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Factors governing the Toxicity of Cultures containing the Phytoflagellate *Prymnesium parvum* Carter

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SUMMARY: Methods for the laboratory maintenance and purification of cultures of *Prymnesium parvum* are detailed. The upper limit of temperature for the growth of the organism is 30°. Light exerts an augmenting effect on the production of the toxin by *Pr. parvum*. Rapid and economical procedures for the bioassay of the toxin are based on the use of *Gambusia* minnows or tadpoles. Cell-free centrifugates of the cultures contain heat-labile toxic material which is non-diffusible through cellophan, sensitive to oxidizing agents, and reversibly inactivated by mild acidity. The toxin is rapidly inactivated by ubiquitous bacterial species (*Bacillus subtilis* and *Proteus vulgaris*). Charcoals, clays and calcium sulphate are efficient adsorbents of the toxic material. The concentration levels of toxin in cultures of *Prymnesium parvum* appear to reflect a dynamic equilibrium between toxin destruction and production; a similar equilibrium may prevail in nature.

A phytoflagellate toxin was first implicated in an occurrence of mass mortality among fish in Holland by Liebert & Deerns (1920). Subsequent study of a similar occurrence in Denmark by Otterstroem & Steeman-Nielsen (1939) identified the responsible organism as *Prymnesium parvum* Carter, and confirmed that the toxic effect was caused by an extracellular thermolabile toxin. Reich & Aschner (1947) described the widespread occurrence of this organism in Israel and proposed measures for its control in fishponds.

The present communication describes methods for the maintenance and purification of laboratory cultures of *Prymnesium* spp. and procedures for the bioassay of the toxin. An analysis of the effects of certain environmental factors on the production and persistence of the toxin in pond water has been attempted. The experiments show the extracellular occurrence of the prymnesium toxin and suggest that it may be a protein.

EXPERIMENTAL

Bioassay of the toxin of Prymnesium parvum

Estimations of toxin content of culture fluids and of pond water were made by using *Gambusia* minnows or tadpoles of *Rana* or *Bufo* as the test organism. These species were readily available and could be handled in relatively small volumes of fluid. The tests are carried out as follows. For tadpole tests, five animals are immersed in 5 ml. volumes in 15 mm. diam. test-tubes. Cell-free centrifugates of the toxic samples are used for the assay. The dilution medium was sodium chloride solution (0.16%), 9 volumes + Sorensen's phosphate

buffer pH 8, 1 volume, the mixture being adjusted to final pH value of 7.5–8.5; temperature of test 15–25°. Bacterial contamination was prevented with streptomycin (1 mg./ml.). Assays were based on direct determinations of the minimum lethal dose or on observations of the time of onset of toxic reactions, particularly the loss of equilibrium sense in minnows (equilibrium-loss time), or of tail curvature in tadpoles (see Pl. 1). As the *Gambusia* minnow is the more sensitive test organism, it was preferred for work with dilute toxin. The response is, however, observed sooner in the tadpole, and this is therefore the preferable species for rapid assay work.

A prymnesium toxin unit (T.U.) is defined as the minimum amount of toxin/20 ml. which causes death of *Gambusia* minnows within 12 hr. in standard test conditions, specified for the *Gambusia* test as two minnows about 1.5 cm. long immersed together in 20 ml. test fluid in a 50 ml. beaker 40 mm. diameter. It should be noted that 1 T.U. in the standard volume of 20 ml. will kill more than the two *Gambusia* minnows, but twofold dilution of the fluid renders it non-toxic even to one minnow. The response observed in these conditions appears to depend on the toxin concentration rather than on the absolute amount of toxin present.

