BIOCHEMICAL CHARACTERIZATION OF MICROCYSTIN TOXICITY IN RAINBOW TROUT (*ONCORHYNCHUS MYKISS*)

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F. Tencalla and D. Dietrich. Biochemical characterization of microcystin toxicity in rainbow trout (*Oncorhynchus mykiss*). *Toxicol* **35**, 583-595, 1997.—The kinetics and biochemical effects of microcystins in rainbow trout were studied with freeze-dried toxic cells of *Microcystis aeruginosa*, strain PCC 7806. Following *in vivo* exposure the changes in liver histology were observed over a 72 hr period and the absorption of microcystins from the gastrointestinal tract into the blood and liver, as well as the inhibition of hepatic protein phosphatase 1 and 2A activities, were recorded using the protein phosphatase inhibition assay. The interaction between microcystins and trout liver phosphatases was further tested *in vitro* using the protein phosphatase inhibition assay. The *in vivo* experiments demonstrated a high organotropy of microcystins for the liver, where rapid and total inhibition of protein phosphatase 1 and 2A activity was observed. Maximal inhibition of phosphatases was observed 3 hr after gavage. At that time-point, approximately 63% of the toxin present in the liver was refractive to detection via the phosphatase inhibition assay and therefore most likely covalently bound to cellular proteins. The inhibition of hepatic protein phosphatases 1 and 2A proved to be transient only, as a progressive increase in phosphatase activity was observed beginning 12 hr after gavage of the fish, reaching approximately 50% of the control activity at 72 hr. In contrast, liver damage continued to progress despite this renewed protein phosphatase activity. © 1997 Elsevier Science Ltd. All rights reserved

INTRODUCTION

Microcystins are a family of potent liver toxins produced by certain strains of aquatic cyanobacteria (blue-green algae). The members of this group of cyclic heptapeptides differ mainly at two sites where the amino acids can vary (Kondo *et al.*, 1992; Sivonen *et al.*,

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1992). The toxin containing L-leucine and L-arginine in these sites is the most extensively studied form and has been designated microcystin-LR. Periodic massive growths of microcystin-producing cyanobacteria occur worldwide in fresh and brackish waters subject to eutrophication. Exposure to contaminated water has been responsible for cases of illness in humans (Chorus, 1992), and caused sickness or even death in cattle and wild animals (Codd *et al.*, 1989).

In mammals, microcystins act specifically in the liver, where they are thought to be taken up into the hepatocytes via multispecific bile acid transport systems (Runnegar *et al.*, 1981, 1991; Eriksson *et al.*, 1990a; Hooser *et al.*, 1991). At acutely toxic doses, microcystins cause rounding of the hepatocytes and loss of normal hepatocyte structure. This disorganization of the tissue leads to massive intrahepatic hemorrhage, often followed by death of the animals from hypovolemic shock or hepatic insufficiency (Carmichael, 1992). The changes observed in the hepatocytes have been shown to result from a collapse of the intermediate filament and microfilament network of the cells (Eriksson *et al.*, 1989, 1990b; Falconer and Yeung, 1992; Carmichael, 1994). This process appears to be triggered when microcystins bind to and thereby inhibit important regulatory enzymes, e.g. protein phosphatases 1 and 2A (PP1 and 2A) in the cytosol of the hepatocytes (Eriksson *et al.*, 1992).

In the last few years, evidence has accumulated pointing to the fact that not only mammals but also fish are susceptible to this class of natural toxins. Fish kills involving algae-feeders such as carp (*Cyprinus carpio*) (Viala *et al.*, 1986), as well as omnivorous stages of carnivorous fish or strictly carnivorous fish, have been reported in conjunction with toxic cyanobacterial blooms or Aufwuchs in net pens of salmon farms (Andersen *et al.*, 1993; Rodger *et al.*, 1994). It is indeed surprising that carp can be affected by toxic cyanobacterial blooms, as algae feeders such as carp and tilapia (*Oreochromis niloticus*) are known to graze on non-toxic *Microcystis aeruginosa* strains in environmental conditions (Northcott *et al.*, 1991; Moriarty and Moriarty, 1973) and have been shown to avoid ingestion of cyanobacteria (Dewan *et al.*, 1991) as well as to decrease grazing activity in conjunction with increasing percentage of toxic cyanobacteria (Keshavanath *et al.*, 1994). However, in intensive aquaculture and thus highly confined conditions or during an extensive bloom of toxic cyanobacteria in small lakes and ponds, avoidance or decreasing grazing activity may not suffice to protect algae feeders and other fish from acute intoxications with cyanobacterial toxins. The fact that the efforts in aquaculture of tilapia, carp and salmonids, which represent an important aspect of securing nutrition for the ever-increasing world population, may be jeopardized by blooms of toxic cyanobacteria underlines the necessity to further the understanding of the factors involved in the induction of toxic cyanobacterial blooms and the effects of these toxins on various fish species.

Laboratory experiments have shown that, at the histological level, the effects produced by cyanobacterial hepatotoxins in fish are comparable to those observed in mammals (Phillips *et al.*, 1985; Råberg *et al.*, 1991; Tencalla *et al.*, 1994). However, since many differences exist between the metabolism of homeotherms and poikilotherms, it may not a priori be assumed that protein phosphatase inhibition is the major underlying biochemical mechanism in microcystin toxicity to fish as well.

