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The LD_{50} (25 hr; i.p.) for microcystin-LR in fed rats (122 µg/kg) was significantly higher than that in fasted rats (72 µg/kg). At doses of 100, 150 and 200 µg/kg microcystin-LR, the median times to death were 31.9, 18.2 and 11.2 hr for fed rats, and 1.8, 1.7 and 1.5 hr for fasted rats. Biochemical and ultrastructural changes resulting from microcystin-LR (100 µg/kg, i.p.) were compared in fed and fasted rats 1 hr after injection. In both groups, liver weight and serum levels of sorbitol dehydrogenase and glucose significantly increased. Plasma membranes, isolated from livers of fed or fasted rats, exhibited similar toxin-induced changes in associated cytoskeletal elements. Liver mitochondria from toxin-treated, fasted rats exhibited complete inhibition of state 3 respiration, while those from toxin-treated, fed rats had ADP/O ratios and respiratory control indices comparable to control values. A sublethal dose of microcystin (50 µg/kg) afforded protection to fasted, but not fed, rats against a subsequent lethal dose (200 µg/kg) challenge given 72 hr later. Keywords: Phalloidin; Cyl (KT)

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HEPATOTOXICITY OF MICROCYSTIN-LR IN FED AND FASTED RATS*

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The views of the authors do no purport to reflect the positions of the Department of the Army or the Department of Defense (para.4-3, AR 360-5). In conducting research described in this report, the investigators adhered to the "Guide for the Care and Use of Laboratory Animals," as promulgated by the Committee on the Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council. The facilities are fully accredited by the American Association for Accreditation of Laboratory Animal Care.

ABSTRACT

G. A. MIURA, N. A. ROBINSON, W. B. LAWRENCE, and J. G. PACE. Hepatotoxicity of microcystin-LR in fed and fasted rats. *Toxicon* xx, xxx-xxx, 1990.--The LD_{50} (25 hr; i.p.) for microcystin-LR in fed rats (122 μg/kg) was significantly higher than that in fasted rats (72 μg/kg). At doses of 100, 150 and 200 μg/kg microcystin-LR, the median times to death were 31.9, 18.2 and 11.2 hr for fed rats, and 1.8, 1.7 and 1.5 hr for fasted rats. Biochemical and ultrastructural changes resulting from microcystin-LR (100 μg/kg, i.p.) were compared in fed and fasted rats 1 hr after injection. In both groups, liver weight and serum levels of sorbitol dehydrogenase and glucose significantly increased. Plasma membranes, isolated from livers of fed or fasted rats, exhibited similar toxin-induced changes in associated cytoskeletal elements. Liver mitochondria from toxin-treated, fasted rats exhibited complete inhibition of state 3 respiration, while those from toxin-treated, fed rats had ADP/O ratios and respiratory control indices comparable to control values. A sublethal dose of microcystin (50 μg/kg) afforded protection to fasted, but not fed, rats against a subsequent lethal dose (200 μg/kg) challenge given 72 hr later.

INTRODUCTION

Certain freshwater cyanobacteria synthesize a group of related cyclic heptapeptides called microcystins (CARMICHAEL *et al.*, 1988; BEASLEY *et al.*, 1989) which have novel features, such as D-amino acids, isolinkages and a β-amino acid residue (BOTES *et al.*, 1985; RINEHART *et al.*, 1988; MERILUOTO *et al.*, 1989). One of these variants is microcystin-LR (MCYST-LR), a potent hepatotoxin (CARMICHAEL *et al.*, 1985) which accumulates in liver, disrupting sinusoidal endothelial cells and causing hemorrhagic shock and rapid death in mice (SLATKIN *et al.*, 1983; ADAMS *et al.*, 1988; DABHOLKAR and CARMICHAEL, 1987; THEISS *et al.*, 1988; ROBINSON *et al.*, 1989).

The changes reported in rats after a lethal dose of MCYST-LR include: dilation of rough ER, whorls of rough ER, loss of sinusoidal architecture, hydropic mitochondria, loss of desmosomal tonofilaments and necrotic hepatocytes (HOOSER *et al.*, 1988; BERG *et al.*, 1988; MIURA *et al.*, 1989). Rats fasted 16 hr prior to experiments died 2 hr after an i.p. injection of 100 μg/kg toxin (MIURA *et al.*, 1989). In contrast, the time to death of fed rats was 16 hr for the same dose of toxin (THEISS *et al.*, 1988).

In the present study, we compared the hepatotoxic effects of MYCST-LR in fed and fasted Fischer 344 rats. Toxin induced biochemical and ultrastructural alterations previously defined in fasted rats (MIURA *et al.*, 1989) were measured in toxin-treated, fed rats to probe the enhanced toxicity caused by fasting. In addition, we investigated the effect of a sublethal dose of toxin

in fed and fasted rats later challenged with a lethal dose.

