PATHOPHYSIOLOGY AND TOXICOKINETIC STUDIES OF BLUE-GREEN ALGAE INTOXICATION IN THE SWINE MODEL

ANNUAL REPORT

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19. ABSTRACT (Continue on reverse if necessary and identify by block number)
Nonlabelled and tritium labelled dihydromicrocystin-IR (2H-MCLR) were produced by reacting MCLR with sodium borohydride and 3H-sodium borohydride, respectively. Chemical purity in excess of 99% and radiochemical purity of over 98% were demonstrated with TLC, HPLC, and mass spectrometry. The labelled toxin was stable for two wk in ethanol at -20°C and significant, biological instability of the label was not apparent as indicated by the presence of tritiated water in urine of dosed animals.

Male mice were dosed ip with 2H-MCLR at 200 μg/kg or 3H-MCLR + 2H-MCLR at a total of either 100 or 200 μg/kg. At the low dose, > 90% of the radiolabel was in the liver by 60 min, and > 50% was
there at 72 hr. At the lethal dose, 50% of the label was taken up by the liver, with maximal concentrations present by 30 min. Toxin uptake at the lethal dose was higher than at the low dose, but a threshold is reached beyond which uptake is limited, probably due to liver damage. Shortly after dosing, radiolabel appears in the duodenum, suggesting biliary secretion. Elimination of radiolabel via the urine occurred only during the first 12 hr, and roughly 3.6% of the dose was eliminated by this route. Fecal elimination gradually increased over 72 hr and accounted for 34% of the dose. In other studies, 'H-2H-MCLR was rapidly taken up by isolated perfused rat livers and rat hepatocytes in suspension at 37C. Toxin uptake by hepatocytes in suspension was slowed and decreased by either incubation at 0C or rifampicin. In suspensions, 13 to 18%, and in isolated perfused livers, 0 to 7% of the radiolabel was present in plasma membrane/nuclear fractions. Over 67% of the radiolabel was present in cytoplasmic fractions. In perfused livers, roughly 15% of the label was in microsomal fractions. Precipitation of cytosolic fractions suggested that 50 to 60% of the toxin was bound to cytoplasmic protein in cells in suspension and in perfused liver, 78 to 88%. These findings suggest that uptake of 'H-2H-MCLR is energy dependent, involves a bile acid carrier, and once inside the cell, 2H-MCLR binds to a cytoplasmic protein.

Free and protected forms of 3-amino-9-methoxy-2,6,8-trimethyl-10-phenyl-4,6-decadienoic acid (ADDA), were non-toxic when administered ip at approximately 18 times the lethal dose (on a mM/kg basis) of MCLR. Alteration in or cleavage of the ADDA side group may result in effective detoxification.

Decreases in hepatic and renal perfusion of anesthetized swine given MCLR occurred within 18 and 24 min, respectively, of a lethal iv dose (72 ug/kg) as indicated by temperature pulse decay data. At the sublethal dose (25 ug/kg), hepatic and renal perfusion were reduced at 39 and 51 min. The livers of lethal dose animals were enlarged, dark red-purple, and exuded blood. Gallbladder edema was severe, and straw- to brown-colored ascitic fluid was noted. Almost 38% of the blood of high dose pigs was in the liver. Deaths of swine were associated with reduced hepatic perfusion, lethal hemorrhage into the liver, and terminal hypovolemic shock.

Human plasma cholinesterase activity and its inhibition by antx-a(s) were virtually unchanged over 9 mo of observation. The dried toxin was stored at -20C under nitrogen gas. In anesthetized rats continuously infused with antx-a(s), declines in heart rate and blood pressure occurred before large decreases in respiratory minute volume. Atropine antagonized these effects, but animals still died after about 25% more antx-a(s) than those given toxin alone. Antx-a(s)-dosed rats had increased phrenic nerve activity despite reduced tidal and minute volumes and a terminal lack of coordinated diaphragmatic electromyographic activity. Rats given artificial respiration survived 4.7 lethal doses of antx-a(s). Thus, nicotinic paralysis is of highly important in lethality.

Behavioral effects of a subclinical (0.75), threshold (1.5), or moderately toxic (3.0 ug/kg) dose of antx-a(s) were compared with those of paraoxon in rats dosed ip. Spontaneous figure 8 maze activity and responses to tactile stimuli were reduced by higher doses of antx-a(s). Accelerating rotarod performance was reduced by the highest dose of antx-a(s). The finding of behavioral decrements occurred only at doses causing clinical signs is compatible with effects in the periphery. Behavioral effects abated by 48 hr, although blood cholinesterase was still inhibited. No lesions were evident in the antx-a(s) - or paraoxon-dosed rats.
FOREWORD

In conducting research using animals, the investigator(s) adhered to the "Guide to the Care and Use of Laboratory Animals," prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources Commission on Life Sciences, National Research Council (DHHS, PHS, NIH Publications No. 86-23, Revised 1985).

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SUMMARY

Sodium borohydride was used to synthesize nonlabelled dihydro-microcystin-LR (2H-MCLR), and tritium-labelled sodium borohydride was used to produce $^{3}$H-2H-MCLR. Similarly prepared 2H-MCLR was approximately one-half as toxic as the parent compound and had induced the same qualitative toxic effects as MCLR in a previous trial. To purify either the 2H-MCLR or $^{3}$H-2H-MCLR, the reaction mixture was loaded onto C18 cartridges, rinsed with water and 10% methanol, and eluted with methanol; and the eluate was chromatographed using reversed phase HPLC with a mobile phase of dibasic potassium phosphate and methanol. The identities of the purified reaction products were confirmed by HPLC, TLC, and fast atom bombardment mass spectrometry (FABMS). The 2H-MCLR was > 99% chemically and the $^{3}$H-2H-MCLR was > 99% chemically and > 98% radiochemically pure. Chemical stability of the tritium labelled compound was established by replicate injections of the material on the HPLC. No loss of radiolabel was detected from toxin stored for 2 weeks in ethanol at -20°C.

Male mice were dosed ip with 2H-MCLR (at 200 µg/kg), $^{3}$H-2H-MCLR + 2H-MCLR (total of 100 µg/kg), or $^{3}$H-2H-MCLR + 2H-MCLR (total of 200 µg/kg) or the saline vehicle. Each mouse dosed with labelled compound was given 0.2 µCi of total radioactivity regardless of the total dose of dihydro-MCLR (labelled + nonlabelled), and the animals were killed at predetermined intervals. In a second experiment, euthanasia of mice given the lower dose occurred after time for collection of urine and feces. Biological loss of tritium to form tritiated water was < 0.01% in urine samples, indicating biological stability. Over 95% of the radiolabel from the low dose was detected in the liver within 60 minutes of dosing and over 58% remained in that organ at 72 hours postdosing, suggesting comparatively slow elimination. At the lethal
(200 µg/kg) dose, only approximately 50% of the dose was detected in the liver and peak concentrations were reached at around 30 minutes after dosing. Nevertheless, total toxin uptake was still slightly greater at the high than at the low dose. These findings seem to suggest that, after the liver has reached a threshold concentration at which massive damage is induced, further toxin uptake is greatly diminished. Shortly after dosing, toxin accumulated in the most orad portion of the small intestine in greater concentrations than in other portions of the gut suggesting biliary excretion. At the lethal dose, small amounts of radiolabel accumulated in the lungs and kidney over time, which is consistent with passage of hepatocytes and hepatic debris to the vasculature of these organs from the damaged liver as previously demonstrated. Urinary elimination was significant only during the first 12 hours postexposure, and approximately 3.6% of the dose was eliminated by this route; whereas fecal elimination over 72 hours gradually increased and accounted for 34% of the dose.

Tritiated 2H–MCLR was taken up rapidly by isolated perfused rat livers and by rat hepatocyte suspensions at 37°C. However, toxin uptake by hepatocytes in suspension was slowed and reduced by incubation at 0°C, and by pretreatment with rifampicin. In suspensions, only approximately 13 to 18%, and in isolated perfused livers 0 to 7% of the radiolabel was present in the plasma membrane/nuclear fraction. In contrast, at least two-thirds of the radiolabel was consistently present in the cytoplasmic fraction. In perfused liver, approximately 15% of the radioactivity was detected in the microsomal fraction. Trichloroacetic acid precipitation of cytosolic fractions from cells in suspension suggested that 50 to 60% of the tritiated compound was bound to cytoplasmic protein; and in perfused liver, 78 to 88% of the
radiolabel was similarly bound. Overall, these findings seem to suggest that the uptake of \(^3H\)-2H-MCLR is an energy requiring process which relies upon a bile acid carrier(s) in the hepatocyte plasma membrane and, once inside the cell, the toxin binds to a cytoplasmic protein.

The unusual C-20 amino acid, 3-amino-9-methoxy-2,6,8-trimethyl-10-phenyl-4,6-decadienoic acid (ADDA), is a constituent of all known microcystins and nodularin; and when ADDA is altered, toxicity of MCLR and nodularin is greatly diminished. However, free and protected forms of ADDA were non-toxic when administered ip at approximately 18 times (on a nM/kg equivalent basis) the lethal dose of MCLR. Thus, water treatments that cause similar alterations in or cleavage of the ADDA side group may form the basis for effective detoxification methods.

Analysis of temperature pulse decay data indicative of the perfusion of the livers and kidneys of anesthetized swine given a sublethal (25 µg/kg) or lethal (72 µg/kg) dose of MCLR indicated that hepatic perfusion was significantly decreased at 15 minutes after administration of the higher dose. Renal perfusion was significantly decreased at 21 minutes after the lethal dose. At the sublethal dose, hepatic perfusion was reduced at 39 and renal perfusion at 51 minutes postdosing. The livers of the lethal dose animals were markedly enlarged, dark red-purple, and readily exuded blood on cut surface. Edema of the gallbladder was severe, and there was straw- to brown-colored fluid in the peritoneal cavity. Liver weights (as percent of body weight), liver iron, and liver hemoglobin were significantly elevated in the high dose animals as compared to the control and toxic-sublethal groups. A mean of almost 38% of the estimated blood volume of the pigs was lost into the liver in animals of the high dose group. Acute death of swine with MCLR
toxicosis is associated with reduced perfusion of the liver, lethal hemorrhage into the damaged liver, and terminal hypovolemic shock.

The first recognized case of microcystin-LA toxicosis in the USA involved a herd of Hereford cattle in Wisconsin. The animals displayed kicking at the abdomen, intense rubbing of the abdomens against the ground, opisthotonus, dyspnea, and bright red bloody diarrhea. Hepatic lesions and serum enzyme profiles typical of microcystin toxicosis were observed in affected animals. Also, 3 to 5 days after the onset of clinical signs, photosensitization of unpigmented areas of the skin was evident. A mouse dosed ip with an algal extract died after developing lesions highly compatible with those of other microcystins such as MCLR.

The stability study of anatoxin-a(s) [antx-a(s)] was continued after 3 additional months of storage. Both human plasma cholinesterase activity and its inhibition by antx-a(s) were virtually unchanged as compared to values observed during previous trials from 0 to 6 months of storage.

In anesthetized rats continuously infused with antx-a(s), there was a rapid decline in heart rate and blood pressure, which generally occurred before a large decrease in respiratory minute volume. These findings seemed to suggest a very important role in the lethal toxicosis for muscarinic effects of the antx-a(s) on the circulatory system. A subsequent trial, however, demonstrated that atropine could counteract these effects, but the animals still died after being given only about 25% more antx-a(s) than those given toxin alone. Moreover, antx-a(s)-dosed rats consistently displayed an increase in phrenic nerve activity despite gradual reductions in tidal and minute volumes, and ultimately there was a lack of coordinated diaphragmatic electromyographic activity. In addition, rats provided artificial respiration
survived a dose of antx-a(s) that was 4.7 times the lethal dose for positive control rats not given respiratory assistance. Thus, although the muscarinic effects of antx-a(s) are of importance in the lethal toxicosis, and atropine is currently recommended, a highly important aspect in lethality from this syndrome is nicotinic paralysis of the muscles of respiration, and atropine alone is unlikely to be of great benefit when this occurs. It appears at this time that therapy for antx-a(s) toxicosis should consist of gastrointestinal evacuation, an absorbent such as superactivated charcoal, atropine, and artificial respiration.

Using rats dosed ip, the acute behavioral effects of a subclinical (0.75), threshold (1.5), or moderately toxic (3.0 μg/kg) dose of antx-a(s) were compared with those of the well-characterized central and peripheral acting organophosphorus cholinesterase inhibitor, paraoxon (400 μg/kg). Controls were given normal saline ip. Spontaneous activity in a figure 8 maze was reduced from 20 to 80 minutes postdosing with paraoxon or either of the two higher doses of antx-a(s). A significant reduction in the amplitude of the startle response to tactile stimuli was caused by either of the two higher doses of antx-a(s), but only a more modest and non-significant reduction was observed with paraoxon. Accelerating rotarod performance, which involves coordination, was significantly reduced only by the highest dose of antx-a(s) at 85 minutes postdosing. The fact that behavioral decrements with antx-a(s) occurred only at doses causing overt clinical signs is compatible with the effects of an agent that works strictly in the periphery. Behavioral responses had returned to baseline by approximately 48 hours postdosing, which seems to suggest that the animals had developed an ability to compensate for the presence of antx-a(s) or paraoxon since blood cholinesterase, with either...
antx-a(s) or paraoxon, and brain cholinesterase, with paraoxon only, were still inhibited at that time. No histologic lesions were detected in either the antx-a(s)- or paraoxon-dosed rats.

The sudden death of a dog in Illinois within 30 minutes of licking algae from its hair coat was attributed to a cholinesterase inhibiting *Anabaena*. Toxicity and peripheral cholinesterase inhibition was demonstrated in mice dosed ip with an algal extract from the implicated bloom. Recognition of spontaneous algal toxicoses in domestic animals with known exposure (such as cattle or working dogs in the field) can serve as an inexpensive and highly efficient indicator of the hazards of algal contamination of water supplies.
INTRODUCTION

We had previously proposed a method for the production of (isotopically labelled) tritiated microcystin-LR (\([^{3}H]\)-MCLR); however, when this method was utilized by New England Nuclear, the toxin was degraded (Third Annual Report, pp. 9-19). Methods for the saturation and toxicity assessment of dehydroamino acids of nodularin (First Annual Report, pp. 9-14) and MCLR (Second Annual Report, pp. 44-52; Dahlem et al., 1990) using sodium borohydride have been reported previously. These studies had indicated that nonlabeled dihydro-MCLR retained potent, selective toxicity for the liver; saturation of the double bond in the N-methylaminobutyric acid residue decreased the lethal toxicity of the compound by a factor of only 50%; and lesions induced by the dihydro-MCLR were not different from those caused by the parent compound. The goal of the present study, therefore, was to produce and characterize the fate in mice dosed ip of highly purified tritiated dihydro-microcystin-LR (\([^{3}H]\)-2H-MCLR). We also produced nonlabeled 2H-MCLR for use in diluting the radiolabelled material.

MATERIALS AND METHODS

Toxin

MCLR from \textit{Microcystis aeruginosa} strain PCC 7820 was produced in the laboratory of W. Carmichael at Wright State University in Dayton, OH. Toxin
purity was evaluated using Whatman K6F normal phase high performance silica TLC plates (Baxter Scientific Products, McGaw Park, IL; Harada et al., 1988a) and HPLC with UV detection at 238 nm (Harada et al., 1988b). Toxin was confirmed to be >99% pure by each of these methods prior to chemical reactions or administration to laboratory animals.

Other Chemicals

Tritium-labelled sodium borohydride ([3H]NaBH₄; specific activity = 14 Ci/mole) was obtained from ICN Radiochemicals (Division of ICN Biomedicals, Irvine, CA) and was determined by be >97% radiochemically pure by TLC prior to chemical reactions. All other chemicals were reagent grade or, in the case of solvents, HPLC grade.

Synthesis of Nonlabelled 2H-MCLR

Microcystin-LR (2.0 mg) was reacted with excess NaBH₄ (10 mg) in 70% 2-propanol (1.0 ml, aqueous) at room temperature (22°C) for 24 hours. The reaction was then quenched with 10% acetic acid (0.5 ml, pH 4) and evaporated to dryness in vacuo using a rotary evaporator. The reaction products were evaluated using silica TLC (MCLR Rf = 0.30; 2H-MCLR Rf = 0.31, 0.33) and a solvent development system consisting of the organic (upper phase of a mixture of ethyl acetate, 2-propanol, and water (4:3:7). Since no parent MCLR was detected, the reaction was determined to have gone to completion.

Synthesis of [3H]-2H-MCLR

A total of 2.5 mg of MCLR was reacted with [3H]NaBH₄ (12.5 mCi) in 70% 2-propanol (1.0 ml) at room temperature for 24 hours then quenched with 10% acetic acid (0.5 ml, pH 4). The reaction product was then evaporated to dryness in vacuo on a rotary evaporator.
Purification of 2H-MCLR and [\(^3\)H]-2H-MCLR

The reaction mixture in water (0.5 ml) was loaded onto preconditioned 1 g C\(_{18}\) cartridges (Analytichem International, Harbor City, CA). The reaction products were then rinsed with water (3 x 2 ml) and 10\% methanol (3 ml) and eluted with 100\% methanol (3 x 2 ml). This step removed the bulk of the excess tritium when the radiolabelled materials were used.

Isolation and purification of a single enantiomer was accomplished by reversed-phase HPLC using a Spectra Physics 8800 HPLC system equipped with a Whatman Partisphere C\(_{18}\) column (4.6 x 120 mm), a mobile phase of 0.05 M dibasic potassium phosphate and methanol (58:42, pH 3) at a flow rate of 1.0 ml/minute, and detection at 238 nm. The fraction containing the labelled dihydro-toxin was subsequently dried \textit{in vacuo} and purified using the same C\(_{18}\) method described previously to remove the buffer from the mobile phase. The 2H-MCLR obtained was determined by be > 99\% chemically pure by HPLC and TLC methods, and the [\(^3\)H]-2H-MCLR was found to be > 99\% chemically pure and > 98\% radiochemically pure by HPLC and TLC (Dahlem, 1989).

Confirmation of Reaction Products

- The identity of the purified reaction product was confirmed by low and high resolution fast atom bombardment mass spectrometry (FABMS) using a VG ZAB-SE 10 kV mass spectrometer. The compounds were mixed with magic bullet matrix (1:3, dithiotreitol-dithioerythritol) and 10\% oxalic acid (1 \(\mu\)l) and analyzed using a source temperature of 30\(^\circ\)C, and an acceleration potential of 8 kV. In low resolution FABMS, characteristic pseudomolecular ions of 995 and 997 (M + H\(^+\)) were observed for MCLR and 2H-MCLR, respectively. High resolution FABMS confirmed structural formulae of C\(_{49}\)H\(_{74}\)N\(_{10}\)O\(_{12}\) (M + H, ± 1.6 mmu) and C\(_{49}\)H\(_{76}\)N\(_{10}\)O\(_{12}\) (M + H, ± 1.6 mmu), respectively. The identity of
[\textsuperscript{3}H]-2H-MCLR was confirmed indirectly by comparison of the HPLC and TLC behaviors of the labelled compound with those of previously characterized 2H-MCLR. The chemical stability of the tritium label in [\textsuperscript{3}H]-2H-MCLR was determined by replicate injections of the toxin on the HPLC system described above at weekly intervals for 2 weeks.

Mice

Male Swiss Webster mice of 20 to 22 g were obtained from Harlan Sprague-Dawley, Inc., Indianapolis, IN. In the initial study, mice were given food and water ad libitum, allowed 1 week to become acclimated, and then randomly assigned to one of four treatment groups. Each group was dosed ip with one of the following: 2H-MCLR (200 µg/kg), \textsuperscript{3}H-2H-MCLR + 2H-MCLR (total of 100 µg/kg), \textsuperscript{3}H-2H-MCLR + 2H-MCLR (total of 200 µg/kg), or the saline vehicle. Following dosing, animals were housed individually and killed by decapitation at predetermined time intervals (N = 3/time interval). Each mouse dosed with \textsuperscript{3}H-2H-MCLR was given 0.2 uCl of total radioactivity regardless of the toxin dose.

Following euthanasia, the animals were subjected to a midline incision and fluids in the peritoneal cavity were blotted onto absorptive paper. Organs were removed, blotted dry, weighed, homogenized, and stored at -20°C. At the conclusion of the dosing phase, aliquots of tissue (approximately 100 mg) were removed and placed in preweighed glass scintillation vials, reweighed, and suspended in 500 µl of Protosol Tissue and Gel Solubilizer (New England Nuclear Research Products, Boston, MA). The tissues were then mixed on a vortexer, heated at 55°C for 18 hours with intermittent gentle shaking, and then cooled to room temperature. Samples were decolorized by addition of 100 µl of 30% hydrogen peroxide. Aquasol liquid scintillation cocktail (6 ml, New
England Nuclear, Boston, MA) was then added, and the samples were placed in the dark for 72 hours prior to counting on a Packard Tri-carb Model 300 M liquid scintillation counter (Downers Grove, IL). Cpm were converted to dpm for all calculations of organ distribution of radioactivity.