In the present study, microcystin poisoning in rainbow trout was analysed from both the histological and the biochemical point of view. Trout were administered acutely toxic doses of the microcystin-producing cyanobacteria *M. aeruginosa* PCC 7806 via gavage. Uptake of the toxin into the liver and the effects on protein phosphatase 1 and 2A activities were analysed. The results were compared to the concurrent development of hepatic tissue

damage. The goal was to study the first steps of the toxicity mechanism of microcystins in fish and to compare these observations with the data presently available for mammals.

MATERIALS AND METHODS

Gavage trial

Trout. Yearling rainbow trout *Oncorhynchus mykiss* ($n = 18$), mean weight 60 g, were purchased at the Thedy Waser Commercial Fish Hatchery (Andelfingen, Switzerland). Fish were acclimated for 1 week in 100 liter flow-through aquaria containing 11°C dechlorinated tap water and fed commercial trout food (Hokovit No. 506, Hoffman AG, Switzerland) *ad libitum*. Feeding was interrupted 24 hr before experiments began. Before gavaging, trout were lightly anesthetized by being placed less than 1 min in a 100 mg/ml solution of Tricain (Product No. 06955, Fluka, Switzerland). At the end of the experiments, fish were killed by being exposed for approximately 5 min to the same solution until no opercular activity was visible.

Gavage procedures. The freeze-dried cyanobacterial material (*M. aeruginosa*, strain PCC 7806) used for the experiments was provided by the Swiss Federal Institute for Water Resources and Water Pollution (EAWAG). Before use, the material was analysed for toxin content via reversed-phase high-performance liquid chromatography (HPLC) following the method of Meriluoto *et al.* (1990a). The toxic fraction was shown to contain two peaks possessing similar UV spectra and eluting close together at around 10 min. The area of the first peak was approximately half of that of the second one. Based on the analysis of Martin *et al.* (1990) as well as on the retention time compared to pure microcystin-LR (Product No. M5407, Sigma, Switzerland), the major peak was identified as microcystin-LR. The minor peak may be a slightly different microcystin or a conformational variant of microcystin-LR (Martin *et al.*, 1990). Since Dierstein *et al.* (1988) demonstrated that the substances contained in each peak possessed comparable toxicity to mice, the two peaks were grouped and referred to as microcystin-LR* in the present study. The cyanobacterial material used contained a total of 4.6 µg microcystin-LR*/mg dry weight.

Gavage was carried out using 9 × 0.7 cm 1 ml syringes (Asik, Denmark). Approximately 1 ml of freeze-dried *M. aeruginosa* suspended in dechlorinated water was introduced directly into the trout stomachs. A dose of cyanobacteria equivalent to 5700 µg microcystin-LR*/kg body weight (bw), known to cause acute effects within 96 hr (Tencalla *et al.*, 1994), was chosen. Following treatment, the fish were returned to the flow-through aquaria and observed to see whether any of the gavaged cyanobacteria suspension was coughed up or expelled via the gills.

After 1, 3, 12, 24, 48 and 72 hr, three control and three test fish were killed. Liver samples were collected and immediately frozen at -80°C for determination of protein phosphatase activity and toxin content. Samples of the major organs (liver, spleen, digestive tract, kidney and heart) were fixed in 4% neutral-buffered formalin. Sections of 3 µm were stained with hematoxylin and eosin for histological analysis. Liver samples were also fixed in osmium tetroxide for electron-microscopic analysis. Blood samples were taken via heart puncture for determination of the activities in plasma of lactate dehydrogenase (LDH), alanine aminotransferase (ALT) and aspartate aminotransferase (AST). Plasma samples were furthermore frozen at -80°C for the analysis of toxin content.

Biochemical analysis of samples

Preparation of liver homogenates. Liver samples (200 mg) were rinsed and then homogenized in 600 ml extraction buffer (50 mM Tris-HCl, pH 7.0, 250 mM sucrose, 4 mM EDTA, and 0.1% mercaptoethanol) using two 5 sec strokes of a polytron homogenizer (Kinematica GmbH, Kriens, Switzerland). Cellular debris was eliminated by 10 min centrifugation at 15,000 g. The supernatant fraction was collected and small aliquots were stored at -80°C for determination of the protein phosphatase activity and methanol extraction of microcystin-LR*. The protein content of each sample was measured with a COBAS FARA chemistry analyser (Roche, Switzerland) using a standard kit for the Bradford method (Boehringer, Mannheim, Germany). Average protein content was 20 mg/ml.

Protein phosphatase activity in liver homogenates. The protein phosphatase 1 and 2A activity in the livers was determined using a method adapted from Cohen *et al.* (1988) and described in Sahin *et al.*, 1995. Briefly, liver homogenates were incubated at 30°C with ³²P-radiolabelled glycogen phosphorylase- α . The dephosphorylation of this substrate, which is proportional to the activity of the protein phosphatase present in the sample, was then measured. Incubation lasted for 10 min, during which time the release of ³²P was linear.

Control incubations were carried out in parallel without the addition of liver extracts. Over a period of several weeks, phosphatase activities (measured in picomoles ³²P released/mg protein/10 min) were observed to vary by a factor of 2 to 4 for the same liver extracts. This was due to a gradual loss of activity of the radiolabelled

phosphorylase-a. Therefore, samples from treated animals were always analysed in parallel with samples from control fish gavaged with water only. The results obtained were expressed as a percentage of the activities found in the livers of the control animals.