MATERIALS AND METHODS

MCYST-LR (molecular weight 994), purified >95% from cultures of *M. aeruginosa* strain PCC-7820, was obtained from Wayne W. Carmichael (Wright State Univerisity, Dayton, OH, U.S.A.). The toxin was dissolved in normal saline (1 mg/ml) and stored at -10°C.

Male, Fischer 344 rats (Charles River, Wilmington, MA, U.S.A.), weighing 180 to 200 g, were maintained on a 12-hr light/dark cycle and fed NIH open formula chow (Agway Inc., Waverly, NY, U.S.A.) *ad libitum* for at least 1 week prior to experimentation. Rats, fasted overnight (16 hr) prior to the experiments, were allowed water *ad libitum*. Fed rats were not given food after toxin injections.

Sample collection

Fed and fasted rats were injected i.p. with MCYST-LR (100 μg/kg); control rats were injected with normal saline. After 1 hr, rats were killed by cervical dislocation, livers were excised, and blood was collected by cardiac puncture. For light microscopy, liver samples from the right lobe were fixed in 10% formalin. For electron microscopy, liver samples from the right central lobe (1 mm^3 cubes) were fixed in 2.0% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.4 (2 hr). After a wash in buffer at 4° C (overnight), the tissues were post-fixed in 1% osmium

tetroxide (1 hr), and block stained with 0.5% uranyl acetate (1 hr). After sequential dehydration in 10%, 50%, 80%, 95% and 100% ethanol and propylene oxide, the tissue was embedded in Epon 812, sliced into sections with a diamond knife, and stained with 5% uranyl acetate and lead citrate. Micrographs, made from a JEOL 100CX electron microscope (JEOL, Tokyo, Japan) at a plate magnification of 10,000X and enlarged 2.75 times photographically, were analyzed with a Videoplan 2 (Carl Zeiss, Thornwood, NY, U.S.A.).

Preparation of isolated mitochondria from rat liver

Minced liver was homogenized with a Potter-Elvehjem tissue homogenizer in 220 mM mannitol and 75 mM sucrose, pH 7.4 (DIMARCO and HOPPEL, 1975). The homogenate was centrifuged (700 x g, 10 min) and the pellet was discarded. After centrifugation of the supernatant (8000 x g, 10 min), the pellet was resuspended in 2.5 ml of the homogenizing solution. Respiratory rates were determined at 28°C with a Clark-type oxygen electrode (Yellow Springs Instruments Co., Yellow Spring, OH, U.S.A.) in 3 ml of assay medium containing mitochondria (2 to 4 mg protein), 150 mM KCl, 10 mM potassium phosphate, 20 mM Tris and 3 mM $MgCl_2$ (pH 7.4). Substrates (10 µmoles glutamate, 30 µmoles malate plus 30 µmoles pyruvate or 25 µmoles succinate) were added 1 min before ADP (1 µmole).

Liver plasma membrane-associated filaments

Plasma membranes, prepared by the method of HUBBARD *et al.*

(1983) were extracted with 2% Triton X-100 in 150 mM NaCl, 20 mM phosphate buffer, pH 7.4, and 5 mM EDTA. Extraction-resistant membrane proteins were dissolved in 2.5% sodium dodecyl sulfate, 5% 2-mercaptoethanol, 10 mM phosphate buffer (pH 7.0), 0.02% bromphenol blue, and 20% glycerol, and analyzed with a Phastgel electrophoresis system (Pharmacia, Piscataway, NJ, U.S.A.). A Camag thin-layer chromatography plate scanner (Muttenz, Switzerland) was used to scan the Coomassie blue-stained gels.

Chemical analyses

Sorbitol dehydrogenase (SDH), 5'nucleotidase and Lowry total protein were assayed with kits purchased from Sigma Chemical Company (St. Louis, MO, U.S.A.). Glucose and alanine aminotransferase (ALT) kits were purchased from Roche Analytical Instruments (Nutley, NJ, U.S.A.). Analyses were performed with the COBAS BIO Centrifugal Analyzer (Roche).

Determination of LD_{50}

LD_{50} (25 hr; i.p.) of MCYST-LR in fed or fasted rats was calculated by probit analysis. A minimum of six doses of MCYST-LR (12.5 to 200 µg/kg) in normal saline and ten rats per dose were used.

