A Sensitive Assay System for Determination of the Ichthyotoxicity of *Prymnesium parvum*

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SUMMARY

The activity of *Prymnesium parvum* ichthyotoxin and its dependence on various cofactors has been studied. Spermine or other polyamines have a marked synergistic effect on the ichthyotoxic activity. Of the cations studied, Ca^{2+} and Mg^{2+} increased ichthyotoxic activity, while Na^+ inhibited. Changes in pH value had a profound influence, ichthyotoxicity increasing at least 400-fold as the pH value was increased from 7 to 9. A sensitive bioassay for the estimation of *P. parvum* ichthyotoxin has been developed for use in the laboratory and under field conditions.

INTRODUCTION

Prymnesium parvum, a halophilic Chrysomonad, has been the cause of extensive fish mortalities in many parts of the world (Shilo, 1964). This organism produces a number of soluble toxic principles including an ichthyotoxin, a haemolysin and a cytotoxin (Otterstrøm & Steeman-Nielson, 1940; Shilo & Rosenberger, 1960; Yariv & Hestrin, 1961). In spite of its widespread endemic occurrence in Israel in brackish water fish ponds, mortality of fish due to *P. parvum* intoxication is observed only sporadically. It has been postulated that the lack of correlation between numbers of phytoflagellates and the toxicity of the pond water was due to a dynamic state of equilibrium existing between toxin formation and destruction (Shilo & Aschner, 1953), since the ichthyotoxin is readily adsorbed on various colloids and inactivated by external factors such as light (Parnas, Reich & Bergmann, 1962), heat, high pH value or by the action of various bacteria (Shilo & Aschner, 1953). It was further shown that the expression of ichthyotoxic activity was dependent on the presence of cationic cofactors such as Ca^{2+} , Mg^{2+} or streptomycin (Yariv & Hestrin, 1961). The lability of the ichthyotoxin and its cofactor requirements pose special problems in its quantitative estimation. The present paper deals with the effect of different factors on fish toxicity and with the development of a sensitive bioassay for ichthyotoxin estimation.

METHODS

Chemicals. The chemicals used were obtained from the following sources: streptomycin sulphate (Merck and Co., U.S.A.); spermine tetrahydrochloride and propane-1,3-diamine (Fluka AG. Buchs SG Switzerland); neomycin sulphate (Uclaf, Paris, France); 3,3-diaminodipropylamine (Eastman Kodak Co., U.S.A.); diethyl-

enetriamine, triethylenetetramine, tetraethylenepentamine (L. Light and Co., Ltd., England); putrescine dihydrochloride and cadavarine dihydrochloride (Nutritional Biochemicals Corp., U.S.A.); spermidine trihydrochloride (California Corp. for Biochemical Research, U.S.A.).

Prymnesium parvum culture. The strain of *P. parvum* used in these experiments had been isolated from brackish-water fish ponds in Israel by Reich & Kahn (1954).

Medium and conditions of growth. The growth medium for *Prymnesium parvum* used in this work was the defined medium developed by Droop (1958) in which the amounts of trace elements were modified as follows (per litre medium): FeCl_3 , 5 mg.; MnCl_3 , 500 $\mu\text{g.}$; ZnSO_4 , 50 $\mu\text{g.}$; CaSO_4 , 50 $\mu\text{g.}$; CoSO_4 , 5 $\mu\text{g.}$; $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$, 5 $\mu\text{g.}$ The medium was sterilized by autoclaving at 120° for 20 min. Cultures were grown in Erlenmeyer flasks of 250 ml. capacity containing 100 ml. liquid medium in continuous 'fluorescent white' daylight, 220–260 foot-candles, at $26\text{--}28^\circ$. Cultures were harvested after a period of 30–40 days of cultivation.

