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EFFECTS OF SALINITY ON SYNTHESIS OF DNA, ACIDIC POLYSACCHARIDE, AND ICHTHYOTOXIN IN *GYMNODINIUM BREVE*

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Key Word Index—*Gymnodinium breve*; Pyrrophyta; algae; dinoflagellate; ichthyotoxin; toxigenesis; Florida red tide; biosynthesis.

Abstract—A Florida red tide organism, *Gymnodinium breve* Davis, an unarmored dinoflagellate, was grown in enriched sea water media at salinities 20–43‰ and constant illumination. Use of lowest (23‰) and highest (43‰) salinities resulted in death within 24 hr of inoculation, though good growth was obtained at all intermediate salinities (29–39‰), in accord with field observation. Rates of synthesis of DNA, acidic polysaccharide and ichthyotoxin were determined as a function of salinity and growth constant (K_{10}). The relative rate of synthesis of DNA or polysaccharide increased linearly with growth constant. Mean cell volumes, determined during log-phase growth, showed a positive correlation with doubling time. Hemolytic activity was detected in cell extracts only at high toxin concentrations (0·35–2·05 mg of ichthyotoxin). No significant difference was noted in hemolytic activity of extracts of cells grown in high (34‰) or low (26‰) salinity. The rate of toxin synthesis showed a linear decrease with the rate of DNA or polysaccharide synthesis.

INTRODUCTION

THE TERM 'Florida red tide' has been applied to areas of sea water on the west coast of Florida from Cape Sable to Cape San Blas that are discolored by populations (250000 cells/l.) of the unarmored dinoflagellate *Gymnodinium breve* Davis.[1-4] These blooms have been sporadic since the first was noted in 1884 by Walker,[2] but the impact of the effects of catastrophic fish kills in areas of burgeoning human populations has been greater in recent years.

Following the identification of *G. breve* as the causative agent of catastrophic fish kills,[1] many attempts have been made to determine the conditions necessary for sudden blooms, but it appears that no single biological, chemical, or hydrological factor is responsible.[2,5,6] However, several workers have noted the possible significance of the trace metals and organic compounds[6-8] in river effluents.

The mass marine mortalities associated with *G. breve* blooms are due to two effects: di-

[1] DAVIS, C. C. (1948) *Botan. Gaz.* **109**, 358.
[2] ROUNSEFELL, G. A. and NELSON, W. R. (1966) *U.S. Fish Wildl. Serv. Spec. Sci. Report. Fish.* 535.
[3] BRYDON, G. A., MARTIN, D. F. and OLANDER, W. K. (1971) *Environ. Letters* **1**, 235.
[4] SASNER, JR., J. J. (1973). in *Marine Pharmacognosy* (MARTIN, D. F. and PADILLA, G. M., eds.), Ch. 5, Academic Press, New York.
[5] ROUNSEFELL, G. A. and DRAGOVICH, A. (1966) *Bull. Mar. Sci.* **16**, 402.
[6] COLLIER, A., WILSON, W. B. and BORKOWSKI, M. (1969) *J. Phycol.* **5**, 168.
[7] INGLE, M. and Martin, D. F. (1971) *Environ. Letters* **1**, 69.
[8] MARTIN, D. F. and MARTIN, B. B. (1973) in *Trace Metals and Metal-Organic Interactions in Natural Waters* (SINGER, P. C., ed.), Chap. 12, Ann Arbor Science Publishers, Ann Arbor.

rectly because of a neurotoxin,[9-15] and indirectly because of anaerobiosis (due to increased respiratory activity of the bloom organism or by intense bacterial activity associated with decomposition of affected organisms).[15,16]

