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Formation and Mode of Action of Algal Toxins

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INTRODUCTION

When I decided to choose as the subject of this lecture a review on the phycotoxins, their formation and action, it was clear to me that it would be difficult to present a panoramic view of the field since our knowledge of it is at best fragmentary.

This would seem surprising since algal intoxications certainly make dramatic appearances. Explosive multiplication of these algae frequently reaches tremendous proportions, causing the appearance of toxic blooms or tides in large oceanic areas, in freshwater bodies, or in artificial fish ponds. The "red tides" common to the Pacific as well as the Atlantic coast of the United States are just such blooms which often develop high toxicity. Some of the algal poisons are among the most potent known to man. For example, the product of the dinoflagellate *Gonyaulax catenella* is 160,000 times more potent than cocaine in producing action potentials in nerves and voluntary muscles and approaches botulinus toxin in lethal activity.

On the other hand, bacterial toxins, because of their role in the physiopathology of many human and animal diseases, had already attracted the interest of microbiologists at the end of the last century. Since that time an extraordinary store of information has been gathered on their biosynthesis and their chemical nature and mode of action. Products of fungal metabolisms are similarly of great importance in causing intoxication, in induction of tumors, and in their antibiotic properties. These are powerful tools in pharmacological studies and in approaching basic problems in molecular biology.

We do know that toxic substances formed by many and different algal species represent health hazards to human beings, and cause severe economic damage in intoxication of cattle and other domestic animals. Extensive mortalities of fish and other aquatic animals and severe losses to fisheries and shellfish and clam industries are other effects of algal intoxication.

Finally, the possible role of algal metabolites as antibiotics cannot be overlooked. Recent studies have indeed shown that products of planktonic algae in mixed and axenic cultures may exhibit antibiotic activities in experimental conditions against other algae and bacteria. This could account for the well-known bactericidal activity of sea water (3), and play an important role in the succession of species in different aquatic ecosystems.

With this in mind, I have chosen to present a review of the work of our own group and other groups around the world who have devoted themselves to the subject, in the hope that, with the formulation of some of the more important problems, new interest and ideas will be stimulated and attention will be focused on a neglected field of research.

HISTORICAL BACKGROUND

The natural history of poisonous waters has an orthodox literary tradition from most ancient times, being mentioned in the Bible as one of the plagues which came on Egypt. It is written that "all the waters that were in the river were turned to blood. And the fish that was in the river died; and the river stank, and the Egyptians could not drink of the water of the river. . . . (Exodus 7:21). This description is strongly reminiscent of the poisonous red tides. Darwin, in his description of discolored water in 1832 during his voyage on the "Beagle," graphically described blooms of algae, which clearly must have been dinoflagellates similar to those appearing in toxic blooms.

Death and illness caused by consumption of poisonous mussels and clams, which we now know are caused by toxic dinoflagellates, were reported nearly 200 years ago by Captain Cook and by Captain George Vancouver during their expeditions to the coast of the Pacific Northwest. An old custom among Indian tribes along the coast of Alaska, particularly among the Poma, was to station sentries to watch for the marine luminescence occurring during hot weather, which they understood as being associated with Kal-Ko-o, as they called mussel poisoning (28, 34).

The main reasons for our present fragmentary knowledge of phycotoxins and the factors influencing their biosynthesis and action in nature is that toxic blooms are sporadic, they are short-lived, and they appear at various widely separated geographical locations. Furthermore, it was very difficult to draw conclusions from ecological relations participating in formation of the toxic outbreak. By the use of pure cultures, an experimental approach has become possible only recently.

Evidence for the causal relationship between algal blooms and animal intoxications usually rests on the seasonal and geographic correlation of these events. For example, extensive fish mortalities amounting to thousands of tons along the shores of the Gulf of Mexico are related to blooms of *Gymnodium breve* and *G. monilata*, the "red tide" organisms (19, 21, 87). Death of cattle in the thousands appears as a result of drinking water heavily infested with the blue green *Microcystis* (20, 80) and the fish mortalities described in Denmark (37), Bulgaria (85), Britain (27), and Israel (48) are connected with the phytoflagellate *Prymnesium parvum*.

The appearance of toxic algae together with various toxic phenomena suggested, but did not prove unequivocally, that the algae themselves are entirely responsible for toxin formation. The role of bacteria accompanying the toxic algae or the possibility of synergistic effects between different organisms could not be excluded (6). The finding that bacterial species accompanying toxic tides may have ichthyotoxic properties stresses the importance of this point (6).

Many of the toxigenic algae long defied efforts to isolate and grow them in pure culture. This was most often due to their delicate structure, slow growth rate, fastidious growth requirements, and coverings of epiphytic bacteria or slime layers. The availability of several of the algal forms involved in toxic tides and blooms in axenic culture now allows (12, 17, 47, 50, 79, 86) for the resolution of many of the problems which could not be approached directly up to the present. Among these are the evaluation of algal nutritional requirements and effects of environmental factors on growth and toxin formation, and, of course, the identification of the toxic principles.

Toxigenic algae often become serious public health problems, when they are ingested and concentrated in the organs of filter-feeding marine animals (28). Most important among these are the intoxications known as "mussel poisoning" and "paralytic shellfish poisoning." These are caused by consumption of molluscs which have fed on toxic dinoflagellates. In human intoxication, the molluscs served merely as vectors of the toxin. The injested poison tends to accumulate in the digestive gland of mussels and clams, as well as in the gills of the soft-shell clam and the syphons of butterclams (28). The amount of toxigenic algae concentrated and stored by the bivalves can be considerable, even when the density of *G. catenella* in the water is relatively low (200 per ml) and certainly is so during periods of blooms when densities reach up to 10^4 to 10^5 organisms per ml of seawater.