In the second study, mice were housed individually in metabolism cages (Nalge Company, Rochester, NY) and allowed to acclimate to the cages for 24 hours prior to dosing. Urine and feces were separated during the acclimation period and subsequently served as control material for the study. Animals (N = 6) were administered (3H-2H-MCLR + 2H-MCLR (100 μg/kg) and their fecal and urinary excreta collected separately at 12-hour intervals for 72 hours. Feces were weighed and urine volumes recorded. and samples were stored at -20°C until assayed. At 72 hours, animals were killed and tissues collected as described above. Urine was diluted with water to a volume of 20 ml and counted directly in liquid scintillation cocktail. Feces were diluted with water (5 ml/g feces), digested, and processed as described above for tissues. Biological stability of the tritium label was determined by evaporating subsamples of the collected urine and measuring the tritium associated with the distillate.

Statistical Analysis

Analyses of variance for a factorial design (F-ANOVA) were used to compare radiolabel in organs of the animals of the various treatment groups and to compare the radiolabel in the first 10 cm of small intestine with that in the remainder of the intestines in the two toxin-treated groups. The factors analyzed in this design were: 1) dose of radiolabeled material, 2) time interval between treatment and euthanasia, and 3) organ accumulation of radiolabel. Additional F-ANOVA analyses were used to compare the liver
weights of various treatment groups over time. When statistically significant differences between groups were found for radiolabel uptake over time, linear contrasts were used within the F-ANOVA analyses to compare the radioactivity of livers and small intestines of various treatment groups at specific times. A level of $\alpha = 0.05$ was chosen a priori to detect statistically significant differences between groups of treated animals.

**RESULTS**

**Toxins**

The treatment of MCLR with NaBH$_4$ and subsequent purification by HPLC yielded 1.4 mg of 2H-MCLR. The conversion of MCLR to the tritiated product yielded 0.24 mg of purified labeled product with a specific activity of 5.3 $\mu$Ci/µmol.

No detectable spontaneous loss of radiolabel was noted in toxin stored at a concentration of 1 µg/µl in 0.1% ethanol and a temperature of -20°C for 2 weeks. Biological loss of radioactivity to tritiated water was < 0.01% in all of the urine samples analyzed.

**Distribution Study**

The maximum dose of toxin which had been uniformly sublethal in preliminary studies (100 µg/kg) was compared with the minimum dose of toxin which had been uniformly lethal (200 µg/kg) in animals (whether given either labeled or unlabeled toxin). The calculated total amounts of toxin equivalents and the concentrations of toxin equivalents in each organ at the two doses evaluated are shown in Table 1. The fractions of the doses of toxin present in each organ over time are shown in Figure 1. Greater than 99% of the low dose and > 90% of the high dose were accounted for by the 60-minute time point.
The liver accumulated the radiolabel over time with > 95% of the low dose found in the liver within 60 minutes of toxin administration. The majority of the toxin (58.0% ± 5.2%) remained in the liver of low dose animals up to 72 hours after toxin administration. By contrast, the high dose animals accumulated only approximately 50% of the toxin in the liver, and the uptake appeared to result in peak concentrations at approximately 30 minutes postdosing. Although the relative percent of administered radioactivity was less, the concentrations of toxin equivalents in the liver were significantly higher in the livers of the 200 µg/kg group than the 100 µg/kg group at both 30 and 60 minutes. The mean relative liver weights of the high dose group were also significantly higher than the low dose group at 60 minutes (8.9% ± 0.8 versus 6.7% ± 0.6; mean ± standard deviation), thus the total toxin uptake at the lethal dose was even higher than reflected in the concentration figures.

At most time points, the second highest concentration of toxin equivalents was found in the first 10 cm of the small intestine (the most oral 10 cm of small intestine). Radiolabel in this portion of intestine was initially elevated, then tended to decline, and finally became elevated again over time. As compared to the remainder of the small intestine, significantly higher concentrations of label were found in the first 10 cm of the small intestine at 10, 30, and 60 minutes in both groups given the radiolabeled toxin.

Concentrations of toxin equivalents became elevated in the kidneys and lungs of the high dose animals at the 60-minute time point. The relative amounts of the label present in the kidneys increased from < 2% of the administered dose to > 4% between 30 and 60 minutes, and the lung concentrations increased from less than 0.3% to greater than 0.8% in the same time period.
Elimination Study

In the second study, measurable radioactivity was found in the urine of toxin-dosed animals for only the first 12 hours after administration (Table 2). In animals dosed at 100 μg/kg, urine volumes were uniformly less than 100 μl at the 12-hour collection point. Thereafter, urine volumes increased to predosing levels of 0.5 to 1.0 ml at every 12-hour sampling interval for the duration of the observation period. Although fecal elimination of radiolabel from toxin was minimal during the first 12 hours after dosing, cumulative elimination in feces exceeded 34% of the administered dose over 72 hours. As urinary elimination decreased, fecal elimination increased and was maintained at a relative high level throughout the 72-hour observation period.

DISCUSSION

The reaction of tritium-labeled sodium borohydride with MCLR forms a mixture of diastereomers which are epimeric at the α-carbon of the dehydro amino acid. Tritium is also incorporated at the α-carbon. Two principal reduction products are formed, and they were separated from each other and the parent (unreacted) toxin by HPLC. One of the diastereomers (the one with the shortest retention under the HPLC conditions described) retains approximately one-half of the original toxicity of the parent compound. The toxic effects of this diastereomer and the time course of toxicity were found to be virtually identical to the parent compound in related studies (Dahlem et al., 1989a). Unfortunately, the configuration of the specific isomeric structure used in these studies could not be directly determined.

A steep threshold for toxicity is observed for MCLR between doses which cause minimal toxic effects and acutely lethal doses (First Annual Report,
This threshold was also observed in animals treated with 2H-MCLR in preliminary experiments. The doses compared in this study were selected in order to evaluate the accumulation of toxin in mouse tissues at doses that bracket this critical threshold of toxicity.

The liver is the major target organ for microcystins (Falconer et al., 1981; Hooser et al., 1989). In the present study, the liver accumulated the majority of the doses, and a large concentration of radiolabel remained in the liver for up to 72 hours after toxin administration. A larger percentage of the dose of toxin reached the liver in the sublethal group, but the lethal group accumulated higher absolute concentrations of the compound at the 30-minute time point than the sublethal group did even at 60 minutes. This suggested that either saturation of sites of uptake occurred or damaged hepatic cells had lost their ability to take up additional toxin. The toxic effects which often culminate in lethal intrahepatic hemorrhage in heavily exposed animals are likely to be initiated by a threshold intracellular concentration of algal peptide toxin.

It appeared that the liver played a major role in the elimination of the toxin. The high concentrations of radioactivity in the first 10 cm of the small intestine over the observation period suggested that biliary excretion had occurred. The presence of high concentrations of radioactivity in the feces but not in the urine is also compatible with excretion via the bile, an important route of elimination. The high concentrations of label found in the intestines at early time points in both groups is likely to have been a result of intraperitoneally administered toxin that adhered to the intestine prior to absorption.
The increase in concentration of toxin in the kidneys and lung tissue of the lethal treatment group at the 60-minute time point correlates with lesions seen in these organs in separate studies (Hooser et al., 1989). The arterioles of the kidneys and lung contained eosinophilic debris at the 60-minute time point that on light and electron microscopy was highly suggestive of intact or fragmented degenerative or necrotic hepatocytes. The increase in the radioactivity associated with these organs is therefore presumed to be secondary to the hepatotoxic effects of the lethal dose of 2H-MCLR, rather than as a primary result of direct binding.

Most previous studies with labeled algal peptide hepatotoxins have had significant shortcomings. They have failed to: determine the structure of the labeled compound, identify the specific location of the label(s) within the peptide structure, or establish the stability of labeled compounds (Falconer et al., 1986; Runnegar et al., 1986; Brooks and Codd, 1987). Absence of such information diminishes the assurance of validity of any studies of toxin fate. The microcystins are a diverse group of heptapeptides and determination of the specific toxin used in distribution studies is essential if comparisons are to be made with other members of this group. In this study, the identity of the microcystin used and the site of label incorporation were established.

Brooks and Codd (1987) found that accumulation of label in the liver reached 75% of the administered dose in sublethally treated groups within 1 minute of toxin administration by the intraperitoneal route. In our study, accumulation was dose dependent and did not reach 75% of the dose in sublethal groups until more than 30 minutes after toxin administration. Runnegar et al. (1986) did not find accumulations of 125I-labeled peptide in the liver at
concentrations higher than 63% of the administered dose, either when animals died acutely or when killed at the end of a 24-hour observation period. Perhaps the differences among these reports and the present study may be explained by differential absorption of the various toxin(s) investigated associated with differences in polarity of the compounds. Regardless of differences in the absolute uptake or time course of accumulation, all of the investigations reported to date identify the liver as the target organ, and the site where the majority of the toxin accumulates.

Fecal elimination of toxin began within 12 hours of dosing and continued through the 72-hour observation period. Passage of feces was slightly decreased in the first 12 hours but increased to predosing rates within 24 hours of dosing. It is not known whether the reduction in fecal output was due to stress caused by handling the animals in order to dose them or a response to the toxin.

Indirect evidence exists for the involvement of a bile acid transport system(s) in the uptake of microcystin into isolated hepatocytes (Runnegar et al., 1981). Our studies are also compatible with a role for bile acid transport systems in the uptake of MCLR from the ileum (Dahlem et al., 1989b). Biliary excretion appears to be important in removing toxin from the liver since toxin appears rapidly in the duodenum of toxin-treated animals and is presumably transported there via bile. It is not known whether the toxin is excreted unchanged or in a conjugated form and this will be the subject of a future investigation. Whether or not enterohepatic recirculation occurs remains to be demonstrated.

It is concluded from these studies that: 1) saturation of the dehydroportion of microcystin-LR with tritium-labeled sodium borohydride is practical
and the resultant material retains toxicity and organ specificity, 2) the labeled toxin has reasonable chemical and biological stability, 3) the liver is the target organ for toxicity, 4) accumulation of toxin by the liver is rapid and elimination occurs slowly probably via the bile, 5) accumulation of toxin in the liver is a dose-dependent phenomenon and hepatic toxin uptake reaches a maximum after administration of a lethal dose, 6) accumulation of radiolabel from the toxin occurs in the lungs and kidneys to a greater degree when lethal doses are administered, which is compatible with passage of toxin bound to hepatocytes and hepatic debris from the damaged liver, and 7) toxin initially leaves via the urine but soon this virtually ceases and fecal elimination predominates.
Table 1. Mean tissue concentrations of toxin equivalents based on tritium-labeled dihydro microcystin-LR (± SD) over time. N = 3 animals/treatment/time in all groups with the exception of the 4,320-minute group where N = 6.

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Dose (µg/kg)</th>
<th>Time (min)</th>
<th>Total toxin equivalents (µg/organ)</th>
<th>Concentration of toxin equivalents (µg/g)</th>
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<td>0.09 ± 0.01</td>
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<td>0.04 ± 0.01</td>
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<td>Total toxin equivalents (µg/organ)</td>
<td>Concentration of toxin equivalents (µg/g)</td>
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<td>----------------</td>
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<td>-----------------------------------</td>
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<td>0.28 ± 0.07</td>
<td>0.41 ± 0.14</td>
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<tr>
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<td>0.17 ± 0.06</td>
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<td>0.92 ± 0.20</td>
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Table 1. Continued

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<th>Tissue</th>
<th>Dose (µg/kg)</th>
<th>Time (min)</th>
<th>Total toxin equivalents (µg/organ)</th>
<th>Concentration of toxin equivalents (µg/g)</th>
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<td>0.12 ± 0.02</td>
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<tr>
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<td></td>
<td>4,320</td>
<td>0.01 ± 0.00</td>
<td>0.12 ± 0.02</td>
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<td>4,320</td>
<td>0.01 ± 0.00</td>
<td>0.12 ± 0.02</td>
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</tbody>
</table>

*Mean ± standard deviation.
Table 2. Elimination of tritium-labeled dihydro microcystin-LR as a function of time in the urine and feces of intraperitoneally dosed mice.

<table>
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<tr>
<th>Sample</th>
<th>Time</th>
<th>Percent of Dose*</th>
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</thead>
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<td>Urine</td>
<td>0 to 12 hours</td>
<td>3.63 ± 2.25</td>
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<tr>
<td></td>
<td>12 to 24 hours</td>
<td>&lt; 0.10</td>
</tr>
<tr>
<td></td>
<td>24 to 36 hours</td>
<td>&lt; 0.10</td>
</tr>
<tr>
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<td>36 to 48 hours</td>
<td>&lt; 0.10</td>
</tr>
<tr>
<td></td>
<td>48 to 60 hours</td>
<td>&lt; 0.10</td>
</tr>
<tr>
<td></td>
<td>60 to 72 hours</td>
<td>&lt; 0.10</td>
</tr>
<tr>
<td>Feces</td>
<td>0 to 12 hours</td>
<td>2.51 ± 2.37</td>
</tr>
<tr>
<td></td>
<td>12 to 24 hours</td>
<td>7.19 ± 6.48</td>
</tr>
<tr>
<td></td>
<td>24 to 36 hours</td>
<td>6.89 ± 6.77</td>
</tr>
<tr>
<td></td>
<td>36 to 48 hours</td>
<td>9.70 ± 9.35</td>
</tr>
<tr>
<td></td>
<td>48 to 60 hours</td>
<td>13.87 ± 5.51</td>
</tr>
<tr>
<td></td>
<td>60 to 72 hours</td>
<td>13.16 ± 12.87</td>
</tr>
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</table>

*Mean ± standard deviation.
Figure 1. Percent of the administered sublethal (100 μg/kg) (squares) or lethal (200 μg/kg) (triangles) labeled and nonlabeled toxin in mouse tissues over time. N = 3 animals - treatment - 1 - time - 1.
II. TOXICITY OF FREE AMINO DECA DIENOIC ACID (ADDA) AND A PROTECTED DERIVATIVE THEREOF

A. M. Dahlem, M. Namikoshi, K. L. Rinehart, and V. R. Beasley

ABSTRACT

The toxicity of two forms of one of the substituent amino acids, 3-amino-9-methoxy-2,6,8-trimethyl-10-phenyl-4,6-decadienoic acid (ADDA), a component in the structures of both the heptapeptide hepatotoxin, microcystin-LR (MCLR), and the pentapeptide hepatotoxin, nodularin, was investigated. These derivatives were formed synthetically and the toxicities of purified products were compared with that of parent MCLR by mouse bioassay. The component and its "protected" derivative were without apparent toxicity when evaluated by mouse bioassay at the doses tested.

INTRODUCTION

Microcystin-LR (MCLR) is the predominant toxin produced by Microcystis aeruginosa strain PCC-7820 (Dahlem et al., 1989a). This hepatotoxin has been isolated from field collections and laboratory isolates (Botes, et al., 1985; Harada et al., 1988a,b) and its complete structure, including the absolute stereochemistry, has recently been established (Rinehart et al., 1988) (Figure 1).

Nodularin is a cyclic pentapeptide produced by the cyanobacteria Nodularia spumigena Mertens emend. L57S from New Zealand (Carmichael et al., 1988b). The structure of this compound has also been recently elucidated (Figure 1) and found to share many structural similarities with MCLR (Rinehart et al., 1988). The structures of both MCLR and nodularin have in common: the unusual hydrophobic amino acid, 3-amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4,6-dienolic acid (ADDA); two acidic amino acids in the stereospecific "D"
configuration, which are involved in peptide linkages not involving the α-carboxyl; L-arginine; and one dehydro amino acid.

The toxicities of purified MCLR and nodularin were compared in related studies and found to be nearly identical despite the fact that nodularin contains two fewer amino acids (Carmichael et al., 1988b; Dahlem et al., 1989a). It therefore appears that the D-alanine and L-leucine contained in MCLR contribute little to the toxic action of these molecules since their absence in nodularin did not notably alter the observed toxicity.

Other shared structural similarities were then investigated in order to find out which portions of the molecules were essential for the observed potent toxicity. In separate studies, the roles of the dehydro amino acids in MCLR and nodularin in the hepatotoxicity of these compounds were evaluated by mouse bioassay after selective hydrogenation of these components with sodium borohydride (Dahlem et al., 1989a). In those studies, saturation of the dehydro portion of the molecule modestly increased the lethal dose (reduced toxicity) but did not alter the hepatospecificity or clinical signs associated with the parent toxins. It was concluded that the dehydro amino acids contributed to the toxicity but were not essential for the toxic and hepatospecific properties of these compounds.

In related studies, the toxicity of an MCLR derivative which had the bulk of the hydrophobic portion of ADDA removed, but which retained the cyclic peptide nature of the compound, was investigated (Third Annual Report, pp. 42-48). In the first of these experiments, the toxin was subjected to ozonolysis, then reduced with excess sodium borohydride to yield a cyclic peptide structure which lacked all points of unsaturation. Additional MCLR was reduced with sodium borohydride to form the dihydro derivative which
results from hydrogen addition to the N-methyl dehydroalanine, then subjected to ozonolysis and reduced again with sodium borohydride to yield another cyclic product which lacked points of unsaturation. These derivatives were without apparent toxicity when bioassayed in mice at doses in excess of 16 times the LD100 of the parent compound (on a nM/kg basis).

The portions of the molecules which are likely to contribute to the overall toxicity of these compounds include: 1) the D-amino acids and the isolinkages of the acidic amino acids which probably retard normal enzymatic degradation, 2) the absence of N and C terminal amino acids due to the cyclic nature of the compound which is also likely to retard enzymatic degradation, and 3) the unusual hydrophobic amino acid ADDA. In the studies described in this report, toxicity of synthetic ADDA in the absence of other components of the molecule was investigated.

MATERIALS AND METHODS

ADDA was synthesized and confirmed to have the same stereochemistry as the natural product (Namikoshi and Rinehart, unpublished data). A protected derivative was also formed (Figure 2). The toxicities of both the free amino acid and the protected amino acid (Table 1) were then compared with parent MCLR by mouse bioassay. Male Swiss Webster mice (Harlin Sprague-Dawley, Indianapolis, IN) were used in all toxicity trials. Animals were dose ip with either parent toxin, appropriate toxin derivative, or saline vehicle and monitored for 24 hours. Animals which survived the observation period were killed by cervical dislocation. Survival times were recorded along with the whole body, liver, and kidney weights. Tissues (liver, kidneys, spleen, small intestine, large intestine, heart) were examined grossly for lesions. Sections of tissue were then fixed by immersion in 10% neutral buffered
formalin for histologic examination. After fixation, the sections were routinely processed, embedded in paraffin, cut at 4 to 6 µm, and stained with hematoxylin and eosin.

RESULTS

ADDA by itself in either the protected or the deprotected form did not demonstrate the lethal potential associated with either MCLR or nodularin. Animals which survived for 24 hours after toxin, derivative, or vehicle administration did not display any gross or histologic lesions associated with MCLR, and organ weights were not significantly different than controls. Positive control animals dosed with 100 µg/kg of MCLR had elevated liver weights and marked hepatic changes associated with MCLR including: severe wide-spread centrilobular and midzonal hepatocyte dissociation and rounding, degeneration, and necrosis of hepatocytes with massive intralesional hemorrhage.

DISCUSSION

Alterations explored to date in the dehydro amino acids and variation in the L-amino acids of algal cyclic peptide hepatotoxins seem to result in modest alterations in toxicity and do not change the hepatospecificity or the qualitative character of the toxic manifestations of the molecule (First Annual Report, pp. 9-14, Second Annual Report, pp. 44-52). Alterations in the ADDA moiety, however, caused marked reductions in both hepatic damage and lethal toxicity (First Annual Report, pp. 14-15, and Third Annual Reports, pp. 42-48). These data seem to indicate that ADDA in its normal configuration is essential for potent hepatotoxicity of microcystins. ADDA is likely to be important in the bioactivity of these compounds for one of two reasons. Either the hydrophobic portion of ADDA in the correct stereospecific
configuration is important for receptor recognition for incorporation of the toxin into the hepatocyte, or the hydrophobic portion of this unusual amino acid has a direct toxic effect. However, the present study strongly suggests that ADDA must function in concert with other components of the molecule for the principal toxic effects of algal cyclic peptide hepatotoxins to occur.
Table 1. Comparative toxicity of microcystin-LR, synthetic ADDA, and its protected derivative.