The ecological limitations of *G. breve* are significant. For example, the optimum salinity was reported to be 27–37‰ based on laboratory studies,[2,17] and Ray and Aldrich[18] have noted that *G. breve* and oysters are normally separated by low salinity barrier, because of poor survival of *G. breve* below 24‰. Once the red tide organism enters an estuarine system, particularly a nutrient-enriched one with tolerable salinity, however, the organisms can be concentrated by several mechanisms and maintained for several days.[19] Though much information is available on the toxin from *G. breve*,[12,14,20,21] little is known of the conditions under which the toxin is produced. The ichthyotoxin is reported to be hemolytically active[22] which gives a convenient assay. The toxin appears to be a degradation product of cell wall constituents[13] so that toxigenesis should be related to the development of cell polysaccharide content, perhaps cell size (area) or cell DNA content. The present investigation was undertaken to determine if toxin production is related to salinity-induced changes in the growth of *G. breve* and how these changes affect biosynthetic capabilities. Specifically, the relationships between salinity, growth rate, cell size, rate of synthesis of DNA and polysaccharide content, toxicity and hemolytic activity were investigated.

RESULTS

Growth

The growth pattern was evaluated for two series of four cultures of *G. breve* at 25 ± 1° in enriched media (salinity range of 22–39‰, see Table 1). After a lag period of 1 week (similar to that noted before),[23] the cells entered log growth until the stationary phase (6–9 × 10^6 cells/l.) was attained at 6–7 weeks. The variation in growth constant, K_{10} (= 2·303 m, where m is the slope of the linear portion of the log-growth phase of the semilogarithmic plot, log cells as a function of time) and doubling time, D.T. (= $\log_{10}2/K_{10}$), with salinity is indicated in Table 1. For two separate series of experiments, the optimum salinities appear to be 30–34‰, and agreement between the two series of experiments was good (Table 1). The growth constant at salinity 34–36‰ was 0·078 ± 0·009 day^{-1} which agrees with a previously reported value[23] of 0·083 ± 0·01 for salinity 33–35‰ for similar media.

[9] SASNER, JR., J. J. (1965) Doctoral Dissertation, Univ. Calif., Los Angeles.
[10] CUMMINS, J. M., JONES, A. C. and STEVENS, A. A. (1971) *Trans. Am. Fisheries Soc.* **100**, 112.
[11] SIEVERS, A. M. (1970) *J. Protozool.* **16**, 401.
[12] MARTIN, D. F. and CHATTERJEE, A. B. (1970) *Fish. Bull.* **68**, 433.
[13] DOIG, III. M. T. and MARTIN, D. F. (1972) *Environ. Letters* **3**, 279.
[14] SASNER, JR., J. J., IKAWA, M., THURBERG, F. and ALAM, M. (1972) *Toxicon* **10**, 163.
[15] SIMON, J. L. and DAUER, D. M. (1972) *Environ. Letters* **3**, 229.
[16] STEIDINGER, K. A. and INGLE, R. M. (1972) *Environ. Letters* **3**, 271.
[17] ALDRICH, D. V. (1960) *U.S. Fish Wildl. Serv. Circ.* **92**, 40.
[18] RAY, S. M. and ALDRICH, D. V. (1967) in *Toxic Animals* (RUSSEL, F. E. and SAUNDERS, P. R., eds.), p. 75, Pergamon Press, Oxford.
[19] RYTHER, J. H. (1955) in *The Luminescence of Biological Systems* (JOHNSON, F. H., ed.), p. 387 AAAS, Washington.
[20] TRIEFF, N. M., VENKATASUBRAMANIAN, M. M. and RAY, S. M. (1972) *Texas Rept. Biol. Med.* **30**, 97.
[21] SPIEGELSTEIN, M. Y., PASTER, Z. and ABBOTT, B. C. (1973) *Toxicon* **11**, 85.
[22] PASTER Z. and ABBOTT, B. C. (1970) *Science* **169**, 600.
[23] MARTIN, D. F. and OLANDER, W. K. (1971) *Environ. Letters* **2**, 135.
[24] CROXTON, F. E. (1959) *Elementary Statistics, with Applications in Medicine and the Biological Sciences*, Chap. 7, Dover, New York.

