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.

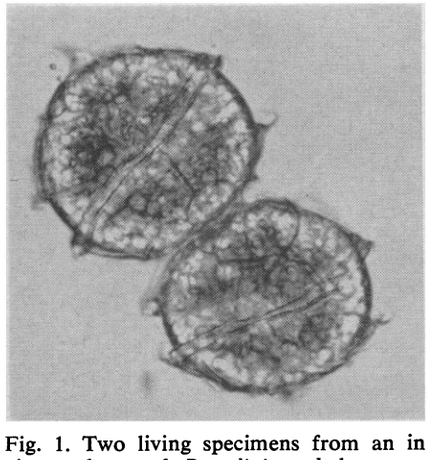

Fig. 1. Two living specimens from an in vitro culture of *Pyrodinium bahamense*. This luminescent armored dinoflagellate is responsible for the "fiery waters" of Phosphorescent Bay, Puerto Rico (× 1300). [Photograph by John J. Lee and Stanley Pierce]

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)*.

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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

HeLa cell cultures for comparison. Culture fluid harvested from the infected cultures, showing characteristic cellular degeneration, was centrifuged at 3000 rev/min for 15 minutes to remove crude debris masses. The supernatant fluid, undiluted or diluted with tissue-culture fluid, was loaded onto a column containing ECTEOLA-cellulose and eluted with 0.07*M* phosphate buffer solution (*p*H 7.2) (*4*). Some supernatant fluid was treated with fluorocarbon (*5*). By either procedure practically 100 percent of the virus is recovered, and more than 90 percent of the nitrogen contained in the original virus materials is removed. Nitrogen contents of purified virus suspensions, as measured by the micro-Kjeldahl method, ranged mainly from 0.1 to 0.2 mg/ml.

The enzymes used were trypsin (crystalline) (*6*), papain (powdered) (*7*), bacterial proteinase (crystalline) (*8*), pancreatic lipase (powdered) (*6*), bacterial lipase (liquid) (*8*), wheat-germ lipase (powdered), α-amylase (crystalline), ribonuclease (crystalline), and lysozyme (crystalline) (*6*). The enzymes, except for bacterial lipase, were dissolved in sterile phosphate buffer solution (*p*H 7.2) at a final concentration of 0.125 percent and tested without further sterilization. Bacterial lipase was diluted with phosphate buffer solution (*p*H 6.0) to a final concentration of 125 units per milliliter; one unit decomposes 1 percent of 2 g of olive oil after exposure of 2½ hours at *p*H 5.6 and temperature of 30°C.

The partially purified viruses were mixed with enzymes, incubated at 37°C (except for bacterial lipase, which was incubated at 30°C) for 1 to 2 hours, diluted decimally with Hanks' salt solution, and titrated for active virus content by inoculation into either mice or tissue culture tubes. The LD_{50} or $TCID_{50}$ values were calculated by the method of Reed and Muench (*9*). In controls, phosphate buffer solution was used in place of enzyme solution.

The results from several experiments are summarized in Table 1. The infectivity of these arbor viruses decreases markedly, or is lost completely, on exposure to trypsin or pancreatic lipase. Bacterial proteinase or lipase also reduce (though less than trypsin or pancreatic lipase do) the infectivity of purified Japanese B encephalitis virus, whereas wheat-germ lipase, α-amylase, ribonuclease, and lysozyme have little effect. When purified Japanese B encephalitis virus is treated with trypsin for 1 hour at 37°C and then exposed to ribonuclease for 2 hours, there is no reduction in viral infectivity.

Table 1. The effect of various enzymes on the infectivity of partially purified viruses. For Western equine encephalitis and Japanese B encephalitis the unit of virus titer was mouse intracerebral LD_{50} (log) per 0.02 ml; for dengue, yellow fever, and poliomyelitis, it was $TCID_{50}$ (log) per 0.2 ml plus 1.8 ml of culture medium.