Experimental proof for the algal origin of the shellfish poisoning was adduced by Sommer and his collaborators in California, when nontoxic mussels became toxic after experimental feeding with *G. catenella* in the laboratory (78) and by isolation of the algal toxin directly from the dinoflagellate cells from blooms (53). Transplanting of nontoxic clams into beds of toxic bivalves and vice versa (63) served as additional proof, and the time necessary for toxin accumulation and loss was thus measured. Final proof of the role of the algae in shellfish poisoning was obtained when the mussel toxin from *Mytillus californicus* and saxitoxin from the syphon of the butterclam (*Saxidomus giganteus*) were isolated and characterized (61, 64), and were shown to be chemically identical with the toxin from axenic *Gonyaulax catenella* cultures (62). *G. catenella* known from the west coast of the U. S. seems to be not the only toxic dinoflagellate concentrated by molluscs; on the Atlantic coast, toxicity has been traced to *Gonyaulax tamarensis* (36). Other cases of shellfish poisoning associated with dinoflagellates have been reported: *Prorocentrum* was isolated from the intestine of toxic littleneck clams (*Tapes semidecussata*) during an outbreak of shellfish poisoning in 1942 in Japan (2), and *Pyrodinium phoneus* has been associated with paralytic shellfish poisoning in Europe (26). It has been suggested that the fish poisoning known as Ciguatera may also be caused by toxigenic algae, since toxicity of the fish varies seasonally (30).

CHARACTERIZATION OF THE MAIN ALGAL TOXINS

The known toxigenic algae belong to different taxonomic groups. The most prominent of these are some species of dinoflagellates (19, 28, 87); certain blue-green algae, in particular *Microcystis aeruginosa* and *Anabena flos-aquae* (20, 68, 80); and several species of the Chrysophyta, such as *Prymnesium parvum* (37) and *Ochromonas danica* (52).

Only two of the algal toxins have been chemically characterized. The toxin of *Gonyaulax catenella* (identical to saxitonin), having a relatively low molecular weight of 372, yields on hydrolysis various guanidine derivatives (65). Its structure has been proposed by Rapoport et al. (67) to be a purine base with a three-carbon bridge linking positions 3 and 9 and a methyl carbamate at position 6. The other algal toxin is that of the blue-green *Microcystis aeruginosa*. It is a cyclic polypeptide made up of 10 amino acid units with a molecular weight of about 1,200, and it contains a residue of the unnatural amino acid D-serine (10, 20).

The unique pharmacological properties of several of the algal toxins have attracted special interest and have made these toxins valuable tools in physiological research and as potential therapeutic agents.

The toxin of *Gymnodinium venificum* (1, 4) was found to act as a depolarizing agent of the neuromuscular junction, whereas that of *Prymnesium parvum* acted as a nondepolarizing blocking agent at neuromuscular junctions (41).

The closely related, probably identical toxins of *Gonyaulax catenella*, the shellfish poison and saxitoxin, block axonal and muscle conduction but show little if any effect on the neuromuscular junction (18, 24, 25). The action of these toxins at a cellular level was suggested to be due to the specific blocking of increase of sodium conductance normally associated with excitation, without affecting membrane conductance attributed chiefly to potassium and chloride permeability.

In contrast to *Gonyaulax catenella* toxin, the blocking action of *Prymnesium parvum* toxin on excitable membranes does not seem to involve changes in sodium conductance, since membranes in which excitation involves sodium movement are unaffected by *Prymnesium* toxins (9, 41). The blocking by these toxins in sensitive tissues may be associated with calcium movement.

Consequently, a study of the action of these toxins is of importance because of their usefulness for the elucidation of excitation phenomena and the molecular structure of excitable membranes.

ACTION OF ALGAL TOXINS IN NATURE

Another intriguing problem which has been dealt with by many investigators is the study of the factors which trigger algal blooms or which cause in nature the succession of blooms of different species (82). The practical outcome of these studies would be to prevent mass development of toxic algae, or at least to predict its development.

In a recent study by Rounsefell and Dragovich (56), the degree of association of the abundance of the "red-tide" organism *Gymnodinium breve* with certain environmental factors measured over a period of 8 years was tested. In the multiple curvilinear analysis of four of the major variables—salinity, temperature, wind, and the previous month's abundance ranking of the alga, the importance of these factors was clearly shown, but the predictive value of the data was rather low and additional factors must be involved in algal abundance. In certain cases, and the "red tide" seems to be among them, the mass development of the algae is not sufficient to account for the formation of the blooms. Passive concentration of the floating organisms by offshore winds (57) and physical holding together of water masses of suitable salinity also seem to be involved in formation of "red tide" (75). Once formed, such "red tides" may persist largely by recycling of their own metabolites, since the cell concentrations found in such heavy "red-tide" patches cannot possibly be sustained by the nutrients in the surrounding sea (81).

A major problem not yet satisfactorily explained was the lack of correlation between the level of toxicity and the density of organisms in the natural milieu or even in cultures. Often, high toxin levels were reached with very low algal numbers, whereas in many other cases mass development of toxigenic algae did not produce any observable toxicity in the milieu. This phenomenon has been described for every one of the known toxigenic algae (14, 20, 70).

The ability to form toxins may be determined by genetic factors, as has been shown in blue-green algae by J. A. Peary and P. R. Gorham in Canada (Abstr. Phycological Society of America, 1966). Different strains of *Microcystis aeruginosa* and *Anabena flos-aquae* in monoalgal cultures were shown to differ markedly in their toxin-forming capacity. In additon, nontoxigenic strains of these algae have been found (20).

It might, however, be equally important to study the role of physiological and environmental factors in the biosynthesis of algal toxins and to understand the steps leading to extra-

cellular accumulation of the toxin in the aquatic milieu. These include factors which affect excretion and stability of the toxin, or which enhance or inhibit the activity of extracellular toxin. Recently, Peary and Gorham (Abstr. Phycological Society of America, 1966) have proved with an axenic clone of the toxigenic blue-green alga *Anabena flos-aquae* (Lyngb.) that physiological factors such as the growth phase as well as environmental growth conditions may indeed determine the toxicity of these algae.