Concentration of extractable toxin in the livers and plasma samples. In the fish liver samples, the analysis of microcystin content was carried out using a protein phosphatase inhibition assay. The liver homogenates were first extracted with methanol in order to remove interfering substances. With this method, only the extractable toxin fraction could be detected in the livers. Microcystin which was irreversibly bound to cellular proteins, and therefore rendered unable to inhibit the phosphatases, was not measured. For the tests, liver homogenates (400 μ l) were freeze-dried, dissolved in 750 μ l of pure methanol, and shaken overnight at 4°C. Following 5 min centrifugation at 1000 g and 4°C, the supernatant was collected and the pellet resuspended in 750 μ l of pure methanol. The mixture was ultrasonicated for 5 min, shaken for 1 hr at 4°C, and recentrifuged. The supernatant was then added to the one collected after the first extraction step, and freeze-dried. Before analysis, the extracts were redissolved in 100 μ l of phosphatase buffer (50 mM Tris-HCl, pH 7.0, 0.1 mM EGTA, and 0.1% 2-mercaptoethanol).

In the plasma, microcystin concentrations were measured directly. Samples were diluted in phosphatase buffer before use in the tests.

The amount of toxin present in the various samples was determined with a test system consisting of the substrate 32 P-glycogen phosphorylase-a, to which PP1 and PP2A extracted from rapeseed were added (modified from Mackintosh *et al.*, 1990, as described in Sahin *et al.*, 1996). Plasma or methanol liver extracts at different dilutions (1 10^4 -fold) were then introduced into the system and inhibition of substrate dephosphorylation was measured. Controls were carried out with plasma or methanol extracts of animals gavaged with water only. As described earlier, results were expressed as protein phosphatase activity in per cent of the activity found in control fish having received no toxic cyanobacteria. Based on these results, toxin concentrations in the tissue of origin could be deduced. Analyses carried out with pure microcystin-LR (Product No. 5407, Sigma, St Louis, MO, U.S.A.) showed that, under the conditions of this test, 50% inhibition of the PP1 and PP2A activities of the rapeseed extracts occurred with 7.4 pg (0.25 nM) toxin. Toxin concentrations in each liver sample were therefore calculated after determination of the sample dilution causing 50% rapeseed phosphatase inhibition.

In vitro analysis of trout liver protein phosphatase inhibition by microcystin-LR

In order to characterize more clearly the interaction between the cyanobacterial microcystins and the trout PP1 and PP2A present in liver homogenates, a series of *in vitro* analyses was carried out. Liver homogenates containing 14 mg protein/ml were prepared as described above with tissue from a control animal gavaged with water only. Different concentrations of pure microcystin-LR (Sigma) diluted in 100 μ l phosphatase buffer were added to 200 μ l of homogenate (corresponding originally to 50 mg liver tissue and 2.8 mg protein) on ice. The mixture (300 μ l) was vigorously vortexed, divided into aliquots, and stored at -80°C until further analysis. Control samples received buffer with no toxin.

For each toxin dose, the protein phosphatase activity and the toxin content of the samples were analysed as described above. The methanol extraction was carried out with 240 μ l of extracts and 450 μ l of methanol.

For each microcystin-LR concentration, determinations were carried out twice. The amount of toxin added and recovered was expressed in ng microcystin-LR/g liver tissue. Protein phosphatase activities were expressed as percentage of the activity found for control fish treated with water only.

Before use, the microcystin-LR obtained from Sigma was tested for purity with the method of Meriluoto *et al.* (1990a). The signal obtained at 238 nm showed a single peak and a UV spectrum corresponding to that described for microcystin-LR by Eriksson *et al.* (1988).

RESULTS

Gavage trials

Trout recovered quickly from anesthesia and began to swim again in less than 1 min after handling. Only two individuals (from the 1 and 3 hr groups) were observed to expel algae suspension via the gills. Since the amounts expelled appeared to be minute, the gavage was considered successful and the fish were not eliminated from the test.

No mortality of the test animals was observed within the 72 hr test period at a cyanobacteria dose equivalent to 5700 μ g microcystin-LR*/kg bw. However, this dose caused massive necrosis of the liver. As soon as 1 hr after exposure, changes in small, well-defined areas could be observed. In these areas, the typical chord structure of trout liver disappeared and the cytoplasm of the hepatocytes was condensed. Between 3 and

12 hr, the loss of structure increased across the tissue, and limited punctual microhemorrhaging via ruptured vessels was observed. The hepatocyte nuclei began to condense and the cytoplasm of these cells was highly vacuolated (Fig. 1). After 24–48 hr,

Fig. 1. Ultrastructural changes in trout following gavage of rainbow trout (*Oncorhynchus mykiss*) with toxic *Microcystis aeruginosa* PCC 7806 at 5700 μg microcystin-LR*/kg body weight. (a) Structure of a normal hepatocyte from a control trout gavaged with water only; (b) appearance of trout hepatocytes 12 hr postgavage. Microcystin-LR* refers to the approximately 2:1 combination of microcystin-LR, and a structural or conformational variant of comparable toxicity to mice produced the cyanobacteria strain used. Scale bars = 5 μm .

tissue damage involved the whole liver. Hepatocyte membranes were lysed and hepatocyte nuclei were pyknotic. Macroscopically, the livers appeared shrunken and yellow in color 48 hr after gavage although no weight differences were noted when compared to control fish.

