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PHASE I AND PHASE II METABOLISM OF LITHOCHOLIC ACID IN HEPATIC ACINAR ZONE 3 NECROSIS

EVALUATION IN RATS BY COMBINED RADIOCHROMATOGRAPHY AND GAS–LIQUID CHROMATOGRAPHY–MASS SPECTROMETRY

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Abstract—In the present study, lithocholic acid (LCA) metabolism was assessed by radiochromatography and gas–liquid chromatography–mass spectrometry, and its relationship to cholestasis was investigated. In addition, the role of the perivenous zone in LCA-induced cholestasis and LCA biotransformation was examined by using bromobenzene (BZ), a chemical that causes selective necrosis of hepatocytes in this zone. LCA injection induced cholestasis of comparable amplitude in both control and BZ-treated rats. The biliary recovery of bile salts (BS) was 65–70% 2 hr after LCA injection. Excretion of LCA and its cholestatic metabolite, LCA glucuronide, was similar in both groups, although LCA excretion was delayed in BZ-treated animals. The appearance of LCA and LCA glucuronide in bile occurred early, and their proportion decreased with time. Concentrations of choleretic hydroxylated metabolites were low immediately after LCA injection but increased with time. 3α,6β-Dihydroxy-5β-cholanoic and 3α,6β,7β-trihydroxy-5β-cholanoic acids were the major species arising from LCA, indicating the importance of 6β hydroxylation in LCA detoxification in rats. Other metabolites were found, but their contribution was either minor or negligible. Overall amounts of hydroxylated metabolites were comparable in both groups, but trihydroxylated metabolites predominated over their dihydroxylated counterparts in control rats, whereas the production of dihydroxylated forms was more pronounced in BZ-treated animals. These results suggest that the destruction of perivenous hepatocytes does not exacerbate LCA-induced cholestasis, and that there may be an acinar zonation of LCA biotransformation to trihydroxylated metabolites in the rat liver.

Key words: lithocholate; cholestasis; bile acids; GC–MS

LCA‖ and its taurine conjugate are known to induce cholestasis in experimental animals [1–3]; however, their relevance in the pathogenesis of human cholestasis is still being debated [4–11]. Nevertheless, the cholestasis produced by LCA represents a suitable model for studies on the pathogenesis of defective bile formation. Available evidence indicates that LCA can only be detoxified by phase I reactions (hydroxylation), since in phase II reaction amidation with taurine and/or glycine, sulfation and glucuronidation of LCA yield metabolites with greater cholestatic properties than the parent compound [3, 12–14].

Interestingly, when T-LCA is infused retrogradely in the isolated perfused rat liver, cholestasis does not occur [15]. This protection has been attributed partly to extensive T-LCA biotransformation during retrograde perfusion, since functional zonation of many metabolic processes exists between periportal and perivenous hepatocytes within the hepatic acinus [for review, see Ref. 16] and since phase I drug metabolism takes place predominantly in the perivenous zone. This explanation is consistent with the observation that the biotransformation of taurodeoxycholic acid, a dihydroxy bile acid, to cholic acid, a trihydroxy bile acid, is three times greater during retrograde than during antegrade perfusion in isolated rat livers [17]. However, the pathogenesis of LCA-induced cholestasis may differ from that of T-LCA-induced cholestasis, since LCA binds to cellular membranes, but T-LCA does not [18]. In addition, LCA undergoes glucuronidation, conjugation and hydroxylation [14], but T-LCA undergoes only hydroxylation [15]. Therefore, the effect of the perivenous zone on LCA-induced cholestasis may differ from that of T-LCA-induced cholestasis. The objective of the present study was to elucidate the role of the perivenous zone in LCA-induced cholestasis and in LCA biotransformation,

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‖ Abbreviations: AUC, area under the curve; BZ, bromobenzene; CA, cholic acid; CDCA, chenodeoxycholic acid; DCA, deoxycholic acid; GC–MS, gas–liquid chromatography–mass spectrometry; HDCA, hyodeoxycholic acid; LCA, lithocholic acid; LCA-GLU, lithocholic acid glucuronide; α-MC, α-muricholic acid; β-MC, β-muricholic acid; MDCA, murideoxycholic acid; MSD, mass selective detector; and T-LCA, taurolithocholic acid.

using selective chemical destruction of the perivenous zone by BZ [19, 20].

MATERIALS AND METHODS

Materials. BZ was purchased from the Aldrich Chemical Co. (Milwaukee, WI). Collagenase was obtained from Cooper Biomed (Malvern, PA). Bovine serum albumin and LCA sodium salt were supplied by Calbiochem (La Jolla, CA). [^{14}C]LCA (56 mCi/mmol) was acquired from Amersham (Oakville, Ontario, Canada). 3α-Hydroxysteroid dehydrogenase was obtained from the Sigma Chemical Co. (St Louis, MO). Bile acid standards were procured from Steraloid (Wilton, NH). Other chemicals were obtained from the Sigma Chemical Co. and local suppliers.

Animals and pretreatment. Male Sprague–Dawley rats weighing 160–190 g (Charles River, St-Constant, Quebec, Canada) were maintained *ad lib.* on a standard rat diet (Prolab, RMH 4020, Agway, Syracuse, NY) and tap water for 4–5 days prior to the experiment. They were kept in a controlled environment with room temperature at 22° and lighting from 7:00 a.m. to 7:00 p.m.

Half of the rat population was injected i.p. with BZ (600 mg/kg in corn oil) and the other half with corn oil alone. The BZ dose was selected on the basis of previous experiments in which 30% of the acinus in the perivenous zone was destroyed by BZ 48 hr after injection [19]. In the present experiments, the extent of necrosis produced by BZ treatment was validated using light microscopy and determination of the activity of certain marker enzymes in isolated hepatocytes. For light microscopy, liver sections were fixed in ethanol/neutralized formaldehyde and were stained with hematoxylin-eosin as previously described [20].

Hepatocytes were isolated following collagenase perfusion [21] with slight modification of the composition of the infusion buffers [22]. Briefly, the liver was perfused *in situ* through the portal vein with Ca^{2+}- and Mg^{2+}-free HEPES buffer containing 0.5 mM EGTA for 10 min at 30 mL/min. A modified Krebs–Henseleit buffer containing 0.05% collagenase and 2.5 mM $CaCl_2$ was then perfused at 20 mL/min for 12–15 min. Solutions were oxygenated and kept at 38°. The cells were suspended, centrifuged and filtered through Nylon mesh. The viability as assessed by Trypan Blue exclusion was $\geq$85%. Cells were used immediately or stored at −40°.

