Influence of Light and Phosphate on Toxin Production and Growth of *Prymnesium parvum*

By ZIPORA DAFNI, S. ULITZUR AND M. SHILO

*Department of Microbiological Chemistry, Hebrew University – Hadassah
Medical School, Jerusalem, Israel*

(Accepted for publication 9 October 1971)

SUMMARY

The formation and release of cytolytic toxins by *Prymnesium parvum* are affected by environmental factors. Under conditions of phosphate limitation toxin biosynthesis and release are markedly enhanced. Light is necessary for maximum toxin production. Toxin production and cell multiplication do not depend on identical factors. Cells grown under phosphate limitation in the light are considerably larger than those grown in phosphate-rich media.

INTRODUCTION

The causal role of a number of algal species in intoxications of aquatic and terrestrial animals has been established. Nevertheless, a striking lack of correlation between algal cell numbers and the degree of toxicity of the milieu has been observed with toxigenic dinoflagellates (Collier, 1958; Spikes, Ray, Aldrich & Nash, 1968) and blue-green algae (Gorham, 1960), both in nature and laboratory cultures. This was similarly observed in *Prymnesium parvum* populations in ponds (and axenic cultures) where algal blooms develop in no constant relation to the degree of ichthyotoxicity. *Prymnesium* toxin is known to be labile to oxidizing agents, adsorbed to soil colloids and destroyed by micro-organisms (Shilo & Aschner, 1953); the ichthyotoxin is known to be inactivated by light in the visible (400 to 510 nm) and u.v. (255 nm) spectra (Parnas, Reich & Bergmann, 1962). Ichthyotoxicity is enhanced by polyvalent cations (Yariv & Hestrin, 1961) and high pH (Ulitzur & Shilo, 1966). Thus, the expression of *Prymnesium* ichthyotoxic activity in nature is affected by many different factors. A whole chain of events, including biosynthesis of the toxin, its release, stability, the presence of activating substances and conditions required for the sensitization of the target organisms, seems to be involved in the accumulation of the algal toxin in the milieu at levels required for intoxication.

In this paper we deal with the production of *Prymnesium* toxin and its release into the medium under different growth conditions. The finding of Rahat & Jahn (1965), that heterotrophic growth of *Prymnesium parvum* occurs in the dark, made it possible to study the influence of light on growth and toxin production. Analysis of highly purified preparations indicated that the *Prymnesium* toxin consists of a group of closely related phosphate-containing proteolipids (Ulitzur & Shilo, 1970). It was decided to investigate the effects of metabolic disturbances of the cell membrane caused by phosphate limitation on toxin biosynthesis and release. In addition, our studies involve the definition of conditions which allow maximal and rapid production of the toxin needed for purification, chemical identification and study of toxic properties, as well as for its use in physiological experiments as an agent affecting cell permeability.

METHODS

Prymnesium culture. Axenic cultures of *Prymnesium parvum*, isolated by Reich & Kahn (1954), were used.

Culture media. S_{50} medium was a modification of the defined medium of Droop (1958). Amounts per litre of twice distilled water: NaCl, 15 g; $MgCl_2 \cdot 6H_2O$, 2.5 g; KCl, 400 mg; $CaSO_4 \cdot 2H_2O$, 500 mg; Na_2EDTA , 50 mg; $FeCl_3$, 5 mg; $MnCl_3$, 500 μ g; $ZnSO_4$, 50 μ g; $CuSO_4$, 50 μ g; $CoSO_4$, 5 μ g; $Na_2MoO_4 \cdot 2H_2O$, 5 μ g; glycylglycine, 500 mg; glycine, 250 mg; KNO_3 , 100 mg; K_2HPO_4 , 10 mg; vitamin B_{12} , 100 ng; thiamine HCl, 1 mg; the pH of the medium was adjusted to pH 8 by addition of 1 M-NaOH. The supplemented S_{50} ($S_{50}S$) medium was identical to the above, except that glycylglycine was omitted, the amount of glycine increased to 750 mg/l, and 30 ml glycerol and 2.6 g DL-serine were added to each litre. The phosphate-poor $S_{50}S$ medium was identical to the above $S_{50}S$ medium, except that the phosphate concentration was reduced to one-fifth of the original (to 0.0114 mM- K_2HPO_4).