<table>
<thead>
<tr>
<th>Toxin</th>
<th>LD_{100} (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Microcystin-LR</td>
<td>90 nM/kg</td>
</tr>
<tr>
<td>Protected ADDA*</td>
<td>&gt; 1,600 nM/kg</td>
</tr>
<tr>
<td>ADDA (Free Animo Acid)*</td>
<td>&gt; 1,600 nM/kg</td>
</tr>
</tbody>
</table>

*ADDA = 3-amino-9-methoxy-2,6,8-trimethyl-10-phenyl-4,6-decadienolic acid.
Figure 1. Structures of microcystin-LR (a) and nodularin (b).
Figure 2. Structures of: 1) free amino decadienoic acid (ADDA), \([R = R' = H]\), and 2) protected ADDA, \([R = \text{CH}_2\text{CH}_3; R' = \text{CO}_2\text{CH}_2\text{C}_6\text{H}_5]\).
III. UPTAKE AND SUBCELLULAR LOCALIZATION OF TRITIATED DIHYDRO-MICROCYSTIN-LR IN PERFUSED RAT LIVERS AND RAT HEPATOCYTE SUSPENSIONS

S. B. Hooser, M. S. Kuhlenschmidt, A. M. Dahlem, and W. M. Haschek

ABSTRACT

Microcystin-LR (MCLR) is a cyclic heptapeptide hepatotoxin (MW = 994) produced by the blue-green alga, *Microcystis aeruginosa*. The uptake of tritiated-dihydro-microcystin-LR ($^3$H-2H-MCLR) in perfused liver and in suspensions of hepatocytes at 0°C, 37°C, and with pretreatment of rifampicin at 37°C and the subcellular distribution of $^3$H-2H-MCLR in these same groups were evaluated. Suspensions of hepatocytes were incubated with $^3$H-2H-MCLR at 0°C, 37°C, or following pretreatment with rifampicin and aliquots removed at various times for determination of uptake. Cells from each group were disrupted mechanically and subfractionated using sucrose gradient centrifugation. Similar suspensions of hepatocytes were solubilized in Triton X-100 following incubation with toxin and ultracentrifuged to pellet the detergent insoluble (actin-containing) fraction. Isolated livers were perfused with $^3$H-2H-MCLR, the uptake determined, the livers homogenized, and subcellular fractions collected. Uptake of $^3$H-2H-MCLR was rapid in isolated perfused livers and hepatocytes in suspension at 37°C but was slowed when hepatocyte suspensions were incubated at 0°C or pretreated with rifampicin. In all groups, 65 to 77% of the radiolabel in the cells was in the cytosolic fraction, and in suspensions, 13 to 18% was present in the plasma membrane/nuclear fraction with lesser amounts in the other fractions. In perfused liver, approximately 15% of the radiolabel in the cells was in the microsomal fraction and 0 to 7% in the plasma membrane/nuclear fraction, with lesser...
amounts in other fractions. Trichloroacetic acid treatment of all cytosolic fractions for cells in suspension indicated that approximately 50 to 60% of the $^{3}$H-$^{2}$H-MCLR was bound to cytosolic protein. In perfused liver, 78 to 88% of the radiolabel precipitated out of cytosolic fractions indicating that it was bound to cytosolic protein. These data suggest that the uptake of $^{3}$H-$^{2}$H-MCLR takes place via an energy-dependent, hepatocyte bile acid carrier and, once inside the hepatocyte, the toxin binds to cytoplasmic protein.

**INTRODUCTION**

Microcystin-LR (MCLR) is a cyclic heptapeptide hepatotoxin (MW = 994) produced by the blue-green alga, *Microcystis aeruginosa*. Toxic blooms of this organism in ponds and water reservoirs have been associated with numerous livestock death and implicated epidemiologically in human illness (Konst et al., 1965; Falconer et al., 1983; Jackson et al., 1984; Galey et al., 1987).

MCLR is specifically hepatotoxic. In rats and mice, it causes rapid, progressive, centrilobular hepatocyte rounding, disassociation, and necrosis, with breakdown of the sinusoidal endothelium. This results in massive intrahepatic hemorrhage and death (Runnegar and Falconer, 1982; Hooser et al., 1990a). In vitro, MCLR causes plasma membrane blebbing in hepatocytes but not in sinusoidal endothelial or Kupffer cells (see Third Annual Report, pp. 49-76). In cultured hepatocytes treated with MCLR, there is aggregation of actin filaments which corresponds with plasma membrane blebbing (see Third Annual Report, pp. 49-76; Hooser et al., 1990b). However, no change in the ratio of polymerized to unpolymerized actin in MCLR-treated hepatocytes has been reported (Runnegar and Falconer, 1986; Eriksson et al., 1989).

Radiolabeled microcystins are rapidly taken up by the liver. Within 30 minutes of $^{125}$I-labeled microcystin being given iv to rats, 21.7% of the
radioactivity was localized in the liver with no more than 5.6% in any other organ (Falconer et al., 1986). Following ip injection of microcystin containing biosynthetically incorporated $^{14}$C into mice, 75.8% of the label was reportedly localized in the liver after 1 minute. After 180 minutes, 88.1% of the label was found in the liver (Brooks and Codd, 1987).

The reasons for hepatocyte specificity and the mechanism of action of MCLR have not been fully elucidated (Frimmer, 1982; Weiland et al., 1984). It has been suggested that MCLR is transported into hepatocytes by a hepatocyte-specific bile acid carrier. When suspensions of isolated hepatocytes were preincubated with noncompetitive inhibitors of these carriers (rifampicin and bromsulfphthalein) (Ziegler and Frimmer, 1986), there were marked reductions in MCLR-induced plasma membrane blebbing (Runnegar et al., 1981; Runnegar and Falconer, 1982). Taken collectively, the results of these studies seem to suggest that MCLR is indeed transported into hepatocytes by a hepatocyte-specific bile acid carrier and, once internalized, interacts with or in some other way causes the aggregation of actin filaments.

The objectives of this study were to: 1) determine and compare the rate and extent of uptake of $^{3}$H-$^{2}$H-MCLR in hepatocytes at 0°C and 37°C with and without pretreatment with rifampicin and in isolated, perfused liver; 2) determine the intracellular localization of $^{3}$H-$^{2}$H-MCLR using subcellular fractionation of hepatocytes and isolated, perfused liver; and 3) determine if $^{3}$H-$^{2}$H-MCLR binds directly to hepatocyte proteins, particularly those of the cytoskeleton.

MATERIALS AND METHODS

Animals

Male, 175 to 250 g, Sprague-Dawley rats (Harlan Sprague-Dawley, Inc., Indianapolis) were allowed to acclimate for 2 weeks or longer before use.
Animals had free access to a commercial laboratory animal ration and water and were maintained on a 12-hour light/12-hour dark cycle. Rats were not fasted prior to use.

Toxin

MCLR from *M. aeruginosa* strain PCC-7820 was produced and purified (approximately 95% pure) in the laboratory of W. W. Carmichael using previously described techniques (Krishnamurthy et al., 1986). The toxin was dissolved in phosphate-buffered saline, pH = 7.4, prior to use.

Synthesis and purification of dihydro-microcystin (2H-MCLR) and tritium-labeled dihydro-microcystin-LR was carried out as described in Chapter I of this section.

Hepatocyte Isolation

Rat hepatocytes were isolated using a modification of a previously described technique (Third Annual Report, pp. 49-76; Kuhlenschmidt et al., 1982).

Uptake of Tritiated-Dihydro-Microcystin-LR in Hepatocytes

Uptake of $^3$H-2H-MCLR was measured in suspensions of hepatocytes at 0°C and 37°C with or without a 10-minute preincubation with rifampicin at 50 μg/ml. Aliquots of hepatocytes in complete Ham's F-10 were centrifuged at 70 x g for 2 minutes and the pellets were resuspended in 2.5 ml of Ham's F-10 media without fetal bovine serum (FBS) but with insulin (incomplete media) to a final concentration of 1.5 x 10^7 hepatocytes/ml, placed in capped suspension culture tubes, and incubated at either 0°C or 37°C under constant end-over-end rotation at 6 rpm. For rifampicin preincubation, rifampicin in PBS was added to the suspended hepatocytes to a final concentration of 50 μg/ml and incubated for 10 minutes before addition of toxin. Cold 2H-MCLR (37.5 μg) and
$^{3}$H-2H-MCLR (12.5 μg) were added to phosphate-buffered saline (PRS) so that the final toxin concentration was 20 μg/ml. This was then added to the suspensions of hepatocytes resulting in a final toxin concentration of 10 μg/ml. Incubation was carried out at 0°C or 37°C. The specific activity of $^{3}$H-2H-MCLR used was 2.2 x $10^{5}$ cpm/μg.

At time 0, at 20, 40, and 60 seconds, and at 2, 4, 6, 8, 10, 15, and 30 minutes, two 100-μl aliquots of each suspension were removed, layered over 500 μl of DC550 (Dow Corning) oil:mineral oil (5:1) and microfuged for 15 seconds to separate cells containing toxin from cell-free toxin in the supernatant. Each supernatant (50 μl) was mixed in 10 ml of liquid scintillation cocktail (LSC). The remaining supernatant and oil from each tube were removed and 100 μl of 1% Triton X-100 (TX-100) was mixed with each pellet until solubilized. The 100-μl pellet in TX-100 was placed in 10 ml of LSC. All supernatants and pellets in LSC were counted with a Packard 460 Tri-Carb scintillation counter. Following removal of the two 100-μl aliquots at 30 minutes, all suspension cultures were placed in an ice bath for no more than 1 hour until subcellular fractionation was begun.

**Uptake of Trinitiated-Dihydro-Microcystin-LR into Isolated, Perfused Liver**

Two rats were anesthetized and their portal veins catheterized in a manner identical to that used to isolate hepatocytes, except that the flow rate of perfusate was 25 ml/minute (Berg et al., 1988). Once catheterized, the livers were perfused for 15 minutes with Ham's F-10 media containing (per 500 ml) 17% FBS, 0.3 ml of insulin, and 1.2 g HEPES buffer. In previous studies using the same strain of rats (see Second Annual Report, pp. 44-52), it was determined at 2H-MCLR has a minimum LD$_{100}$ approximately four times that of MCLR (180 μg/kg). Thus, based on the minimum LD$_{100}$ of 2H-MCLR, a dose
approximately equivalent to 720 μg/kg (2 parts of cold 2H-MCLR:1 part
\(^{3}\text{H}-2\text{H}-\text{MCLR}) was added to 100 ml of Ham's F-10 media (with FBS, insulin, and
HEPES buffer). Following a 15-minute perfusion with Ham's F-10 without toxin,
the perfusate was changed to the 100 ml of Ham's F-10 containing toxin; this
was drained from the liver to allow recirculation of the toxin-containing
media. For the first liver, one 200-μl aliquot was removed from the container
of recirculating media at 0, 20, 40, and 60 seconds, 2, 4, 6, 8, and 10
minutes, and then every 5 minutes for 120 minutes. Each aliquot was placed in
5 ml of LSC and counted. At 120 minutes, the liver was perfused with 100 ml
of Ham's F-10 media without toxin, and then placed on ice in 0.25 M sucrose
for subsequent subcellular fractionation. At 15, 30, 45, and 120 minutes,
small pieces of liver were tied off with surgical suture, removed, and fixed
in 10% neutral-buffered formalin for histologic examination.

Since the maximum uptake of toxin was at 45 minutes following the
beginning of perfusion with \(^{3}\text{H}-2\text{H}-\text{MCLR}, in the second liver, perfusion with
toxin was ended at 45 minutes and liver samples for histologic examination
were taken at 0, 15, 30, and 45 minutes.

Subcellular Fractionation of Isolated Hepatocytes and Perfused Liver
Preparations

Fractionation was performed on the perfused livers and the hepatocyte
suspensions. The sucrose gradient, subcellular fractionation method of Reid
and Williamson (1974) was used. This technique yields 5 fractions: crude
nuclear (which also contains plasma membrane fragments), heavy mitochondrial,
light mitochondrial (which also contains lysosomes), microsomal, and
cytosolic. The only modification was in the preparation of isolated
hepatocytes. Hepatocytes were pelleted at 70 xg for 2 minutes, resuspended in
cold 0.25 M sucrose, and disrupted by passage through a 26-gauge needle 20 to 40 times. Disruption was verified by microscopic examination. All pellets were resuspended in 1 ml of PBS and aliquots placed in Aquasol II (which contains detergent) for scintillation counting.

Detergent Extraction of Isolated Hepatocytes following Uptake of Tritiated-Dihydro-Microcystin-LR

Suspensions of hepatocytes in incomplete Ham's F-10 media were prepared as described above under "uptake of \(^3\)H-2H-MCLR in hepatocytes." Cold 2H-MCLR (37.5 µg) and \(^3\)H-2H-MCLR (12.5 µg) were added to PBS for a total toxin concentration of 20 µg/ml. This was then added to the 2.5 ml of hepatocyte suspension so that the final concentration of toxin was 10 µg/ml. These suspensions were incubated at 37°C for 30 minutes and then placed on ice. The cells were pelleted by centrifugation at 70 x g for 2 minutes at 4°C.

The supernatant was removed and frozen, and 10 ml of 1% TX-100 extraction buffer (1% Triton X-100, 10 mM EDTA, 0.1 M Tris-Cl, pH = 7.4) was mixed with the cell pellet. This mixture was placed on ice and allowed to solubilize for 2 hours with periodic agitation. At the end of 2 hours, the solubilized cells in TX-100 extraction buffer were centrifuged at 150,000 x g for 45 minutes at 4°C in a Beckman L5-50 ultracentrifuge (Fox et al., 1984). The TX-100 soluble supernatant was removed and saved for scintillation counting. The insoluble pellet (containing actin filaments) was added to 1.5 ml of 3N HCl and warmed until dissolved. To this was added an equal amount of 3M KOH, and the samples were saved for scintillation counting.

Trichloroacetic Acid Precipitation of Cytoplasmic Fractions

Two hundred microliters (200 µl) of 30% trichloroacetic acid (TCA) was thoroughly mixed in 1.5-ml microfuge tubes with 400 µl of: 1) pure \(^3\)H-2H-MCLR
in PBS (8,900 cpm/ml), 2) cytosolic fractions from the perfused livers, or 3) cytosolic fractions from the 6 isolated hepatocyte suspensions used in uptake studies (0°C and 37°C with or without rifampicin pretreatment) for a final TCA concentration of 10%. These were immediately placed on ice for 1 hour and then microfuged for 10 minutes. Five hundred microliters (500 µl) of supernatant was placed in 4.5 ml of LSC and counted. The pellets were mixed with 500 µl of Aquasol II and placed in an oven overnight at 53°C to solubilize. A small amount of detergent insoluble material remained. The samples were again microfuged for 10 minutes and the Aquasol soluble supernatant removed and placed in 4.0 ml of LSC for counting. The insoluble pellet was mixed with 250 µl of 3M KOH and dissolved overnight at 53°C. Two hundred fifty microliters (250 µl) of 3N HCl was added for neutralization, and the entire 500 µl was added to the 4.5 ml LSC (containing the Aquasol soluble supernatant) for scintillation counting.

Histologic Examination

The samples of liver from the liver perfusion studies were fixed in 10% neutral-buffered formalin, embedded in glycol methacrylate, sectioned at 2 µ, and stained with hematoxylin and eosin. Histologic examination was performed using an Olympus BH-2 microscope.

RESULTS

Uptake of Tritiated-Dihydro-Microcystin-LR

The uptake of 3H-2H-MCLR in both suspensions of hepatocytes and perfused liver was very rapid. At 37°C, the uptake by hepatocytes in suspension was rapid for the first 4 minutes, after which it remained high but began to level off at about 30 minutes (Figure 1). At this time, the average amount of radioactivity taken up by the 100-µl aliquot of hepatocytes (9 x 10^5
hepatocytes/100 µl) was 1,752 cpm. Since the $^{3}$H-2H-MCLR was one-fourth of the total amount of toxin, this was equivalent to an uptake of 31.2 ng (31.4 pmole) of total toxin per 9 x 10⁵ hepatocytes. A maximum mean of 1,800 cpm was observed at 30 minutes. This would be equivalent to an uptake of 32.1 ng (33.3 pmole) of total toxin per 9 x 10⁵ hepatocytes.

The incubation of hepatocytes at 0°C or preincubation with rifampicin at 37°C markedly slowed the rate and reduced the total uptake of $^{3}$H-2H-MCLR, although it did not completely prevent it (Figure 1). After 30 minutes of incubation at 0°C, the uptake of $^{3}$H-2H-MCLR was reduced by 50% when compared to incubation at 37°C. Preincubation with rifampicin reduced the uptake by 64%. During the first 4 minutes, the rate of uptake at 37°C was 287 cpm/minute, but at 0°C and with rifampicin, the rate was reduced to 93 and 54 cpm/minute, respectively.

In perfused liver at 37°C, the uptake of $^{3}$H-2H-MCLR was equally as rapid. In the first experiment (duration of 120 minutes), there was rapid uptake of toxin for the first 10 minutes, then uptake plateaued (there was no additional uptake) for approximately 40 minutes. This was followed by a release of radioactivity from the liver into the perfusion medium beginning at 50 minutes until the end of the experiment (Figure 2a). At the time of maximum hepatic uptake (50 minutes), there were 3.9 µg equivalents of $^{3}$H-2H-MCLR in this liver, which weighed 11.58 g. Since the $^{3}$H-2H-MCLR was one-third of the total toxin in the media, this was equivalent to an uptake of 1 µg (1 nmole) of total toxin per gram of liver. Assuming 1.2 x 10⁸ hepatocytes per gram of liver (Doust, 1958), this is equivalent to an uptake of 7.6 ng (7.7 pmole) of total toxin per 9 x 10⁵ hepatocytes.
The second experiment was technically superior to the first with good perfusion throughout. Uptake in this liver was even more rapid, with near maximal uptake reached at 4 minutes after the beginning of the perfusion with $^{3}$H-2H-MCLR (Figure 2b). After 4 minutes, uptake virtually stopped and the radioactivity in the perfusion media remained constant until 40 minutes when hepatic radioactivity began to decrease (i.e., radioactivity in media began to increase) (Figure 2b). At the time of maximal uptake (40 minutes), there was the equivalent $11.8 \mu g$ of $^{3}$H-2H-MCLR present in this 12.62-g liver. This is equivalent to an uptake of $2.8 \mu g$ (2.8 nmole) of total toxin per gram of liver or $21.0 \text{ng}$ (21.2 pmole) of total toxin per $9 \times 10^{5}$ hepatocytes.

**Histologic Examination**

Almost all hepatocytes incubated with $^{3}$H-2H-MCLR at 37°C for 30 minutes had numerous large plasma membrane blebs. In contrast, < 50% of hepatocytes preincubated with rifampicin or incubated at 0°C for 30 minutes had multiple large plasma membrane blebs.

In the perfused livers, microscopic lesions were seen beginning 30 minutes after perfusion with $^{3}$H-2H-MCLR was begun (Figure 3). Initial lesions were characterized by hepatocyte disassociation and rounding. This was followed by fragmentation and necrosis of hepatocytes. In the severe lesions, necrosis was observed periportally and midzonal while hepatocyte dissociation was present centrilocularly.

**Subcellular Fractionation**

In suspension cultures, the majority of radioactivity remained in the medium and was not incorporated into hepatocytes. Incubation of hepatocytes at 37°C resulted in uptake of 2.5% of the $^{3}$H-2H-MCLR from the medium, which was 31 and 34% higher uptake than at 0°C (1.7%) and with rifampicin (1.6%).
respectively. In the hepatocytes (2.8 ml) which were fractionated in the 37°C study, this amounts to 24.8 ng (25 pmole) of total toxin taken up per 9 x 10^5 cells (Table 1), which is similar to the amount calculated from the uptake studies.

When fractionated, the largest percentage of radioactivity was present in the cytosolic fraction, followed by the nuclear/plasma membrane fraction. In hepatocytes incubated at 37°C, 70 and 13.5% were found in the cytosolic and nuclear/plasma membrane fractions, respectively (Table 1 and Figure 4). Although the total uptake of 3H-2H-MCLR was reduced at 0°C or with rifampicin pretreatment, the percentages of total cellular radioactivity found in the various fractions were similar to those found in fractions from the hepatocytes incubated at 37°C. With rifampicin pretreatment, 63% was found in the cytosolic fraction and 18% in the nuclear/plasma membrane fraction. At 0°C, the percentages in the cytoplasmic and nuclear/plasma membrane fractions were 66 and 17%, respectively.