Extraction and assay of ichthyotoxin. The ichthyotoxin produced by *Prymnesium parvum* was extracted from the organisms with ethanol as described by Shilo & Rosenberger (1960). The extraction was facilitated by sonic oscillation (Raytheon sonic oscillator Model DF 101, 10 KC) for 7 min at 4° . Such ethanolic extracts were kept at 4° in the dark and were used in concentrations not exceeding 1% to avoid toxic effects of the ethanol on the test minnows. For titration of ichthyotoxin two-fold dilutions of the extracts of *P. parvum* were prepared in tris buffer (0.05 M) containing cofactor. In the experiments to be described three minnows (1.5–2.0 cm. long) served as test organisms for each dilution. The type and concentration of cofactor were as indicated later and the resulting fish toxicity expressed relative to toxicity under standard conditions of test, as described below in the definition of an ichthyotoxic unit. The pH value of the test solution was readjusted when necessary every hour with N-NaOH.

An 'ichthyotoxic unit' of toxin (ITU) was defined as the minimal amount of *Prymnesium parvum* toxin/ml. which killed within 8 hr all the fish in the test system containing tris buffer (0.05 M) and spermine (0.5 mg./ml.) as cofactor, at pH 8.0 and 25° in the dark.

RESULTS

The effect of different cofactors on the ichthyotoxic activity

Cofactors studied included Ca^{2+} , Mg^{2+} and streptomycin, which have been shown by Yariv & Hestrin (1961) to enhance the activity of *Prymnesium parvum* ichthyotoxin, and spermine, neomycin and several polyamines. Figure 1 summarizes the results of these experiments. A marked effect of spermine in enhancement of ichthyotoxicity occurred; this was 5 times greater than the enhancement with neomycin and 10 times greater than that observed with Ca^{2+} or streptomycin when these substances were tested at 0.0045 M, pH 8.0. The effect of other amines on the ichthyotoxic activity is summarized in Table 1. Additional materials tested for their synergistic activity as cofactors included lysine, arginine, histamine, ornithine, chloramphenicol, and erythromycin (0.5 mg./ml. at pH 8.0 and 9.0). None of these materials showed any cofactor activity except for a slight effect with histamine at pH 9.0. A relationship was found to exist between the concentration of every one of the active cofactors tested and the minimal lethal dose (MLD) of

ichthyotoxin of *P. parvum*. This relationship, described by Yariv & Hestrin (1961), can be expressed as cofactor concentration $\times$ MLD = a constant; this held up to a concentration of 0.0035–0.0045 M for all the cofactors tested. On further increase of cofactor concentration no additional decrease in the minimal lethal dose was observed.

Table 1. *The effect of various polyamines on ichthyotoxic activity of Prymnesium parvum*

Cofactor used at concentration of 0.0015 M	Formula of the cofactor	Relative activity
Spermine	$\text{NH}_2-(\text{CH}_2)_3-\text{NH}-(\text{CH}_2)_4-\text{NH}(\text{CH}_2)_3-\text{NH}_2$	10
Spermidine	$\text{NH}_2-(\text{CH}_2)_3-\text{NH}-(\text{CH}_2)_4-\text{NH}_2$	5
3,3-Diaminodipropylamine	$\text{NH}_2-(\text{CH}_2)_3-\text{NH}-(\text{CH}_2)_3-\text{NH}_2$	5
Diethylene triamine	$\text{NH}_2-(\text{CH}_2)_2-\text{NH}-(\text{CH}_2)_2-\text{NH}_2$	2
Triethylene tetramine	$\text{NH}_2-(\text{CH}_2)_2-\text{NH}-(\text{CH}_2)_2-\text{NH}-(\text{CH}_2)_2-\text{NH}_2$	5
Tetraethylene pentamine	$\text{NH}_2-(\text{CH}_2)_2-\text{NH}-(\text{CH}_2)_2-\text{NH}-(\text{CH}_2)_2-\text{NH}-(\text{CH}_2)_2-\text{NH}_2$	7.5
Propane 1,3-diamine	$\text{NH}_2-(\text{CH}_2)_3-\text{NH}_2$	2
Putrescine	$\text{NH}_2-(\text{CH}_2)_4-\text{NH}_2$	2
Cadavarine	$\text{NH}_2-(\text{CH}_2)_5-\text{NH}_2$	1
Without cofactor		0.25