Enzyme	Viral infectivity at times indicated				
	Enzyme-treated			Control	
	Hr 0	Hr 1	Hr 2	Hr 0	Hr 2
	Western equine encephalitis				
Trypsin	6.50	4.50	1.50	6.50	6.50
Lipase	6.50	3.00	0.50	6.50	6.50
	Japanese B encephalitis				
Trypsin	5.00	4.75	0.50	5.25	4.50
Papain	3.50		2.50	3.25	3.25
Proteinase (*B*)*	4.50	4.25	3.25	4.75	4.75
Lipase (*P*)†	3.25	0‡	0	2.75	2.50
Lipase (*B*)*	4.75	3.50	3.25	4.75	4.75
Lipase (*W*)§	4.75		4.50	4.75	4.50
α-Amylase	4.50		4.75	4.75	4.50
Ribonuclease	3.75		3.75	4.25	4.00
Lysozyme	2.75		3.25	2.75	2.50
	Dengue				
Trypsin	5.25		0.50	4.75	4.50
Lipase (*P*)†	5.00		2.50	4.75	4.50
	Yellow fever				
Trypsin	4.25		1.50	4.25	4.25
Lipase (*P*)†	4.25		1.25	4.25	4.25
	Poliomyelitis				
Trypsin	6.75		5.50	6.50	6.50
Lipase (*P*)†	6.25		6.50	6.50	6.50

* *B*, bacterial. † *P*, pancreatic. ‡ No infective virus was detected. § *W*, wheat germ.

One milliliter of the treated or untreated Japanese B enchephalitis virus was injected intravenously into male rabbits three or four times at intervals of about a week. Complement-fixing titers and neutralizing indices were measured. In animals receiving active purified virus, antibodies were produced well and regularly; complement-fixing titers of serum drawn 1 to 2 weeks after the last injection ranged from 1:64 to 1:128. Neutralizing indices ranged from $10^{2.5}$ to $10^{3.0}$. Antibody development in animals inoculated with enzyme-treated virus was significantly lower and more irregular; in some animals no antibody was produced.

The results appear to indicate that trypsin and lipase have an effect on substances controlling the infectivity and immunogenicity of some arbor viruses. Although infective ribonucleic acid can be extracted from tissues infected with arbor viruses (*10*), viral ribonucleic acid in infective (probably mature) virus particles is apparently resistant to ribonuclease.

Partially purified poliovirus is resistant to pancreatic lipase, although it is apparently affected by trypsin to a certain extent. Whether there is a correlation between this differential effect of enzymes and the finding that poliovirus resists ethyl ether whereas arbor viruses are destroyed by it (*11*) may warrant further consideration (*12*).

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7. The papain used in this study was obtained from Merck and Company.
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Insecticide Content of Diet and Body Fat of Alaskan Natives

Abstract. A study was made of the DDT and DDE content of the diet and body fat of native Alaskans who lived in isolated, primitive areas and had minimal contact with insecticides. No DDT or DDE was detected in any of the native Alaskan foods analyzed with the exception of two white owls, both of which contained low levels of DDE. Eskimos store considerably less DDT and DDE in their body fat than the general population in the United States. These low dietary levels and the resultant low levels in body fat are consistent with previously published data on the relationship between intake and storage of DDT.

Analysis of representative restaurant (*1*) and institutional (*2*) meals has indicated that the average person in the United States consumes 184 to 202 μg of DDT and even smaller amounts of DDE in his daily food. Most of the material is found in animal fats, and only small amounts in vegetables and other constituents of meals. The total dietary intake accounts for most if not all of the DDT and its metabolite DDE stored in people without occupational exposure to the insecticide.

Hayes and his co-workers (*3*) reported that analysis of fat from persons who died before 1942, and, therefore, before the use of DDT, revealed no trace of DDT-like material. By contrast, samples of body fat collected from the general population during 1954–56 contained DDT in an average concentration of 4.9 parts per million (ppm). The same authors found that meatless meals served in a cafeteria catering to meat abstainers contained only about one-fourth as much DDT as meals served in ordinary restaurants. Persons abstaining from meat deposited in their fat only about half as much DDT as people with an ordinary diet. Thus, for that study, the storage of the insecticide was not only proportional to dietary dosage but also proportional to the intake of meat. Although dosage was probably the important variable, the data offered no way of evaluating any contribution that animal fat may make to the absorption and eventual storage of DDT present in the food.

