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Toxicity and Kinetics of [^{3}H]Microcystin-LR in Isolated Perfused Rat Livers

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calculated hepatic extraction ratio was low, there was a significant accumulation of microcystin in liver. Many toxic effects of microcystin in the perfused liver mimicked those observed in the whole animal, suggesting that this model can be used as an alternative to whole animals for screening of potential therapeutic agents.

INTRODUCTION

Microcystin-LR (MCYST-LR) is a cyclic hepatotoxin isolated from the cyanobacterium *Microcystis aeruginosa* (Rinehart *et al.*, 1988). In a variety of animals, exposure to a lethal dose of toxin produces blood-engorged livers, which exhibit diffuse centrilobular necrosis (Konst *et al.*, 1965; Jackson *et al.*, 1984; Adams *et al.*, 1988). The hepatotoxic effects of *Microcystis* toxins have been described in both rats and mice (Dabholkar and Carmichael, 1987; Miura *et al.*, 1989). Fasted rats injected ip with 100 μg/kg of MCYST-LR exhibit blood-engorged livers after 60 min and die within 2 hr (Miura *et al.*, 1989). Electron microscopy and biochemistry of the livers from these rats revealed formation of hydropic mitochondria, which exhibited decreased ADP/O ratios and respiratory control indices; formation of rough endoplasmic reticulum whorls; decreased desmosome-associated filaments; dilation of bile canaliculi; disruption of sinusoidal lining; and, ultimately, necrosis of hepatic parenchymal cells.

The plasma elimination half-life of MCYST has been described for rat (Falconer *et al.*, 1986) and mouse (Robinson *et al.*, 1989) with [^{125}I]MCYST-YM and [^{3}H]MCYST-LR, respectively. The biological half-life for MCYST-YM after an iv injection in the rat was 42 min. However, recovery of radiolabel was only 36% of injected dose, suggesting possible de-iodination of the labeled toxin. In mice, the route of administration was ip and elimination from plasma was complicated by slow absorption from

the peritoneal cavity. The plasma half-life was 29 min.

In an effort to develop an *in-vitro* system that could be used to screen for potential therapeutic agents, we selected the isolated perfused rat liver. With this model, Berg *et al.* (1987) and Theiss *et al.* (1988) noted that bile flow ceases 10 min after addition of toxin, the liver becomes engorged with perfusate within 30-40 min, and normal lobular architecture and sinusoidal spaces are obliterated. In this study, we correlated the ultrastructural and biochemical effects of [^{3}H]MCYST-LR in the perfused fasted-rat liver. In addition, we have described, for the first time, the hepatic subcellular distribution, metabolism, and kinetics of radiolabeled MCYST-LR.

METHODS

MCYST-LR (>95% purity by HPLC analysis) was supplied by W. W. Carmichael (Wright State University, Dayton, OH). [^{3}H]MCYST-LR (Amersham Corp, Arlington Heights, IL, 40% radiochemical purity) was produced by chemical tritiation of MCYST-LR (Matsuo and Narita, 1975) and purified by reverse-phase, high-performance liquid chromatography (HPLC) to 90-99% radiochemical purity with a specific activity of 194 mCi/mmol (Robinson *et al.*, 1989).

Liver Perfusion. Male, Fischer 344 rats (200-250g) were fasted overnight in preparation for surgery as described by Pace (1986). Bile duct, inferior vena cava (out-flow) and portal vein

(in-flow) were cannulated and livers were perfused *in situ* in an apparatus described by Zenser *et al.* (1974). Perfusion medium consisted of 30% washed sheep erythrocytes, 3% bovine serum albumin, and heparin (5 units/ml) in Krebs-Ringer bicarbonate buffer, pH 7.4. The atmosphere in the perfusion chamber was 95% O_2 and 5% CO_2. After 30 min of equilibration, 0, 0.3, or 0.5 μg/ml [^{3}H]MCYST-LR was added to the perfusate (119 ml), which was recirculated at a constant flow of 10 ml/min for 60 min.

Analysis of Perfusate and Bile. Reservoir perfusate (1.5 ml) and bile (total produced) were sampled every 10 min. Perfusate was centrifuged at 700*g* for 10 min and radioactivity was measured in a Beckman 5800 liquid scintillation counter. Methanol (60%) was added to erthrocyte-free perfusate and centrifuged in an Eppendorf microfuge. An aliquot of the supernatant (150 μl) was analyzed with a Waters HPLC system (Milford, MA) equipped with a Waters 490 multiwavelength detector, as described by Robinson *et al.* (1989). A C-18 column (Adsorbosphere HS, 4.6 x 250 mm, 5 μm, Alltech, Deerfield, IL), was run at a flow rate of 1 ml/min of 75% 10 mM ammonium acetate (pH 6.0) and 25% acetonitrile for 20 min. Elution of toxin was monitored at 238 nm. Radioactivity was measured with a Flow I β-radiodetector (Radiomatic Instruments and Chemical Co., Tampa, FL), outfitted with a 5-ml cell with Flo-Scint III scintillation fluid (Radiomatic) pumped at 2 ml/min (efficiency 35%). Bile (5-10 μl) was analyzed by HPLC without prior treatment.

A COBAS BIO Analyzer (Roche Analytical Instruments, Nutley, NJ) was used to measure glucose, blood urea nitrogen, alanine aminotransferase, aspartate aminotransferase, lactate dehydrogenase, alkaline phosphatase (Roche), 5'-nucleotidase, and Lowry total protein concentrations (Sigma Chemical Company, St. Louis, MO) in erythrocyte-free perfusate.

Electron Microscopy. After a 60-min toxin or vehicle perfusion, one liver per dose group was perfused with 150 ml saline and perfusion-fixed with 120 ml 2% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.4. Sections from the central region of the right lateral lobe were removed, cut into small cubes (1 mm^3), and further fixed in 2% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.4, for 2 hr. The tissue was washed in buffer overnight at 4°C, post-fixed in 1% osmium tetroxide (1 hr), followed by block staining in 0.5% uranyl acetate (1 hr). Tissue was dehydrated in 10%, 50%, 80%, 95%, and 100% ethanol and propylene oxide and embedded in Epon 812. Ultrathin sections were cut with a diamond knife on a Om U3 ultramicrotome (Reichert Jung, Vienna, Austria), stained with 5% uranyl acetate and lead citrate, and examined with a JEOL 100CX electron microscope (JEOL, Tokyo, Japan).