In elucidation of the ecological problems of toxigenic algal blooms and the action of the toxins, the nature of the release of the toxins into the aquatic milieu and the conditions for their accumulation and action have to be considered.

In certain cases, such as with the naked dinoflagellate *Gymnodinium breve* and all of the toxigenic blue-green algae, the toxins are typical endotoxins. Their release after death and decomposition of the algae is a prerequisite for action of these substances on any sensitive organism in the natural environment. Since in the aquatic milieu any active substance would rapidly diffuse and become dilute, massive release of the toxins by decomposition of large portions of the algal blooms is necessary for intoxication of aquatic animals.

It has been observed that sudden decomposition of algal blooms indeed often precedes mass mortality of fish. A systematic search for microorganisms which might be active in causing lysis of blue-green algae was undertaken in our laboratory. A number of bacteria which cause decomposition of living blue-green algae have been isolated during such natural decomposition cycles from fish ponds (Fig. 1). The lytic action of one of these gram-negative, nonmotile agar-decomposing bacterial strains was found to be specific for all the blue-green species tested, including *Nostoc.* sp., *Plectonema boryanum*, *Anacystis nidulans*, *Coccochloris peniocystis*, and *Synechoccus*, whereas the green alga *Chlorella* was unaffected. An interesting observation made with one particular strain of these bacteria was that, in the decomposition and breakdown of filaments of *Nostoc*, the algal heterocysts remained intact (Fig. 2).

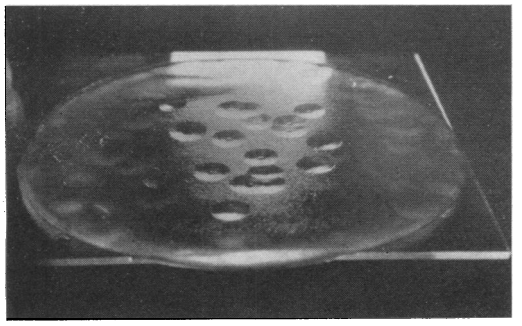

FIG. 1. *Agar-decomposing colonies of blue-green algae lysing bacteria on lawn of Plectonema boryanum.*

Synchronized, massive release of endotoxins to the aquatic environment in excess of critical extracellular concentrations could also be caused by lysis of the algae by viruses, such as the phycovirus active against blue-green algae isolated by Safferman and Morris (58, 59, 60).

We have found that these phycoviruses are present in various natural habitats during the decomposition of blue-green blooms, which suggests that they may play a role in the natural cycles involving breakdown of these algae (39). In their ultramicroscopic morphology (Fig. 3), and in particular in the anatomy of their tail and in the pattern of their infective cycles (11, 39, 76, 77), these viruses strikingly resemble bacteriophages. The similarity between these cyanophages and bacteriophages may thus constitute yet another important characteristic showing the close relationship of the bacteria and the blue-green algae. So far, cyanophages active against *Plectonema* and closely related genera have been found. It is conceivable that cyanophages active against other genera of blue-green algae exist as well.

Autolytic phenomena, often observed in algal cultures (5), may be yet another reason for the release of intracellular algal toxins.

Prymnesium parvum, a Model for Study of Toxigenic Algae

In view of the serious difficulties in drawing conclusions from ecological relations in nature, it seemed to us that a suitable organism which would lend itself to detailed experimental study in pure culture and in nature and which could serve as a model system, should be sought. Studies with such an organism could provide solutions to problems of toxigenic algae in general.

P. parvum, a marine phytoflagellate of the *Chrysomonadinae*, which forms potent exotoxin and which has been the cause of extensive fish mortalities in brackish-water conditons in many countries in Europe and in Israel, seems to us to fulfill this role.

Our studies with this organism started several years ago, soon after *P. parvum* penetrated and