The relationship between toxin concentration and the equilibrium-loss time in minnows is shown in Fig. 1. The relationship between the minimum lethal concentration (M.L.C.) and the equilibrium-loss time was constant in all the toxin solutions tested. The equilibrium-loss time decreased as the amount of toxin increased above 1 T.U., and approached a constant (15–30 min.) at 5 T.U./20 ml. or more. Above 2 T.U./20 ml., the plot of the reciprocal of toxicity against the equilibrium-loss time deviates only slightly from a straight line. The loss of equilibrium sense was always followed by the death of the *Gambusia*. The minimal lethal concentration and the minimal concentration causing the loss of the equilibrium sense are therefore identical. Titrations of the equilibrium-loss time could be converted into T.U. by reference to Fig. 1. It is apparent that whereas direct determination of M.L.C. could only be completed in 24 hr., the observations of equilibrium-loss time afford reliable estimates of toxin concentration within 1 hr., the assay becoming increasingly sensitive and accurate as the equilibrium-loss time is allowed to increase beyond 1 hr.

The relationship of toxin concentration to time of showing tail curvature (curvature-time) in tadpoles is illustrated in Fig. 2. It is similar in general to that of equilibrium-loss time in *Gambusia* minnows to toxin concentration (cf. Fig. 1). The tadpole requires more toxin for a visible response, the minimum effective dose being 2.2 T.U./20 ml. As the toxin dose increased the curvature-time decreased at a decreasing rate and became constant above 50 T.U./20 ml. at a value near 8 min. Curvature-times of 15–30 min. were satisfactory for assay. Curvature response was always followed by the death of the tadpole.

The ratio of the minimum dose causing equilibrium loss to that causing tail curvature response was found to be 2.2 within a fluctuation limit of 10% in ten water samples from different natural sources. The constancy of ratio suggests that the equilibrium-loss in minnows and curvature response in

tadpoles are caused by the same toxin, though in this connexion it should be noted that tail curvature in tadpoles can also be elicited by other agents, e.g. D.D.T. (Schreiman & Rugh, 1949).

Fig. 1

Fig. 1. The relationship between toxin concentration and equilibrium-loss time in *Gambusia* minnows. Toxicity expressed in toxin units (τ.u.)/20 ml. of immersion fluid was determined on graded dilutions of cell-free supernatant fluid of toxic *prymnesium* cultures. Different symbols represent different batches of toxin.

Fig. 2

Fig. 2. The relationship between toxin concentration and tail-curvature time in tadpoles. Toxicity tests were made on young *Rana* tadpoles. Values plotted represent averaged results of experiments with three toxin preparations. Toxin concentrations were converted to standard τ.u. by multiplying the tadpole M.L.C. by the factor 2.2 which relates M.L.C. of minnows to that of tadpoles.

Preparation and storage of cell-free toxin solution

Liebert & Deerns (1920) showed that toxic water samples subjected to moderate heating may kill fish even though they no longer contain viable phytoflagellates. Otterstroem & Nielsen (1939) observed that centrifugation of pond water which resulted in a marked diminution of the flagellate concentration did not render the sample non-toxic and suggested that the toxic principle was extracellular. We found that centrifugation at 10,000 r.p.m. for 15 min. in the Servall angle centrifuge gives a cell-free toxin solution. Benzylpenicillin (500–1000 units/ml.) plus streptomycin (0.5–1.0 mg./ml.) were added to the toxin solutions to prevent bacterial contamination. This cell-free fluid was as toxic as the original cell suspension and could be stored without marked loss of potency in the refrigerator for a period up to 4 weeks.

Microscopical examination of cultures and determination of population densities

Living organisms were examined in hanging-drop preparations. For permanent preparations, the hanging drop was placed above iodine crystals, fixation being effected by the iodine vapour. The drops were spread out in

a thin film, dried, and stained for 1 sec. with undiluted Gram's gentian violet.

The suspensions of organisms were counted in a blood counting chamber which contained a small crystal of iodine in the overflow channel. Fixation with iodine preserved the shape of the rather fragile organisms of *Pr. parvum* better than other fixatives and made it easy to see the third flagellum, which is somewhat difficult to observe in life and which serves as the principal identifying characteristic of the genus.