Mitochondrial Respiration. At the end of the 60-min perfusion, three livers per dose group were excised, minced in 30 ml of 220 mM mannitol and 75 mM sucrose (pH 7.4), and homogenized with a

Potter-Elvehjem tissue homogenizer. After centrifugation, (700*g*, 10 min) the pellet was discarded and the supernatant centrifuged (8000*g*, 10 min). The pellet, containing mitochondria, was resuspended in 2.5 ml of the homogenizing solution. Respiration rates were determined at 28°C with a Clark-type oxygen electrode (Yellow Springs Instruments Co., Yellow Springs, OH). The assay mixture (3 ml) contained mitochondria (2-4 mg protein), 150 mM KCl, 10 mM potassium phosphate, 20 mM Tris-Cl, and 3 mM $MgCl_2$ (pH 7.4). Substrate (10 µmol glutamate or 25 µmol succinate) and ADP (1 µmol) were added sequentially.

Subcellular Distribution. Portions of liver homogenate (prepared as above) were applied to a Percoll step gradient as described by Reinhart *et al.* (1982). After centrifugation at 45,000*g* for 30 sec, nine fractions were obtained and analyzed for marker enzymes. Lactate dehydrogenase (cytosol, fraction 1), 5'nucleotidase (plasma membrane, fractions 2 and 4), and total protein were measured with the COBAS BIO analyzer. Cytochrome oxidase (mitochondria, fraction 7) (Cooperstein and Lazarow, 1950) and cytochrome reductase (microsomes, fractions 1,2 and 4) (Beaufay *et al.*, 1974) were measured as previously described. Radioactivity was measured in a Beckman LS 8501 scintillation counter. Fractions 1, 2 and 4 from the Percoll gradient were recentrifuged at 100,000*g* (Knupp *et al.*, 1987) in a Beckman SW50.1 rotor to sediment microsomes. The pellet and supernatant (S-100) were analyzed for enzymes and radioactivity as above. An

aliquot (300 µl) of the pooled S-100 from fractions 1, 2, and 4 was added to 2.0 ml of 0.1 M potassium phosphate buffer (pH 7.2) and heat denatured at 90°C for 30 min. The sample was treated with 2.5 mg pronase (Calbiochem, La Jolla, CA) for 1 hr at 37°C, cooled, and centrifuged (Eppendorf). The supernatant was applied to a C-18 Sep Pak cartridge (Waters Assoc., Milford, MA) and eluted with two 1-ml aliquots of 100% methanol. The eluant was dried under nitrogen, redissolved in 80% methanol, and analyzed by HPLC as described above.

Calculations and Statistical Analysis. Theoretical considerations for the recirculating perfusion system have been described by Pang and Gillette (1978). The perfusate concentration curves were analyzed with MKMODEL software (Elsevier-BIOSOFT, Cambridge, UK) on an IBM personal computer AT. The single-compartment, bolus-injection model was used to estimate clearance (C_L), and the first-order elimination rate constant (k). The elimination half-life from perfusate was calculated as $T_{1/2} = \ln 2/k$. The steady-state hepatic extraction ratio (E) was calculated as $E = C_L/Q$, where Q is the perfusate flow rate. Results are presented as mean ± SE. Statistical significance was calculated by ANOVA and Duncan's multiple range test. A p value < 0.05 was considered significant.

RESULTS

Effect of MCYST-LR on perfused rat liver

Livers perfused with 0.3 or 0.5 μg/ml (initial concentration) MCYST-LR typically exhibited dark surface mottling 30-40 min after introduction of toxin and began to swell at 40 min. The swelling progressed and, at the higher dose, perfusate leaked from the surface of the liver after 50 min. Bile flow decreased 20-30 min after addition of toxin and stopped 50 min post-toxin exposure (0.5 μg/ml, Fig. 1). Perfusate concentrations of lactate dehydrogenase, blood urea nitrogen, alanine aminotransferase, and 5'nucleotidase were not affected by toxin. The rate of glycogen breakdown, measured as glucose released into perfusate over time, was significantly stimulated, compared to control, from 0.13 ± 0.02 to 0.94 ± 0.14 and 0.56 ± 0.07 (mg/dl)/min for 0.3 and 0.5 μg/ml MCYST, respectively.

Fate and distribution of radiolabeled MCYST-LR

Figure 2 is a representative disposition curve of radiolabel in perfusate and bile. Steady-state elimination via the bile was reached within 20 min. Toxicokinetic parameters (Table 1) were calculated from the perfusate concentration curve (Fig. 3), assuming that no metabolism of the parent toxin (100% of perfusate radiolabel was associated with parent toxin) and <1% toxin associated with red blood cells (data not shown). The

perfusate elimination half-lives, 101 ± 13 and 131 ± 10 min, were not significantly different for the doses studied. The hepatic extraction ratio was low ($E < 0.10$) and C_l was less than 1 ml/min at a constant flow rate of 10 ml/min.

Distribution of radiolabel at the end of the 60-min perfusion was the same, regardless of dose (Table 2). The liver retained 16% of the delivered radiolabel or 0.9 μg [^{3}H]MCYST-LR/g liver, 79% remained in the perfusate, and < 2% was excreted via bile. Two to four percent was recovered in perfusate that leaked from the liver's surface. In bile, 78% of the radiolabel was associated with parent toxin (Fig. 4, A and B). More-polar metabolites comprised 22% of the biliary radiolabel, or < 0.4% of the total delivered dose.