Liver damage was reflected by an increase in LDH, AST, and ALT values in plasma. Twenty-four hours after gavage, LDH concentrations began to rise and reached 4209 ± 240 U/liter at 72 hr, i.e. 7-fold the average control values of 637 ± 117 U/liter. AST and ALT also increased after 24 hr, reaching maximal values of 1406 ± 83 U/liter and 319 ± 26 U/liter, respectively, after 72 hr (5 and 25 times the average control values of 298 ± 47 U/liter and 13 ± 4 U/liter for ALT and AST, respectively).

No damage could be observed histologically in any of the other organs (kidney, spleen, heart) throughout the experimental period. The digestive tract of the fish was inspected macroscopically and found to be free of cyanobacteria 12–24 hr after initial gavage.

Biochemical analysis of the samples

In vivo tests. The protein phosphatase activity of liver homogenates from control fish varied between 70 and 200 pmoles ^{33}P released/mg protein/10 min. When assays were carried out with freshly labelled phosphorylase-a, $0.16 \mu\text{l}$ were necessary per incubation. At 1.5–2 weeks after radiolabelling, $0.33 \mu\text{l}$ phosphorylase-a per incubation was used in order to provide for reproducible results. This was due to the gradual loss of activity of the labelled product.

The analysis showed that protein phosphatase activities in the liver of treated animals began to decrease almost immediately after gavaging [Fig. 2(a)]. One hour after exposure to the toxic cyanobacteria, the activity was approximately 60% of control values. At 3 hr, a peak of inhibition was reached and practically no activity could be detected. Values began to recover gradually after this time-point and reached over 50% of those of controls animals at 72 hr.

Absorption of microcystin-LR* from the gastrointestinal tract into the blood appeared to occur rapidly [Fig. 2(b)]. Toxin reached maximal concentrations in the plasma of 517 ± 38 ng/ml at 3 hr. Values then decreased gradually to below the detection limit after 48 hr. The half-life of microcystin-LR* in plasma was calculated to be approximately 3.3 hr. Determination of the area under the curve (AUC) in Fig. 2(b) indicated that the total amount of microcystin-LR* taken up into the blood during the experiment was 6019 ng/ml. Assuming a circulating blood volume of around 4% the body weight of trout (experimental observation), this amount therefore corresponded to 4.4% of the applied toxin dose of $5700 \mu\text{g}$ microcystin-LR*/kg bw.

Microcystin-LR* could be detected as soon as 1 hr after exposure [Fig. 2(c)] in methanol extracts of liver homogenates. Maximum values of 524 ± 197 ng/g tissue were reached at 3 hr. The concentrations then rapidly dropped to 51 ± 5 ng/g after 24 hr and finally to 44 ± 5 ng/g at the last time point. The half-life of the toxin in the liver was approximated to be 6.3 hr for the first rapid elimination phase. After 72 hr, the total amount of extractable toxin detected in the liver [AUC, Fig. 2(c)] was 8494 ng/g tissue. In trout, the liver accounts for around 1% of the total body weight (experimental observation). Therefore, the total amount of extractable toxin present in this organ corresponded to around 1.5% of the microcystin-LR* dose applied.

Fig. 2. Hepatic protein phosphatase activities.

(a) In trout after gavage with toxic *Microcystis aeruginosa* PCC 7806 at the equivalent dose of 5700 μg microcystin-LR*/kg body weight; (b) microcystin-LR* concentrations found in plasma; and (c) extractable microcystin-LR* concentrations in liver tissue. All points represent averages $\pm$ S.D. and are cited in the order of increasing time (hr): (a) 100 $\pm$ 0, 60 $\pm$ 7, 4 $\pm$ 1, 20 $\pm$ 1, 42 $\pm$ 3, 41 $\pm$ 1, 52 $\pm$ 12; (b) 0 $\pm$ 0, 24 $\pm$ 2, 520 $\pm$ 38, 180 $\pm$ 12, 62 $\pm$ 6, 0 $\pm$ 0, 0 $\pm$ 0; (c) 0 $\pm$ 0, 144 $\pm$ 17, 524 $\pm$ 197, 226 $\pm$ 21, 51 $\pm$ 5, 40 $\pm$ 8, 44 $\pm$ 5.

In vitro tests. Protein phosphatase activities in liver homogenate samples from control fish, determined *in vitro*, were approximately 180 pmoles ^{33}P released/mg protein/10 min. These values compared well with those obtained for the *in vivo* experiments.

Trout liver protein phosphatase 1 and 2A enzyme activities were inhibited by pure microcystin-LR *in vitro* in a dose-dependent fashion, as shown in Fig. 3(a). A 50% inactivation of the activity of the combined enzymes was obtained when 600 ng toxin was added/g extracted liver. Total inhibition occurred at an addition of 2000 ng pure toxin/g liver homogenate.

Fig. 3. (a) Inhibition of protein phosphatases 1 and 2A contained in trout liver homogenates by pure microcystin-LR; (b) microcystin-LR recovery after methanol extraction of the same liver extracts.