It is, of course, clear that occupational exposure to DDT may lead to storage far greater than that ever reported as the result of ordinary dietary intake. An average concentration of 17.1 ppm was found in the fat of agricultural workers who applied DDT (*3*). A concentration as high as 648 ppm was found in the fat of an asymptomatic worker in a formulating plant (*2*).

Further search has been made for groups of people with minimal occupational, environmental, and dietary contact with DDT. Native Alaskans who live in an isolated, primitive area where there is little or no use of insecticides and who eat food of local origin appeared to be a group that might have minimal DDT exposure and at the same time maximal intake of animal fat. The present paper describes a study of DDT and DDE content of the diet and body fat from these Alaskan natives.

The food samples were collected in the villages of Shungnak, Kotzebue, Gambell, Hooper Bay, and Point Hope. A total of 42 samples of food, representing 31 different items of the Eskimo diet, were analyzed. The foods studied included various fresh and dried fish; fat, oil, or meat from beaver, beluga, caribou, eider duck, moose, oogruk, polar bear, seal, walrus, whale, and white owl; and miscellaneous foods including cranberries, salmonberries, and wild rhubarb. These foods make up the major portion of the diet of the village Eskimos. There are also a limited number of imported food items, including cereals, sugar, bacon, lard, and hydrogenated fat, in the diet of these people.

All analyses for DDT and DDE in both food and body fat were carried out by the modifications of the Schechter-Haller spectrophotometric procedure cited by Hayes *et al.* (*3*).

No DDT or DDE was detected in any of the native foods analyzed, with the exception of two white owls from Point Hope, which contained 1.1 ppm DDE in the meat. The source of exposure of these birds to DDT is unknown. It may be accounted for by their migratory habits. It is also possible that some naturally occurring constituent of food stored by these birds may interfere in this analysis.

Samples of human body fat were obtained through the cooperation of the U.S. Public Health Service Hospital in Anchorage. Single samples from 20 patients were analyzed. The subjects chosen were residents of isolated villages. Fat samples were taken from patients who underwent surgery after a minimal period of hospitalization. Sample meals from this hospital for one day were analyzed and found to contain 184 μg of DDT and 26 μg of DDE. A dish of meatballs and spaghetti contained 111 μg of DDT and represented 60 percent of the daily total residue of this insecticide in the diet. These daily totals for DDT and DDE are similar to those found in representative restaurant and institutional meals in the 48 contiguous states (*1*, *2*).

The DDT and DDE content of the body fat of these native Alaskans is shown in Table 1, along with comparative values obtained for the general population and for meat abstainers by

Table 1. Storage of DDT and DDE in the fat of Alaskan natives (as found in this study) in comparison with the general population of the United States and with abstainers from meat (as found by Hayes *et al.*, *3*).

Value	DDT (ppm)		DDE (ppm)	
	Tissue	Extract*	Tissue	Extract*
	Alaskan natives (20 cases)			
Range	0 to 1.9	0.3 to 2.2	0 to 3.9	2.5 to 5.8
Mean ± S.E.	0.8 ± 0.10	1.4 ± 0.16†	2.0 ± 0.41	3.8 ± 0.31†
	General population (61 cases)			
Range	2 to 12	3 to 22	2 to 13	3 to 25
Mean ± S.E.	4.9 ± 0.35	6.8 ± 0.42	6.1 ± 0.42	8.6 ± 0.52
	Abstainers from meat (16 cases)			
Range	0 to 7	0 to 10	0 to 9	0 to 12
Mean ± S.E.	2.3 ± 0.44	3.5 ± 0.63	3.2 ± 0.63	4.9 ± 0.84

* Carbon tetrachloride extract. † Based on 11 samples only.