Subcellular distribution of radiolabel

The homogenate of MCYST perfused rat liver, containing 16% of the total radiolabel, distributed across a Percoll step gradient as shown in Fig. 5. Distribution patterns of lactate dehydrogenase (soluble) and cytochrome reductase (microsomes) were similar to the radiolabel distribution. After centrifugation at 100,000*g*, 90% of the radioactivity, but only 25% of the microsomal marker, remained in the supernatant (S-100). The S-100 fraction was heat denatured and treated with pronase as described in the methods section (recovery 50%). Figure 4 (C and D) shows the separation of radiolabeled peaks by HPLC. Between 65 and 80% of the recovered liver radiolabel was

associated with a more-polar component. Both parent MCYST and thi- polar component appeared to be tightly bound to soluble protein(s).

Electron microscopy

The following ultrastructural changes were noted after a 60-mi.. perfusion with either 0.3 or 0.5 μg/ml [^{3}H]MCYST-LR: (a) disruption of normal stacked appearance of endoplasmic reticulum (er) and formation of er whorls (Fig. 6); (b) mitochondrial swelling (Fig. 6 and 7); and (c) loss of desmosome-associated intermediate filaments, dilation of bile canaliculi, and widening of the gap between hepatocytes (Fig. 7). There was a toxin concentration-dependent widening of the spaces of Disse and rounding of hepatocytes. Erythrocytes were found within the space of Disse, indicating rupture of the sinusoidal lining (Fig. 8).

Mitochondrial respiration

Mitochondria isolated from toxin-perfused livers displayed a 50% decrease in state 3 respiratory rates and respiratory control indices (Table 3). There appeared to be a dose-dependent decrease in the ADP/O ratio ($p < 0.1$).

DISCUSSION

Hepatotoxic effects of MCYST have been studied in isolated hepatocytes, both in suspension (Runnegar *et al.*, 1987) and

monolayers (Thompson *et al.*, 1987), perfused livers (Berg *et al.*, 1988) and several whole animal models (Konst *et al.*, 1965; Jackson *et al.*, 1984; and Miura *et al.*, 1988). The isolated perfused liver has several advantages over other model systems for the study of hepatotoxins. Unlike the *in-vitro* cell systems, the functional and dimensional relationships of various cell types are preserved in the isolated organ. With the isolated liver, it is also possible to differentiate direct organ effects from those modulated by the whole animal. For example, in rats, 1 hr after injection of [^{3}H]MCYST-LR, there were significant increases in serum enzyme levels (lactate dehydrogenase, alanine aminotransferase, aspartate aminotransferase, and sorbitol dehydrogenase) (Miura *et al.*, 1989); while in livers perfused with a comparable dose of MCYST, there were no changes in perfusate enzyme levels.

In addition, with the perfused organ model, we can measure toxic effects that are masked in the whole animal model. For example, serum glucose decreased in the whole animal model (Miura *et al.*, 1989) while in the perfused model, toxin stimulated the rate of glucose release. Stimulation of glucose release is consistent with the observation that MCYST activates phosphorylase a (Runnegar *et al.*, 1987), the rate-limiting enzyme for glycogenolysis. MCYST stimulation of glycogenolysis probably occurs *in vivo* but was not reflected by a rise in serum glucose levels because of increased peripheral utilization of glucose. This possibility is also supported by the observation that *in-*

vivo glycogen stores are rapidly depleted after administration of MCYST toxins (Adams *et al.*, 1988).

Many of the ultrastructural toxic effects of MCYST-LR noted in the fasted rat model (Miura *et al.*, 1989) were also observed in the perfused liver, indicating a direct hepatotoxic effect of MCYST. Morphologic changes included dilation of endoplasmic reticulum, mitochondrial swelling, rounding of hepatocytes, loss of cell-cell contact, and destruction of sinusoidal lining. Isolated mitochondria displayed inhibition of state 3 respiration, indicative of their hydropic state.

The concentration of toxin/g of liver was independent of the perfusion dose, suggesting that a saturable limit was reached. The accumulation of [^{3}H]MCYST-LR in liver appeared to be due to binding of the molecule to one or more cytosolic proteins (Fig. 4 C and D). Hepatic radiolabel (parent and metabolite) was recovered only after heat denaturation and treatment of the S-100 fraction with a non-specific protease (Fig. 4 C and D). When parent toxin was exposed to this procedure, no additional components were formed, indicating that the polar component was not an artifact of the isolation procedure. We are currently attempting to identify the protein(s) to which MCYST-LR bind(s). This toxin-protein interaction may help explain the specificity of MCYST-LR for the liver, as well as its hepatotoxic effects.

Many toxic effects of MCYST-LR in the perfused liver mimic those observed in the whole animal and can be used as indices to screen potential therapeutic agents against MCYST intoxication.

Among these effects are the presence of hydropic mitochondria, decreased desmosomal tonofilaments, destruction of normal sinusoidal architecture, increased glucose release, and cessation of bile flow. Since MCYST-LR displayed low extraction characteristics, the perfusate elimination half-life is theoretically dependent on the liver's ability to metabolize and excrete the toxin (Wilkinson and Shand, 1975). But, because MCYST accumulates in liver, the target organ, and is only minimally eliminated via bile, agents that decrease distribution to liver, such as rifampicin (Pace *et al.*, 1990) and/or increase urinary clearance appear to be better therapeutic candidates than are enzyme inducers.

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TABLE 1

PERCENT DISTRIBUTION OF RADIOLABEL AFTER 60-MIN PERFUSION

Tissue	Dose 0.3 µg/ml	0.5 µg/ml
Bile	1.7 ± 0.4	1.6 ± 0.3
Perfusate	79.2 ± 3.2	78.8 ± 2.1
Liver	16.8 ± 3.6	15.5 ± 1.1
Surface Leak	2.4 ± 0.7	4.1 ± 1.1

TABLE 2

TOXICOKINETIC PARAMETERS

Parameter[a]	Toxin Concentration 0.3 µg/ml	0.5 µg/ml
Dose (dpm) x 10^7	1.7	2.6
V (ml)	122 ± 2	122 ± 3
C_L (ml/min)	0.85 ± 0.10	0.66 ± 0.04
k x 10^{-3}	7.1 ± 0.8	5.4 ± 0.4
C_{max} (dpm/ml) x 10^5	1.45 ± 0.03	2.22 ± 0.18
AUC (dpm/ml)*min x 10^7	2.2 ± 0.2	4.0 ± 0.6
E	0.09 ± 0.01	0.07 ± 0.00
$T_{\frac{1}{2}}$ (min)	101 ± 13	131 ± 10

[a]Apparent volume of distribution (V); clearance (C_L); disposition rate constant (k); concentration at 0 time (C_{max}); area under the perfusate concentration curve (AUC); hepatic extraction ratio (E); and ß phase half-life ($T_{\frac{1}{2}}$).