Pure microcystin-LR (Sigma product no. M5407) was added to liver homogenates from control trout. Inhibition of the protein phosphatase activity in the samples was measured as described in Materials and Methods. Samples were then extracted with methanol and toxin concentrations were determined using the protein phosphatase inhibition assay. Phosphatase activities shown represent average $\pm$ S.D. and are cited in the order of increasing concentrations of toxin added: (a) 100 ± 0 , 100 ± 0 , 100 ± 0 , 98 ± 1 , 95 ± 8 , 72 ± 3 , 25 ± 3 , 5 ± 1 , 0 ± 0 , 0 ± 0 ; (b) 0 ± 0 , 0 ± 0 , 0 ± 0 , 100 ± 4 , 100 ± 1 , 500 ± 17 , 550 ± 42 , 5400 ± 297 , 7500 ± 78 .

The tests showed that, although the recovery of extractable toxin was almost complete (results not shown), methanol extraction did not allow determination of the total amount of toxin present in the liver samples [Fig. 3(b)]. Indeed, no microcystin-LR could be detected in the methanol fraction when as little as 100 ng toxin was added per g of liver homogenate. Increasing amounts of toxin were, however, recovered at concentrations above 100 ng/g, and as of 2000 ng/g, recovery rate increased linearly.

DISCUSSION

In the present experiments, trout were gavaged with acutely toxic doses of the freeze-dried cyanobacteria *M. aeruginosa* PCC 7806, producing microcystin-LR and an equally toxic structural or conformational variant of microcystin-LR (referred to together in this paper as microcystin-LR*). The results of the tests yielded information on the kinetics of microcystins in trout during acute intoxication. Data on the interaction between microcystins and hepatic protein phosphatases, as well as the impact of this interaction on liver structure and function, were also obtained.

Kinetics

In the exposed trout, absorption of microcystin-LR* from the gastrointestinal tract into the blood occurred rapidly. The intestinal tract appeared, however, to represent an important barrier to the toxin since less than 5% of the applied dose reached the blood. Once in the blood, microcystin-LR* was quickly taken up into the liver, where concentrations peaked 3 hr after gavage. The speed of this uptake and the clear specificity of toxic effects to the liver support the hypothesis that, as in mammals (Runnegar *et al.*, 1981, 1991; Eriksson *et al.*, 1990a; Hooser *et al.*, 1991), bile acid carrier systems play an important role in fish for uptake of the toxin into the target organ.

The decrease of toxin concentrations in the liver occurred more slowly than uptake, and toxin could still be extracted from this organ 3 days after gavage. There is evidence that bile plays an important role in the elimination and recirculation of excess microcystin from the liver of fish. Sahin *et al.* (1996) detected microcystins in the gall bladder of trout gavaged with toxic cyanobacteria. Toxin has also been found in the digestive tract of salmon after i.p. dosing with tritiated dihydromicrocystin-LR (Williams *et al.*, 1995). This is comparable to the situation observed in mice and rats (Falconer *et al.*, 1986; Meriluoto *et al.*, 1990b; Pace *et al.*, 1991).

Using the protein phosphatase inhibition assay, it was possible to show that the extractable amount of microcystin-LR accumulated in the livers of treated fish over the 72 hr experimental period was equivalent to 1.5% of the applied dose (530 ng microcystin-LR/g liver tissue). The *in vitro* tests, however, suggested that the extractable toxin was only a part of the total microcystin-LR* present. The importance of the remaining fraction, probably bound to protein phosphatases and eventually other cellular proteins, could be extrapolated from these same *in vitro* experiments. For this estimation, it was assumed that 3 hr after gavage of the trout when PP1 and PP2A activity was close to 0% and an excess of toxin was present in the livers (Fig. 2), the hepatic protein phosphatases were saturated with microcystin-LR*. As suggested by Takai *et al.* (1995) the microcystin-phosphatase bond was considered to be irreversible. It was further postulated that the *in vitro* and *in vivo* exposure situations were comparable. This would mean that, as shown in Toivola *et al.* (1994), the toxin present in the liver *in vivo* was

concentrated in the cytoplasm and only small losses occurred during the preparation of homogenates. Under these assumptions, it could be deduced that, at the 3 hr time point when PP1 and PP2A activity was almost 0%, 2000 ng microcystin-LR*/g tissue were present in the liver *in vivo*, as was the case *in vitro* [Fig. 3(a)]. In the livers of treated fish, 530 ng microcystin-LR*/g could be extracted with methanol 3 hr after gavage. The amount of non-extractable microcystin-LR* therefore corresponded to 1470 ng toxin/g hepatic tissue, i.e. the difference between 2000 and 530 ng/g. The conclusion is that the total amount of toxin which reached the livers, represented by the total extractable fraction [calculated above using the area under the curve of Fig. 2(c)] plus the bound fraction, was equal to 8494 plus 1470 ng/g liver. Considering a liver weight in trout of 1% the body weight, this value corresponds to 1.7% of the applied toxin dose of 5700 µg microcystin-LR/kg bw.