Maintenance and purification of cultures

We used water from brackish fishponds in which the concentration of *Pr. parvum* varied from 10,000 to 5000,000 organisms/ml. Samples were kept at winter room temperature (12–15°) in the laboratory in a northern window or under a 40 W. daylight lamp. Tall cylindrical glass vessels or test tubes half-filled with medium served as culture vessels. Under these conditions the *Pr. parvum* multiplied and maintained toxicity for long periods, whereas most of the other algae present usually diminished in number until they could no longer be detected by direct microscopic examination. The persistence of low number of algal forms which were not *Prymnesium* spp. was demonstrated by adding ammonium sulphate in a concentration lytic to *Prymnesium* spp. or by heating at 35° for short periods; this was followed by reappearance of other algal species in the cultures.

The medium was pond water of suitable salinity (about 1000 mg. Cl/l.) which had been allowed to undergo self-purification in the dark, Chu's medium (1942), or sea water which had been diluted 1:10 with tap water. The pH values of these media ranged from 7.5 to 9.0. The prymnesium cultures were grown with massive transfer (1 volume of inoculum in 2–5 volumes of medium) at intervals of 2–4 weeks.

Typical growth is summarized in Table 1. Marked increases in the final prymnesium counts followed the incorporation of fish peptone or egg yolk (Table 2). Routine addition of these supplements was held to be undesirable, however, since they also promoted the growth of non-prymnesium members of the population, especially bacteria. Excessive proliferation of bacteria may lead to disappearance of *Pr. parvum* from the culture.

Temperatures higher than 30° were markedly inhibitory; exposure to 35° for intervals as short as 5 min. was sufficient to cause death. Caution was thus required when cultures were exposed to direct sunlight, and cooling of the cultures was necessary. *Pr. parvum* survives at temperatures as low as 2° for many days. The seasonal fluctuations of *Pr. parvum* density in ponds throughout Israel correspond broadly with these temperature requirements of the organism, the density rising markedly in winter and falling almost to nil at the beginning of summer when the pond temperatures approach 30°.

Adequate illumination of the cultures was important for cell proliferation and maintenance of culture toxicity. Light affected toxicity more quickly than cell number. The experiment in Table 3 shows the specific effect of light on the toxicity as distinct from its effect on cell proliferation. In cultures transferred in the laboratory under standard conditions there are generally

Table 1. *The multiplication of Prymnesium parvum in different nutrient media*

Inoculation of the nutrient media was by massive transfer of *Prymnesium parvum* after two previous transfers through the same media. The inoculum in these experiments was 1/3–1/10 of the total volume of the medium. Cultures were kept in 1 l. cylindrical containers at room temperature in diffuse northern light (1000–1500 lux).

Medium	Prymnesium counts (organisms/ μ l.) on successive days				
	0	1	2	4	8
Pond water:*					
Culture 1	120	280	500	970	1050
Culture 2	130	340	1070	1090	1660
Culture 3	370	830	—	1360	—
Tap water containing 7% sea water:					
Culture 4	230	—	250	410	500
Culture 5	600	—	—	1200	1500
Chu medium no. 10† containing 7% sea water:					
Culture 4	230	180	580	900	1400
Culture 3	300	660	—	1200	—

* Salinity of approx. 1000 mg. Cl/l.

† As described by Chu (1942).

Table 2. *Influence of nutrients added to pond water on prymnesium counts*

Pond-water cultures containing equilibrium populations of prymnesium were divided into similar glass containers and kept at room temperature in northern diffuse light with nutrient supplements as described below.

Nutrient supplement	Period of cultivation (days)			
	0	4	14	18
Prymnesium counts (organisms/ μ l.)				
Egg yolk: 0	390	500	400	530
1 : 1,100	350	650	2,940	4,000
1 : 5,000	340	480	1,230	1,700
1 : 10,000	350	410	700	800
1 : 50,000	390	280	400	420
1 : 100,000	360	370	250	480
Fish peptone:* 0	820	860	900	—
1 : 500	820	2050	2,300	—
1 : 1,000	920	1910	2,470	—
1 : 5,000	740	—	900	—
1 : 10,000	610	—	690	—
0	120	140	150	—
1 : 1,000	100	480	1,250	—

* As prepared by Snieszko, Griffin & Friddle (1950).

1 to 4 T.U./10⁶ organisms, the concentration of the toxin 2–6 days after transfer being c. 1 T.U./ml. of culture.