TABLE 3

EFFECT OF MCYST-LR ON MITOCHONDRIAL RESPIRATION

Substrate	MCYST (µg/ml)	STATE 3 Rate (nmol O_2/min/mg)	ADP/O	RCI[a]
Succinate	0.0	63.6 ± 5.3	1.82 ± 0.03	5.94 ± 0.66
	0.3	35.1 ± 4.6[b]	1.60 ± 0.09	2.54 ± 0.31[b]
	0.5	41.4 ± 5.0[b]	1.48 ± 0.12	2.92 ± 0.75[b]
Glutamate	0.0	36.0 ± 4.0	2.58 ± 0.08	5.94 ± 0.99
	0.3	22.1 ± 2.3[b]	2.47 ± 0.08	2.32 ± 0.13[b]
	0.5	24.0 ± 3.3[b]	2.13 ± 0.19	2.52 ± 0.40[b]

[a]RCI = Respiratory control index (state 3/state 4)

[b]$p \leq 0.05$ compared to control

Values are mean ± SE, n=3

FIGURE LEGENDS

Fig. 1. Effect of MCYST-LR on bile flow. Livers were perfused with 0 (□), 0.3 (■), or 0.5 (▨) µg/ml [^{3}H]MCYST-LR. Bile was collected at 10-min intervals. Bile volume was measured and average flow rate for 10 min was calculated. Values are mean ± SE, n=4. The * indicates $P < 0.05$ as compared to control.

Fig. 2. Representative curve of the disposition of [^{3}H]MCYST-LR during recirculation through the liver. Toxin was delivered at a constant flow of 10 ml/min. The initial input concentration was 129,000 dpm/ml. The rate out of radioactivity (concentration x flow rate) in perfusate (●) and bile (○) are shown.

Fig. 3. Perfusate concentration curve. Toxin was delivered at a constant flow of 10 ml/min for 60 min in a recirculation experiment. The initial input concentration was 129,000 dpm/ml. Values are mean ± SE, n=3.

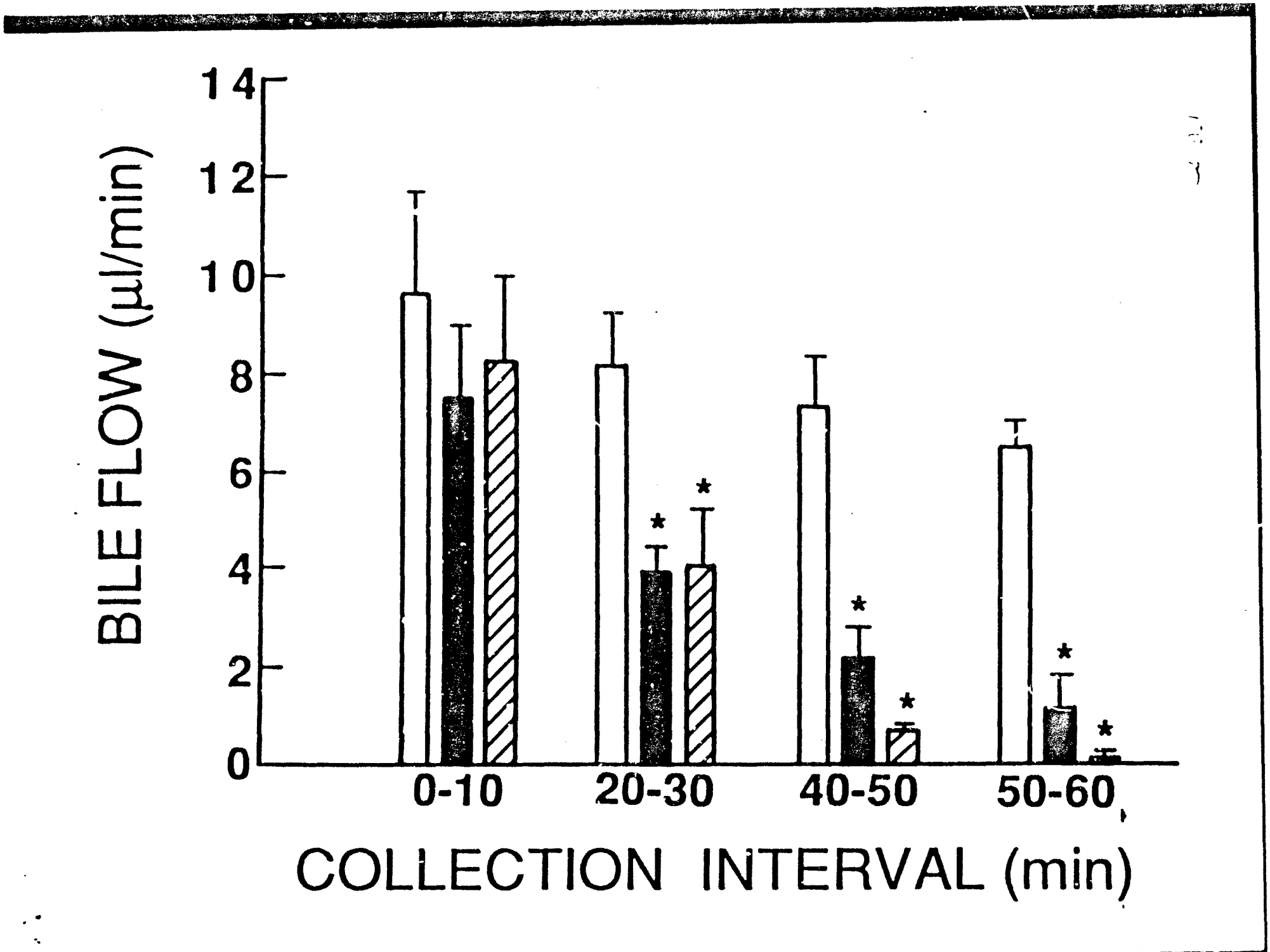
Fig. 4. HPLC analysis of radiolabel in bile and liver. HPLC was performed as described in METHODS. Panel A, bile spiked with [^{3}H]MCYST; panel B, bile collected from 10-20 min after exposure to toxin (0.5 µg/ml); panel C, S-100 fraction of liver spiked with [^{3}H]MCYST-LR; panel D, S-100 fraction of liver that was perfused with [^{3}H]MCYST-LR for 1 hr.