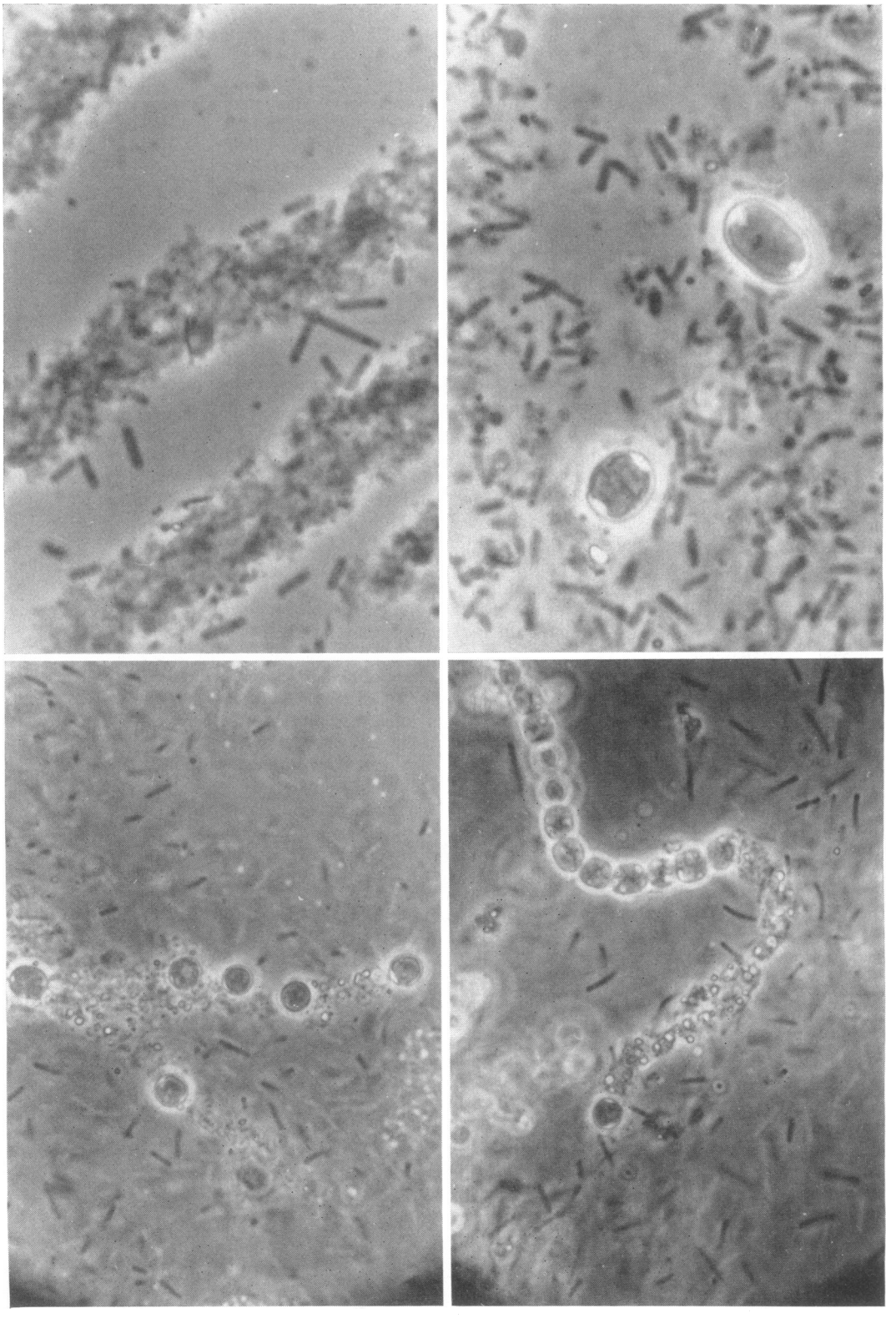

FIG. 2. *Filaments of Nostoc sp. lysed by bacteria. Heterocysts not affected.*

rapidly established itself in all the brackish-water fish ponds in Israel (48). *Prymnesium* has since become endemic, appearing in dense blooms (10^6 to 10^7 cells/ml) in the majority of fish ponds throughout most of the year.

Field studies with this organism could therefore be easily undertaken, and were not dependent on sporadic blooms. That blooms of *Prymnesium parvum* occur in water bodies whose size is in the range of experimentation and in which control can economically be attempted are yet other advantages in the study of this organism.

The further suitability of *P. parvum* as an object of study lies in the ease of culturing it. Axenic cultures have been isolated by Droop in England and by Reich and his group in Jerusalem (17, 50). These cultures can be grown in light on simple mineral media with B_{12} as sole required vitamin and with B_1 as a growth-enhancing factor (29, 45, 46), forming toxins under these conditions (43, 74, 88). Indeed, toxins isolated from pure cultures and from *Prymnesium*-infested pond water exhibited identical properties.

Recently, conditions for heterotrophic growth in the dark in glycerol-rich medium have been developed (44), and we have succeeded in obtaining clones and colonial growth on solid media (38).

Since the hemolysin formed by single colonies of *Prymnesium* can easily be tested directly on plates by the Jerne method (23), the screening for naturally occurring and induced mutants with different toxigenic capacities can be undertaken.

The detailed studies of Manton and her group in Leeds on the morphology and fine structure of the *Prymnesium* cell and its intracellular organelles (30–33) now allow the study of their function in toxin biosynthesis.

Sensitive and reliable methods for the quantitative assay of the different biological activities

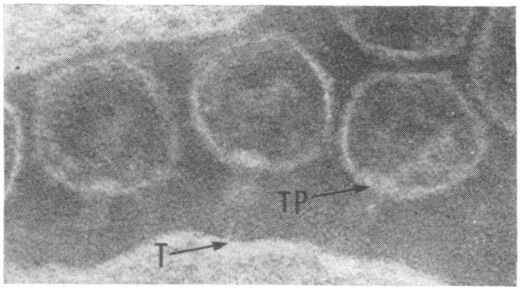

FIG. 3. *Cyanophage G-III attached to photosynthetic vesicles of Plectonema boryanum. Negative staining with phosphotungstic acid.* × *357,000. Tail (T); tail plate (TP).*

of the *Prymnesium* toxins [including an ichthyotoxin (8, 83) a hemolysin (88), and a cytotoxin (15, 16)] have been developed, and form a firm basis for studies on the formation and action of these substances.

A purification technique was developed which allows for a thousandfold increase in specific activity of both the intra- and extracellular toxins, with a yield of 25 to 50% of initial total activity (Fig. 4). Recent purification and analysis of such preparations (Paster, *personal communication*) have shown that they are rich in glucose and do not contain any detectable nitrogen, sulfur, or phosporus.

Spectrum of Biological Activities of P. parvum Toxins

Of the different biological activities of the toxic principles of *P. parvum*, the most striking is the lethal effect on aquatic gill-breathing animals, such as fish and molluscs. In amphibia, only the tadpole stage is sensitive to immersion in solutions containing ichthyotoxin; immediately after metamorphosis, the animals become refractory (70).

In addition to the ichthyotoxic activity, *Prymnesium* toxins exert lytic effects on various cell types, such as erythrocytes (7, 88) and a number of nucleated cells, including Ehrlich ascites cells (Landshutz strain), HeLa cells, normal human liver cells, and amnion cells (74). The treated cells swell, form pseudopodia-like extrusions, stain with trypan blue and finally lyse. The kinetics of this process can be traced conveniently in a Coulter counter by following the changes in volume of Ehrlich ascites cell population. It was found (16) that by changing incubation conditions of the toxin with the Ehrlich ascites cells by lowering of *p*H or of incubation temperature from 37 to 27 C, the Ehrlich ascites cells swell but no leakage of macromolecules, staining by dye, or lysis occurs. It seems, therefore, that the *Prymnesium* cytotoxin induces lysis in consecutive steps which can be separated by environmental conditions.

