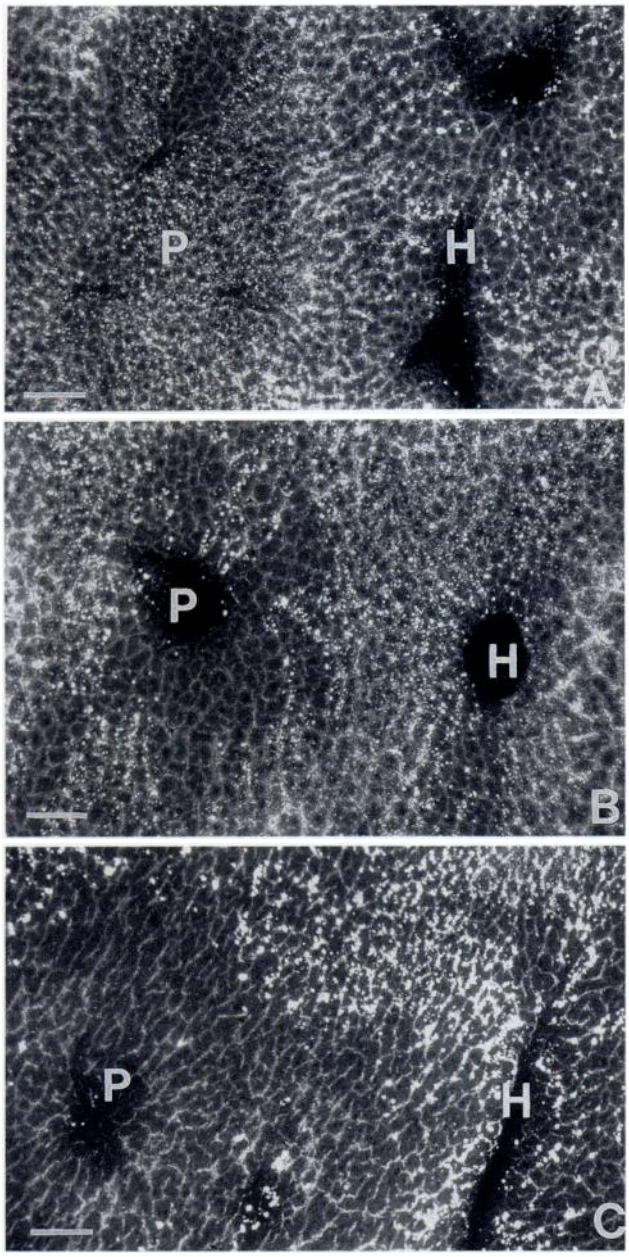

Fig. 3. Influence of TC on the intrahepatic distribution of LU. A, Control; B, the medium contained 9 μM TC (rate of infusion, 0.20 μmol/min); C, 45 μM TC (1 μmol/min). *P*, Terminal portal venule; *H*, terminal hepatic venule. *Bar*, 100 μm.

Without bile salts in the medium, all cells contained LU. Predominantly small dots were seen in zone 1, while a mixed population was present in the other zones (Fig. 3A). With rising TC concentrations (Fig. 3, B and C), a nonfluorescent upstream zone developed towards the hepatic vein to an estimated maximum of 40 to 50% of the acinus length (Fig. 3C). At 4 μmol of TC/min, the pattern of fluorescence was more or less the same as at 1 μmol/min (not shown).

Discussion

In this study, we employed isolated livers perfused in an antegrade or retrograde direction. The data in Table 1 demonstrate that the two preparations were functionally the same. Liver weights were similar which indicates that livers were not swollen after retrograde perfusion. Equal medium flow at equal portal pressures shows that livers had similar intrahepatic resistances. Bile flow is used as a general viability criterion. We previously showed by computer modeling and simulation that clearance parameters should be the same for both perfusion directions, irrespective of intrinsic zonal differences in uptake (9, 10, 14). The numerically different extraction efficiency was caused by a slightly higher medium flow through the retrograde perfused livers, due to a small difference in rat weight between the two experimental groups.

