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Enzymatic analysis of liver samples from rainbow trout for diagnosis of blue-green algae-induced toxicosis

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Summary

Microcystin and related toxic peptides produced by cyanobacteria (blue-green algae) are potent and selective inhibitors of protein phosphatases 1 and 2A. We adapted existing enzymatic techniques to analyze the liver of rainbow trout after oral administration of hepatotoxic cyanobacteria. Liver tissue was removed 3 and 12 hours after treatment, and phosphatase activity was determined in liver extracts, using a specific phosphoprotein substrate. In all samples from fish exposed to toxic cyanobacteria, phosphatase activity was suppressed, whereas the control enzyme, lactate dehydrogenase, present in the same liver extract, was not affected by cyanobacteria. Thus, experimental poisoning by hepatotoxic cyanobacteria resulted in an abnormally low ratio of phosphatase to lactate dehydrogenase activity in the liver extracts. These results indicate that specific inhibition of phosphatases 1 and 2A may provide a useful diagnostic tool to determine the early effects of cyanobacteria toxic peptides directly in liver samples from poisoned animals. Although this test was developed with rainbow trout, it should be possible to extend the analysis of liver phosphatase activity to other species, including sheep and cattle, which are frequently affected by hepatotoxic cyanobacteria.

Microcystin belongs to a class of potent cyclic penta- and heptapeptides produced by freshwater cyanobacteria (blue-green algae), such as the unicellular *Microcystis* sp or the filamentous *Anabaena, Aphanizomenon, Nodularia,* and *Oscillatoria* spp.[1-4] Under appropriate conditions, these cyanobacteria form waterblooms (ie, accumulations of cells at the surface of lakes, rivers, reservoirs, or other water bodies).[5,6] The toxic polypeptides produced by these microorganisms cause poisonings of wild and domestic animals in many parts of the world,[7-9] and cyanobacterial contamination of water supplies leads to potential hazards to human health.[10,11] Toxicity of these cyanobacterial peptides is selectively targeted to the liver,[12] presumably as a result of the bile acid transporter that carries microcystin and related compounds into hepatocytes.[13] The hepatotoxic effect of these peptides is characterized in mammals by coagulative hepatocyte necrosis, breakdown of hepatic endothelium, and massive liver hemorrhage leading to rapid death attributable to hemodynamic shock.[14,15] At the biochemical level, the extreme hepatotoxicity of these compounds involves highly specific inhibition of protein phosphatases 1 and 2A.[16-19] These enzymes remove phosphate groups from proteins and are of prime importance in regulating cellular functions by controlling the phosphorylation state of their substrates.[20,21]

Currently, cyanobacteria poisoning usually is inferred from the presence of toxic components in algal blooms. In most instances, the mouse bioassay is used to determine the toxicity of crude algal biomass.[5,12,14] Frequently, however, animals are seemingly poisoned by cyanobacteria hepatotoxins despite lack of overt algal bloom. Also, environmental conditions that favor cyanobacteria bloom formation may change rapidly, and waterblooms may disappear before samples for toxicologic analysis are collected. As a consequence, poisonings by cyanobacteria are frequently not recognized, particularly in areas where this problem has not been reported, or during periods when weather conditions are apparently not optimal for algal growth. Diagnostic problems also arise when poisonings are correlated with the growth of cyanobacteria species that have not been identified as potential toxin producers. To facilitate identification of cyanobacteria as a cause of disease or death, we decided to adapt widely used enzymatic techniques to detect specific effects induced by cyanobacteria toxins in the liver of affected fish. This method is based on the ability of cyanobacteria hepatotoxins to selectively inhibit protein phosphatases 1 and 2A.

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Materials and Methods

Rainbow trout—Experiments were carried out on 1-year-old rainbow trout (*Oncorhynchus mykiss*), mean body weight of 30 to 60 g, purchased from a commercial fish hatchery.[a] Fish were acclimated 2 weeks in flowthrough aquaria containing 15 to 18 C dechlorinated tap water, then were transferred to 100-L aerated recirculation tanks for the experiments. Trout were fed commercial food ad libitum,[b] and feeding was interrupted 24 hours before exposure to cyanobacteria. Immediately prior to treatment, trout were anesthetized by placement in a solution of 3-aminobenzoic acid ethyl ester (100 mg/L).[c]

Experimental protocol—Crude algal biomass obtained from *M aeruginosa* PCC 7806 was freeze-dried and suspended in dechlorinated water. Fish were orally gavaged with 1.44 g of biomass/kg of body weight in a volume of 1 ml as described.[22] This cyanobacteria dosage induced an acute toxic response within 96 hours after treatment; the oral LD_{50} was estimated to be approximately 1 g of biomass/kg.[22] The liver was removed 3 (n = 3) and 12 hours (n = 2) after treatment and stored at −20 C. Controls (2 groups of 4 fish) were gavaged with 1 ml of plain dechlorinated water.