In addition to the effects described, toxins of *Prymnesium* have been found to manifest a multitude of pharmacological activities on muscle and nerve preparations (7, 9, 41). The question has therefore been posed whether the multiplicity of toxic manifestations observed with *Prymnesium* toxin in diverse biological systems may be different expressions of one and the same principle or are due to a mixture of different toxic principles.

The formation of several active toxins by *Prymnesium* was first indicated by Yariv and Hestrin (88), who separated two hemolytic frac-

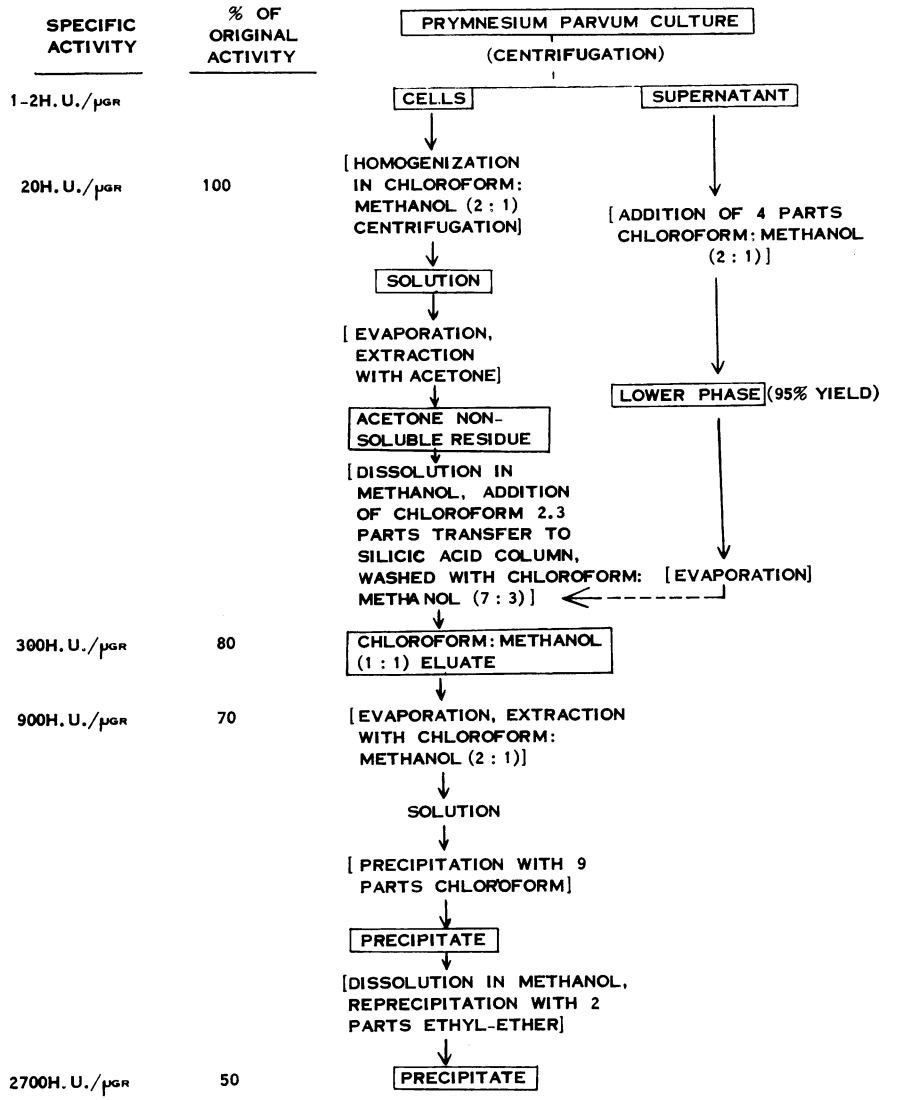

FIG. 4. *Schematic diagram of isolation and purification procedure of Prymnesium parvum hemolysin. Hemolytic units (HU) as described by Yariv and Hestrin (88). Hemolysin test was carried at pH 5.5. Cultures were grown in 1 liter of phosphate-deficient S medium (see Fig. 6). Twenty liters of pooled cultures, containing about 10^8 HU of intracellular toxin after 7 to 8 days of growth, was used for each purification.*

tions by paper chromatography. It was further found that the ratio between different toxic activities may vary under different growth conditions (49, 74), and that some of the hemolytic activity may be selectively removed from *Prymnesium* preparations by adsorption on erythrocyte ghosts (74). Most striking was the differential inactivation of the different toxic activities by alkali (74) and by light (42, 51). Although alkali rapidly inactivated the hemolysin active at 35 C, a hemolysin active at 8 C was only slightly affected (Fig. 5). Ichthyotoxicity was not diminished by the alkali treatment and often was slightly enhanced.

Toxin Formation and Stability Under Different Growth Conditions

In growing cultures in suitable media, synthesis of the toxic principles is greatest during the late stages of the logarithmic phase of growth and continues into the stationary phase. The toxins can first be detected within the cells from which