The data obtained here for microcystin-LR* kinetics in trout are in accordance with findings in Atlantic salmon injected i.p. with tritiated dihydromicrocystin-LR (Williams *et al.*, 1995). Many similarities also exist with the results previously found for mammals (Robinson *et al.*, 1991; Nishiwaki *et al.*, 1994). In all cases, a rapid toxin accumulation in the liver and a slower elimination/degradation were observed. Between the various experiments, differences nevertheless exist in the time at which peak concentrations are reached in liver and plasma. These range from 1 min to 12 hr (Falconer *et al.*, 1986; Lin and Chu, 1994). In addition, no concordance exists concerning the percentage of toxin taken up into the liver for a given application route. Following gavage, an uptake of 1–2% has been found for both trout (present study) and mice (Nishiwaki *et al.*, 1994). The values, however, range from 4.9 to over 70% after i.v. application in salmon and rodents (Falconer *et al.*, 1986; Robinson *et al.*, 1991; Williams *et al.*, 1995). These differences may be due to species-specific factors, or the type of toxin used for the tests. It has, for example, been suggested that the reduction of the double bond of the Mdha residue of radiolabelled dihydromicrocystin-LR might have an effect on uptake and clearance of the peptide, despite similar phosphatase-inhibiting properties to the parent compound (Williams *et al.*, 1995).

Protein phosphatase inhibition

The effects of protein phosphatase 1 and 2A inhibition on hepatocyte degeneration have been demonstrated using cell culture systems (Eriksson *et al.*, 1990b) but the link had not yet been made in living animals. In the present experiments, application of microcystin-LR* proved to have strong effects on hepatic PP1 and PP2A activity of trout *in vivo*, and this inhibition could be shown to correlate with the development of liver damage in the animals. Following gavage of the fish, PP1 and PP2A activities progressively decreased and changes in liver structure appeared as toxin concentrations in the liver increased (Figs 1 and 2).

Surprisingly, 3 hr after treatment, the enzymatic activities recovered and reached almost 50% of control values within 72 hr. This was unexpected, since damage continued to progress and the livers gradually became necrotic. As the phosphatase–microcystin binding is irreversible (Takai *et al.*, 1995), the observed increase in activity may be explained by a new synthesis of enzymes by still functional hepatocytes, in an attempt to counter the imbalance. The fact that this reaction mechanism was, however, ineffective in stopping the progression of liver damage yields information on the events involved in the toxicity mechanism of microcystins. Indeed, it suggests that the time-limited phosphatase

inhibition triggered an apparently irreversible cascade of reactions leading to liver necrosis. That the phosphatases themselves are the key element in this chain of events can be deduced from the type of effects observed in the livers of the gavaged trout. These included loss of hepatocyte structure, as has been described for mammal cells exposed to microcystin (Eriksson *et al.*, 1989, 1990b; Falconer and Yeung, 1992). Also, PP1 and PP2A have a broad spectrum of substrates *in vivo* and are known to play an essential role not only in cytoskeletal integrity, but also in intracellular transport (Davidson *et al.*, 1992) and cell division mechanisms (Wolniak and Larsen, 1992).

Nevertheless, if PP1 and PP2A are important for microcystin toxicity, the exact link between inhibition of these enzymes and mortality remains to be explained. According to the results of i.p. injection tests carried out by Lin and co-workers (Lin and Chu, 1994), PP2A is also partially inhibited in mice after application of sublethal doses of microcystin-LR. In the present study, a total inactivation of PP1 and PP2A in the presence of excess amounts of toxin was lethal. The question thus remains open whether a threshold exists, which must be overcome before irreversible cell damage occurs.

Finally, whether binding to phosphatases is the only mechanism involved in the acute toxicity of microcystin *in vivo* has not yet been definitely proven. Elevated expression of TNF- α has, for example, been noted in mice after exposure to microcystin (Nakano *et al.*, 1991). The possibility that, *in vivo*, such a reaction may also have a role in some of the effects observed in the acute response of fish or mammals to toxic doses of microcystin cannot be eliminated as long as the identity and respective importance of the various proteins involved in the cascade of reactions are not entirely elucidated.

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REFERENCES

- Andersen, R. J., Hue, A. L., Chen, D. Z. H., Holmes, C. F. B., Kent, M. L., Le Blanc, M., Taylor, F. J. R. and Williams, D. E. (1993) Chemical and biological evidence links microcystin to 'salmon netpen disease'. *Toxicon* **31**, 1315–1320.
- Carmichael, W. W. (1992) Cyanobacterial secondary metabolites: the cyanotoxins. *J. appl. Bacteriol.* **72**, 445–459.
- Carmichael, W. W. (1994) The toxins of cyanobacteria. *Scient. Am.* **1994**, 64–72.
- Chorus, I. (1992) Algal metabolites and water quality: toxins, allergens and taste-and-odor substances. *Int. J. Limnol.* **52**, 257.
- Codd, G. A., Bell, S. G. and Brooks, W. P. (1989) Cyanobacterial toxins in water. *Wat. Sci. Tech.* **21**, 1–13.
- Cohen, P., Alemany, S., Hemmings, B. A., Resink, T. J., Stralfors, P. and Lim Tung, H. Y. (1988) Protein phosphatase-1 and protein phosphatase-2A from rabbit skeletal muscle. *Meth. Enzymol.* **159**, 390–408.
- Davidson, H. W., McGowan, C. H. and Balch, W. E. (1992) Evidence for the regulation of exocytic transport by protein phosphorylation. *J. Cell Biol.* **116**, 1343–1355.
- Dewan, S., Wahab, M. A., Beveridge, M. C. M., Rahman, M. H. and Sarkar, B. K. (1991) Food selection, electivity and dietary overlap among planktivorous Chinese and Indian major carp fry and fingerlings grown in extensively managed, rain-fed ponds in Bangladesh. *Aquacult. Fish. Management* **22**, 277–294.
- Dierstein, R., Kaiser, I. and Weckesser, J. (1988) Rapid determination of *Microcystis* sp. toxins by reversed-phase liquid chromatography. *FEMS Microbiol. Lett.* **49**, 143–147.
- Eriksson, J. E., Meriluoto, J. A., Kujari, H. P. and Skulberg, O. M. (1988) A comparison of toxins isolated from the cyanobacteria *Oscillatoria agardhii* and *Microcystis aeruginosa*. *Comp. Biochem. Physiol.* **89**, 207–210.
- Eriksson, J. E., Paatero, G. I., Meriluoto, J. A., Codd, G. A., Kass, G. E., Nicotera, P. and Orrenius, S. (1989) Rapid microfilament reorganisation induced in isolated rat hepatocytes by microcystin-LR, a cyclic peptide toxin. *Exp. Cell. Res.* **185**, 86–100.