In liver perfused for 45 minutes, 13% of the radioactivity was taken up by the liver. This is equivalent to an uptake of 10.5 ng (10.6 pmole) of total toxin per 9 x 10^5 hepatocytes. When fractionated, 78% of the radiolabel was present in the cytosolic fraction with 1 and 16% present in the nuclear/plasma membrane and microsomal fractions, respectively. After perfusion for 120 minutes, 11% of the radioactivity was incorporated into the liver. This is equivalent to an uptake of 6.7 ng (6.7 pmole) of total toxin per 9 x 10^5 hepatocytes. When fractionated, 71% of the radiolabel was in the cytosolic fraction, with 7 and 16% present in the nuclear/plasma membrane and microsomal fractions, respectively (Table 2 and Figure 5).
Detergent Extraction of Isolated Hepatocytes following Uptake of Tritiated-Dihydro-Microcystin-LR

The detergent-soluble fraction contained 97% of the radioactivity associated with the cells, while only 2.5% was associated with the insoluble pellet.

Trichloroacetic Acid Precipitation

When purified $^3$H-2H-MCLR was treated with TCA, 92% of the radioactivity remained in the supernatant. In the cytosolic fraction of hepatocytes incubated at 37°C for 30 minutes, 59% of the $^3$H-2H-MCLR associated radioactivity was present in the precipitated pellet and 41% in the supernatant (Table 2 and Figure 5). In the cytosolic fractions of the hepatocytes preincubated with rifampicin or of those incubated at 0°C, approximately 50% of the radioactivity was present in the precipitate and 50% remained in the supernatant.

The precipitated pellet of the cytosolic fraction of the liver perfused for 45 minutes contained 88% of the radiolabel, while the supernatant contained 12%. In the liver perfused for 120 minutes, the protein bound fraction was slightly reduced since 79% was found in the precipitate and 21% in the supernatant (Table 2 and Figure 5).

DISCUSSION

The uptake of $^3$H-2H-MCLR is rapid both in hepatocyte suspension and in isolated perfused liver. After 4 to 10 minutes, the rapid uptake plateaus. Additional uptake of toxin may be inhibited due to: 1) disruption of actin filaments and/or their plasma membrane connections, 2) an effect on the plasma membrane carrier itself, 3) another MCLR-intracellular protein interaction which then secondarily affects the carrier (or receptor), 4) saturation of
hepatocyte receptors, or 5) establishment of a steady-state where uptake rate is equally balanced by toxin efflux or degradation followed by efflux of the labelled fragment. The release of radioactivity by liver perfused with toxin for 120 minutes is probably due to the severe cellular necrosis which is present by 45 minutes after the beginning of perfusion with $^{3}$H-$^{2}$H-MCLR. It is also possible that some of the release could be due to biliary excretion of the toxin. However, this is less likely because it has been shown that bile flow ceases within 45 minutes after initiating perfusion with a toxic dose of microcystins (Berg et al., 1988).

Incubation of hepatocytes at 0°C reduces the rate and total amount of $^{3}$H-$^{2}$H-MCLR uptake. This suggests that the uptake of $^{3}$H-$^{2}$H-MCLR into hepatocytes requires cellular energy (Frimmer, 1982). Preincubation of hepatocytes with rifampicin causes an even greater reduction in the uptake of $^{3}$H-$^{2}$H-MCLR presumably through noncompetitive inhibition of a hepatocyte bile acid carrier that also transports microcystins (Ziegler and Frimmer, 1986). This is in agreement with the studies of Runnegar (Runnegar and Falconer, 1982) in which pretreatment of hepatocytes in suspension with rifampicin prevented or greatly reduced microcystin-induced plasma membrane blebbing. These studies associate the observed reduction in blebbing with a reduction in $^{3}$H-$^{2}$H-MCLR uptake.

The uptake of $^{3}$H-$^{2}$H-MCLR into hepatocytes at 37°C or into perfused liver was only 2 or 13%, respectively, of the total in the media. When calculated on a nanogram/hepatocyte basis, however, the uptake in each system was very similar. Although the amount of toxin taken up is very small, it is enough to be of biological significance since the morphologic changes in the hepatocytes and in the perfused liver are identical to those seen with unlabeled MCLR.
Even though uptake is slower, morphologic changes eventually occur at 0°C or with rifampicin preincubation. Thus, it appears that it is necessary for a critical amount of microcystin to enter the cell before damage occurs and morphologic alterations are seen, and the requisite amount of toxin is extremely small.

Although the lesions observed in liver perfused with $^3$H-$^2$H-MCLR were identical to those we and others have described with MCLR in vivo, the distribution of the lesions within the hepatic lobule differed in that the earliest and the most severe changes were located periportally rather than centrilobularly (Runnegar and Falconer, 1982; Hoosar et al., 1990a). Since centrilobular hepatic necrosis was observed in mice given 2H-MCLR (see Second Annual Report, pp. 44-52), the altered lesion distribution in this study may have been a response to changes in perfusion or oxygen tension due to the artificial perfusion system used.

In all hepatocyte suspensions and in the perfused livers, the majority of intracellular radioactivity was found in the cytosolic fraction; a smaller percentage was found in the plasma membrane/nuclear fractions. Based on these studies, it is uncertain if the $^3$H-$^2$H-MCLR is associated with the plasma membrane or the nucleus. It may be primarily associated with the plasma membrane bile acid carrier because at 0°C and with rifampicin pretreatment, the percentage in the plasma membrane/nuclear fraction was increased at the expense of the cytosolic fraction; this may be associated with the slower uptake. Almost no radioactivity was observed in the plasma membrane/nuclear fraction in the perfused liver fractionated after 45 minutes. There was substantially more radiolabel associated with the microsomal fraction in
perfused liver than in suspension. This may represent greater metabolism, modification, or other interaction with the endoplasmic reticulum.

When the cytoplasmic fractions from cells exposed in suspension were treated with trichloroacetic acid, 40 to 50% of the radiolabel was found in the supernatant, presumably as free or degraded toxin. However, 50 to 60% of the 3H-2H-MCLR was in the precipitated pellets suggesting it was bound to cytosolic protein. From cells exposed in isolated, perfused liver, even greater amounts (78.5 and 88.3%) were present in the precipitate; this may simply reflect increased cytosolic binding due to the later times of fractionation (45 and 120 minutes) as compared to the cell suspensions (30 minutes).

Based on the detergent-extraction experiments, which separate out insoluble actin and other detergent insoluble elements, it appears unlikely that 3H-2H-MCLR perferentially binds to actin. This is unlike phallolidin which causes toxicosis through direct binding to actin filaments (Weiland, 1977). Previous studies have suggested that microcystins do not cause a change in the polymerization state of actin in toxin-treated hepatocytes (Runnegar and Falconer, 1986; Eriksson et al., 1989). Nevertheless, MCLR does cause morphologic alterations in the actin filaments of hepatocytes in culture and in vivo (see Third Annual Report, pp. 49-76). Therefore, it is possible that MCLR and microcystins in general interact with or otherwise activate a cytoplasmic protein (or proteins) which causes cross linking of actin filaments which are already polymerized; thus the polymerization state would remain constant. Alternatively, MCLR may bind to a cytoplasmic protein which interacts with one of the many proteins responsible for actin-plasma membrane binding. Either mechanism may result in disruption of the attachments of
actin to the plasma membrane and collapse of the microfilaments into the interior of the cell.

The data in the present study, together with that from previous experiments (Runnegar and Falconer, 1982; Weiland et al., 1984; Hooser et al., 1989, 1990a,b), supports the hypothesis that $^3$H-2H-MCLR is taken into hepatocytes by an energy-driven, hepatocyte-specific bile acid carrier. Once inside the hepatocyte, the toxin appears to bind to cytoplasmic protein that directly or indirectly causes aggregation of polymerized actin filaments, plasma membrane alterations, breakdown of the hepatic architecture, hemorrhage, and eventual death of the animal.
### TABLE 1. Hepatic uptake of $^{3}$H-2H-MCLR.

The percent uptake of radioactivity in suspended cells and perfused livers and the percentage of the cellular radioactivity in each fraction.

<table>
<thead>
<tr>
<th>Sample and Treatment</th>
<th>[3H]-2HMC-LR Uptake (percent)</th>
<th>Cell Fractions</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Medium</td>
<td>Cells</td>
</tr>
<tr>
<td><strong>Hepatocyte Suspensions</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>37°C</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>± 0.2</td>
<td>± 0.2</td>
</tr>
<tr>
<td>Rif</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>± 0.1</td>
<td>± 0.1</td>
</tr>
<tr>
<td>0°C</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>± 0.4</td>
<td>± 0.4</td>
</tr>
<tr>
<td><strong>Liver Perfusion</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Liver 1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>120 min</td>
<td>89.5</td>
<td>10.5</td>
</tr>
<tr>
<td>Liver 2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>45 min</td>
<td>86.9</td>
<td>13.1</td>
</tr>
</tbody>
</table>
Table 2. Effect of TCA on solubility of cytosolic $^3$H-2H-MCLR.

<table>
<thead>
<tr>
<th>Sample and Treatment</th>
<th>Supernatant (cpm)</th>
<th>Pellet (cpm)</th>
<th>Supernatant (percent)</th>
<th>Pellet (percent)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Purified $^3$H-2H-MCLR</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0°C</td>
<td>1433.4 ± 7.5</td>
<td>118.0 ± 21.4</td>
<td>92.4 ± 1.3</td>
<td>7.6 ± 1.3</td>
</tr>
<tr>
<td>37°C</td>
<td>143.6 ± 1.7</td>
<td>239.8 ± 83.1</td>
<td>40.9 ± 7.1</td>
<td>59.1 ± 7.1</td>
</tr>
<tr>
<td>Rifampicin</td>
<td>107.3 ± 9.8</td>
<td>108.5 ± 17.6</td>
<td>49.7 ± 6.4</td>
<td>50.3 ± 6.4</td>
</tr>
<tr>
<td>0°C</td>
<td>127.9 ± 30.6</td>
<td>122.7 ± 1.4</td>
<td>51.0 ± 6.4</td>
<td>49.0 ± 6.4</td>
</tr>
<tr>
<td>Liver Perfusion</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Liver 1 120 min</td>
<td>87.0</td>
<td>318.4</td>
<td>21.5</td>
<td>78.5</td>
</tr>
<tr>
<td>Liver 2 45 min</td>
<td>118.9</td>
<td>900.2</td>
<td>11.7</td>
<td>88.3</td>
</tr>
</tbody>
</table>

Aliquots of pure and cytosolic $^3$H-2H-MCLR were treated with TCA as described in Materials and Methods. The data are expressed as the amount (cpm) of radioactivity and percentage found in TCA soluble and pellet fractions.
Figure 1. Kinetics of uptake of $^3$H-2H-MCLR by hepatocytes in suspension. Each point represents mean of four replicates. Bar = SEM. (O-O), uptake at 37°C; (Δ-Δ), uptake at 0°C; (○-○), uptake at 37°C by rifampicin-treated hepatocytes.
Kinetics of uptake of $^3$H-2H-MCLR by perfused liver. Data are expressed as total radioactivity in the liver at each time point.

A. Liver #1; B. Liver #2.
Figure 3. 
a. Central vein and surrounding hepatocytes of a control rat liver perfused with media. Bar = 100 µm.
b. Central vein and surrounding hepatocytes of a rat liver perfused for 45 minutes with media containing \(^{3}\)H-\(^{2}\)H-MCLR. Prominent rounding and disassociation of hepatocytes is present. Bar = 100 µm.
c. Portal vein and surrounding hepatocytes of a rat liver perfused for 45 minutes with media containing \(^{3}\)H-\(^{2}\)H-MCLR. In addition to hepatocyte rounding and disassociation, there is prominent hepatocyte necrosis characterized by cell fragmentation as well as nuclear pyknosis and karyorrhexis. Bar = 100 µm.
Figure 4. Subcellular distribution of $^3$H-2H-MCLR. The data are presented as the percentage of radioactivity found in each subcellular fraction of hepatocytes incubated with $^3$H-2H-MCLR at 37°C, 0°C, or 37°C following preincubation with rifampicin (Rifam); and in the two rat livers perfused for 45 minutes (45' Perf) or 120 minutes (120' Perf) with media containing $^3$H-2H-MCLR. PM/NUC, plasma membrane plus nuclear fraction; H. MIT., heavy mitochondrial fraction; L. MIT., light mitochondrial fraction; MICRO, microsomal fraction; CYTO, cytosolic fraction.
Figure 5. Effect of TCA on the solubility of $^3$H-2H-MCLR in cytosolic fractions. The percentage of radioactivity in supernatants and pellets was determined following treatment of purified $^3$H-2H-MCLR and cytosolic fractions with 10% trichloroacetic acid. Cytosolic fractions were obtained from hepatocytes incubated with $^3$H-2H-MCLR at 37°, 0°, or 37°C following preincubation with rifampicin (Rif) and from two rat livers perfused for 45 minutes (45') or 120 minutes (120') with media containing $^3$H-2H-MCLR.
INTRODUCTION

Information on the goals and selected findings of this study have been reported previously in the Second (pp. 96-122) and the Third Annual Reports (pp. 77-100). Here we describe grossly evident lesions and provide data on organ weights, liver iron and hemoglobin concentrations, as well as on statistical analysis of the hepatic and renal perfusion data.

MATERIALS AND METHODS

Postmortem Observations

Gilts still alive 5 hours after dosing were killed by exsanguination, and a complete gross examination was performed. The liver, kidneys, adrenals, and spleen were removed and weighed. The renal capsule was removed and adipose tissue around the adrenal, kidney, and spleen was excised prior to weighing. The gallbladder was considered part of the liver weight. Portions of the liver (> 100 g) were frozen at -5°C until the study was complete and then iron (Oshelm and Ross, 1985) and hemoglobin concentrations were determined.

Statistical Analysis

A nested analysis of variance patterned on a univariate repeated measures analysis (Wilkinson, 1988) was used to identify significant differences (α = 0.05) in the organ perfusion data due to treatment, time, or treatment×time interaction. The number of surviving animals in the lethal dose group decreased with time which prevented the use of a multivariate
repeated measures analysis. Prior to statistical analysis, all postdosing values were subtracted from the predose mean of that animal to obtain the corrected response values. The model used was: corrected response (corresp) = constant + treatment (trt) + subjects in control group (sub1) + subjects in toxic-sublethal dose group (sub2) + subjects in lethal dose group (sub3) + time + treatment*time interaction (trt*time).

Comparisons between treatment groups at postdosing time points were performed using linear contrasts at a level of $\alpha = 0.05$ (Wilkinson, 1988), except at the time points where only one pig was alive in the lethal dose group. These included the 186-, 198-, 210-, 222-, 236-, 248-, 260-, 272-, 284-, and 294-minute time points for organ perfusion parameters. The denominator of the $F$ statistic in these contrasts was the error (time) term in the nested ANOVA analysis. At time points where only one pig was alive in the lethal dose group, a one-way analysis of variance (Wilkinson, 1988) was used to compare the lethal dose group with the other two groups. Due to the small sample size and a desire to increase power, $\alpha$ was set at 0.15 in these analyses. A one-way analysis of variance (Wilkinson, 1988) was used to identify statistically significant ($\alpha = 0.05$) differences between treatment groups for organ weights (percent of live body weight) and liver iron and hemoglobin concentrations.

RESULTS

Hemodynamic and Organ Perfusion Effects

Mean hepatic perfusion (Figure 1) in the lethal dose group was significantly less than that of controls at 15 minutes (12- to 18-minute mean) after MCLR administration. At 21 minutes (18- to 24-minute mean), mean renal
perfusion (Figure 2) was significantly decreased in the lethal dose group as compared to controls. Similarly, the hepatic and renal perfusion of the lethal dose group were first significantly decreased in comparison to the toxic-sublethal dose group at 15 and 21 minutes, respectively.

The organ perfusion parameters of the toxic-sublethal dose group exhibited the same direction of change as in the lethal dose group, but these changes took longer to become significantly different (liver perfusion = 39 minutes; renal perfusion = 51 minutes) and were not of as great a magnitude.

**Gross Lesions**

The most striking gross lesions were in the livers of the lethal and toxic-sublethal dose groups. The livers of the lethal dose group were markedly swollen, dark red-purple, and readily exuded blood on cut surface. There was severe edema of the gallbladder, excessive straw- to brown-colored peritoneal fluid, slight to moderate subendocardial hemorrhage, and a pale carcass. The livers in the toxic-sublethal dose group varied from slightly mottled to dark red and exuded blood from the cut surface. Slight to moderate edema of the gallbladder and increased peritoneal fluid was present in the three pigs of this group with the most marked hepatic lesions. No compound-related lesions were evident in the control group.

Significant increases in liver weight (or percent body weight), liver iron, and liver hemoglobin were present in the lethal dose group when compared to the toxic-sublethal dose or control groups. The kidney weights of the toxic-sublethal dose group were significantly higher than those of the lethal dose group and nearly significantly more than in the control group \( p = 0.051; \) Table 1). No difference in spleen or adrenal weights was detected between groups.
DISCUSSION

The temperature pulse decay data showed that organ perfusion is one of the first parameters affected following MCLR administration. Perfusion of the liver decreased more rapidly than in the kidneys in both the lethal and toxic-sublethal groups which provides further support for a primary MCLR effect on the liver. The perfusion to these vital organs did not return to near predose levels for 5 hours postdosing in either the lethal or toxic-sublethal dose group, while in the controls, liver and kidney perfusion increased. These data indicate that the liver and kidney are deprived of normal perfusion following a lethal or toxic-sublethal dose of MCLR and that local hypoxic changes are likely, particularly in animals exposed to lethal doses.

The decreases in renal perfusion and mild renal damage are the most likely cause(s) for the significant increases documented in serum urea nitrogen, creatinine, and inorganic phosphorus (Third Annual Report, pp. 77-100). Renal tubular nephrosis has been reported previously in several species (Ashworth and Mason, 1946; Louw and Smit, 1950; Konst et al., 1965; Edler et al., 1985; Hooser et al., 1989; Lovell et al., 1989) and is probably secondary to hypoxia in part due to hepatic emboli, although direct microcystin effects cannot be ruled out.

Gross findings in the livers of the pigs in this study were similar to those previously described in other species. In most previous reports, histologic findings included centrilobular to panlobular hemorrhage and hepatocyte necrosis. Similar lesions in the livers of microcystin-dosed pigs (Second Annual Report, p. 28) probably account for the increase in mean portal venous pressure and the decline in mean central venous and aortic pressures.
(Third Annual Report, pp. 77-100) and contributed to the terminal reduction in hepatic perfusion.

Mild pulmonary edema, straw-colored and/or serosanguinous fluid in the peritoneal and/or thoracic cavities, enteritis often with hemorrhage, marked distension of the gallbladder with edema often involving the adjacent tissues, and wide-spread petechial and/or ecchymotic hemorrhaging have been documented in many reports on exposures of animals to hepatotoxins from *Microcystis* spp. Gross evidence of extrahepatic hemorrhage and enteritis was not observed in pigs in this study. This apparent lack of agreement with some of the field reports might be explained by differences between iv dosing with purified toxin versus ingestion of intact algal cells, the time courses of the toxicoses, and/or the species of animals involved.

Marked increases in liver weight (> 8% of total body weight) in mice following parenteral microcystin administration are well documented. This finding is used as part of the criteria to establish the hepatotoxicity of cyanobacterial blooms (Beasley et al., 1989). Sasner et al. (1984) estimated that 36% of the blood volume of lethally dosed mice was present in the liver within 3 hours of microcystin exposure. Assuming that the estimated blood volume of swine is 8.23% (Bossone and Hannon, 1985) and that this blood contains 11 g of hemoglobin (Hb)/dl, then the estimated blood volume lost in the liver of the average pig in the lethal group was 37.3% after accounting for normal liver blood volume (control group). This conclusion is based on the following computation:

\[
\frac{((4.40\% \times 9.19 \text{ g Hb/dl}) - (2.43\% \times 2.53 \text{ g Hb/dl})}{(8.23\% \times 11 \text{ g Hb/dl})} \times 100 = 37.9\%
\]

where the 4.40% and 2.43% represent the mean liver weight (percent live body weight), and the 9.19 and 2.53 g Hb/dl represent the mean concentrations of
hemoglobin in liver of the lethal and control groups, respectively. The acute loss of 25 to 30% of the estimated blood volume leads to death in 50% of animals experiencing rapid hemorrhage (Phyllis, 1976). The higher liver Hb and Fe concentrations and significantly increased (23.6%) liver weight in the toxic-sublethal group versus controls are suggestive of a lesser degree of intrahepatic hemorrhage in this group (Table 1).

