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13. ABSTRACT *(Maximum 200 words)*

Tritiated dihydromicrocystin-LR ([^{3}H]2H-MCLR) plus 2H-MCLR were given to anesthetized pigs IV or via an ileal loop. Over half the radiolabel in blood at one minute after dosing IV was cleared by 6 minutes; clearance at 25 μg/kg was faster than at 75 μg/kg. At 75 μg/kg via the ileum, the blood concentration peaked at 90 minutes, and the concentration in portal blood was 3.6 times greater than in peripheral blood. Radioactivity was first detected in bile at 12 minutes post-dosing. At 4 hours after dosing IV at 25 or 75 μg/kg, and at 5 hours after dosing via the ileal loop at 75 μg/kg, radiolabel was distributed as follows: liver (64.6, 46.99, and 49.5 percent of total dose [%TD]), kidneys (1.2, 2.19, and 1.04 %TD), lungs (1.75, 0.55, and 0.65 %TD), heart (0.22, 0.23, and 0.81 %TD), ileum (0.13, 0.20, and 33.94 %TD), and spleen (0.04, 0.07, and 0.16% %TD), respectively. Most hepatic radiolabel was attributable to parent compound, although two minor radioactive components were isolated. Previous evidence indicating inhibition of protein phosphatases by intact microcystins and the observation that nearly all [^{3}H]2H-MCLR in the liver was parent compound during lesion development suggest that microcystins are toxicologically active *in vivo* as parent compounds.

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Val R. Bradley 14/May/94

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ABSTRACT

The toxicokinetics of tritiated dihydromicrocystin-LR ([^{3}H]2H-MCLR) were studied in anesthetized, specific pathogen free (SPF) pigs. Three dosage groups were studied. Two doses of the radiolabeled plus nonlabeled 2H-MCLR were administered IV and one dose was given via an isolated ileal loop. The IV doses of 25 μg/kg and 75 μg/kg were rapidly removed from the blood. At either IV dose, more than half the radiolabel from [^{3}H]2H-MCLR present in the blood at one minute post-dosing was cleared by 6 minutes. The blood clearance at the 75 μg/kg dose was slower than that at the 25 μg/kg dose. Accordingly, at the high dose, the concentrations of the toxin in blood were disproportionately higher from 10 minutes after dosing until the study ended 4 hours later. Following administration of [^{3}H]2H-MCLR at 75 μg/kg via the ileum, the peak concentration of toxin in blood was achieved at 90 minutes after dosing, when [^{3}H]2H-MCLR in portal venous blood was 3.6 times higher than in peripheral venous blood. Although bile production varied, following iv dosing, radioactivity in bile from the gall bladder was detected as early as 12 minutes postdosing in one animal. At 4 hours after IV dosing at 25 μg/kg, 64.6 percent of the total dose (%TD) was located in the liver tissue, with lesser amounts in the kidneys (1.2 %TD), lungs (1.75 %TD), heart (0.22 %TD), ileum (0.13 %TD), and spleen (0.04 %TD). A similar distribution was found at 4 hours post-dosing in pigs dosed at 75 μg/kg, with the liver

containing somewhat less at 46.99 %TD, and the kidneys containing slightly more at 2.19 %TD. The value for the lungs (0.55 %TD) was slightly lower at the high dose; whereas the radioactivity in the heart (0.23 %TD), ileum (0.20 %TD), and spleen (0.07 %TD) were more similar to the sublethal dose. The livers of the pigs given 75 μg/kg via the ileal loop at 5 hours post-dosing, contained 49.5% TD and the ileum had 33.94 %TD. Lesser amounts were distributed to kidneys (1.04 %TD), lungs (0.65 %TD), heart (0.81 %TD) and spleen (0.16 %TD). Thus, the livers of both groups dosed with 75 μg/kg contained higher concentrations of toxin, but lower percentages of total dose. Larger increases in serum arginase and more severe histopathological evidence of disruption of the hepatic organization were noted in the 75 μg/kg groups as compared to the 25 μg/kg group. The majority of radiolabel in the liver could be accounted for by parent compound, but two minor radioactive components were also isolated. This study demonstrates the rapid removal of [^{3}H]2H-MCLR from the blood of anesthetized swine, the appearance of the radiolabel in the bile within minutes after dosing, and the selective uptake of [^{3}H]2H-MCLR in the liver of swine. Previous evidence indicating the potent inhibition of protein phosphatases by intact microcystins and the fact that nearly all of the toxin in the liver of these swine was parent compound during the time of lesion development indicate that microcystins are probably toxicologically active *in vivo* largely as the parent compounds.

INTRODUCTION

Cyanobacteria (blue-green algae) are found in many regions of the world. Cyanobacteria in fresh or brackish waters may suddenly and unexpectedly produce a toxic bloom containing low molecular weight toxins capable of causing injury or death to man and other animals (Carmichael *et al.*, 1984, 1985) One species often associated with bloom formation and subsequent toxin formation is *Microcystis aeruginosa* (Carmichael *et al.*, 1984, 1985; Beasley *et al.*, 1989). The principle toxic peptide produced by the laboratory isolate of *M. aeruginosa* strain PCC-7820 is microcystin-LR (MCLR), which has a molecular weight of 994 daltons (Botes *et al.*, 1982; Krishnamurthy *et al.*, 1989). Ingestion of *M. aeruginosa* cells has resulted in toxicoses in wildlife, sheep, pigs, cattle, dogs, and man (Carmichael *et al.*, 1984, 1985; Sykora and Keliti, 1981; Galey *et al.*, 1987; Carmichael and Falconer 1993).

The absorption and distribution of MCLR or a radiolabelled derivative in rats and mice have been described based on the findings of preliminary studies (Dahlem, 1989; Dahlem *et al.*, 1989). There is evidence to suggest that MCLR is poorly absorbed by the rat and mouse when administered orally (Dahlem, 1989; Dahlem *et al.*, 1987, 1989). When administered directly into an ileal loop of the small intestine of the rat, however, the toxin more readily produces a syndrome similar to naturally occurring

toxicoses (Dahlem *et al.*, 1989). The major fraction of a dose of parenterally administered microcystin or [^{3}H]2H-MCLR is taken up by the liver, with a much smaller amount deposited in the kidneys (Falconer *et al.*, 1986; Dahlem, 1989; Robinson *et al.*, 1989, 1991).

When given at sufficient doses, algal cyclic peptide toxins cause extreme enlargement of the liver, due in significant measure to intrahepatic hemorrhage (Carmichael *et al.*, 1984, Lovell *et al.*, 1989; Lovell, 1989). Death appears to result from hepatic necrosis and hypovolemic shock, hypoglycemia, and/or hyperkalemia (Theiss *et al.*, 1988; Dahlem, 1989; Lovell *et al.*, 1989). Hepatocytes are initially rounded and disassociated; and later become necrotic. Hepatic necrosis often becomes sufficiently severe that only two or three rows of periportal hepatocytes survive. Hepatocytes may be found in the central veins of the liver lobules and in the pulmonary veins as the necrosis of the liver continues. After death, histologic examination may indicate the presence of debris from necrotic hepatocytes in pulmonary vessels (Slatkin *et al.*, 1983; Hooser *et al.*, 1989).

Mice tend to die much sooner after toxin administration than do other laboratory or domestic species. It has been suggested that, in the mouse, the endothelial cells of hepatic sinusoids are the first affected cells (Dabholkar and Carmichael, 1987).

Studies in our laboratory with rats, however, seem to indicate that structural effects on the endothelium occur after initial deformation of hepatocytes, and endothelial cells but not hepatocytes are tolerant of MCLR *in vitro* (Hooser *et al.*, 1989, 1990).

Certain physiologic effects of MCLR have been demonstrated using small laboratory rodents; and work performed in our laboratory using swine dosed intravascularly with the toxin has demonstrated effects on hepatic and renal blood flow. Using temperature pulse decay methods in lethally dosed swine, we found that hepatic blood flow declined 10 to 20 minutes before a precipitous fall in arterial blood pressure. The decline in renal blood flow more closely paralleled the decrease in aortic blood pressure, suggesting that MCLR does not directly modify overall renal perfusion (Holmes and Lovell, 1987). In a preliminary group of control and MCLR treated, anesthetized swine, our group also demonstrated the feasibility of a protocol involving sequential wedge biopsies and concurrent monitoring of perfusion with temperature pulse decay probes.

We have demonstrated that serum arginase activity is a sensitive indicator of severe hepatic necrosis. In addition, because it has relative short half-life of about two hours, the decline of serum arginase can be used to evaluate the persistence of enzyme leakage from the damaged liver (Lovell *et al.*, 1987).

The algal cells and purified toxins are of comparatively low toxicity in orally exposed mice and rats, but the cells are quite toxic to cattle (Galey *et al.*, 1987) and swine (Lovell, 1989) dosed by gavage. This suggests that the inherent susceptibility to, or the absorption, distribution, and elimination of the toxin is not the same in all species.

Further studies in our laboratory have shown that saturation of N-methyldehydroalanine to form dihydromicrocystin LR causes only a modest reduction in toxicity and hepatic specificity is retained. In a subsequent study, reduction of MCLR using tritium labeled sodium borohydride yielded [^{3}H]2H-MCLR. A mixture of labeled and unlabeled MCLR was then administered IP to mice at sublethal and lethal doses (but equalized on the basis of radiolabel/kg of body weight) in order to characterize its distribution and elimination. Ninety percent of a sublethal dose (100 μg/kg) became localized in the liver after one hour. The toxin was also present in the most orad ten centimeters of the small intestine, suggesting biliary excretion. At a lethal dose of 200 μg/kg, a lesser fraction of the total dose, but a greater overall amount of toxin was taken up by the liver. When the two groups were compared, the radiolabel in the kidneys and lungs of the lethal dose group was much higher than in the sublethal dose group. This difference seemed to suggest that the toxin had been retained by hepatocyte debris that was carried to these areas

(Dahlem 1989; Hooser *et al.*, 1989, 1990). The predominant route of excretion of labeled compound was via the feces. When the sublethal dose was given IP, sixty percent of the radiolabel remained in the liver at seventy-two hours after administration.

In other studies, we have shown that both superactivated charcoal and the ion-exchange resin cholestyramine(CTR) bind to MCLR, but, on an equal weight basis of the adsorbents, the latter was more effective, both *in vitro* and *in vivo* (Dahlem *et al.*, 1987; Dahlem, 1989). Although pretreatments with various agents have increased survival and/or survival time (Adams *et al.*, 1989; Hermansky *et al.*, 1990a, 1990b; Nakano *et al.*, 1991; Mereish *et al.*, 1989, 1991; Stoner *et al.*, 1990) in mice given a lethal dose of toxin, studies have not identified an effective treatment regimen in any species after the onset of toxicosis, and essentially no therapy studies have yet been performed to evaluate larger monogastrics which may be a more reliable model of the human pathophysiology associated with microcystin toxicosis.

SYNTHESIS OF TRITIATED DIHYDRO-MICROCYSTIN-LR

INTRODUCTION

At the time of the design of the study there was not a method of directly measuring MCLR in the blood or tissues of animals. Dr. Fun Sun Chu (Research Institute of the Department of Food Microbiology and Toxicology, University of Wisconsin-Madison WI.) developed an immunoassay for MCLR during the course of this research. Preliminary testing of the assay with the MCLR in swine blood gave inconsistent results at the extremely low concentrations anticipated for the swine kinetic studies. It was therefore necessary to label MCLR to quantify toxin concentrations in blood by another method. It was also necessary to label the toxin in a manner that did not markedly alter the bioactivity of the molecule, and in a way that the label was retained in the molecule in a living animal. The method that had been developed in our laboratory by Dr. Andrew M. Dahlem, and which addressed both of these requirements, was employed with modifications to achieve a higher specific activity and to produce larger quantities of the labelled compound (Dahlem, 1989).

OBJECTIVE

To produce an analog of MCLR that is measurable at very low concentrations in biological systems and that retains the bioactivity of MCLR.

MATERIALS AND METHODS

Thirty gallons of frozen blue-green algae cells that were collected from Homer Lake were dehydrated by freeze-drying and the MCLR toxin was extracted and purified as described on page 23. MCLR of > 95% purity was reacted with tritiated sodium borohydride (NaB^3H_4) and the products purified. Four reactions were carried out in order to produce a pooled source of 10.37 mCi of [^{3}H]2H-MCLR (figures 1-3). A molar ratio of MCLR:NaB^3H_4 of 1:3.7 was used in the first reaction performed with 100 mCi of NaB^3H_4 purchased from American Radiochemical, St. Louis, MO. The reaction was carried out for 24 hours in 0.5 ml of 70% isopropanol. The reaction products were dried under nitrogen gas, redissolved in H_2O and loaded on a C_{18} reversed phase column. The column was eluted with methanol and the eluate dried with nitrogen gas. Ten microliters of this eluate were loaded on a 5 cm X 20 cm fluorescent silica gel thin layer chromatography plate and developed with a chloroform:methanol:water (65:35:10) mobile phase. The silica gel plate was scanned on a TLC radio scanner. The scan revealed a substantial amount of the radioactivity located at the origin of the plate. The dried products were then dissolved in a mobile phase of chloroform:methanol:water (65:35:10), and loaded on a 200 ml chromatography column packed with 9 grams of silica gel. The fractions were collected and counted with a scintillation counter. Ten microliter fractions were also loaded on a 20 cm X 20 cm fluorescent silica gel thin

layer chromatography plate and developed in a tank with a chloroform:methanol:water (65:35:10) mobile phase. Fractions were combined that had the same retention factor (Rf) as the standard. Ten μl of the combined fractions were loaded on a TLC plate as described for the methanol eluate from the C18 column. The TLC plate scan showed two distinct radioactive peaks which corresponded with Rfs from the standard 2H-MCLR. A second reaction using 100 mCi from the same batch of NaB^3H_4 as used in the first reaction was performed with a molar ratio of MCLR:NaB^3H_4 of 1:4.5. A third reaction was preformed using 250 mCi of NaB^3H_4 supplied by Amersham Inc., Arlington Heights, IL, with a molar ratio of MCLR:NaB^3H_4 of 1:5. A fourth reaction was performed using 250 mCi of NaB^3H_4 also supplied by Amersham Inc., with a molar ratio of MCLR:NaB^3H_4 of 1:4. The last three reaction products were purified in the same manner as described for the first reaction.

The purified reaction products from all four reactions were dissolved in 10% ethanol forming 60 ml of a pooled [^{3}H]2H-MCLR solution. The concentration of [^{3}H]2H-MCLR was determined to be .166 mg/ml using regression analysis to compare area under the curve of this toxin solution to a standard curve of 2H-MCLR. The purity of the final reaction product was determined to be over 90% [^{3}H]2H-MCLR by HPLC (Figure 4) and TLC (Figure 5). The specific activity of the final reaction product was determined by liquid scintillation counting to be 173 μCi/ml.

FIGURE LEGENDS

Figure 1-MCLR was reacted with tritiated sodium borohydride (NaB^3H_4) for 24 hours in 70% isopropanol to form [^{3}H]2H-MCLR (*2HMCLR) and was then dried in N_2. The reaction products were dissolved and loaded onto a C_{18} reversed phase column. The column was eluted with methanol and the methanol elutation was dried with N_2.

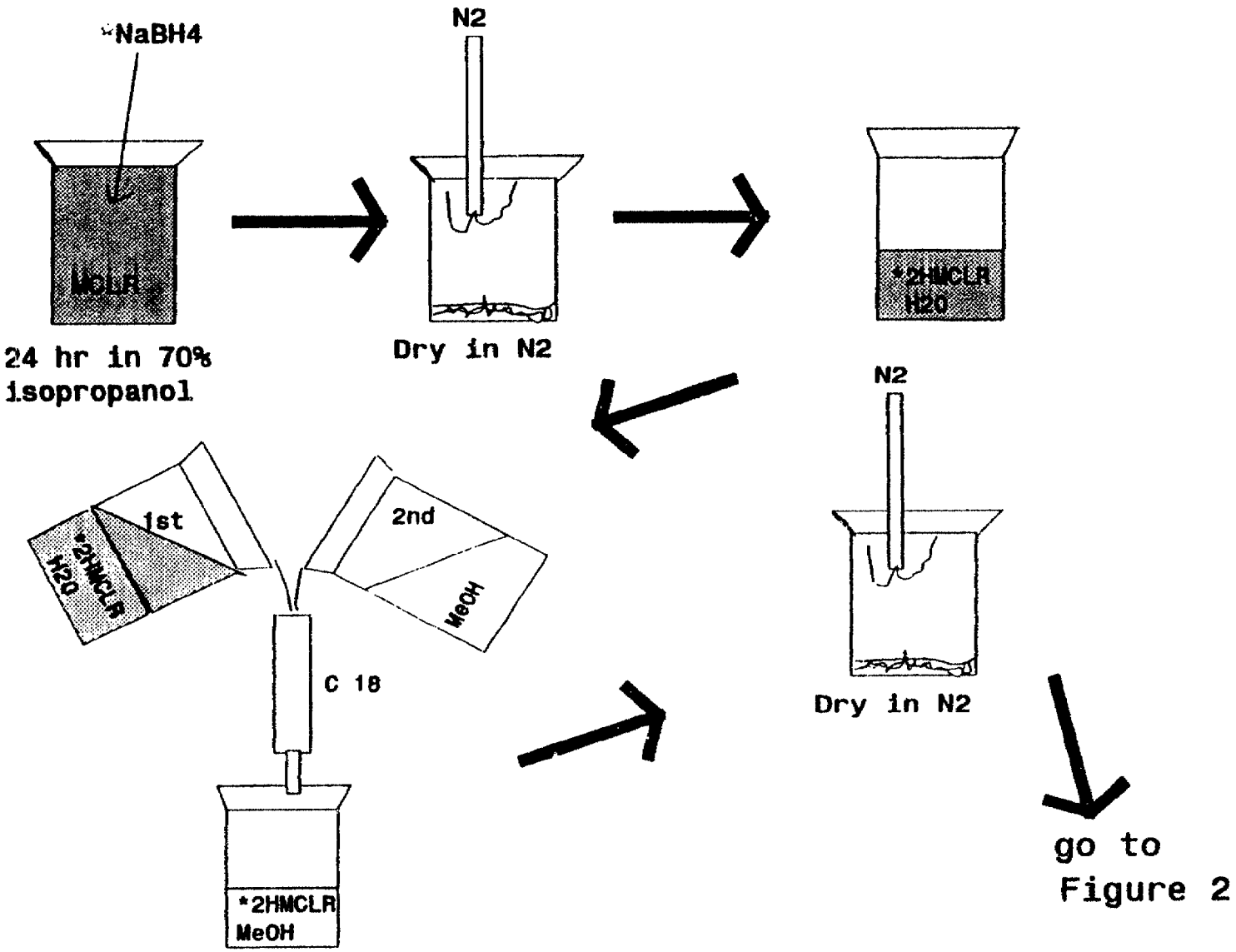
Figure 2-The reaction products including [^{3}H]2H-MCLR (*2HMCLR) in the methanol eluate were dissolved in a mobile phase of chloroform:methanol:water (65:35:10) and loaded on a 200 ml chromatography column packed with 9 g of silica gel. Samples from each fraction were loaded on a TLC plate and were analyzed for radioactivity. Fractions that contained radioactivity and that had the same Rf as the non-labelled 2H-MCLR standard were combined.

Figure 3-The reaction products were combined and the purity of the combined final product was evaluated by HPLC and TLC.

Figure 4-High performance liquid chromatogram of the [^{3}H]2H-MCLR dosing solution using a Radiomatic FLO-ONE\Beta Model IC radioactive flow detector. One radioactive peak containing 100% of the radioactivity was found. This peak had a retention time from 8.60 minutes to 10.90 minutes which coincided with a single peak identified by UV detection at 238 nm which eluted between

9.40 minutes and 10.60 minutes.

Figure 5-Thin layer chromatography plate scan of the [^{3}H]2H-MCLR dosing solution obtained using a Radiomatic MODEL RS Radio-thin layer chromatography scanner. Peaks 2 and 3 correspond to the 2H-MCLR standard.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*NaBH4
MbLR
24 hr in 70% isopropanol
N2
Dry in N2
*2HMCLR H20
N2
Dry in N2
1st
*2HMCLR H20
2nd
MeOH
C 18
*2HMCLR MeOH
go to Figure 2

Figure 1

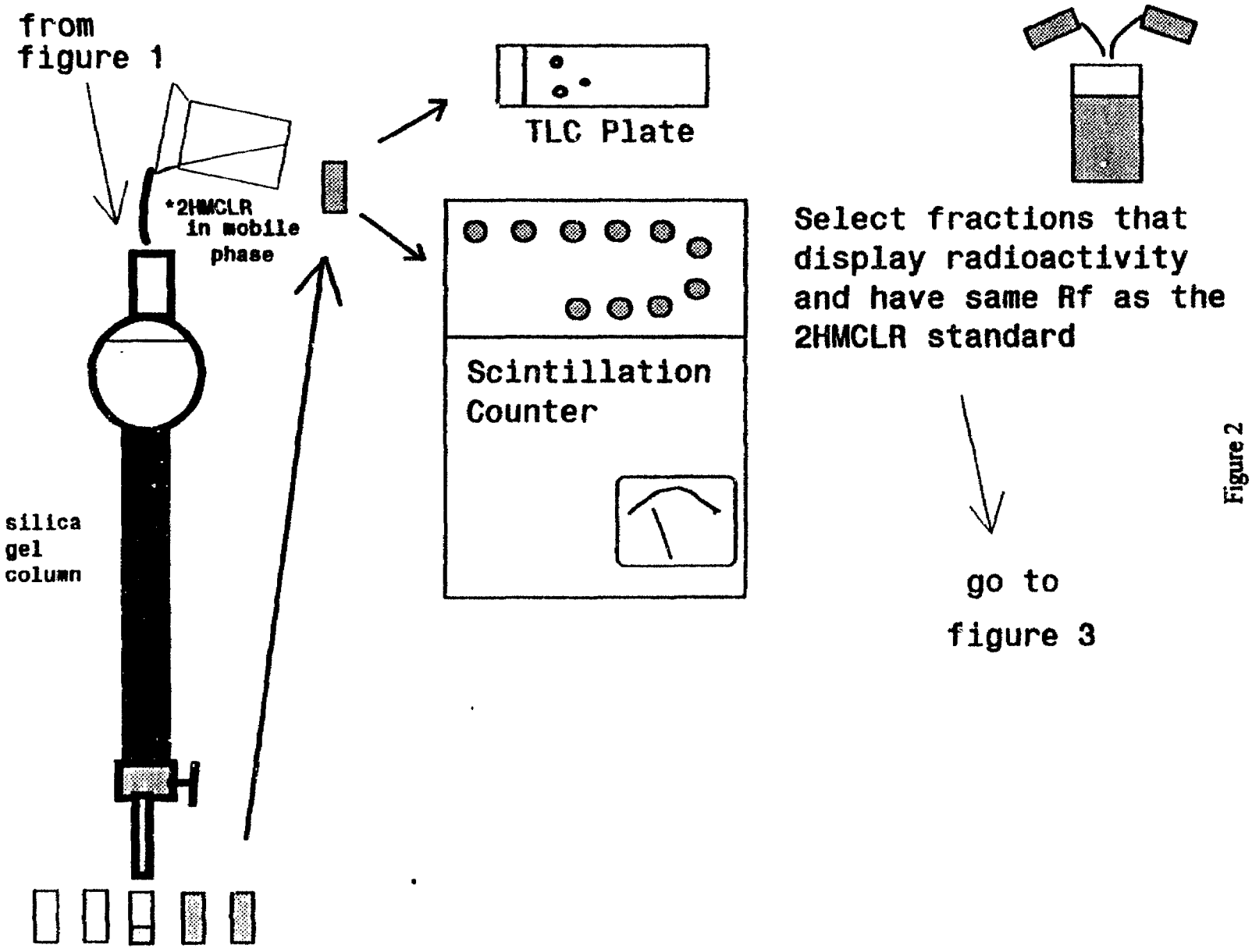
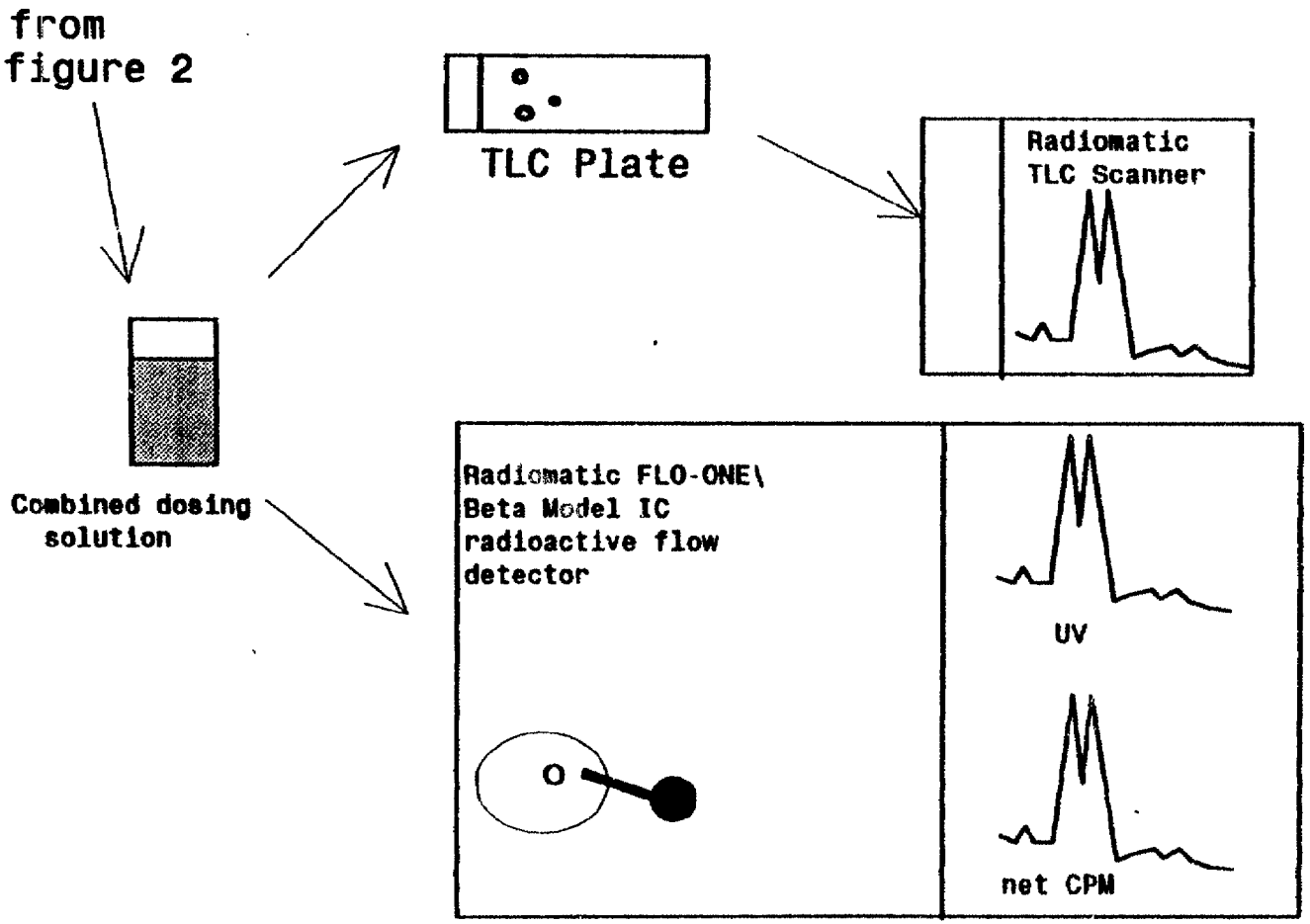

from
figure 1
*2HMCLR
in mobile
phase
TLC Plate
Scintillation
Counter
silica
gel
column
Select fractions that
display radioactivity
and have same Rf as the
2HMCLR standard
go to
figure 3

Figure 2



Evaluation of Dosing Solution

Figure 3

Figure 4

NET CPM
H Scale: 1000000
UV Scale: 1000
100
0
100
0
0
5
10
15
20
Highest point: 188949
Background: 0
CH3 Delay: 0: 0:54
Highest point: 393
Noise Level: 0
NET CPM
H
UV
I PEAK INTEG FROM TO RET %RUN II PEAK INTEG FROM TO RET %RUN I
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I -- 2421297 ---------- TOTAL II -- 5363 ---------- TOTAL I