Below salinity 22‰, cells were rapidly lysed (i.e. within 24 hr) and good growth was never observed; growth constants at salinities 26 and 39‰ were significantly lower than those at salinity 30–34‰ (Table 1).

TABLE 1. GROWTH AND BIOSYNTHESIS FOR *Gymnodinium breve* GROWN IN ENRICHED SEA WATER (NSW medium) AT VARIOUS SALINITIES*

Salinity S‰	Growth constant, K_{10} (day^{-1})	Doubling time (days)	Mean cell dia.†(μ)	DNA μg/10^6 cells	DNA Rate	DNA Rate ratio‡	Polysaccharide μg/10^6 cells	Polysaccharide Rate‖	Polysaccharide Rate ratio	Rate of synthesis of ichthyotoxin (mg/10^6 cells/day)
20	0									
22	0									
	—									
26	0·060‡	5·0	33	142	8·52	0·79	5·1	0·31	0·76	
	0·057§	5·3		117	6·67	1·10	34·6	1·97	1·57	0·058
30	0·075	4·0	30	143	10·73	1·00	5·4	0·41	1·00	
	0·081	3·7		75	6·06	1·00	15·5	1·26	1·00	0·067
34	0·066	4·6	31	119	7·85	0·73	5·0	0·33	0·82	
	0·089	3·4		78	6·94	1·14	20·9	1·86	1·48	0·064
36	—	—		—	—	—	—	—	—	
	0·097§	3·1		88·9	8·62	0·80	8·0	0·78	0·62	0·099
38	—	—		—	—	—	—	—	—	
	0·068	4·4		64·4	4·38	0·72	7·2	0·49	0·39	0·114
39	0·050	6·0	34	87·0	4·35	0·41	1·8	0·09	0·22	
	—	—		—	—	—		—	—	
43	—	—		—	—	—		—	—	
	0									

* Enrichments/l. sea water: $FeCl_3 \cdot 6H_2O$, 2·0 mg; Na_2EDTA, 50·0 mg; 5·0 ml of sulfides mixture. (stock solution, g/lr: NH_4Cl, 0·2; K_2HPO_4, 0·1; $NaHCO_3$, 0·2; $MgCl_2 \cdot 6H_2O$, 0·4; $Na_2S \cdot 9H_2O$, 0·15); Vitamin B_{12}, 1 μg; biotin, 0·5 μg; thiamine·HCl, 10·0 mg.

† Expressed as spherical equivalents.

‡ First and second entries refer to first and second series of experiments.

§ Results omitted in calculation of R_{DNA} vs K_{10} and $R_{polys.}$ vs K_{10}.

‖ μg of constituent/10^6 cells/day.

These results were compared with the variation of cell size with salinity. Mean cell volumes, expressed as spherical equivalents, varied with salinity, and minimum cell diameters were observed at optimum salinities. Quantitatively, the linear correlation coefficient, r, relating cell volume (or cell diameter) and doubling time is statistically significant ($r = 0{\cdot}962$, $P = 2{\cdot}5$–5%).

In addition, the cell DNA and acidic polysaccharide content were measured during log-phase growth for two series of experiments (Table 1). Qualitatively, as the salinity decreased, or increased from the optimum range, nucleic acid and anionic polysaccharide content decreased, and the mean DT increased. Quantitatively, rates of biosynthesis of cellular components and rates of growth can be related by means of the growth equation: $N_t = N_0 2^{t/a}$ (where a = DT, t is the time interval between measurements, N_0 and N_t are the number of cells initially and after time t).