- Eriksson, J. E., Gronberg, L., Nygård, S., Slotte, J. P. and Meriluoto, J. A. (1990a) Hepatocellular uptake of 3H-dihydromicrocystin-LR, a cyclic peptide toxin. *Biochim. biophys. Acta* **1025**, 60–66.
- Eriksson, J. E., Toivola, D., Meriluoto, J. A., Karaki, H., Han, Y. G. and Hartshorne, D. (1990b) Hepatocyte deformation induced by cyanobacterial toxins reflects inhibition of protein phosphatases. *Biochem. biophys. Res. Commun.* **173**, 1347–1353.
- Eriksson, J. E., Brautigan, D. L., Vallee, R., Olmsted, J., Fujiki, H. and Goldman, R. D. (1992) Cytoskeletal integrity in interphase cells requires protein phosphatase activity. *Proc. natn. Acad. Sci. U.S.A.* **89**, 11093–11097.
- Falconer, I. R. and Yeung, D. S. (1992) Cytoskeletal changes in hepatocytes induced by *Microcystis* toxins and their relation to hyperphosphorylation of cell proteins. *Chem.-Biol. Interact.* **81**, 181–196.
- Falconer, I. R., Buckley, T. and Runnegar, M. T. (1986) Biological half-life, organ distribution and excretion of 125-I-labelled toxic peptide from the blue-green alga *Microcystis aeruginosa*. *Aust. J. Biol. Sci.* **39**, 17–21.
- Hooser, S. B., Kuhlenschmidt, M. S., Dahlem, M. S., Beasley, V. R., Carmichael, W. W. and Haschek, W. M. (1991) Uptake and subcellular localisation of tritiated dihydromicrocystin-LR in rat liver. *Toxicol.* **29**, 589–601.
- Keshavanath, P., Beveridge, M. C. M., Baird, D. J., Lawton, L. A., Nimmo, A. and Codd, G. A. (1994) The functional grazing response of a phytoplanktivorous fish *Oreochromis niloticus* to mixtures of toxic and non-toxic strains of the cyanobacterium *Microcystis aeruginosa*. *J. Fish Biol.* **45**, 123–129.
- Kondo, F., Ikai, Y., Oka, H., Ishikawa, N., Watanabe, M. F., Watanabe, M., Harada, K. and Suzuki, M. (1992) Separation and identification of microcystins in cyanobacteria by frit-fast atom bombardment liquid chromatography. *Toxicol.* **30**, 227–237.
- Lin, J.-R. and Chu, F. S. (1994) Kinetics of distribution of microcystin-LR in serum and liver cytosol of mice: an immunochemical analysis. *J. Agric. Fd Chem.* **42**, 1034–1040.
- Mackintosh, C., Beattie, K. A., Klumpp, S., Cohen, P. and Codd, G. A. (1990) Cyanobacterial microcystin-LR is a potent and specific inhibitor of protein phosphatases 1 and 2A from both mammals and higher plants. *Fedn Eur. biochem. Socs Lett.* **264**, 187–192.
- Martin, C., Sivonen, K., Matern, U., Dierstein, R. and Weckesser, J. (1990) Rapid purification of the peptide toxins microcystin-LR and nodularin. *FEMS Microbiol. Lett.* **56**, 1–5.
- Meriluoto, J. A., Eriksson, J. E., Harada, K., Dahlem, A. M., Sivonen, K. and Carmichael, W. W. (1990a) Internal surface reversed-phase high-performance liquid chromatographic separation of the cyanobacterial peptide toxins microcystin-LA, -LR, -YR, -RR and nodularin. *J. Chromat.* **509**, 390–395.
- Meriluoto, J. A., Nygård, S. E., Dahlem, A. M. and Eriksson, J. E. (1990b) Synthesis, organotropism and hepatocellular uptake of two tritium-labeled epimers of dihydromicrocystin-LR, a cyanobacterial peptide toxin analog. *Toxicol.* **28**, 1439–1445.
- Moriarty, D. J. W. and Moriarty, C. M. (1973) The assimilation of carbon from phytoplankton by two herbivorous fishes: *Tilapia niloticus* and *Haplochromis nigripinnis*. *J. Zool. Lond.* **171**, 41–55.
- Nakano, Y., Shirai, M., Mori, N. and Nakano, M. (1991) Neutralisation of microcystin shock in mice by tumor necrosis-alpha factor antiserum. *Appl. envir. Microbiol.* **57**, 327–330.
- Nishiwaki, R., Ohta, T., Sueoka, E., Suganuma, M., Harada, K., Watanabe, M. F. and Fujiki, H. (1994) Two significant aspects of microcystin-LR: specific binding and liver specificity. *Cancer Lett.* **83**, 283–298.
- Northcott, M. E., Beveridge, M. C. M. and Ross, L. G. (1991) A laboratory investigation of the filtration and ingestion rates of the tilapia *Oreochromis niloticus*, feeding on two species of blue-green algae. *Envir. Biol. Fishes* **31**, 75–85.
- Pace, J. G., Robinson, N. A., Miura, G. A., Matson, C. F., Geisbert, T. W. and White, J. D. (1991) Toxicity and kinetics of 3H-microcystin-LR in isolated perfused rat livers. *Toxic. appl. Pharmac.* **107**, 391–397.
- Phillips, M. I., Roberts, R. J., Stewart, J. A. and Codd, G. A. (1985) The toxicity of the cyanobacterium *Microcystis aeruginosa* to rainbow trout, *Salmo gairdneri* Richardson. *J. Fish Dis.* **8**, 339–344.
- Råberg, C. M. I., Bylund, G. and Eriksson, J. E. (1991) Histopathological effects of microcystin-LR, a cyclic peptide toxin from the cyanobacterium (blue-green alga) *Microcystis aeruginosa*, on common carp (*Cyprinus carpio* L.). *Aquat. Toxic.* **20**, 131–146.
- Robinson, N. A., Pace, J., Matson, C. F., Miura, G. A. and Lawrence, W. B. (1991) Tissue distribution, excretion and hepatic biotransformation of microcystin-LR in mice. *J. Pharmac. exp. Ther.* **256**, 176–181.
- Rodger, H. D., Turnbull, T., Edwards, C. and Codd, G. A. (1994) Cyanobacterial (blue-green algal) bloom associated pathology in brown trout, *Salmo trutta* L., in Loch Leven, Scotland. *J. Fish Dis.* **8**, 177–181.
- Runnegar, M. T., Falconer, I. R. and Silver, J. (1981) Deformation of isolated rat hepatocytes by a peptide hepatotoxin from the blue-green algae *Microcystis aeruginosa*. *Naunyn-Schmiedeberg's Arch. Pharmac.* **317**, 268–273.
- Runnegar, M. T., Gerdes, R. G. and Falconer, I. R. (1991) The uptake of the cyanobacterial hepatotoxin microcystin by isolated rat hepatocytes. *Toxicol.* **29**, 43–51.
- Sahin, A., Tencalla, F. G., Mez, K., Dietrich, D. and Naegeli, H.-P. (1995) Enzymatic analysis of liver samples from rainbow trout for the diagnosis of blue-green algae-induced toxicosis. *Am. J. vet. Res.* **56**, 1110–1115.