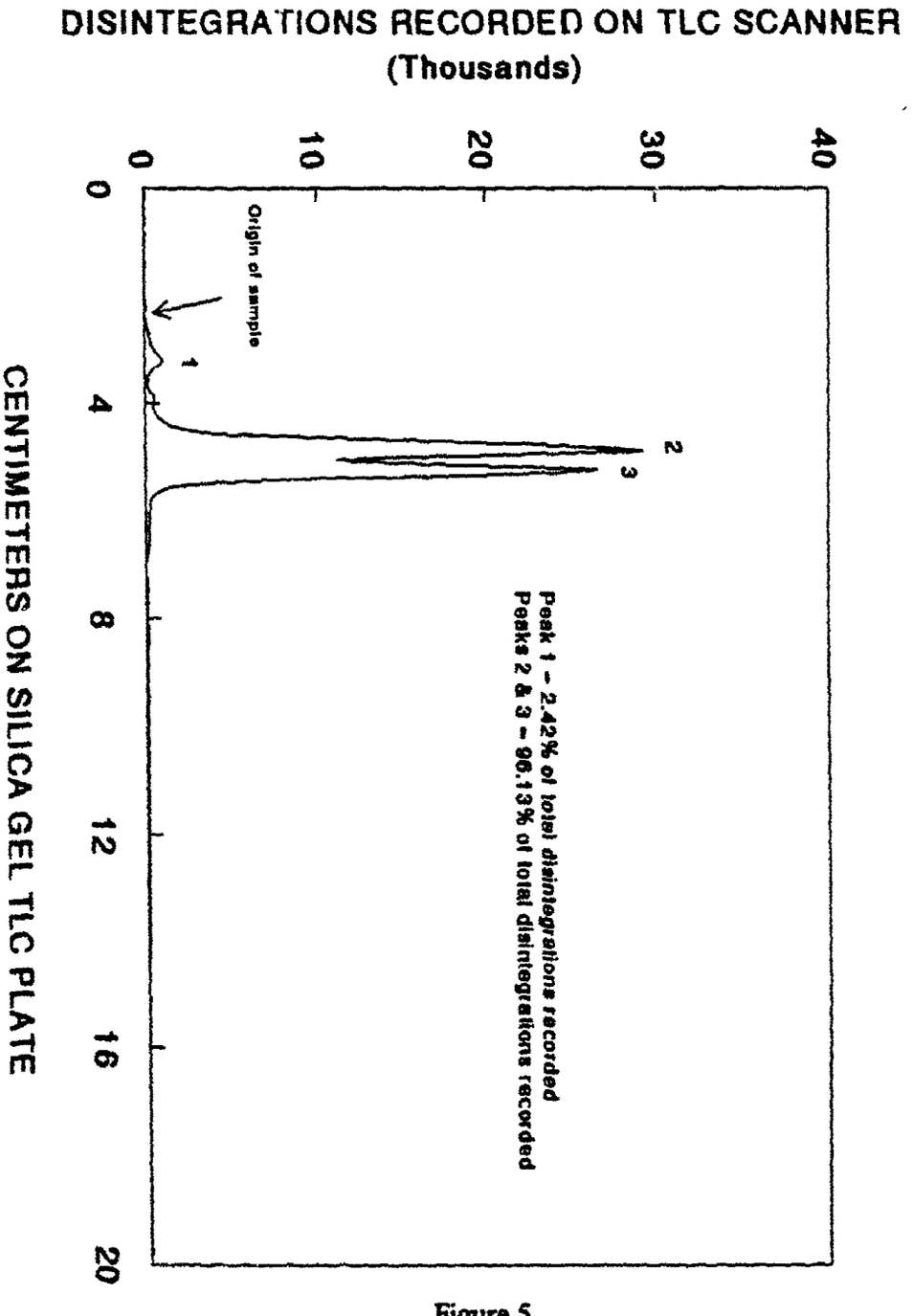

DISINTEGRATIONS RECORDED ON TLC SCANNER
(Thousands)
0
10
20
30
40
Origin of sample
1
2
3
Peak 1 = 2.42% of total disintegrations recorded
Peaks 2 & 3 = 96.13% of total disintegrations recorded
0
4
8
12
16
20
CENTIMETERS ON SILICA GEL TLC PLATE

Figure 5

TOXICOKINETICS OF TRITIATED DIHYDROMICROCYSTIN IN SWINE

SUMMARY

The toxicokinetics of tritiated dihydromicrocystin-LR ([^{3}H]2H-MCLR) were studied in anesthetized, specific pathogen free (SPF) pigs. Two doses of the radiolabeled plus nonlabeled 2H-MCLR were administered IV and one dose was given via an isolated ileal loop. The IV doses of 25 μg/kg and 75 μg/kg were rapidly removed from the blood. At either IV dose, more than half the radiolabel from [^{3}H]2H-MCLR present in the blood at one minute post-dosing was cleared by 6 minutes. The blood clearance at the 75 μg/kg dose was slower than that at the 25 μg/kg dose. Accordingly, at the high dose, the concentrations of the toxin in blood were disproportionately higher from 10 minutes after dosing until the study ended 4 hours later. The decreased clearance is presumably related to decreased elimination as a consequence of the hepatic injury that was observed histologically. Following administration of [^{3}H]2H-MCLR at 75 μg/kg via the ileum, the peak concentration of toxin in blood was achieved at 90 minutes after dosing, at which time [^{3}H]2H-MCLR concentration in portal venous blood was 3.6 times higher than in peripheral venous blood.

Although bile production varied significantly between animals, following IV dosing, radioactivity in bile from the gall

bladder was detected as early as 12 minutes post-dosing in one animal. This study demonstrates the rapid removal of [^{3}H]2H-MCLR from the blood of anesthetized swine and the presence of the radiolabel in the bile within minutes after dosing.

INTRODUCTION

Species of several genera of cyanobacteria (blue-green algae) including, *Microcystis, Anabaena, Nostoc,* and *Oscillatoria,* produce cyclic heptapeptide hepatotoxins that have been termed microcystins (Carmichael *et al.*, 1988; Beasley *et al.*, 1989). Microcystins from *Microcystis aeruginosa* often pose hazards to livestock, and sometimes to public health, in many regions of the world (Carmichael *et al.*, 1985). Toxic blooms of this organism usually occur in eutrophic still waters during warm months of the year (Carmichael *et al.*, 1984). The occurrence of toxic blooms is likely to increase with expansion in the use of fertilizers, pesticides, animal-based agriculture, and construction of water holding facilities such as ponds, lakes, and reservoirs.

Microcystins, contain three D-amino acids, two L-amino acids, N-methyldehydroalanine, and one unusual 3-amino-9-methoxy-2,6,8-trimethyl-10-phenyl-4,6-decadienoic acid (ADDA) component (Botes *et al.*, 1982; Rinehart *et al.*, 1988).

The LD_{50} of microcystin-LR (MCLR) is approximately 75 μg/kg IP in mice (Robinson *et al.*, 1989). Following IP or IV dosing, the livers of mice given a fatal dose rapidly become dark and enlarged, and the mucous membranes of such mice become pale before death (Lovell *et al.*, 1987). Centrilobular hepatic

necrosis and hemorrhage are characteristic histologic lesions of acute MCLR toxicosis in all mammalian species reported to date, including swine (Lovell *et al.*, 1989; Lovell, 1989). Following a lethal parenteral dose, mice usually die within three hours. Death is believed to be caused by shock largely attributable to intrahepatic hemorrhage.

Microcystins affect the cytoskeleton of hepatocytes (Hooser *et al.*, 1991a; Eriksson *et al.*, 1989; Wicksrom *et al.*, 1993, 1994). Microfilaments, intermediate filaments, and microtubules are disrupted and hepatocyte plasma membranes undergo severe blebbing. The loss of hepatocyte structural integrity is accompanied by disruption of sinusoids and intrahepatic hemorrhage. Cytoskeletal disorganization may be due to intracellular hyperphosphorylation as a consequence of toxin-induced inhibition of protein phosphatases (Falconer and Yeung, 1992).

Studies of the fate of radiolabeled microcystins given to rats and mice have shown accumulation primarily in the liver with lesser amounts in the kidneys (Runnegar *et al.*, 1986; Dahlem *et al.* 1989;, Robinson *et al.*, 1989). The concentration of the labeled toxin by the liver is believed to be due to uptake of microcystins by hepatocytes via rifampicin-sensitive bile acid carriers (Hooser *et al.*, 1991b). Microcystin labelled with ^{125}I disappeared biphasically from the blood of rats dosed IV with an

initial phase half-life of 2.1 minutes, followed by a later phase half-life of 42 minutes (Falconer *et al.*, 1986). The disposition of radioactivity in the blood of anesthetized fasted mice given tritiated microcystin IV followed a similar biphasic curve; however, disposition was more rapid in mice with first and second phase half-lives of 0.8 minutes and 6.9 minutes, respectively (Robinson *et al.*, 1991). The disposition of tritiated microcystin from the perfusate of isolated perfused rat livers was slower with a half-life of 130 minutes (Pace *et al.*, 1991). Thus, radiolabeled microcystin in rats and mice is removed quickly from the blood, and the majority of the radioactivity is concentrated in the liver.

Dihydromicrocystin-LR (2H-MCLR) causes the same clinical signs and lesions in rodents as does MCLR (Dahlem, 1989; Hooser *et al.*, 1991b). Isolated perfused rat livers developed microscopic lesions characteristic of microcystin toxicosis within 15 minutes after exposure to [^{3}H]2H-MCLR (Hooser *et al.*, 1991b). In mice, 2H-MCLR given IP was consistently lethal at 200 μg/kg with the time course of the toxicosis being similar to that of the parent toxin, whereas MCLR was lethal at 100 μg/kg (Dahlem, 1989). It is unknown whether the modest reduction in toxicity of 2H-MCLR results from a reduced rate or extent of uptake by hepatocytes or from reduced interactions with intracellular receptors. The tritiated dihydro compound seems to be an appropriate derivative to investigate the absorption and

disposition of microcystins, because the syndrome caused by 2H-MCLR is virtually identical to that induced by MCLR; a relatively high specific activity has been obtained; and the location of the inserted tritium in [^{3}H]2H-MCLR is known and is biologically stable as indicated by absence of the radiolabel in the distillate of urine from dosed mice (Dahlem, 1989). The objectives of the study reported here were to determine the clearance of [^{3}H]2H-MCLR from the blood of swine and its biliary excretion, as well as to determine the rapidity of absorption of [^{3}H]2H-MCLR from the ileum using an isolated ileal loop model.

MATERIALS AND METHODS

Animals-Landrace-cross, specific pathogen free female swine weighing 18 to 24 kg were given free access to feed and water until 12 hours before surgery when feed, but not water was withheld. One hour before anesthesia was induced, the animals were fed 0.5 kg of ground corn mixed with 50 ml of corn oil in an attempt to stimulate bile production.

Toxin-MCLR was purified from a naturally occurring algal bloom collected from Homer Lake, Illinois. The algae water mixture was frozen within hours of removal from the lake, then lyophilized and stored frozen at -40 °C. The crude microcystin was extracted from the lyophilized cells in methanol. The extract was dried,

then redissolved in water, passed through a reversed phase C-18 column, and eluted with methanol. The elution products were separated further via liquid chromatography using a series of two silica gel columns. The first column employed a mobile phase of chloroform, methanol, and water (65:35:10) which was shaken and allowed to separate before discarding the top phase. The second column employed a mobile phase of ethylacetate, isopropanol, and water (4:3:7) and was similarly prepared, discarding the bottom phase. The final purification step was achieved with a size exclusion column (Toyopearl HW40) with methanol as the mobile phase. The purity of the MCLR was determined to be greater than 95% by HPLC, TLC, and fast atom bombardment-mass spectrometry.

Radiolabeling-[^{3}H]2H-MCLR was produced by reacting MCLR with tritiated sodium borohydride ($NaB^{3}H_{4}$) and the products purified. Four reactions were carried out in order to produce the radiolabelled toxin. Molar ratios of MCLR:$NaB^{3}H_{4}$ (100 mCi) (American Radiolabel Chemical, St. Louis MO.) of 1:3.7 and 1:4.5, respectively, were used in the first two labelling reactions. The reactions were carried out for 24 hours in 0.5 ml of 70% isopropanol then quenched with acetic acid. The third and fourth reactions were performed using 250 mCi of $NaB^{3}H_{4}$ (Amersham Inc., Arlington Heights, IL.) with molar ratios of MCLR:$NaB^{3}H_{4}$ 1:4 and 1:5.

The reaction products were dried under nitrogen gas,

redissolved in H_2O and loaded on a C_{18} reversed phase column. The labelled toxin was eluted with methanol and dried with nitrogen gas. The dried products were then dissolved in a mobile phase of chloroform:methanol:water (65:35:10) prepared as described above and passed through a 200 ml chromatography column packed with 9 gm of silica gel. Fractions were collected and samples from each fraction counted with a scintillation counter. Ten μl samples from each fraction were also loaded on a fluorescent silica gel thin layer chromatography plate and developed using a chloroform:methanol:water (65:35:10) mobile phase prepared as previously described. Fractions were combined that had the same retardation factors (Rfs) as the standard 2H-MCLR. Ten μl of the combined fractions were loaded on a TLC plate and developed as described above for the methanol fraction from the C18 column.

The purified reaction products from all four reactions were combined and dissolved in 10% ethanol forming a final reaction product. The concentration of [^{3}H]2H-MCLR was determined to be 0.166 mg/ml based on a comparison to a standard HPLC curve of 2H-MCLR. The final reaction product was determined to be greater than 90% [^{3}H]2H-MCLR or 2H-MCLR by HPLC and TLC (Figures 4 and 5). The specific activity of the final reaction product used in preparing the dosing solutions was 1.04 mCi/mg as measured by liquid scintillation counting.

Dosing solutions-The pigs given 25 μg/kg of toxin were dosed

with the final reaction product of [^{3}H]2H-MCLR. In order to conserve the [^{3}H]2H-MCLR the swine dosed IV with 75 μg/kg of toxin were administered 25 μg/kg of [^{3}H]2H-MCLR and 50 μg/kg of 2H-MCLR. Because lower blood concentrations were anticipated in the swine dosed with 75 μg/kg of toxin via the ileal loop, they were given of [^{3}H]2H-MCLR at 75 μg/kg to improve quantification in the blood. The toxin was dissolved in 10 % ethanol prior to dosing.

Anesthesia-Each pig was anesthetized with isoflurane (Anaquest Inc., Liberty Corner, NJ.) by mask and a cuffed endotracheal tube was inserted. A combination of xylazine (0.66 mg/kg) and lidocaine (3.6 mg/kg) was then administered to the pigs by epidural injection. Anesthesia was maintained with isoflurane at 2.5% during surgery, and 1.5% during the dosing and sampling period.

Surgical procedures-Pigs were placed on a circulating water heated pad. An incision was made in the lateral-ventral cervical skin, and the jugular vein and carotid artery were catheterized. A second skin incision was made over the right femoral vein which was then catheterized. A midventral abdominal incision was made, and a catheter placed in the caudal vena cava cranial to the renal veins with the tip advanced to the hepatic sinus. A second catheter was placed in the hepatic portal vein. The common bile duct was ligated to stop bile flow to the intestine. The gall

bladder was emptied with a 20 gauge needle and syringe, and the site of perforation was closed using a pair of hemostats. The urinary bladder was evacuated in the same manner.

In the 3 pigs to be dosed via the ileal loop, the ileum was clamped with two bowel clamps placed 4 cm orad to the ileocecal junction, the blood vessels immediately adjacent to the clamps were ligated and then the ileum was transected between the clamps. The procedure was repeated 15 cm rostrally leaving an isolated ileal loop. The isolated ileal loop was then flushed with 0.9% NaCl in water to remove lumen contents and each end closed with an inverting suture pattern. The integrity of the loop was determined by injecting 0.9% NaCl into the lumen and observing for signs of leakage. The abdominal cavities of the pigs dosed IV and via the ileal loop were temporarily closed with towel clamps.

Dosing-In the pigs dosed IV, the toxin containing solutions were injected via the jugular catheter, over a one minute period, and the catheter was then flushed with 5 ml of 0.9% NaCl. The remaining pigs were given the dosing solution by direct injection into the lumen of the ileal loop using a syringe and needle.

Sampling-Blood was taken from the femoral vein of pigs at 1, 3, 5, 7, 10, 20, 30, and 40 minutes, as well as at 1, 1.5, 2, 2.5, 3, 3.5, and 4 hours after dosing IV. Blood samples were drawn

from the portal vein and hepatic sinus at 20, and 40 minutes, and 1, 1.5, 2, 2.5, 3, 3.5, and 4 hours after dosing IV.

The pigs dosed via the ileal loop were sampled for one hour longer than the pigs dosed IV. Blood samples were taken from the femoral, hepatic, and portal veins at 5, 20, and 40 minutes, as well as at 1, 1.5, 2, 2.5, 3, 3.5, 4, 4.5, and 5 hours from the pigs dosed via the ileal loop.

Bile samples were taken with a syringe and needle when the gall bladder filled, and the aperture was closed with hemostats between sampling periods to prevent leakage into the abdominal cavity. The time of sampling and volume of bile evacuated were recorded.

Histopathology-At the end of the experiment, sections of liver were fixed in 10% neutral buffered formalin, routinely processed, embedded in paraffin, sectioned at 4 μm, stained with hematoxylin and eosin, and examined by light microscopy.

Scintillation counting-Whole blood and bile samples were counted with a Packard Tri-Carb model B2450 scintillation counter (Packard Instrument Co., Meriden, CT.). Samples of blood or bile (500 μl) along with an equal volume of Solvable (NEN, Du Pont Co., Boston, MA.) were placed in 20 ml polypropylene scintillation vials. The vials were then incubated for one hour

at 50°C in a shaking water bath. To reduce foaming, 100 μl of 100 mM ethylenediaminetetraacetic acid (EDTA) were added to each vial followed by three 100 μl aliquots of 30% H_2O_2 to decolorize each sample. Samples were incubated again for one hour at 50°C in a water bath. The vials were cooled to room temperature, after which 15 ml of Aquasol II were added. The samples were stored in darkness for 72 hours before they were counted.

Computation of disintegrations per min(DPM)-A quench curve was established using a tritium standard which was quenched with eleven dilutions of carbon tetrachloride. The external standard ratio (ESR) and percent quenching were determined for each dilution and the values compared using regression analysis. Regression was used to determine the percent quenching based upon the ESR for all of the swine blood samples. A tritium standard was used to establish the efficiency of the scintillation counter which was consistently in the range of 69.8% to 69.9%.

Pharmacokinetic analysis-The DPM in blood versus time following the IV doses were fitted by mono-, bi-, and triexponential equations using the Autoan computer program (Sedman and Wagner, 1974). The intercepts of the equations were subsequently converted from DPM to ng/ml.

RESULTS

IV dose study-Concentrations of toxin in femoral venous blood decreased rapidly (Figure 6). Disposition was biphasic with the 25 μg/kg dose being cleared more rapidly than the 75 μg/kg dose. A biexponential equation of the form ng/ml = $Ae^{-\alpha t}+Be^{-\beta t}$ was determined by F test to fit the data best. The early disappearance rate constant (alpha) at the 25 μg/kg dose was 11.4/hour and the later disappearance rate constant (beta) was 0.311/hour (Table 1). The value for alpha at the 75 μg/kg dose was 13.8/hour and the beta value was 0.155/hour. Therefore, the half-life ($T_{1/2}$) values for the alpha phase of the low dose and high dose, respectively, were 0.61 hour and 0.05 hour, and those for the beta phases of the low and high doses were 2.23 hours and 4.48 hours, respectively. The blood clearance (Cl) of the low dose (0.203 l/kg/hour) was approximately 3 times greater than the clearance of the high dose (0.0674 l/kg/hour).

The concentrations of toxin in the portal vein and hepatic sinus area of the caudal vena cava were very similar to the concentrations of toxin in the femoral vein (Figures 7 and 8).

The production of bile was inconsistent and therefore, so were the times of bile collection. One pig in the low dose group (N=2) and one pig in the high dose group (N=3) failed to produce any collectable bile during the four hour observation period. The second pig given the low dose produced 53 ml of bile which

contained 4% of the total radioactivity given IV with a significant portion of the radiolabel (0.5% of the dose) present in the bile at 35 minutes after dosing. The second and third pigs given the high IV dose produced 35 ml and 75 ml of bile, respectively. The second high dose pig, which produced 75 ml of bile yielding 5.9% of radioactivity given IV, had measurable activity (1.12% of the dose) in the bile at 12 minutes after dosing. The third high dose pig yielded 45 ml of bile containing 1.27% of the dose with the first measurable sample (0.08% of the dose) collected at 120 minutes after dosing.

Ileal loop study-Peripheral blood concentrations of toxin were not as high as with the IV doses (Figure 9). The portal values were consistently higher than those of other blood samples at all sampling times in the 5 hour study. The maximum concentration was present in the portal blood at 90 minutes after dosing. Three pigs were dosed via the ileal loop. The first pig produced 51 ml of bile which contained 12.8% of the total dose. The initial bile sample was collected at 90 minutes after dosing and contained 2.6% of the total dose. The second pig produced 102 ml of bile containing 15.3% of the total radioactivity, with the first measurable radioactivity, accounting for 0.17% of the total dose, being obtained at 90 minutes after dosing. The third pig dosed via the ileal loop eliminated 5.26% of the total dose in the collected bile; and the first sample which contained detectable radioactivity, accounting for 1.3% of dose, was

collected at 120 minutes after dosing.

Histopathology-All pigs treated with 2H-MCLR developed liver lesions that are characteristic of MCLR toxicosis: swelling, disassociation and early fragmentation of centrilobular and, in more severe cases, midzonal hepatocytes. The pigs given the high dose had the most severe and extensive lesions with associated hemorrhage (2 of the 3 pigs). The pigs dosed with the toxin via the ileal loop had a very uneven distribution of the lesions, with large areas of liver being unaffected. These latter pigs had background lesions consistent with pericholangitis.

DISCUSSION

The alpha phases of the high and low doses were similar, however, there was a difference between the beta phases. The $T_{1/2}$ for the alpha phase, which lasted about 20 minutes after dosing, was slightly less for the high dose (3.0 minutes) as compared to the low dose (3.6 minutes). For the beta phase, which continued from 20 minutes postdosing until the end of the 4 hour study, the $T_{1/2}$ at the low dose of 133.5 minutes was considerably less than that at the high dose of 268.6 minutes. The biphasic disposition of [^{3}H]2H-MCLR in this study with pigs is similar to that reported by Robinson *et al.*(1991) who administered tritiated MCLR to mice; however, the blood clearance in mice was more rapid. The difference in blood clearance may be due to species

variation, an effect of anesthesia or surgery, or differences in the toxins. The production of tritiated MCLR, however, has not been consistently achievable because the process often tends to degrade the toxin (Dahlem, 1989). Whether the greater toxicity of MCLR, as compared to 2H-MCLR, is related to more rapid uptake of the toxin from the blood by the liver remains to be assessed.

The peak concentration of toxin in the blood of swine dosed via the ileal loop occurred at 90 minutes after dosing. During the entire five hour monitoring period, the toxin concentration in the peripheral blood was significantly lower than in the portal venous blood. Although blood flow rates in sampled vessels and hepatic extraction ratios were not measured, the difference in peripheral venous concentrations of toxin in ileal loop-dosed pigs compared to the IV-dosed pigs, suggests that a first pass effect is, in part, responsible for clearance of the toxin. This is in concurrence with previous studies which demonstrated that the liver preferentially accumulates and is a major target organ for the toxin (Dahlem, 1989; Runnegar *et al.*, 1986; Robinson *et al.*,1989).

Data from Table 1 indicate that the blood clearance (Cl) of the toxin at the high IV dose was only 36% of the low IV dose value. The slower clearance at the high dose was due primarily to impaired elimination of the toxin as reflected in the decreased elimination rate constant (k10) of the toxin; ie., k10

at the high dose was 37% of the k10 at the low dose. The apparent volume of distribution at steady state (V_{ss}) and apparent volume of distribution based on area under the curve (V_{area}) at the high dose actually decreased while the apparent volume of the central compartment (V_c) remained about the same, suggesting that the toxin did not distribute as well to the peripheral tissues at the high dose or that the animals were more dehydrated. Previous hemodynamic studies in swine have shown that MCLR causes a decrease in mean aortic pressure and a decrease in blood flow through the liver resulting in decreased central venous pressure with a corresponding increase in portal venous pressure (Lovell, 1989). The same investigation showed that hepatic perfusion decreased more rapidly than renal perfusion and that approximately 37.9% of the estimated total blood volume was sequestered in the liver of pigs given a lethal dose of MCLR. The decreased volume of distribution at the high dose would be expected to increase the clearance of the toxin had it not been for impaired elimination. Thus, at the high dose, the decreased clearance of the toxin was probably due to decreased elimination as a consequence of hepatic damage, which was more severe in the pigs given the high IV dose. The hepatic lesions induced by 2H-MCLR were similar to those observed in naturally occurring and experimentally induced toxicosis associated with MCLR containing cyanobacteria.

In conclusion, the clearance of [^{3}H]2H-MCLR from the blood of anesthetized swine is rapid and follows a biphasic pattern. This study indicates that the liver rapidly clears [^{3}H]2H-MCLR from the blood and secretes it into the bile. Also, at a potentially lethal dose, clearance is reduced. Following exposure via an ileal loop, data were indicative of marked first pass effect.

FIGURE LEGENDS

Figure 6-Cartesian plot of [^{3}H]2H-MCLR concentrations in femoral vein blood samples collected for 240 minutes from pigs dosed at 25 μg/kg (n=2) or 75 μg/kg (n=3) IV and for 300 minutes from pigs dosed at 75 μg/kg (n=3) via the ileal loop. Error bars represent standard errors. Where error bars are not visible the standard error was graphically within the width of the symbols.

Figure 7-Semilog plot of [^{3}H]2H-MCLR concentrations in femoral, portal, and hepatic venous blood of pigs(n=2) dosed IV at 25 μg/kg. The first samples from the portal and hepatic veins were obtained at 20 minutes after dosing. Error bars represent standard errors. Where error bars are not visible the standard error was graphically within the width of the symbols.

Figure 8-Semilog plot of [^{3}H]2H-MCLR concentration in blood of pigs (n=3) dosed IV at 75 μg/kg. The first sample was obtained from the portal and hepatic veins at 20 minutes after dosing. Error bars represent standard errors. Where error bars are not visible the standard error was graphically within the width of the symbols.

Figure 9-Semilog plot of 2H-MCLR concentrations in blood of pigs (n=3) dosed via the ileal loop at 75 μg/kg. Error bars represent standard errors. Where error bars are not visible the standard error was graphically within the width of the symbols.

TABLE LEGENDS

Table 1-Toxicokinetic parameters (means) for the disposition of ^{3}H from [^{3}H]2H-MCLR following intravenous administration to swine.

