Proc., 1956, p. 70.

10. Szilagyi, D. E., Overhulse, P. R., Shonnard, C. P., and LoGrippo, G. A., *Surgical Forum of the Am. Coll. Surg.*, 1954, p. 244.

11. Trafas, P. C., Carlson, R. E., LoGrippo, G. A., and Lam, C. R., *A.M.A. Arch. Surg.*, 1954, v69, 415.

12. LoGrippo, G. A., Overhulse, R. P., Szilagyi, D. R., and Hartman, F. W., *Lab. Invest.*, 1955, v4, 217.

13. Smolens, J., and Stokes, J., Jr., PROC. SOC. EXP. BIOL. AND MED., 1954, v86, 538.

14. Havens, W. P., Watson, D. W., Green, R. H., Lavin, G. I., and Smadel, J. E., *J. Exp. Med.*, 1943, v77, 139.

15. Sulkin, S. E., Wallis, C., and Allen, R., PROC. SOC. EXP. BIOL. AND MED., 1956, v93, 79.

16. Casals, J., *J. Immunol.*, 1947, v56, 337.

17. Reed, L. J., and Muench, H., *Am. J. Hyg.*, 1938, v27, 493.

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Studies in Marine Biology. II. *In vitro* Culture of Zooxanthellae.* (23139)

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In November, 1956, the authors examined a wide variety of marine invertebrates from the waters around Bimini, B.W.I. We were interested in the algal cells—the so-called symbiotic zooxanthellae—which live within the tissues of many radiolarians, coelenterates, molluscs, bryozoa, worms, ascidians, etc. (1,2). With a few exceptions, firm taxonomic placement of zooxanthellae has been difficult because of their somewhat generalized morphology and because of absence or elusive nature of a motile phase(3,4); only Kawaguti(5) has reported seeing motile forms. Furthermore, whether zooxanthellae of various host animals fall into one or into multiple taxonomic groups has not been established. As to their role as symbionts, one school(6,7) holds that the zooxanthellae do not appreciably contribute to nutrition of the host; another, based largely on recent work of Sargent and Austin(8) and of Odum and Odum (9), suggests that the abundant presence in coral reef communities of photosynthetic algae (including both interstitial zooxanthellae and intraskeletal filamentous algae) may well be crucial to growth and maintenance of the entire reef biotope.

If, following on the work of Kawaguti, zooxanthellae could be isolated from the host animal, grown serially in bacteria-free culture, and their nutritive and reproductive characteristics studied, not only should their identity in each case be ascertainable, but also possibly clues as to their trophic relationship with the host animal. The aim of the present study was to isolate certain coelenterate zooxanthellae and to culture them *in vitro*.

Materials and methods. A large zooxanthellae-containing Scyphozoan (*Cassiopeia* sp.) and a large zooxanthellae-containing anemone (*Condylactis* sp.) were selected. *Cassiopeia* abounded in waters around S. Bimini, B.W.I. *Condylactis* was similarly abundant at E. Bimini. The brown-green coloration of both these animals reflects the presence, mainly in endodermal tissues, of densely packed layers of zooxanthellae (Plate I, Fig. 1 and 2). To secure zooxanthellae free from host tissue for *in vitro* culture, an entire live specimen of either *Cassiopeia* or *Condylactis* was washed successively in tapwater and sterile seawater, then macerated in a Waring blendor with approximately 100 ml of sterile seawater. Within a few minutes tissues were

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reduced to a soupy fluid, which was immediately filtered through glass wool. The filtrate was centrifuged at moderate speed until a sediment, consisting of packed algal cells largely freed from host tissue, collected on the bottom of the tubes. The supernatant was discarded, and the sediment resuspended in sterile seawater. Centrifugation and resuspension were repeated 6 times, with the last 3 washings made in synthetic seawater media. Finally, the sediment was resuspended in media containing different concentrations of