The R medium was similar to that described by Rahat & Jahn (1965) with the addition of 30 ml glycerol and 2.6 g DL-serine/litre, and 15 g NaCl/l (instead of 10 g NaCl/l as in the original medium). The phosphate-poor R medium was identical to the above R medium, except that the phosphate concentration was reduced to 0.0114 mM- Na_2HPO_4 .

Growth conditions. Cultures were grown, stationarily in 250 ml Erlenmeyer flasks containing 100 ml medium. Starter cultures were grown, in the same media and conditions as in experiments, for 7 days before inoculation into fresh media at an initial density of 2.5 to 3×10^5 organisms/ml. Cultures were grown at 26° to 28° in the light (continuous fluorescent light at an incident intensity of 200 to 250 ft-candles) or in total darkness.

Measurement of growth parameters. Cultures were sampled and numbers of organisms counted periodically in a Coulter counter (Model B, Coulter Electronics, Hialeah, Florida, U.S.A.); the relative mean cell volume was determined from the plot of the Coulter counter from the integral curve at 50% population number.

Toxin extraction. A 40 ml sample of culture was centrifuged (12,000 g) for 10 min. The supernatant served for determination of the extracellular toxin and was stored at -20° until testing. The pellet was resuspended in 5 ml absolute ethanol, and then treated for 2 min in a Servall OmniMixer at 200 V in the cold. After overnight storage at 4°, the extract was separated from cell debris by centrifugation.

Haemolysin determination. The haemolytic activities of cell extracts and supernatants were determined as described by Shilo (1971). The minimum amounts of haemolysin detectable with this assay system and extraction procedure were 12.5 H.U. (haemolytic units)/ml of culture for intracellular toxin and 4 H.U./ml of culture for extracellular toxin. Before the assay, the materials were kept at room temperature for at least 15 min.

Release of intracellular macromolecules. For determination of 260 nm absorbing substances released by the growing organisms, a sample of the culture supernatant was measured in the spectrophotometer at 260 nm wave length (Gilford Model 2400, Oberlin, Ohio, U.S.A.).

RESULTS

Growth and toxin production in S_{50} , $S_{50}S$ and R media

In S_{50} medium, 1200 to 1300 H.U./ml of culture were obtained, but only on the 46th day after inoculation when the culture reached the decline phase. Heterotrophic growth of

Prymnesium parvum in the dark proceeds in the R medium, which differs from S₅₀ mainly in the addition of a high concentration of glycerol.

Table 1 compares the growth and haemolysin production in S₅₀, S₅₀S and R media and their phosphate-poor variants in the light and dark. Tests were made of the cytotoxicity to Ehrlich ascites cells (Dafni, 1970; Shilo, 1971) of the various culture supernatants and cell extracts. The similarity of this cytotoxic activity to the haemolytic activity made it possible to consider the haemolysin a valid parameter of *Prymnesium* toxicity; therefore, only haemolytic activity is listed here.

Light had a greater influence on growth of the R cultures than of the S₅₀S cultures. More toxin was produced on the R than on the S₅₀ and S₅₀S media. There was a lower cell yield and toxin production than in R medium in the light. Although light had little influence on cell yield in S₅₀S medium, no detectable amounts of toxin were produced in the dark.

In all light conditions, cell yields were lower in phosphate-poor media than in phosphate-rich media. However, toxin yields were increased. It is interesting to compare the extra- and intracellular toxicities developed in the illuminated phosphate-poor S₅₀S and R media. Though total toxin was high in both cases, the extracellular amounts in S₅₀S were much higher than intracellular amounts, while this relation was reversed in R medium.

Although the phosphate-poor media supported much poorer growth in the dark than the corresponding phosphate-rich media, toxin yields were nevertheless increased. Toxin was produced in the dark in the phosphate-poor S₅₀S medium, whereas no toxin was detected in phosphate-rich S₅₀S in the dark.

The rate of growth and toxin production in R medium in the presence and absence of light are shown in Fig. 1; similar results have been obtained in S₅₀S media (Shilo, 1967; Dafni & Ulitzur, 1968; Dafni, 1970).

The rate of intracellular toxin production in phosphate-rich R medium in the light was constant during the log phase, and rose toward the end of this phase and in the stationary phase (Fig. 1*a*). In the dark (Fig. 1*b*) after a short decline toxin was produced at a continuously low rate similar to the initial (2 to 6 days) rate in illuminated cultures (Fig. 1*a*).