Low-dose of MCYST-LR followed by a high-dose challenge

Fed and fasted rats (20 per group) were injected i.p. with a sublethal dose of MCYST-LR (50 µg/kg). Food was made available 13 hr after the toxin injection. After 72 hr, the rats (16 per

group) were injected (i.p.) with a lethal dose of toxin (200 μg/kg). Twenty four hr after the high-dose challenge, the surviving animals were killed with CO_2, sections of the left lobe of liver were removed, fixed for 24 hr in 10% neutral buffered formalin, and processed by routine paraffin-embedding techniques. Sections (4-6 μm) were stained with hematoxylin and eosin, and examined by light microscopy.

Statistics

Statistical differences were determined with Student's t-test. All values are presented as mean ± S.E.M.

RESULTS

LD_{50} and median time to death

The LD_{50} of MCYST-LR in fed and fasted rats (25 hr LD_{50}; i.p., 95% confidence levels) were 122 μg/kg (106-141) and 72 μg/kg (60-83), respectively (Fig. 1). At 100, 150 and 200 μg/kg doses, the respective median times to death were 31.9, 18.2 and 11.2 hr for fed rats, and 1.8, 1.7 and 1.5 hr for fasted rats.

Liver weight; serum chemistries

In both fed and fasted rats, MCYST-LR (100 μg/kg; i.p.; 60 min) produced the following: (a) typical signs of toxicity, such as lethargy, piloerection and paleness of the ears; (b) blood-engorged livers; liver weight increased 83% in the fasted and 50% in the fed group (Table 1; n = 7); (c) increased serum SDH;

five fold in fasted rats and nine fold in fed rats (Table 1); and (d) elevated serum glucose; two fold in fed rats and 17% in fasted rats (Table 1). The absence of hepatic glycogen in both groups was confirmed by electron microscopy. ALT increased in fed animals only (Table 1).

Electron transport

In mitochondria isolated from liver of intoxicated fasted rats state 3 respiration was completed inhibited 60 min post toxin exposure (MIURA *et al.*, 1989). In contrast, mitochondria from intoxicated fed rats displayed normal state 3 respiration, ADP/O ratios and respiratory control indices (RCI; state 3/state 4) 60 min after injection of MCYST-LR (Fig. 2). Compared to intoxicated fasted rats, electron microscopy revealed denser and more turgid mitochondria as evidenced by smooth rounded outer membranes.

Plasma membrane-associated cytoskeletal filaments

SDS gel scans of plasma membrane-associated filaments from fed control and toxin-treated, fed rats are depicted in Fig. 2. Expressed as a ratio of areas under the curve (toxin treated/control), cytokeratins decreased (0.32 ± 0.02; n = 4), actin increased (1.75 ± 0.12; n = 4), and an unknown protein increased (4.95 ± 0.55; n = 4). Electron microscopy showed that, in livers from fed toxin-treated rats, sinusoidal spaces were enlarged and interhepatocyte contact was progressively degraded. These results are similar to those from intoxicated, fasted rats

(MIURA *et al.*, 1989).

Low-dose pretreatment with MYCST-LR

A low-dose pretreatment with 50 µg/kg MCYST-LR (i.p.) protected fasted, but not fed rats from a high-dose challenge of 200 µg/kg MCYST-LR (i.p.; Table 2) given 72 hr later. In control fasted rats, hepatic and renal tissues were histologically normal. In challenged, fasted rats (Fig. 4A), hepatic lesions due to the initial low dose of MCYST (50 µg/kg) included: (a) a chronic inflammatory response, (b) diffuse coagulative necrosis intimately associated with the chronic inflammatory response, and (c) mitotic figures. Consistent with damage due to the high-dose challenge were modest numbers of necrotic hepatocytes which were not associated with inflammatory cells (Fig. 4A). Livers from fasted rats displaying regeneration (mitotic figures, Fig. 4A) were not swollen and averaged 6.48 ± 0.75 g (n = 6). Four of these rats had histologically normal kidneys; one exhibited diffuse vacuolation of the tubular epithelium, and one showed necrosis of the cortical tubular epithelium.

Only one of the six fed, pretreated, then challenged rats showed prior hepatic injury due to the initial low-dose treatment. Hepatic lesions present in the other five rats were characteristic of acute MCYST intoxication: massive hepatocellular necrosis and loss, with concommitant congestion and hemorrhage (Fig. 4B). In contrast to livers from their fasted counterparts, these livers were blood engorged and weighed

13.59 ± 0.76 g (n = 6). Renal lesions in this group were characteristic of acute MCYST intoxication.