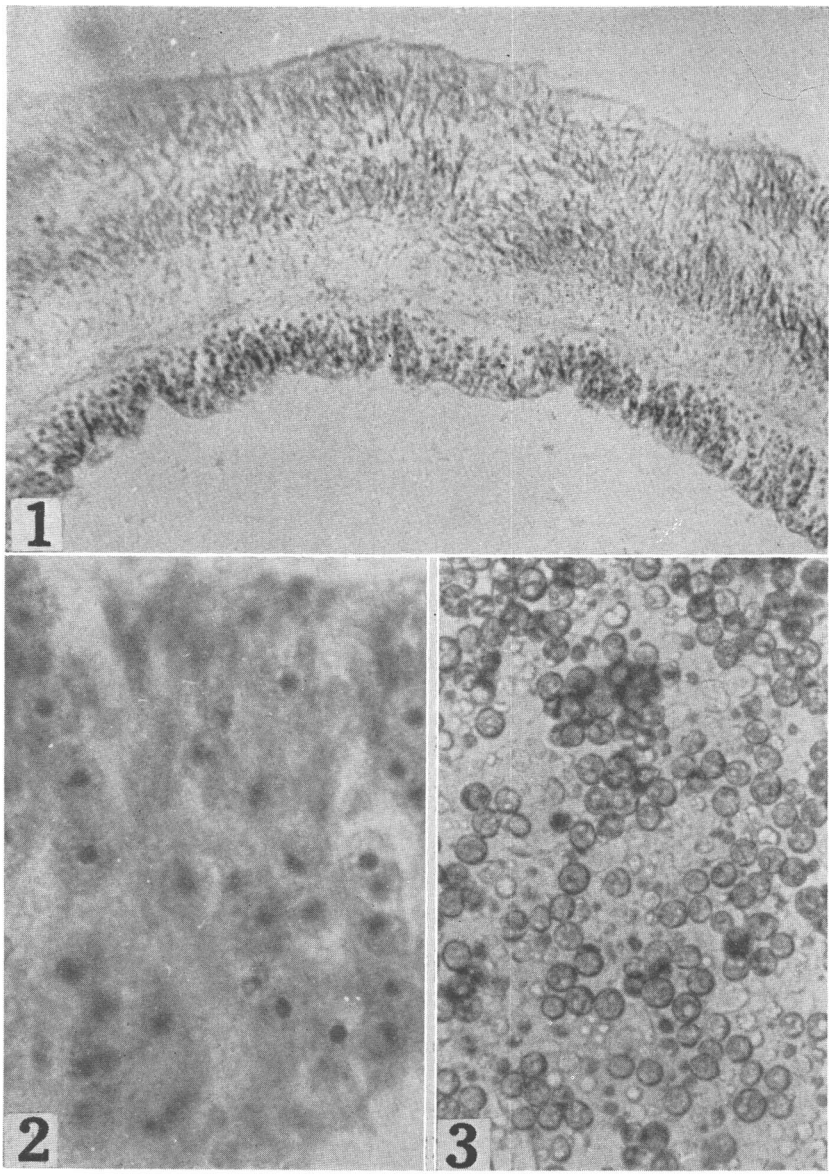

Plate I.

FIG. 1. Cross-section of portion of anemone *(Condylactis)* tentacle to show endodermal location of zooxanthellae cells. Bouin's, H & E. Mag. × 140.

FIG. 2. Detail of endoderm of anemone tentacle to show interstitial position of zooxanthellae cells. Bouin's, H & E. Mag. × 1000.

FIG. 3. Living vegetative zooxanthellae cells removed from anemone tissue by methods described in text. Mag. × 400.