Table 1. *Influence of phosphate concentration and light on growth and haemolysin production by Prymnesium parvum in S₅₀, S₅₀S and R media*

Culture conditions	Duration of log phase (days)	Doubling time, (h)	Maximal yield cells/ml (days after inoculation)	Cells begin to disintegrate (days after inoculation)	Maximum intracellular haemolysin. H.U./ml (days after inoculation)	Maximum extracellular haemolysin. (H.U./ml)
Light S ₅₀	7	63.0	11.6 × 10 ⁶ (16)	46	1250 (46)	1500
Phosphate-rich						
Light S ₅₀ S	5	31.0	5.6 × 10 ⁶ (7)	7	800 (11)	500
Light R	4	20.6	11.9 × 10 ⁶ (7)	13	2100 (8)	30
Dark S ₅₀ S	7	37.5	5.8 × 10 ⁶ (11)	16	*	*
Dark R	6	44.2	7.0 × 10 ⁶ (10)	21	160 (8)	12
Phosphate-poor						
Light S ₅₀ S	3	23.2	2.4 × 10 ⁶ (5)	9	1300 (5-7)	8000
Light R	3	23.5	2.84 × 10 ⁶ (4)	10	3125 (8)	1300
Dark S ₅₀ S	11	20.0	1.25 × 10 ⁶ (25)	29	32 (25)	65
Dark R	6	47.8	3.0 × 10 ⁶ (12)	16	700 (21)	64

* Toxin level was below the amount detectable by the assay system.

Fig. 1. Kinetics of growth, toxin production and leakage of 260 nm absorbing material from *Prymnesium parvum* grown in phosphate-rich (*a, b*) and phosphate-poor (*c, d*) R media in the light (*a, c*) and dark (*b, d*). Culture media and assays are described in Methods. Samples with no detectable amounts of toxin (by the assay system used) are noted neither on this graph nor in Fig. 2 and are considered to be below 1 H.U./ 10^6 cells. Intracellular (Δ) and extracellular ($\blacktriangle$) toxins, in H.U./ 10^6 cells; growth ($\circ$), in number cells/ml; leakage of 260 nm absorbing material ($\square$), in o.d. units/ 10^6 cells; dagger marks cell disintegration.

In the phosphate-poor media, an early (by the second day) increase in the rate of toxin production was shown in both light and dark, but toxin yields per organism were 10 times greater in the light (Fig. 1 *c, d*).

At both high and low phosphate concentrations, illuminated cultures showed a rapid decrease in toxin formation in the late stationary phase (Fig. 1 *a, c*); disintegration of the algae was observed in these cultures at this time.

Only traces of extracellular toxin were found in the culture medium of phosphate-rich cultures (Fig. 1 *a, b*). However, toxin accumulated in the culture medium of phosphate-poor cultures at the beginning of the stationary phase of growth (Fig. 1 *c, d*). Extracellular toxin disappeared rapidly from the illuminated cultures when the rate of intracellular toxin production dropped (Fig. 1 *c*) initially (0 to 2 days) and finally (after 10 days). This loss was probably due to the photoinactivation of extracellular toxin (Parnas *et al.* 1962).

Other substances besides toxin accumulated in the culture medium. To demonstrate this, the extracellular appearance of 260 nm absorbing substances was measured (Fig. 1).

Fig. 2. Effect of shift from dark to light conditions on kinetics of growth and toxin production of *Prymnesium parvum* in phosphate-poor $S_{50}S$ medium. Intracellular haemolysin in the dark (▲) and after shift to light (△) and extracellular toxin in the dark (■) and after shift to light (□), in H.U./ 10^6 cells; growth in dark (●) and after shift to light (○), in number cells/ml; arrow indicates shift to light; dagger indicates disintegration of cells.

Leakage of cellular constituents started in phosphate-poor R medium after the log phase and was most striking in the illuminated cultures (Fig. 1c).

To test the effect of a shift from dark to light, some cultures grown in the phosphate-poor $S_{50}S$ medium in the dark were illuminated on the seventh day (Fig. 2). A significant increase in intracellular toxin production was noted within 24 h of illumination, whereas considerable amounts of extracellular toxin appeared after 48 h. In the controls grown in darkness no toxin was detected before the 20th day of growth.