DISCUSSION

The toxicity of MCYST-LR was potentiated by overnight fasting of male rats. FRANZ *et al.* (1988) found the same phenomenon in mice that were fasted for 24 hr and then were administered MCYST-LR s.c. No differences in liver weights, serum SDH concentrations, and plasma membrane-associated cytoskeletal filaments were noted between fasted (MIURA *et al.*, 1989) and fed toxin-treated rats. However, in fed, but not fasted rats, the mitochondrial electron transport chain was functional.

Fasting is known to effect many biochemical pathways profoundly (KRISHNAN and STENGER, 1966; EL-REFAI and CHAN, 1982; FREIDENBERG *et al.*, 1985; OSUNA *et al.*, 1987). Hence, the task of correlating the responsible biochemical event(s) in fasting that produce(s) enhanced hepatotoxicity is a difficult one. Research with other hepatotoxins, such as carbon tetrachloride, acetaminophen and bromobenzene, has led others to suggest that bioactivation is a common denominator in fasting-induced potentiation (PLAA, 1980); hence, altered enzyme activities may be critical for the enhanced effects. With acetaminophen, fasting perturbed uridine-5'-diphospho-α-D-glucuronic acid pools to lower glucuronidation, a pathway that detoxified the toxic metabolite (PRICE *et al.*, 1987). In our laboratory, we found

that [^{3}H]MCYST-LR was biotransformed in perfused rat livers (PACE *et al.*, 1989) and was strongly associated with cytosolic protein (MATSON *et al.*, 1990). Thus, perturbations in enzymatic reactions, whether resulting in bioactivation and/or detoxification of MCYST-LR, may be important consequences of fasting.

In the case of acetaminophen, the mechanism of enhanced toxicity appears to be an increase in a toxic metabolite, resulting from a drop in hepatic glutathione pools (WALKER *et al.*, 1982). A similar explanation for MCYST-LR's toxicity was not supported by a study showing that depressed glutathione levels enhanced mean survival time of mice (DAHLEM *et al.*, 1989). With a related *Microcystis* toxin, MCYST-YM, hepatocytes showed dose- and time-dependent drops in glutathione pools (RUNNEGAR *et al.*, 1987); and a free radical scavenger, silymarin, protected mice against MCYST-LR (MEREISH *et al.*, 1989).

One of the more dramatic changes in liver after exposure to MCYST-LR is depletion of glycogen stores (this study and PACE *et al.*, 1990). Because cortisone-induced glycogen synthesis was found to protect rats treated with the hepatotoxin dimethylnitrosamine (DE MAN, 1964), carbohydrate metabolism may be an interesting area to explore.

In this study, the protective effect of a sublethal injection of MCYST-LR in fasted, but not fed, rats was correlated with a regeneration of hepatocytes, an effect reported by SLATKIN *et al.* (1983). A possible explanation for this observation is that fasting prior to the initial low-dose of MCYST resulted in

greater hepatocellular damage; in essence, a chemical partial hepatectomy was performed. The subsequent regenerative response resulted in a greater population of immature hepatocytes which were resistent to the high-dose challenge, possibly due to lack of enzyme systems necessary to metabolize MCYST into toxic metabolites (ADAMS *et al.*, 1985), or lack of specific transport systems (bile acid transport system; RUNNEGAR et al., 1981). In addition to alterations in hepatocytes, it is reasonable to assume that mediators of inflammation and repair are present in abundance in the "partially hepatectomized" animals, and these may significantly reduce the lesions induced by MCYST-LR. In contrast, fed rats suffered less initial damage to the low-dose of MCYST-LR, and consequently had a greater population of mature hepatocytes at the time of high-dose challenge. We hypotheize that these animals were, therefore, more susceptible to the effects of the toxin.

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TABLE 1. Effects of MCYST-LR on liver weight and serum chemistries of fed and fasted rats.

	Fasted Rats		Fed Rats	
	Toxin	Control	Toxin	Control
Liver Wt (% BW)	5.3 ± 0.3*	2.9 ± 0.1	5.7 ± 0.4*	3.8 ± 0.2
Glucose (mg/dl)	90.4 ± 0.6*	77.0 ± 0.9	259 ± 5.4*	119 ± 0.4
SDH (U/l)	109 ± 21**	21 ± 5	185 ± 20*	20 ± 2
ALT (U/l)	352 ± 95	144 ± 42	501 ± 39*	102 ± 17

Rats were injected ip with 100 μg/kg MCYST-LR, and blood was collected 1 hr post injection. * : $p<0.001$ when compared to the respective controls; ** : $p<0.05$ when compared to the respective controls.

TABLE 2. Effect of low-dose MCYST-LR on survival of fed and fasted rats challenged with a lethal dose of toxin.