Experimental protocol. The jugular vein and common bile duct were then cannulated with PE-90 and PE-10 catheters (Clay Adams, NJ), respectively, under pentobarbital (Somnotol, 48 mg/kg) anesthesia. Bile was collected on ice for 60 min in 30-min samples, after which LCA (50 mg/kg) was injected slowly over a period of 5 min. LCA was suspended in 1 mL of 3.5% bovine serum albumin in saline solution with 3×10^5 dpm [^{14}C]LCA. Bile was then collected for 3 hr in 15- or 30-min samples. All treatments and surgical procedures were performed between 9:00 and 11:00 a.m. The animals remained under anesthesia during the experiment; body temperature was maintained at 37° with a rectal probe and a thermostatically controlled heat lamp. Saline was infused during the experiment at a rate of 1 mL/hr to compensate for the loss of body fluids resulting from biliary drainage.

Assays. Lactate dehydrogenase (EC 1.1.1.27) and γ-glutamyltransferase (EC 2.3.2.2) activities were determined kinetically with a kit provided by Sigma (340-LD) and according to the recommendations of the Enzyme Committee of the Scandinavian Society for Clinical Chemistry and Physiology [23] after solubilization of the freshly isolated cells with 0.5% Triton X-100. Glucose-6-phosphatase (EC 3.1.3.9) and glutamine synthetase (EC 6.3.1.2) were measured according to the methods described by Schwartz and Bodansky [24] and Wellner and Meister [25], respectively. Proteins were determined according to Lowry *et al.* [26].

Bile flow was measured gravimetrically, assuming a density of 1 g/mL. Total bile salts were assessed by the 3α-hydroxysteroid dehydrogenase procedure [27]. Radioactivity in bile was measured by the addition of 10 mL of Universal scintillation fluid (ICN, Costa Mesa, CA) to an aliquot of bile, in a Beckman LS 5000 TD counter equipped with an external standard for automatic correction after quenching.

Thin-layer chromatography. Bile samples were deproteinized with methanol at 80°. Aliquots of the methanol extract were analysed by TLC using a solvent system of ethanol : ethyl acetate : ammonium hydroxide (3 : 3 : 1, by vol.) for the separation of glucuronides [28] and chloroform : methanol : acetic acid : water (65 : 24 : 15 : 9, by vol.) for the separation of amidates. Other samples were hydrolysed overnight in 2.5 N NaOH at 115°, extracted with diethyl ether, and separated by TLC in a solvent system containing isooctane : ethyl acetate : acetic acid (10 : 10 : 2, by vol.). Bile acids were visualized on TLC plates using phosphomolybdic acid. The bile acid spots (identified by comparison to standards as well as by gas chromatography–mass spectrometry using spots visualized by iodine vapor) were scraped from the plates and transferred to vials containing 1 mL of methanol : water (9 : 1, v/v). The vials were agitated for 30 min, and 10 mL of Universal scintillation fluid was added. Radioactivity was measured as described above.

Gas–liquid chromatography–mass spectrometry (GC–MS). Bile samples were extracted with ethanol : methanol (95 : 5, v/v) at 80° for 5 min. After the addition of 100 μg of 5β-cholanic acid as internal standard, bile acids were hydrolysed overnight in 2.5 N NaOH in sealed tubes. The tubes were then cooled on ice and acidified with 2.5 N HCl to pH < 1. Free bile acids were extracted with diethyl ether. Methyl esters were prepared by the addition of 2 mL methanol, 2.2 mL dimethoxypropane, and 10 drops of concentrated HCl [29]. After 1 hr, bile acid methyl esters were extracted with diethyl ether. Acetate derivatives were prepared by the addition of 0.5 mL of a mixture of 7 mL glacial acetic acid, 5 mL acetic anhydride and 5 drops of perchloric acid [30]; the tubes were sealed and kept at room temperature for 1 hr, after which methyl ester acetate derivatives were extracted with diethyl ether, dried under nitrogen, dissolved in 100–200 μL of chloroform, and then injected into a Hewlett–Packard 5890 gas

chromatograph equipped with a 12-meter fused silica capillary column cross-linked with 5% phenyl-methyl silicone, having an internal diameter of 0.22 mm [31]. The chromatograph was connected to a Hewlett–Packard 5971A MSD, and bile acids were scanned between m/z 100 and 650. The bile acids were assessed with a database obtained from the analysis of various bile acid standards, using the MSD Chemstation software supplied by Hewlett–Packard. Other identities were ascertained by comparing the obtained spectra with previously reported spectra, employing methyl ester acetate derivatives of the bile acids. The methodology for bile acid analysis by the GC–MS was validated in the following manner:

(1) Complete hydrolysis of the bile acids was determined by using authentic standards of various conjugated bile acids. In all cases, hydrolysis was complete as checked for the absence of conjugated bile acids by a TLC system using butanol:acetic acid:water (10:1:1, by vol.); no degradation occurred after alkaline saponification when checked by GC.

(2) Extraction of bile acids was determined using tracer amounts of radioactive CA or CDCA; in each case, the extraction efficiency was greater than 95%.

(3) The GC response (detector response) for various bile acids in relation to the internal standard was found to be 100% for various bile acids; thus, no correction for the GC detector was needed. The MSD detector response factor, however, varied between different bile acids, and appropriate correction factors were applied.

(4) The GC and the MSD detector responses were linear in the range of concentrations present in the sample analysed.

Calculations and statistics. Results are expressed as means ± SEM. To minimize inter-animal variations, the data were normalized per 100 g body weight. Further standardization was achieved by expressing some of the results as a percentage of basal values. The bile salt secretion rate is the product of bile salt concentration and bile flow. The AUC was calculated by adding the percent value for bile flow or bile salt secretion rate versus time, according to the trapezoidal procedure. The excretion of exogenous bile salts during a given time period (Q_{exot}) was assumed to be equal to the difference between the total amount of bile acid excreted during a given time period (Q_{sect}) and the amount of endogenous bile acid excreted during the same period (Q_{endt}):

$$Q_{exot} = Q_{sect} - Q_{endt}$$

Q_{endt} was calculated as follows:

$$Q_{endt} = Q_0 \cdot Q_{CAt}/Q_{CA0}$$

where Q_0 equals the basal amount secreted before LCA injection, Q_{CAt} equals CA secreted during the period of interest and Q_{CA0} is CA secreted during the basal period. In this calculation it is assumed that the amount of CA secreted at any given time is related to the secretion of endogenous bile acids.