In conclusion, the hemodynamic, blood-gas, clinical pathology (Third Annual Report, pp. 77-100), organ perfusion, and gross findings of the present study indicate that the liver is the target organ for MCLR. Acute death is caused by hypovolemic shock that results from partial blockage of blood flow through the liver and accumulation of approximately 38% of the estimated blood volume in the liver.
FOOTNOTE

aDMA Cyanmethemoglobin Procedure, Data Medical Associates, Inc., Arlington, TX.
Table 1. Liver and kidney weights (% live body weight) and liver hemoglobin and iron concentrations in swine intravenously administered a normal saline vehicle (N = 4) or a toxic-sublethal (25 µg/kg; N = 5 or 6) or lethal (72 µg/kg; N = 6) dose of microcystin-LR.

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Toxic-Sublethal</th>
<th>Lethal</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liver weight (mean ± SD)</td>
<td>2.43 ± 0.22a</td>
<td>3.00 ± 0.149b</td>
<td>4.40 ± 0.298c</td>
</tr>
<tr>
<td>(range)</td>
<td>2.15 to 2.68</td>
<td>2.87 to 3.26</td>
<td>4.02 to 4.77</td>
</tr>
<tr>
<td>Kidney weight (mean ± SD)</td>
<td>0.247 ± 0.0148a,b</td>
<td>0.271 ± 0.0344b</td>
<td>0.231 ± 0.0151a</td>
</tr>
<tr>
<td>(range)</td>
<td>0.233 to 0.272</td>
<td>0.208 to 0.314</td>
<td>0.209 to 0.256</td>
</tr>
<tr>
<td>Liver Hb (mean ± SEM)</td>
<td>2.5 ± 0.33a</td>
<td>3.3 ± 0.73a</td>
<td>9.2 ± 1.30b</td>
</tr>
<tr>
<td>(range)</td>
<td>1.9 to 3.4</td>
<td>1.9 to 5.7</td>
<td>5.4 to 13.2</td>
</tr>
<tr>
<td>Liver iron (mean ± SEM)</td>
<td>34 ± 33.5a</td>
<td>112 ± 68.8a</td>
<td>331 ± 125.8b</td>
</tr>
<tr>
<td>(range)</td>
<td>28 to 106</td>
<td>46 to 226</td>
<td>200 to 492</td>
</tr>
</tbody>
</table>

SD = standard deviation.
SEM = standard error of the mean.
Liver Hb = liver hemoglobin and is expressed in g/dl.
Liver iron is expressed in parts per million.
Means with different letters are significantly different (p < 0.05).

Liver iron is expressed in parts per million.
Means with different letters are significantly different (p < 0.05).
Figure 1. Six- and 12-minute means ± standard error of the mean of the change from the predose mean for liver perfusions in gilts intravenously administered a lethal 72 µg/kg; N = 6 and decreases with time) or toxic-sublethal (25 µg/kg; N = 6) dose of microcystin-LR or the normal saline vehicle (N = 4).

Predose Mean ± SEM

<table>
<thead>
<tr>
<th>Dose (µg/kg)</th>
<th>Mean ± SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.0</td>
<td>2.65 ± 0.530</td>
</tr>
<tr>
<td>25</td>
<td>2.53 ± 0.140</td>
</tr>
<tr>
<td>72</td>
<td>3.17 ± 0.228</td>
</tr>
</tbody>
</table>

*Indicates the first time point where the lethal group is significantly different than both the control and toxic-sublethal groups (p < 0.05).

#Indicates the first time point where the toxic-sublethal group is significantly different than controls (p < 0.05).
Figure 2. Six- and 12-minute means ± standard error of the mean of the change from the predose mean for kidney perfusions in gilts intravenously administered a lethal (72 µg/kg; N = 6 and decreases with time) or toxic-sublethal (25 µg/kg; N = 6) dose of microcystin-LR or the normal saline vehicle (N = 4).

*Indicates the first time point where the lethal group is significantly different than both the control and toxic-sublethal groups (p < 0.05).

#Indicates the first time point where the toxic-sublethal group is significantly different than controls (p < 0.05).
V. SUMMARY REPORT OF THE FIRST RECOGNIZED CASE OF 
MICROCYSTIN-LA (MCLA) TOXICOSIS IN THE USA; 
EFFECTS OF MCLA ON MICE DOSED INTRAPERITONEALLY


ABSTRACT

Hepatotoxicosis due to microcystin-LA (MCLA) was diagnosed in a herd of 60 Hereford cattle. Algal material was obtained from the bloom and submitted to Dr. Wayne Carmichael for algal identification and analysis for algal hepatotoxins. The organism was identified as *Microcystis aeruginosa*, and microcystin-LA was found to be the predominant toxin. The two-letter suffix designates the identity of the two variant L-amino acids found in all cyclic heptapeptide hepatotoxins examined to date. The suffix "LA" stands for leucine and alanine (Carmichael et al., 1988a).

CASE STUDY

The herd of 60 Hereford cattle had access to an algal bloom of the blue-green algae *Microcystis aeruginosa* during August 1987 in a 2.5-acre pond in Eau Claire, Wisconsin. All of the forty 11-month-old to 6-year-old heifers and cows in the herd were affected, but none of the twenty 4- to 6-month-old calves showed evidence of being poisoned.

The primary clinical sign in the cattle was abdominal pain as indicated by animals kicking at their abdomens and intensely rubbing their abdomens against the ground. Opisthotonus, dyspnea, and bright red, bloody diarrhea were also observed, and 3 to 5 days after the onset of clinical signs, photosensitization occurred.

Gross lesions in the only animal that died, a 450-kg heifer, included reduced size and black discoloration of the liver, approximately 4 liters of
brownish-orange fluid in the peritoneal cavity, and petechial hemorrhages in the abomasal mucosa.

Clinical pathologic examination of serum from one affected animal was consistent with severe liver damage: serum activities of aspartate aminotransferase, gamma glutamyl transpeptidase, creatinine phosphokinase and lactate dehydrogenase, and concentrations of total bilirubin, direct bilirubin, and indirect bilirubin were markedly increased, while serum concentrations of calcium and phosphorous were decreased.

Oral and intraperitoneal (ip) mouse bioassays were performed using algae and algal extracts from the pond. Only the mouse dosed ip with an algal extract died. The liver of this animal was enlarged, purple-black in color, and friable on cut section.

The algae was identified as M. aeruginosa. Chemical analysis (Harada et al., 1988b) of field-collected algal material revealed MCLA. Mice dosed with purified toxin had grossly evident liver lesions similar to those observed with extracts. The liver from one mouse dosed ip with purified toxin and examined histologically had severe centrilobular and midzonal disassociation and rounding up of hepatocytes with individual cell necrosis and hemorrhage.

The structure of MCLA, originally called BE-4 and later cyanoginosin LA, was first reported by Botes from South Africa during 1982 to 1986 and was the first Microcystis hepatotoxin to be structurally characterized. The source of the toxin from which Botes determined the structure was a natural bloom of M. aeruginosa that occurred in Hartbeespoort Dam, South Africa, in 1974. To our knowledge, this is the first time MCLA toxicosis has been recognized in North America.
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ANATOXIN-A(S)

I. CONTINUATION OF STABILITY STUDY OF ANATOXIN-A(S)

W. O. Cook

INTRODUCTION

The study described in the Third Annual Report, pp. 111-127, was extended for an additional 3 months.

MATERIALS AND METHODS

The materials and methods employed were described in the Third Annual Report.

RESULTS

There was no appreciable loss in either the activity of the human plasma cholinesterase enzyme or its inhibition by anatoxin-a(s) over the 9 months of monitoring (Table 1).
Table 1. Results of assays run after 0, 1, 2, 3, 6, and 9 months of storage to determine the stability of anatoxin-a(s) using human plasma cholinesterase in vitro.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Month</th>
<th>Mean (mmole/l/min)</th>
<th>SD</th>
<th>% CV</th>
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N = 27 for all assays.
II. EFFECTS OF ANATOXIN-A(S) ON BLOOD PRESSURE, HEART RATE, RESPIRATORY RATE, TIDAL VOLUME, MINUTE VOLUME, AND PHRENIC NERVE ACTIVITY IN RATS

W. O. Cook, G. A. Iwamoto, D. J. Schaeffer, and V. R. Beasley

ABSTRACT
The effects of anatoxin-a(s) [antx-a(s)], an organophosphorus cholinesterase inhibitor from the cyanobacterium Anabaena flos-aquae NRC-525-17, on mean arterial blood pressure, heart rate, respiratory rate, tidal volume, minute volume, and phrenic nerve activity were evaluated in Sprague-Dawley rats. Anatoxin-a(s) was administered to anesthetized animals by continuous iv infusion. The initial effect of the toxin was to decrease the heart rate and reduce the arterial blood pressure. These early effects were followed by severe reductions in the same two parameters. The marked decline in heart rate and blood pressure frequently occurred before there was a large decrease in respiratory minute volume, suggesting that antx-a(s) exerts an important muscarinic action on the cardiovascular system in vivo. Although phrenic nerve amplitude increased, there was a progressive decrease in tidal and minute volumes. These findings indicate that antx-a(s), unlike many organophosphorus compounds which inhibit cholinesterase, does not exert its lethal effect via depression of centrally mediated respiratory drive.

INTRODUCTION
Anatoxin-a(s) [antx-a(s)] is a highly polar (as indicated by solubility only in polar solvents such as water, methanol, and ethanol), low-molecular-weight (< 400 daltons), alkaloid organophosphorus cholinesterase (ChE)-inhibiting neurotoxin produced by the blue-green alga Anabaena.
flos-aquae strain NRC-525-17 (Carmichael and Gorham, 1978; Mahmood and Carmichael, 1986, 1987). A. flos-aquae has been associated repeatedly with poisoning of domestic and wild animals (Carmichael and Gorham, 1978; Carmichael et al., 1985) and is known to produce additional neurotoxins as well as a cyclic peptide hepatotoxin (Carmichael, 1988). Deaths of dogs, pigs, and ducks in field cases have been diagnosed as having been due to ingestion of ChE-inhibiting neurotoxins from Anabaena flos-aquae (Mahmood et al., 1988; Cook et al., 1989a).

The lethal effect of low-molecular-weight anticholinesterase agents is often attributable to respiratory failure arising from effects on the central nervous system (de Candole et al., 1953; Rickett et al., 1986; Beers et al., 1987). Measurement of phrenic nerve activity is an effective means of discerning peripherally versus centrally mediated respiratory failure (de Candole et al., 1953; Wright, 1954; Rickett et al., 1986). Clinical signs in mice and rats given antx-a(s) include dyspnea, seizures, and death from apparent respiratory failure (Mahmood and Carmichael, 1986; Cook et al., 1988). In vitro, antx-a(s) has been demonstrated to cause direct muscarinic stimulation and indirect nicotinic and muscarinic stimulation via inhibition of ChE and build-up of acetylcholine (Hyde and Carmichael, 1988, 1989). Mice dosed ip with antx-a(s) at 0.5 or 1.0 mg/kg body weight after pretreatment with atropine sulfate ip at 50 mg/kg had longer survival times than mice given toxin alone and had no parasympathetic signs of toxicosis, but the animals still died (Mahmood and Carmichael, 1986). Following ip administration of antx-a(s), no inhibition of whole brain ChE activity of mice was observed, suggesting that the toxin is unable to cross the blood-brain barrier (Cook et al., 1988).
The present study of antx-a(s) assessed the occurrence of: 1) effects of the toxin on centrally mediated respiratory drive by monitoring phrenic nerve potentials, respiratory rate, and tidal volume; and 2) effects on heart rate and mean arterial blood pressure.

MATERIALS AND METHODS

Animals

Four male Sprague-Dawley rats (478 ± 35 g) were housed in air-conditioned quarters on a 12-hour light/12-hour dark cycle and provided food and water ad libitum. Animals were evaluated after being anesthetized and were not allowed to recover from anesthesia.

Anesthesia, Surgical Procedures, and Physiological Measurements

Anesthesia was induced by halothane and subsequently maintained by ip administration of a mixture of alpha chloralose (Aldrich Chemical, Milwaukee, WI) at 60 mg/kg and urethane (Aldrich Chemical, Milwaukee, WI) at 800 mg/kg prior to surgical procedures. This regimen produced stable anesthesia throughout the study. The temperature of rats during surgical procedures was maintained via a heating pad.

From a ventral midline incision in the cervical area of each rat, the trachea was dissected free, incised, and intubated with a 14-gauge, 6.4-cm Teflon catheter (Longdwell, Becton Dickson, Rutherford, NJ). Silk sutures placed around the trachea secured the catheter and provided an air-tight seal. By further dissection ventrally and laterally, the right carotid artery was isolated, separated from the vagus, ligated, and catheterized with PE 50 polyethylene tubing (Clay Adams, Parsippany, NJ). The carotid catheter was employed for measurement of arterial blood pressure. The left jugular vein was isolated by lateral dissection from the initial incision and cannulated
with PE 50 tubing such that the tip of the catheter was in the anterior vena cava. The jugular and arterial catheters were kept patent by flushing with heparinized Ringer's solution.

From the initial incision, dissection caudally to the clavicle at the base of the neck was used to expose the right phrenic nerve where it diverged from the fifth cervical nerve. The phrenic nerve was transected and the cranial end laid across bipolar silver hook electrodes for recording action potentials. The phrenic nerve preparation was stabilized by tightly suturing the surrounding skin to a solid ring support. This construction also formed a pocket such that the phrenic nerve and electrodes could be continually maintained in a pool of mineral oil. The phrenic nerve signal was amplified using a Grass P511 AC preamplifier and a bandpass of 100 Hz-10 KHz. The signal was full wave rectified and integrated (Gould integrator) using a reset time of 0.033 seconds (integrate sample/hold).

Tidal volumes were determined from flow measurements made with a Fleisch 0000 pneumotachograph (OEM Medical, Richmond, VA) attached to the tracheal catheter and a Valdyne 45/16 transducer (Northridge, CA). In order to reduce the dead space and ensure sufficient oxygenation of the animal, medical grade air was blown (0.2 l/minute) into the tracheal catheter by way of a three-way connector thereby providing a bias flow of air. Heart rate, blood pressure, phrenic nerve activity, and tidal volume signals were recorded on a Gould 2400 chart recorder. Changes in phrenic nerve activity were determined by comparing the amplitude of the integrated signal during toxin administration with that of the predosing period.

**Toxicant Preparation and Administration**

Antx-a(s) was isolated by the method of Harada et al. (1988) from batch cultures of *A. flos-aquae* strain NRC-525-17 that were grown by the method of
Mahmood and Carmichael (1986) in the laboratory of W. Carmichael at Wright State University, Dayton, OH. Analysis using thin-layer chromatography indicated purity of 90 to 95%. In addition to antx-a(s), the majority of the remaining material used in this study was low-molecular-weight salts from the culture media and purification process (with which toxicity has not been associated upon repeated testing). The 2-hour mouse ip LD₅₀ of the toxin was 21 μg/kg. Antx-a(s) was stored under nitrogen gas at -20°C prior to use and brought into solution for dosing with physiologic saline (0.9% Sodium Chloride Injection, USP, Abbott Laboratories, North Chicago, IL) such that the dosing solution contained 50 μg of antx-a(s)/ml. Physiologic saline was also used as the control solution in this study. The saline control or toxin solution was administered iv by a Harvard (Mills, MA) Model 907 infusion pump at a constant rate of 0.033 ml/minute.

Rats were infused with saline for 3 minutes prior to administration of toxin in order that they could serve as their own controls. Rats were subsequently infused with antx-a(s) until they died as indicated by a cessation of phrenic nerve activity. In preliminary studies, rats were instrumented as above and continuously infused iv with saline for 20 minutes. These rats did not exhibit appreciable changes in heart rate, mean arterial blood pressure, tidal volume, minute volume, respiratory rate, or amplitude of integrated phrenic nerve signals.

Statistical Methods

Multivariate repeated measures analyses were performed to detect significant changes over time in: blood pressure, heart rate, tidal volume, respiratory rate, minute volume, and phrenic nerve amplitude. When overall trends were deemed significant by these repeated measures analyses, linear
contrasts were used to determine significant differences at 1-minute intervals postdosing as compared to the control period. In all analyses, a level of $\alpha = 0.05$ was chosen \textit{a priori} to detect significant differences.

**RESULTS**

The mean survival time (from initial toxin exposure to death) was $8.7 \pm 0.2$ minutes (mean $\pm$ SE). Blood pressure, heart rate, tidal volume, respiratory rate, minute volume, and phrenic nerve activity are presented in Figures 1 to 6. Significant ($p < 0.01$) decreases over time occurred in blood pressure, heart rate, tidal volume, and minute volume. Mild reductions in heart rate and mean arterial pressure were the first observed effects of the toxin. Subsequently, as measured over 30 seconds, there were marked reductions in heart rate to the point at which there was an average reduction of $165 \pm 14$ beats/minute (mean $\pm$ SE), which was 47% of the value during the predose period, and a reduction in arterial blood pressure by $24.1 \pm 8$ mmHg (mean $\pm$ SE), which was 22% of the mean value for the predose period. These changes in heart rate and blood pressure occurred by $3.47 \pm 0.3$ minutes (mean $\pm$ SE) after the initiation of toxin infusion and took place over a period of time when respiratory minute volume had decreased by only $15.4 \pm 3\%$ (mean $\pm$ SE) of the predose mean value. The majority of the decrease in mean arterial blood pressure resulted from a reduction in diastolic pressure.

Although decreases occurred in respiratory rate, tidal volume, and minute volume, only with respiratory rate were values at none of the observation times after dosing significantly less than those of the control period. Phrenic nerve amplitudes were increased significantly despite the decline in respiratory parameters.
During toxin administration, rats developed hypersalivation, chromodacryorrhea, and later muscle tremors and fasciculations. Visual inspection of the animals indicated that respiratory effort initially increased over time in response to the infusion of toxin and then decreased just prior to death.

DISCUSSION

The results of the present study seem to suggest that antx-a(s) has no centrally mediated inhibitory effect on respiration since there was such a marked and consistent increase in phrenic nerve activity. These results were compatible with the previous observation that the toxin does not inhibit whole brain ChE activity in mice dosed ip. It is likely that antx-a(s) has only peripheral activity due to inability to cross the blood-brain barrier, but this remains to be conclusively demonstrated (Cook et al., 1988).

The changes in heart rate and blood pressure suggest that antx-a(s) exerts important effects on the cardiovascular system. Such effects are a likely result of accumulation of acetylcholine due to inhibition of ChE. Alternatively, direct muscarinic agonist effects of antx-a(s) have been observed using isolated denervated guinea pig ileum in vitro (Hyde and Carmichael, 1988, 1989). Thus, the fact that mice and rats given antx-a(s) display muscarinic signs of defecation, lacrimation, hypersalivation, and urination after toxin exposure could be a result of a combination of primary muscarinic effects and, probably to a greater degree, accumulation of acetylcholine (Mahmood and Carmichael, 1986; Cook et al., 1988). Whether primary or secondary, the muscarinic actions of antx-a(s) are important since mice that survived significantly longer when given atropine prior to toxin administration as compared to mice given toxin alone (Mahmood and Carmichael,
Thus, whether by virtue of inhibition of ChE, direct agonist activity, or a combination of the two, it would appear that antx-a(s) causes muscarinic stimulation and that atropine is able to block most if not all such effects of the toxin at least temporarily.

Antx-a(s) did not cause hypertension in the rat, unlike many other ChE-inhibiting agents such as isopropyl methyl phosphonofluoridate (sarin), diisopropylfluorophosphate (DFP), and diethylaminophenylphosphate (paraoxon) (Holmstedt, 1959). Hypertension due to these compounds in rats has been postulated to be a result of an action in the central nervous system. A similar effect by antx-a(s) may not be possible if the toxin cannot penetrate the blood-brain barrier. The observed decrease in diastolic blood pressure that accounted for most of the decrease in mean arterial blood pressure suggests a loss of vascular tone.

In the present study, toxin-induced inhibition of ChE resulting in nicotinic effects of excessive acetylcholine was suspected to have been at least partially responsible for the observed gradual reduction in tidal and minute volumes. These changes occurred despite a marked increase in phrenic nerve activity. Also, much of the observed decrease in minute volume appeared to be a result of a decrease in tidal volume and not respiratory rate. Indirect nicotinic effects of antx-a(s) due to inhibition of ChE have been observed previously in vitro in frog rectus abdominis muscle and on electrically stimulated rat phrenic nerve-diaphragm preparations, and there is evidence from these and similar studies in vitro to suggest that the toxin is not a direct neuromuscular blocking agent. For example, in the frog rectus abdominis preparation, antx-a(s) did not change the dose response curve of the neuromuscular blocking agent antx-a, and antx-a(s) antagonized d-tubocurarine
blockade in the chick biventer cervicis and frog rectus abdominis muscle preparations (Mahmood and Carmichael, 1986; Hyde and Carmichael, 1988). Thus, in the present study, observations in vivo were consistent with previous evidence indicative of an absence of direct neuromuscular blockade in vitro. The present study also seems to indicate that this indirect neuromuscular blockade effect of antx-a(s) would ultimately be very likely to contribute to the toxin-associated lethality.