Titration were carried out in the dark at 28°, in 0.05 M tris buffer at pH 8.0. Results were recorded after 4 hr. The toxin employed was 'S' extract having 4000 ITU/ml. Identical results were obtained in all of the five assays made with each cofactor.

Combined effects of various cofactors on ichthyotoxic activity

The possibility that more than one cofactor may be active in the natural environment of the fish led us to investigate the effect of combining different cofactors. Experiments on the effect of increasing concentrations of CaCl_2 , in presence or absence of spermine, on the ichthyotoxic activity are summarized in Fig. 2. It can be seen that with increasing concentrations of Ca^{2+} , the ichthyotoxicity in the presence of spermine decreased rapidly and approached the value obtained with Ca^{2+} alone. The overall cofactor activity of the Ca^{2+} and spermine mixtures tested on the ichthyotoxicity was found to approach the sum of the activities of the individual cofactors in the mixture each multiplied by its relative concentration (expressed as percentage of total molar cofactor concentration) in the system. No difference in final ichthyotoxicity was found between tests of simultaneous addition of spermine and Ca^{2+} and tests where one of the cofactors was added 30 min. before adding the other.

The study of the effect of increasing concentrations of Ca^{2+} on ichthyotoxicity in presence of a constant concentration of streptomycin (0.001 M) as cofactor showed (Fig. 3) an increase in ichthyotoxicity up to 0.003 M- Ca^{2+} , a concentration at which the maximal effect is obtained when Ca^{2+} served as the single cofactor.

The effect of increasing concentrations of different salts (NaCl; sea water; NaCl + CaCl_2 in a ratio of 26:1 as found in sea water; brackish pond water) and of different concentrations of sucrose on the ichthyotoxicity of *Prymnesium parvum* extracts was studied. Results of a typical experiment in presence and absence of spermine are summarized in Fig. 4. It can be seen that with increasing NaCl con-

Fig. 1

Fig. 2

Fig. 1. The effect of concentration of spermine, neomycin, CaCl_2 and streptomycin on activity of *Prymnesium parvum* ichthyotoxin. Titrations were carried out in the dark at 25° with tris buffer (pH 8.0). Results were recorded after 8 hr. The toxin used was extract 'S' having 10,000 ITU/ml.

Fig. 2. The combined effect of spermine and CaCl_2 on activity of *Prymnesium parvum* ichthyotoxin. Tests were carried out at pH 9.0 in 0.05M-tris buffer. Solutions contained various concentrations of CaCl_2 and a constant concentration of spermine (0.0014M). Control solutions contained various CaCl_2 concentrations only in 0.05M-tris buffer (pH 9.0). Tests were carried out at 25° in the dark. Results were recorded after 8 hr. Extract 'S' having activity 6400 ITU/ml. was used.

Fig. 3. The combined effect of streptomycin and CaCl_2 on activity of *Prymnesium parvum* ichthyotoxin. Tests were made at pH 8.0 in 0.05M-tris buffer solutions containing various concentrations of CaCl_2 and a constant concentration of streptomycin 0.001M. Control solutions contained various CaCl_2 concentrations only, in 0.05M-tris buffer (pH 8.0). Tests were carried out at 25° in the dark. Results were recorded after 8 hr. Extract 'S' of activity 12,800 ITU/ml. was used.