Differences between the means were analysed by Student's t-test, and P values of less than 0.05 were considered to be statistically significant.

RESULTS

Histological changes caused by BZ. Figure

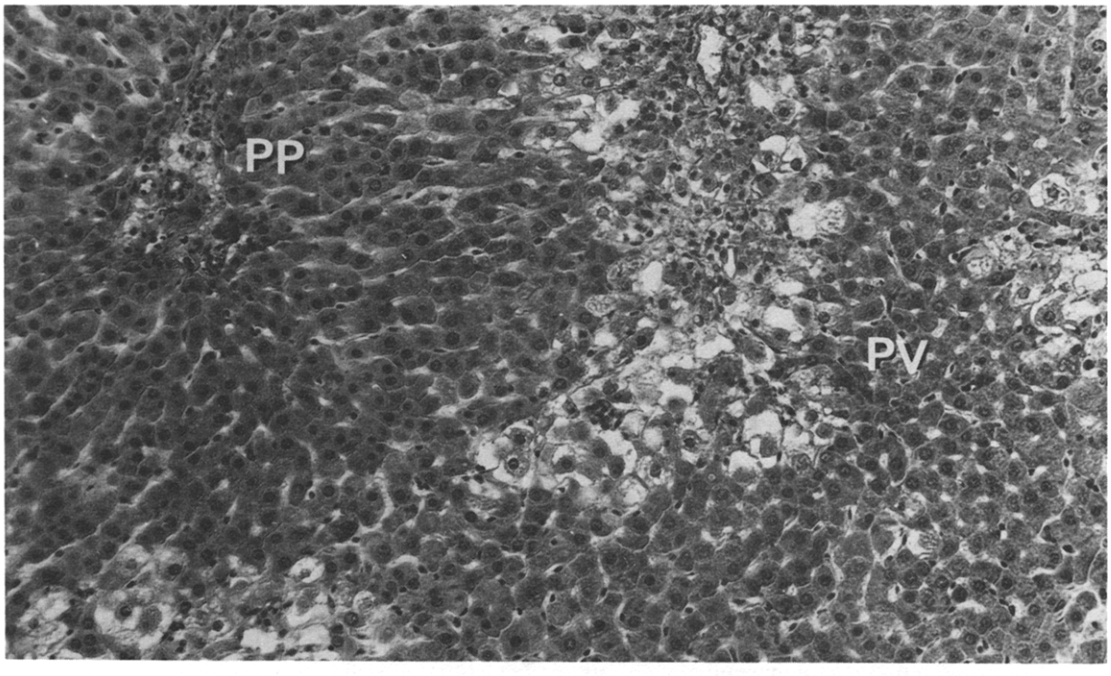

Fig. 1. Light microscopy of rat liver, 48 hr after bromobenzene administration. Note swollen and necrotic cells in the perivenous zone (PV). No apparent necrosis is seen in the periportal (PP) and intermediate zones. (Magnification: 160×.)

Table 1. Effect of perivenous necrosis on activities of enzymes with different acinar distribution in isolated hepatocytes

Enzyme	Activity	
	Control	BZ
γ-Glutamyltransferase	1.33 ± 0.60	2.43 ± 0.13*
Glutamine synthetase	13.8 ± 3.9	0.51 ± 0.34*
Lactate dehydrogenase	2514 ± 385	2196 ± 306
Glucose-6-phosphatase	2.70 ± 0.81	2.54 ± 0.72

Cells were isolated 48 hr after bromobenzene (BZ) or corn oil administration. Activities are expressed as nmol · min^{-1} · (mg protein)$^{-1}$ for γ-glutamyltransferase and lactate dehydrogenase and μmol · hr^{-1} · (mg protein)$^{-1}$ for glutamine synthetase and glucose-6-phosphatase. Values are means ± SEM from four to six hepatocyte preparations.
* Significantly different from controls ($P < 0.05$).

1 illustrates the liver damage 48 hr after BZ administration. All the animals developed extensive perivenous necrosis. The damaged area was 30.4 ± 4.3% of the acinar volume. As seen in Fig. 1, the necrosis was confined to the perivenous zone, and no morphological abnormalities were detected in the other part of the acinus. These results are consistent with more detailed studies from our laboratory [20, 32] and others [19, 33].

Functional characterization of isolated hepatocytes. Since cell function can be affected without detectable morphological modifications and since a functional readaptation of the non-damaged cells is possible due to the dynamics of metabolic zonation, the activities of four acinar marker enzymes were measured in isolated hepatocytes (Table 1). The specific activity of the predominantly periportal enzyme γ-glutamyltransferase [33] was higher in BZ-treated rats than in controls. This indicates an enrichment of periportal cells in the preparation. When the perivenous zone was destroyed, the activity of the perivenous restricted enzyme glutamine synthetase [34] was decreased by 96.4%. The activities of the rather homogenously distributed enzymes, lactate dehydrogenase and glucose-6-phosphatase, were not changed by BZ treatment. The ratio of the activity for each enzyme between BZ-treated and control animals was similar to that obtained by other investigators using different methods [34–37]. Moreover, the present results are consistent with other reports indicating the absence of adaptable changes for several enzymes after perivenous damage [38, 39].

Effect of LCA injection on bile flow and bile salt secretion rate. Figure 2 shows the effect of LCA injection on bile flow (panel a) and bile salt secretion rate (panel b) in rats treated with BZ and controls given only corn oil. In the control group, bile flow and bile salt secretion rate (measured enzymatically and thus, not including LCA secreted as 3-*O*-glucuronide) were reduced by a maximum of 65 and 45%, respectively, within 60 min after LCA injection. In the BZ-treated group, they were also reduced but to a lesser extent (by 40 and 25%, respectively).