The cultures also contained protozoa and bacteria. The relationship between the different species is not well understood. As is shown in Table 4, *Pr. parvum* possess a relatively high degree of resistance to several antibiotics, and

Table 3. *Influence of light on cell density and toxicity of prymnesium cultures*

In this experiment a sample of non-toxic pond water was dispensed into three cylindrical vessels which were placed at room temperature under different lighting conditions as indicated below. Toxicity was tested with *Gambusia minnows*. Illumination was measured with the help of an exposure-meter and expresses average intensity in each condition at noon.

Illumination of culture	Relative light intensity	Age of culture								
		0 hr.			24 hr.			72 hr.		
		Count of prymnesium (organism/ μ l.)	Toxicity (units/20 ml.)	T.U./10 ⁶ cells	Count of prymnesium (organism/ μ l.)	Toxicity (units/20 ml.)	T.U./10 ⁶ cells	Count of prymnesium (organism/ μ l.)	Toxicity (units/20 ml.)	T.U./10 ⁶ cells
Diffuse day-light	12	250	<1	<0.2	260	5	1	420	10	1.2
Fluorescent light*	9	250	<1	<0.2	350	<1	<0.3	240	4	0.8
Laboratory interior	7	250	<1	<0.2	260	<1	<0.2	140	<1	<0.3

* 40 W. lamp operated for 12 hr./day.

Table 4. *Survival of prymnesium in cultures containing antibacterial concentrations of selected antibiotics*

Pond-water cultures with specified antibiotic were kept in northern diffuse light at room temperatures (22°). The prymnesium counts are indicated as follows:

++++ 500–1000/ μ l.; +++ 250–500/ μ l.; ++ 50–250/ μ l.; + 10–50/ μ l.; – <10/ μ l.

Antibiotic added	Time of assay after addition of antibiotic (days)	Concentration of antibiotic (mg./ml.)								
		6.5	3.3	2.0	1.0	0.5	0.25	0.1	0.05	0.01
		Survival of prymnesium								
Penicillin G*	1	++	++++	++++	++++	++++	++++	++++	++++	++++
	3	–	++++	++++	++++	++++	++++	++++	++++	++++
	7	–	++++	++++	++++	++++	++++	++++	++++	++++
Streptomycin†	1	.	.	++++	++++	++++	++++	++++	++++	++++
	3	.	.	+	++++	++++	++++	++++	++++	++++
	7	.	.	–	++++	++++	++++	++++	++++	++++
Terramycin	1	.	.	–	–	–	+	+	++	++++
	3	.	.	–	–	–	–	+	+	++
	7	.	.	–	–	–	–	–	+	+
Chloramphenicol	1	.	.	–	–	–	++	++	+++	++++
	3	.	.	–	–	–	–	–	++	++++
	7	.	.	–	–	–	–	–	–	++++
Aureomycin	1	.	.	–	–	–	–	–	++	+++
	3	.	.	–	–	–	–	–	–	++
	7	.	.	–	–	–	–	–	–	++

* 1430 units/mg.

† Similar results were also observed with dihydrostreptomycin.

advantage may be taken of this to purify cultures. The *Pr. parvum* survived and even multiplied in the presence of bactericidal concentration of streptomycin and penicillin. When 0.1 ml. samples of culture fluid containing these antibiotics were inoculated into nutrient broth, brain and heart infusion, and into glucose agar media, they often failed to show bacterial growth within 24–48 hr. after the addition of the antibiotics. Cultures of *Pr. parvum* which

were bacteria-free by this criterion were carried in an antibiotic-free medium for several transfers. Such cultures tended to die after 2–3 weeks. The causes of the decline are being studied.

Population density of Prymnesium parvum in pond-water samples and laboratory cultures; relationship to the toxicity of the culture fluids

Counts of *Pr. parvum* and toxicity assays of pond-water samples taken from selected locations are shown in Table 5; the counts are arranged in order of size.