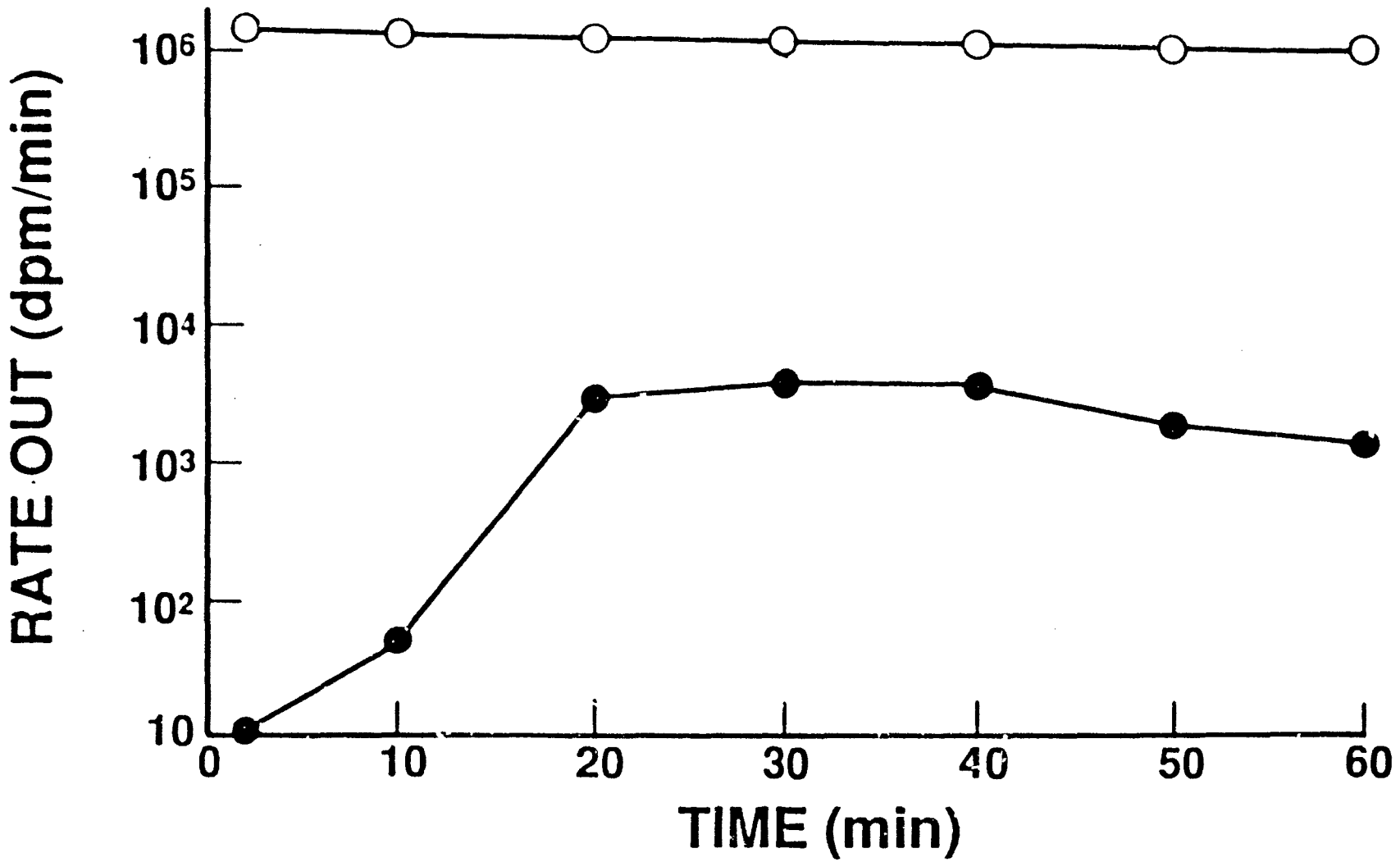
Fig. 5. Distribution pattern of [^{3}H]MCYST-LR on Percoll step gradients. Homogenates of [^{3}H]MCYST-LR (0.5 μg/ml, initial concentration) perfused liver were applied to Percoll step gradients. Marker enzymes and radioactivity were measured in each of the nine fractions.