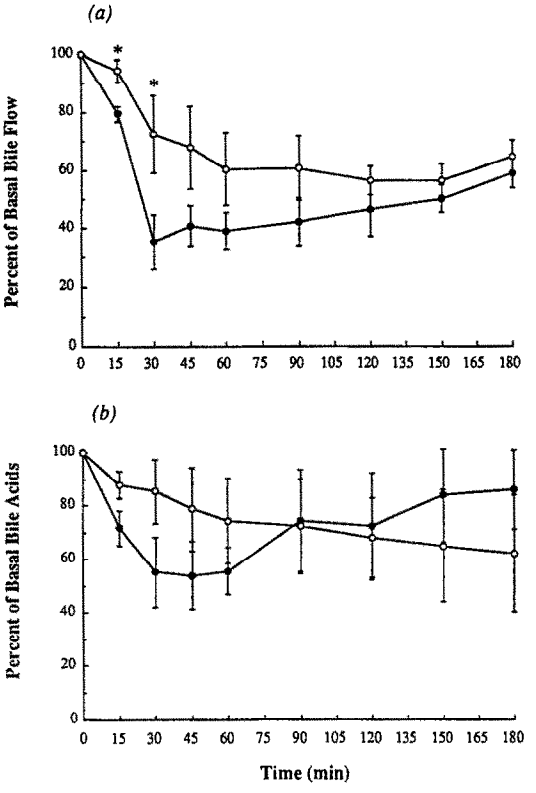

Fig. 2. Effect of bromobenzene treatment on variations of bile flow (a) and bile salt secretion rate (b) induced by LCA. Values are means ± SEM of seven control (●) and five BZ-treated (○) rats. Bile was collected for 1 hr to assess basal values, after which LCA (50 mg/kg) was injected intravenously and bile collected for 3 hr. The results are expressed as a percentage of basal value. Initial values were 7.70 and 6.61 μL·min^{-1}·(100 g)$^{-1}$ for bile flow and 206.3 and 119.8 nmol·min^{-1}·(100 g)$^{-1}$ for bile salt secretion rate in control and BZ-treated animals, respectively. Key: (*) indicates the time at which bile flow differed significantly ($P < 0.05$) from the corresponding control value.

Control rats entered a post-cholestatic recovery phase for bile flow and bile salt secretion rate, but the values remained lower than during the basal period. BZ-treated rats did not experience this post-cholestatic recovery; instead, a slow, progressive decline was observed. Although the bile flow decrease induced by LCA tended to be greater in the control group, a statistically significant difference was found only with the first two measurements.

When the AUC for bile flow or bile salt secretion rate was calculated until the end of the experiment (2 hr after LCA injection), there were no significant differences between control and BZ-treated rats (Table 2). During the first 60 min after LCA injection, however, the AUC for bile flow was significantly higher in the BZ group.

[^{14}C]LCA excretion and metabolism measured by TLC. After [^{14}C]LCA injection, the excretion of radioactivity was more pronounced in the early phase of cholestasis (Fig. 3a). There were no

Table 2. Effect of LCA on the cumulative AUC for bile flow and bile salt secretion rate in control and BZ-treated rats

Group	Bile flow		Bile salt secretion rate	
	AUC_{60}	AUC_{120}	AUC_{60}	AUC_{120}
Control	3318 ± 255	5838 ± 507	3791 ± 506	7876 ± 1099
BZ	4721 ± 518*	8118 ± 1038	5083 ± 546	9403 ± 1299

The AUC values from 0 to 60 and 120 min were calculated from the results presented in Fig. 2. Values are means ± SEM for seven control and five BZ-treated animals.

* Cumulative bile flow was decreased to a lesser extent in BZ-treated rats during the first hour post-LCA.

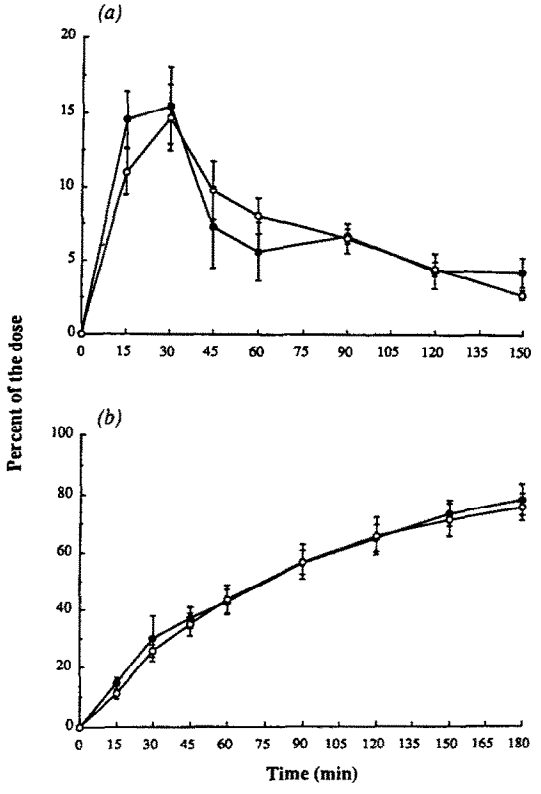

Fig. 3. Biliary excretion of radioactivity following [^{14}C]-LCA injection. The means ± SEM of biliary output (a) and cumulative biliary excretion (b) are shown for control (●, N = 7) and BZ-treated (○, N = 5) rats. Radioactivity was measured in bile samples obtained after [^{14}C]LCA (50 mg/kg, 3×10^5 dpm) injection and expressed as percentages of the injected dose.

differences between control and BZ-treated animals (Fig. 3b). Figure 4 shows the distribution of radioactivity secreted in bile 0–15 (Fig. 4a), 15–60 (Fig. 4b) and 60–120 (Fig. 4c) min after LCA injection, in various bile acids separated by TLC. In 0–15 min (Fig. 4a), LCA and glucuronidated LCA (LCA-GLU) each accounted for an average of 40% of radioactivity; 10–15% was present in MDCA (3α,6β-dihydroxy-5β-cholanoic acid) and β-MC (3α,6β,7β-trihydroxy-5β-cholanoic acid), and the rest was distributed between CDCA and other unidentified bile acids. When non-hydrolysed bile salts were separated by TLC, 90–95% of the LCA was found in the tauroamidate form in both groups; the rest was secreted as free LCA and glycolithocholic acid.

LCA and LCA-GLU were reduced in control and BZ-treated rats to 25 and 30%, respectively, at 15–60 min and to 15 and 20%, respectively, at 60–120 min. The decrease in LCA and LCA-GLU with time was compensated for by an increase in MDCA and β-MC (Fig. 4, b and c). Although no significant differences were noted between control and BZ values, the contribution of trihydroxylated bile acids tended to be higher in control as compared with BZ-treated rats, whereas the opposite was true for dihydroxylated bile acids.