Table 5. *Population of Prymnesium spp. and toxicity of fishponds*

Location and serial number of pond		Date of sample	Prymnesium count (organisms/ μ l.)	Toxicity (units/20 ml.)
Sdeh Nachum	4	25. xi. 46	1080	1.0
	7	28. x. 46	960	1.0
Messiloth	6	10. xii. 46	870	1.0
	6	15. xii. 46	600	1.0*
	12	11. v. 49	550	1.0
	12	17. v. 49	400	1.0*
Tel Amal	Z	20. xi. 50	300	1.0
	Z	26. xi. 50	300	1.0*
Messiloth	6	6. vi. 52	240	10–20
Sdeh Nachum	7	26. viii. 48	60	1.0*
Tel Amal		1. iii. 47	50	1.0*

1.0* indicates toxin concentration which killed carp, i.e. = 1.3 T.U.

As Reich & Aschner (1947) showed there is no simple relationship between the *Pr. parvum* count and the toxicity of the pond-water sample. In fact, very high prymnesium counts are sometimes associated with absence of toxicity (e.g. 7×10^6 organisms/ml.; < 0.05 T.U./ml.). On the other hand, several highly toxic samples of pond water gave very low prymnesium counts (5×10^4 organisms/ml.; > 0.5 T.U./ml.). The wide variation of the toxicity:count ratio might be due to the occurrence of variants of *Pr. parvum* or to differential effects of environmental factors on the production and destruction of the toxin. The notion that the low toxicity:count ratios are characteristic for non-toxigenic variants could be ruled out by the observation that pond-water samples of low or undetectable toxicity and a high count for prymnesium regularly yield highly toxic cultures when maintained in the laboratory under standard conditions for 2–3 days. During this interval the increase in the flagellate population is less than twofold, and it seems unlikely that a process of natural selection favouring a toxigenic variant is responsible for the increase in toxicity.

When the population density of laboratory cultures was increased by growth-promoting supplements to numbers in excess of 5×10^6 flagellates/ml., an inverse relationship between cell count and toxicity was sometimes observed. Dilution of such dense cultures with sea water or pond water rapidly re-established the normal toxicity:count ratio. An example of such an experiment is given in Table 6. Cultures of high cell count have a deep brown colour

and considerable turbidity. It remains to be ascertained to what extent the decrease in toxicity which is associated with excessive cell proliferation may be due to the inadequate illumination of the interior of the culture.

Table 6. *Effect of dilution on toxicity of dense prymnesium cultures*

A static culture of *Prymnesium parvum* which had been fortified with a soil supplement and contained 7.3×10^6 prymnesium organisms/ml. was diluted in the proportions shown below with 1:10 strength sea water and held for 24 hr. in cylindrical vessels at room temperature under a 40 W. daylight lamp.

Dilution of culture with 1:10 strength sea water	Organisms $\times 10^6$ /ml.		T.U./20 ml.	
	0 hr.	24 hr.	0 hr.	24 hr.
1:0	7.30	7.30	<1.0	<1
1:4	1.46	1.66	<1.0	2.5
1:0	0.73	0.90	<1.0	5.0

Fig. 3. The effect of temperature on prymnesium toxin. The toxicity expressed in T.U./20 ml. was tested with *Gambusia*. Curves 1, 2 and 3 show effects of exposure to 62, 80 and 97° respectively.

Observations on general properties of the toxin of Prymnesium parvum cultures

Effect of temperature. At 97 and at 80°, the toxicity of the culture supernatant fluid declined rapidly; at 62° the decline was relatively slow. Thermal inactivation became progressively slower as the concentration of the toxin fell (Fig. 3). At room temperature and at 4° there was no detectable loss of toxicity for at least 7 days, provided that bacterial growth did not occur and oxidation was prevented.

Diffusibility. Solutions of toxin in cellophan tubing showed no significant fall in titre when suspended in frequently renewed distilled water or in isotonic sodium chloride solution (0.16 %) for 12–24 hr. at 4°.

Fig. 4

Fig. 5

Fig. 4. Influence of pH on activity of toxin. The toxin solution contained 28 t.u./20 ml. The pH was adjusted with phosphate buffer 0.02 M in the range pH 6.0–8.0 and with borate buffer (Clark) at a final concentration of 1 : 10 in the range pH 7.8–9.0. The toxicity was measured on *Gambusia* 1 hr. after the adjustment of the pH of the toxin solution. Buffer solutions containing 10 % sea water of the same pH and concentration were used for dilution.