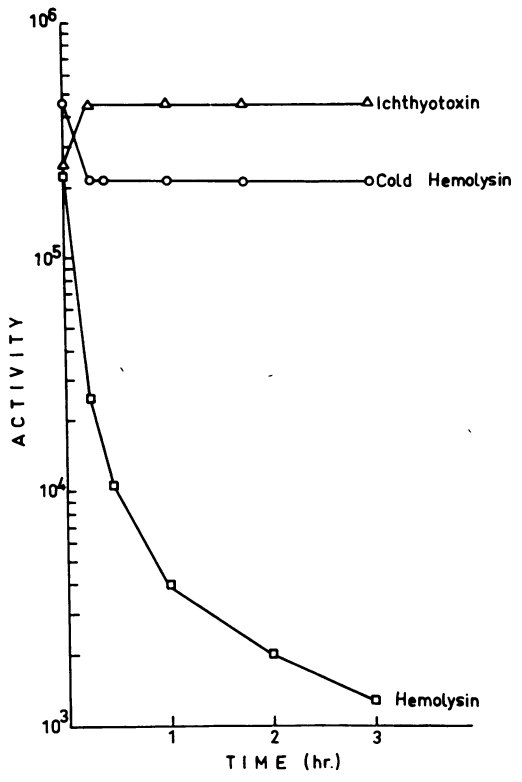

FIG. 5. *Effect of alkaline treatment on hemolytic and ichthyotoxic activity. Purified Prymnesium toxin preparation dissolved in methanol treated with methanolic NaOH to give a final concentration of 0.5 N was incubated at room temperature. Ichthyotoxicity (83), hemolytic activity (88) at pH 5.5, and in addition hemolytic activity at 8 C (cold hemolysin) were estimated at the times specified.*

they can easily be extracted with methanol. Later, the toxins are excreted and accumulate in the surrounding medium.

For ichthyotoxin, cytotoxin, and hemolysin formation, light (Fig. 6) is absolutely essential: no toxin synthesis is observed in heterotrophic conditions in the dark, although cell multiplication continues undistrubed. *Prymnesium* colonies grown in the dark exhibit hemolytic activity after 24-hr exposure to light. Limitation of phosphate in the medium enhances toxin formation even before markedly affecting growth. A 10- to 20-fold increase in hemolysin, cytoxin, and ichthyotoxin content was found in phosphate-starved cells, and high titers of these toxins are obtained in the culture fluid. High intracellular hemolysin and cytotoxin levels are obtained when dark-grown, toxinless cells from liquid phosphate-limited media are exposed to light for 24 hr.

Comparison of the capacity to form toxins in different media and under different environmental conditions clearly shows that growth and toxin biosynthesis have different optimal requirements.

Toxins obtained from culture supernatant fluids or cell extracts are readily inactivated by changes in *p*H of test solutions (70). Adsorption of the toxins on various colloids removes activity (70), as does exposure to light in the 400 to 510 mμ and ultraviolet ranges (42, 51). Thus, not only biosynthesis but extracellular stability of toxin as well are affected by environmental conditions.

Mode of Action of Ichthyotoxin

A unique property of *Prymnesium* ichthyotoxin is its requirement for cofactors (84, 88). When ichthyotoxin solutions were dialyzed for prolonged periods or passed through a cation-exchange column, they were rendered nontoxic to fish. The ichthyotoxic activity was restored on addition of the dialysate to the dialyzed fluid, and similarly when a successive washing displaced by 1 N HCl was added to the original eluant recovered from the cation-exchange column.

Appropriate salts of divalent cations, such as calcium or magnesium, when added to dialyzed culture fluid also restored its toxic activity. In addition, streptomycin, spermine, and many other polyamines were found to enhance the ichthyotoxic activity of crude *Prymnesium* extracts and of highly purified preparations as well (83).

In the presence of more than one cofactor, the resulting toxicity was not necessarily additive, but depended on the specific activity of each cofactor and its concentration relative to other cofactors. Thus, the addition of a cofactor of low activity, such as calcium, to a system containing a cofactor of high activity, such as spermine, drastically lowers the degree of toxicity to fish. The competitive inhibitory effect of NaCl in replacing a potent cofactor could similarly explain the inverse relationship between toxicity and salinity of the test medium or pond (83).

For the toxin to act on immersed sensitive organisms, the cofactor has to be present at the same time. No sequence of treatment with cofactor prior to toxin or vice versa was found effective. Hence, it appears that the cationic cofactor combines with the ichthyotoxin to form an active toxic complex. On the basis of electrophoretic mobility and separation on Sephadex G-50 columns with tritium-labeled spermine as

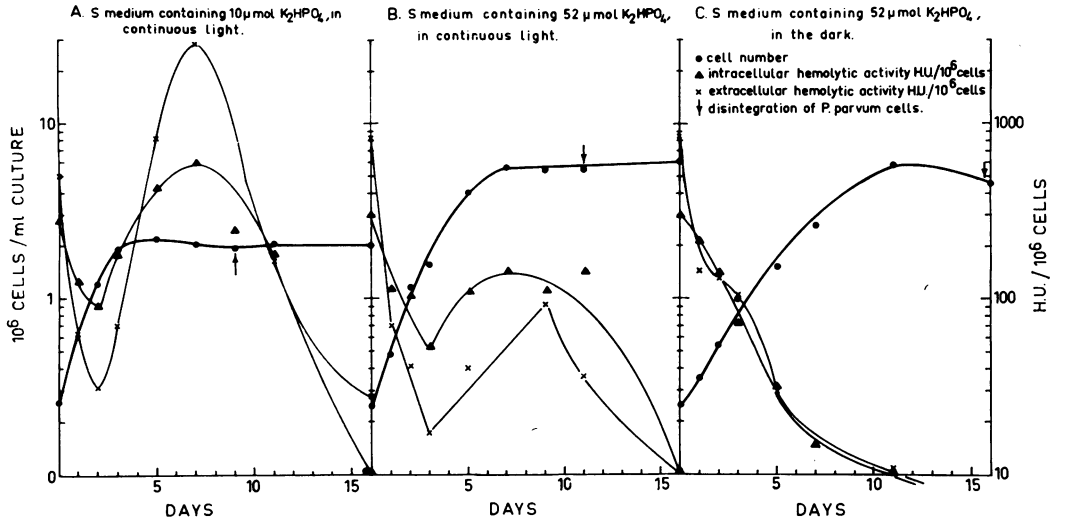

FIG. 6. *Effect of growth conditions on formation of Prymnesium parvum hemolysin. S medium is a synthetic medium described previously (83) with omission of glycyl-glycine and addition of the following ingredients (per liter): 30 ml of glycerol, 2.6 g of DL-serine, and 0.5 g of glycine. The phosphate concentration varied (10 or 52 µmoles/ml), as indicated. Light was "fluorescent white" of 220 to 260 ft-ca. Growth temperature was 26 to 28 C. Cells were counted with a Coulter counter (88). Hemolysin estimation as described (14). The pH of the test mixture was 5.5. Ethanolic extracts of cells (74) or culture supernatant fluid were taken for hemolysin determination at times specified. Arrow (↓) indicates the commencement of detectable cell autolysis and disintegration in the culture.*