Organism size during growth in various conditions

It was found that growth conditions affected final size of the organisms (Fig. 3). In some media, their volume also changed during growth. The largest organisms were developed in the phosphate-poor media where they started enlarging after the log phase and reached maximal size before disintegration. The rate of increase in their volume was lower in the dark than in the light. Organisms from autotrophic S_{50} and R media cultures (except the phosphate-poor R medium in the light) were the smallest and most stable in mean volume. The observed differences in volume are illustrated in Fig. 4.

In an experiment with phosphate-poor $S_{50}S$ medium, cultures in the log phase were shifted from dark to the light. An increase was noted in size of the algae after 2 days (Fig. 3a).

Separation of the toxic extracts on thin-layer chromatography (t.l.c.)

Samples of the intracellular extracts obtained from $S_{50}S$ cultures at different phases of growth were separated on t.l.c. (after Ulitzur & Shilo, 1970). In all cases where toxicity appears, the six haemolytic spots obtained on separation of 'toxin B' were found, although their relative quantities varied. This indicates that the active substances are produced by *Prymnesium parvum* in all the tested growth conditions.

Fig. 3. Relative mean cell volume of *Prymnesium parvum* populations in S_{50} and $S_{50}S$ media (a) and R media (b). Culture media and conditions and counting techniques are described in Methods. S_{50} in light ($\times$): phosphate-rich $S_{50}S$ in light ($\circ$) and in dark ($\bullet$); phosphate-poor $S_{50}S$ in light ($\triangle$), in dark ($\blacktriangle$) and after shift from dark to light ($\diamond$); arrow indicates shift to light. (b) Phosphate-rich, R in light ($\circ$) and dark ($\bullet$); phosphate-poor in light ($\triangle$) and dark ($\blacktriangle$).

DISCUSSION

The present work shows that the toxicity of *Prymnesium parvum* is influenced by specific factors during its biosynthesis, and is not solely an expression of toxin stability and activating factors. Moreover, toxin production and algal multiplication do not depend on identical factors. Toxin concentration increased rapidly in illuminated suspensions of dense *Prymnesium* populations where no multiplication took place (Shilo & Rosenberger, 1960). Light, which is essential for toxin production and excretion (Padan, Ginzburg & Shilo, 1967) can be dispensed with for growth when media containing glycerol and serine are used (Rahat & Jahn, 1965; Paster, Reich, Bergmann & Rahat, 1966).

The experiments with R medium resulting in low amounts of toxin produced in the dark suggest that the slightly different concentration of microelements in this medium as compared with supplemented S_{50} medium may have resulted in toxin production.

A general phenomenon observed in this work was the rise in toxin production at the end of the logarithmic phase, reaching a peak during the deceleration and stationary phases. This suggests that toxin production might be related to growth limitation. This is strikingly demonstrated with respect to phosphate limitation: in phosphate-poor media where toxin production is high even at the beginning of growth, it is further increased during later

Fig. 4. Variation in cell size of *Prymnidium parvum* after 8 days of growth. After 8 days of growth, four typical *Prymnidium parvum* cells are shown from each of the different media. S₅₀ (rich-phosphate-light); R medium, rich phosphate, light (RPL); R medium, poor phosphate, light (PPL); R medium, rich phosphate, dark (RPD); R medium, poor phosphate, dark (PPD). Photographed in phase-contrast microscope under identical magnification ($\times 2750$).

phases, and finally reaches very high levels. This effect is emphasized on comparing growth in the dark in phosphate-rich and phosphate-poor media of the S₅₀S series: while no toxin is detected in the former medium, a low but distinct toxicity appears at the end of the stationary phase in the latter medium. This effect is so far peculiar to phosphate; limiting levels of nitrogen, thiamine and vitamin B₁₂ do not cause any increase of the toxigenic capacity of the alga (Shilo, 1971). Since concentrations of soluble phosphate are usually low in nature, this effect of phosphate limitation might well be decisive in *Prymnesium* toxin production in fishponds. The study of this condition in nature is complicated by the continuous and variable equilibrium balance of soluble and insoluble phosphates in the pond.

The accumulation of 260 nm absorbing substance in phosphate-poor conditions or in the stationary phase could be the result of membrane damage. A disturbance in the formation of membrane phospholipids resulting from phosphate limitation could lead to leakiness. The increased volume of organisms in the phosphate-poor media may either be a result of swelling due to impaired osmotic balance caused by membrane damage or to a disturbance in the regular division of the algae.