The observation that LU is not metabolized ensures that the acinar distribution of LU is the consequence of transport phenomena and not of metabolic processes. Because LU was taken up in periportal cells (Fig. 1B) as well as in perivenous hepatocytes (Fig. 1A), we concluded that all parenchymal cells were capable of internalizing LU. Hence, the lack of uptake in upstream areas could not be due to an intrinsic difference but should have been caused by one or more external factors.

The perfusion medium is the most probable external factor. It contained inorganic salts, glucose, bovine serum albumin, oxygen, and sodium taurocholate. The latter substance was infused into the medium to stabilize bile flow by compensating for loss of bile salts via the bile cannula (12). The steady state concentration of 10 μM TC was chosen because it stabilized bile flow rapidly and was low enough to avoid (toxic) side effects on the liver. Of all medium constituents, only oxygen (15, 16) and TC (11) are known to display an acinar gradient, decreasing in the downstream direction.

The influence of TC on kinetics and distribution of LU was investigated in single-pass perfusions. The advantage over a recirculating protocol is that the composition of the medium remains constant during the experiment. Thus, accumulation of metabolic waste and substances still synthesized and secreted after isolation of the liver is prevented.

The effect of TC on LU was obvious; both clearance (Fig. 2) and intrahepatic distribution (Fig. 3) were affected. Although clearance only diminished to 80%, this value is compatible with the observed distribution pattern. With a previously presented model (10), extraction efficiencies can be calculated. When the liver is divided into three zones and all zones are supposed to extract an equal percentage of LU (25%) from the medium, tissue and medium gradients are calculated as follows. The first zone extracts 25%, leaving 75% in the medium; the second zone extracts 25% of 75%, leaving 56% in the medium; then the remaining zone extracts 25% of 56%, which leaves 42% and, hence, the extraction is 58%. When the first third of the liver cannot take up LU, the second zone will extract 25% and leave 75%, while the third zone completes the extraction to a total of 44% (100% − 56%). In short, the fall out of one third of the acinus is expected to result in a decrease in extraction of only 24%. In other words, part of the inhibited uptake in the upstream zone is obviously compensated for by the remaining cells. When these experiments were carried out with recirculating medium, the effects were more variable, which confirmed our notion that the medium composition is less constant in a recirculating perfusion, compared with a single-pass perfusion.

The inhibition of clearance of LU reaches a maximum at about 50 μM TC. About the same concentration was reported by Vonk *et al.* (17) to cause 50% inhibition of the uptake of the cation N^4-acetyl procainamide ethobromide, the zwitterion indocyanin green, and the organic anion dibromosulphthalein.

The similarity of the inhibitory TC concentration in the uptake of widely varying substances handled by the liver via very different transport systems indicates that a competitive mechanism is not very likely. Vonk *et al.* (17) suggested a detergent-like effect, but this was contradicted by the observation that bile acids with different detergent properties exert the same inhibitory capacity. On the other hand, a change in hepatic plasma membrane fluidity is induced by several bile acids, such as TC (18).

A common effect of TC on membrane conformation is not unlikely to have an influence on clearance and distribution of LU, because LU is internalized via adsorptive endocytosis (1) and a strong adsorption to the plasma membrane precedes uptake. In addition, TC appeared to accelerate bile canalicular contractions (19), a phenomenon that can be inhibited by cytochalasin B. The effect of TC may be related to interference with the function of actin filaments, which suggests a possible action on actin-dependent LU uptake. TC (250–900 μM) also has an inhibitory effect on hepatic receptor-mediated endocytosis of asialo intestinal alkaline phosphatase (20). Inversely, uptake of TC into the liver is clearly decreased by cytoskeleton-disrupting agents, such as colchicine (21), cytochalasin B, and phalloidin (22). A vesicular intracellular transport mechanism has been suggested for bile salts (22), which is supported by the finding that bile salts probably are localized in vesicular intracellular structures (23). Once the mechanism of the interaction between TC and LU is clarified, an explanation may be offered for the "saturability" of the effect of TC.