Glucuronidated bile acid excretion was maximal immediately after LCA injection and then declined progressively (Fig. 5a). Cumulative glucuronide excretion was similar in both groups and averaged 17–19% of the [^{14}C]LCA dose administered (Fig. 5b). Glucuronidated bile acids were identified by treatment of samples with β-glucuronidase, which resulted in the disappearance of radioactivity in the glucuronide zone (38.6 vs 0.9%) and an increase in the radioactivity in LCA (21.2 vs 59%, data not shown).

Quantification of the metabolites of LCA. Analysis of biliary bile acids by GC showed that after LCA injection, the proportion of LCA in bile increased rapidly, while that of the other bile acids was reduced markedly with the exception of MDCA (Table 3). When the actual excretion of LCA and its metabolites was calculated (see Materials and Methods), total LCA secretion averaged 339, 487 and 288 μg/100 g body wt in the controls at 0–15, 15–60 and 60–120 min, respectively, compared to 178, 515 and 298 in BZ-treated rats. MDCA was present at 0–15 min (46 μg/100 g body wt) and increased to 288 and 276 μg/100 g body wt at 15–60 and 60–120 min, respectively, in the controls and to 355 and 327 μg/100 g body wt in the BZ group. β-MC was present only in trace amounts at 0–15 min in both groups, rising to 150 and 111 μg/100 g body wt during the cholestatic period (15–60 min) and to 320 and 213 μg/

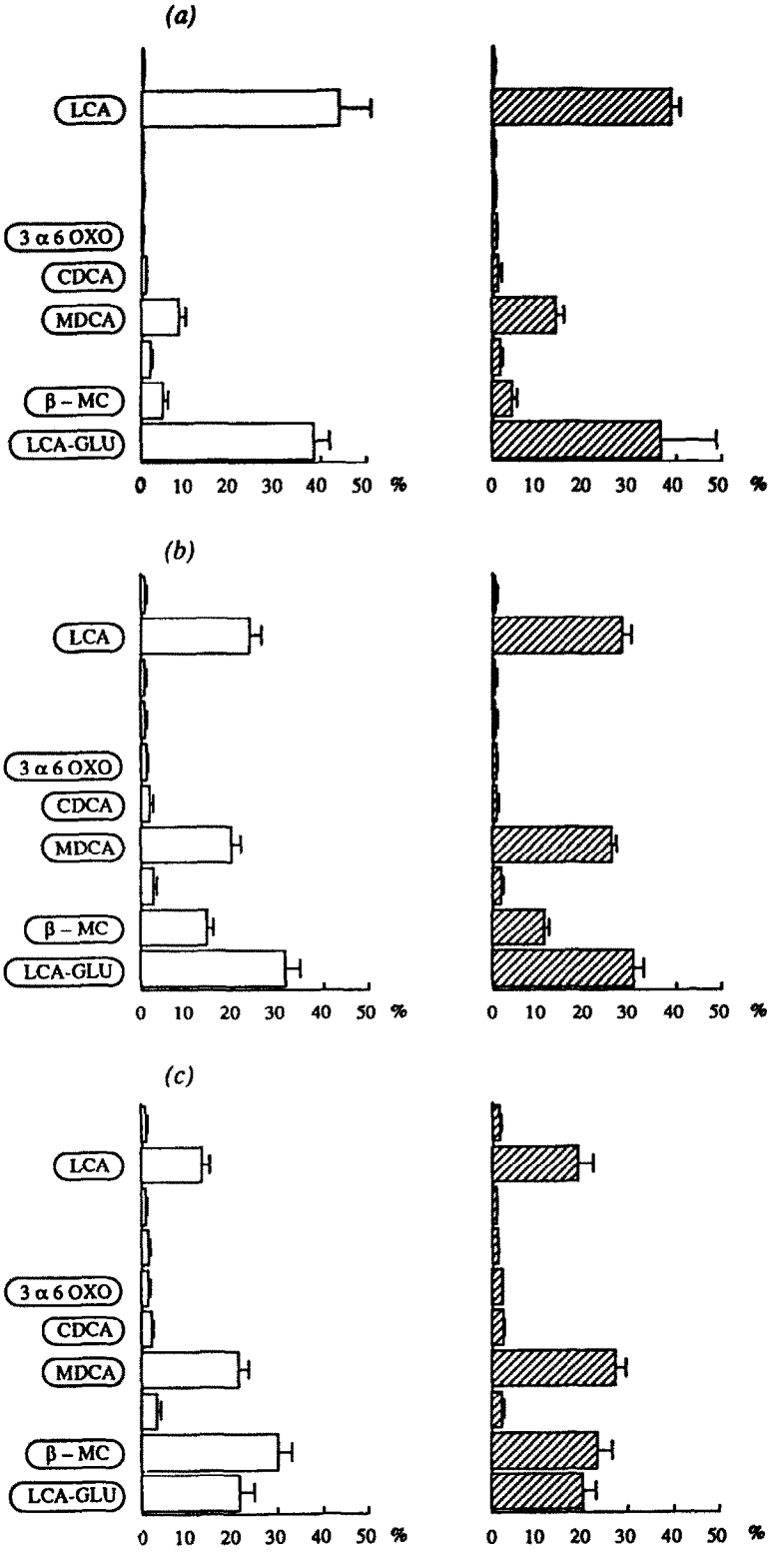

Fig. 4. TLC analysis of the distribution of radioactivity in LCA and its metabolites in bile after [^{14}C]-LCA injection. Bile collected from 0 to 120 min after [^{14}C]LCA injection was pooled as pre-cholestatic (0–15) (a), cholestatic (15–60) (b) and post-cholestatic (60–120) (c) samples. After alkaline hydrolysis, bile acids were extracted, dissolved in methanol:chloroform (9:1), and applied on TLC plates. They were separated in isooctane: ethyl acetate:acetic acid (10:10:2). Zones were scraped and counted for radioactivity. The values represent means ± SEM of the percentage of ^{14}C radioactivity secreted in bile during defined periods for control (□, N = 7) and BZ-treated (▨, N = 5) rats.