Fig. 5. Reversible inactivation of toxin at acid pH values. Toxicity is expressed as the reciprocal of the equilibrium-loss time in minutes. pH was adjusted as indicated by addition of 0.5 N-hydrochloric acid or sodium hydroxide.

Effect of pH value. The influence of the pH value on toxicity is illustrated in Fig. 4. Toxicity was independent of pH value within the range 7.5–9.0. The toxicity decreased rapidly at pH values below 7.5, and was zero at pH 6.0. At moderate acidities, the inactivating effect of hydrogen ion was almost completely reversible for at least several days (Fig. 5). This behaviour resembles that of different enzyme proteins. Assuming that the variation of the pH value is modifying the nature of the toxin, it would follow that the toxin possibly possesses a functional group of pK near 6–7. However, it should not be overlooked that the variation of the pH value might also be affecting the susceptibility of the test animal.

Oxidizing agents. Toxicity declined when oxygen was bubbled through a solution of toxin; air produced the same result though somewhat more slowly. Potassium permanganate (2×10^{-4} M) or sodium hypochlorite (2.0 p.p.m.) added to culture supernatant fluid containing 0.5 t.u./ml. immediately destroyed toxicity.

Adsorbents. A wide variety of adsorbents removed the toxic material from culture supernatant fluid. At room temperature, the toxicity of 50 ml. containing 2.5 t.u. was completely removed within 5 min. by 2–5 g. of kaolin, Norit A (acid washed), activated charcoal, or calcium sulphate ($\text{CaSO}_4 \cdot 2\text{H}_2\text{O}$).

Under the same conditions, Decalco, calcium carbonate, kieselguhr, Fuller's earth and activated alumina (Eimer & Amend) removed little or no toxin. Highly toxic cultures were rapidly detoxified by stirring with pond-bottom soils. When such cultures are then allowed to remain in contact with the soil, they continue non-toxic for periods up to a fortnight in conditions which are otherwise favourable to toxin production.

Destruction of toxin by micro-organisms. A destructive action of micro-organisms on the toxin was suggested by the observation that the maintenance of the toxicity of stored toxin solution at all temperatures compatible with bacterial growth was conditional on the addition of suitable antibiotics to the toxin sample. Washed suspensions of *Proteus vulgaris* and of *Bacillus subtilis* decreased the potency of prymnesium toxic culture supernatant fluid by at least 50 % in 1 hr., whereas *Bacterium coli* was almost without effect under the same conditions.

DISCUSSION

The prymnesium toxin appears to be a substance of high molecular weight with some of the properties of a protein. It is readily differentiated from other algal toxins, notably those found in marine dinoflagellates (Sommer *et al.* 1948*a, b*; Connell & Gross, 1950) and in *Microcystis* (Prescott, 1948; Ashworth & Mason, 1946; Shelubsky, 1951) which are intracellular constituents of cells and not excreted into the medium. Some explanation can now be offered of the puzzling lack of correlation between the population density of *Prymnesium parvum* and the toxicity of the pond water. The pronounced lability of the prymnesium toxin to oxidizing agents, its ready adsorption by bottom soil and its rapid destruction by micro-organisms may explain non-toxic pond waters. Light, by augmenting toxin production, works in the opposite direction, and is probably one of many factors which regulate toxin production in nature. The concentration of toxin observed represents, therefore, a dynamic equilibrium between production and destruction. The dramatic increases in toxicity which may be observed in fish ponds with stable prymnesium populations can thus represent either the withdrawal of a toxin-removing agency or an enhancement of a factor concerned in toxin production.

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Fig. 1

Fig. 2

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EXPLANATION OF PLATE

Fig. 1. Tadpoles of *Rana* sp. after 15 min. in toxin solution. Typical curvature of the tail is the first visible effect of the intoxication.

Fig. 2. Mass-mortality of fish in a carp-breeding pond caused by toxin of *Prymnesium parvum*.

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