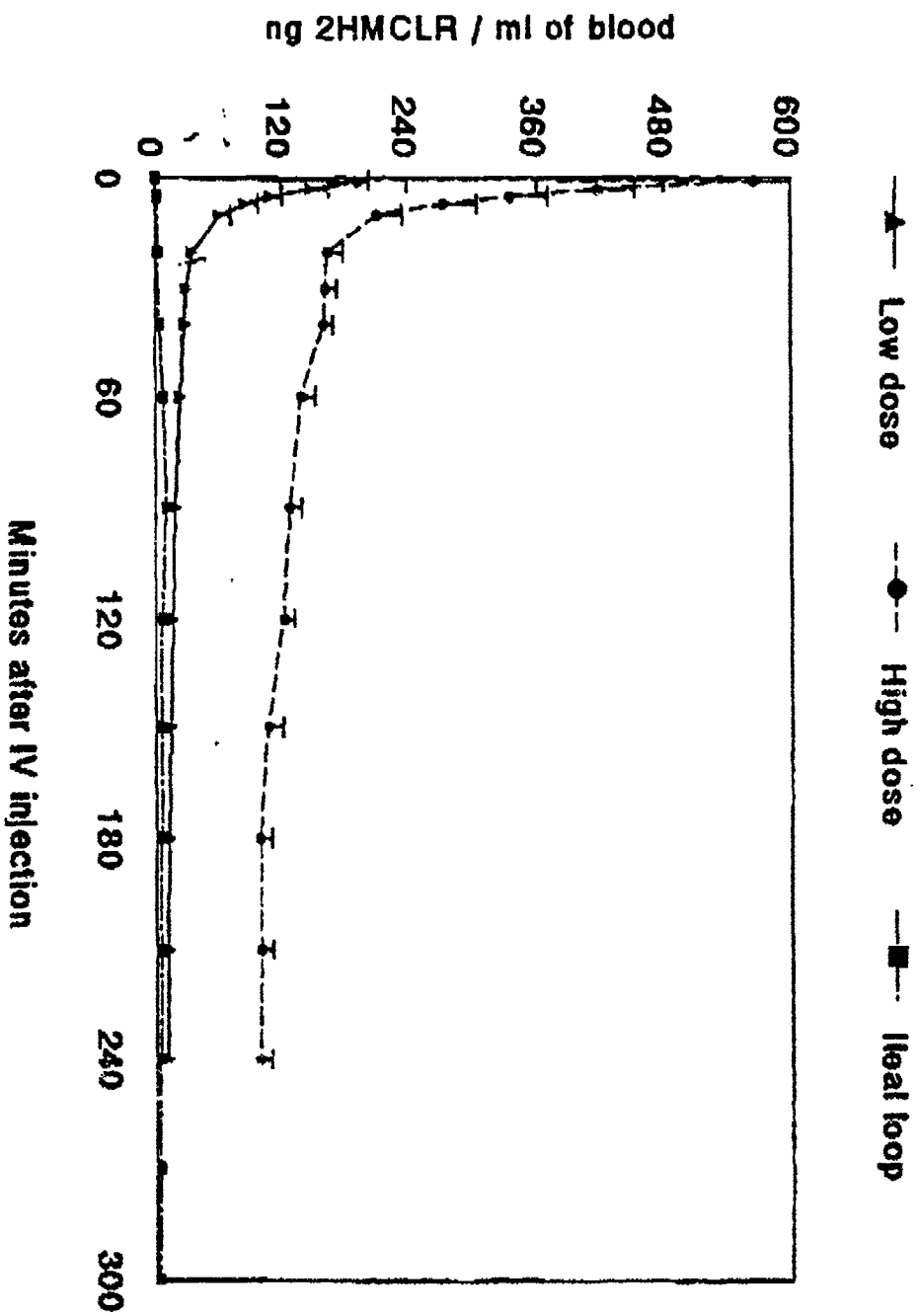

ng 2HMCLR / ml of blood
0
120
240
360
480
600
0
60
120
180
240
300
Minutes after IV injection
Low dose
High dose
Ileal loop

Figure 6

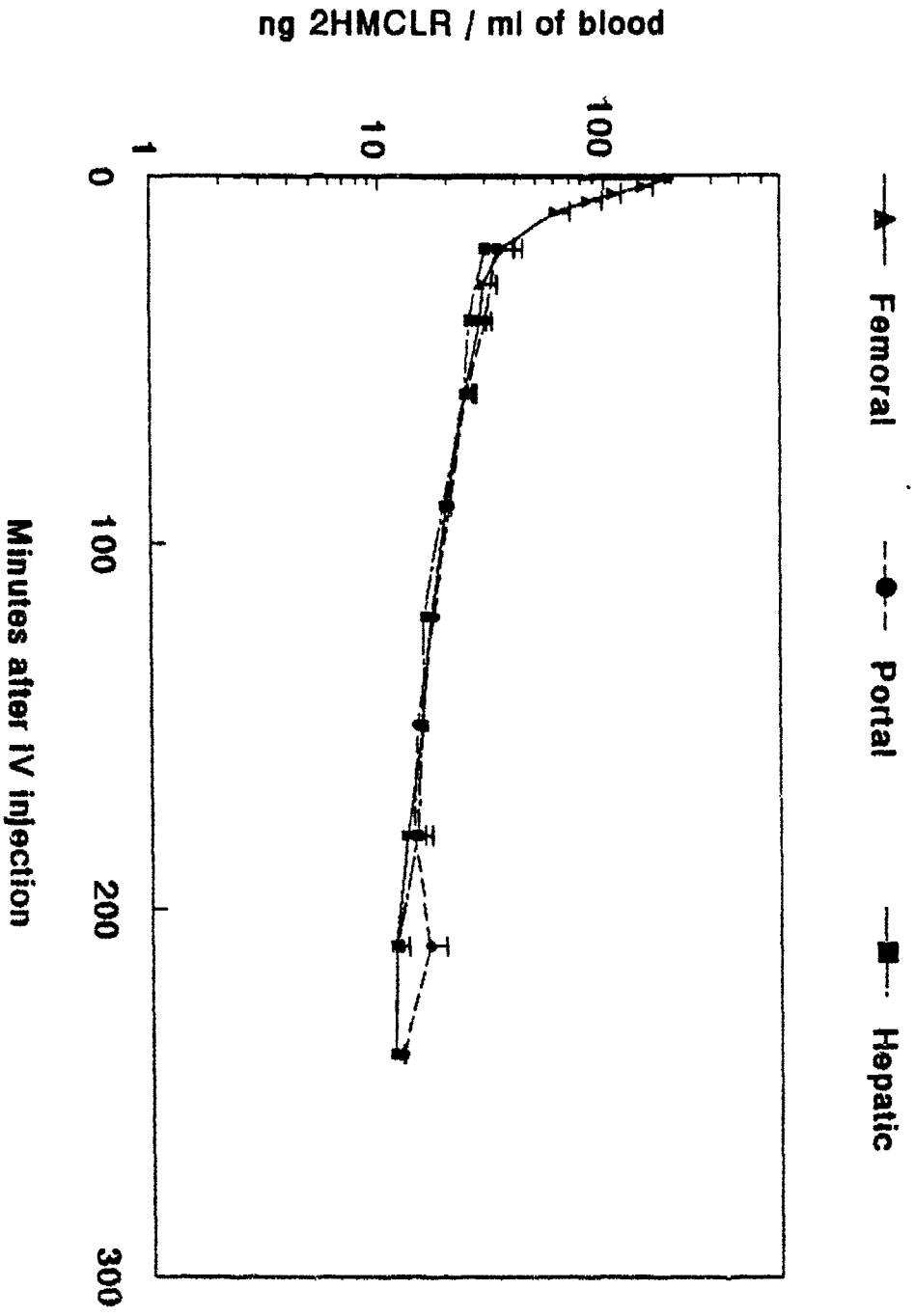

ng 2HMCLR / ml of blood
1
10
100
0
100
200
300
Minutes after IV injection
Femoral
Portal
Hepatic

Figure 7

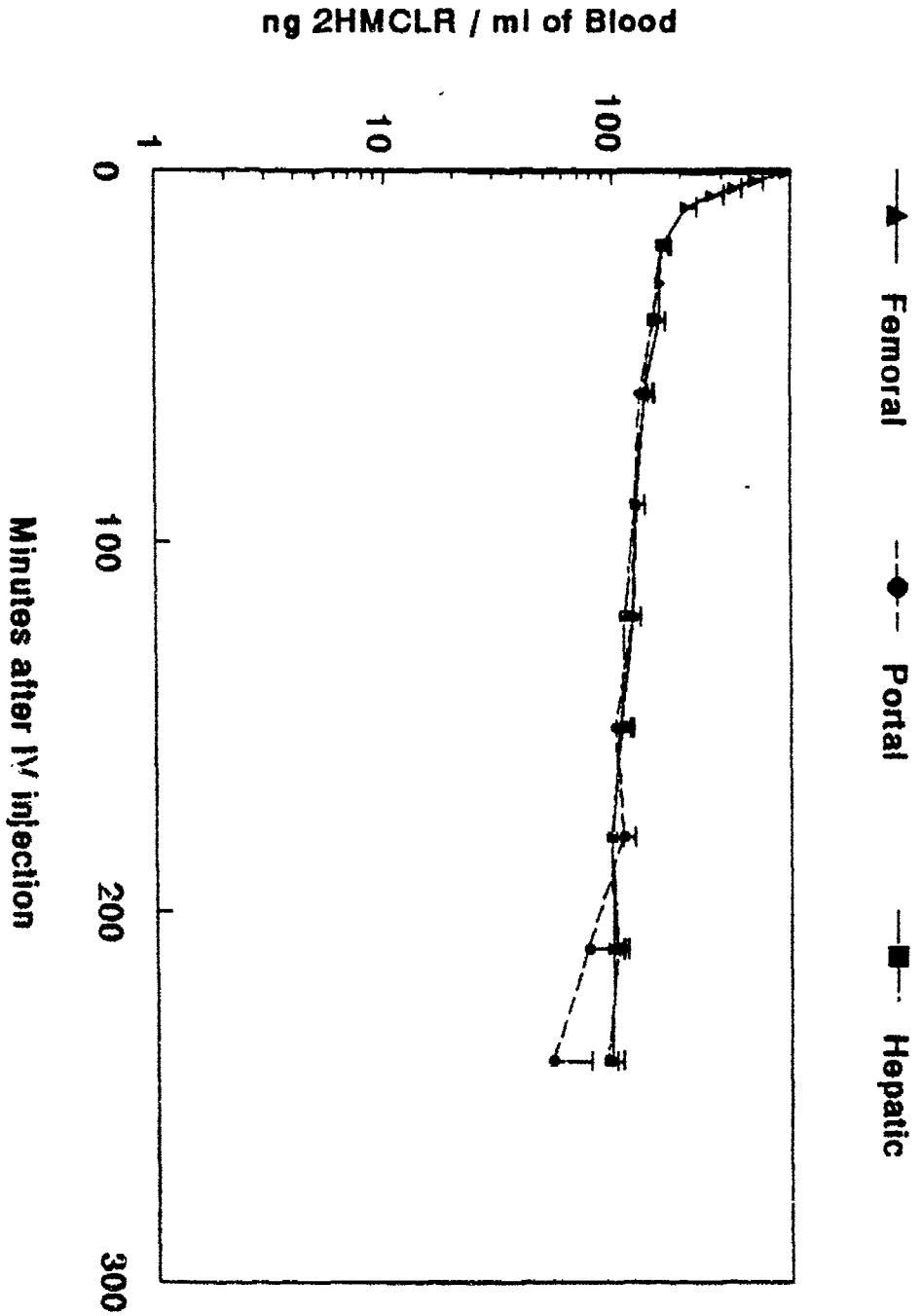

ng 2HMCLR / ml of Blood
1
10
100
0
100
200
300
Minutes after IV injection
Femoral
Portal
Hepatic

Figure 8

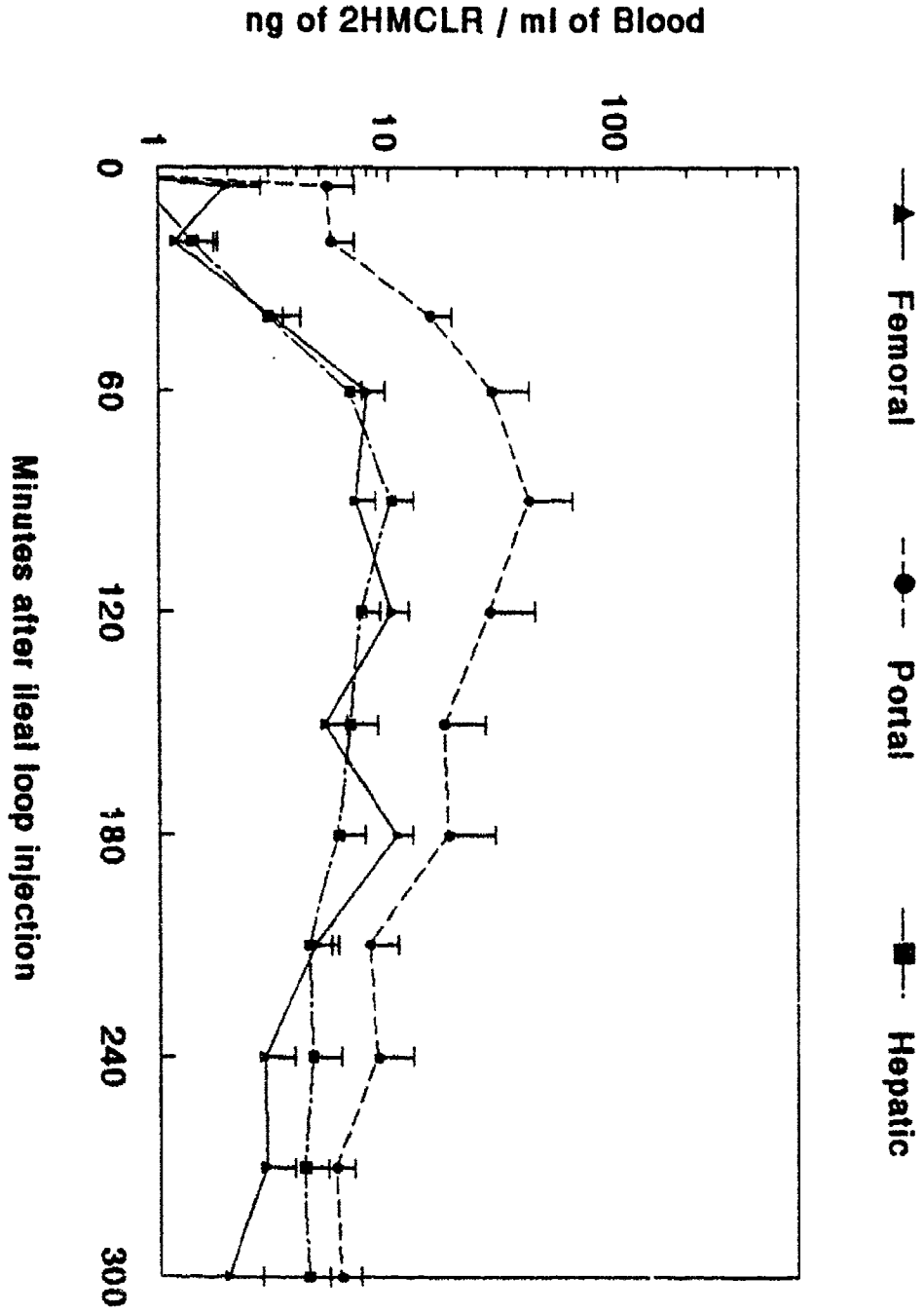

ng of 2HMCLR / ml of Blood
1
10
100
0
60
120
180
240
300
Minutes after ileal loop injection
Femoral
Portal
Hepatic

Figure 9

TABLE 1

	Low Dose (25μg/kg)	High dose (75μg/kg)
A	198 ng/ml	516 ng/ml
B	33 ng/ml	167 ng/ml
α	11.4 hr^{-1}	13.8 hr^{-1}
β	0.311 hr^{-1}	0.155 hr^{-1}
$\beta t_{\frac{1}{2}}$	0.061 hr	0.050 hr
$\alpha t_{\frac{1}{2}}$	2.23 hr	4.48 hr
k12	7.94 hr^{-1}	9.87 hr^{-1}
k21	2.17 hr^{-1}	3.50 hr^{-1}
k10	1.64 hr^{-1}	0.620 hr^{-1}
V_c	0.1080 l/kg	0.1099 l/kg
V_{ss}	0.5043 l/kg	0.4216 l/kg
V_{area}	0.6512 l/kg	0.4353 l/kg
Cl	0.2030 l/kg/hr	0.0674 l/kg/hr

DISTRIBUTION OF TRITIATED DIHYDROMICROCYSTIN IN SWINE TISSUE

SUMMARY

The distribution of tritiated dihydromicrocystin [^{3}H]2H-MCLR was studied in anesthetized specific pathogen free (SPF) pigs. Two doses were administered IV and one dose was given via an isolated ileal loop. At 4 hours after IV administration of the toxin at 25 μg/kg, 64.6 % of the total dose (%TD) was located in the liver tissue, with lesser amounts distributed to the kidneys (1.2 %TD), lungs (1.75 %TD), heart (0.22 %TD), ileum (0.13 %TD), and spleen (0.04 %TD). A similar distribution was found at 4 hours post-dosing in pigs given 75 μg/kg, although the liver contained a lower percentage of the toxin total dose, at 46.99 %TD, and the kidneys had somewhat more, at 2.19 %TD, as compared to the low dose. The lungs (0.55 %TD), heart (0.23 %TD), ileum (0.20 %TD), and spleen (0.07 %TD) had amounts similar to those at the low dose. The livers of the pigs given 75 μg/kg via the ileal loop, at 5 hours post-dosing, contained 49.5% TD and the ileum had 33.94 %TD. Lesser amounts were distributed to kidneys (1.04 %TD), lungs (0.65 %TD), heart (0.81 %TD) and spleen (0.16 %TD). The livers of both groups dosed at 75 μg/kg contained higher concentrations of toxin, but lower percentages of the total dose as compared to the 25 μg/kg dosed pigs. Larger increases in serum arginase in the two 75 μg/kg groups were associated with histological evidence of more severe disruption

of the hepatic organization than at the 25 μg/kg dose. Analysis of radiolabeled compounds from hepatic tissue using fast atom bombardment mass spectrometry determined that the primary constituent was parent compound, but two minor radioactive components were also isolated. These findings indicate that [^{3}H]2H-MCLR is actively concentrated in the hepatic tissue of swine and is probably toxicologically active as the parent compound.

INTRODUCTION

Microcystins are cyclic heptapeptide hepatotoxins containing three D-amino acids, two L-amino acids, N-methyldehydroalanine, and one unusual ADDA component (Botes *et al.*, 1982; Rinehart *et al.*, 1988). Several variations of the toxin have been reported which induce hepatotoxicity in laboratory animals (Kirshnamurthy *et al.*, 1989; Namikoshi *et al.*, 1992; Stotts *et al.*, 1993).

The LD_{50} of microcystin-LR (MCLR) is approximately 75 μg/kg IP in mice (Robinson *et al.*, 1989). Following IP or IV dosing, the livers of mice given a fatal dose rapidly become dark and enlarged, and the mucous membranes of such mice become pale before death (Lovell *et al.*, 1987). Centrilobular hepatic necrosis and hemorrhage are characteristics histologic changes of MCLR toxicosis in all mammalian species reported to date, including swine (Lovell *et al.*, 1987; Lovell *et al.*, 1989;

Lovell, 1989). Following a lethal parenteral dose, mice usually die within three hours. Death is believed to be caused primarily by shock largely attributable to intrahepatic hemorrhage.

Microcystins affect the cytoskeleton of hepatocytes by disrupting microfilaments, intermediate filaments, and microtubules. Blebbing of the cell membranes is prominent in cells exposed *in vitro*. The loss of hepatic structural integrity causes the centrilobular and midzonal regions to accumulate large volumes of blood. More recent studies suggest that the cytoskeletal disruption is related to hyperphosphorylation of intracellular proteins probably due to inhibition of protein phosphatases (Eriksson *et al.*, 1990; Falconer and Yeung, 1992).

Studies using rats and mice have shown that radiolabelled microcystins accumulate primarily in the liver with lesser amounts in the kidneys (Runnegar *et al.*, 1986; Falconer *et al.*, 1986; Robinson *et al.*, 1989; Dahlem, 1989a; Robinson *et al.*, 1991). The concentration of the labeled toxin in the liver is believed to be due to uptake of the microcystins by rifampicin-sensitive hepatic bile acid carriers (Hooser *et al.*, 1991).

The objective of this study was to determine the distribution of [^{3}H]2H-MCLR in swine dosed IV and via an ileal loop.

MATERIALS AND METHODS

Animals-Landrace cross specific pathogen free female swine weighing from 18 to 24 kg were group housed and given free access to feed and water until the day before surgery. Feed was withheld for 12 hours before surgery. In an attempt to stimulate bile production, pigs were fed one pound of ground corn with 50 ml of added corn oil one hour before induction of anesthesia.

Microcystin-MCLR was purified as described on page 23.

Radiolabeling-[^{3}H]2H-MCLR was produced as described on page 24.

Dosing solutions-Dose formulations are described on page 25.

Anesthesia-Pigs were anesthetized as described on page 26.

Surgical procedures-Surgery was performed as described on page 26.

Experimental groups:

Group 1-Three pigs were given 25 μg of toxin per kg of body weight IV by injecting the dosing solution over a one minute period via the jugular catheter. The jugular catheter was then flushed with 5 ml of 0.9% NaCl solution.

Group 2-Three pigs were given 75 μg of toxin per kg of body weight IV in the same manner as in group 1.

Group 3-Three pigs were given 75 μg of toxin per kg of body weight by injection directly into the lumen of the ileal loop with a syringe and needle.

Sampling-Bile samples were taken with a syringe and needle when the gall bladder filled. The perforation initially employed to evacuate the gall bladder was used for each sampling and the aperture closed with hemostats between sampling periods to prevent leakage of bile into the abdominal cavity.

One pig that was dosed IV with [^{3}H]2H-MCLR and 2H-MCLR at a total of 25 μg/kg of toxin was selected to evaluate the distribution of toxin in the liver. Samples were taken at 14 locations in the liver using a 5 mm biopsy punch, cores which penetrated completely through the liver were obtained at each site (Figure 10).

The concentration of toxin was measured in the tissues of the pigs dosed with [^{3}H]2H-MCLR. The pigs were killed by exsanguination while anesthetized and the tissues immediately removed. Approximately 500 g of tissue were collected from the right medial liver lobe, spleen, lung, kidney, ileum, and heart of each pig for scintillation counting. Tissue specimens to be

examined histologically were placed in 10% formalin and the remaining tissue was frozen.

Scintillation counting-Samples were counted with a Packard Tri-Carb Model B2450 scintillation counter (Packard Instrument Co., Meriden, CT.). Bile (500 μl) and an equal volume of Solvable (NEN, Du Pont Co. Boston, MA.) were placed in 20 ml polypropylene scintillation vials. The vials were then incubated for one hour at 50°C in a shaking water bath. To reduce foaming, 100 μl of 100 mM EDTA were added to each vial followed by three 100 μl aliquots of 30% H_2O_2 to decolorize each sample. Samples were then incubated for one hour at 50°C in a water bath. The vials were cooled to room temperature, after which 15 ml of Aquasol II (NEN, Du Pont Co. Boston, MA.) were added. The samples were stored in darkness for 72 hours before they were counted. Urine samples were processed following the same procedures as described for the bile samples.

Samples of tissues (≈ 500 mg) were placed in a glass tube and distilled water equal to 2 times the weight of the tissue was added to the vessel. After the tissue and water mixtures were homogenized, 500 μl of the homogenate were pipetted into 20 ml polypropylene scintillation vials and processed as described above for the bile samples.

A quench curve was established using a tritium standard

which was quenched with eleven dilutions of carbon tetrachloride. The external standard ratio (ESR) and percent quenching were determined for each dilution and the values compared using regression analysis. Regression was used to determine the percent quenching based upon the ESR for all of the swine blood samples. A tritium standard was used to establish the efficiency of the scintillation counter which was consistently in the range of 69.8% to 69.9%.

Histologic evaluation of tissues-Sections from liver, kidney, spleen, heart, and lung were fixed in a 10% buffered neutral formalin, embedded in paraffin, sectioned at 4μ, stained with hematoxylin and eosin, and evaluated by light microscopy.

Analysis of serum arginase-The concentration of serum arginase in venous blood was analyzed at 1, 2, 3, 4 and 5 hours after being given [^{3}H]2H-MCLR IV. Pigs that were dosed via the ileal loop were sampled at one, two, three, four, and five hours. The blood samples were refrigerated and allowed to clot. As soon as the clot was well formed, serum was removed and 50 μl of serum were evaluated for arginase activity as described by Mia and Koger (1978).

Chromatography-Thin layer chromatography (TLC) of aqueous extracts of individual livers was performed on precoated silica gel plates (Kieselgel 60 F_{254}, 0.25 mm thickness, EM Science,

Gibbstown, NJ). Adsorbed spots were detected under UV light at 254 nm and by spraying phosphomolybdic acid (10% in ethanol) followed by heating. The usual TLC developing solvent was chloroform-methanol-water (16:14:3). For column chromatography, silica gel was purchased from E. Merck (Darmstadt, Germany) and C-18 reversed phase bulk packing material from Fuji-Davison Chemical Ltd. (Tokyo, Japan). High performance liquid chromatography was performed on a system comprised of Beckman 114M pumps (Beckman Corp.; Berkeley, CA) a Rheodyne injector (Rheodyne Inc.; Colati, CA) and a Beckman 153 8μL analytical UV detector fixed at 254 nm unless otherwise noted.

Radioisotope measurement of fractions separated by column chromatography-Small aliquots of radiolabeled samples were dissolved in 7 or 15 ml of Aquasol scintillant (NEN, Boston, MA) and the radioactivity was measured with a Tracor Analytical Betatrac 6895 (TM Analytic; Brandon, FL) liquid scintillation counter. Background counts were subtracted from all samples counted. The radiochemical purity (RTLC analysis) was measured with a Radiomatic Instruments and Chemical Co., Inc. RTLC scanner (Packard, Meriden, CT) using silica gel TLC plates.

Mass spectroscopy-Fast atom bombardment mass spectroscopy (FABMS) spectra and high resolution FABMS data were recorded using either a VG Analytical ZAB-SE or a VG 70 SE-4F four sector spectrometer with a xenon fast atom gun (VG Analytical;

Manchester, England) using magic bullet (dithiothreitol-dithioerythritol) matrix (Witten *et al.*, 1984). Collisionally-induced tandem MS/MS spectra were obtained on a VB 70 SE-4F four sector spectrometer using helium as a collision gas.

Isolation of metabolites from liver tissue-The livers of the pigs dosed with [^{3}H]2H-MCLR were removed immediately after the pigs were killed and frozen. The frozen livers were individually homogenized in a blender and extracted with methanol (500 ml x 3). The extracts were pooled and concentrated. After filtering to remove undissolved solids, hexane (3L) was added to the filtrate and the radioactive methanol layer was separated. This methanol solution was partitioned again using petroleum ether:ethyl acetate:methanol:water (7:4:4:3) and the radioactive lower layer was concentrated to remove the organic solvents resulting in an aqueous solution. This aqueous solution was purified by solid phase partition using a C-18 column (2.2 cm x 22 cm). The solution was passed through the column, which was then washed with deionized water, and eluted using a gradient of acetonitrile and 0.1% ammonium acetate solution, yielding three radioactive fractions, PLC-1, PLC-2, and PLC-3. PLC-2, the most radioactive fraction, was purified again, first using Sephadex LH-20 column (Pharmaceutica Fine Chemicals; Piscataway, NJ) (2.2 cm x 70.4 cm) and a mobile phase of methanol to yield a fraction termed PLC-2-2. PLC-2-2 was purified further first by silica gel chromatography, and then by preparative reversed phase HPLC and

the peaks corresponding to radioactivity were collected to give a radioactive oil. This material was then analyzed by FABMS.

RESULTS

Distribution of toxin equivalents in the liver-Samples were analyzed from 17 locations in the liver of the pig given the radiolabeled toxin IV at a dose of 25 μg/kg. The toxin equivalents measured by scintillation counting were later confirmed to be almost entirely parent toxin, as described in the section entitled *Characterization of toxin in liver tissue* (page 57). Minute quantities of other products are suspected to be minor metabolites. The toxin was distributed throughout the liver (Figures 10 & 11). The concentration varied from the lowest in sample number 12 (351 μg/kg of tissue) which was located in the quadrate lobe, to the highest in sample number 5 (573 μg/kg) which was taken from the left lateral lobe. The mean concentration of toxin equivalents in liver samples was 494 μg/kg. All sample concentrations except for number 12 were within 2 standard deviations of the mean (Table 2).

Concentration of toxin equivalents in the tissues

Group 1-One of the three pigs dosed at 25 μg/kg died because of anesthetic compilations before the end of the 4 hour sampling period, therefore the data from that animal were not included. In the other pigs of this dose, the liver had the highest concentration of toxin equivalents (mean of 633 μg/kg of tissue)

and contained 64.6% of the total dose (Figure 12). The next highest concentration was found in the kidneys (mean of 121 μg/kg) which accounted for 1.2% of the total toxin administered to the pigs. The lungs (62 μg/kg), heart (17 μg/kg), ileum (11 μg/kg) and spleen (9 μg/kg) accounted for 1.75%, 0.22%, 0.13%, and 0.04%, respectively, of the total toxin administered to the pigs.

Group 2-The distribution of toxin equivalents in all three pigs dosed IV at 75 μg/kg was similar to that in the pigs of group 1. The concentration of toxin equivalents in liver tissue (1,110 μg/kg) was the highest of any organ and accounted for 46.99% of the total toxin dose (Figure 12). The kidney tissue at 654 μg/kg had the second highest concentration and accounted for 2.19% of the total [^{3}H]2H-MCLR administered. The lungs (59 μg/kg), heart (54 μg/kg), ileum (57 μg/kg), and spleen (41 μg/kg) contained 0.55%, 0.23%, 0.20%, and 0.07%, respectively, of the administered radiolabel.