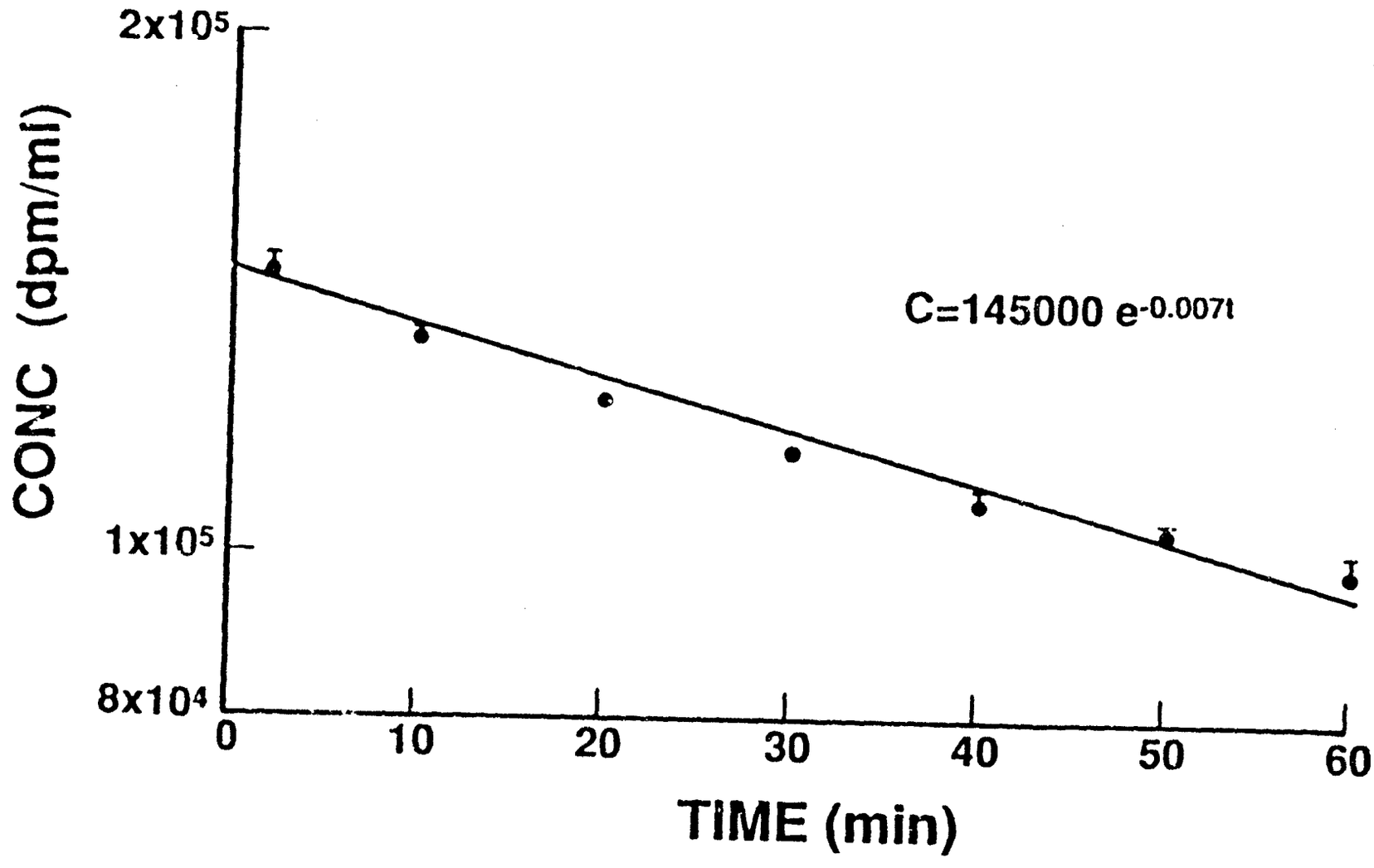
Fig. 6. Effect of toxin treatment on endoplasmic reticulum (er) and mitochondria (M). After a 60-min perfusion with 0 (A) or 0.5 (B) μg/ml MCYST-LR, the liver was processed for electron microscopy as described in METHODS. Bar = 1 μm.

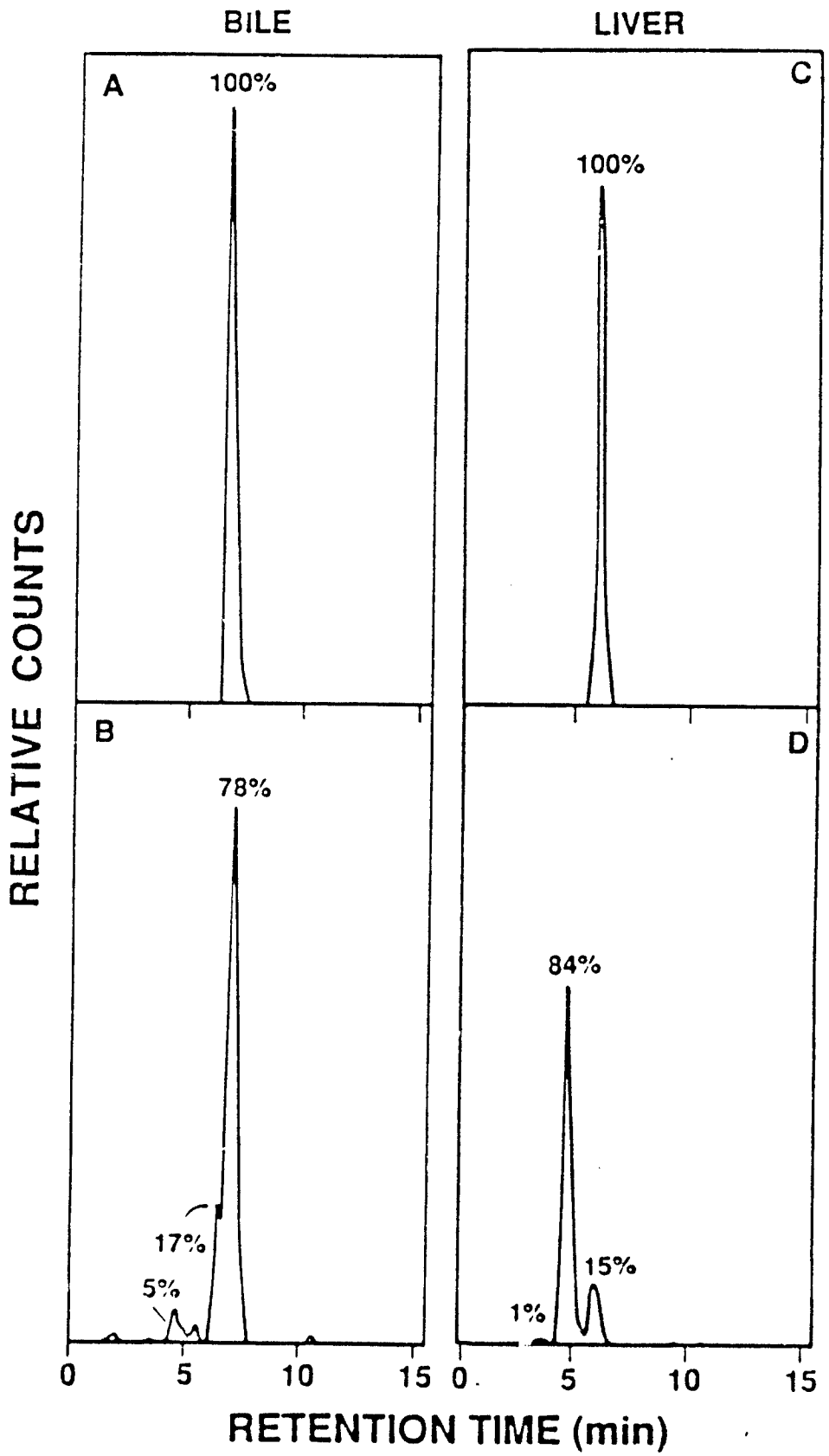
Fig. 7. Effect of MCYST-LR on desmosome-associated filaments (D) and bile canaliculi (BC). After a 60-min perfusion with 0 (A) or 0.3 (B) μg/ml MCYST-LR, the liver was processed for electron microscopy. Note the abscence of desmosomes in the area of the bile canaliculi after toxin exposure (B). Bar = 1 μm.