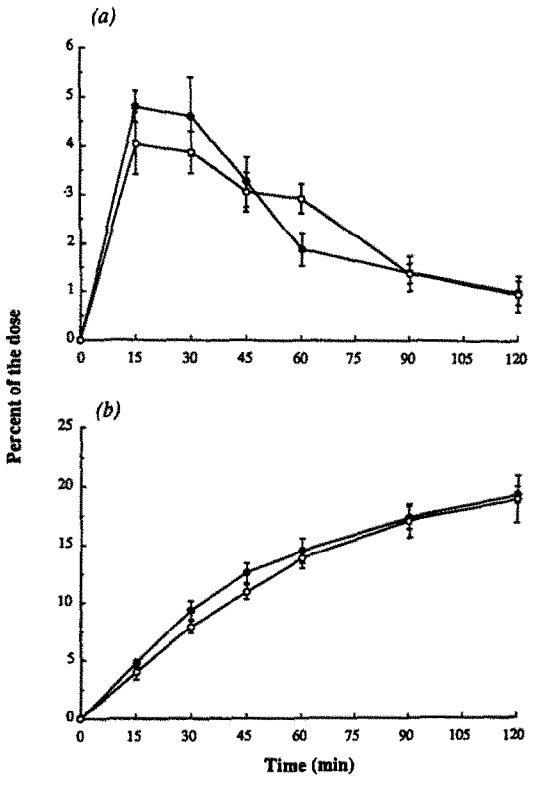

Fig. 5. Time course (a) and cumulative (b) biliary excretion of glucuronides following [^{14}C]LCA injection. The results were obtained by multiplying [^{14}C] activities in bile for a given period by the glucuronide fraction determined by TLC in the same period. Values are means ± SEM of seven control (●) and five BZ-treated (○) rats.

100 g body wt during the early period of recovery in the controls and BZ-treated rats, respectively.

Other bile acids, mainly α-MC, CDCA, HDCA and keto bile acids, were present in small amounts ranging from 1 to 10 μg. The cumulative secretion of LCA and its two principal hydroxylated metabolites was 2196 μg/100 g body wt in control and 2043 μg/100 g body wt in BZ-treated rats, which represents 43.9 and 40.9%, respectively, of the dose injected into the two groups. The proportion of hydroxylated metabolites (as evaluated by the ratio of LCA/(MDCA + β-MC) increased with time and was comparable in both groups throughout the experiment with the exception of the first 15 min sample, where more LCA was secreted in the controls than in the BZ group. Conversely, the proportion of LCA-GLU (measured separately after isolation by TLC, hydrolysis by β-glucuronidase and determination by GC–MS) in bile decreased with time. The secretion of LCA-GLU paralleled that of LCA, since the LCA/LCA-GLU ratio remained between 0.9 and 1.4 (data not shown).

Identification of LCA metabolites by GC–MS. To identify other LCA metabolites, bile samples were analysed by GC–MS. The two major LCA metabolites were identified as MDCA: *m/z* 415

Table 3. Biliary bile salt composition before and after LCA administration

Period*	LCA	CDCA	HDCA	MDCA†	β-MC	α-MC	Keto‡	CA	DCA	Others§
					(% of total)					
					Control					
Basal	1.6 ± 0.4	12.3 ± 0.6	4.9 ± 0.4	2.8 ± 0.5	11.9 ± 1.4	9.6 ± 1.0	2.0 ± 0.8	44.7 ± 3.8	3.8 ± 0.6	6.4 ± 0.9
0–15	29.1 ± 4.9	9.6 ± 0.9	2.2 ± 0.5	3.9 ± 0.9	7.6 ± 1.6	5.3 ± 0.6	1.0 ± 0.4	32.4 ± 6.2	2.8 ± 0.6	6.0 ± 1.2
15–60	23.9 ± 3.1	7.4 ± 0.4	2.5 ± 0.4	14.0 ± 1.6	13.0 ± 1.7	5.8 ± 0.6	2.3 ± 0.5	25.5 ± 3.4	2.0 ± 0.2	3.5 ± 0.8
60–120	10.1 ± 2.0	9.2 ± 0.9	3.3 ± 0.2	10.6 ± 1.5	18.6 ± 2.0	7.5 ± 0.6	1.3 ± 0.5	30.3 ± 3.5	2.2 ± 0.4	6.9 ± 1.8
					Bromobenzene					
Basal	1.8 ± 1.0	8.3 ± 2.7	7.3 ± 1.6	1.0 ± 0.3	6.8 ± 2.1	3.3 ± 0.7	3.8 ± 0.8	54.8 ± 5.1	5.7 ± 0.9	7.1 ± 1.0
0–15	25.8 ± 5.3	4.8 ± 1.3	5.3 ± 0.8	7.2 ± 1.0	5.7 ± 1.5	2.4 ± 0.6	1.2 ± 0.4	38.9 ± 4.3	4.2 ± 0.7	5.3 ± 0.9
15–60	31.1 ± 6.0	4.4 ± 1.3	2.4 ± 0.3	18.0 ± 1.6	8.6 ± 1.7	2.4 ± 0.6	1.0 ± 0.5	24.0 ± 3.0	1.9 ± 0.2	6.2 ± 0.9
60–120	16.0 ± 2.3	3.8 ± 0.8	4.3 ± 0.6	16.7 ± 2.0	15.8 ± 2.4	2.1 ± 0.6	0.4 ± 0.3	31.4 ± 2.4	2.4 ± 0.4	7.1 ± 1.4

Data were obtained by GC analysis of pooled samples. Results are means ± SEM from seven control and five BZ-treated animals. See the legend of Fig. 2 for the experimental protocol.

* Basal refers to the bile collected between 30 and 60 min after canulation, whereas indicated periods correspond to times after LCA injection.

† This peak contains also some ursodeoxycholic acid.

‡ Refers to a ketone bile acid whose structure remains uncertain.

§ Contains some identified (see text) as well as unidentified bile acids.