Group 3-The pigs dosed via the ileal loop at 75 μg/kg demonstrated a somewhat different distribution pattern from the pigs dosed IV (Figure 13). The pigs in group 3 had a much higher concentration of toxin equivalents in the ileal loop at the end of the five hour sampling period. The hepatic concentration (1,408 μg/kg) was lower than the ileal concentration (9,165 μg/kg), however, because of the greater mass of hepatic tissue

compared to the mass of ileal tissue and contents, the liver contained 49.59% of the total dose, while the ileum contained only 33.94% of the total dose. The distribution in the other tissues sampled relative to the concentration in the liver was similar to that found in the pigs dosed IV. The kidneys (31 μg/kg), lungs (69 μg/kg), heart (19 μg/kg), and spleen (94 μg/kg) accounted for 1.04%, 0.65%, 0.81%, and 0.16%, respectively, of the total toxin administered to the pigs.

Concentration of toxin equivalents in the bile and urine-The production of bile during the experiments was inconsistent (Figure 13). Two of the pigs produced only 0.5 ml of bile, but the highest producing pig yielded 102 ml of bile during the sampling period. The percent of dose recovered in the bile corresponded closely to the quantity of bile produced by each pig (Figure 14). Neither the radiolabelled toxin nor any radiolabelled products were detected in the urine of the pigs dosed by either route.

Pathology

Group 1 - The liver tissue of pigs dosed IV at 25 μg/kg had centrilobular and midzonal hepatocytes that were pale, dissociated, and beginning to undergo fragmentation. Increased numbers of red blood cells were noted in the sinusoids. The heart showed multifocal areas of myofiber changes that included cytoplasmic hypereosinophilia, nuclear hyperchromasia and

perinuclear vacuolization. The lungs had mild perivascular edema and peribronchial lymphatic dilation. The bronchi were constricted and surrounded by atelectatic parenchyma. One of the pigs had some areas of scattered mononuclear infiltrates. The kidneys contained areas with dilated cortical tubules. The spleens were normal in appearance.

Group 2 - The pigs dosed IV at 75 μg/kg had lesions similar to, but more severe than in the pigs given 25 μg/kg. Livers were uniformly affected by centrilobular and individual hepatocellular dissociation, degeneration, necrosis, and hemorrhage. The heart and pulmonary lesions were identical to the pigs that were given the toxin at 25 μg/kg. The spleens were not remarkable. The kidneys of two animals given the high dose appeared normal, but the third had scattered proximal tubules that were dilated due to thinning of the epithelium with some segmental sloughing of degenerative cells into the lumen.

Group 3 - The pigs dosed at 75 μg/kg via the ileal loop had no lesions in the kidney or spleen but had cardiac and pulmonary lesions identical to those in pigs of groups 1 and 2. The liver lesions were different from those in the pigs from the first two groups. Pericholangitis with associated coagulative necrosis and some areas of severe centrilobular and midzonal dissociation were noted in the livers of these pigs. There were some multinucleated hepatocytes observed in one liver.

Serum Arginase Activity-The serum arginase activity in the blood of the pigs dosed at 25 μg/kg increased from a predose mean of 10.3 units to a mean of 13.7 units during the four hour monitoring period (Figure 15). Serum arginase in the pigs dosed IV at 75 μg/kg began to increase after two hours (17.1 units) and was the highest at four hours (92.8 units) after dosing. With the ileal loop dose, serum arginase increased steadily throughout the five hour testing period with the mean serum arginase activity of the final samples (236.4 units) exhibiting a ten fold increase as compared to the mean activity of the predose samples (23.1 units).

Characterization of toxin in liver tissue-Extracts from all of the individual livers showed similar distributions of radioactivity on the TLC plates. The initial combined concentrated extract contained 2.0 mCi of radioactivity which concentrated in the lower level of the petroleum ether:ethyl acetate:methanol:water partitioning. The majority of the radioactivity was concentrated in the two peaks at Rf 0.37 and 0.43 which were identical to those of the parent toxin (Figure 16).

The lower phase was concentrated to yield an aqueous solution and separated by reversed phase chromatography (OD, 2.2cm X 22cm) into three fractions (PLC-1, 2.8g, containing 90 μCi; PLC-2, 3.3 g, containing 1.9 mCi; and PLC-3, 4.5 g,

containing 6 μCi.) (Figure 17). The first fraction, PLC-1 showed two peaks at Rf 0.37 and 0.43, which are suspected to be parent toxins. In addition, there were other peaks showing less polarity. The most radioactive fraction, PLC-2, similarly showed two major peaks corresponding to parent toxins. The last fraction, PLC-3, showed mainly four peaks, which might be minor metabolites.

When fraction PLC-2 was purified again using LH-20 and methanol, 1.5g of a subfraction, PLC-2-2, accounting for 1.9 mCi was isolated. When PLC-2-2 was purified further using silica gel chromatography, a fraction weighing 0.2 g was isolated which accounted for 1.8 mCi. After preparative reversed phase HPLC, the peaks corresponding to the radioactivity were collected to yield 6.2 mg of a radioactive oil accounting for 1.6 mCi which had a radiochemical purity of 90%. Although the fraction was sill crude, its low resolution fast atom bombardment (LRFAB) mass spectrum (Figure 18) showed a molecular ion *m/z* 997.2 (HRFABMS, $C_{49}H_{77}N_{10}O_{12}$, Δ=1.8mmu), which strongly indicated that the radioactive compounds were identical to the parent toxins. Attempts to purify and identify the other metabolites from PLC-1 and PLC-3 fractions failed due to the small amounts recovered.

DISCUSSION

In order to determine the distribution and excretion

characteristics of a microcystin, MCYM, it was radiolabeled with ^{125}I using the lactoperoxidase method which yielded a peptide with 4-4.6 X 10^6 c.p.m. activity (Runnegar *et al.*, 1986). The radiolabeled MCYM was given to anesthetized female rats via the femoral vein. Elimination characteristics were based on blood samples collected at 8 time points from the tail vein and the distribution was determined by serial killing and analysis of tissues (Falconer *et al.*, 1986). The radiolabel was eliminated in a biphasic pattern with the first phase, lasting from 0 to 10 minutes, having a half-life of 2.1 minutes; and the second phase, lasting from 10 to 30 minutes, a half-life of 42 minutes. At 30 minutes after dosing, the radioactivity was located primarily in the liver (21.7 % of total equivalent dose [%TD]), gut (7 %TD), kidney (5.6 %TD), and urine (0.9 %TD). At 120 minutes after dosing, the distribution in tissues was similar with radioactivity in the liver (19.2 %TD), gut (9.4 %TD), and urine (1.9 %TD).

Microcystis aeruginosa cells grown in the presence of sodium ^{14}C-bicarbonate produced a ^{14}C radiolabeled microcystin (Brooks WP and Codd GA, 1987). A sub-LD_{50} dose of this radiolabeled toxin (specific activity of 2.6 μCi/mg of toxin) was given IP to mice. At one minute post-dosing, 75.8 %TD was reportedly located in the liver with lesser amounts in the kidneys (1.9 %TD), lungs (5.2 %TD), heart (4.7 %TD), large intestine (4.8 %TD), ileum (3.2 %TD), and spleen (4.3 %TD). Overtime, radiolabel in mice

continued to move to the liver and at the end of the study at 180 minutes, the mean liver content accounted for 88.1 %TD.

Reaction of MCLR with tritiated borohydride has been used to produce [^{3}H]2H-MCLR (Dahlem, 1989; Meriluoto *et al.*, 1990). The product had a specific activity of 5.3 μCi/mg. Male mice dosed with a sublethal dose of [^{3}H]2H-MCLR via the tail vein had the majority of the toxin equivalents distributed to the liver (35 %TD) in 45 minutes with lesser amounts found in the intestines (5 %TD), kidneys (4 %TD), spleen (<2 %TD), muscle (<2 %TD), brain (<2 %TD), and plasma. The administration of [^{3}H]2H-MCLR) resulted in hepatotoxic effects in mice, inducing the same lesions and clinical signs in rodents as did MCLR (Meriluoto *et al.*, 1990). Mice dosed IP with [^{3}H]2H-MCLR at 200 ug/kg consistently died as compared to MCLR which was consistently lethal at 100 ug/kg. The lesions and clinical syndrome induced by [^{3}H]2H-MCLR and MCLR were indistinguishable (Hooser *et al.*, 1991). Isolated perfused rat livers demonstrated microscopic lesions characteristic of microcystin toxicosis within 15 minutes after exposure to [^{3}H]2H-MCLR.

At one hour after IV dosing with tritiated MCLR ([^{3}H]MCLR), mice had 67 %TD in the liver and had much lower concentrations in the kidney (0.8 %TD), intestine (8.6 %TD), carcass (6.0 %TD), plasma (<0.1 %TD) (Robinson *et al.*, 1991). The same study found that 6% of the radiolabel was in urine and 5 %TD was in feces by

6 hours after dosing.

The distribution of [^{3}H]2H-MCLR in swine dosed IV is similar to that found in small rodents dosed by the same route. There appears to be relatively uniform distribution of the toxin throughout the liver. The lower concentration of the sample taken from the quadrate lobe was probably because of a higher ratio of connective tissue to parenchymal tissue. In anesthetized swine, we found that percentage of toxin that was concentrated in the liver at five hours after dosing IV (low dose 64 %TD, high dose 47 %TD) fell between the values of the two mouse IV studies (35 %TD at 45 minutes after dosing [Meriluoto *et al.*, 1990] and 67 %TD at 60 minutes after dosing [Robinson *et al.*, 1991]). The high concentration of [^{3}H]2H-MCLR in the liver of swine as compared to other tissues, supports clinical observations and experimental data (Lovell, 1989) that suggest that swine also actively transport MCLR into hepatocytes. The high first pass effect reported with [^{3}H]2H-MCLR in swine also suggests that the toxin is actively transported into hepatocytes (Stotts *et al.*, 1994). Although there was a higher concentration of toxin in the livers of the pigs given the high dose IV than in the low IV dose pigs, there was a lower %TD in the former. The lower percentage of the toxin in the livers of the high dosed pigs is probably due to the more severe parenchymal damage noted at the high dose which may impair the ability of the liver to actively transport [^{3}H]2H-MCLR. The serum arginase values

support the histopathological observations. The serum arginase of the low dose pigs increased only slightly during the exposure period, while at the high dose arginase increased nine fold indicating severe hepatocyte damage. However, pigs dosed via the ileal loop had the largest increase in serum arginase, reaching ten times the predose values which may have been because of the direct route of the toxin from the portal venous system to the liver as compared to the wider distribution of the toxin in animals dosed IV.

None of the pigs dosed via the ileal loop or IV with [^{3}H]2H-MCLR had elevated radioactivity in the urine collected at the end of the experiment. By contrast, miced dosed IP with [^{3}H]2H-MCLR excreted 3.25% of the administered radioactivity in the urine by 12 hours after dosing (Dahlem, 1989). Moreover, at 6 hours after dosing mice IP with [^{3}H]MCLR, the urine contained 6% of the radiolabel given.

In mice given [^{3}H]2H-MCLR, 63% of the radiolabel was bound to a component which had the same Rf by HPLC evaluation as the parent toxin, (Robinson *et al.*, 1991). Since there were significant amounts of labeled toxin found in the bile of the pigs and in the intestine of mice dosed IV (Robinson *et al.*, 1989) the route of excretion of MCLR in these species appears to be predominately via the bile.

Two radiolabeled compounds in addition to the parent toxin ([^{3}H]MCLR) were discovered in the cytosol of isolated rat livers exposed via the perfusate (Pace *et al.*, 1990). Two radiolabeled components as well as [^{3}H]MCLR were also found in the hepatic cytosol and feces of mice given a mildly toxic dose of the toxin IV (Robinson *et al.*, 1991). The evaluation of swine tissue by FABMS indicates that, in contrast to mice given [^{3}H]MCLR (Robinson *et al.*, 1991), nearly all of the [^{3}H]2H-MCLR in swine remains as parent compound in the parenchyma of the liver for the first several hours post-dosing. It is not clear whether the small amount of radioactive materials detected from PLC-1 and PLC-3 are truly metabolites that might correspond to those found in rodents or impurities from the parent toxin, since the radiolabeled toxin administered contained up to 5% unidentified impurities.

Further studies in unanesthetized swine should be conducted involving sampling of bile over a longer period of time to determine the quantity and composition of [^{3}H]2H-MCLR metabolites ultimately excreted by this route. It is clear from these studies, that during the time of significant lesion development in the livers of swine, nearly all of the toxin was in the form of the parent [^{3}H]2H-MCLR. This suggests that the parent toxin rather than a toxic metabolite is primarily responsible for the toxic syndrome seen in microcystin exposed swine.

FIGURE LEGENDS

Figure 10-Location of liver samples taken by punch biopsy in a pig given [^{3}H]2H-MCLR IV.

Figure 11-Concentrations of toxin equivalents from [^{3}H]2H-MCLR in tissue specimens taken from locations depicted in Figure 10 of a pig which was dosed IV with [^{3}H]2H-MCLR at 25 μg/kg.

Figure 12-The percent of total dose (as toxin equivalents) recovered in spleens, kidneys, ileums, hearts, and livers of pigs given [^{3}H]2H-MCLR IV or via the ileal loop. Error bars represent standard errors.

Figure 13-Quantity of bile collected during from gall bladders of pigs given [^{3}H]2H-MCLR IV or via the ileal loop. Error bars represent standard errors.

Figure 14-Percent of the total dose of [^{3}H]2H-MCLR given pigs IV or via an ileal loop that was collected in the bile.

Figure 15-Arginase activities in serum of pigs given [^{3}H]2H-MCLR either IV or via the ileal loop. Error bars represent standard errors.

Figure 16-Thin layer chromatography plate scan of radioactive

material extracted from the the upper layer of the extraction solvent of the livers from 8 pigs given [^{3}H]2H-MCLR either IV or via the ileal loop.

Figure 17-Thin layer chromatography plate scans of radioactive material extracted from the lower layer of the extraction solvent of the livers from 8 pigs given [^{3}H]2H-MCLR either IV or via the ileal loop.

a) First fraction (PLC-1) separated by reversed phase chromatography.

b) Second fraction (PLC-2) separated by reversed phase chromatography.

c) Third fraction (PLC-3) separated by reversed phase chromatography.

Figure 9-Low resolution fast atom bombardment mass spectrum of radioactive material extracted from the livers of 8 pigs given [^{3}H]2H-MCLR either IV or via the ileal loop.

TABLE LEGENDS

Table 1-Distribution of [^{3}H]2H-MCLR as depicted in Figure 10 of the liver of a pig given [^{3}H]2H-MCLR IV.

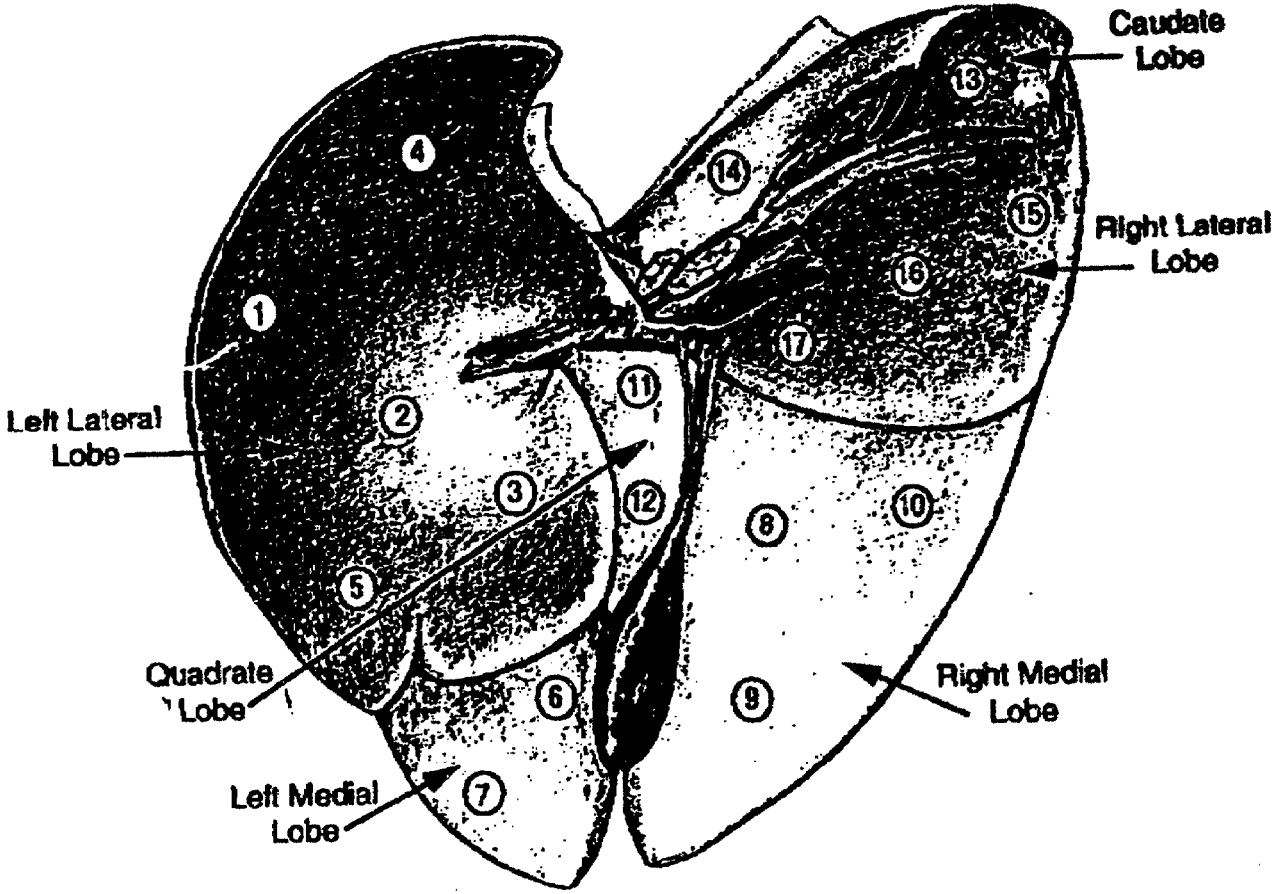

Caudate Lobe
Right Lateral Lobe
Left Lateral Lobe
Quadrate Lobe
Left Medial Lobe
Right Medial Lobe
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17

Figure 10

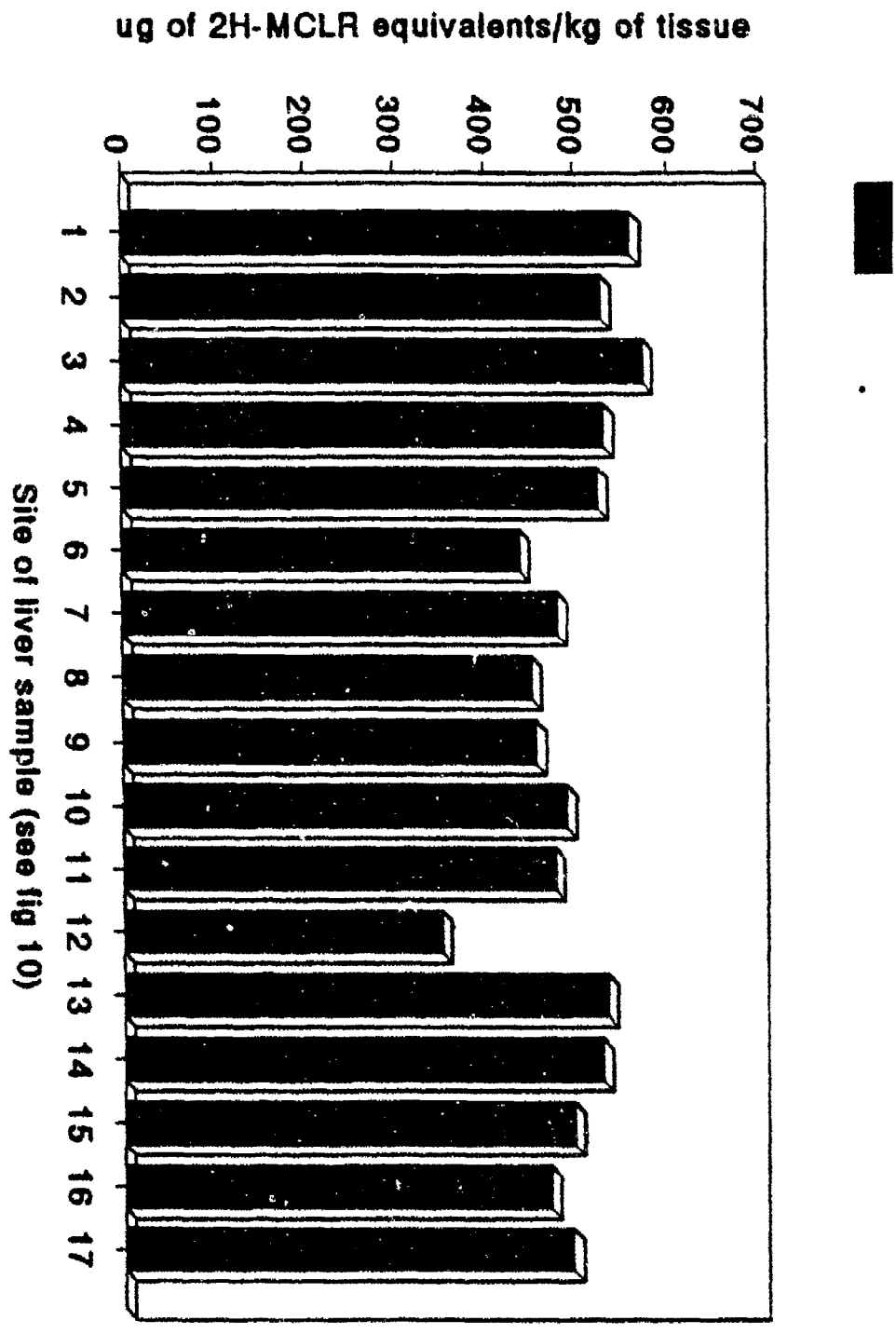

ug of 2H-MCLR equivalents/kg of tissue
0
100
200
300
400
500
600
700
Site of liver sample (see fig 10)
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17

Figure 11

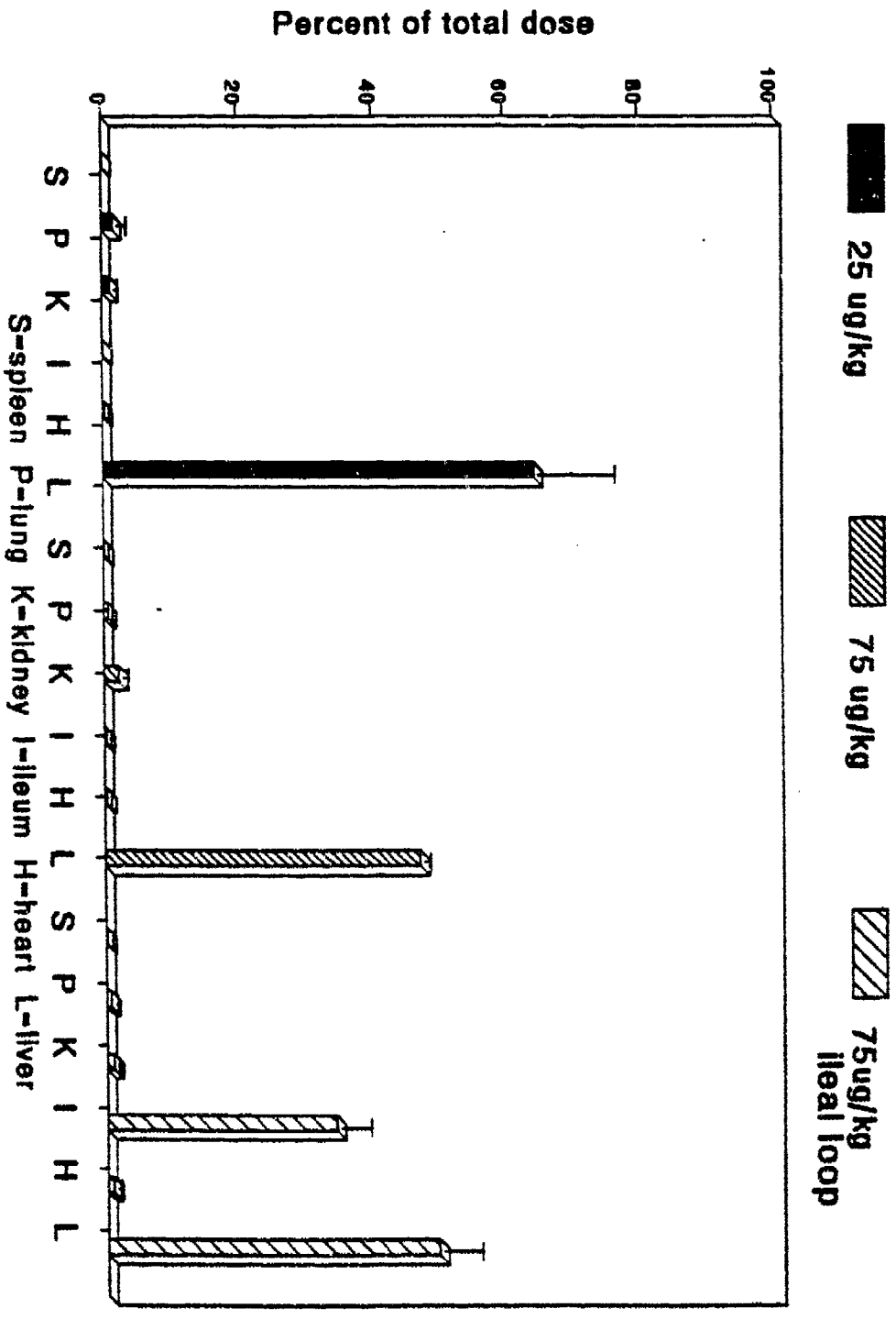

Percent of total dose
0
20
40
60
80
100
S P K I H L S P K I H L S P K I H L
S-spleen P-lung K-kidney I-ileum H-heart L-liver
25 ug/kg
75 ug/kg
75ug/kg ileal loop

Figure 12

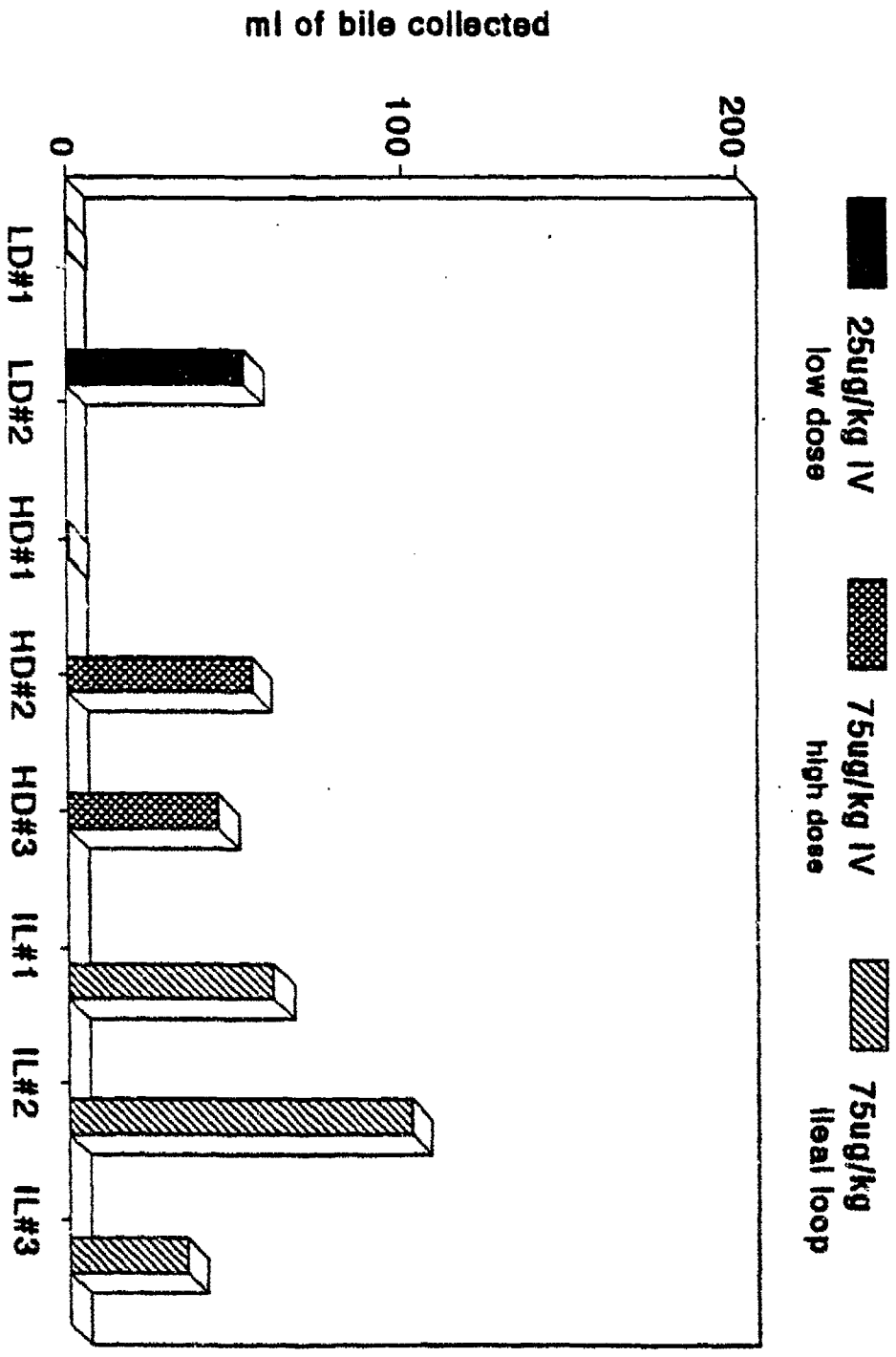

ml of bile collected
0
100
200
LD#1
LD#2
HD#1
HD#2
HD#3
IL#1
IL#2
IL#3
25ug/kg IV
low dose
75ug/kg IV
high dose
75ug/kg
ileal loop