The growth equation was utilized by substituting for N_t and N_0 the values of DNA, polysaccharide, or other cellular component that must double from division to division.[25]

Using the data in Table 1, the rate of synthesis of DNA or polysaccharide was calculated as μg of component/10^6 cells/day. In order to normalize the data from two series of experiments, the relative biosynthetic rates were calculated [= (rate at $S = n$‰)/(rate at S = 30‰) where n is 26, 34, etc.]. Data for $S = 30$‰ were used as standard because maximum growth constants were observed for the range 30–34‰. The relative rate of biosynthesis of DNA (R_{DNA}) or polysaccharide ($R_{polys.}$) was a function of growth constant, K_{10}. The least-squares treatment gave the following results: $R_{DNA} = -0{\cdot}40 \pm 0{\cdot}19 + 17{\cdot}6 \pm 2{\cdot}7\ K_{10}$; $R_{polys.} = -1{\cdot}2 \pm 0{\cdot}5 + 28 \pm 6{\cdot}8\ K_{10}$.

[25] PADILLA, G. M. (1970) *J. Protozool.* **17**, 456.

Toxigenisis

The ichthyotoxicity of extracts of *G. breve* culture (salinity, 34‰) has been measured.[13] One toxin unit was defined[13] as the amount of toxin needed to kill a fish, *Poecilia sphenops*, (4–6 cm, 1–2·5 g) in 20 ± 3·8 min. Our study indicates that one toxin unit was equal to 0·3 mg of crude toxin. The amount of toxin (mg/10^6 cells) obtained and the rate of synthesis varies parabolically with salinity. The minimum in amount or rate was observed at salinity 30–34‰; amount and rate of synthesis of toxin approximately doubled at $S = 39‰$ and was slightly greater at suboptimum salinities (27‰) (see Fig. 1).

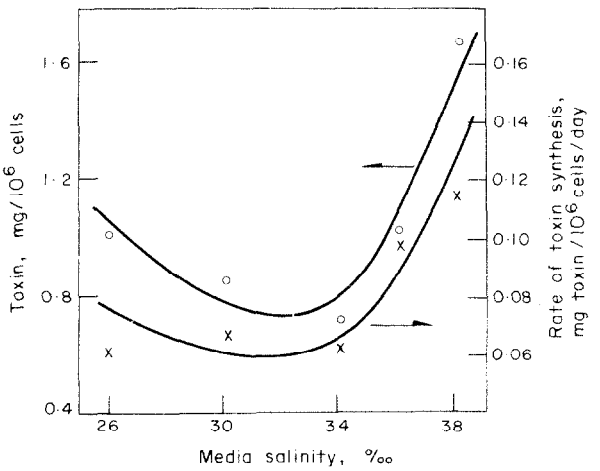

FIG. 1. AMOUNT OF ICHTHYOTOXIN AND RATE OF ICHTHYOTOXIN SYNTHESIS AS A FUNCTION OF THE MEDIA SALINITY FROM WHICH THE TOXIN WAS ISOLATED.

The rate of toxin synthesis (mg/10^6 cells/day) was a linear function of DNA and polysaccharide synthesis rates (R'_{DNA} and $R'_{polys.}$, respectively, expressed as $\mu g/10^6$ cells/day). A least-squares treatment gave the following results (± indicates s.d.): Rate of toxin synthesis = 0·205 ± 0·026 − 0·021 ± 0·004 R'_{DNA}; Rate of toxin synthesis = 0·127 ± 0·010 − 0·0362 ± 0·007 $R'_{polys.}$

Toxigenesis was also studied by determining the hemolytic activity of extracts from two *G. breve* cultures (salinity, *S*, 34‰ and 26‰). Red blood cells were incubated with the methanolic solution of toxin and the absorbance of the supernatant at 540 nm (A_{540}) was measured, relative to control. Applying a least-squares treatment, we found that $\log_e A_{540} = -a + b$ (mg toxin), where *a* and *b* are the intercept and the slope, respectively, of the linear relationship, and where the concentration of toxin is expressed as mg of toxin in 5 ml of red blood cell suspension (2% v/v, MeOH-blood buffer). The values of *a* and *b* were 4·03 ± 0·31 and 2·20 ± 0·23 respectively, ($S = 34‰$) 3·75 ± 0·08 and 1·82 ± 0·006, respectively ($S = 26‰$). Thus, no significant differences in hemolytic activity seemed evident at these salinities (mg toxin = 0·35–2·05).