Preparation of liver extracts—Extracts were prepared from fish liver by use of the method of Ingebritsen et al.[23] All steps were performed on ice. Liver tissue (typically 200 mg) was washed with 1 ml of extraction buffer (50 m*M* Tris-HCl, pH 7.0, 250 m*M* sucrose, 4 m*M* EDTA, 0.1% 2-mercaptoethanol), and was homogenized in 0.6 ml of the same buffer, using two 5-second strokes of a homogenizer.[d] Homogenates were centrifuged at 15,000 × g for 10 minutes, and the supernatants were stored in small aliquots at −80 C. The average protein concentration of fish liver extracts was 19.8 mg/ml, as determined by the method of Bradford.[24]

Purification of microcystin—Microcystin was extracted and purified from *M aeruginosa* PCC 7806, as described by Martin et al.[25] Lyophilized cells were extracted with 5% (v/v) acetic acid, and the material was centrifuged 25 minutes at 10,000 × g and 4 C. The supernatant was processed through an octadecylsilyl silica gel (C_{18}) cartridge,[e] followed by passage through an ion-exchange cartridge.[f] After washing with 30% methanol, microcystin was eluted from this column with 0.02*M* ammonium bicarbonate in 30% methanol, dried by rotary evaporation, and dissolved in 1 ml of 50 m*M* Tris/HCl, pH 7.0, containing 0.03% (w/v) polyoxyethylene 23 lauryl ether.[g] On analysis by high-performance liquid chromatography (HPLC), 85% of the obtained material eluted with the same retention time as pure microcystin-LR.[g]

Quantification of microcystin in crude algal biomass—The *M aeruginosa* biomass was analyzed for toxin content as described.[22] Briefly, crude algal extracts were obtained by suspending the freeze-dried material in 0.1*M* potassium phosphate buffer, pH 6.8, containing 15% (v/v) acetonitrile, followed by sonication and centrifugation. The supernatant was then resolved by reversed phase HPLC,[h] using commercially available microcystin-LR as a standard. The HPLC separation was monitored by absorbance at 238 nm. This quantification yielded an average microcystin content of 4.6 mg of microcystin-LR/g dry weight of crude algal biomass.[22]

Preparation of phosphoprotein substrate—The preparation of [^{33}P]phosphorylase *a* was adapted from Cohen et al.[26] Phosphorylase *b*[g] (10 mg) was incubated with phosphorylase kinase[g] (200 U) for 60 minutes at 30 C in 1 ml of buffer containing 100 m*M* Tris-HCl, pH 8.2, 100 m*M* sodium glycerol-1-phosphate, 0.1 m*M* $CaCl_2$, 10 m*M* magnesium acetate, and 0.2 m*M* [γ-^{33}P]ATP[i] (5 × 10^6 counts per minute/nmol). The reaction was stopped by addition of 1 ml of 90% saturated ammonium sulfate (adjusted to pH 6.0 with NaOH), and, after incubation on ice for another 60 minutes, the suspension was centrifuged for 10 minutes at 17,500 × g and 4 C. The supernatant was discarded, and the precipitate was resuspended in 0.5 ml of phosphatase buffer (50 m*M* Tris-HCl, pH 7.0, 0.1 m*M* EGTA, 0.1% 2-mercaptoethanol) and 0.5 ml of 90% saturated ammonium sulfate. After centrifugation, the supernatant was again discarded, and [^{33}P]phosphorylase *a* was resuspended in 0.5 ml of phosphatase buffer containing 15 m*M* caffeine and 0.5 ml of 90% saturated ammonium sulfate. The phosphoprotein substrate was stored at 4 C, and could be used for phosphatase assays for up to 4 months. Just prior to use, the substrate was washed by diluting a 20-µl aliquot of the suspension (corresponding to 200 µg of [^{33}P]phosphorylase *a* and sufficient for 20 reactions) with 200 µl of 45% saturated ammonium sulfate (pH 6.0). After centrifugation for 10 minutes at 15,000 × g and 4 C, the washed precipitate was dissolved in 200 µl of phosphatase buffer containing 15 m*M* caffeine.