Figure 13

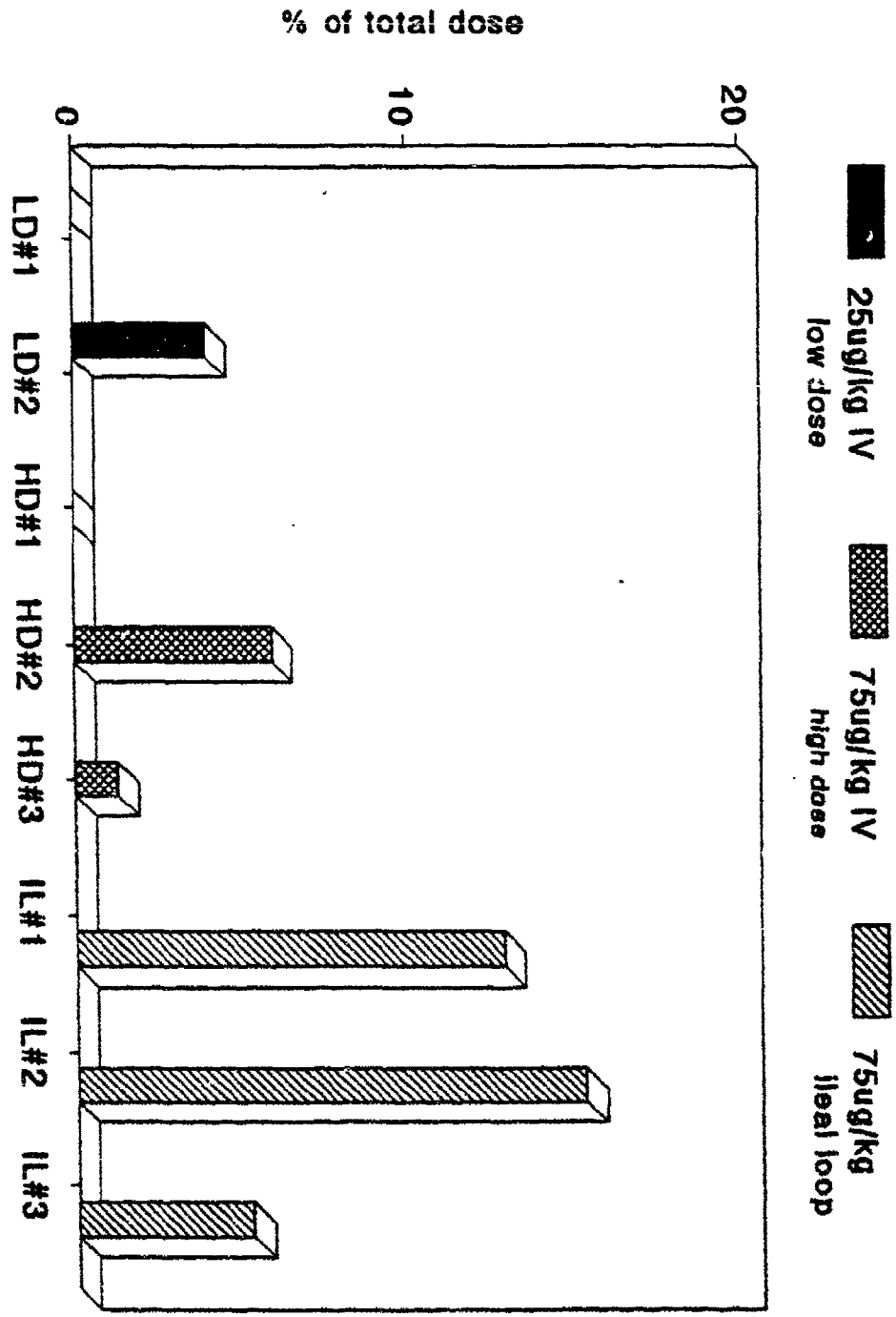

% of total dose
0
10
20
LD#1
LD#2
HD#1
HD#2
HD#3
IL#1
IL#2
IL#3
25ug/kg IV
low dose
75ug/kg IV
high dose
75ug/kg
ileal loop

Figure 14

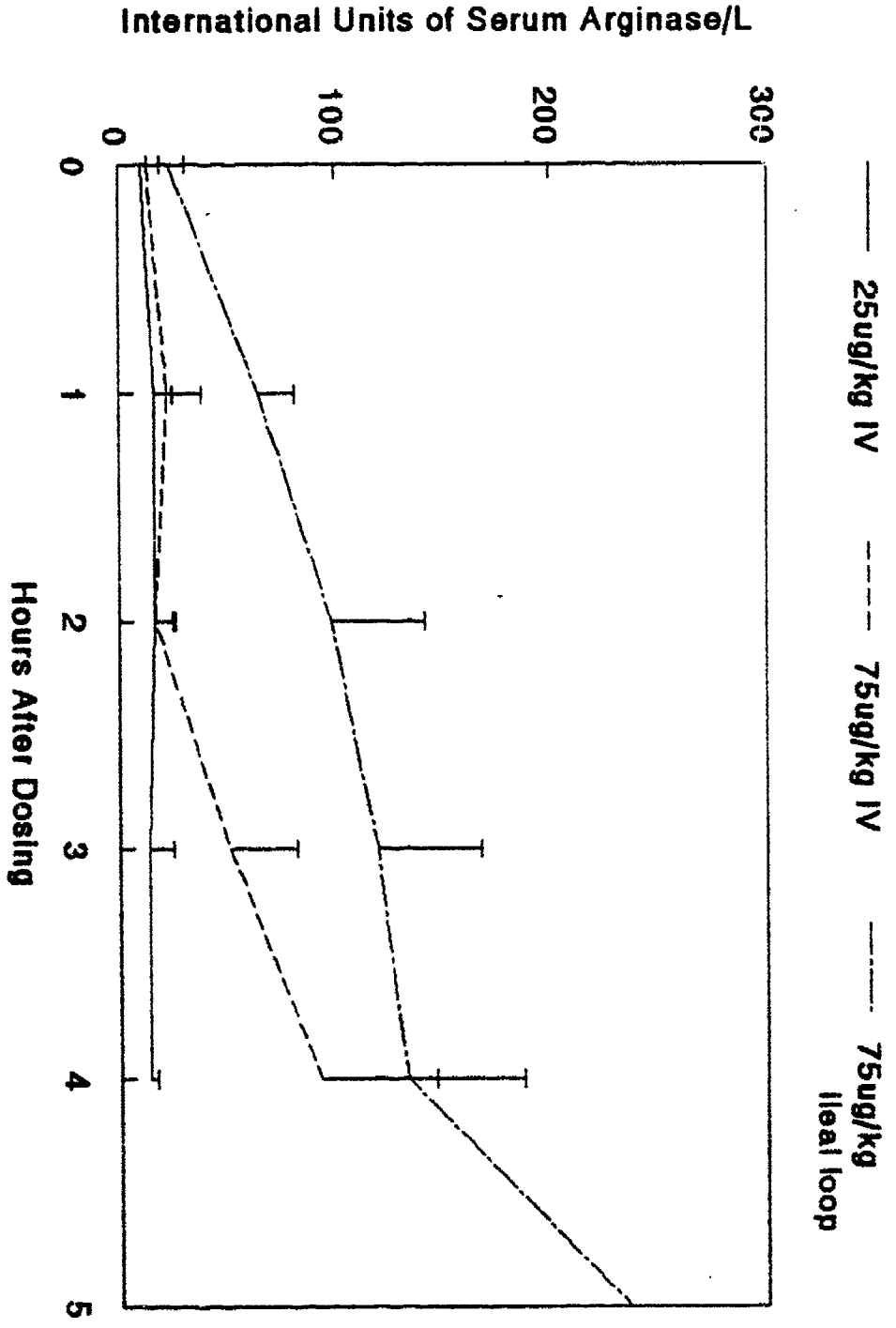

International Units of Serum Arginase/L
0
100
200
300
0
1
2
3
4
5
Hours After Dosing
25ug/kg IV
75ug/kg IV
75ug/kg
Ileal loop

Figure 15

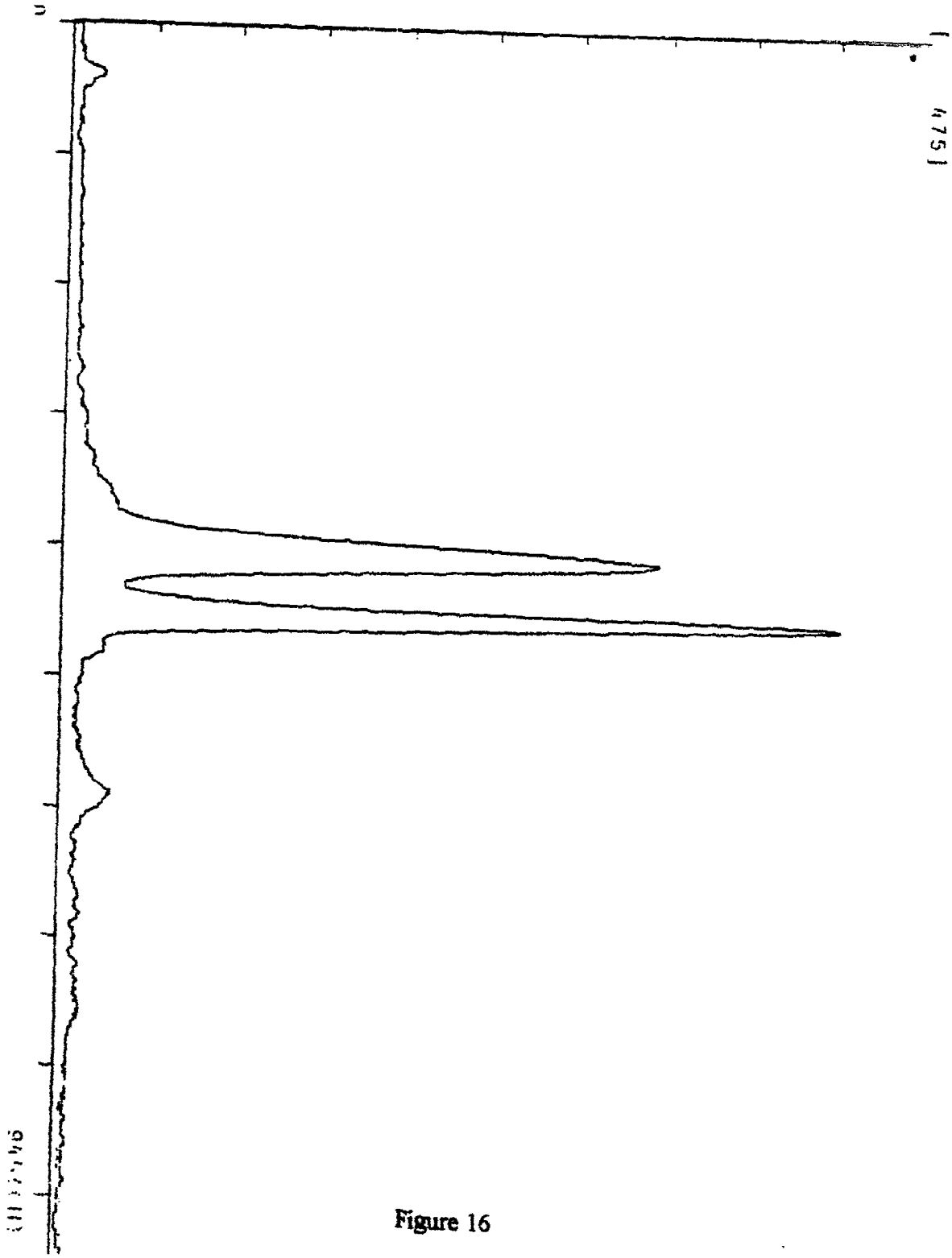
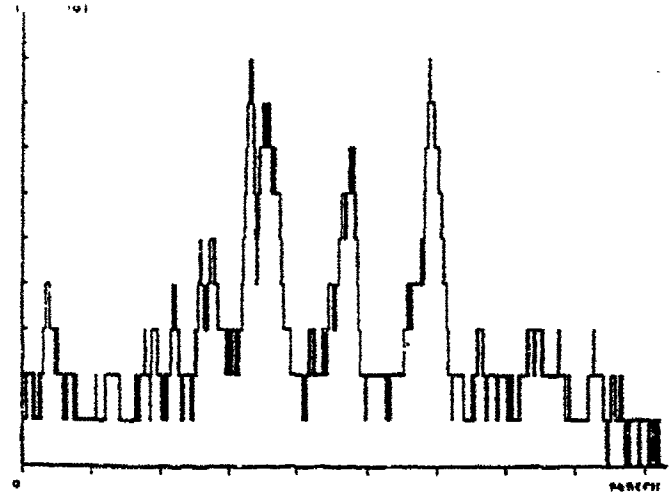
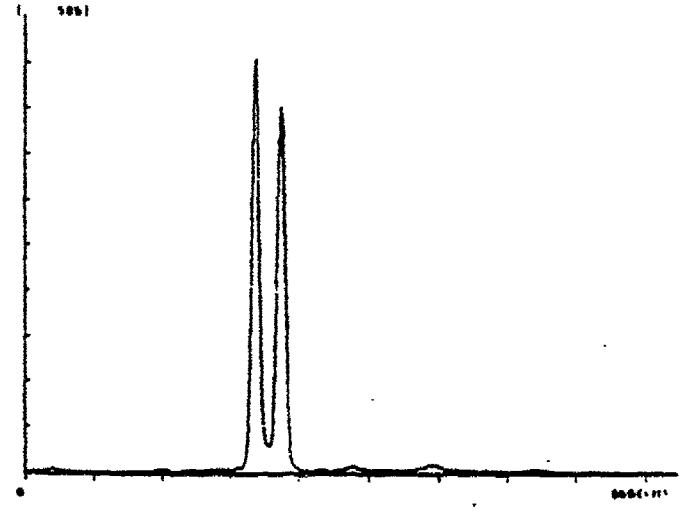


Figure 16

PLC-1
2.8 g
90 μCi



PLC-2
3.3 g
1.9 mCi



PLC-3
4.5 g
6 μCi

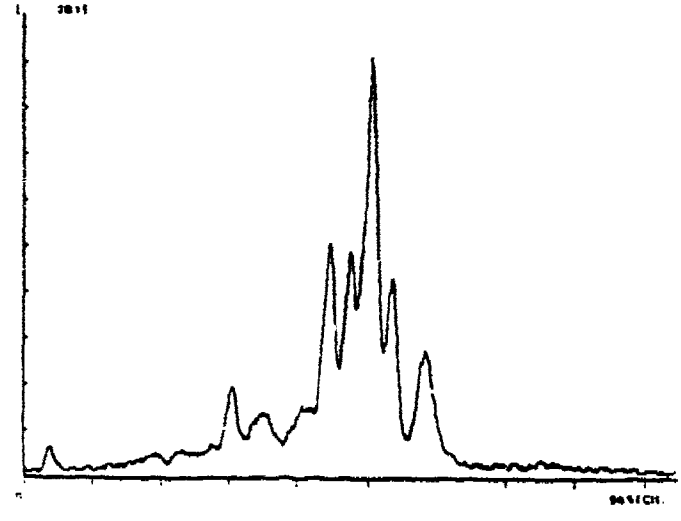


Figure 17

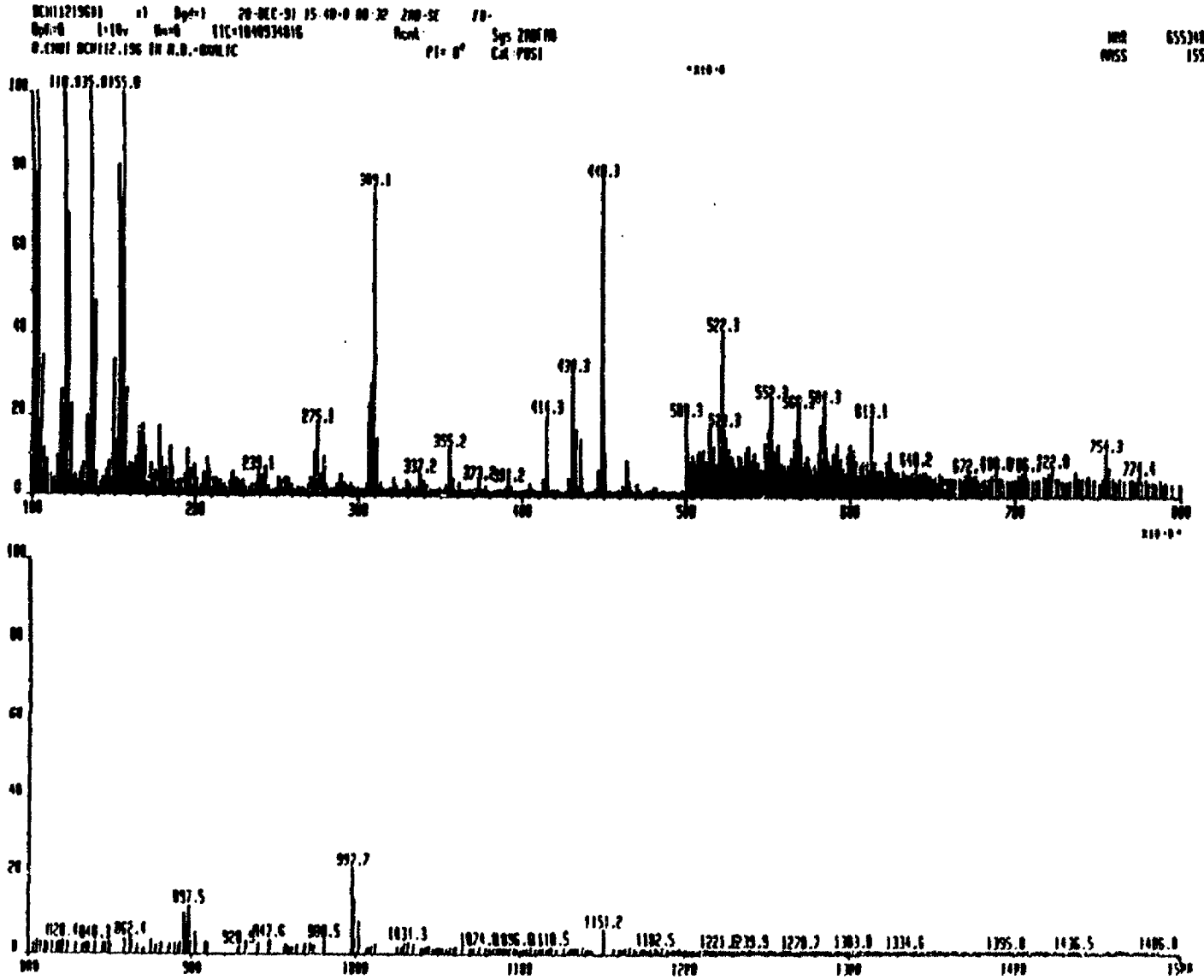


Figure 18

TABLE 2

Distribution of [3H]2H-MCLR equivalents in the liver of a pig dosed IV at 25 μg/kg

Site #	μg[3H]2H-MCLR/kg
1	560
2	528
3	572
4	530
5	524
6	439
7	481
8	452
9	457
10	491
11	478
12	351
13	534
14	528
15	498
16	472
17	495
Mean	493.5294
Std. Dev.	52.49181
95% C.I.	26.99

TABLE 2

A PILOT STUDY OF THE EFFECTIVENESS OF CHOLESTYRAMINE IN PROTECTING SWINE DOSED INTRAGASTRICALLY WITH LYOPHILIZED MICROCYSTIS CELLS

INTRODUCTION

Superactivated charcoal and cholestyramine resin (CTR) have been tested *in vitro and in vivo* as treatments for MCLR toxicosis. Previou_ work in our laboratory showed that CTR was effective in preventing liver weight increases when dosed at a ratio of 1:100 (MCLR:CTR) via an *in situ* rat ileal loop preparation. Activated charcoal was effective *in vitro* in reducing the availability of the free toxin, however, when given to rats *in vivo,* it failed to reduce the increase in liver weight associated with MCLR toxicosis (Dahlem,1989). The effect of CTR upon blue-green algae exposures in domesticated food animals has not been reported.

OBJECTIVE

The objective of the study was to determine if CTR was useful in treating swine that had ingested hepatotoxic blue-green algae cells.

METHODS AND MATERIALS

Blue-green algae cells-The blue-green algae cells were collected from Homer Lake, Illinois in the summer of 1988.

Approximately 400 liters of cells and water were collected. The concentrated cells and lake water were frozen at -40°C. During the winter and summer of 1989, the cells were dried in several batches in a lyophilizer. The dried cells were stored at -40°C in sealed polyethylene containers until removed for extraction of toxin. There was a large variation of the amount of toxin extracted from different aliquots of the dried cells. There seemed to be a trend toward decreasing yields of toxin over the two years that toxin was extracted and purified. The decrease in yields may have been due to variations of the amount of toxin present in the cells when collected, breakdown of the toxin during storage, or binding of the toxin to cellular constituents which would decrease extraction efficiency. The cells used in the intragastric study came from a batch of cells which yielded from 1.15 to 4.2 mg of pure toxin from 15 g of dried cells. This is equivalent to from 0.076 to 0.24 mg of pure MCLR/g of dried cells.

Animal Procedures-Four female mixed breed SPF pigs from the University of Illinois Veterinary Research Farm, weighing from 19.1 to 20.9 kg were received and acclimated for 24 hours. They were anesthetized by inhalation of halothane. Catheters were placed aseptically in one jugular vein and one carotid artery in each of the pigs and tunneled under the skin so as to expose the ends on the dorsal cervical skin surface. The skin was closed with sutures and the ends of the catheters were covered with a

resealable plastic bag which was taped and glued to the skin.

Two days later each of the pigs was dosed intragastrically with the lyophilized blue green algae at 6 g/kg. The dose was suspended in 2 liters of filtered deionized water prior to administration. Due to the large volume, the algae was given in three divided doses over a thirty minute period. Two of the pigs(#3 and #4) were given 4 g of cholestyramine immediately after each dose of algae such that a total of 12 g of cholestyramine were administered per pig. Blood samples were drawn hourly from each pig for 48 hours using the jugular catheter. Serum samples, collected predosing and every six hours postdosing, were analyzed for hepatic enzymes. After the last blood sample was collected from each pig, they were killed with 8 ml of T61 euthanasia solution (Hoechst-Roussel, Agrivet Co., Summerville, NJ).

Analysis of MCLR in blood-The blood samples were immediatedly packed in ice bags and forwarded to Dr. F.S. Chu (Food Research Institute of the Department of Food Microbiology and Toxicology, University of Wisconsin, Madison). Dr Chu's laboratory determined the concentration of MCLR in the swine blood using ELISA techniques (Chu *et al.*, 1989).

Serum Enzyme Analysis-Samples of blood for serum enzyme activities were collected via the jugular catheter every six

hours. The samples were allowed to clot and the serum remove for analysis by the University of Illinois College of Veterinary Medicine Clinical Pathology Laboratory.

Histopathology-At the end of the experiment, sections of the liver, heart, lungs, kidneys, and spleen from pigs #2, #3, and #4 were fixed in 10% neutral buffered formalin, routinely processed, embedded in paraffin, sectioned at 4 μm, stained with hematoxylin and eosin, and examined by light microscopy.

RESULTS

Pig #1-When a neoprene stomach tube was passed, and pig #1 was given approximately one liter of the blue-green algae solution was given via the tube, the pig became extremely distressed. Dosing of the other three pigs was therefore delayed in order to observe the reaction of the first pig. The pig became recumbent and died at 35 minutes after dosing. Necropsy revealed that the stomach tube had perforated the esophagus and the blue-green algae suspension had been deposited in the thoracic cavity. The neoprene tube was then replaced with a softer rubber (#6) dog feeding tube before dosing the other three pigs. It was also decided at this time to divide the doses into three parts, because the volume seemed excessive for one dose.

Pig #2-The second pig moved restlessly around it's pen after dosing. One hour and 45 minutes after dosing, the pig urinated.

Nine hours and 18 minutes after dosing, the pig passed feces containing the color of the algae. Sixteen hours after dosing the pig ate ground mixed feed and drank water.

Pig #3-This pig appeared restless after dosing but did not demonstrate any clinical signs of shock. At one hour and 10 minutes postdosing the pig vomited about 400 ml of vomitus containing large amounts of the cholestyramine and algae suspension. At three hours and 45 minutes post-dosing, the pig drank water. Sixteen hours after dosing, the pig ate ground mixed feed and drank water.

Pig #4-This pig managed to pull out the jugular catheter, and thus all blood samples were withdrawn via the carotid artery. As the first sample of blood was withdrawn the pig went into clonic-tonic seizures. The subsequent blood samples were drawn more slowly, but the pig exhibited seizures each time the blood was drawn. The catheter failed to function after seven hours. At seven hours and 15 minutes after dosing, the pig vomited. Nine hours after dosing the pig defecated and algae was evident in the feces. The pig seemed more depressed and weaker than either pig #2 or pig #3.

Analysis of MCLR in blood-The concentrations of MCLR in the blood of pig #2 were very low. There was no clear cut elimination curve during the course of the study. Concentrations of MCLR in

pig #3 were even lower with most values below the limit of detection. Pig #4 had the highest concentrations, but the loss of the catheter prevented sampling after 7 hours (Figure 19).

Serum Enzyme Analysis-Three of the enzymes; alkaline phosphatase, creatine phosphokinase, and serum dehydrogenase; were elevated during the course the study. All three of the enzymes were more elevated in the pig that was not given cholestyramine (Figures 20 to 22).

Histopathology-Samples of the liver, heart, lung, kidney, and spleen of pigs #2, #3, and #4 were submitted for histological evaluation. There were no differences noted between the pigs. All livers demonstrated mild to moderate pericholangitis. Hepatocytes were swollen diffusely with a granular cytoplasmic appearance and, in some subcapsular areas, there was dissociation of hepatocytes. Heart tissues demonstrated multifocal areas of myofiber changes that include cytoplasmic hypereosinophilia, nuclear hyperchromasia, and perinuclear vacuolization. The spleens of all the pigs showed marked congestion. A moderate number of cortical proximal tubules were moderately dilated in the kidneys of all three pigs.

DISCUSSION

The concentrations of MCLR in the blood of the pigs were near the detection limit of Dr. Chu's assay. The proximity to this limit probably contributed to the non-linear character of the blood concentration curve. Dr. Chu stated that, if future studies were performed, serum samples might be better, because of the difficulty of extracting the MCLR from whole blood and the fact that his assay can be used to measure serum MCLR directly. Despite the limitations of this study, the results suggest that cholestyramine resin reduced the amount of MCLR entering the blood from the digestive tract.