Figure 1 consists of two line graphs. The y-axis for both is 'Ichthyotoxic activity (ITU)' with values 6,400, 12,800, 19,200, and 25,600. The x-axis is 'Salt concn. (mg. Cl⁻/ml.)' with values 5, 10, and 15. The left graph includes four data series: NaCl + spermine (open triangles), NaCl + CaCl₂ (Na⁺/Ca²⁺ = 26/1) + spermine (filled circles), Sea water + spermine (open circles), and Pond water + spermine (filled triangles). The right graph includes two data series: NaCl alone (open triangles) and Sea water alone (open circles). In both graphs, activity is highest at 15 mg/ml salt and lowest at 5 mg/ml salt.

Salt concn. (mg. Cl ⁻ /ml.)	Left Graph: With Spermine				Right Graph: Alone	
	NaCl + spermine (Δ)	NaCl + CaCl ₂ + spermine (●)	Sea water + spermine (○)	Pond water + spermine (▲)	NaCl alone (Δ)	Sea water alone (○)
15	25,600	25,600	19,200	25,600	19,200	19,200
10	12,800	12,800	12,800	12,800	12,800	12,800
5	6,400	6,400	6,400	6,400	6,400	6,400

In experiments with streptomycin+Ca²⁺ as cofactors, increasing amounts of NaCl up to 3.0 mg. Cl⁻/ml. caused a progressive inhibition of the ichthyotoxicity, similar to that observed with spermine. Addition of sucrose up to 0.2 M did not

affect the activity of ichthyotoxin in presence of spermine, thus excluding the possibility that the suppression of ichthyotoxic activity by different salts was caused by increased osmotic pressure of the suspension medium.

*The effect of pH value on the activity of *Prymnesium parvum* ichthyotoxin*

Experiments on the effect of the pH value of the medium on the activity of ichthyotoxin in the presence or absence of added cofactors are summarized in Table 2. An increase in ichthyotoxicity was observed with an increase in pH value of the test medium with all the cofactors studied, and even in the absence of added cofactor. The activity at pH 9.0 was four times greater than that at pH 8.0; no ichthyotoxic activity was observed in the system at pH 7.0.

Table 2. *The effect of pH on ichthyotoxic activity*

Cofactor used at concentration of 0.0015M	Activity at pH 7.0	Activity at pH 8.0	Activity at pH 9.0
CaCl ₂	< 100	1000	4000
Streptomycin	< 100	1000	4000
Spermine	< 100	10.000	40.000
3,3-Diaminodipropylamine	< 100	5000	20.000
Neomycin	< 100	2000	4000
Without cofactor	< 100	250	1000

Titration were carried out at pH 7.0, 8.0 and 9.0 with 0.05M-tris buffer at 20–25° in the dark. Results were recorded after 8 hr. Identical results were obtained in all of the five assays made with each cofactor.

*The effect of temperature on the ichthyotoxic activity of *Prymnesium parvum**

The effect of temperature in the range of 10–30° at pH 8.0 on the killing of minnows by *Prymnesium parvum* ichthyotoxin in the presence of spermine is shown in Fig. 5. It can be seen from this figure that an increase in temperature caused an increase in the rate of mortality but did not affect the final titre (MLD) of the toxin. Experiments at pH 9.0 showed a similar effect of temperature on the kinetics of killing, but the rate was 30–50 % greater than that observed at pH 8.0. Temperatures higher than 30° were not tolerated by the fish under the test conditions.

ASSAY OF ICHTHYOTOXIN

On the basis of the experiments reported above we have developed the following test for assaying the ichthyotoxin produced by *Prymnesium parvum*. The procedure is essentially the same as described under Methods, with 0.5 mg. spermine/ml. as a cofactor and tris buffer (0.05 M) adjusted to pH 9.0 with HCl. The titration, with three standard size minnows for each test condition, is carried out at 26–28° in the dark, and the results recorded after 3 hr. In cases where the samples tested contain high concentration of salt (e.g. brackish pond water or growth media of *P. parvum*) the medium is dialysed against distilled water for several hours before the test. An examination of the reproducibility of this test under the standard conditions at pH 8 and 9 showed the results to be highly dependable with a standard deviation ranging from 10 to 30 %.