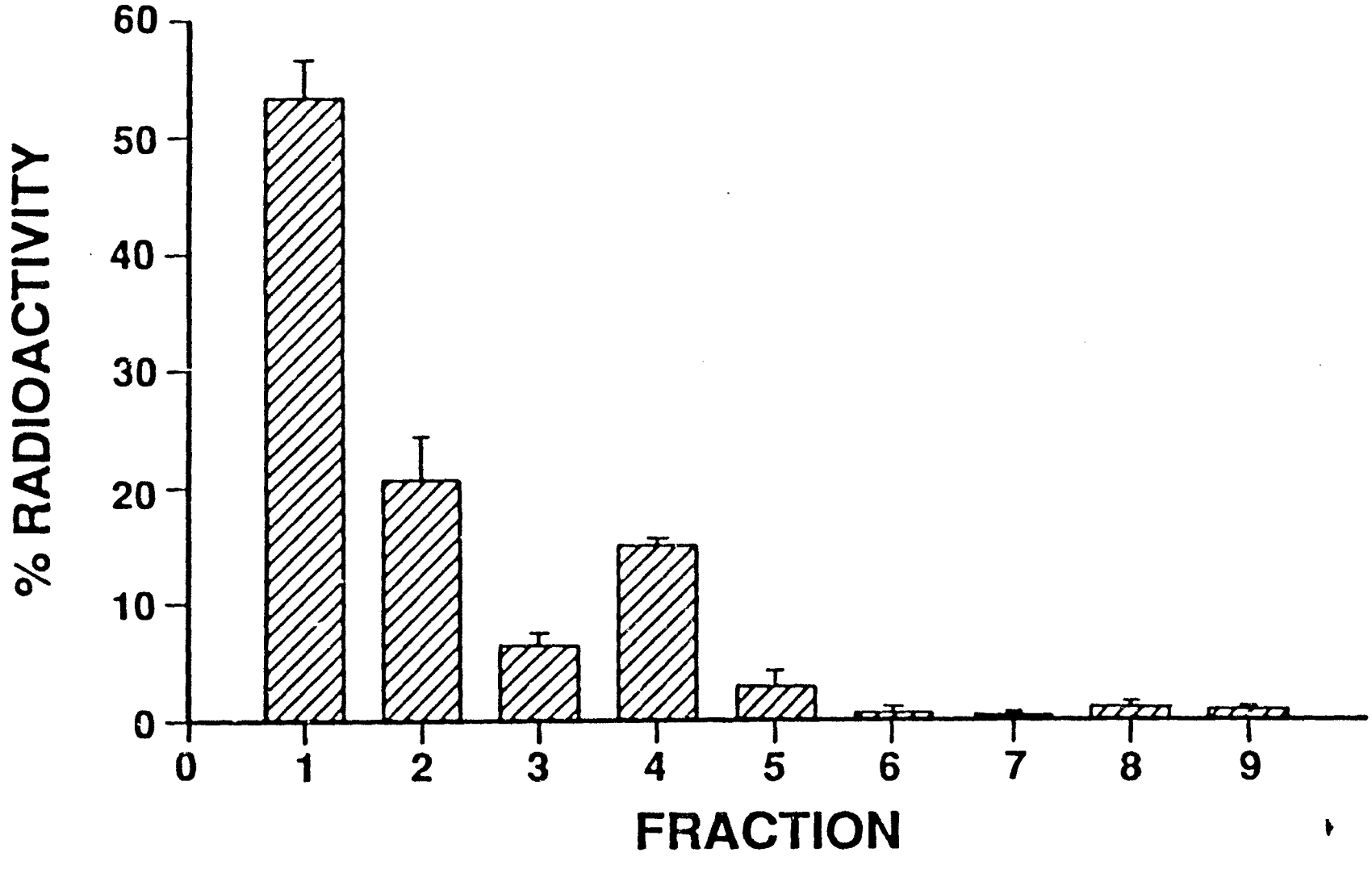
Fig. 8. Effect of MCYST-LR on sinusoidal architecture. Livers were perfused with 0 (A), 0.3 (B), or 0.5 (C) μg/ml MCYST-LR and processed for electron microscopy. Abbreviations: Sn-sinusoids, *-space of Disse, and E-erythrocyte. Bar = 1 μm.