The pigs were given very large doses of blue-green algae cells. Even if the amount of MCLR in the cells was 0.076mg of MCLR per g of algae cells, the lowest concentration found in that batch of cells, the dose would have at least 2.736 mg of MCLR/kg of pig body weight. This is much higher than the consistently lethal IV dose in swine established by Lovell of 75 μg/kg (Lovell, 1989). This suggests that the bioavailability of the toxin dosed in swine dosed intragastrically is low.

The histologic evaluation of the livers showed lesions typical of sublethal MCLR toxicosis in all three pigs. The cholestyramine-dosed pig did not show any reduction in the

quantity or severity of the lesions. However, the lesions were mild in all pigs. The engorgement of the spleens was typical of animals euthanized with T61.

All three of the serum enzymes measured were higher in the pig that did not receive cholestryramine suggesting that there was more damage to the liver of the pig not protected by the resin. These data suggest that the cholestyramine may have provided some protection, but the difference may also be attributed to vomiting by both of the cholestyramine-treated pigs. Because of the small number of pigs dosed and successfully monitored, coupled with the highly variable blood concentrations, it can not be proven that cholestryamine had a protective effect. The data seem to suggest that cholestryamine might be a useful tool in treating MCLR toxicosis. This study should be repeated with a substantially larger group of pigs to determine the effectiveness of cholestyramine as a treatment for MCLR toxicosis.

FIGURE LEGENDS

Figure 19-MCLR concentrations in the blood of pigs dosed intragastrically with blue-green algae.

Figure 20-Serum alkaline phosphatase (ALP) concentrations in pigs dosed intragastrically with blue-green algae (N=1).

Figure 21-Creatine phosphokinase (CPK) concentrations in pigs dosed intragastrically with blue-green algae (N=1).

Figure 22-Sorbitol dehydrogenase (SDH) concentrations in pigs dosed intragastrically with blue-green algae (N=1).

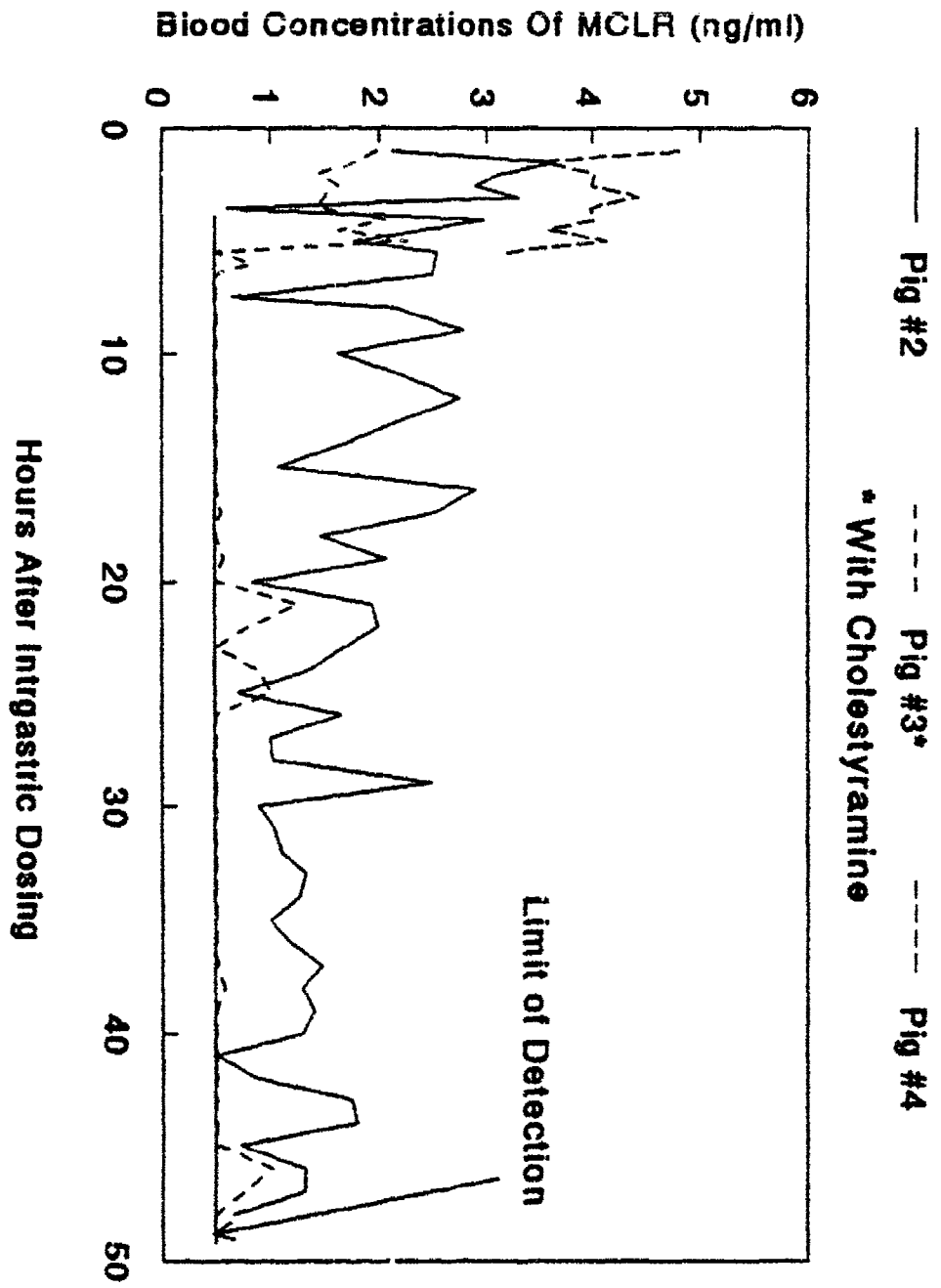

Blood Concentrations Of MCLR (ng/ml)
0
1
2
3
4
5
6
0
10
20
30
40
50
Hours After Intragastric Dosing
Limit of Detection
Pig #2
Pig #3*
Pig #4
* With Cholestyramine

Figure 19

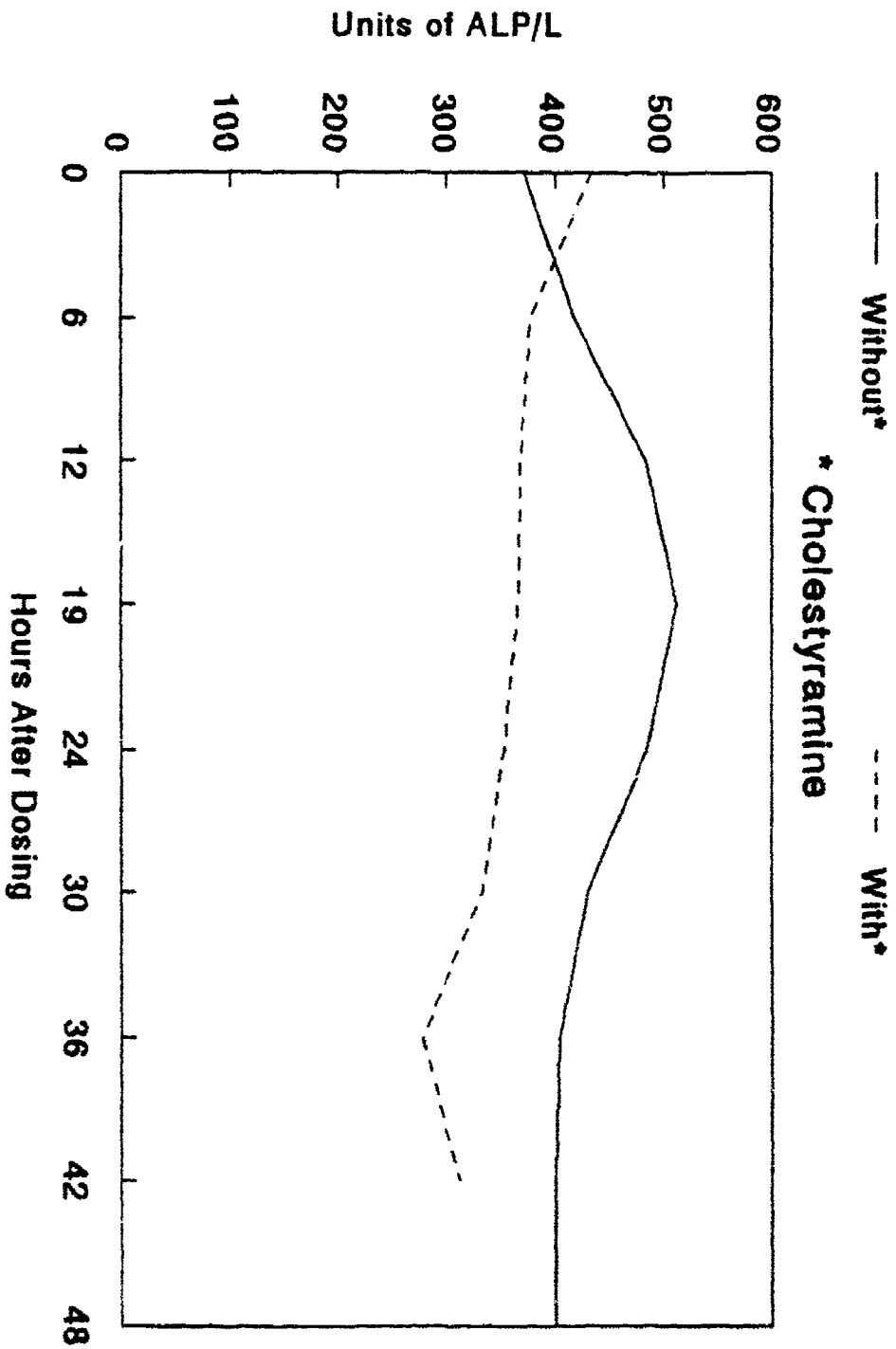

Units of ALP/L
0
100
200
300
400
500
600
0
6
12
19
24
30
36
42
48
Hours After Dosing
Without*
With*
* Cholestyramine

Figure 20

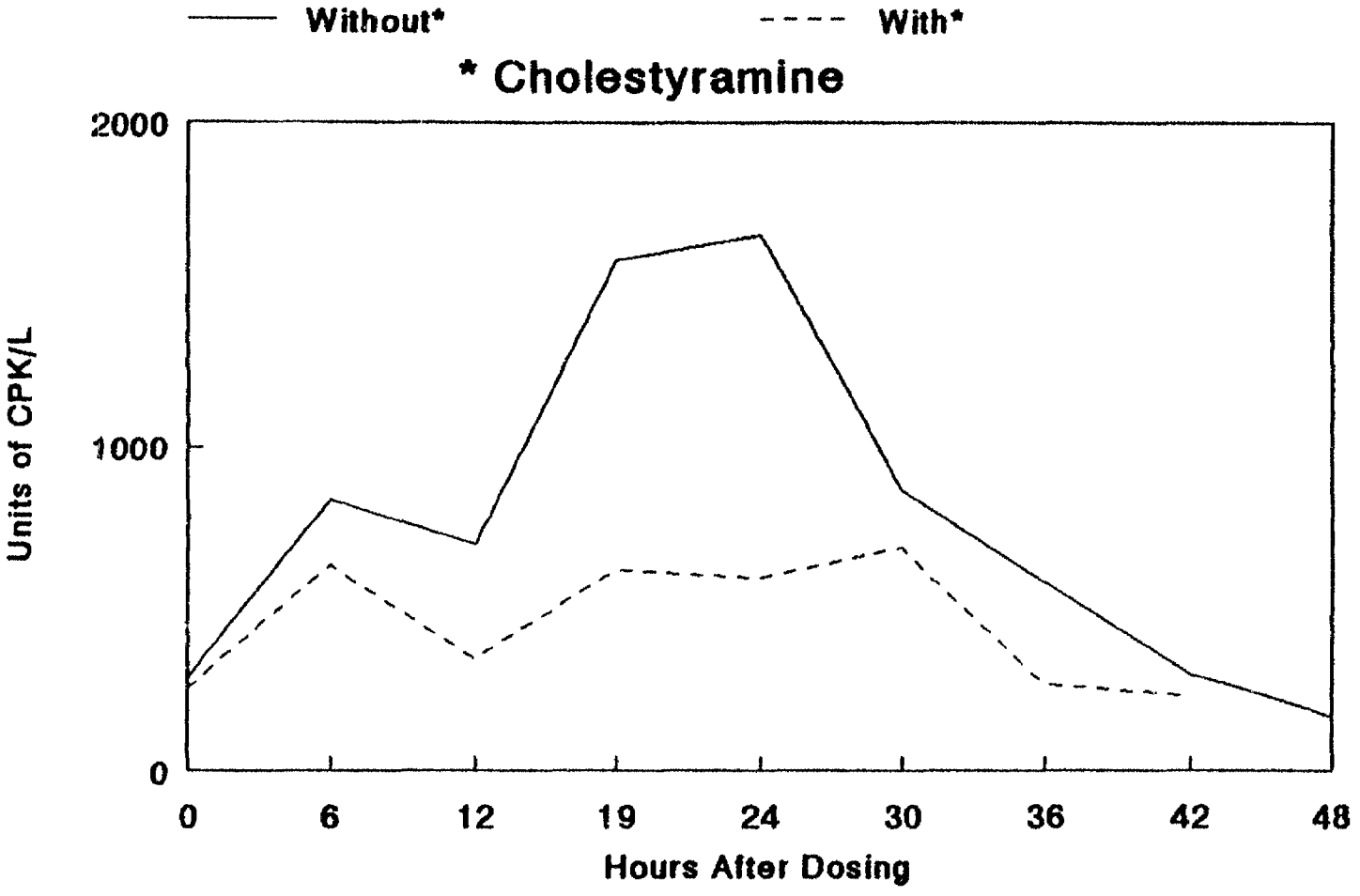

Without*
With*
* Cholestyramine
2000
1000
0
Units of CPK/L
0
6
12
19
24
30
36
42
48
Hours After Dosing

Figure 21

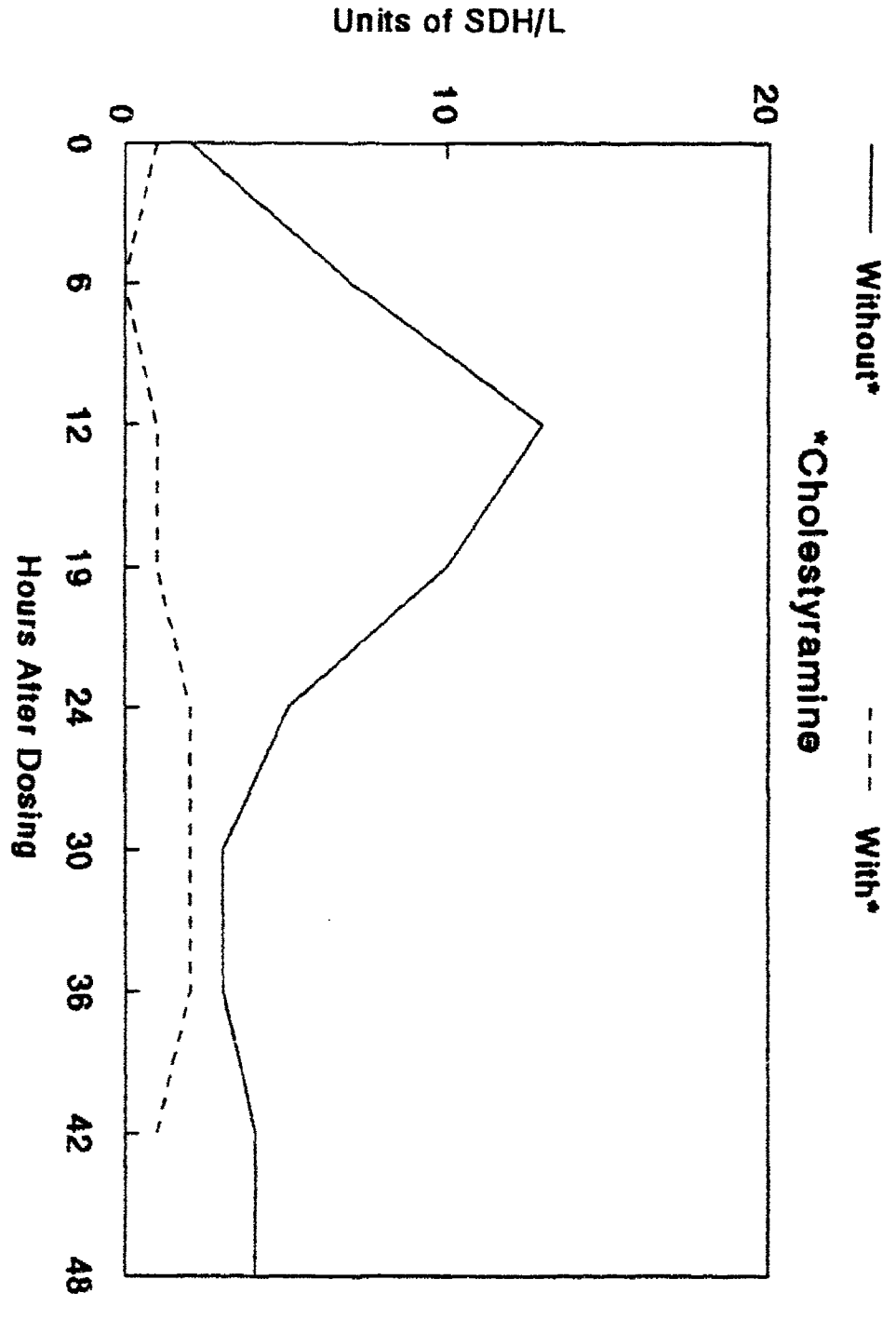

Units of SDH/L
0
10
20
0
6
12
19
24
30
36
42
48
Hours After Dosing
*Cholestyramine
Without*
With*

Figure 22

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Experimental (Refereed)

Stotts, R.R., Namikoshi, M., Haschek, W.M., Rinehart, K.L., Carmichael, W.W., Dahlem, A.M., and Beasley, V.R. Structural modifications imparting reduced toxicity in microcystins from *Microcystis* spp. Toxicon 31:783-789, 1993.

Submitted for Publication (to date)

Stotts, R.R., Twardock, R.A., Koritz, G.D., Haschek, W.M., Manuel, R.K., Hollis, W.H., and Beasley, V.R. Toxicokinetics of tritiated dihydromicrocystin-LR in swine.

Theses

Stotts, R.R. Toxicokinetics of Dihydromicrocystin-LR in Swine. PhD Thesis. College of Veterinary Medicine, University of Illinois at Urbana-Champaign. Currently in draft form.

PERSONNEL RECEIVING CONTRACT SUPPORT

Researchers

William Hollis

PhD Degrees

Richard R. Stotts (Anticipated Fall, 1994)

Abstract # 18, Toxicologist, 13:31 (1993).

STRUCTURAL MODIFICATIONS IMPARTING REDUCED TOXICITY IN MICROCYSTINS FROM *MICROCYSTIS* SPP. V R Beasley, R R Stotts, M Namikoshi, W M Haschek, K L Rinehart, W W Carmichael, A M Dahlem. Departments of Veterinary Biosciences, Veterinary Pathobiology, and Chemistry, University of Illinois, Urbana, IL and Biological Sciences, Wright State University, Dayton, OH.

An algal bloom containing *Microcystis aeruginosa* (dominant), *M. viridis*, and *M. wesenbergii*, was collected from Homer Lake, IL and 12 microcystins (MCs) were isolated (Namikoshi *et al.*, 1992). The major toxin was microcystin-LR (MCLR); two minor components were MCRR and MCYR; and nine MCs were new to us including 1) [DMAdda^5] MCLR, where DMAdda = *O*-demethyl-Adda = (2*S*,3*S*,8*S*,9*S*)-3-amino-9-hydroxy-2,6,8-trimethyl-10-phenyldeca-4,6-dienoic acid, 2) [Dha^7]MCLR, where Dha = dehydroalanine, 3) MCFR, 4) MCAR 5) MC-M(O)R, where M(O) = methionine *S*-oxide, 6) [Mser^7]MCLR, where Mser = *N*-methylserine, 7) MC analogue but structure not fully determined, 8) [D-Glu(OC_3H_7O)^6]MCLR, where D-Glu(OC_3H_7O) = α-monoester of D-glutamic acid, and 9) MCWR; although 2) was recently isolated, characterized, and toxicity tested by Harada *et al.* (1991). Mice were dosed ip to determine approximate LD_{50}s (≈LD_{50}s) and thus identify structural characteristics important in toxicity. When L-leu was replaced by other hydrophobic amino acids, Phe, Ala, or Trp, the ≈LD_{50}s ranged from 171 to 249 μg/kg. Replacing L-Leu with more hydrophilic Tyr gave an ≈LD_{50} of 171 μg/kg. When L-Leu was changed to the basic Arg, the ≈LD_{50} declined to 650 μg/kg. A similar effect was observed when L-Leu was changed to a sulfoxide of methionine with an ≈LD_{50} of 750 μg/kg. Although Adda is critical to the toxicity of microcystins, toxicity was not markedly reduced by demethylation at its C-9 unit. Formation of an α-monoester of D-glutamic acid reduced toxicity such that the compound ([$C_3H_7O_2$]MCLR) was nonlethal at 1 mg/kg.

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STRUCTURAL MODIFICATIONS IMPARTING REDUCED TOXICITY IN MICROCYSTINS FROM *MICROCYSTIS* SPP.

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Departments of [1]Veterinary Biosciences, [2]Veterinary Pathobiology, and [3]Chemistry, University of Illinois, Urbana, IL 61801, U.S.A.; and [4]Department of Biological Sciences Sciences, Wright State University, Dayton, OH 45435, U.S.A.

(*Received* 19 *October* 1992; *accepted* 1 *December* 1992)

R. R. STOTTS, M. NAMIKOSHI, W. M. HASCHEK, K. L. RINEHART, W. W. CARMICHAEL, A. M. DAHLEM and V. R. BEASLEY. Structural modifications imparting reduced toxicity in microcystins from *Microcystis* spp. *Toxicon* **31**, 783–789, 1993.—A cyanobacterial (blue-green algal) bloom containing *Microcystis aeruginosa* (dominant), *M. viridis*, and *M. wesenbergii*, was collected from Homer Lake (Illinois, U.S.A.) in the summer of 1988 and microcystins were isolated. One microcystin of substantially reduced toxicity was isolated, together with ten hepatotoxic microcystins. The compound with reduced toxicity was nonlethal at 1 mg kg (i.p. mouse) and was determined to have a ($C_3H_7O_2$) mono-ester of the α-carboxyl on the Glu unit of microcystin-LR. The other nine microcystins apart from MCLR had approximate LD_{50}s ranging from 97 μg/kg to 750 μg kg.

INTRODUCTION

A GROUP of cyclic heptapeptide hepatotoxins produced by various species of *Microcystis*, *Anabaena*, *Oscillatoria*, and *Nostoc* have been termed microcystins (CARMICHAEL *et al.*, 1988; BEASLEY *et al.*, 1989). Toxins produced by the blue-green alga *M. aeruginosa* often pose hazards to livestock, and sometimes to public health, in many regions of the world (CARMICHAEL *et al.*, 1988, 1990). Toxic blooms of this organism usually occur in eutrophic still waters during warm months of the year (CARMICHAEL and MAHMOOD, 1984). The occurrence of toxic blooms is likely to increase with expansion in the use of fertilizers, irrigation, animal-based agriculture, and construction of water holding facilities such as ponds, lakes, and reservoirs. Recent reports suggest that the potent toxicity of the microcystins is attributable to marked inhibition of protein phosphatases type 1 and type 2A (ERIKSSON *et al.*, 1990) making them important biochemical probes. The potent toxicity of these algal peptides and the likelihood of an increased incidence of toxic blooms has stimulated the study in our laboratories of structure toxicity relationships.

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Recently, HARADA *et al.* (1990*a*,*b*) described geometrical isomers of microcystins-LR (MCLR) and -RR, which were nonlethal when administered i.p. to mice at 1.2 mg/kg. We recently described the isolation and structural characterization of 12 microcystins from a water bloom of *Microcystis* sp. taken from Homer Lake, Illinois (NAMIKOSHI *et al.*, 1992). Here we describe the toxicity of these compounds in mice.

MATERIALS AND METHODS

Organisms and initial screening for toxicity

A water bloom of cyanobacteria collected from Homer Lake (Illinois, U.S.A.) in the summer of 1988 consisted primarily of *M. aeruginosa* with small amounts of *M. viridis* and *M. wesenbergii*. I.p. injection of mice with 0.5 ml of lysate from aqueous cell suspensions revealed hepatotoxicity as indicated by acute death (within hours) and marked distension of the liver with hemorrhage.

Separation and purification of microcystins

The algal cells were lyophilized and extracted with methanol. The microcystins were isolated by Sephadex LH-20, Toyopearl HW-40, and reversed-phase (C-18) silica gel column chromatography followed by preparative thin-layer chromatography (NAMIKOSHI *et al.*, 1992). Microcystins were identified using high-resolution fast atom bombardment (FAB) mass, tandem FAB mass spectrometry, ^{1}H NMR spectroscopy, and amino acid analysis using a Waters Pico-Tag HPLC system, in conjunction with chiral capillary gas chromatography. The principal toxin (approximately 90% of the toxic components) was determined to be MCLR. Two minor components were revealed to be microcystin-RR (MCRR) and microcystin-YR (MCYR), but nine microcystins were new to us (NAMIKOSHI *et al.*, 1992), including: (1) [DMAdda^5]microcystin-LR ([DMAdda^5]MCLR), where DMAdda = *O*-demethyl-Adda = (2*S*,3*S*,8*S*,9*S*)-3-amino-9-hydroxy-2,6,8-trimethyl-10-phenyldeca-4,6-dienoic acid; (2) [Dha^7]microcystin-LR ([Dha^7]MCLR), where Dha = dehydroalanine; (3) microcystin-FR (MCFR); (4) microcystin-AR (MCAR); (5) microcystin-M(O)R [MCM(O)R], where M(O) = methionine *S*-oxide; (6) [Mser^7]microcystin-LR ([Mser^7]MCLR), where Mser = *N*-methylserine; (7) microcystin analogue but structure not fully determined; (8) [D-Glu(OC$_3$H$_5$O)^6]microcystin-LR ([C$_3$H$_5$O$_2$]MCLR) where D-Glu(OC$_3$H$_5$O) = α-monoester of D-glutamic acid; and (9) microcystin-WR (MCWR), although [Dha^7]MCLR was recently isolated, characterized, and toxicity tested by HARADA *et al.* (1991).

Determination of approximate LD_{50} *for each microcystin*

Toxicity tests were performed using male Swiss–Webster mice (22–28 g) purchased from Charles River. The mice were housed four to a cage and allowed to acclimatize for 7 days prior to dosing. The mice were dosed with 0.1 ml of 0.09% saline solution i.p. containing a dose at 1 mg/kg body weight of the respective microcystin and were observed for 1 week for signs of illness. If the mice died, the times of death were recorded, necropsies performed, and liver weights determined as a percentage of total body weights. One week after dosing, surviving mice were killed by ether anesthesia and evaluated in the same manner as the mice that had died. The mice given (C$_3$H$_5$O$_2$)MCLR lived for 1 week; all other dosed mice died. The compounds given to the mice that died were then given to additional mice at a dose of 0.1 mg/kg. Only one mouse died at the 0.1 mg/kg dose. Approximate LD_{50} ($\simeq LD_{50}$) doses were then determined using the up-and-down method as described by BRUCE (1985, 1987) using doses of 0.8, 0.7, 0.5, 0.4, 0.3, 0.25, 0.2, 0.15, 0.1, 0.09, 0.08, or 0.05 mg/kg (Fig. 1). The $\simeq LD_{50}$s were computed using a SAS Probit analysis program; however, data were insufficient to compute the $\simeq LD_{50}$s for three of the microcystins, such that it was necessary to estimate these values. Additional dosing was precluded as a result of limited toxin that could be isolated from the lyophilized bloom material.

Comparison of liver weights

The livers of the mice were removed and weighed immediately after death. The fractional liver weights of mice that died following dosing were compared with those of mice that lived for 1 week after dosing using a two sample *T*-test of unequal variance at a value of alpha = 0.01. Control values, provided for an additional comparison, were based on historical control mice dose given the vehicle under the same conditions (Fig. 2).