Fig. 5. The effect of temperature on activity at pH 8.0 of *Prymnesium parvum* ichthyotoxin. Tests were carried out at pH 8.0 in 0.05M-tris buffer+spermine 0.0014M in the dark. The minnows were incubated in tap water for 12 hr before test at temperatures corresponding to those used in the tests. Average time for loss of equilibrium of all three minnows was recorded. Control fish remained unaffected for 3 hr after the death of all test fishes. Extract 'S' having 12,800 ITU/ml. was used in these tests.

DISCUSSION

The present study confirms and extends the findings of Yariv & Hestrin (1961) on the cofactor requirements of *Prymnesium parvum* ichthyotoxin. In addition to Ca^{2+} , Mg^{2+} and streptomycin described by these workers, other cationic substances, e.g. neomycin, spermine and certain other polyamines, were shown to enhance the ichthyotoxicity of *P. parvum* extract. All the compounds so far found to have cofactor activity in the *P. parvum* ichthyotoxic system have been reported to act as stabilizing agents in a variety of biological systems such as bacterial spheroplasts and protoplasts, mitochondria and microsomes (Tabor, Tabor & Rosenthal, 1961); in all these systems, spermine was the most active.

It has been confirmed that the quantitative relationship between cofactor and ichthyotoxin expressed by Yariv & Hestrin (1961) as $a \times b = Q$ (where a is the ichthyotoxin concentration, b the minimum concentration of cofactor which elicits a lethal effect in presence of a , and Q is a cofactor-specific constant) holds for all the cofactors tested, but that this relationship has an upper limit at cofactor concentration of 0.0045 M.

In the presence of more than one cofactor the resulting toxicity is not necessarily additive but depends on the specific activity of each cofactor and its concentration relative to the other cofactor. Thus, the addition of a cofactor of low activity, e.g.

Ca^{2+} , to a system containing a cofactor of high activity, e.g. spermine, drastically lowers the degree of toxicity to fish. On the other hand, the addition of Ca^{2+} to a system containing a limiting concentration of streptomycin (a cofactor of roughly equivalent activity) increases fish toxicity. This has the result that in high concentrations of Ca^{2+} the effects of spermine and streptomycin are masked. This relationship and the phenomenon of cofactor saturation might be due to competition of cofactors for similar sites.

It has already been emphasized (Shilo & Aschner, 1953) that no correlation was found between the population density of *Prymnesium parvum* in brackish fish ponds and the concentration of ichthyotoxin in the water. It has been suggested that this phenomenon can be explained partly through the continuous destruction of excreted toxin by various inactivating factors. The present findings on the effects of pH value, salt concentration and temperature on the activity of the ichthyotoxin show that various environmental factors may in this manner play a decisive role in determining the overall picture of toxicity. In fact, a survey of the appearance of fish intoxication in brackish-water fish ponds in Israel has shown that a correlation exists between toxicity and elevated pH values and/or low salinity of pond water. Furthermore, the widely differing activity of the various cofactors tested, such as Ca^{2+} , streptomycin and some polyamines, in enhancing ichthyotoxicity suggests that the concentration and nature of cofactor or cofactors in the milieu may have a profound influence on final toxic effect of *P. parvum* in a pond or culture.

The bioassay for the ichthyotoxin developed was suitable for the determination of extracellular, as well as intracellular ichthyotoxin from axenic cultures and from *Prymnesium parvum* blooms in fish ponds. By using this assay method it was possible to detect sublethal amounts of ichthyotoxin in ponds (as low as $\sim 1/30$ of the lethal dose for carp). Since only a small percentage of *Prymnesium*-infested ponds in nature develop lethal concentrations of ichthyotoxin, this method has economic importance since it limits the need for control of the phytoflagellate to those ponds containing detectable sublethal amounts of toxin.

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