In previous studies, unanesthetized rats that were given antx-a(s) ip developed clonic seizures and died (Cook et al., 1989b). In the present study, rats that were anesthetized did not develop seizures but still died, indicating that seizures are not the sole cause of death in animals exposed to antx-a(s).

In conclusion, antx-a(s) unlike most known low-molecular-weight organophosphorus ChE-inhibiting agents did not have a profound inhibitory action on centrally mediated respiration but did appear to have an important effect on the peripheral responses of the cardiovascular and respiratory systems. Based on the present study, it would appear that, if the cardiovascular effects of antx-a(s) are not sufficiently severe to cause lethality, then progressive neuromuscular blockade of the muscles of respiration may, alone or in combination with the cardiovascular alterations, ultimately lead to death.
Figure 1. Effects of anatoxin-a(s) on heart rate in anesthetized rats. Data are presented as the mean ± SE (N = 4 rats). Observations postdosing which were significantly different (p < 0.05) from the predose period (average of 16 predose observations [four animals at four time points spaced 1 minute apart]) are indicated by asterisks (*).
Figure 2. Effects of anatoxin-a(s) on mean arterial blood pressure in anesthetized rats. Data are presented as the mean ± SE (N = 4 rats). Observations postdosing which were significantly different (p < 0.05) from the predose period (average of 16 predose observations [four animals at four time points spaced 1 minute apart]) are indicated by asterisks (*).
Figure 3. Effects of anatoxin-a(s) on tidal volume in anesthetized rats. Data are presented as the mean ± SE (N = 4 rats). Observations postdosing which were significantly different (p < 0.05) from the predose period (average of 16 predose observations [four animals at four time points spaced 1 minute apart]) are indicated by asterisks (*).
Figure 4. Effects of anatoxin-a(s) on respiratory rate in anesthetized rats. Data are presented as the mean ± SE (N = 4 rats). Observations postdosing which were significantly different (p < 0.05) from the predose period (average of 16 predose observations [four animals at four time points spaced 1 minute apart]) are indicated by asterisks (*).
Figure 5. Effects of anatoxin-a(s) on minute volume in anesthetized rats. Data are presented as the mean ± SE (N = 4 rats). Observations postdosing which were significantly different (p < 0.05) from the predose period (average of 16 predose observations [four animals at four time points spaced 1 minute apart]) are indicated by asterisks (*).
Figure 6. Effects of anatoxin-a(s) on phrenic nerve amplitude in anesthetized rats. Data are presented as the mean ± SE (N = 4 rats). Phrenic nerve amplitude data are expressed as the percent change from the predose period (average of 12 observations: four animals at three time points spaced 1 minute apart [-3, -2, and -1 minutes]). Observations postdosing which were significantly different (p < 0.05) from time 0 are represented by asterisks (*).
III. EFFECTS OF ANATOXIN-A(S) ON HEART RATE, MEAN ARTERIAL BLOOD PRESSURE, PHRENIC NERVE AMPLITUDE, AND ELECTROMYOGRAPHIC ACTIVITY OF THE DIAPHRAGM OF RATS: THE INFLUENCE OF ATROPINE AND ARTIFICIAL RESPIRATION

W. O. Cook, G. A. Iwamoto, D. J. Schaeffer, and V. R. Beasley

ABSTRACT

The pathophysiologic effects of anatoxin-a(s) [antx-a(s)] from the cyanobacterium Anabaena flos-aquae NRC-525-17 were investigated using anesthetized adult male Sprague-Dawley rats given the toxin by continuous iv infusion until death. Rats (N = 6) pretreated with atropine ip at 50 mg/kg survived significantly longer (p < 0.05) than nonatropinized rats (N = 6), suggesting that the muscarinic effects of antx-a(s) were very important in the lethal syndrome. In contrast to rats given toxin alone which experienced an early reduction in heart rate and mean blood pressure, those pretreated with atropine had a decrease in heart rate and mean blood pressure that occurred only after profound reductions in tidal and minute volume, suggesting that neuromuscular blockade of the muscles of respiration was the cause of death. Even when survival times of rats were increased by pretreatment with atropine, phrenic nerve amplitudes increased, indicating a lack of effect of antx-a(s) on central mediation of respiration. Rats (N = 3) continuously ventilated during toxin infusion survived a dose more than four-fold greater than a consistently lethal dose of the toxin as determined above. This suggests that the cardiovascular effects of antx-a(s) alone do not account for the death of rats. Electromyograms (EMG) recorded from the diaphragms of rats (N = 5) during continuous toxin administration revealed an increase in muscular electrical activity that became more random and finally decreased prior to
death. These findings suggest that toxin-induced neuromuscular blockade is ultimately the lethal mechanism in antx-a(s)-dosed rats.

INTRODUCTION

The goal of the present study was to evaluate the relative importance of peripheral mechanisms involved in antx-a(s) toxicosis by assessing effects of the toxin on: 1) cardiovascular and respiratory parameters in atropinized and nonatropinized rats, 2) cardiovascular and respiratory function in rats provided artificial ventilation, and 3) transmission of impulses to the muscles of respiration by measuring phrenic nerve and diaphragm electromyographic (EMG) activity during toxicosis.

MATERIALS AND METHODS

Rats

Twenty male Sprague-Dawley rats weighing 485 ± 34 g were housed in air-conditioned quarters on a 12-hour light/12-hour dark cycle and provided food and water ad libitum.

Toxicant Preparation and Administration

The toxin employed was prepared and handled as described in the previous chapter. Antx-a(s) was brought into solution for dosing with physiologic saline (0.9% Sodium Chloride Injection, USP, Abbott Laboratories, North Chicago, IL). Physiologic saline (0.9%) was also used as the control solution in this study. The toxin or control solution was administered to rats by means of a Harvard (Southnatick, MA) Model 907 infusion pump. Toxin-dosed rats were given 3.5 µg of antx-a(s)/kg/minute using a volume of 0.033 ml/minute. The same volume of the saline vehicle alone was given to the control rats.
Anesthesia

The anesthetic protocol was the same as that described in the previous chapter. This anesthetic regimen provided a stable level of anesthesia for the duration of the experiment.

Surgical Procedures and Physiological Measurements

The techniques for maintenance of body temperature; tracheal, carotid artery, and jugular vein catheterizations; and measurement of tidal volumes were described in the previous chapter. From the initial incision, dissection caudally to the clavicle at the base of the neck was used to expose the right phrenic nerve where it diverged from the fifth cervical nerve. The phrenic nerve was transected and the cranial end laid across bipolar silver hook electrodes for recording action potentials. The phrenic nerve signal was amplified with a Grass P511 AC preamplifier using a bandpass of 100 Hz to 10 KHz. The signal was full-wave rectified and integrated (Gould integrator) using a reset time of 0.033 seconds (integrate sample/hold).

Diaphragm electromyograms (EMG) were recorded by placing two hook-shaped, Teflon-coated, 25-gauge silver wire electrodes into the left lateral aspect of the diaphragm. The hook electrodes were inserted into the diaphragm by way of a 25-gauge needle that was inserted behind the last rib, through the skin and abdominal wall, and into the diaphragm. The needles were subsequently withdrawn leaving the electrodes in the diaphragm. The positions of the electrodes in the diaphragm were verified at necropsy. The EMG signal was amplified with a Grass P511 AC preamplifier using a bandpass of 100 Hz to 10 KHz. The signal was full-wave rectified and integrated (Gould integrator) using a reset time of 0.033 seconds (integrate sample/hold).
In rodents that were ventilated, a Harvard (Southnatick, MA) Model 683 ventilator was used with the rate and volume adjusted so that phrenic nerve amplitude activity was recordable. Heart rate, blood pressure, phrenic nerve activity, and tidal volume signals were recorded on a Gould 2400 chart recorder. Diaphragm, phrenic nerve, and tidal volume signals were integrated prior to recording. Changes in phrenic nerve and EMG amplitudes were calculated based on measurements of the amplitude of the integrated signals in mm and computing the percent change from the control period. A schematic diagram of the various equipment and measurements used is presented in Figure 1.

Experimental Design

In the first study, in order to assess the significance of muscarinic effects of antx-a(s), rats (N = 6) were pretreated with atropine sulfate at 50 mg/kg 15 minutes before toxin administration. Positive control rats (N = 6) were given only toxin. Measurements included arterial blood pressure, heart rate, and tidal volume. Rats served as their own controls; baseline parameters were recorded while saline was infused in each rat for 3 minutes immediately prior to toxin administration. Thereafter, toxin was infused iv until rats died (as indicated by cessation of phrenic nerve activity). Survival time to the nearest second and the amount of toxin administered were recorded.

In the second study, the effects of ventilation (as a means of pulmonary support) on antx-a(s) toxicosis were evaluated. Rats (N = 3) were ventilated with air and concurrently infused iv with toxin. The predicted end point of the experiment was to be either animal death or delivery of a total of 170 μg of antx-a(s)/kg (over 4.7 times the median lethal dose of 36 μg of
antx-a(s)/kg as determined in the first study]. Rats served as their own controls and the same parameters were measured as in the first study except that tidal and minute volumes were not recorded.

In the third study, toxin was infused iv continuously until the rats died (N = 5). Respiration was not supported. In addition to diaphragm EMG activity, the same parameters were measured as in the second study and rats served as their own controls as described above.

Statistical Analysis

Comparison of survival time between rats given toxin and those pretreated with atropine prior to toxin administration was performed using Bartlett's test for homogeneity of variances followed by a two-tailed T-test. With regard to heart rate, blood pressure, respiratory rate, and tidal and minute volume data, comparisons between rats given toxin and those given atropine and toxin were analyzed by multivariate repeated measures analyses, and when overall treatment differences were detected, univariate comparisons (linear contrasts) were used to identify significant differences between the two groups at each time point. In rats that were ventilated, multivariate repeated measures analyses were used, and for parameters in which significant trends over time were present, linear contrasts were used to detect differences between observations at 3-minute intervals postdosing and the control period observations. Because of the small number of animals used in these studies, a level of $\alpha = 0.10$ was chosen a priori to detect significant differences in the above comparisons; however, comparisons using $\alpha = 0.05$ are also presented.

RESULTS

Figures 2, 3, 4, 5, 6, and 7 represent findings with regard to mean arterial blood pressure, heart rate, respiratory rate, tidal volume, minute...
volume, and phrenic nerve amplitude, respectively. The Bartlett's test for homogeneity of variance for survival times did not reveal a significant difference ($p < 0.05$). Rats pretreated with atropine sulfate prior to toxin exposure had significantly longer ($p < 0.05$) survival times than rats given toxin alone: $12.8 \pm 1.0$ minutes (mean $\pm$ SE) and $10.1 \pm 0.5$ minutes, respectively. Furthermore, atropine-treated rats received a higher mean lethal dose of toxin ($45.2 \pm 3.5$ µg/kg [mean $\pm$ SE], range of 34.2 to 55.6 µg/kg) than rats given toxin alone ($35.9 \pm 1.7$ µg/kg [mean $\pm$ SE], range of 30.1 to 42.6 µg/kg).

There were overall trends such that the following parameters were characterized by lesser values in rats given toxin alone as compared to rats given atropine and toxin: mean arterial blood pressure ($p = 0.095$), heart rate ($p = 0.001$), respiratory rate ($p = 0.001$), tidal volume ($p = 0.098$) and minute volume ($p = 0.016$) (Figures 2 to 6). Blood pressure and heart rate in atropinized rats decreased markedly only after profound reductions had occurred in tidal and minute volumes. Phrenic nerve amplitude increased faster in rats given toxin alone than in rats given atropine and toxin ($p = 0.058$). For comparative purposes, the arterial blood pressure, heart rate, tidal volume, and integrated phrenic nerve signal responses of a single rat given antx-a(s) are presented in Figure 8.

In the second study, rats survived administration of 170 µg of antx-a(s)/kg, 4.7 times the average lethal dose and over 4 times the highest dose needed to cause lethality in the first study. The animals lived (as indicated by continued phrenic nerve activity) despite sudden and marked reductions in heart rate and blood pressure (Figure 9 to 11), similar to those observed in rats given toxin alone in the first study. Blood pressure and heart rate eventually stabilized at low values.
In the third study, diaphragm EMG activity in toxin-dosed rats increased and then became more nonsynchronous until EMG activity was not matched in any way to phrenic nerve activity. Despite a progressive increase in the amplitude of the bursts of phrenic nerve activity (Figure 12), the EMG activity was eventually markedly decreased (Figure 13), although it was again somewhat more synchronous prior to death (Figure 14). The arterial blood pressure, heart rate, raw diaphragmatic EMG signal, and integrated phrenic nerve signal at predosing and postdosing times in two of the rats are presented in Figures 14 and 15.

**DISCUSSION**

Lethality from most low-molecular-weight, highly toxic organophosphorus cholinesterase inhibitors has been attributed primarily to reduced stimulation of the muscles of respiration via action(s) on the central nervous system (Beers et al., 1987; de Candole et al., 1953; Rickett et al., 1986). However, even when rats survived significantly longer as a result of atropine pretreatment, antx-a(s) did not inhibit central mediation of respiration due to ChE inhibition or any other mechanism as indicated by there having been an increase in phrenic nerve amplitude. The absence of a centrally mediated effect was compatible with the previous observation that this toxin does not inhibit whole brain ChE activity in mice dosed ip and the probably inability of the compound to cross the blood-brain barrier (Cook et al., 1988).

In the first study, the muscarinic effects of the toxin were found to be of importance in the lethal syndrome, since atropinized rats survived significantly longer than nonatropinized rats. Thus, because the atropinized rats lived longer, they were infused with more toxin before death occurred. Mahmood and Carmichael (1986) observed a similar increase in survival time in
mice pretreated ip with atropine at 50 mg/kg 15 minutes before dosing ip with antx-a(s) at 0.5 or 1.0 mg/kg, but in that study, the mechanism of atropine protection was not investigated. In the present study, atropine treatment stabilized the heart rate, blood pressure, and respiratory rate of antx-a(s)-dosed rats, and it diminished the severity of the reductions in tidal and minute volumes. These findings seem to suggest that muscarinic effects of antx-a(s) on the cardiovascular system are an important mechanism of action. The observed cardiovascular effects could have resulted from a direct muscarinic action of the toxin on the heart or indirectly through acetylcholine as a result of ChE inhibition, but were unlikely to have been a product of a centrally mediated mechanism.

The less severe reductions in tidal and minute volumes in response to atropine were likely to have been a result of antagonism of bronchial constriction and glandular secretion into the lung. Bronchoconstriction and increased secretions in the airways are anticipated in the presence of excessive concentrations of acetylcholine or from direct muscarinic stimulation. As a probable result of the beneficial effect of cholinergic blockade, phrenic nerve activity did not increase as rapidly in the animals given toxin plus atropine as in those given toxin alone. The decrease in respiratory rate in nonatropinized rats given antx-a(s) may have been a result of fatigue of the stretch receptors in the lung or an increase in the inspiratory and/or expiratory time of respiration.

Since relatively large doses of antx-a(s) were used in the present survival studies and studies done by others (Mahmood and Carmichael, 1986), it cannot be said that atropine could not be lifesaving in antx-a(s) toxicoses. Atropine is considered a reasonably effective therapy for anticholinesterase
poisoning in experimental animals given doses of ChE inhibitors up to 2.0 to 2.5 times the iv LD₅₀ (Holmstedt, 1959). At the present time, based on the consistent and repeated findings of significantly increased survival times in rats and mice given antx-a(s), atropine should be recommended as part of a treatment plan for antx-a(s) toxicosis.

Despite the benefits of atropine in the present study, since all the rats pretreated with the drug still died after having survived only an average of about 2 minutes longer than rats given toxin alone and after having received approximately 25% more antx-a(s) and nonatropinized rats, blockade of muscarinic receptors afforded only a modest protective effect. Assuming that the parenterally dosed rat is an appropriate model, other animals exposed to large quantities of algae and antx-a(s), such as in natural exposures where sudden death is the primary clinical sign, may not survive with atropine treatment alone.

In the second study, animals survived when artificially ventilated during continuous toxin infusion and retained central respiratory drive (as indicated by the presence of phrenic nerve activity) despite being given more than 4 times the lethal dose. This seems to suggest that the effects of antx-a(s) on the cardiovascular or other nonpulmonary systems alone were not lethal. Also, inhibition of diffusion of oxygen across the pulmonary membranes and into the pulmonary capillaries as a result of possible secretions was not a highly lethal mechanism of antx-a(s), since animals survived when on the ventilator. Artificial ventilation has been observed previously to keep animals alive that have been given anticholinesterase agents, provided that much higher doses that caused acute cardiovascular failure were not given (Meeter and Wolthuis, 1968; Adams et al., 1976).
The third study conclusively demonstrated paralysis of the diaphragm in the lethal syndrome caused by intravenously administered antx-a(s) in vivo. It has been demonstrated previously that phrenic nerve impulses are bilaterally emitted (Wright, 1954). Thus, the asynchronous and marked decrease in contralateral EMG activity that occurred despite the progressive increase in phrenic nerve amplitude strongly indicated that these EMG changes were the result of a peripheral action of antx-a(s). It would appear that the paralysis of the diaphragm and muscles of respiration was the most important lethal mechanism in this study. Changes in the EMG noted during toxin infusion, the increased amplitude of activity, then the more random activity, and finally a decrease in amplitude were comparable to what has been observed in rat phrenic nerve-diaphragm preparations exposed to other ChE inhibitors in vivo and in vitro (Barnes and Duff, 1953; Holmstedt, 1959). Indirect nicotinic effects of antx-a(s) caused by inhibition of ChE have also been observed in vitro, in which antx-a(s) was demonstrated to cause twitch potentiation in the rat phrenic nerve-diaphragm preparation, and caused tetanic fade in conjunction with a high (100 Hz) rate of electrical stimulation.