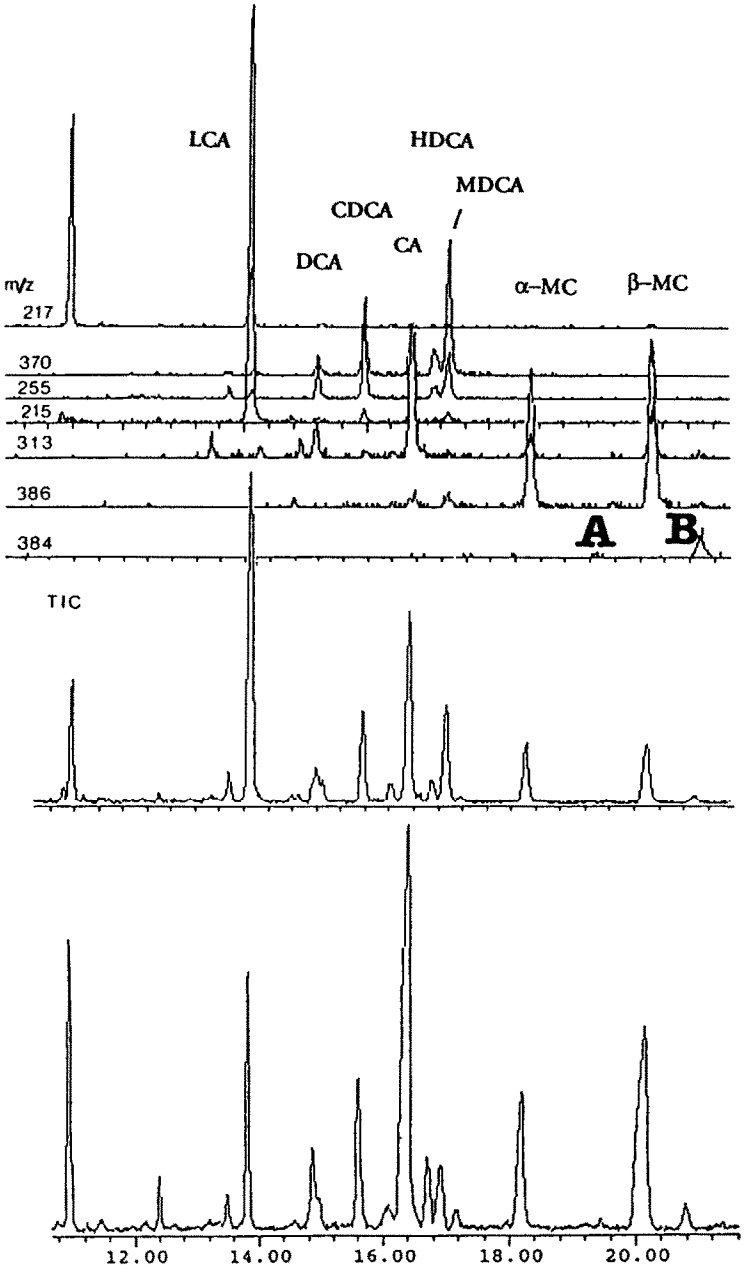

Fig. 6. Repetitive scanning GC–MS of bile acids excreted in bile during cholestatic and post-cholestatic phases after LCA injection. Bile acids were analysed as their methyl ester acetate derivatives. Upper panel: repetitive scanning GC–MS analysis. The presented ions m/z included 217 (internal standard), 370 and 255 (dihydroxy bile acids), 215 (monohydroxy), 313 (nuclear fragment of trihydroxy), and 386 and 384 (trihydroxy bile acid with side chain). The presence of m/z 384 in bile acids A and B indicates muricholic acids having a double bond in the chain side. This ion, as well as m/z 426, was not found when authentic standards of muricholic acids were analysed (data not shown). Middle panel: total ion current (TIC) during the cholestatic phase. Lower panel: TIC during the post-cholestatic phase.

(M^+ − (AcO + Me), 0.5%), 386 (1.5%), 370 (M^+ − 2xAcO, 100%), 355 (M^+ − (2xAcO + Me), 17%), 315 (ABCD + AcO, 6%), 283 (M^+ − (2xAcO + C_{22} ... C_{24}), 3%), 262 (M^+ − (C_1 ... C_6 + CH_3), 3.5%), 255 (ABCD, 40%), 249 (M − C_1 ... C_7, 9%), 228 (M^+ − (2xAcO + C_{16} ... C_{24}), 21%), 213 (ABC, 47%), 208 (M^+ − C_1 ... C_{11}, 4%), 173 (M^+ − (AcO + C_{16} ... C_{24}), 14%); and β-MC: m/z 446 (M^+ − AcO + 42, 3%), 428 (M^+ − 2xAcO, 10%), 386 ("428" − 42, 100%), 371 (M^+ − 2 AcO + CH_3 + 42, 7.5%), 368 (M^+ − 3xAcO, 14%), 353 (M^+ − (3xAcO + Me), 6%), 331 (ABC + 2xAcO, 1.1%), 313 (ABCD + AcO, 23%), 281 (M^+ − (3xAcO + C_1 . . . C_{22}), 2%), 271 (ABC + AcO, 4.5%), 253 (ABCD, 35%), 244

(12%), 211 (ABC, 11%). Other minor metabolites consisted of α-MC: 446 (6%), 428 (13.5%), 386 (100%), 371 (6.2%), 368 (13%), 353 (6%), 331 (<0.5%), 313 (7.5%), 281 (3.5%), 271 (3%), 253 (18%), 244 (12%), 211 (11%); CDCA, an oxo bile acid whose structure has not been confirmed; and two other bile acids (A and B, Fig. 6). The spectra of the latter: m/z 546 (M^+, 2 and 0%), 444 (1.5 and 1.4%), 426 (1.5 and 7%), 386 (33 and 16%), 384 (100 and 100%), 369 (10 and 3%), 366 (4 and 2%), 354 (36 and 3%), 327 (10 and 1%), 313 (95 and 6%), 281 (84 and 30%), 271 (18 and 20%), 269 (14 and 3%), 253 (99 and 90%), 211 (10 and 10%) are compatible with muricholates having a double bond in the side chain, since ions where the side chain was intact, but not the others, showed two units less. These bile acids were present before LCA injection and accounted for 0–1 and 1–2% of bile acids, respectively.

DISCUSSION

In this study, we investigated (1) LCA metabolism in the rat and (2) the effect of destruction of the perivenous zone on LCA biotransformation and development of cholestasis. Previous attempts to examine bile acid metabolism by GC in intact animals were hampered by the confounding influence of exogenous and endogenous bile acids. In the present investigation, the endogenous (bile acid pool prior to the injection of LCA) and exogenous (LCA and its metabolites) contribution to individual bile acid species was determined. These calculations allowed us to quantify phase I (hydroxylated) and phase II (conjugated) LCA metabolites *in vivo*. It was considered appropriate for the following reasons: (1) the calculated exogenous excretion of bile acids not derived from LCA remained close to zero during the experiment; (2) a significant positive correlation was obtained between the excretion of radioactivity and the calculated exogenous mass excreted ($Y = 1.028x + 10.54$, $r = 0.915$ for control; $Y = 1.195x + 9.663$, $r = 0.951$ for BZ-treated rats); and (3) the difference in the cumulative recovery of mass and of radioactivity in bile corresponded to glucuronide production, which accounted for an average of 19% of the injected dose.