Phosphatase assay—Fish liver extract (9 µg of protein) was mixed on ice with 1 µg of [^{33}P]phosphorylase *a* (10 µl of the washed substrate solution) and incubated at 30 C in 30 µl of phosphatase buffer containing 100 µg of bovine serum albumin and 0.01% (w/v) polyoxyethylene 23 lauryl ether. In some experiments (Fig 1), the indicated concentrations of purified microcystin-LR were added to these reactions. After various incubation times, phosphatase activity was stopped by addition of 0.15 ml of ice-cold 20% (w/v) trichloroacetic acid. The suspension was allowed to stand on ice for 10 minutes, then was centrifuged for 10 minutes at 13,500 × g and 4 C. A fraction of the supernatant (0.1 ml) was removed, mixed with 5 ml of cocktail,[j] and quantified in a liquid scintillation counter.[k] All values were corrected for background radioactivity obtained in the supernatant when control samples were incubated on ice. In other control reactions performed in the absence of liver extracts, only background amounts of radioactivity were released from the phosphoprotein substrate. Phosphatase activities were expressed as the quantity (picomoles) of ^{33}P released per minute of incubation time and milligrams of protein in the liver extract.

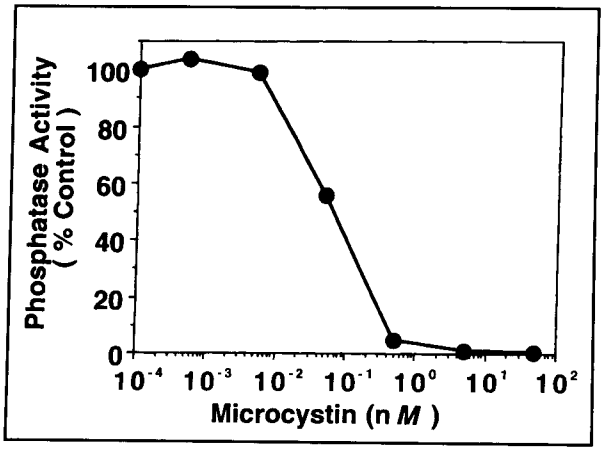

Figure 1—Inhibition of fish phosphatase activity by microcystin. Phosphatases were extracted from the liver of a control fish, and incubated with [^{33}P]phosphorylase *a* and microcystin-LR at the indicated concentrations. Phosphatase activity is expressed as percentage of phosphate released during control reactions performed in the absence of microcystin (mean values of 3 independent determinations). The microcystin-LR concentration inducing 50% inhibition of phosphatase activity was 0.05 n*M*.

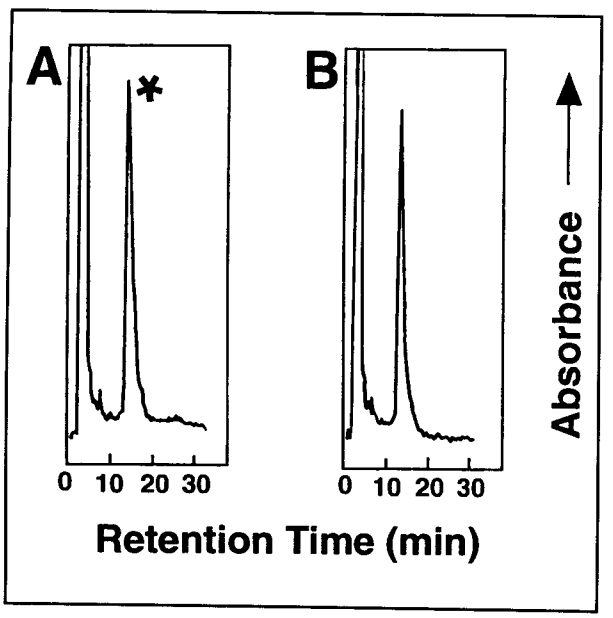

Figure 2—Chromatograms of *Microcystis aeruginosa* crude extracts.
A—Reversed phase separation monitored by absorbance at 238 nm. The material indicated by the asterisk eluted with the same retention time as a pure microcystin-LR standard. Quantitative evaluation of this chromatogram yielded 4.6 mg of microcystin-LR/g dry weight.
B—Addition of pure microcystin-LR to the tenfold diluted crude extract. This analysis confirms that the observed peak coeluted with microcystin-LR.