Nutritional Status	Initial Dose Toxin (μg/kg)	Challenge Toxin (μg/kg)	# dead/ # total	Median Time to death (hr)
Fed	50	0	0/10	-
Fasted	50	0	1/10	2.2
Fed	0	200	5/5	8.8
Fasted	0	200	3/3	1.3
Fed	50	200	15/16	13.4
Fasted	50	200	6/16	17.3

LEGENDS FOR FIGURES

Fig. 1. TWENTY FIVE-HR LD_{50} OF MCYST-LR IN FED AND FASTED RATS
Dose-response relationship of MCYST-LR in fed (○) and fasted rats (●). A minimum of six doses of MCYST-LR (12.5 to 200 μg/kg; i.p.) and ten animals per group were used to determine the median lethal dose by probit analysis.

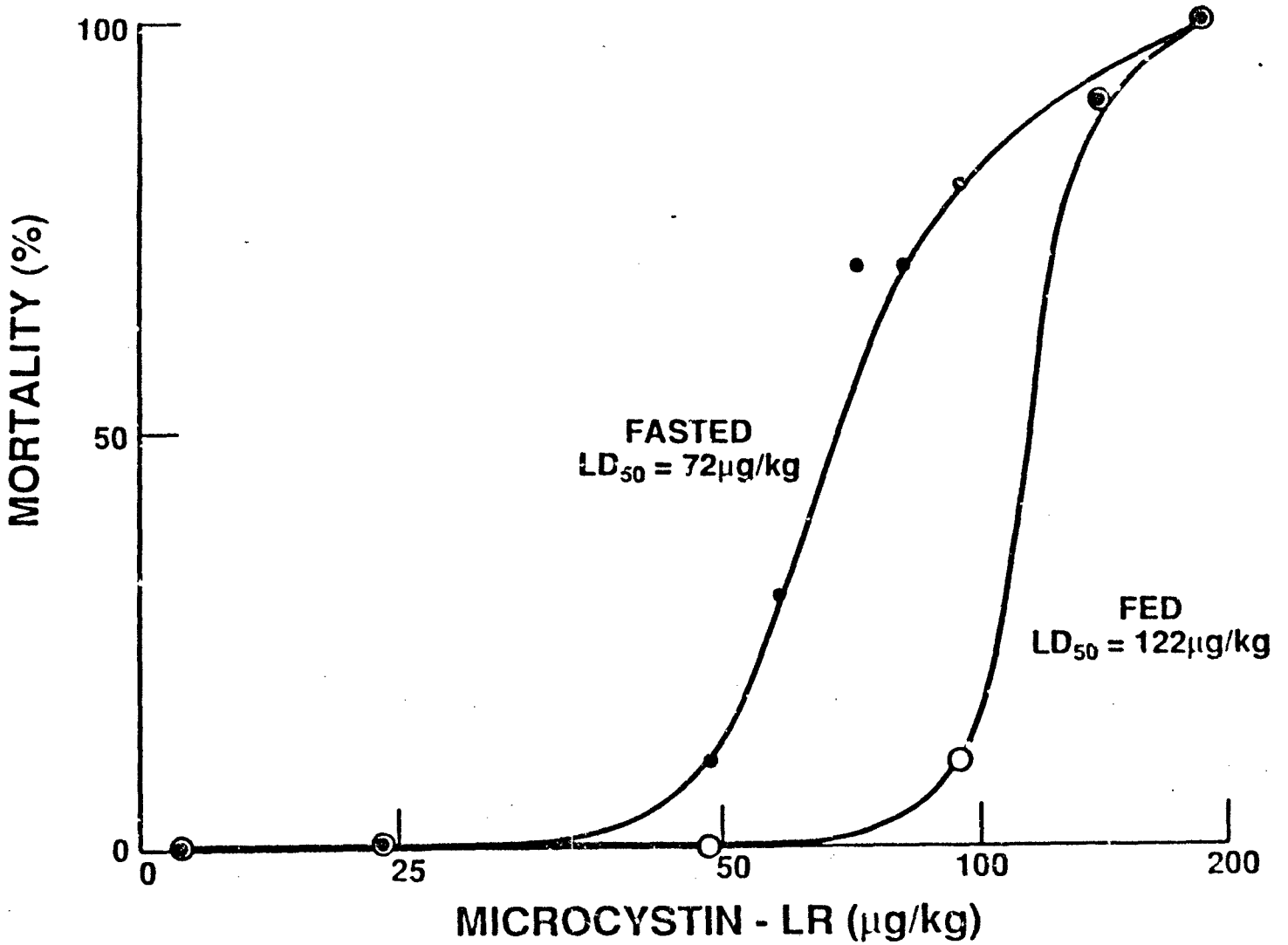
Fig. 2. ADP/O RATIOS AND RESPIRATORY CONTROL INDICES OF LIVER MITOCHONDRIA ISOLATED FROM FED-CONTROL AND TOXIN-TREATED RATS.
Effects of 100 μg/kg MCYST-LR (i.p., 60 min) (▨) on the ADP/O ratios (A) and RCI (B) of liver mitochondria with various substrates. Control rats were injected with saline (▨). n = 3.

