In vitro Culture of Pyrodinium

Abstract. Water from Puerto Rico's Phosphorescent Bay, when enriched with vitamin B_{12}, thiamine, biotin, yeast autolysate, and bay-mud acid hydrolysate, has been found to support vigorous in vitro growth of the luminescent dinoflagellate *Pyrodinium bahamense*. Cultures of *Pyrodinium* are being maintained through serial passage.

Bioluminescence displayed so spectacularly in waters of Phosphorescent Bay, Puerto Rico, is produced by the dinoflagellate *Pyrodinium bahamense* (*1, 2*). The late E. Newton Harvey lamented that chemiluminescence studies of this brilliantly flashing organism would have to await solution of the in vitro culture problem (*2*). Fulfilling this desideratum, we report the successful isolation and maintenance of *P. bahamense* under both the axenic and bacterized conditions.

From water samples collected during midday hours from depths to 2 meters near the center of Phosphorescent Bay, some 8000 *Pyrodinium* cells were miropipetted to petri dishes containing Seitz-filtered bay water. From these, groups of 300 to 600 cells were transferred to the first well of a nine-well depression plate. The most vigorously swimming cells were then micropipetted successively from well to well, 2 ml of sterile bay water having previously been added to each well. This technique had previously been found effective in lessening or eliminating bacterial contamination during isolation procedures with other motile single-cell algae and protozoa (*3*). From the final well, groups of 1 to 100 cells were transferred to 20-ml plastic-capped culture tubes containing 10 ml of sterile bay water.

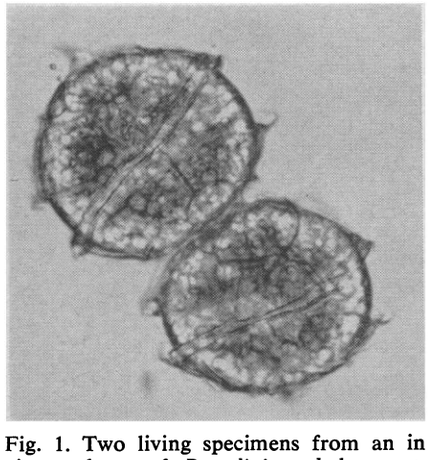

Fig. 1. Two living specimens from an in vitro culture of *Pyrodinium bahamense*. This luminescent armored dinoflagellate is responsible for the "fiery waters" of Phosphorescent Bay, Puerto Rico (× 1300). [Photograph by John J. Lee and Stanley Pierce]

Guided by our earlier experience in isolating and culturing zooxanthellae (*3*), we tested some 2000 variations and permutations of enrichment and antibiotic materials before a suitable formula was achieved for support of *Pyrodinium* growth. Optimal growth-supporting media comprised filtered bay water, 90 percent; B_{12}, 0.1 μg/100 ml; thiamine HCl, 1.0 mg/100 ml; biotin, 0.1 μg/100 ml; yeast autolysate, 0.0001 percent; acid hydrolyzed bay-mud extract, 1 to 10 ml/100 ml. Enrichment materials were used in conjunction with an antibiotic solution containing K penicillin G, 1.0 percent; novobiocin, 0.001 percent; polymyxin B, 0.001 percent; Vancomycin, 0.1 percent; Ilotycin, 0.1 percent; and Tylosine, 0.1 percent (*3*). The antibiotic mix was added to culture tubes over a concentration range of 0.05 to 2.0 ml per 10 ml of culture media. Previously derived synthetic media (*3*) failed to support *Pyrodinium* growth. Before inoculation, medium was autoclaved at 5 lb pressure for 2 hours.

Culture tubes were maintained at 24° to 28°C in a light-dark cabinet (14 hours light; 10 hours dark), with light supplied by three 40-watt white cool fluorescent lamps and one 20-watt tungsten bulb.

Within 20 days after inoculation, abundant cell division was observed. Transfers to fresh media are now made routinely at 30-day intervals. Such cultures have gone through multiple serial passage, with no apparent loss of motility or reproductive vigor. Two culture lines are currently in an axenic or bacteria-free state; three lines, although bacterized, support a vigorously proliferating population of *P. bahamense*.

Luminescence appears to be greatest when observed 4 to 6 hours after the beginning of the daily dark period. A sharp tap on the culture vessel invariably results in bright luminescence of the entire liquid contents, with a myriad of conspicuous starlike flashes. The luminosity pattern of *P. bahamense* appears to resemble that observed by Sweeney and Hastings for *Gonyaulax polyedra (4; 5)*.

JOHN J. A. MCLAUGHLIN
PAUL A. ZAHL
Haskins Laboratories, New York

References and Notes

1. P. R. Burkholder and L. M. Burkholder, *Bull. Marine Sci.* **8**, 201 (1958); P. A. Zahl, *Natl. Geographic Mag.* **118**, 120 (1960).
2. E. N. Harvey, *Bioluminescence* (Academic Press, New York, 1952).
3. P. A. Zahl and J. J. A. McLaughlin, *Nature* **180**, 199 (1957); J. J. A. McLaughlin and P. A. Zahl, *Proc. Soc. Exptl. Biol. Med.* **95**, 115 (1957) ——, *Ann. N.Y. Acad. Sci.* **77**, 55 (1959); P. A. Zahl and J. J. A. McLaughlin, *J. Protozool.* **6**, 344 (195 J. J. A. McLaughlin, P. A. Zahl, A. Nowak, J. Marchisotto, J. Prager, *Ann. N.Y. Acad. Sci.* **90**, 856 (1960).
4. B. M. Sweeney and J. W. Hastings, *J. Cellular Comp. Physiol.* **49**, 115 (1957).
5. We express gratitude to the University of Puerto Rico's Institute of Marine Biology for laboratory facilities made available to us; to Dr. Luis R. Almodóvar and Andrew Nowak for field and technical assistance; and to Dr. John J. Lee and Stanley Pierce, Department of Micropaleontology, American Museum of Natural History, New York, for photos made of our in vitro *Pyrodinium*. We give special thanks to the National Geographic Society, Washington, D.C., for support of initial field work by one of us (P.A.Z.) leading to the development of this program. Other aspects of the work were supported by the U.S. Public Health Service, under grants B-1198 and R.G. 7022. Acknowledgment is made of a gift from the Eli Lilly Co. of Vancomycin, Ilotycin, and Tylosine. A more detailed account of isolation and culture techniques, together with the results of present nutritional and physiological studies on *Pyrodinium*, is in preparation.

3 August 1961

Effect of Enzymes on Partially Purified Japanese B Encephalitis and Related Arbor Viruses

Abstract. Japanese B encephalitis and some other arbor viruses were partially purified by cellulose column chromatography or by fluorocarbon deproteinization and tested for sensitivity to enzymes. Infectivity decreased markedly when the viruses were mixed with trypsin or pancreatic lipase at 37°C. The enzymes also impair the immunogenicity of the virus in rabbits. Poliovirus is resistant to the enzymes.

The effect of enzymes on viruses is different from one enzyme-virus combination to another (*1*). Reduction of the infectivity of arbor (*ar*thropod-*bor*ne) viruses by proteolytic enzymes has been described (*2*). The virus materials used in these previous studies were relatively crude. We report experiments in which arbor viruses grown in tissue cultures and then partially purified were examined for sensitivity to enzymes.

The viruses used were Japanese B encephalitis, strain G1; dengue type 1, Mochizuki strain; yellow fever, strain 17 D; and Western equine encephalitis, Rockefeller Institute stock strain. The viruses were grown in trypsinized hamster-kidney cell cultures (*3*). Poliovirus, strain MEF-1, was grown in