Histologic evaluation of the tissues

Sections from liver, kidney, spleen, heart, and lung were removed from mice immediately after they died or were killed. They were fixed in 10% buffered formalin, embedded in paraffin, sectioned at 4 μm, stained with

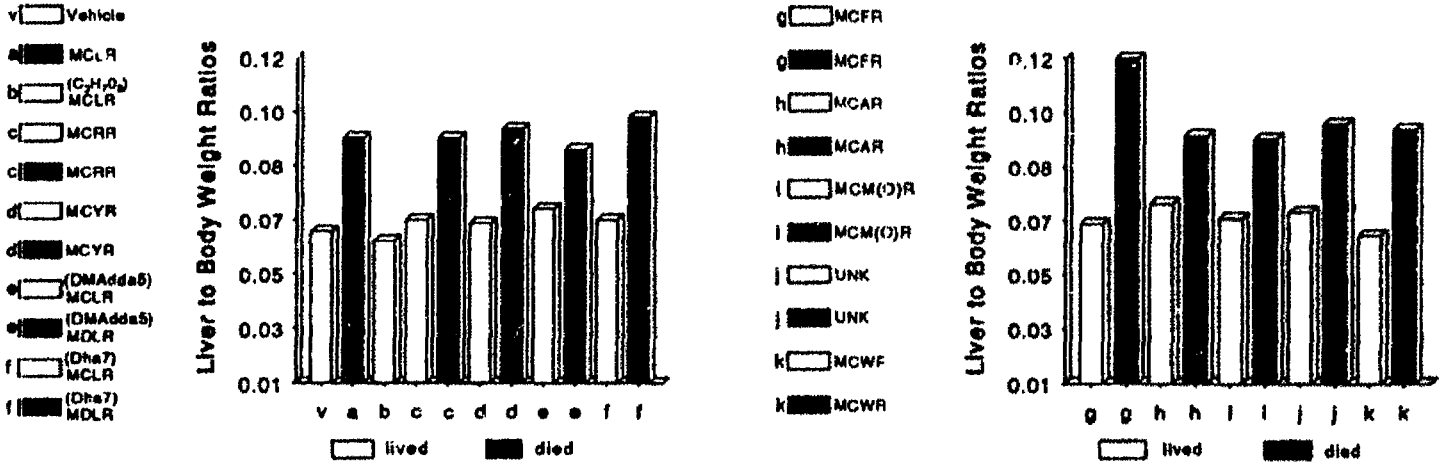

FIG. 1. APPROXIMATE LD_{50}S OF MICROCYSTINS TESTED IN THIS STUDY.
All compounds were highly potent except for $C_4H_4O_2$ MCLR. The LD_{50} value for microcystin-LR was based on historical data using the same strain of mice and method of administration.

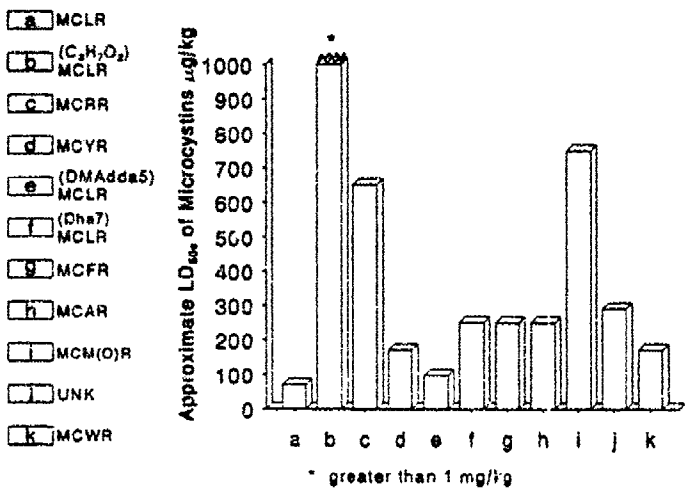

FIG. 2. LIVER TO BODY WEIGHT RATIOS IN MICE THAT LIVED OR DIED FOLLOWING DOSING WITH THE MICROCYSTINS TESTED IN THIS STUDY.

hematoxylin and eosin, and evaluated by light microscopy. When lesions typical of microcystin toxicosis were present, i.e. centrilobular dissociation, necrosis and hemorrhage, a score was assigned based on extent of lobule involvement: e.g. 25% of lobule affected = 2, 50% = 4, and 100% = 8.

RESULTS

Microcystin not lethal at a dose of 1 mg/kg

The mice given $(C_3H_7O_2)$MCLR [$(C_3H_7O_2)$ mono-ester of the alpha-carboxyl on the Glu unit of MCLR (NAMIKOSHI *et al.*, 1992)] at 1 mg/kg lived for 1 week. The compound failed to induce any clinical signs of toxicosis during the week of observation. These mice continued to eat, drink, sleep, and move about their cages in the same manner as the historical control mice and necropsy revealed no grossly visible lesions. Similarly, histopathologic evaluation of the liver, kidney, and spleen revealed no difference between the mice dosed with $(C_3H_7O_2)$MCLR and historical control mice, and both groups had a pathology score of 0.00.

Microcystins lethal at doses less than 1 mg/kg

The most potent MC apart from MCLR was [DMAdda^5]MCLR which had an $\simeq LD_{50}$ of 97 µg/kg. Two microcystins which as compared to MCLR, had substitutions in place of the leucine of MCLR had $\simeq LD_{50}$s of 171 µg/kg. The first was MCYR which had a tyrosine, and the second was MCWR which had a tryptophan, in place of leucine. There were two microcystins with $\simeq LD_{50}$s of 249 µg/kg: MCFR and MCAR which had substitutions of phenylalanine and alanine, respectively, in place of leucine. The $\simeq LD_{50}$ of [Dha^7]MCLR which had the methyl group removed from *N*-methyldehydroalanine (Mdha), the seventh amino acid, was 250 µg/kg. One microcystin demonstrated an $\simeq LD_{50}$ of 290 µg/kg, but the structure has not been totally determined. It has the sequence of MeAsp–Arg–Adda–Glu–Mdha–Ala and contains an unknown dehydroamino acid in

place of L-leucine in MCLR. The $\simeq LD_{50}$ for MCRR, with arginine substituted for leucine, was 650 μg/kg. Among the least potent of the microcystins exhibiting lethality in this study was MCM(O)R which had a methionine sulfoxide in place of leucine and an $\simeq LD_{50}$ of 750 μg/kg.

Due to the limited amount of toxin available, the $\simeq LD_{50}$ for [Dha⁷]MCLR was estimated by selecting a dose at which one animal lived and one died, while all animals given higher doses (300 μg/kg or more) died and all at lower doses (200 μg/kg or less) survived. MCRR and MCM(O)R $\simeq LD_{50}$s were estimated by selecting values midway between the highest doses at which all animals lived, 500 μg/kg and 700 mg/kg, and the lowest doses at which all animals died, 800 μg/kg and 800 μg/kg, respectively.

The liver to body weight ratios of mice which died following dosing were significantly higher than those of mice killed one week after dosing (Fig. 2). Liver lesions were present only in mice that died and were consistent with the hepatotoxic effects of algal toxins (HOOSER *et al.*, 1989).

The pathology scores for the mice that died averaged 4.95 and there was no significant difference between the compounds when compared using analysis of variance and a level of significance of $P < 0.05$. All of the mice that lived for 1 week had a pathology score of 0.00.

DISCUSSION

Liver weight as a percentage of body weight in mice has been used as an indicator of acute algal peptide hepatotoxicity (LOVELL *et al.*, 1989; DAHLEM *et al.*, 1989). The narrow range between doses at which no animals died and those at which all members of the group died is consistent with the tendency of microcystins to exhibit steep dose–response curves (LOVELL *et al.*, 1989). When given at sufficient doses, algal cyclic peptide toxins cause extreme enlargement of the liver, due in significant measure to intrahepatic hemorrhage (CARMICHAEL *et al.*, 1985). However, for changes in liver weights to have remained evident at 1 week after dosing, damage would have to have been severe. Previous studies in this laboratory have indicated that even when mice display acute signs of hepatoxicity shortly after dosing with MCLR, there may not be significant increases in liver weights of survivors 10–11 days later (LOVELL *et al.*, 1989). The liver weights of mice given nonlethal doses of microcystins may have increased after dosing, but were near normal by the time they were killed 1 week later. All compounds causing death in mice produced lesions consistent with those previously described in mice following dosing with microcystin (HOOSER *et al.*, 1989). Although lesions and increases in relative liver weights may have been missed as a result of waiting until day 7 postdosing to euthanatize the mice, it was deemed of greater importance to allow time for any potential toxicosis to either develop fully or display its reversibility than to identify only peracute toxic effects.

Modifications at different sites of the microcystin molecule were found to produce mild to marked changes in toxicity. Other investigators have described retention of hepatotoxicity in microcystins despite modifications at the site of the second amino acid, leucine in MCLR, such as with MCRR and MCYR (BOTES *et al.*, 1982; HARADA *et al.*, 1988; WATANABE *et al.*, 1988). In the present study, hepatotoxicity was maintained despite replacement of L-Leu with several individual amino acids. When L-Leu, which is a hydrophobic amino acid, was replaced by other hydrophobic amino acids, i.e. Phe, Ala, or Trp, the resulting microcystins retained $\simeq LD_{50}$s from 171–249 μg/kg. Replacing L-Leu with a somewhat more hydrophilic Tyr gave a MC with an $\simeq LD_{50}$ of 171 μg/kg, which is

slightly higher than reported by WATANABE *et al.* (1988) of 68 μg/kg. There was a larger decrease in the hepatotoxicity when L-Leu was changed to the basic Arg giving a MC which had an $\simeq LD_{50}$ of 650 μg/kg, which is close to the value reported by WATANABE *et al.*, of 600 μg/kg. A similar effect was observed when the L-Leu was changed to a sulfoxide of methionine resulting in a compound with an $\simeq LD_{50}$ of 750 μg/kg.

Other nearby amino acids may also be modified giving MCs that exhibit hepatotoxicity. One of these amino acids is the third AA, D-MeAsp. MCs containing demethylated MeAsp and retaining hepatotoxicity include [D-Asp^3]MCLR, [D-Asp^3]MCRR, [D-Asp^3,Dha^7]MCRR, and [D-Asp^3,ADMAdda^5]MCLR (KRISHNAMURTHY *et al.*, 1989; HARADA *et al.*, 1991; NAMIKOSHI *et al.*, 1990).

Another amino acid that can be modified without eliminating hepatotoxicity is the fourth amino acid, L-Arg. Investigators have reported toxic MCs with Ala, Met, and homoargine replacing L-Arg (BOTES *et al.*, 1982; CARMICHAEL, 1988; NAMIKOSHI *et al.*, 1990).

The presence of the fifth AA, Adda, appears to be of great importance in hepatotoxicity. Previous studies have demonstrated that removal or saturation of the Adda structure greatly reduces the toxicity of MCLR (DAHLEM, 1989). Certain slight modifications of the Adda unit, however, appear to be tolerated such that the molecule still exhibits hepatotoxicity. NAMIKOSHI *et al.* (1990) describe three hepatotoxic MCs from *Nostoc* spp. which retained toxicity similar to MCLR despite the fact that they contained an acetoxyl group instead of a methoxy group at the C-9 in Adda. Similarly in this study, demethylation of the C-9 unit of the Adda did not significantly reduce toxicity.

This study demonstrated that the toxicity of MCLR was reduced substantially by the addition of the ($C_3H_7O_2$) unit to the D-glutamic acid. The structure assignment of the ($C_3H_7O_2$) unit is now being carried out, but clearly the free carboxylic acid on the Glu unit seems be important in toxicity. All MCs reported at this time with significant hepatotoxicity have conserved the D-Glu in the position of the sixth AA.

Removal of the *N*-methyl group from Mdha as found in this study and as reported by HARADA *et al.*, (1991) did not markedly alter hepatotoxicity. Moreoever, reduction of the double bond in methyldehydroalanine did not significantly reduce the toxicity of MCLR (MERILUOTO *et al.*, 1990; DAHLEM *et al.*, 1989; STOTTS *et al.*, unpublished data).

This study along with evidence presented by previous investigators, suggests that the Adda and D-glutamic acid portions of the MCLR molecule play highly important roles in the hepatotoxicity of microcystins. Possible explanations are that these portions of the molecule may provide a necessary steric configuration which is directly involved in a carrier protein conveying hepato-specificity and/or at an active site involving intracellular inhibition of protein phosphatase.

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- Submitted Draft -

Toxicokinetics of tritiated dihydromicrocystin-LR in swine

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Summary

The toxicokinetics of tritiated dihydromicrocystin-LR ([^{3}H]2H-MCLR) were studied in anesthetized, specific pathogen free (SPF) pigs. Three dosage groups were studied. Two doses of the radiolabeled plus nonlabeled 2H-MCLR were administered IV and one dose was given via an isolated ileal loop. The IV doses of 25 μg/kg and 75 μg/kg were rapidly removed from the blood. At either IV dose, more than half the radiolabel from [^{3}H]2H-MCLR present in the blood at one minute post-dosing was cleared by 6 minutes. The blood clearance at the 75 μg/kg dose was slower than at the 25 μg/kg dose. Accordingly, at the high dose, toxin concentrations in blood were disproportionately higher from 10 minutes after dosing until the study ended 4 hours later. The decreased clearance is presumably due to decreased elimination as a consequence of the hepatic injury observed histologically. Following administration of [^{3}H]2H-MCLR at 75 μg/kg via an ileal loop, the peak concentration of toxin in blood was achieved at 90 minutes after dosing, when [^{3}H]2H-MCLR in portal venous blood was 3.6 times higher than in peripheral venous blood. Although bile production varied, following iv dosing, radioactivity in bile was detected as early as 12 minutes postdosing in one animal. This study demonstrates the rapid removal of ^{3}H-2H-MCLR from the blood of anesthetized swine and the appearance of radiolabel from the toxin in bile within minutes after dosing.

Species of several genera of cyanobacteria (blue-green algae) including, *Microcystis*, *Anabaena*, *Nostoc*, and *Oscillatoria* produce cyclic heptapeptide hepatotoxins that have been termed microcystins[1,2]. Microcystins from *Microcystis aeruginosa* often pose hazards to livestock, and sometimes to public health, in many regions of the world[3]. Toxic blooms of this organism usually occur in eutrophic still waters during warm months of the year[4]. The occurrence of toxic blooms is deemed likely to increase with expansion in the use of fertilizers, insecticides which harm zooplankton, animal-based agriculture, and construction of water holding facilities such as ponds, lakes, and reservoirs.

Microcystins, contain three D-amino acids, two L-amino acids, N-methyldehydroalanine, and one unusual 3-amino-9-methoxy-2,6,8-trimethyl-10-phenyl-4,6-decadienoic acid (ADDA) component[5,6]. The medial lethal dose of microcystin-LR (MCLR) is approximately 75 μg/kg IP in mice[7]. Following IP or IV dosing, the livers of mice given a fatal dose rapidly become dark and enlarged, and the mucous membranes of such mice become pale before death. Centrilobular hepatic necrosis and hemorrhage are characteristic histologic changes of MCLR toxicosis in all mammalian species reported to date, including swine[8,9]. Following a lethal parenteral dose, mice usually die within three hours. Death is believed to be caused by shock largely attributable to intrahepatic hemorrhage.

Microcystins affect the cytoskeleton of hepatocytes[10,11]. Microfilaments and intermediate filaments are disrupted and hepatocyte plasma membranes undergo severe deformation. The loss of hepatocyte structural integrity is accompanied by disruption of hepatic sinusoids and intrahepatic hemorrhage. Cytoskeletal disorganization may be due to intracellular hyperphosphorylation due to toxin-induced inhibition of protein phosphatases[12].

Studies of the fate of radiolabeled microcystins given to rats and mice have shown accumulation primarily in the liver with lesser amounts in the kidneys[7,13,14]. The concentration of the labeled toxin by the liver is believed to be due to uptake of microcystins by hepatocytes via rifampicin-sensitive bile acid carriers[15]. Microcystin labelled with ^{125}I disappeared biphasically from the blood of rats dosed IV with an initial phase half-life of 2.1 minutes, followed by a later phase half-life of 42 minutes[16]. The disposition of radioactivity in the blood of anesthetized fasted mice given tritiated MCLR IV followed a similar biphasic curve; however, disposition was more rapid in mice with first and second phase half-lives of 0.8 minutes and 6.9 minutes, respectively[17]. The disposition of tritiated microcystin from the perfusate of isolated perfused rat livers was slower with a half-life of 130 min[18]. Thus, radiolabeled microcystin in rats and mice is removed quickly from the blood, and the majority of the radioactivity is concentrated in the liver.

Dihydromicrocystin-LR (2H-MCLR) causes the same clinical signs and lesions in rodents as does MCLR[14]. Isolated perfused rat livers developed microscopic lesions characteristic of microcystin toxicosis within 15 minutes after exposure to tritiated dihydromicrocystin-LR ([^{3}H]2H-MCLR)[15]. In mice, 2H-MCLR given IP was consistently lethal at 200 ug/kg, whereas MCLR was lethal at 100 ug/kg[14]. The time course of the toxicosis was similar with the dihydro-derivative and the parent toxin. It is unknown whether the reduction in toxicity of 2H-MCLR results from a reduced rate or extent of uptake by hepatocytes or from reduced interactions with intracellular receptors. The tritiated dihydro compound seems to be an appropriate derivative to investigate the absorption and disposition of microcystins, because a) the syndrome caused by 2H-MCLR is virtually identical to that

induced by MCLR; b) a relatively high specific activity has been obtained; c) the location of the inserted tritium in [^{3}H]2H-MCLR is known; and d) the radiolabel is biologically stable as indicated by its absence in distillate of the urine of dosed mice[14]. The objectives of the study reported here were to determine the clearance of [^{3}H]2H-MCLR from the blood of swine and its biliary excretion, as well as to determine the rapidity of absorption of [^{3}H]2H-MCLR from the ileum using an isolated ileal loop model.

Materials and Methods

Animals—Landrace-cross, specific pathogen free female pigs weighing 18 to 24 kg were given free access to feed and water until 12 hours before surgery when feed, but not water was withheld. One hour before anesthesia was induced, the animals were fed 0.5 kg of ground corn mixed with 50 ml of corn oil in an attempt to stimulate bile production.

Toxin—MCLR was purified from a natural bloom collected from Homer Lake, Illinois. The algae water mixture was frozen within hours of removal from the lake, then lyophilized and stored frozen at -40 C. The crude microcystin was extracted from the lyophilized cells in methanol. The extract was dried, then redissolved in water, passed through a reversed phase C-18 column, and eluted with methanol. The elution products were separated further via liquid chromatography using a series of two silica gel columns. The first column employed a mobile phase of chloroform, methanol, and water (65:35:10) which was shaken and allowed to separate before discarding the top phase. The second column employed a mobile phase of

ethylacetate, isopropanol, and water (4:3:7) and was similarly prepared, discarding the bottom phase. The final purification step was achieved with a size exclusion column (Toyopearl HW40) with methanol as the mobile phase. The purity of the MCLR was determined to be greater than 95% by HPLC, TLC, and fast atom bombardment-mass spectrometry.

Radiolabeling—[^{3}H]2H-MCLR was produced by reacting MCLR with tritiated sodium borohydride ([^{3}H]$NaBH_4$) and the products purified. Four reactions were carried out in order to produce a pooled dosing solution. Molar ratios of MCLR:[^{3}H]$NaBH_4$[a](100 mCi) of 1:3.7 and 1:4.5, respectively, were used in the first two labelling reactions. The reactions were carried out for 24 hours in 0.5 ml of 70% isopropanol then quenched with acetic acid. The third and fourth reactions were performed using 250 mCi of [^{3}H]$NaBH_4$[b] with molar ratios of MCLR:[^{3}H]$NaBH_4$ 1:4 and 1:5. The reaction products were dried under nitrogen gas, redissolved in H_2O and loaded on a C_{18} reversed phase column. The labelled toxin was eluted with methanol and dried with nitrogen gas. The dried products were then dissolved in a mobile phase of chloroform:methanol:water, (65:35:10) prepared as described above and passed through a 200 ml chromatography column packed with 9 gm of silica gel. Fractions were collected and samples from each fraction counted with a scintillation counter. Ten μl samples from each fraction were also loaded on a fluorescent silica gel thin layer chromatography plate and developed using a chloroform:methanol:water, (65:35:10) mobile

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[a]American Radiolabel Chemical, St. Louis MO.

[b]Amersham Inc., Arlington Heights, IL.

phase prepared as previously described. Fractions were combined that had the same retardation factors (R_fs) as the standard 2H-MCLR. Ten μl of the combined fractions were loaded on a TLC plate and developed as described above for the methanol fraction from the C18 column.

The purified reaction products from all four reactions were combined and dissolved in 10% ethanol forming a pooled dosing solution. The concentration of [^{3}H]2H-MCLR was determined to be 0.166 mg/ml based on a comparison to a standard HPLC curve of 2H-MCLR. The dosing solution was determined to be greater than 90% [^{3}H]2H-MCLR or 2H-MCLR by HPLC and TLC (Figs 1 and 2). The specific activity of the toxin used in preparing the dosing solution was 1.04 mCi/mg as measured by liquid scintillation counting.

Anesthesia—Each pig was anesthetized with isoflurane[c] by mask and a cuffed endotracheal tube was inserted. A combination of xylazine (0.66 mg/kg) and lidocaine (3.6 mg/kg) was then administered to the pigs by epidural injection. Anesthesia was maintained with isoflurane at 2.5% during surgery, and 1.5% during the dosing and sampling period.

Surgical procedures—Pigs were placed on a circulating water heated pad. An incision was made in the lateral-ventral cervical skin, and the jugular vein and carotid artery were catheterized. A second skin incision was made over the right femoral vein which was then catheterized. A midventral abdominal incision was made, and a catheter placed in the caudal

[c]Anaquest Inc., Liberty Corner, NJ.

vena cava cranial to the renal veins with the tip advanced to the hepatic sinus. A second catheter was placed in the hepatic portal vein. The common bile duct was ligated to stop bile flow to the intestine. The gall bladder was emptied with a 20 gauge needle and syringe, and the site of perforation was closed using a pair of hemostats. The urinary bladder was evacuated in the same manner.

In the 3 pigs to be dosed via the ileal loop, the ileum was clamped with two bowel clamps 4 cm from the ileocecal junction, the blood vessels in the clamped area were ligated and then the ileum was transected between the clamps. The procedure was repeated 15 cm rostrally leaving an isolated ileal loop. The isolated ileal loop was then flushed with 0.9% NaCl in water to remove the lumen contents and each end closed with an inverting suture pattern. The integrity of the loop was determined by injecting physiological saline into the lumen and observing for signs of leakage. The abdominal cavities of the pigs dosed IV and via the ileal loop were temporarily closed with towel clamps.

Dosing—In the pigs dosed IV, the toxin containing solutions were injected over a one minute period via the jugular catheter, and the catheter was then flushed with 5 ml of 0.9% NaCl. The remaining pigs were given the dosing solution by direct injection into the lumen of the ileal loop using a syringe and needle.

Sampling—Blood was taken from the femoral vein of pigs at 1, 2, 5, 7, 10, 20, 30, and 40 minutes, as well as 1, 1.5, 2.5, 3, 3.5, and 4 hours after dosing IV. Blood samples were drawn from the portal vein and hepatic sinus at 20 and 40 minutes as well as 1, 1.5, 2, 2.5, 3,

3.5, and 4 hours after dosing IV.

The pigs dosed via the ileal loop were sampled for one hour longer than the pigs dosed IV. Blood samples were taken from the femoral, hepatic, and portal veins at 5, 20, and 40 minutes and at 1, 1.5, 2, 2.5, 3, 3.5, 4, 4.5, and 5 hours post-dosing from the pigs given the toxin via the ileal loop.

Bile samples were taken with a syringe and needle when the gall bladder filled, and the aperture was closed with hemostats between sampling periods to prevent leakage into the abdominal cavity. The time of sampling and volume of bile evacuated were recorded.

Histopathology—At the end of the experiment, sections of liver were fixed in 10% neutral buffered formalin, routinely processed, embedded in paraffin, sectioned at 4 μm, stained with hematoxylin and eosin, and examined by light microscopy.

Scintillation counting—Whole blood and bile samples were counted with a Packard Tri-Carb model B2450 scintillation counter[d]. Samples of blood or bile (500 μl) along with an equal volume of Solvable[e] were placed in 20 ml polypropylene scintillation vials. The vials were then incubated for one hour at 50 C in a shaking water bath. To reduce foaming, 100 μl of 100 mM ethylenediaminetetraacetic acid (EDTA) were added to each vial followed by three 100 μl aliquots of 30% H_2O_2 to decolorize the samples. Samples were incubated again for one

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[d]Packard Instrument Co., Meriden, CT.

[e]NEN, Du Pont Co., Boston, MA.

hour at 50 C in a water bath. The vials were cooled to room temperature, after which 15 ml of Aquasol II[e] were added. The samples were stored in darkness for 72 hours before they were counted.

A quench curve was established using a tritium standard which was quenched with eleven dilutions of carbon tetrachloride. The external standard ratio (ESR) and percent quenching were determined for each dilution and the values compared using regression analysis. Regression was used to determine the percent quenching based upon the ESR for all of the swine blood samples. A tritium standard was used to establish the efficiency of the scintillation counter which was consistently in the range of 69.8% to 69.9%.

Pharmacokinetic analysis—The DPM in blood versus time following the IV doses were fitted by mono-, bi-, and triexponential equations using the Autoan computer program[n]. The intercepts of the equations were subsequently converted from DPM to ng of toxin equivalents/ml.

Results

IV dose study—Concentrations of toxin equivalents in femoral venous blood decreased rapidly (Fig 3). Disposition was biphasic with the 25 μg/kg dose being cleared more rapidly than the 75 μg/kg dose. A biexponential equation of the form $ng/ml = Ae^{-\alpha t} + Be^{-\beta t}$ was determined by F test to fit the data best. The early disappearance rate constant (alpha) at the 25 μg/kg dose was 0.1908/minute and the later disappearance rate constant (beta) was

0.005185/minute (Table 1). The value for alpha at the 75 μg/kg dose was 0.2302/minute and the beta value was 0.002581/minute. Therefore, the half-life ($T_{1/2}$) values for the alpha phase of the low dose and high dose, respectively, were 3.632 minutes and 3.014 minutes, and those for the beta phases of the low and high doses were 133.5 minutes and 268.6 minutes, respectively. The blood clearance (Cl) at the low dose (0.002853 l/kg/min) was approximately 2.8 times greater than the clearance at the high dose (0.001028 l/kg/min).
Toxin concentrations in the portal vein and hepatic sinus area of the caudal vena cava were very similar to those in the femoral vein (Figs 4 and 5).

Rates of bile production varied, and thus bile collection times were unevenly spaced. One pig in the low dose group (N=2) and one pig in the high dose group (N=3) failed to produce any collectable bile during the four-hour observation period. The second pig given the low dose produced a total of 53 ml of bile containing 4% of the radioactivity given IV; with the first specimen of bile, accounting for 0.5% of the administered radiolabel, being collected at 35 minutes after dosing. The second high dose pig, which produced 75 ml of bile including 5.9% of the total radioactivity given IV, had measurable radioactivity accounting for 1.12% of the dose in the bile at 12 minutes after dosing. The third high dose pig produced 45 ml of bile containing 1.27% of the dose, and the first measurable radioactivity, equivalent to 0.08% of the dose, was collected at 120 minutes after dosing.

Ileal loop study—Peripheral blood concentrations of toxin were not as high in the three pigs dosed via the ileal loop as in the pigs dosed IV (Fig 6). In pigs dosed via an ileal loop, toxin concentrations in portal venous blood were consistently higher than in other blood

samples at all sampling times. The maximum concentration was present in the portal blood at 90 minutes after dosing. The first pig produced 61 ml of bile which contained 12.8% of the total dose. The initial bile sample was collected at 90 minutes after dosing and contained 2.6% of the total dose. The second pig produced 102 ml of bile containing 15.3% of the radiolabel from the administered dose with the first measurable radioactivity, accounting for 0.17% of total dose, being obtained at 90 minutes after dosing. The third pig produced 35 ml of bile accounting for 5.26% of the total dose. The first sample from this pig which contained detectable radioactivity accounted for 1.3% of dose and was collected at 120 minutes post-dosing.