DISCUSSION

The use of the growth equation has certain limitations, notably that the systems must be in the exponential phase and that the cells be in balanced growth. That these conditions obtained was indicated by the low standard errors associated with repetitive samples from the same or separate cultures of the same time in log phase. Applications of the growth

curves in analyses similar to the present study were done with *Astasia longa*,[26] a bleached strain of *Euglena gracilis*,[27] the dinoflagellate *Gonyaulax tamarensis*,[28] and the euryhaline chrysomonad *Prymnesium parvum*.[25]

The application of these data must be made with some caution. Whether the results mean that the ability to adapt to salinity is initially mediated through a change in cell division, with a parallel, but secondary change in rate of synthesis of DNA, is uncertain. No obvious breaks or changes in the relative rate of biosynthesis appear for the growth constants obtained, and the natural range of salinity was covered. For the chrysomonad *Prymnesium parvum*, however, Padilla[25] found that below a certain division rate, the rate of DNA synthesis was independent of division rate. For example, our measurements were made with cells that had been adapted for several generations to an optimum salinity (*ca* 35‰), though in the same medium (NSW), but rapid change to higher and lower salinities was characterized by a slightly longer, slightly more erratic lag phase than would be typical of the optimum salinity. It appears that controls for DNA synthesis for this organism (though not for *P. parvum*) relate fairly closely to the kinetics of cell division.[25]

Our results have practical implications. Many others believe that during outbreaks, high populations of *G. breve* do not occur overnight because of high division rates[16] but that concentrating mechanisms (including prevailing onshore winds, convergence of water masses, and convection cells[19]) must be invoked to account for appearance and maintenance of the high populations of cells. It is easier to apply these results to account for the appearance of *G. breve* in Tampa Bay (normal salinity *ca* 20‰). During a severe drought (1970–1971), the salinities in the Bay increased to 28–30‰ and approached values for optimum growth.

In the second part of the study, analysis of ichthyotoxicity and hemolytic activity, no variation of hemolytic activity with salinity of media was noted, though we found a variation of ichthyotoxicity with salinity of the media from which the toxin was extracted. In contrast, Padilla[25] found the highest level of hemolysin in *P. parvum* was induced at a particular salinity, though cells had a similar protein and nucleic acid content over a range of salinities above and below the critical one. Also, in our study, ichthyotoxin activity in extracts was a minimum when obtained from cells grown in the salinity of optimum growth. There seems to be no obvious correlation between ichthyotoxin activity and hemolytic activity for crude *G. breve* toxin, but the relationships between rate of synthesis for ichthyotoxin, DNA, and polysaccharide are statistically (and ecologically) significant.

The implications require some consideration. Analyses of DNA and polysaccharide content were based on separation of cells, though the toxin analysis is based on extraction of the media, following lysis of cells. The two procedures are probably equivalent, because previous workers[12] have found that, within experimental error, all the toxin is associated with cells in log growth; following careful filtration of the cells, no toxic activity could be extracted from the filtrate.[12] The opposing rates of biosynthesis for toxin vs DNA or polysaccharide could indicate that toxin production occurs at the expense of normal growth.

The extrapolation of laboratory results of the present study to environmental conditions must be done cautiously, but available evidence suggests that the extrapolation is justified in some instances. As noted earlier, the salinity optimum observed in the present study is in accord with previously reported values, as are salinity limitations. The division times

[26] KAHN, V. and BLUM, J. J. (1967) *Biochemistry* **6**, 817.
[27] BLUM, J. J. and BUETOW, D. E. (1963) *Exp. Cell. Res.* **29**, 407.
[28] PRAKASH, A. (1967) *J. Fish. Res. Bd. Can.* **24**, 1589.

observed for the salinity optimum observed are similar to those in outbreaks.[16] If the trends in biosynthesis can be extrapolated, there would be reason for some optimism: blooms of *G. breve* that extend into an estuary could grow under conditions that would favor the rate of biosynthesis of DNA or RNA at the expense of toxin, and physical uptake of toxin by shellfish would be a less serious problem than if there were a parallel relationship between the biosynthetic rates for toxin and DNA. The results therefore suggest that the accumulation of toxin by shellfish, though a problem,[10] is probably much less serious than it might otherwise be.