cofactor, the activated toxin-cofactor complex could indeed be separated from both ichthyotoxin and cofactor moieties (Fig. 7). Equilibrium dialysis further proved the formation of the complex. Conditions, such as *p*H levels, necessary for the activation step differ from those required for the toxic activity and allow for separation of the two steps.

It is important when examining the mechanism involved in *Prymnesium* ichthyotoxicity to determine which effect is primary and results directly from the action of the toxin.

The rapidity of the action of *Prymnesium* toxin on immersed fish suggested that the immediate target must be an exposed organ, probably the gill. The fact that all gill-breathing animals tested were sensitive to the toxin was an additional strong support to this supposition. The first observable symptom upon immersion of test minnows in a solution containing lethal amounts of ichthyotoxin is a drastic change in gill permeability towards trypan blue, their uptake of radioiodine-labeled serum albumin, and increased sensitivity towards many nonspecific toxicants (84). The changes in gill permeability depend upon the presence of cofactors and occur only in those specific environmental conditions known to be optimal for lethal ichthyotoxic effects on test fish. In fish which are removed promptly from such toxin solutions, the gill damage is repaired withing hours. This fact supports the hypothesis that intoxication of gill-breathing animals immersed in ichthyotoxin solution consists of two stages. Initially, there is reversible specific damage to the gill tissues, resulting in the loss of their selective permeability; then a second stage leads to mortality, a response of the sensitized fish to nonspecific toxicants present in the milieu in concentrations sublethal to normal fish. The various stages of the activation of ichthyotoxin by different cofactors and of intoxication are shown diagrammatically in Fig. 8. The similarity of the ichthyotoxin of *Prymnesium* to surface-active agents in many of their chemical and physical properties raised the question whether a similar mechanism of ichthyotoxicity might not be found on treatment with certain detergents. So, indeed, many detergents have been shown to affect gill permeability (13). A number of anionic detergents (such as sodium dodecyl sulfate and sodium sulfasuccinate) and saponins (such as holothurin) exerted ichthyotoxic activity, which is enhanced by the cationic synergists of *Prymnesium* ichthyotoxin.

The properties which characterize the toxins of *P. parvum*, namely, their nondialyzability in neutral aqueous solutions, their typical solubility pattern in organic solvents, their proneness to

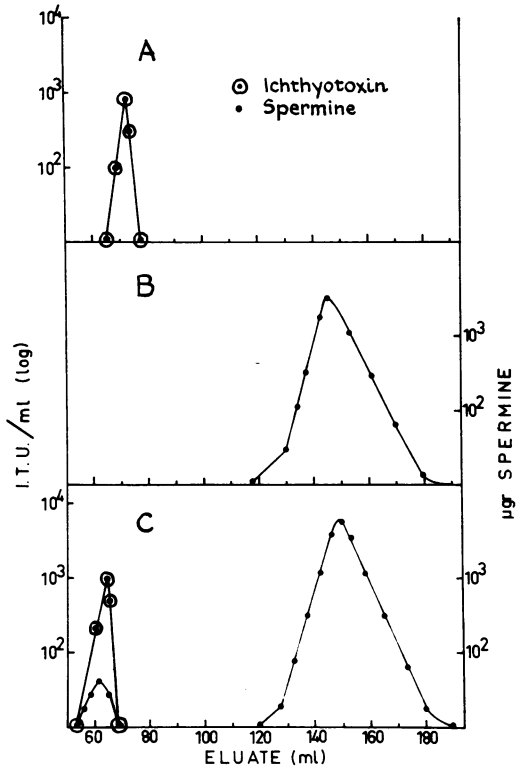

FIG. 7. *Separation of ichthyotoxin-spermine complex on Sephadex G-50. Purified Prymnesium ichthyotoxin and/or radioactive (^3H) spermine (0.01 μc/μmole) in 0.02 M tris(hydroxymethyl)aminomethane buffer (pH 9) were put on a column of Sephadex G-50, 37 × 2.5 cm; elution was carried out with same buffer. A: 2,500 international toxin units (ITU) of Prymnesium toxin in 2 ml of buffer. B: 10 mg of ^3H-spermine in 2 ml of buffer. C: Mixture of 2,500 ITU of Prymnesium toxin and 10 mg of ^3H-spermine in 2 ml of buffer. Ichthyotoxicity as described (83); radioactivity measured in a Tri-Carb scintillation counter.*

inactivation at gas-water interphases, and their similarity to various surfactants, are consistent with the view that the toxic material contains nonpolar and polar moieties, both of which contribute significantly to overall behavior.

Therefore, the possibility that the colloidal behavior of the ichthyotoxins in water may be due to a polymolecular micellar structure rather than to macromolecularity must be considered; indeed Yariv and Hestrin (88) suggested a similarity of this substance to plant saponins.

The difficulties in separation and the close chemical properties of the different active fractions seems to indicate that all the *Prymnesium* toxins belong to a closely related family of chemical substances.

Since homogeneity of even our most potent *Prymnesium* preparations has not yet been established beyond doubt, it seems premature at this point to draw more detailed inference concerning the chemical nature of the toxin. Nor is it known to what extent the different biological activities of *Prymnesium* toxin should be ascribed to chemical change at the molecular level or to physical change at a micellar level.