The results obtained in this experiment confirm those published by Zimniak *et al.* [40, 41], although different approaches were used in both cases. LCA is biotransformed principally to LCA-GLU, MDCA and β-MC. Smaller amounts are produced by other metabolic pathways, leading to CDCA and α-MC. Other potential metabolites were identified by GC–MS, but their proportion was too small to ascertain that they were derived from LCA. A monohydroxy, monoketo bile acid was identified during the basal period by the following ions obtained by the MSD: 428 ($M^+ - 18$, 1.5%), 386 ($M^+ - AcO$, 30%), 313 ($M^+ -$ side chain $- 18$, 14%), 271 (ABCD, 100%), 253 (ABCD $- 18$, 92%), 229 (ABC, 10%), 211 (ABC $- 18$, 11%), 159 (42%), 133 (88%), 121 ($C_1 \ldots C_6 + C_{10} + C_{19}$, 82%) (data not shown). However, after LCA injection, the same ions were present but the major fragments were 311 ($M^+ - (AcO +$ side chain $+ 18)$, 100%) and 371 ($M^+ -$ (side chain + 18), 90%), 269 (ABCD, 80%) and 251 (ABCD $- 18$, 30%), indicative of a dihydroxy, monoketo bile acid or of an unsaturated monohydroxy, monoketo bile acid. The lack of high mass ions did not allow us to determine the exact structure of this bile acid. As pointed out by Baumgartner *et al.* [15], 3α,6β-dihydroxy,7-keto-5β-cholanoic acid is a probable metabolite of LCA. Using microsomal incubations, Zimniak *et al.* [40] found 3α-hydroxy,6-keto-5β-cholanoic acid to be the only ketonic acid derived from LCA. Since mitochondrial, peroxisomal and cytosolic compartments are likely to be implicated in the *in vivo* biotransformation of LCA, some discrepancies in the metabolic pattern from that obtained with microsomes alone may be expected.

In the present study, the presence of CDCA and ketonic bile acid paralleled that of MDCA, while α-MC appeared later, at the same time as β-MC. This suggests that LCA is metabolized to MDCA, which then undergoes 7β-hydroxylation to β-MC. Although earlier studies have shown that β-MC is formed from epimerization of α-MC, it can also arise from MDCA [42, 43]. This pathway was also suggested by Zimniak *et al.* [40] in their study of LCA metabolism by rat liver microsomes. The results presented here also agree with those obtained by the same group during LCA infusion in rats [41].

The present study further demonstrates that acinar zone 3 does not modify significantly the development of LCA-induced cholestasis. Despite a decreased functional mass and a smaller bile acid pool [see Ref. 20] in BZ-treated rats, the severity of the cholestasis induced by LCA was less than that induced in control rats, particularly in the early period after LCA injection. There was also a delay in LCA and LCA-GLU excretion, which resulted in the excretion of smaller amounts of these toxic bile acids during the first 15 min following LCA injection in the BZ group. Perhaps this delay was caused by the decreased bile acid pool and secretion in BZ-treated animals [20], since it has been proposed that secretion of LCA depends on its solubilization by bile acid micelles [44–47].

The present findings further demonstrate that there was no significant difference between BZ-treated rats and control rats in LCA biotransformation. Control rats excreted 22.4% of the LCA injected dose as T-LCA, 19.4% as LCA-GLU, 12.4% as MDCA and 9.5% as β-MC, while the corresponding values in BZ-treated animals were 20.0, 19.0, 14.7 and 6.9%, respectively. The other metabolites each accounted for less than 1.5%. The only difference between the presence of the perivenous zone and its absence was in the di- to trihydroxylated bile acids synthesized from LCA. This ratio was higher in the absence of the perivenous zone (1.3 vs 2.2), and suggests that the perivenous compartment is engaged in the processing of infused LCA in control experiments. A trend towards more β-MC formation in retrograde than in antegrade perfusion was also observed with cholestatic doses of T-LCA [15]. Takikawa *et al.* [48] showed that hydroxylation was greater in retrograde than in prograde perfusion when a tracer dose of LCA was infused. This was due in large part to a 2-fold increase of trihydroxylated metabolite formation. The present study further suggests that the 7-hydroxylase activity is higher in the perivenous zone than in the periportal zone, since CDCA, α-MC and β-MC formation (all

containing a hydroxyl group in the 7 position) is lower in BZ-treated animals. The zonation of 7α-hydroxylase was already suggested in our earlier work [20], in which it was shown that CDCA synthesis was decreased by 45% after perivenous zone destruction. Our present data indicate that there are no changes in the 6-hydroxylation and glucuronidation of LCA when the perivenous zone is destroyed. The possibility that zonal susceptibility to LCA-induced cholestasis may be due to differences in the glucuronidation rate of LCA has not been investigated thus far. The acinar location of glucuronosyltransferases is still a matter of debate [49, 50], and if one takes into account the strong cholestatic properties of LCA-GLU, a predominant perivenous localization could have resulted in milder cholestasis when this zone was destroyed by BZ. Our data, however, show that LCA-GLU formation was not different with or without the perivenous zone, which agrees with a report [51] that glucuronidation of bile acids is evenly distributed in the acinus.

Baumgartner *et al.* [15] observed that retrogradely perfused rat livers were less susceptible to T-LCA-induced cholestasis than antegradely perfused livers. Two mechanisms were postulated: one implicates a higher perivenous biotransformation of T-LCA, and the other relies on the acinar location of canalicular obstruction in relation to the bile flow axis (perivenous to periportal). The importance of the site of bile canalicular obstruction in the development of cholestasis could not be verified with our model, since any canalicular abnormalities must be localized in zones 1 and 2 in both control and BZ-treated animals. Thus, the prevention of cholestasis during retrograde perfusion with T-LCA might be explained by the zonal differences in T-LCA metabolism.

In conclusion, these data demonstrate that destruction of perivenous hepatocytes does not increase the susceptibility of the rat to LCA-induced cholestasis and that there is no significant acinar localization for LCA metabolism.

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