In conclusion, the muscarinic effects of antx-a(s) on the cardiovascular system appeared to play a role in lethal mechanism of antx-a(s) toxicosis. Whether inhibition of cholinesterase and/or direct agonist activity is responsible for the muscarinic effects of the toxin, it is apparent that atropine can still compete effectively for the muscarinic receptor during antx-a(s) toxicosis. Antx-a(s)-induced lethality is likely to be a result of a combination of the effects of the toxin on the cardiovascular and respiratory systems, although overall, the nicotinic effects on the muscles of respiration appear to be the most important mechanism.
Figure 1. Schematic diagram of the various measurements and equipment used in studies of electrophysiologic responses of rats.
Figure 2. Study 1: Mean arterial blood pressure in anesthetized rats (N = 6) pretreated with atropine ip at 50 mg/kg 15 minutes before initiation of a continuous iv infusion of anatoxin-a(s) compared to rats given only the latter. Data are presented as the percent change from the control period (average of observations at seven time points at 30-second intervals). When multivariate repeated measures analysis detected significant treatment effects (p < 0.10), linear contrast analyses were performed. The symbols + and * represent time points at which treatment groups were significantly different from each other at p < 0.10 and p < 0.05, respectively. Both curves are truncated at the time point at which the first animal of either treatment died.
Figure 3. Study 1: Heart rate in anesthetized rats (N = 6) pretreated with atropine ip at 50 mg/kg 15 minutes before initiation of a continuous iv infusion of anatoxin-a(s) compared to rats given only the latter. Data are presented as the percent change from the control period (average of observations at seven time points at 30-second intervals). When multivariate repeated measures analysis detected significant treatment effects (p < 0.10), linear contrast analyses were performed. The symbols + and * represent time points at which treatment groups were significantly different from each other at p ≤ 0.10 and p ≤ 0.05, respectively. Both curves are truncated at the time point at which the first animal of either treatment died.
Figure 4. Study 1: Respiratory rate in anesthetized rats (N = 6) pretreated with atropine ip at 50 mg/kg 15 minutes before initiation of a continuous iv infusion of anatoxin-a(s) compared to rats given only the latter. Data are presented as the percent change from the control period (average of observations at seven time points at 30-second intervals). When multivariate repeated measures analysis detected significant treatment effects (p < 0.10), linear contrast analyses were performed. The symbols + and * represent time points at which treatment groups were significantly different from each other at p < 0.10 and p < 0.05, respectively. Both curves are truncated at the time point at which the first animal of either treatment died.
Figure 5. Study 1: Tidal volume in anesthetized rats (N = 6) pretreated with atropine ip at 50 mg/kg 15 minutes before initiation of a continuous iv infusion of anatoxin-a(s) compared to rats given only the latter. Data are presented as the percent change from the control period (average of observations at seven time points at 30-second intervals). When multivariate repeated measures analysis detected significant treatment effects (p < 0.10), linear contrast analyses were performed. The symbols + and * represent time points at which treatment groups were significantly different from each other at p < 0.10 and p < 0.05, respectively. Both curves are truncated at the time point at which the first animal of either treatment died.
Figure 6. Study 1: Minute volume in anesthetized rats (N = 6) pretreated with atropine ip at 50 mg/kg 15 minutes before initiation of a continuous iv infusion of anatoxin-a(s) compared to rats given only the latter. Data are presented as the percent change from the control period (average of observations at seven time points at 30-second intervals). When multivariate repeated measures analysis detected significant treatment effects (p < 0.10), linear contrast analyses were performed. The symbols + and * represent time points at which treatment groups were significantly different from each other at p ≤ 0.10 and p ≤ 0.05, respectively. Both curves are truncated at the time point at which the first animal of either treatment died.
Figure 7. Study 1: Phrenic nerve amplitude in anesthetized rats (N = 5 to 6) pretreated with atropine ip at 50 mg/kg 15 minutes before initiation of a continuous iv infusion of anatoxin-a(s) compared to rats given only the latter. Data are presented as the percent change from the control period (average of observations at seven time points at 30-second intervals). When multivariate repeated measures analysis detected significant treatment effects (p < 0.10), linear contrast analyses were performed. The symbols + and * represent time points at which treatment groups were significantly different from each other at p < 0.10 and p < 0.05, respectively. Both curves are truncated at the time point at which the first animal of either treatment died.
Figure 8. Study 1: Arterial blood pressure, heart rate, tidal volume, and integrated phrenic nerve signal (amplitude) in rat #1 predose and at 3, 6, and 8 minutes after the start of a continuous iv infusion of anatoxin-a(s).
Figure 9. Study 2: Heart rate (mean ± SE) in anesthetized rats (N = 3) ventilated during continuous iv infusion of anatoxin-a(s). When multivariate repeated measures analysis detected significant differences over time (p < 0.10), linear contrast analyses were performed. The symbols + and * represent time points at which observations postdosing were significantly different from the control period at p < 0.10 and p < 0.05, respectively.
Figure 10. **Study 2**: Mean arterial blood pressure (mean ± SE) in anesthetized rats (N = 3) ventilated during continuous iv infusion of anatoxin-a(s). When multivariate repeated measures analysis detected significant differences over time (p < 0.10), linear contrast analyses were performed. The symbols + and * represent time points at which observations postdosing were significantly different from the control period at p < 0.10 and p < 0.05, respectively.
Study 2: Phrenic nerve activity (mean ± SE) in anesthetized rats (N = 3) ventilated during continuous iv infusion of anatoxin-a(s). Despite the modest decrease in the rate of phrenic nerve impulses, no significant trend was detected by multivariate repeated measures analysis.
Figure 12. Study 3: Phrenic nerve amplitudes (mean ± SE) in anesthetized rats (N = 5) continuously infused iv with anatoxin-a(s) until death. Data are presented as the percent change from the control period versus the percentage of the time between the initiation of dosing and death of the animal (percent of survival time).
Figure 13. **Study 3:** Amplitudes of integrated electromyographic signals (mean ± SE) in anesthetized rats (N = 5) continuously infused iv with anatoxin-a(s) until death. Data are presented as the percent change from the control period versus the percentage of the time between the initiation of dosing and death of the animal (percent of survival time).
Figure 14. Study 3: Arterial blood pressure, heart rate, raw diaphragmatic electromyogram, and integrated phrenic nerve signal (amplitude) in rat #14 predose and at 5, 7, 10, 11, and 12 minutes after the start of a continuous iv infusion of anatoxin-a(s).
Figure 15. **Study 3:** Arterial blood pressure, heart rate, raw diaphragmatic electromyogram, and integrated phrenic nerve signal (amplitude) in rat #13 predose and at 3, 6, and 10 minutes after the start of a continuous iv infusion of anatoxin-a(s).
ABSTRACT

Anatoxin-a(s) [antx-a(s)], an organophosphorus cholinesterase (ChE)-inhibiting neurotoxin from the freshwater cyanobacterium Anabaena flos-aquae was evaluated for acute behavioral effects in male Long-Evans rats. For a comparison to antx-a(s), paraoxon, an organophosphorus agent with known central and peripheral ChE-inhibiting activity, was also used in the study. Rats were dosed ip with antx-a(s) at 0.75, 1.5, or 3 µg/kg, which caused no observed, mild, and moderate clinical signs, respectively, or paraoxon at 400 µg/kg that produced mild clinical signs. Rats were tested with instrumentation to evaluate grip strength (GS), accelerating rotarod (AR), figure 8 maze (F8M), and startle response (SR) performance 48 hours prior to dosing, within 2 hours after dosing, and at 48 hours postdosing. At 50 hours postdosing, inhibition of ChE in whole blood was detected in rats given antx-a(s) and paraoxon, whereas inhibition of brain ChE was detected only in rats given paraoxon, suggesting that the highly polar antx-a(s), unlike most previously characterized organophosphorus cholinesterase inhibitors may not cross the blood-brain barrier. Significant behavioral decrements were observed in the F8M performance of animals dosed with paraoxon or with antx-a(s) at 28 or 56% of the LD50 dose of antx-a(s) (1.5 or 3.0 µg/kg, respectively). AR performance was decreased with the highest dose of antx-a(s). A significant decrease in amplitude of response to tactile stimuli...
was observed at the two highest doses of antx-a(s), but with paraoxon, the
effect was less marked and not significantly different from the control
value. The significant behavioral decrements caused by antx-a(s) were dose
dependent. Significant behavioral decrements were observed only at or above a
relatively high dose (28% of the LD50) of antx-a(s) and only at doses causing
overt clinical signs. These results are compatible with the hypothesis that
behavioral effects of antx-a(s) are a result of effects at peripheral sites of
action.

INTRODUCTION

The degree of cholinesterase (ChE) inhibition in tissues of animals
exposed to ChE-inhibiting toxicants is not an all-encompassing indicator of
nervous system toxicity (Frawley et al., 1952; Hoskins et al., 1986), and
while the effect of antx-a(s) on brain and blood ChE has been examined
(Mahmood and Carmichael, 1987; Cook et al., 1988), no information is available
on the behavioral toxicity of this naturally occurring and potent compound.

At low doses, considerable differences exist between ChE inhibitors in
their ability to cause acute behavioral toxicity (Wolthuis and Vanwersch,
1984). TEPP (tetraethylpyrophosphate) and sarin at doses up to 30% of the
LD50 were without effects in six different behavioral tests, whereas soman,
physostigmine, and pyridostigmine caused effects at doses < 3 and 10%,
respectively, of the LD50 despite the absence of any clinical signs (Wolthuis
and Vanwersch, 1984).

The ability to inhibit ChE in the central nervous system (CNS) has been
implicated as an important mechanism of behavioral toxicity with
ChE-inhibiting agents (Wolthuis and Vanwersch, 1984; Harris et al., 1984).
The fraction of the LD50 necessary to cause behavioral toxicity and the
severity of clinical signs evident at doses that cause behavioral deficits have been correlated with the ability of anticholinesterase compounds to inhibit ChE in the CNS (Wolthuis and Vanwersch, 1984). The relatively high dose of sarin needed to produce behavioral decrements (30 to 56% of the LD₅₀) in comparison to other agents was suggested to have been a result of the primarily peripheral ChE-inhibiting action of the compound (Landauer and Romano, 1984; Clement and Copeman, 1984). In gerbils, the purely peripheral ChE-inhibiting action (Sharma et al., 1973) of phosdrin (mevinphos) was suggested (Wolthuis and Vanwersch, 1984) to have been responsible for the occurrence of behavioral decrements which were noted only at doses sufficient to cause overt clinical signs (Mertens et al., 1974).

Even if an inhibitor has poor ability to affect ChE in the CNS, it cannot be assumed that the toxicant is devoid of CNS activity since anticholinesterase agents are also known to affect serotonergic, catecholaminergic, and gabanergic systems (Karczmar, 1984). For example, the carbamate ChE inhibitor pyridostigmine which does not affect brain ChE activity or brain acetylcholine levels (Stitcher et al., 1978) produced neurobehavioral decrements (Wolthuis and Vanwersch, 1984) at very low doses (< 10% of the LD₅₀) in two-way shuttlebox avoidance learning, open field behavior, and the hurdle-stepping task, suggesting that this compound or a metabolite thereof may have some CNS action despite its inability to inhibit ChE in the CNS. Thus, it is theoretically possible that a metabolite of a parent compound (which acts via ChE inhibition in the periphery) could, unlike the parent compound, gain entry into the CNS and exert effects by an unrelated mechanism resulting in neurobehavioral alterations.

The purpose of this paper was to establish dose-response relationships for the acute effects of antx-a(s) on four behavioral measures and to assess the
reversibility of any detected deficits within 48 hours. The behavioral tests consisted of evaluations of the effects of antx-a(s) on grip strength (GS), locomotor activity using the figure maze (F8M), motor coordination as assessed by an accelerating rotarod (AR), and startle response (SR) to both audio and tactile stimuli. The tests for GS, F8M activity, and AR coordination were chosen because one of the primary targets of anticholinesterase agents is the neuromuscular junction where these compounds cause muscular weakness (Ariens et al., 1969; Karczmar, 1967; Karczmar and Ohta, 1981) and alterations in motor behavior (Koehn and Karczmar, 1978; Lennox et al., 1983). The tests for SR were chosen in order to evaluate the effect of antx-a(s) on sensory responses. For a comparison to antx-a(s), paraoxon, which has central and peripheral ChE-inhibiting actions, was also used. The LD50 of antx-a(s) in rats was determined so that the results of these behavioral tests could be correlated to a standard index of toxicity.

MATERIALS AND METHODS

Animals

Male Long-Evans rats (Charles River, Raleigh, NC) weighing 315 to 430 g were housed in pairs in air-conditioned quarters on a 12-hour light/12-hour dark cycle and provided food (Rodent Laboratory Chow 5001, Purina Mills, Inc., Richmond, IN) and water ad libitum.

Toxicant Preparation and Administration

Antx-a(s) was isolated and prepared as described in Anatoxin-a(s) Section II of this report. Paraoxon (Sigma Chemical, St. Louis, MO) stored at a concentration of less than 10 mM at -20°C in dry acetone was diluted with physiologic saline such that the final dosing solution contained less than 1% acetone. Saline was also used as the control solution.
Rats were dosed ip with one of three levels of antx-a(s), paraoxon, or saline. The doses of antx-a(s) were: 1) 3 µg/kg that produced moderate clinical signs of hypersalivation, lacrimation, diarrhea, and ataxia; 2) 1.5 µg/kg that was a threshold dose and produced barely detectable clinical signs consisting of reduced activity; and 3) 0.75 µg/kg that was a dose producing no clinical signs. Paraoxon was given at 400 µg/kg which produced clinical signs similar to the threshold dose of antx-a(s). Saline was given to the control animals at a volume/body weight equal to that used with the highest dose of antx-a(s).

Accelerod Training and Experimental Procedures

**Accelerod.** The experiment was performed with two groups of 20 rats. Rats were trained (Omnitech Electronics Inc., Omni-Rotor Treadmill, Model RR, Columbus, OH) until they could maintain balance on the AR (from standstill, the rod was accelerated at 1 rpm/second from 0 to a maximum of 20 rpm, and continued at that rate for 2 minutes) (Bogo et al., 1981). The 20 rats in each group were preranked into four groups by the number of seconds that each animal could maintain balance on the AR and randomized to the five treatment groups so that all rank groups were present in all treatment groups.

After dosing, rats were tested by accelerating the rod from standstill at 1 rpm/second and recording to the tenth of a second the time that they could maintain balance on the rod. Rats that fell off the rod prior to 20 second were retested to ensure that their performance was caused by the treatments and not by any external factors (Bogo et al., 1981).

**Figure 8 maze.** Locomotor activity was recorded as the frequency of movement (the number of infrared beam interruptions) in an automated F8M (Photobeam Activity System, San Diego Instruments, San Diego, CA) (Reiter,
The frequency of movement was recorded by summation of beam interruptions over twelve 5-minute intervals (intervals 1 through 12) for a total time of 60 minutes at each of the three observation times as described below under Experimental Design.

**Grip strength.** A hindlimb GS apparatus (designed by Dr. Joel Mattsson, Dow Chemical, Midland, MI) was used. The hindlimbs of each animal were placed squarely on a wire mesh attached to a strain gauge (Chatillon, Model DPP-1.0 kg) and the rat pulled backwards by the tail in a swift movement until the hindlimbs extended. The resistance to this pull was measured as force on the strain gauge. The procedure was repeated three times and the greatest force was taken as the measure for the test.

**Startle response.** Whole body startle response was measured by a startle reflex system (Startle Reflex Laboratory System, San Diego Instruments, San Diego, CA) using maximum amplitude (recorded in units where 1 unit = 2.4 millivolts) and latency (milliseconds) as dependent measures. In the first paradigm, the response to an acoustic stimulus (a 20-millisecond burst of white noise at 120 dB) was measured. In the second paradigm, the response to a tactile stimulus (a 1 kg/cm² puff of air for a 20 millisecond time period) was recorded. In a third paradigm, the response was measured after a prepulse inhibition (a prepulse 5-millisecond-long stimulus of low intensity white noise [100 dB] was presented 95 milliseconds before the tactile stimulus [as described above]). Each of the three paradigms were presented six times in a random manner. The time interval between two paradigms was randomly varied between 5 and 9 seconds. The amplitude and latency responses from the first presentation of each paradigm were disregarded, and the subsequent values were reported as the mean of the remaining five responses.
Experimental Design

A total of eight rats were used per treatment group. Rats were tested with the neurobehavioral equipment in groups of four (ten groups). In eight of the ten groups, each of the three doses of antx-a(s) and either the control or paraoxon group was represented. Only control and paraoxon treatments were represented in the two additional groups to increase the N of these treatment to eight animals. Two groups of rats were dosed and tested with the neurobehavioral equipment starting at the same times (8 or 10 AM) each day. Rats were subjected to the test protocols beginning at three times: 1) at 48 hours prior to testing at time 2 (time 1), 2) at 20 minutes after dosing (time 2), and 3) at 48 hours after testing at time 2 (time 3). Rats were tested with the F8M, GS, AR, and SR equipment at the same times each day (i.e., the times equivalent to 20, 80, 85, and 90 minutes postdosing, respectively). Rats were weighed before each testing session.

Animals were observed for clinical signs (recorded as present or absent) including hypersalivation, diarrhea, respiratory difficulty, fasciculations, depression, and tremors predosing, at 10 minutes postdosing, and prior to and after being evaluated with each component of the neurobehavioral test battery.

After rats were tested at 48 hours postdosing, six rats were randomly selected from each treatment group, anesthetized with carbon dioxide gas for collection of blood via the abdominal vena cava and brain tissues for ChE assays, and then killed by exsanguination. The remaining two rats from each treatment group were bled similarly and then perfusion fixed for later histologic examination.

Paraoxon is a known inhibitor of ChE centrally. In order to correlate the results of neurobehavioral testing performed at 2 hours postdosing with the
degree of inhibition of brain ChE on days when paraoxon was being given to a trained rat in a parallel manner, an additional nontrained rat was dosed similarly with paraoxon, anesthetized 2 hours later with carbon dioxide gas for collection of blood and brain for ChE assays, and then killed by exsanguination.

Cholinesterase Assays

For whole blood ChE assays, 10 μl of heparinized blood was diluted to 25 ml with 0.1 M phosphate buffer (pH 8) and then 3 ml aliquots were used for determination of ChE activity by the addition of 50 μl of 0.01 M dithiobisnitrobenzoic acid (DTNB) and 20 μl of 0.075 M acetylthiocholine iodide (ACTI). ChE activity was determined by measurement of absorbance at 412 nm for 18 minutes on a Shimadzu (Shimadzu Co., Columbia, MD) 160 UV/VIS Spectrophotometer (Ellman et al., 1961).

Brain ChE activity was assayed after addition of 0.05 ml of 0.1 M phosphate buffer (pH 8) containing 1% octyl phenoxy polyethoxyethanol (Triton X-100, Sigma Chemical Co., St. Louis, MO) per mg of tissue and homogenization with a Biohomogenizer (Biospec Products, Bartlesville, OK). Two hundred μl aliquots of the homogenate were then added to 2.5 ml of phosphate buffer containing 1% Triton 100 and ChE activity was assayed with DTNB and ACTI as above, except that absorbance was measured for only 3 minutes (Harlin and Ness, 1986).

Histologic Examination of Tissues

Rats were anesthetized with sodium pentobarbital (40 mg/kg ip) and then perfusion fixed in preparation for histologic examination. Flushing and fixative solutions were administered through an 18-gauge needle inserted into the left ventricle. Blood and solutions exited the circulatory system by way
of an incision made in the right auricle. First, blood was flushed out of the circulatory system with 500 ml of 0.9% physiological saline containing 1,000 international units of heparin and 1 g of sodium nitrite. Subsequently, by the same route, rats were given 500 ml of modified Karnovsky's fixative (30 ml of 50% glutaraldehyde, 50 ml of 20% paraformaldehyde, 250 ml of 0.2 M cacodylate buffer, and 170 ml of distilled water). Brain, diaphragm, gastrointestinal tract, heart, kidney, liver, lung, pancreas, sciatic nerve, spinal cord, and spleen were removed, placed in fresh Karnovsky's fixative, and later sectioned at 6 μ and stained with hematoxylin and eosin for light microscopic examination.

Statistical Analysis

Comparisons among treatment groups for brain and whole blood ChE activities were performed using the SAS General Linear Model with Tukey's test (SAS Institute, 1985). A level of α = 0.05 was chosen to identify statistically significant differences.

GS, AR, F8M (total activity over 60 minutes for each animal), and SR results were evaluated using a multivariate analysis of variance (SAS Institute, 1985). Covariates included chamber, group (first 20 rats versus second 20 rats), rank, time (8 versus 10 AM when tests were performed), and treatment. When significant (p < 0.05) overall treatment differences were detected, multivariate repeated measures analyses were used to assess differences among treatment groups over any two of the three test times. When significant differences among treatment groups were again noted, additional comparisons at each individual observation time were evaluated by univariate analysis of variance and Tukey's test at a level of α = 0.05 (SAS Institute, 1985).
The F8M data was analyzed a second time to assess trends during the 60 minutes of monitoring. In order to include the twelve 5-minute intervals in an analysis of F8M data, the data were transformed logarithically and analyzed using a repeated measures analysis with time (observation) and interval as the two trial factors. The model was expressed in linear form: time 1 (interval 1 through 12) time 2 (interval 1 through 12) time 3 (interval 1 through 12) - constant + treatment. Data were corrected for variation of the control group between times by subtracting the activity of the control group from the toxicant-dosed groups at each trial time. The significance of differences between selected control-corrected toxicant-dosed groups over time was assessed using contrasts at $\alpha = 0.05$.

**LD$_{50}$ Determination**

The LD$_{50}$ of antx-a(s) in this study was determined using eight rats by the up and down method of Bruce (1985) and calculated by the procedure of Salsburg (1984).

**RESULTS**

**LD$_{50}$, Cholinesterase Activities, and Histologic Lesions**

The LD$_{50}$ of antx-a(s) was 5.3 µg/kg. The doses of antx-a(s) employed in this study, 3.0, 1.5, and 0.7 µg/kg, therefore, correlated to 56, 28, and 14% of the LD$_{50}$, respectively. Brain ChE activities in rats given antx-a(s) were not significantly inhibited ($p < 0.05$), but brain ChE activities in rats given paraoxon at 2 and 50 hours postdosing were significantly inhibited ($p < 0.05$) with the ChE activity decreased by 44 and 27%, respectively, compared to the controls. By contrast, whole blood ChE activity was significantly inhibited in rats given either antx-a(s) or paraoxon (Table 1). Histologic lesions were not observed in tissues from rats given any of the treatments.
Body Weight, Grip Strength, and Accelerod

Significant overall treatment effects were detected in body weight data ($F_{[12,74,37]} = 5.19, p < 0.04$). Significant treatment effects were detected between time 3 and times 1 and 2 ($F_{[4,30]} = 14$ and $23, p < 0.01$) but not between times 1 and 2 ($F_{[4,30]} = 0.6, p = 0.7$). Despite the results of Tukey's analysis of weights as presented in Table 2, the mean values and analyses of variance seem to indicate that, at the high doses of antx-a(s), body weight was reduced at 48 hours postdosing.