Histopathology—All pigs treated with [^{3}H]2H-MCLR developed liver lesions characteristic of MCLR toxicosis. These included swelling, disassociation, and early fragmentation of centrilobular and, in more severe cases, midzonal hepatocytes. The pigs given the high IV dose had the most severe and extensive lesions with two of three exhibiting intrahepatic hemorrhage. The pigs given the toxin via the ileal loop had a very uneven distribution of lesions, with large areas of liver being unaffected. These latter pigs had background lesions indicative of pericholangitis.

Discussion

The alpha phases at the high and low IV doses were similar, however, there was a difference in the beta phases of these dose groups. The $T_{1/2}$ for the alpha phase, which lasted

about 20 minutes after dosing, was slightly less at the high dose (3.0 minutes) as compared to the low dose (3.6 minutes). For the beta phase, which continued from 20 minutes postdosing until the end of the 4 hour study, the $T_{1/2}$ at the low dose of 133.5 minutes was considerably less than that at the high dose of 268.6 minutes. The biphasic disposition of [^{3}H]2H-MCLR in this study with pigs is similar to that reported by Robinson *et al.* who used tritiated MCLR in mice[17]; however, the blood clearance in mice was more rapid. The difference in blood clearance may be due to species variation, an effect of anesthesia or surgery, or differences in the toxins. Production of tritiated MCLR, unlike synthesis of [^{3}H]2H-MCLR, has not been consistently achievable because the procedure used to produce the former has a great tendency to degrade the toxin[14]. Whether the higher toxicity of MCLR as compared to 2H-MCLR is related to more rapid uptake of the toxin from the blood by the liver remains to be assessed.

The peak concentration of toxin in the blood of swine dosed via the ileal loop occurred at 90 minutes after dosing. Throughout the five hour monitoring period, the toxin concentration in the portal venous blood was significantly higher than in the peripheral blood. Although blood flow rates in sampled vessels and hepatic extraction ratios were not measured, the difference in peripheral venous concentrations of toxin in ileal loop-dosed pigs compared to the IV-dosed pigs, suggests that a first pass effect is, in part, responsible for clearance of the toxin. This is in concurrence with previous studies which demonstrated that the liver preferentially accumulates and is the major target organ for the toxin[7,13,14].

Data from Table 1 indicate that the blood clearance (Cl) of the toxin at the high dose was only 36% of the low dose value. The slower clearance at the high dose was due primarily to impaired elimination of the toxin as reflected in the decreased elimination rate constant

(k10) of the toxin; ie., k10 at the high dose was 37% of the k10 at the low dose. The apparent volume of distribution at steady state (V_{ss}) and apparent volume of distribution based on area under the curve (V_{area}) at the high dose actually decreased while the apparent volume of the central compartment (V_c) remained about the same, suggesting that the toxin did not distribute as well to the peripheral tissues at the high dose or that the animals were more dehydrated. Hemodynamic studies of swine have shown that MCLR causes a decrease in mean aortic pressure and a decrease in blood flow through the liver resulting in decreased central venous pressure with a corresponding increase in portal venous pressure[9]. The same investigation showed that liver perfusion decreased more rapidly than renal perfusion and that approximately 37.9% of the estimated total blood volume was sequestered in the liver of pigs given a fatal dose of MCLR. The decreased volume of distribution at the high dose would have a tendency to increase clearance of the toxin had it not been for impaired elimination. Thus, at the high IV dose, the decreased clearance of the toxin is probably due to decreased elimination as a consequence of hepatic damage, which was more severe in these pigs. Hepatic lesions induced by 2H-MCLR were similar to those observed in natural and experimental toxicosis induced by MCLR-containing cyanobacteria.

In conclusion, the clearance of [^{3}H]2H-MCLR from the blood of anesthetized swine is rapid and follows a biphasic pattern. This study indicates that the liver rapidly clears microcystins from the blood and secretes them into the bile. Also, at a potentially lethal dose, clearance is reduced. Following exposure via an ileal loop, data were indicative of marked first pass effect.

Figure Legends

Figure 1—Thin layer chromatography plate scan of the [^{3}H]2H-MCLR dosing solution obtained using a Radiomatic Model RS Radio-thin layer chromatography plate scanner. Peaks 2 and 3 correspond to the 2H-MCLR standard.

Figure 2—High performance liquid chromatogram of the [^{3}H]2H-MCLR dosing solution using a Radiomatic Flo-One\Beta Model IC radioactive flow detector. The chromatogram reveals single coincident radiation and UV peaks which correspond to the 2H-MCLR standard peak.

Figure 3—Cartesian plot of [^{3}H]2H-MCLR concentrations in femoral vein blood samples collected for 240 minutes from pigs dosed at 25 μg/kg (n=2) or 75 μg/kg (n=3) IV and for 300 minutes from pigs dosed at 75 μg/kg (n=3) via the ileal loop. Error bars represent standard errors.

Figure 4—Semilog plot of [^{3}H]2H-MCLR concentrations in femoral, portal, and hepatic venous blood of pigs (n=2) dosed IV at 25 μg/kg. The first samples from the portal and hepatic veins were obtained at 20 minutes after dosing. Error bars represent standard errors.

Figure Legends (cont.)

Figure 5—Semilog plot of [^{3}H]2H-MCLR concentrations in blood of pigs (n=3) dosed IV at 75 μg/kg. The first sample was obtained from the portal and hepatic veins at 20 minutes after dosing. Error bars represent standard errors.

Figure 6—Semilog plot of 2H-MCLR concentrations in blood of pigs (n=3) dosed via the ileal loop at 75 μg/kg. Error bars represent standard errors.

Table Legends

Table 1—Toxicokinetic parameters (means) for the disposition of ^{3}H from [^{3}H]2H-MCLR following intravenous administration to swine.

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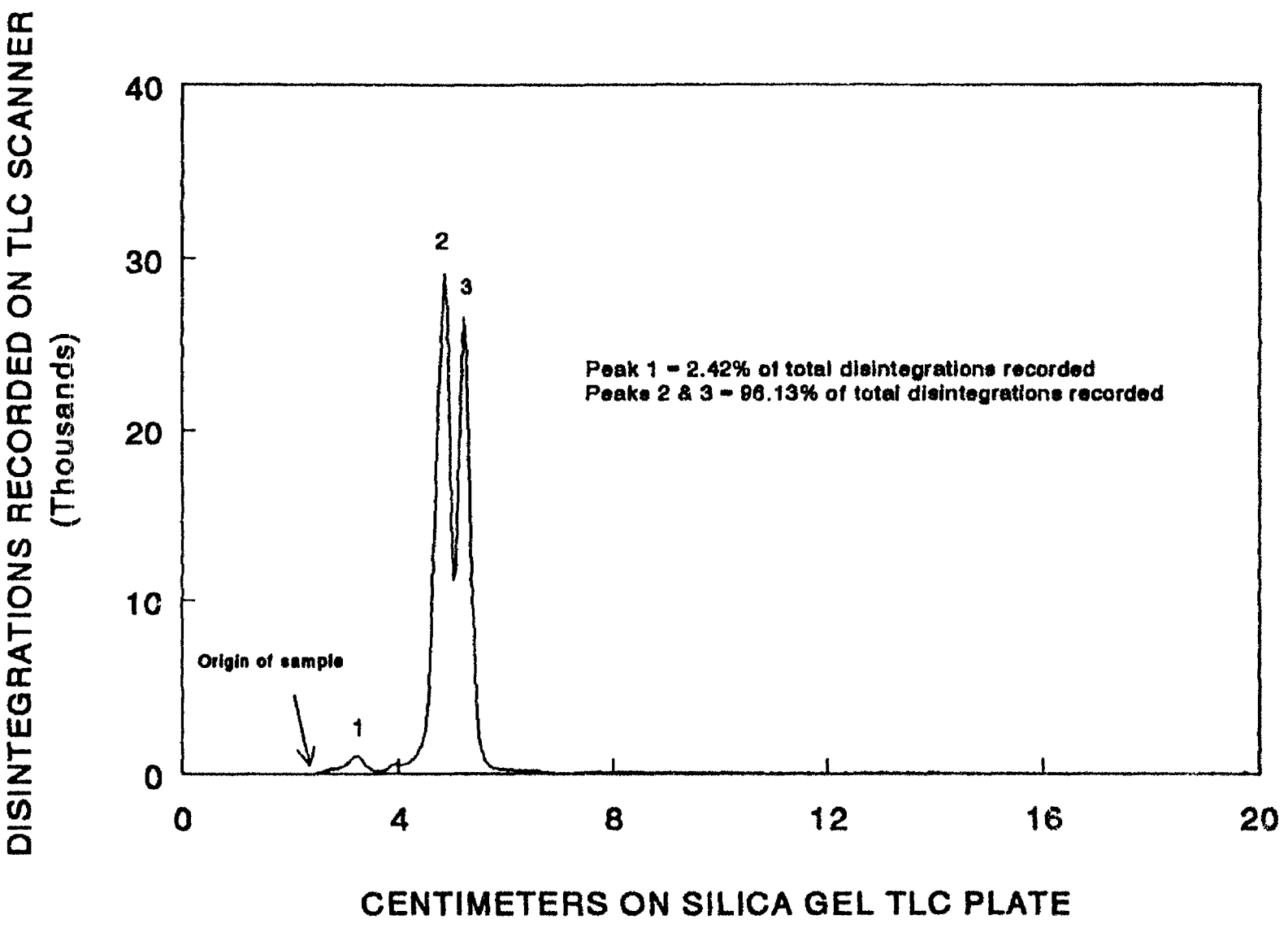
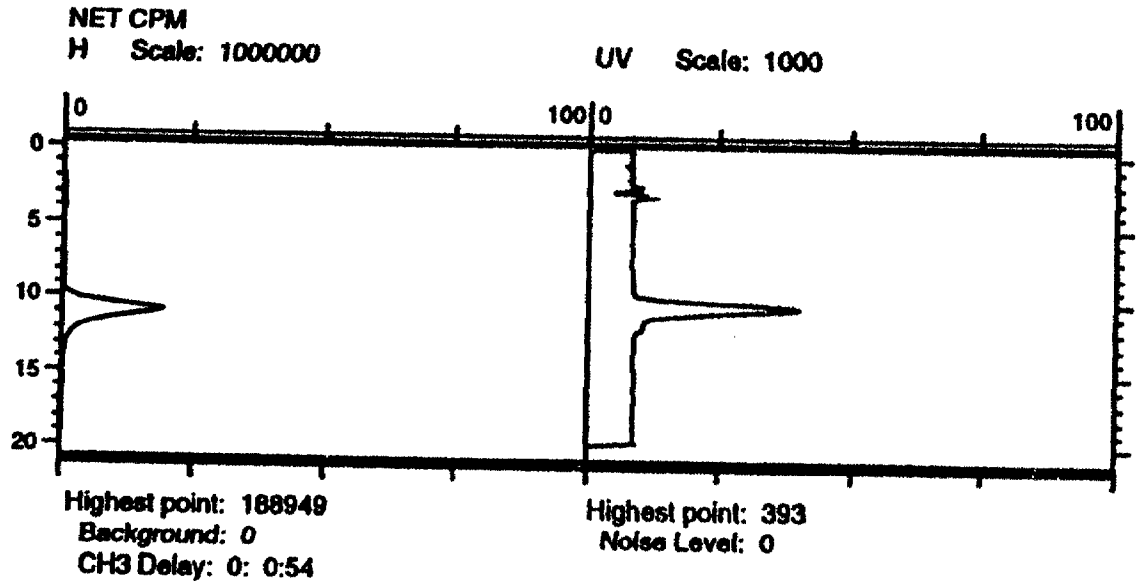
Table I

	Low Dose (25µg/kg)	High dose (75µg/kg)
A	198.4 ng/ml	515.7 ng/ml
B	33.0 ng/ml	166.5 ng/ml
α	.190800 min^{-1}	.230200 min^{-1}
β	.005185 min^{-1}	.002581 min^{-1}
t½ α	3.632 min	3.014 min
t½ β	133.655 min	268.501 min
k12	.036121 min^{-1}	.058038 min^{-1}
k10	.027389 min^{-1}	.010327 min^{-1}
Vc	.1080 l/kg	.1099 min^{-1}
V_{ss}	.5043 l/kg	.4216 l/kg
V_{area}	.6512 l/kg	.4353 l/kg
Cl	.003376 l/kg/min	.001124 l/kg/m


DISINTEGRATIONS RECORDED ON TLC SCANNER
(Thousands)
40
30
20
10
0
0
4
8
12
16
20
CENTIMETERS ON SILICA GEL TLC PLATE
Origin of sample
1
2
3
Peak 1 = 2.42% of total disintegrations recorded
Peaks 2 & 3 = 96.13% of total disintegrations recorded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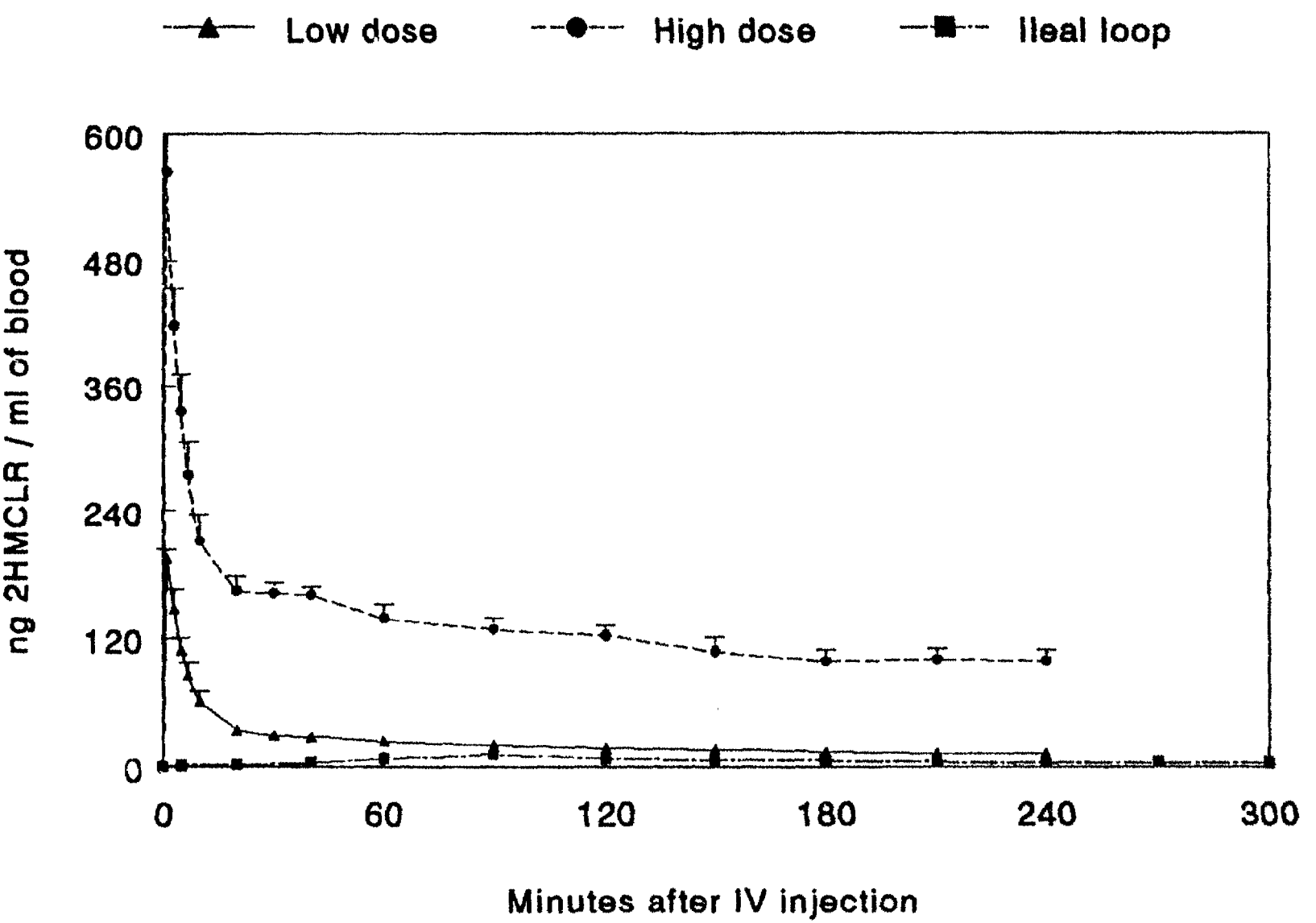
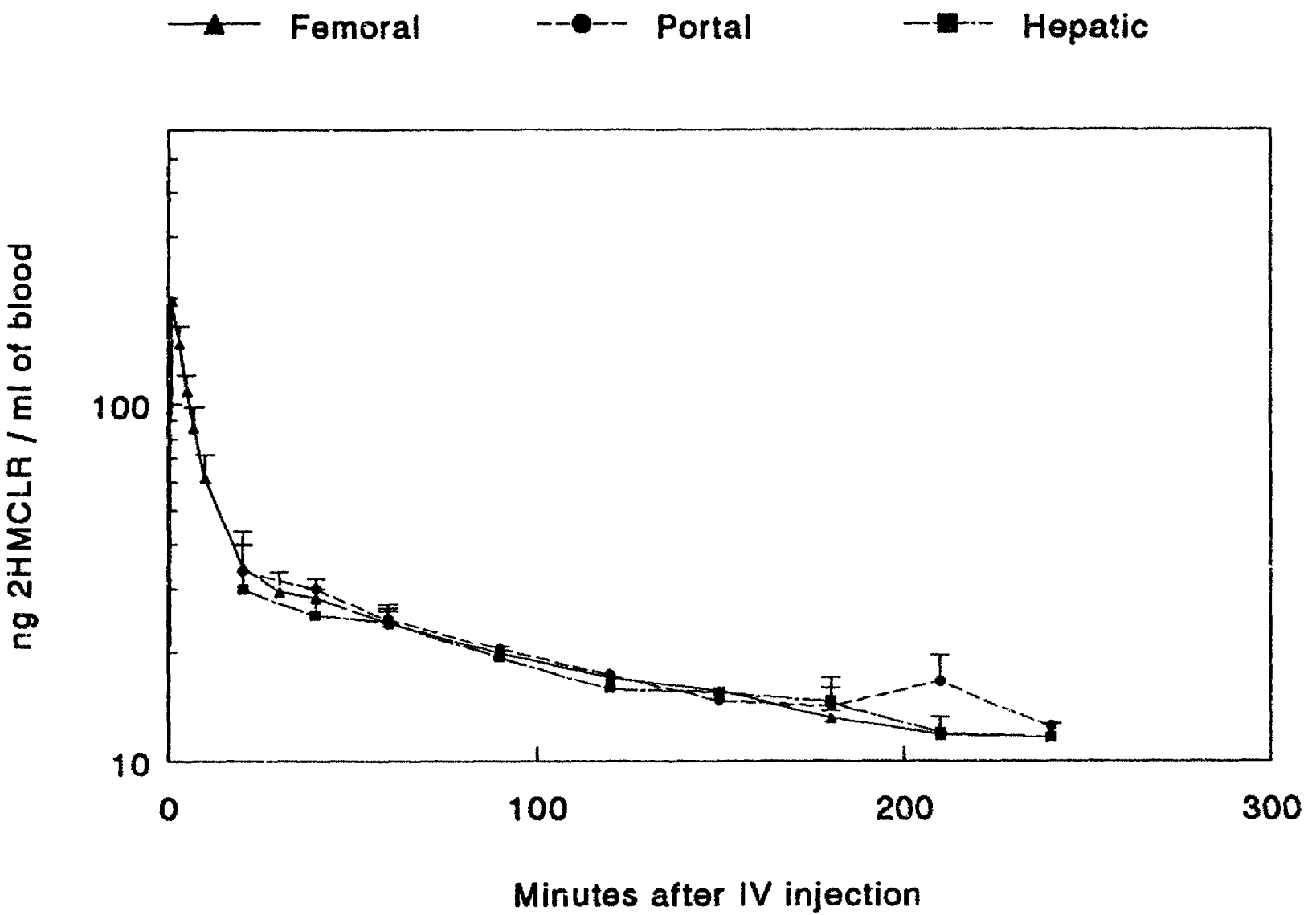
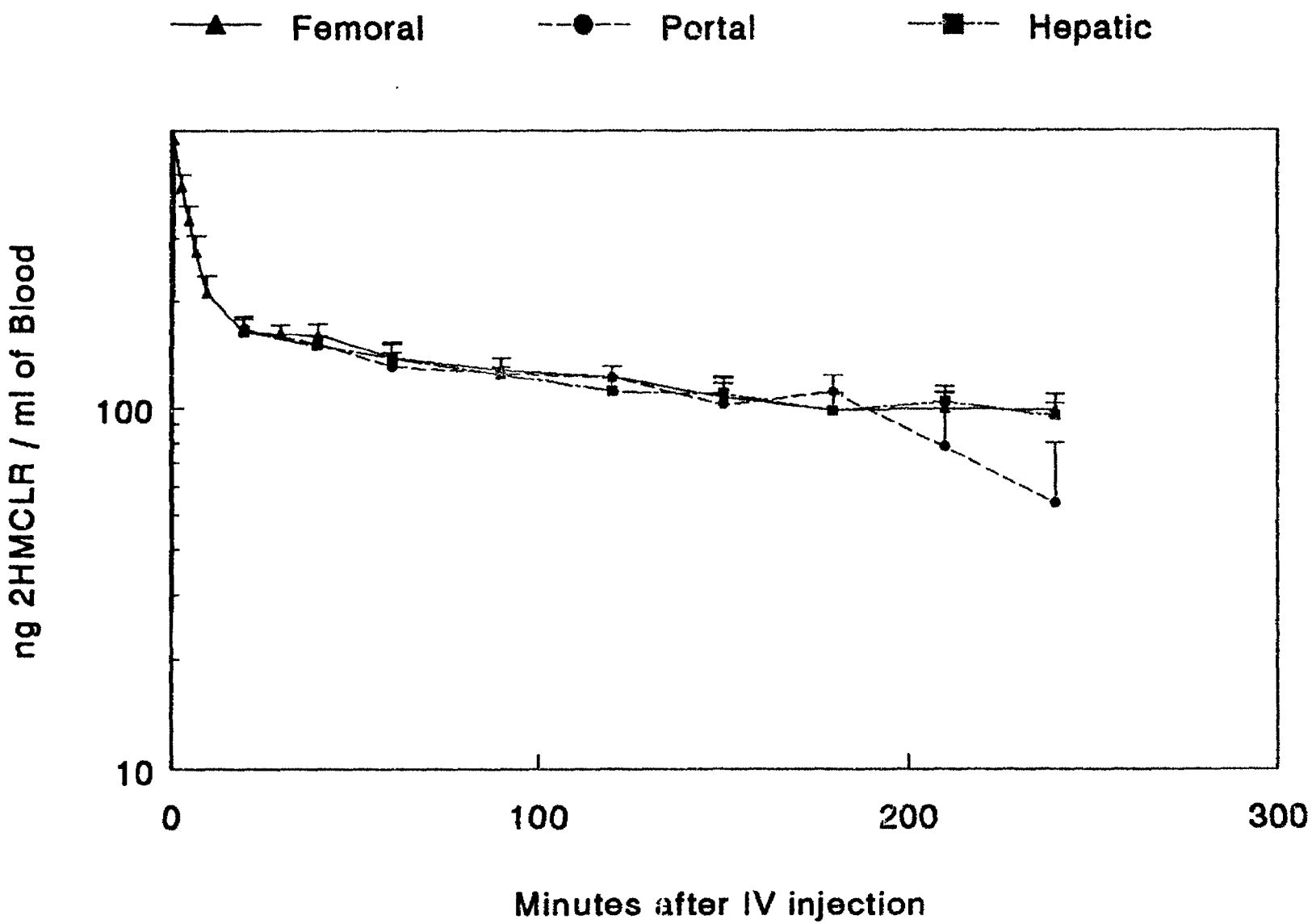
H Scale: 1000000
UV Scale: 1000
0
100
0
100
0
5
10
15
20
Highest point: 188949
Background: 0
CH3 Delay: 0: 0:54
Highest point: 393
Noise Level: 0


Low dose
High dose
Ileal loop
600
480
360
240
120
0
ng 2HMCLR / ml of blood
0
60
120
180
240
300
Minutes after IV injection


Femoral
Portal
Hepatic
ng 2HMCLR / ml of blood
100
10
0
100
200
300
Minutes after IV injection


Femoral
Portal
Hepatic
ng 2HMCLR / ml of Blood
100
10
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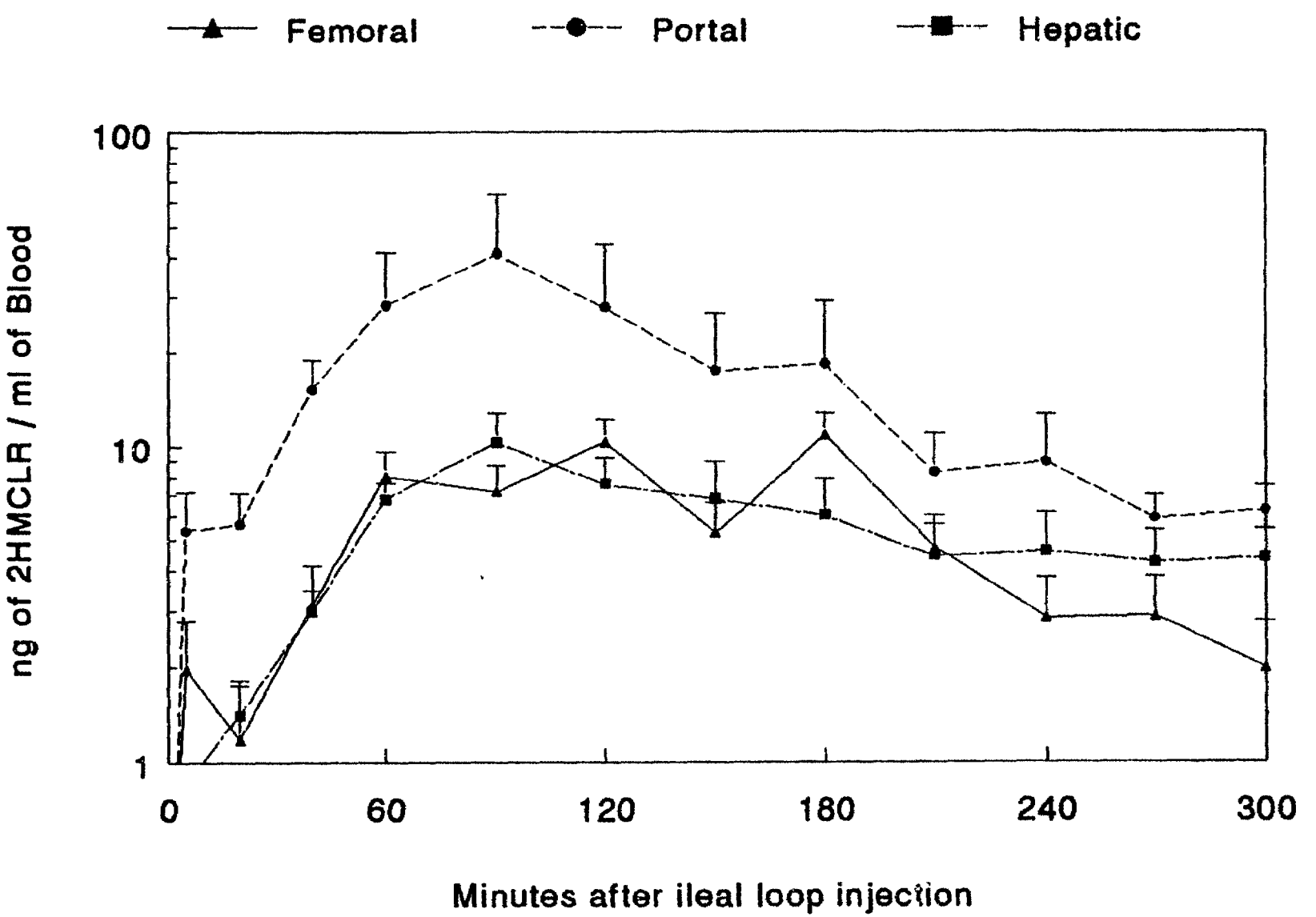
100
200
300
Minutes after IV injection


Femoral
Portal
Hepatic
100
10
1
ng of 2HMCLR / ml of Blood
0
60
120
180
240
300
Minutes after ileal loop injection

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(Apr 1991)

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