Lysis of P. parvum by Weak Electrolytes

The ultimate goal of many investigators working with toxigenic algae is to obtain control measures to combat or prevent the growth of the organism. This is quite often difficult to achieve, since many of these algal blooms cover extensive areas of water. In smaller bodies of water, however, control measures are economically feasible (54, 56, 71). In the case of *P. parvum*, it was observed during the early attempts of Aschner and Reich to maintain unialgal cultures that even extremely low concentrations of ammonia were lethal to the phytoflagellate (48). This unique sensitivity to lysis by ammonia formed the basis for the development of an effective control measure in field conditions, which has allowed for the development of intensive fish breeding in brackish-water conditions.

It was found that ammonia is not the only agent that lyses *P. parvum*, but that other weak electrolytes such as acetic acid are active as well (69, 72, 73). In both cases, the activity was found to be *p*H-dependent, suggesting that the nondissociated molecules, rather than the ion, represent the active agents involved in lysis. The mechanism underlying the lysis of *P. parvum* cells by weak electrolytes is similar to that described for erythrocytes by Jacobs (22), namely, an osmotic mechanism based on penetration and internal concentration of weak electrolytes activated by the difference between the internal *p*H of the cell and that of the external milieu. The osmotic imbalance is created by trapping the penetrating undissociated weak electrolyte after its intracellular dissociation. When the *p*H of the suspension fluid is equal to that of the interior of the cell or of intracellular compartments involved in lysis, no lysis should be possible with weak bases or acids.

When the morphological changes were observed by directly following the process with a phase microscope, the cell showed a marked swelling prior to its lysis, suggesting an osmotic phenomenon. Change in the volume of the *P. parvum* cells could also be followed directly, and the kinetics of swelling could be studied by use of a Coulter counter. The detailed morphology of

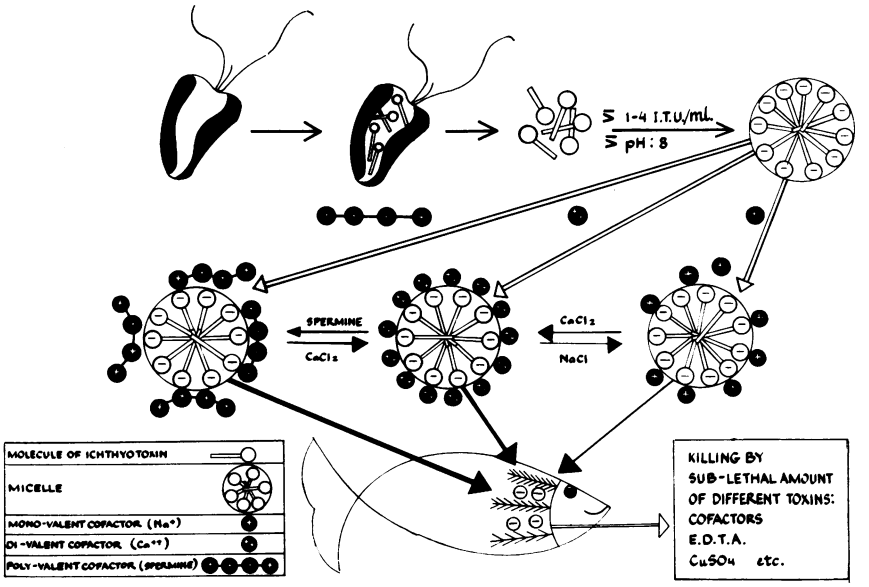

FIG. 8. *Mode of action of Prymnesium toxin on fish.*

lysis further confirmed the osmotic nature of the lytic phenomena induced by the weak electrolytes penetrating the cell. A marked difference was found between lysis by ammonia and that by acetic acid. In ammonia lysis, two lateral vacuoles could be seen to swell quickly, causing the rupture of the cell. In acetic acid-induced lysis, the posterior vacuole was the site of swelling, and the lateral vacuoles were not visible at all.

A satisfactory hypothesis for the mechanism of lysis must explain the strange uniqueness of *Prymnesium* in its sensitivity to the weak electrolytes studied. Later, it became evident that different microorganisms, such as *Dunaliella salina* and *Syracosphaera carterae*, and also certain marine luminous bacteria, will swell and lyse when brought into contact with ammonia or acetate, provided the external *p*H is appropriate and the osmotic pressure of the surrounding milieu is close to the lower limit at which these halophilic cells can survive. These findings indicate that lysis of microbial cells by weak electrolytes may in fact be a widespread and general phenomenon, provided the cells are already under conditions of severe osmotic stress. The sensitivity to weak electrolytes thus seems to be an expression of the halophilic nature of *Prymnesium*, and is shared similarly by other halophiles.

SUMMARY

We have seen that with *P. parvum* a large number of physiological and environmental factors are concomitantly involved in the processes of toxin formation, excretion, and toxin activation. In addition, a dynamic equilibrium was shown to exist between toxin production and its extracellular inactivation in the aquatic milieu. It is, therefore, only when the precise combination of many necessary factors operate in concert that conditions for intoxication of sensitive organisms are created. The possibility of isolating and defining individual steps involved in the accumulation and action of the toxin has certainly justified our expectation regarding *P. parvum* as a model system for the study of phycotoxicity. Furthermore, many of the most important factors shown to affect toxin formation and stability in cultures have been shown to be active in natural conditions as well, and thus the analysis of a complex ecological situation has now become possible. The multitude of steps influencing, and absolutely indispensable for, the chain of events in *Prymnesium* intoxication may certainly be adduced to be similar for other phycotoxic systems, and may therefore guide us in future studies on different toxigenic algae. The study of *Prymnesium* has given us the key for its effective control, and has made it possible to prevent nearly completely the heavy damage inflicted by it on fish breeding. The development of new algicidal agents and the potential use of bacteria and viruses which actively lyse toxigenic algae may open the way for new approaches to the control of additional toxigenic algal species.

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