Assay for lactate dehydrogenase—Lactate dehydrogenase activity was determined by incubating liver extract (2.5 µg of protein) in 1 ml of 50 m*M* phosphate buffer, containing 0.6 m*M* sodium pyruvate and 0.2 m*M* NADH, as described.[27] Reactions were performed at 25 C, and enzyme activity was determined by monitoring the changes in the absorption peak at 340 nm, using a spectrophotometer.[1] Lactate dehydrogenase activity was expressed as units (µmol/min) per milligram of protein in liver extracts.

Data analysis—Student's *t*-test was used for analysis of phosphatase and lactate dehydrogenase activity data. A probability value of $P < 0.005$ was considered significant.

Results

Phosphoprotein substrate—We have considerably simplified the published procedure for preparation and storage of radiolabeled phosphorylase *a*, which is the specific substrate used to test protein phosphatase 1 and 2A activities.[20] Our method requires only 3 hours of laboratory work, and the obtained substrate can be stored and used for up to 4 months. In particular, we replaced ^{32}P as the radioactive isotope with ^{33}P, which is now widely available and has a half-life of 25.4 days. In addition, we found that [^{33}P]phosphorylase *a* can be stored at 4 C as an ammonium sulfate suspension for up to 4 months without loss of enzymatic activity in the phosphatase assay, thus avoiding the dialysis and crystallization step described in literature.[26]

Inhibition of fish phosphatases by microcystin—We first determined that fish liver protein phosphatases are potently inhibited by the peptide toxin (microcystin-LR) purified from *M aeruginosa*. The activity of fish liver phosphatases was tested, using the specific substrate [^{33}P]phosphorylase *a*, in the presence of various concentrations of purified microcystin-LR. As indicated (Fig 1), phosphatase activity was inhibited to approximately 50% at a microcystin-LR concentration of 0.05 n*M*, corresponding to approximately 5×10^{-8} mg/ml. These values are similar to those reported previously for phosphatases from rabbit skeletal muscle, and rat or mouse liver.[16-18] Thus, phosphatases in the fish liver appear to be equally sensitive to microcystin as those from mammalian sources. We decided to develop this inhibition as a potential method for diagnosis of poisonings by hepatotoxic cyanobacteria.

Analysis of crude algal biomass—To establish a quantitative relation between exposure to *M aeruginosa* and toxic effects, we quantified the amount of microcystin contained in the algal biomass, using reversed phase HPLC.[22] A representative chromatogram showing microcystin-LR in crude algal extracts was obtained (Fig 2A). The identity of the observed microcystin peak was confirmed by addition of pure microcystin-LR to the tenfold-diluted crude extract. The microcystin in the crude extract had the same retention time as the microcystin-LR standard (Fig 2B). Quantification of the area under the curve of the peak resulted in an approximate microcystin-LR content of 4.6 mg/g dry weight in the algal biomass.

Suppression of liver phosphatase activity by toxic cyanobacteria—Fish were orally dosed with 1.44 g of *M aeruginosa* algal biomass/kg, corresponding to 6.6 mg of microcystin-LR/kg, as outlined previously. The liv-

ers from experimentally poisoned fish were collected 3 and 12 hours after oral administration of the toxic biomass. Phosphatase activity was then tested in liver extracts prepared from these poisoned fish and from controls maintained under identical conditions. Results of typical phosphatase assays with control and 12-hour postdosing samples were compared (Fig 3). In all instances, the release of inorganic phosphate from the phosphoprotein substrate was linear for incubation times of up to 30 minutes, but the activity was severely reduced in extracts prepared from poisoned fish. Phosphatase activity was then calculated from this linear portion of the reactions and was expressed as the amount of inorganic phosphate (picomoles) released per minute of incubation and milligrams of protein in the liver extract. The mean value of phosphatase activity in extracts from liver collected 3 hours after cyanobacteria administration (0.19 ± 0.11 pmol/min/mg; n = 3) was more than tenfold lower than that in controls (2.28 ± 0.73 pmol/min/mg; n = 4; Fig 4). When analyzed by Student's *t*-test, this difference in phosphatase activity between samples from poisoned and control fish was found to be highly significant ($P < 0.005$). Phosphatase activity determined in extracts from liver collected 12 hours after cyanobacteria administration (0.16 pmol/min/mg; n = 2) was approximately 14-fold lower than the value in controls. In parallel, we tested lactate dehydrogenase in the same liver extracts, but activity of this enzyme was not reduced by treatment with toxic cyanobacteria. On statistical analysis, the apparent increase in lactate dehydrogenase activity (from 4.11 ± 0.66 U/mg to 5.68 ± 0.37 U/mg) was not significant.