Fig. 3. SDS GEL OF PLASMA MEMBRANE ASSOCIATED CYTOSKELETAL PROTEINS.
Representative SDS gel scan of plasma membrane- associated cytoskeletal proteins from livers of a fed control (upper panel) and a fed, toxin-treated rat (lower panel). MCYST-LR (100 μg/kg) was injected i.p., and membranes were isolated one hr after toxin exposure.

Fig. 4A. PHOTO MICROGRAPHS OF LIVER FROM RATS GIVEN A LOW-DOSE OF MCYST-LR PRIOR TO A HIGH-DOSE CHALLENGE.

A. Liver from fasted-intoxicated, then challenged rat. Note the presence of chronic centrolobular (C) hepatitis demonstrated by fibroblasts, collagen, and variable numbers of macrophages, lymphocytes, and neutrophils (I), coagulative necrosis (*), and mitotic figures (arrow). P = portal vein.

B. Liver from fed-intoxicated, then challenged rat. Note massive necrosis extending from the central vein (C) to the portal triad (P) and loss of hepatic architecture. H = congestion and hemorrhage.


100
50
0
MORTALITY (%)
FASTED
LD50 = 72µg/kg
FED
LD50 = 122µg/kg
0
25
50
100
200
MICROCYSTIN - LR (µg/kg)