- Sahin, A., Tencalla, F. G., Dietrich, D. and Naegeli, H.-P. (1996) Biliary excretion of biochemically active cyanobacteria (blue-green algae) hepatotoxins in fish. *Toxicology* **106**, 123–130.
- Sivonen, K., Namikoshi, M., Evans, W. R., Carmichael, W. W., Sun, F., Rouhiainen, L., Luukkainen, R. and Rinehart, K. L. (1992) Isolation and characterisation of a variety of microcystins from seven strains of the cyanobacterial genus *Anabaena*. *Appl. envir. Microbiol.* **58**, 2495–2500.
- Takai, A., Sasaki, K., Nagai, H., Mieskes, G., Isobe, M., Isono, K. and Yasumoto, T. (1995) Inhibition of specific binding of okadaic acid to protein phosphatase 2A by microcystin-LR, calyculin-A and tautomycin: method of analysis of interactions of tight-binding ligands with target proteins. *Biochem. J.* **306**, 657–682.
- Tencalla, F. G., Dietrich, D. R. and Schlatter, C. (1994) Toxicity of *Microcystis aeruginosa* peptide toxin to rainbow trout (*Oncorhynchus mykiss*). *Aquat. Toxic.* **30**, 215–224.
- Toivola, D. M., Eriksson, J. E. and Brautigan, D. L. (1994) Identification of protein phosphatase 2A as the primary target for microcystin-LR in rat liver homogenates. *Fedn Eur. biochem. Socs Lett.* **334**, 175–180.
- Viala, I., Montecino, V., Mühlhauser, H. and Cabrera, S. (1986) Diagnóstico y evaluación del potencial biológico de lagos naturales y artificiales de Chile Central. *Ambiente Desarrollo* **2**, 127–137.
- Williams, D. E., Kent, M. L., Andersen, R. J., Klix, H. and Holmes, C. F. B. (1995) Tissue distribution and clearance of tritium-labelled dihydromicrocystin-LR epimers administered to Atlantic salmon via intraperitoneal injection. *Toxicon* **33**, 125–130.
- Wolniak, S. and Larsen, P. (1992) Changes in the metaphase transit times and the pattern of sister chromatid separation in the stamen hair cells of *Tradescantia* after treatment with protein phosphatase inhibitors. *J. Cell Sci.* **102**, 691–715.