EXPERIMENTAL

Organisms and culture condition. Bacteria-free cultures of *G. breve* were obtained from Ray and Wilson (Texas A & M Marine Station, Galveston). Cells were maintained in B-5 medium as previously described[3] (see Table 1). All inoculations were aseptically transferred to 1 liter culture tubes containing enriched natural sea water media (NSW) when the parent culture had reached the stationary phase of growth. The inoculated flasks (initial cell count 30 ~ 45/ml) were maintained at 25 ± 1° with constant illumination of *ca* 6000 Lx, provided by dual banks of 40 W cool white fluorescent lamps.

Media (NSW) of varying salinities were prepared as follows from offshore sea water (typical composition: salinity *ca* 35‰; other, ppb; total PO_4–P, 360; NO_3–NO_2 nitrogen, 7·5, NH_4–N < 1·0). The sea water was mixed with activated charcoal (Merck, 0·2 g/l), filtered through Whatman No. 1 paper, then through Millipore type HA 0·45 μ membrane filter. The filtrate was concentrated under red. pres. to give a salinity of 40‰. Dilution with 3 × -dist. H_2O gave 1 liter samples of desired salinity (39, 34, 30‰, etc.) that were supplemented with Fe-EDTA and sulfides mixture (see Table 1) and autoclaved at 1 atm. for 15 min. After 3 days, vitamins (see Table 1) in 3 × -dist. H_2O were sterilized by filtration through Swinex filter (0·22 μ, Millipore, Inc.) and added aseptically prior to inoculation. Salinities were determined with a Goldberg T/C refractometer (American Optical Co., Buffalo, N.Y.).

Cells were counted with a Coulter counter (Model B; 100-μ aperture). The instruments was calibrated using latex polystyrene DVB spheres (Dow Chemical Co., Bioproducts Division) of known size.

Analyses. DNA determinations were made using a procedure of Holm-Hansen *et al.*[29] with the following modifications. Culture samples (5 ml) were filtered through 0·45 μ Millipore membrane filters (25 mm dia.) that had been treated with 1 ml 0·25% diatomaceous earth suspension. The residue and membrane were dissolved in 6 ml acetone in a 10 ml centrifuge tube and analyzed as described.[29] HCl (5 ml, 1 M) was used after hydrolysis instead of 0·6 M perchloric acid.[30]

Anionic polysaccharide content was determined by a spectrophotometric method[31] that has been modified[32] for marine dinoflagellates, using sodium heparin standards and expressing the polysaccharide content as heparin-equivalent weight.

Ichthyotoxin was isolated as before[12] from *G. breve* cultures, lyophilized and dissolved in MeOH. The toxicity of the MeOH solns was assayed using *Poecilia sphenops.*[13] The hemolytic potency of MeOH solns was determined by exposing 4·9 ml of 2% suspensions of washed rabbit erythrocytes in normal Ringers to 0·1 ml soln of toxin in MeOH. The extent of hemolysis was determined by measuring the absorbance of the supernatant fluid at 540 nm with a Beckman DB GT spectrophotometer after 45 min incubation at 37° using a Sorval G76 gyrotatory water bath shaker. Control samples contained 0·1 ml MeOH.

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[30] HINEGARDNER, R. T. (1971) *Anal. Biochem.* **39,** 197.
[31] JANDER, J. and WORK, E. (1971) *FEBS Letters* **16,** 343.
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