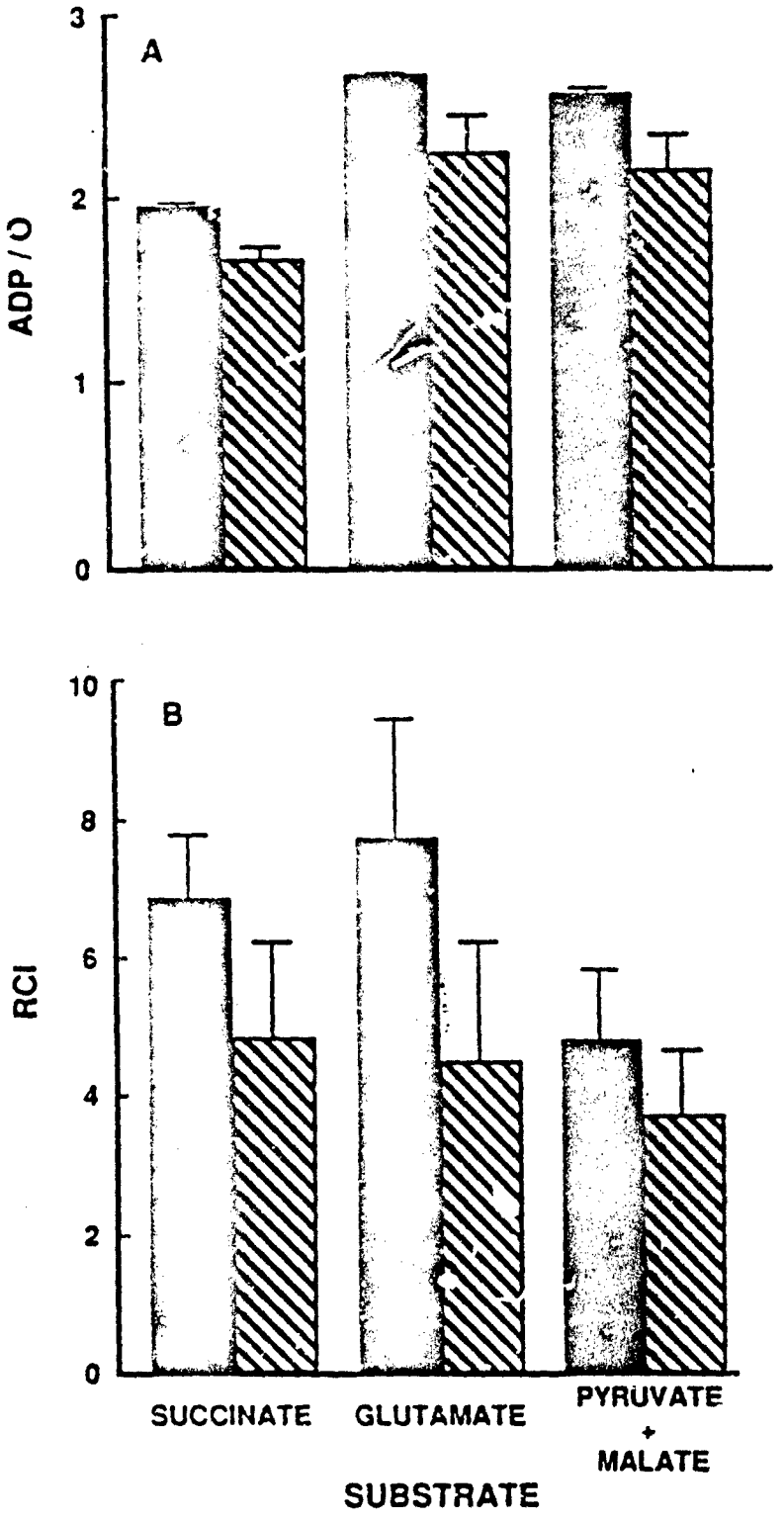

A
ADP / O
3
2
1
0
B
RCI
10
8
6
4
2
0
SUCCINATE
GLUTAMATE
PYRUVATE
+
MALATE
SUBSTRATE

Fig 2

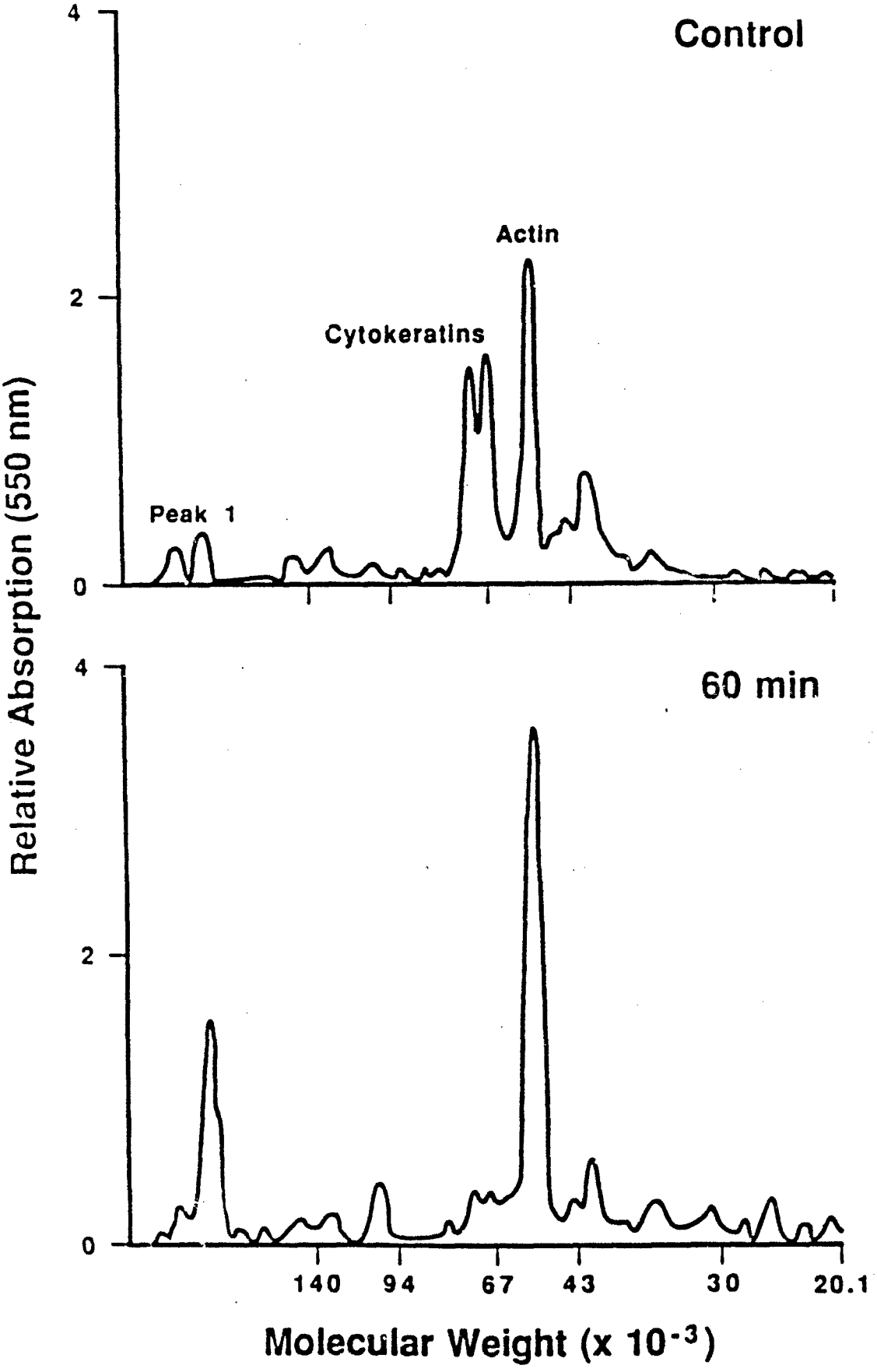