In its proteolipid composition, the toxin is similar to cell membrane components. Padilla's studies (1970) suggest that the toxic entity is located in the cell membrane. Subcellular fractions of *Prymnesium* banded in discontinuous sucrose density gradients showed a close correlation between toxin (haemolysin) content and presence of membrane vesicles. The glycerol-induced toxin synthesis found by Padilla (1970) further supports this notion that the toxin is a structural component of the cell membranes. These observations, together with our findings, indicate that the *Prymnesium* toxin may be a product of imbalanced metabolism of the cell membrane, since it appears at stages and in conditions where growth factors are limited and growth disturbed.

REFERENCES

- COLLIER, A. (1958). Some biochemical aspects of the red tide and related oceanographic problems. *Limnology and Oceanography* **3**, 33-39.
- DAFNI, Z. (1970). *The Cytotoxic Activity of Prymnesium parvum Extracts*. Ph.D. Thesis, The Hebrew University, Jerusalem.
- DAFNI, Z. & ULITZUR, S. (1968). Influence of growth conditions on the production of toxic principles of *Prymnesium parvum*. *Journal of Protozoology* **15** (Suppl.), 183.
- DROOP, M. R. (1958). Requirement for thiamine among some marine and supralittoral protista. *Journal of Marine Biological Association of the United Kingdom* **37**, 323-329.
- GORHAM, P. R. (1960). Toxic waterblooms of blue-green algae. *Canadian Veterinarian Journal* **1**, 235-245.
- PADAN, E., GINZBURG, D. & SHILO, M. (1967). Growth and colony formation of the phytoflagellate *Prymnesium parvum* Carter on solid media. *Journal of Protozoology* **14**, 477-480.
- PADILLA, G. M. (1970). Growth and toxigenesis of the chrysomonad *Prymnesium parvum* as a function of salinity. *Journal of Protozoology* **17**, 456-462.
- PARNAS, I., REICH, K. & BERGMANN, F. (1962). Photoinactivation of ichthyotoxin from axenic cultures of *Prymnesium parvum* Carter. *Applied Microbiology* **10**, 237-239.
- PASTER, Z., REICH, K., BERGMANN, F. & RAHAT, M. (1966). Studies on the growth of *Prymnesium parvum* Carter, and the formation of its toxin. *Experientia* **22**, 790-791.
- RAHAT, M. & JAHN, T. L. (1965). Growth of *Prymnesium parvum* in the dark; note on ichthyotoxin formation. *Journal of Protozoology* **12**, 246-250.
- REICH, K. & KAHN, J. (1954). A bacteria-free culture of *Prymnesium parvum* (Chrysomonadina). *Bulletin of the Research Council of Israel* **4**, 144-149.
- SHILO, M. (1967). Formation and mode of action of algal toxins. *Bacteriological Reviews* **31**, 180-193.
- SHILO, M. (1971). Toxins of Chrysophyceae. In *Microbial Toxins*. Edited by S. J. Ajl, A. Ciegler, S. Kadis, G. C. Montie & G. Weinbaum. New York: Academic Press.

- SHILO, M. & ASCHNER, M. (1953). Factors governing the toxicity of cultures containing the phytoflagellate *Prymnesium parvum* Carter. *Journal of General Microbiology* **8**, 333–343.
- SHILO, M. & ROSENBERGER, R. F. (1960). Studies on the toxic principles formed by the chrysomonad *Prymnesium parvum* Carter. *Annals of the New York Academy of Science* **90**, 866–876.
- SPIKES, J. J., RAY, S. M., ALDRICH, D. V. & NASH, J. B. (1968). Toxicity variations of *Gymnodinium breve* cultures. *Toxicon* **5**, 171–174.
- ULITZUR, S. & SHILO, M. (1966). Mode of action of *Prymnesium parvum* ichthyotoxin. *Journal of Protozoology* **13**, 332–336.
- ULITZUR, S. & SHILO, M. (1970). Procedure for purification and separation of *Prymnesium parvum* toxins. *Biochimica et biophysica acta* **201**, 350–363.
- YARIV, J. & HESTRIN, S. (1961). Toxicity of the extracellular phase of *Prymnesium parvum* cultures. *Journal of General Microbiology* **24**, 165–175.