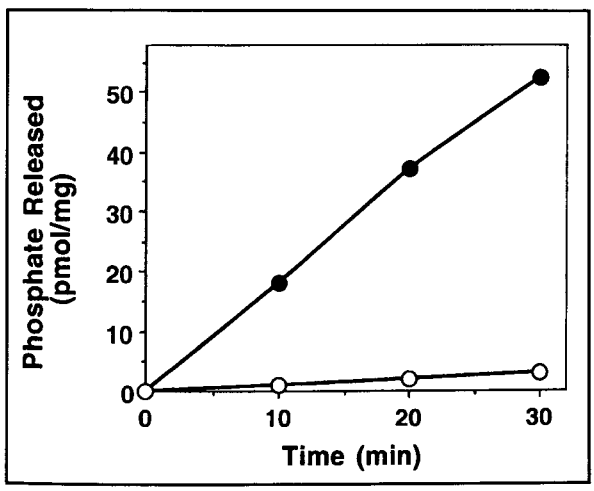

Figure 3—Phosphatase activity observed in extracts from liver tissue collected from a control (●) and a poisoned fish (○) 12 hours after administration of toxic material. Fish liver extracts were incubated with the radioactive phosphoprotein substrate for the indicated periods. Each time point represents the mean value of at least 4 independent determinations. The phosphatase activity measured in samples from treated fish was typically 10- to 20-fold lower than the value in control samples.

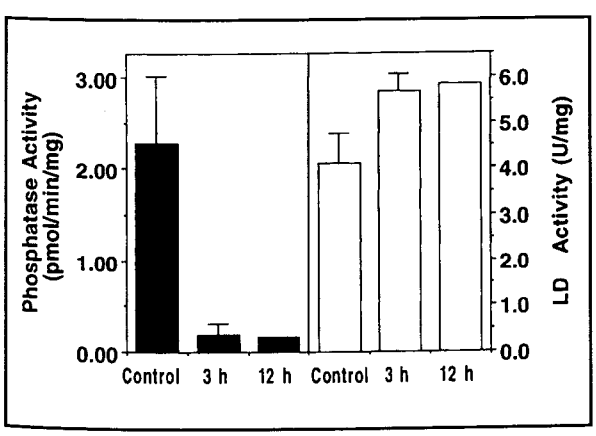

Figure 4—Comparison of phosphatase and lactate dehydrogenase (LD) activities in extracts from control livers or from livers removed 3 and 12 hours after administration of toxic cyanobacteria. Black bars = phosphatase activity; white bars = LD activity. The control, 3-hour, and 12-hour groups consisted of 4, 3, and 2 fish, respectively. The difference in phosphatase activity between control and poisoned fish was highly significant ($P < 0.005$).

Ratio of phosphatase to lactate dehydrogenase activity—We observed a considerable degree of variability in phosphatase and lactate dehydrogenase activities between control samples from different experiments. For example, in a group of controls of the same age, but obtained during a different time of the year, we detected two- to threefold higher phosphatase and lactate dehydrogenase activities. To facilitate interpretation of data, we therefore calculated the ratio (R) of phosphatase to lactate dehydrogenase activity for each sample (Fig 5). On average, this value was R = 0.59 in the first control group, which was not treated with cyanobacteria (Fig 5, samples 6–9). A remarkably similar value (R = 0.86) also was found in the second