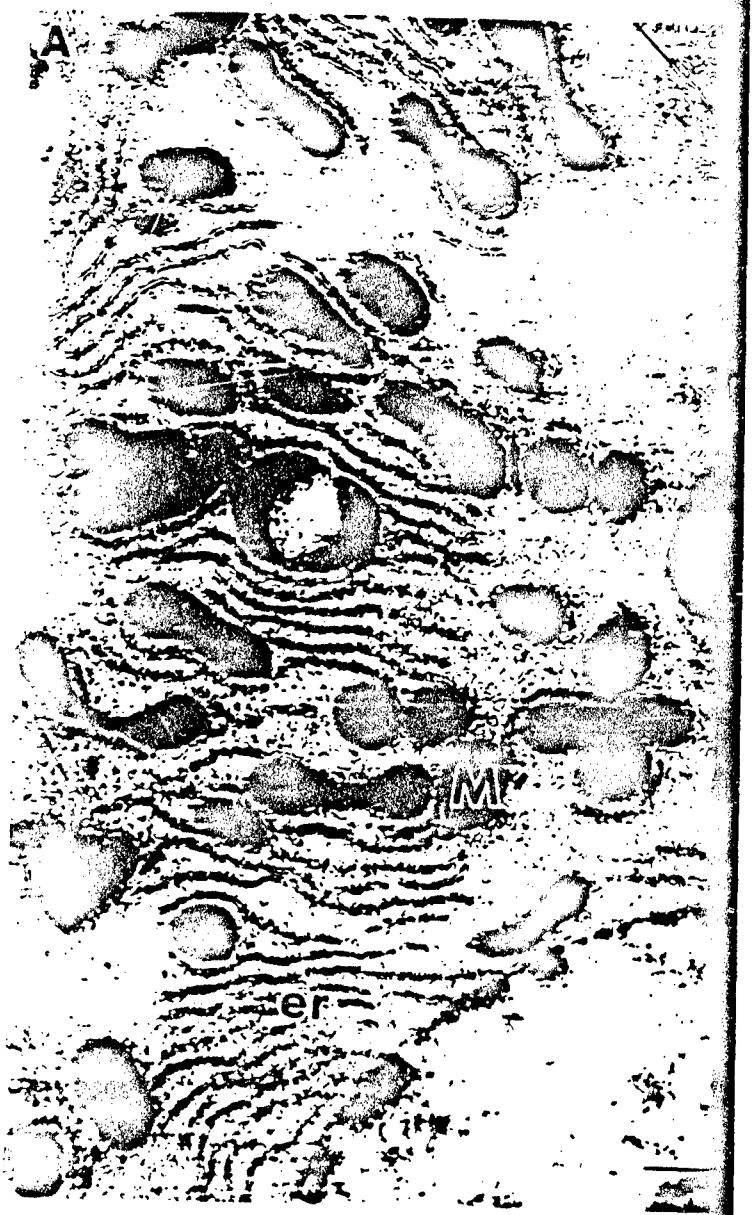

BILE FLOW (µl/min)
14
12
10
8
6
4
2
0
0-10
20-30
40-50
50-60
COLLECTION INTERVAL (min)


RATE OUT (dpm/min)
10⁶
10⁵
10⁴
10³
10²
10
0
10
20
30
40
50
60
TIME (min)


2x10⁵
1x10⁵
8x10⁴
CONC (dpm/ml)
C=145000 e-0.007t
0
10
20
30
40
50
60
TIME (min)


BILE
LIVER
A
100%
C
100%
B
78%
17%
5%
D
84%
15%
1%
0
5
10
15
0
5
10
15
RELATIVE COUNTS
RETENTION TIME (min)


% RADIOACTIVITY
60
50
40
30
20
10
0
0
1
2
3
4
5
6
7
8
9
FRACTION


A
M
er

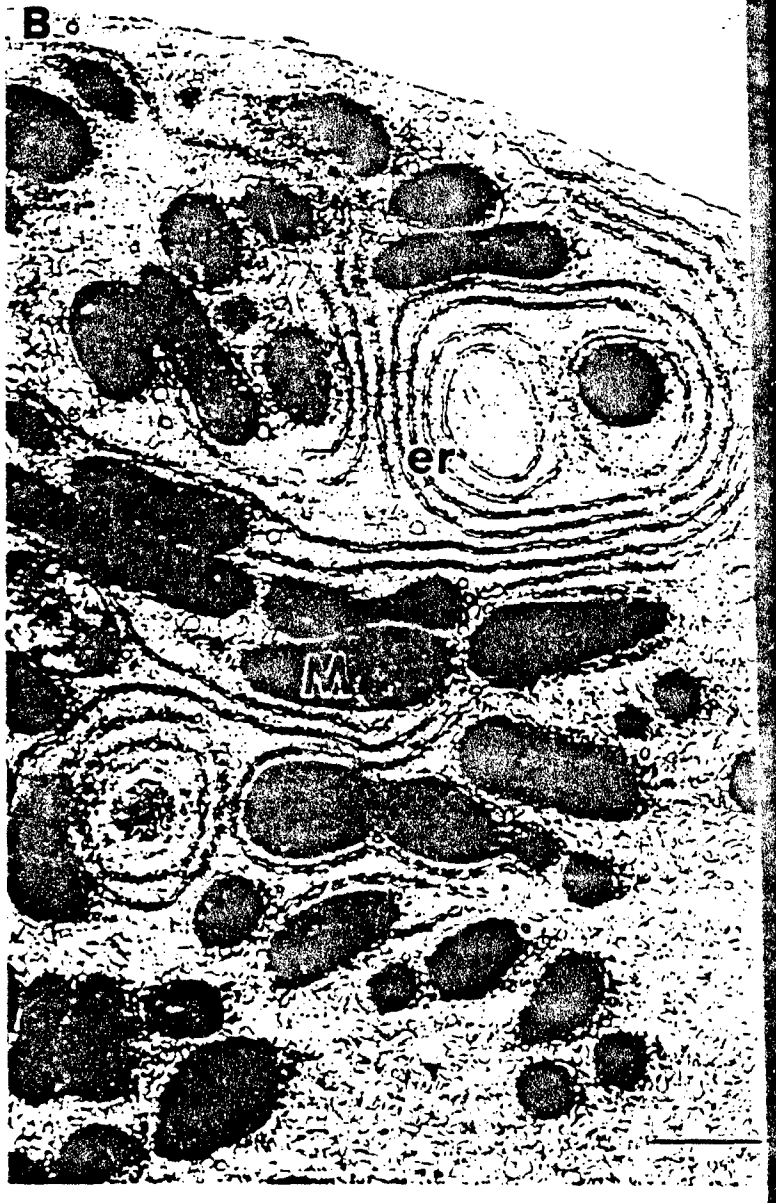

B
er
N

Fig 7


B
BC

A
E
Sn

B
Sn
*

C
E
*
Sn