Significant overall differences within treatment groups were not detected in GS data ($F_{[12,74,37]} = 0.98, p = 0.48$) but were apparent in the AR data ($F_{[12,74,37]} = 2.88, p < 0.01$). In the AR data, significant differences among treatment groups were detected between times 1 and 2 ($F_{[4,30]} = 7.25, p < 0.01$) and times 2 and 3 ($F_{[4,30]} = 4.67, p < 0.01$) but not between times 1 and 3 ($F_{[4,30]} = 0.87, p = 0.5$). Results of Tukey's analysis of the AR data as presented in Table 3 suggest that only the animals given the high dose of antx-a(s) had poorer performance in the AR test, and this occurred only at the first postdosing time point (i.e., the effect at time 2 was reversible).

Figure 8 Maze

F8M activity data are presented in Figures 1, 2, and 3. Analysis of total F8M activity (total activity over 60 minutes) indicated significant overall differences in response to treatments ($F_{[12,66,44]} = 3.05, p < 0.01$). Significant differences among groups were also detected between times 1 and 2 ($F_{[4,27]} = 6.47, p < 0.01$) and times 2 and 3 ($F_{[4,27]} = 6.04, p < 0.01$) but not between times 1 and 2 ($F_{[4,27]} = 0.77, p = 0.55$). Results of Tukey's analysis of total F8M data as presented in Table 4 indicate that at 20 minutes...
postdosing, activity was markedly reduced in the rats given antx-a(s) at the two higher doses and in those given paraoxon.

In the F8M analysis that included the twelve 5-minute intervals, time, interval, and time*interval were significant at (F [2,70] = 28, p < 0.01), (F [11,365] = 73, p < 0.01), and (F [22,770] = 1.9, p = 0.03), respectively. Differences associated with treatment, treatment*interval, and treatment*time*interval components of the model were not highly significant, (F [4,35] = 2, p = 0.11), (F [44,385] = 1.3, p = 0.14), and (F [88,770] = 1.1, p = 0.08), respectively. At time 2, the activity (activity curves) of the rats given antx-a(s) at 3.0 or 1.5 µg/kg or paraoxon were decreased as compared to time 1 (with values of F [1,35] = 17.6, 3.4, and 6.5, and values of p < 0.01, 0.07, and 0.015, respectively). No significant differences in activity between times 1 and 3 were detected among the toxicant-dosed groups.

**Startle Response**

Significant (F [12,66,44] = 1.96, p = 0.04) differences among treatment groups were detected in the amplitudes of response to the tactile stimulus. Marginally significant differences among treatment groups were present in the amplitude of tactile response between times 1 and 2 (F [4,27], p = 0.06) and significant differences were found between times 2 and 3 (F [4,27] = 3.89, p = 0.01) but not between times 1 and 3 (F [4,27] = 0.37, p = 0.8). When data of individual time points were compared, responses of the animals were significantly diminished only at 20 minutes postdosing by the two highest doses of antx-a(s). Results of Tukey's analysis of the SR data for the tactile stimulus are presented in Table 5. Marginally significant (F [12,66,44] = 1.7, p = 0.09) differences among treatment groups were also observed in the amplitude of response during the inhibitory prepulse paradigm;
however, comparisons between treatment groups at any two of the three time points did not reveal any significant differences (p > 0.10).

No significant (F [12,66,44] = 1.33, p = 0.22) differences among treatment groups were present in the amplitude of response to the audio stimulus alone. Also, significant differences among treatment groups were not observed in latency time of response for audio, prepulse, inhibition, or tactile paradigms: (F [12,66,44] = 1.56, p = 0.12), (F [12,66,44] = 0.8, p = 0.6), and (F [12,66,44] = 1.41, p = 0.18), respectively.

**DISCUSSION**

The LD₅₀ of antx-a(s) in rats (5.3 μg/kg) indicated that antx-a(s) in an extremely potent ChE-inhibiting neurotoxin with parenteral toxicity similar to some of the most toxic ChE-inhibiting agents known, such as sarin (isopropylmethylphosphonofluoridate), soman (pinacolymethylphosphonofluoridate), and tabun (dimethylamidoethoxyphosphorylcyanide) (Registry of Toxic Effects of Chemicals, 1979).

In the present study, at 50 hours postdosing, antx-a(s) inhibited ChE activity in whole blood but not brain tissue. Similarly, in Long-Evans rats of the same age that were given antx-a(s) ip at doses of 3.0 and 1.5 μg/kg and killed after 2 hours, no significant inhibition of brain ChE (tested in multiple areas) was detected, but again at these doses, significant inhibition (56 and 86% inhibition, respectively) of whole blood ChE was observed, as compared to controls (Cook et al., 1989b). These results tend to confirm the findings of previous studies (Cook et al., 1988) that antx-a(s) may be unable to cross the blood-brain barrier (BBB). If antx-a(s) does not enter the brain, it would be unable to cause any primary centrally mediated behavioral effects and unlikely to cause behavioral deficits at doses not causing clinical signs due to its action in the periphery.
Antx-a(s) and paraoxon did not cause a decrease in GS. However, in a study by Haggerty et al. (1986), soman, which has a ChE-inhibiting action on the CNS, when given to rats intramuscularly (im) at 54 to 81% of the im LD₅₀, caused a decrease in hindlimb grip strength at 2 hours postdosing, and the effect persisted for 14 days.

In rats, motor coordination, as measured by AR performance, was significantly decreased by the highest dose (58% of the LD₅₀) of antx-a(s). In addition, although significant differences were not detected between treatment groups, AR performance may have been decreased with paraoxon and increased with the subclinical dose of antx-a(s). Effects on motor coordination did not appear to be due to a loss of muscle strength, since strength as evaluated by GS was not remarkably affected in rats given either antx-a(s) or paraoxon. Decrement in AR performance caused by the toxicants appeared to have reversed by 48 hours postdosing. Landauer and Roman (1984) gave rats sarin subcutaneously (sc) at equivalent or greater percentages of the LD₅₀ (59 and 70% of the sc LD₅₀) and also observed significant AR decrements. In addition, decreases in motor coordination have also been demonstrated in rats with relatively high doses of the organophosphorus nerve agent soman and the insecticide dichlorvos (Costa et al., 1983; Lennox et al., 1983).

Motor activity, as measured with the FBM apparatus, when analyzed over the entire 60-minute test period or the twelve 5-minute intervals, was significantly decreased by paraoxon and either of the two highest doses of antx-a(s) but not by the subclinical dose of antx-a(s). As compared to control rats, decrements in motor activity caused by either antx-a(s) or paraoxon appeared to have reversed by 48 hours postexposure.
A biphasic effect on motor activity was not observed with antx-a(s). In contrast, a biphasic change in motor activity was observed previously in rats given sarin sc at 37% of the LD50 (Landauer and Romano, 1984). In that study, a significant increase in spontaneous activity (in the absence of any clinical signs) was detected in the first 10 minutes after dosing, but spontaneous activity was significantly decreased at sarin doses of 51 and 70% of the LD50. A similar biphasic effect in locomotor activity has been observed with the organophosphorus insecticide disulfoton (Clark et al., 1971). The lack of a biphasic effect on locomotor activity by antx-a(s) may be a result of its inability to gain access to the CNS.

Antx-a(s) significantly reduced startle response amplitude to the tactile stimulus only at doses that caused motor deficits detected by the AR and FBM tests. Therefore, the decrements in startle amplitude detected with the two highest doses of antx-a(s) were suspected to have been at least in part due to the known motor deficits. The decrements in response to the tactile stimulus caused by the toxin appeared to have reversed by 48 hours postexposure. The lack of significant effects of either antx-a(s) or paraoxon on the latency of response time to any of the stimuli presented would seem to indicate a lack of centrally mediated effect of these toxicants (at these doses) on neurobehavior.

Various forms of prepulse inhibition serve to evaluate the effect of the toxicants on the integration of additional sensory inputs in the CNS and subsequent modification of the normal response to the eliciting stimulus. Antx-a(s) and paraoxon had no effects on prepulse inhibition. Despite evidence that paraoxon gained entrance into the CNS, neither paraoxon nor antx-a(s) caused alterations in prepulse inhibition response.
The role of acetylcholine on modulation of startle responses is not well understood (Davis, 1980). Diisopropylfluorophosphate (DFP) which increases brain concentrations of acetylcholine when given at 1 mg/kg slightly increased the acoustic startle response, but physostigmine, which is also an effective inhibitor of brain cholinesterase, given at 0.1 to 4 mg/kg did not alter these responses (Davis, 1980). When tested at 2 hours after dosing, rats given soman im at 54 to 81% of the LD50 had a dose-related decrease in the amplitude of response to an acoustic startle stimulus and an increased latency of response (Haggerty et al., 1986). In some rats given soman (50 or 85 μg/kg), an increase in startle response was observed and correlated with an increase in the number of brain lesions (Raffaele et al., 1987). The absence of effects on acoustic startle response amplitude and its latency and the absence of lesions are compatible with the data indirectly indicating that antx-a(s) dose not cross the BBB.

In conclusion, antx-a(s) seemed to cause dose-dependent neurobehavioral deficits in AR, F8M, and SR. However, behavioral deficits did not appear to be due to a loss of muscle strength, since strength as determined by the GS test was not affected. Significant behavioral decrements were observed with antx-a(s) only at relatively high doses (at least 28% of the LD50) which caused overt clinical signs, suggesting that the behavioral effects induced in rats are a result of strictly peripheral actions of the compound. Despite evidence that the toxin is an irreversible inhibitor of ChE, the behavioral effects caused by antx-a(s) appeared to be reversible within 48 hours. This could be explained by possible modulation of the postsynaptic acetylcholine receptor and/or synthesis of new ChE enzyme.
Table 1. Blood cholinesterase activity at 2 and 50 hours postdosing in rats given antx-a(s) or paraoxon.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Time (hours)</th>
<th>Cholinesterase Activity (μmoles/g/minute) Mean ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Antx-a(s)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3.0 μg/kg</td>
<td>50</td>
<td>0.47 ± 0.04&lt;sup&gt;cd&lt;/sup&gt;</td>
</tr>
<tr>
<td>1.5 μg/kg</td>
<td>50</td>
<td>0.58 ± 0.15&lt;sup&gt;bc&lt;/sup&gt;</td>
</tr>
<tr>
<td>0.75 μg/kg</td>
<td>50</td>
<td>0.79 ± 0.21&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Control</td>
<td>50</td>
<td>1.2 ± 0.14&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Paraoxon</td>
<td></td>
<td></td>
</tr>
<tr>
<td>400 μg/kg</td>
<td>2</td>
<td>0.37 ± 0.18&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>400 μg/kg</td>
<td>50</td>
<td>0.76 ± 0.08&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

N = 6 to 8 for all treatments. Different superscript letters indicate significant differences among treatment groups (p < 0.05).
Table 2. Effects of anatoxin-a(s) and paraoxon on body weight.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Time 1 (Predose)</th>
<th>Time 2 (Weight g)</th>
<th>Time 3 (+ 48 hr)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean ± SE</td>
<td>Mean ± SE</td>
<td>Mean ± SE</td>
</tr>
<tr>
<td>Anatoxin-a(s)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3.0 µg/kg</td>
<td>365 ± 7a</td>
<td>370 ± 8a</td>
<td>348 ± 7a</td>
</tr>
<tr>
<td>1.5 µg/kg</td>
<td>352 ± 9a</td>
<td>352 ± 10a</td>
<td>352 ± 9a</td>
</tr>
<tr>
<td>0.75 µg/kg</td>
<td>366 ± 8a</td>
<td>369 ± 9a</td>
<td>372 ± 8a</td>
</tr>
<tr>
<td>Control</td>
<td>367 ± 8a</td>
<td>372 ± 8a</td>
<td>376 ± 8a</td>
</tr>
<tr>
<td>Paraoxon</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>400 µg/kg</td>
<td>370 ± 10a</td>
<td>372 ± 10a</td>
<td>372 ± 11a</td>
</tr>
</tbody>
</table>

*N = 8 for all treatments. Different superscript letters indicate significant differences among treatment groups (p < 0.05).

Time 1 is 48 hours predose. Time 2 is just prior to dosing. Time 3 is 48 hours postdosing.
Table 3. Accelerod performance in rats given anatoxin-a(s) or paraoxon.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Time 1 − 48 hr Mean ± SD (seconds)</th>
<th>Time 2 + 85 min Mean ± SD (seconds)</th>
<th>Time 3 + 49.5 hr Mean ± SD (seconds)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anatoxin-a(s)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3.0 µg/kg</td>
<td>45.9 ± 11.2a</td>
<td>30.1 ± 10.4b</td>
<td>42.5 ± 8.2a</td>
</tr>
<tr>
<td>1.5 µg/kg</td>
<td>46.6 ± 8.2a</td>
<td>49.2 ± 9.5a</td>
<td>49.7 ± 9.9a</td>
</tr>
<tr>
<td>0.75 µg/kg</td>
<td>37.2 ± 10.5a</td>
<td>46.4 ± 13.4a</td>
<td>40.8 ± 8.3a</td>
</tr>
<tr>
<td>Control</td>
<td>36.8 ± 4.1a</td>
<td>39.4 ± 5.0ab</td>
<td>38.4 ± 9.5a</td>
</tr>
<tr>
<td>Paraoxon</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>400 µg/kg</td>
<td>46.0 ± 10.1a</td>
<td>36.4 ± 8.0ab</td>
<td>48.5 ± 5.9a</td>
</tr>
</tbody>
</table>

N = 8 for all treatments. Different superscript letters indicate significant differences among treatment groups (p < 0.05).

Time 1 is 48 hours prior to time 2. Time 2 is 85 minutes postdosing. Time 3 is 49.5 hours after time 2.
Table 4. Figure 8 maze activity in rats given anatoxin-a(s) or paraoxon.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Time 1 - 48 hr</th>
<th>Time 2 + 20-80 min</th>
<th>Time 3 + 48.33hr</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean ± SD</td>
<td>Mean ± SD</td>
<td>Mean ± SD</td>
</tr>
<tr>
<td>Anatoxin-a(s)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3.0 µg/kg</td>
<td>574 ± 143&lt;sup&gt;a&lt;/sup&gt;</td>
<td>131 ± 150&lt;sup&gt;b&lt;/sup&gt;</td>
<td>434 ± 100&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>1.5 µg/kg</td>
<td>434 ± 192&lt;sup&gt;a&lt;/sup&gt;</td>
<td>198 ± 133&lt;sup&gt;b&lt;/sup&gt;</td>
<td>412 ± 159&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>0.75 µg/kg</td>
<td>492 ± 176&lt;sup&gt;a&lt;/sup&gt;</td>
<td>429 ± 128&lt;sup&gt;a&lt;/sup&gt;</td>
<td>399 ± 149&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Control</td>
<td>493 ± 124&lt;sup&gt;a&lt;/sup&gt;</td>
<td>430 ± 146&lt;sup&gt;a&lt;/sup&gt;</td>
<td>394 ± 164&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Paraoxon</td>
<td>498 ± 105&lt;sup&gt;a&lt;/sup&gt;</td>
<td>147 ± 76&lt;sup&gt;b&lt;/sup&gt;</td>
<td>405 ± 159&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

N = 8 for all treatments. Different superscript letters indicate significant differences among treatment groups (p < 0.05).

Time 1 is 48 hours prior to time 2. Time 2 is 20 to 80 minutes postdosing. Time 3 is 48 hours after time 2.
Table 5. Amplitude of startle response to tactile stimuli in rats given anatoxin-a(s) or paraoxon.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Time 1  - 48 hr</th>
<th>Time 2 + 90 min</th>
<th>Time 3 + 49.5 hr</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean ± SE</td>
<td>Mean ± SE</td>
<td>Mean ± SE</td>
</tr>
<tr>
<td>Anatoxin-a(s)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3.0 μg/kg</td>
<td>848 ± 116a</td>
<td>390 ± 42b</td>
<td>830 ± 164a</td>
</tr>
<tr>
<td>1.5 μg/kg</td>
<td>1000 ± 299a</td>
<td>398 ± 60b</td>
<td>812 ± 149a</td>
</tr>
<tr>
<td>0.75 μg/kg</td>
<td>1098 ± 195a</td>
<td>691 ± 83ab</td>
<td>779 ± 144a</td>
</tr>
<tr>
<td>Control</td>
<td>805 ± 131a</td>
<td>885 ± 154a</td>
<td>612 ± 65a</td>
</tr>
<tr>
<td>Paraoxon</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>400 μg/kg</td>
<td>882 ± 111a</td>
<td>566 ± 60ab</td>
<td>598 ± 56a</td>
</tr>
</tbody>
</table>

N = 8 for all treatments. Different superscript letters indicate significant differences among treatment groups (p < 0.05).

Time 1 is 48 hours prior to time 2. Time 2 is 90 to 110 minutes postdosing. Time 3 is 48 hours after time 2.
Figure 1. Figure 8 maze activity in rats given anatoxin-a(s) or paraoxon at time 1 (48 hours prior to time 2, which was 20 to 80 minutes postdosing).
Figure 2. Figure 8 maze activity in rats given anatoxin-a(s) or paraoxon at 20 minutes postdosing (time 2).
Figure 3. Figure 8 maze activity in rats given anatoxin-a(s) or paraoxon at time 3 (48.33 to 49.33 hr postdosing).
V. APPARENT NATURALLY OCCURRING TOXICOSES IN A DOG DUE TO AN ANTICHOLINESTERASE TOXIN FROM ANABAENA SPP.

W. O. Cook, R. A. Lovell, and A. M. Dahlem

The sudden death of a 5-month-old, female, 16-kg golden retriever dog was associated with an algal bloom in a 12-acre lake (Colleens Koats Lake) in Huntley, Illinois (a suburban area 45 miles west of Chicago), in September 1987. The pond contained an algal bloom with a "pea soup" consistency and had been wind-concentrated against the shore line. Another dog, a 1.5-year-old, 41-kg golden retriever, and the dog mentioned above were observed to be wading (not swimming) in the algal bloom for less than 30 seconds (dogs had been let outside recently). The owner was constantly observing the dogs, and they did not drink from the pond. Both dogs had an algal film on their legs when they came ashore. The owner washed off the older dog with a hose before it could lick itself, and this animal did not develop any clinical signs. While the owner was washing the older dog, the younger dog licked its legs and then within 15 minutes developed vomiting, profuse hypersalivation, tremors, fasciculations, and severe respiratory difficulty. The animal became recumbent with its head lowered. Cyanosis and terminal convulsions were observed before death which occurred within 30 minutes of the animal leaving the water. The history was suggestive of oral exposure as the route of poisoning since only the dog that licked itself became ill and died. Postmortem examination revealed only hemorrhage in the duodenum.

A frozen sample of algae was identified as Anabaena spp. and structurally appeared similar to Anabaena flos-aquae. Algae was freeze-dried and 0.04 g reconstituted in 4 ml of distilled water, frozen, thawed, and centrifuged at
10,000 rpm for 5 minutes. Three Swiss-Webster mice weighing 27 to 30 g were dosed ip with 0.2 or 1.0 ml of the algal supernatant or with 1 ml of distilled water. Survival time, organ weights, and blood and brain ChE activities are presented in Table 1. Whole blood ChE but not brain ChE was inhibited in the two mice given the algae supernatant compared to the control mouse. The cause of death in this dog appeared to be exposure to a peripheral-acting, ChE-inhibiting toxin(s) in the algae.Ten and 100 µl of algal supernatant produced approximately 50 and 100% inhibition of ChE activity when added to the Fischer human plasma ChE control, suggesting the toxin(s) were direct-acting inhibitor(s) of ChE.
Table 1. Cholinesterase activities in whole blood and brain tissue and kidney and liver weights of mice dosed ip with a supernatant of freeze-dried blue-green algae from a bloom in Huntley, Illinois, associated with the death of a dog.

<table>
<thead>
<tr>
<th></th>
<th>Cholinesterase Activity</th>
<th>Organ Weights</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Whole Blood (µmole/1/minute)</td>
<td>Brain (µmole/g/minute)</td>
</tr>
<tr>
<td>Control</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 ml DW&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.81</td>
<td>14.0</td>
</tr>
<tr>
<td>Algal supernatant</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.2 ml&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.33</td>
<td>13.6</td>
</tr>
<tr>
<td>1 ml&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.27</td>
<td>13.6</td>
</tr>
</tbody>
</table>

DW = distilled water.

<sup>a</sup>Mouse was killed by cervical dislocation at 48 minutes.

<sup>b</sup>Mouse died 10 minutes after dosing.

<sup>c</sup>Mouse died 5 minutes after dosing.
VI. REFERENCES