At least one point needs further consideration; the uptake of LU in zone 3 is not expected to occur at a TC medium concentration of 180 μM in contrast to what was observed in the present study. At this TC level, uptake of the bile salt into zone 1 is normally saturated. TC, therefore, is present in sinusoidal medium in zone 3 at a concentration higher than 10 μM (11), which is the TC level that prohibited upstream uptake of LU in the recirculating (Fig. 1) as well as in the single-pass (Fig. 3B) perfusion. Two explanations can be given; either the normal TC tissue concentration gradient in the acinus was influenced by LU itself or the acinar distribution of TC was not the only factor involved in the observed heterogeneous pattern of LU. The uptake of LU is obviously largely determined by the presence of TC (Fig. 3), but oxygen gradients may be another factor involved, because its concentration sharply decreases along the acinus (15, 16). The large vacuoles containing LU were only seen in the downstream zones upon antegrade and retrograde perfusion (Fig. 3A), constituting the part of the tissue that is exposed to sinusoidal blood or medium containing the lowest oxygen concentration (15, 16). Vacuolization of hepatocytes occurs under hypoxic conditions (24), so that LU may be trapped in these vesicles, possibly upon fusion of endosomes.

After injection *in vivo*, the downstream increasing LU gradient with the clustered fluorescence pattern was also present. The large vacuoles were absent, however, which may be due to a different oxygen supply *in vivo* and *in vitro*. On the other hand, *in vivo* many other factors may contribute to the observed LU pattern. Therefore, the precise role of oxygen and other factors in the kinetics and distribution of LU remains to be elucidated.

The pronounced effect of TC on the hepatic distribution of LU, however, may have implications for the *in vivo* situation. Other bile salts may exert even stronger effects on the acinar gradient as well as on the extraction behavior of LU. The bile salt concentration and composition in blood varies largely with gall bladder emptying as well as in pathological conditions such as cholestasis. As a consequence, such variations might affect the hepatic uptake rate and distribution of various organic cations, resulting in a (diurnal) variation in the pharmacokinetics of cationic drugs.

Acknowledgments

We thank the Departments of Histology and Pediatrics for giving us access to their fluorescence microscopes and cameras. Angelie and Marike Braakman are acknowledged for typing the manuscript. Roelof Oosting is thanked for technical assistance.