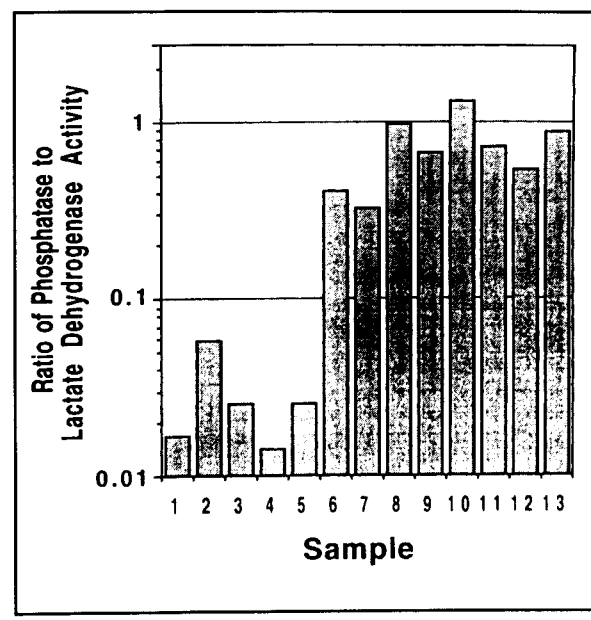

Figure 5—Ratios of phosphatase to LD activity in individual liver samples. Samples 1–5, poisoned fish; samples 6–9, controls; and samples 10–13, controls from another experiment. Data are plotted on a logarithmic scale; the difference between poisoned fish (1–5) and controls (6–13) is of at least 1 order of magnitude.

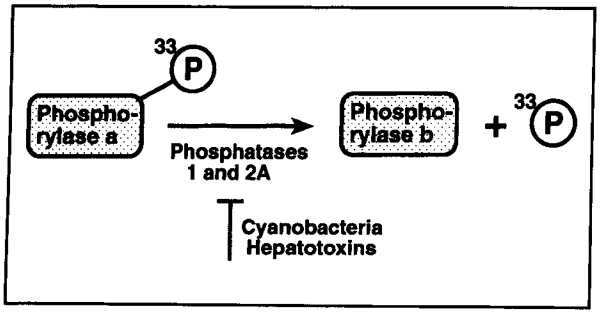

Figure 6—Schematic diagram illustrating the biochemical reaction catalyzed by protein phosphatases. Only phosphatases 1 and 2A are known to be able to remove phosphates from [^{33}P]phosphorylase *a*, and these enzymes are inhibited by microcystin and other hepatotoxins produced by cyanobacteria.

group of controls that had consistently higher enzyme activities (Fig 5, samples 10–13). In contrast, the phosphatase-to-lactate dehydrogenase ratio was reduced to R = 0.03 in fish poisoned by toxic cyanobacteria (Fig 5, samples 1–5). The logarithmic scale used indicates that the R values obtained with samples from poisoned fish were more than 1 order of magnitude lower than those from controls. Thus, an abnormally low ratio of phosphatase to lactate dehydrogenase activity in liver extracts (R < 0.10) may be indicative of poisonings by microcystin and related hepatotoxins.

Discussion

Protein phosphatases 1 and 2A cleave the covalent bond between protein substrates and their phosphate groups, thereby generating dephosphorylated proteins and inorganic phosphate (Fig 6).[20,21] Although many phosphatases have been identified in living cells, only protein phosphatases 1 and 2A are capable of removing phosphates from phosphorylase *a* under the in vitro conditions used in this study.[20,26] In addition, it has been documented that phosphatases 1 and 2A are highly sensitive to cyanobacteria hepatotoxins, such as microcystin, whereas other known phosphatases are not or only moderately inhibited by these compounds.[16-18] Also, it has been reported that intraperitoneal administration of lethal doses of highly purified microcystin markedly decreases liver phosphatase activity in mice.[19] We concluded from these studies that phosphorylase *a* may constitute a specific probe to test for presence of cyanobacteria hepatotoxins in the liver of animals that are suspected of being poisoned by cyanobacteria. This view was confirmed when we found, in our fish model system, that protein phosphatase activity was severely inhibited in liver samples after oral administration of hepatotoxic cyanobacteria (Fig 3 and 4). Importantly, lactate dehydrogenase used as a control enzyme was not inhibited as a consequence of cyanobacteria treatment. Although all fish were obtained from the same hatchery, we observed considerable seasonal variations in the enzyme activities in liver extracts. Both enzymes, phosphatase and lactate dehydrogenase, were affected by these variations. We, therefore, recommend measurement of phosphatase and lactate dehydrogenase activities in the same extract and determination of the ratio (R) between the 2 enzymes. The wide difference between controls, with R values in the range between R = 0.59 and R = 0.86, and cyanobacteria-treated fish (R = 0.03) indicates that this method may be useful when adapted to clinical situations. In rainbow trout, a ratio of phosphatase to lactate dehydrogenase activity of 0.10 or less (Fig 5) is indicative of poisonings by hepatotoxic cyanobacteria.