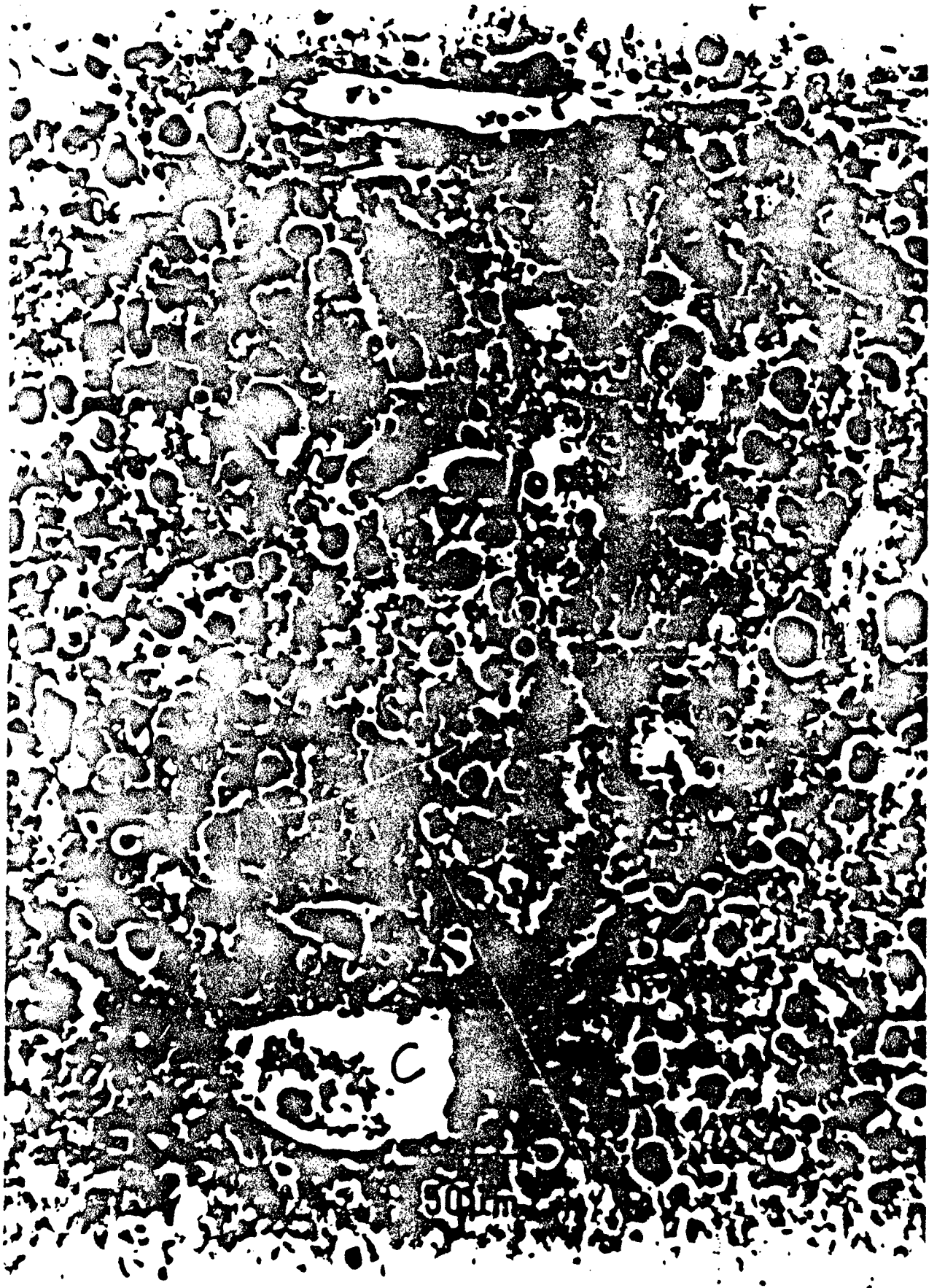

Control
Actin
Cytokeratins
Peak 1
4
2
0
60 min
4
2
0
Relative Absorption (550 nm)
140
94
67
43
30
20.1
Molecular Weight (x 10-3)

Fig 3



4A

4B