References

1. Braakman, I., T. Pijning, O. Verest, B. Weert, D. K. F. Meijer, and G. M. M. Groothuis. Vesicular uptake system for the cation lucigenin in the rat hepatocyte. *Mol. Pharmacol.* **36**:537–542 (1989).
2. Van der Sluijs, P., H. H. Spanjer, and D. K. F. Meijer. Hepatic disposition of cationic drugs bound to asialoorosomucoid: lack of coendocytosis and evidence for intrahepatic dissociation. *J. Pharmacol. Exp. Ther.* **240**:668–673 (1987).
3. Wimmer, M., and D. Pette. Microphotometric studies on intraacinar enzyme distribution in rat liver. *Histochemistry* **64**:23–33 (1979).
4. Gebhardt, R., and D. Mecke. Heterogeneous distribution of glutamine synthetase among rat liver parenchymal cells *in situ* and in primary culture. *EMBO J.* **2**:567–570 (1983).
5. Thurman, R. G., and F. C. Kauffman. Sublobular compartmentation of pharmacologic events (SCOPE): metabolic fluxes in periportal and pericentral regions of the liver lobule. *Hepatology* **5**:144–151 (1985).
6. Häussinger, D., and W. Gerok. Metabolism of amino acids and ammonia, in *Regulation of Hepatic Metabolism. Intra- and Intercellular Compartmentation.* (R. G. Thurman, F. C. Kauffman, and K. Jungermann, eds.) Plenum Press, New York, 253–291 (1986).
7. Jungermann, K. Functional heterogeneity of periportal and perivenous hepatocytes. *Enzyme* (*Basel*) **35**:161–180 (1986).
8. Gumucio, J. J., and J. Chianale. Liver cell heterogeneity and liver function, in *The Liver. Biology and Pathobiology* (I. M. Arias, W. B. Jakoby, H. Popper, D. Schachter, and D. A. Shafritz, eds.) 2nd Ed. Raven Press, New York, 931–947 (1988).
9. Braakman, I., G. M. M. Groothuis, and D. K. F. Meijer. Acinar redistribution and heterogeneity in transport of the organic cation rhodamine B in rat liver. *Hepatology* **7**:849–855 (1987).
10. Van der Sluijs, P., I. Braakman, D. K. F. Meijer, and G. M. M. Groothuis. Heterogeneous acinar localization of the asialoglycoprotein internalization system in rat hepatocytes. *Hepatology* **8**:1521–1529 (1988).
11. Groothuis, G. M. M., M. J. Hardonk, K. P. T. Keulemans, P. Nieuwenhuis, and D. K. F. Meijer. Autoradiographic and kinetic demonstration of acinar heterogeneity of taurocholate transport. *Am. J. Physiol.* **243**:G455–G462 (1982).
12. Meijer, D. K. F., K. Keulemans, and G. J. Mulder. Isolated perfused rat liver technique. *Methods Enzymol.* **77**:81–94 (1981).
13. Snedecor, G. W., and W. G. Cochran. Statistical methods, 7th Ed. Iowa State University Press, Ames, IA (1980).
14. Groothuis, G. M. M., K. P. T. Keulemans, M. J. Hardonk, and D. K. F. Meijer. Acinar heterogeneity in hepatic transport of dibromosulphthalein and ouabain studied by autoradiography, normal and retrograde perfusions and computer simulations. *Biochem. Pharmacol.* **32**:3069–3078 (1983).
15. Lemasters, J. J., S. Ji, and R. G. Thurman. Centrilobular injury following hypoxia in isolated, perfused rat liver. *Science* (*Wash. D. C.*) **213**:661–663 (1981).
16. Matsumura, T., F. C. Kauffman, H. Meren, and R. G. Thurman. O_2 uptake in periportal and pericentral regions of liver lobule in perfused liver. *Am. J. Physiol.* **250**:G800–G805 (1986).
17. Vonk, R. J., P. A. Jekel, D. K. F. Meijer, and M. J. Hardonk. Transport of drugs in isolated hepatocytes: the influence of bile salts. *Biochem. Pharmacol.* **27**:397–405 (1978).
18. Scharschmidt, B. F., E. B. Keeffe, D. A. Vessey, N. M. Blankenship, and R. K. Ockner. *In vitro* effect of bile salts on rat liver plasma membrane, lipid fluidity, and ATPase activity. *Hepatology* **2**:137–145 (1981).
19. Miyairi, M., C. Oshio, S. Watanabe, C. Smith, I. M. Yousef, and M. J. Phillips. Taurocholate accelerates bile canalicular contractions in isolated rat hepatocytes. *Gastroenterology* **87**:788–792 (1984).
20. Russel, F. G. M., J. G. Weitering, R. Oosting, G. M. M. Groothuis, M. J. Hardonk, and D. K. F. Meijer. Influence of taurocholate on hepatic clearance and biliary excretion of asialo intestinal alkaline phosphatase in the rat *in vivo* and in isolated perfused rat liver. *Gastroenterology* **85**:225–234 (1983).
21. Crawford, J. M., C. A. Berken, and J. L. Gollan. Role of the hepatocyte microtubular system in the excretion of bile salts and biliary lipid: implications for intracellular vesicular transport. *J. Lipid Res.* **29**:144–156 (1988).

22. Erlinger, S. Bile flow, in *The Liver. Biology and Pathobiology* (I. M. Arias, W. B. Jakoby, H. Popper, D. Schachter, and D. A. Shafritz, eds.) 2nd Ed. Raven Press, New York, 643–661 (1988).
23. Suchy, F. J., W. F. Balistreri, J. Hung, P. Miller, and S. A. Garfield. Intracellular bile acid transport in rat liver as visualized by electron microscope autoradiography using bile acid analogue. *Am. J. Physiol.* **245**:G681–G689 (1983).
24. Marotto, M. E., R. G. Thurman, and J. J. Lemasters. Early midzonal cell death during low-flow hypoxia in the isolated, perfused rat liver: protection by allopurinol. *Hepatology* **8**:585–590 (1988).

Send reprint requests to: Geny M. M. Groothuis, Department of Pharmacology and Therapeutics, Groningen University, Ant. Deusinglaan 2, 9713 AW Groningen, The Netherlands.