The available experimental evidence indicates that selective inhibition of protein phosphatases 1 and 2A represents a unique mechanism of hepatotoxicity. Treatment of isolated hepatocytes with microcystin has been documented to induce characteristic morphologic changes associated with contraction and aggregation of actin microfilaments.[16,28-30] Similar effects also were observed in the presence of okadaic acid, another phosphatase 1 and 2A inhibitor produced by marine dinoflagellates and responsible for shellfish poisoning.[16] The agents that have been recognized to generate pathologic changes which most resemble those induced by microcystin, okadaic acid, and their analogs are α-amanitin and phalloidin. These bicyclic peptides, from the mushroom *Amanita phalloides*, also cause hepatic hemorrhagic necrosis associated with disruption of microfilament organization, but these toxins do not inhibit phosphatase activity.[16,19] In addition, many other hepatotoxic compounds of a wide range of chemical structures have been reported to induce microfilament disruption through depletion of glutathione or ATP and an increase in cytosolic free Ca^{2+} concentration.[31-33] However, microfilament disruption by microcystin does not involve changes in the intracellular glutathione, ATP, or Ca^{2+} concentrations,[29] indicating a distinctly different pathway of hepatotoxicity. The inhibition of endogenous liver phosphatases 1 and 2A appears to be specific for the toxic effects of microcystin and related cyclic peptides, or okadaic acid and other polyether-like phosphatase inhibitors.[34]

The possible diagnostic significance of the enzymatic analysis of liver samples presented here is indicated by the fact that, in many areas of the world, poisoning by hepatotoxic cyanobacteria remains unrecognized. Frequently, algal biomass is not available or available only in insufficient amounts at sites where cyanobacteria toxicosis may have occurred, so that the presence of algal toxin cannot be positively determined. In addition, only a few laboratories are equipped with instrumentation to perform toxicologic analysis of algal blooms. Unless the mouse bioassay is used, it is often difficult to correlate the laboratory findings with disease or death in affected animals, particularly if a completely new or less well studied hepatotoxin is involved. Furthermore, intraperitoneal administration of algal extracts does not prove the causal relation between existence of toxins and observed effects, because quantitative data have not been obtained on actual exposure and uptake. On the contrary, the test described here consists of a simple combination of enzymatic assays that can be performed without sophisticated equipment and does not require animals for toxicity tests. Another main

advantage of this enzymatic test is that specific effects attributable to the presence of cyanobacteria hepatotoxins can be determined directly in small liver specimens obtained from recently poisoned animals. In addition, previous studies and our own unpublished results indicate that the extent of liver phosphatase 1 and 2A inhibition is directly related to the dose and hepatotoxic potency of the particular microcystin derivative tested.[19,35] Poisonings by hepatotoxic cyanobacteria have also been reported in sheep, cattle, horses, pigs, and dogs.[5,6,36-38] We are planning to exploit this inhibition for diagnosis of hepatotoxic cyanobacteria poisonings in agricultural livestock or other domestic animals. Efforts in our laboratories are now principally directed to the further development of this assay to test bovine liver samples.

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[a] Thedy Waser, Andelfingen, Switzerland.
[b] Hokovit Nr 506, Hoffman AG, Switzerland.
[c] Fluka, Buchs, Switzerland.
[d] Polytron, Kinematica GmbH, Kriens, Switzerland.
[e] Macherey and Nagel, Düren, Germany.
[f] Accell QMA, Waters, Milford, Mass.
[g] Sigma Chemie, Buchs, Switzerland.
[h] GFF-S5-80 ISRP column, Regis Chemical, Morton Grove, Ill.
[i] DuPont NEN, Regensdorf, Switzerland.
[j] Ready Safe, Beckman Instruments Int, Palo Alto, Calif.
[k] Beckman Instruments Int, Palo Alto, Calif.
[l] Ultrospec Plus, Pharmacia Biotech, Brussels, Belgium.

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