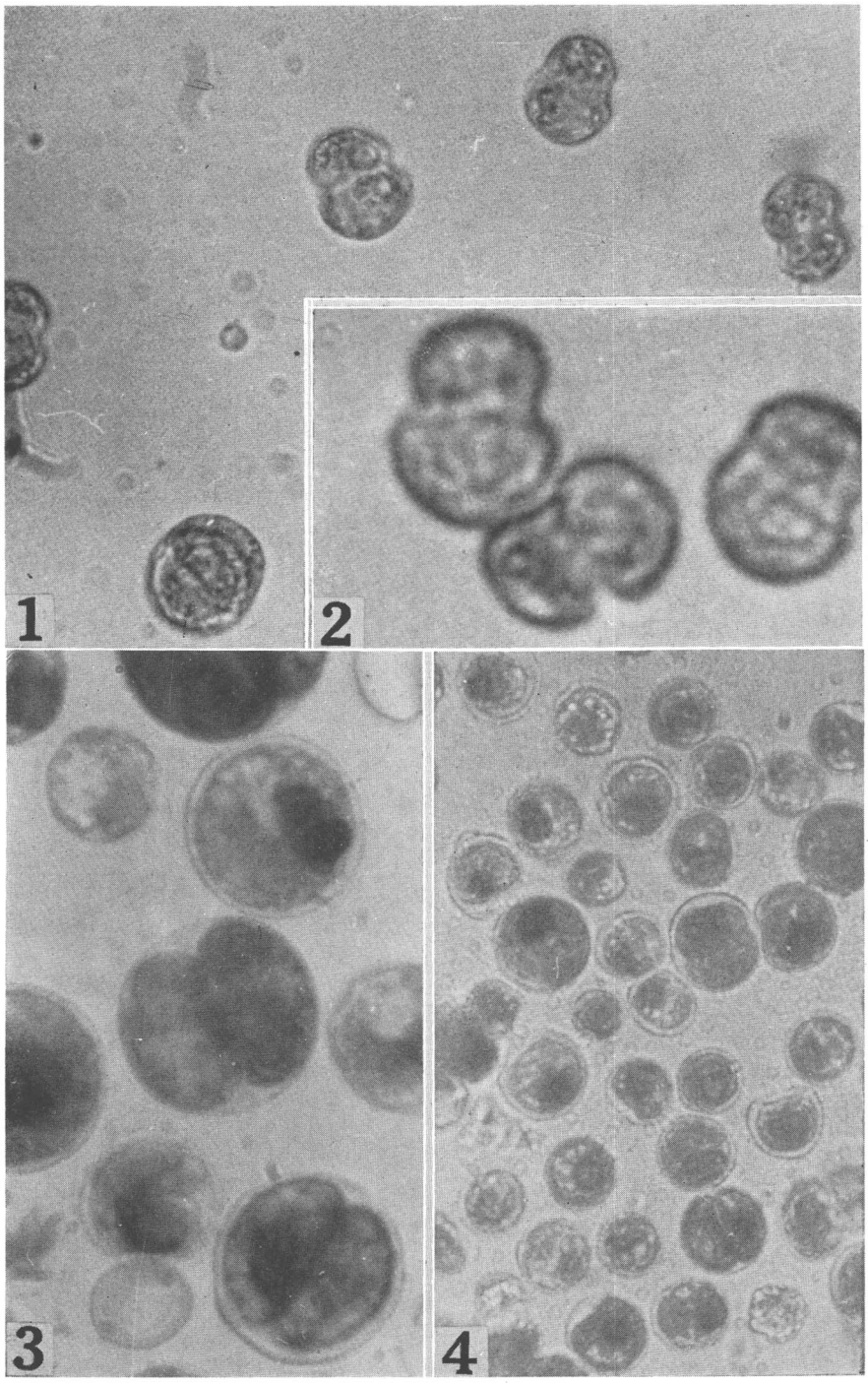

Plate II.

FIG. 1. Living motile cells derived *in vitro* from *Cassiopeia* zooxanthellae. Flagella not visible. Cell in lower part of picture had reverted from motile to spherical non-motile form only a few seconds before photograph was made. Mag. × 3750.

FIG. 2. Enlargement of 3 living motile cells of type shown in Fig. 1 (above). Mag. × 7500.

FIG. 3 & 4. Living vegetative zooxanthellae (from *Cassiopeia*) to show various division stages. Mags. × 2100 and × 1000.

an antibiotic mixture (1 ml of mixture contained: penicillin G, 46,000 units; dihydrostreptomycin, 2,000 μg; chloromycetin, 5,300 μg; neomycin, 12,000 μg) sterilized through a Seitz filter. The most suitable non-toxic concentration of the antibiotic mixture in the wash medium was 5 ml %. Centrifugation from and resuspension in such antibiotic-containing media was repeated 3 times; the cells were allowed to stand in the last wash 1½ hours. A wet-slide preparation of this suspension revealed algal cells in dense concentration. Some showed division stages (Plate II, Fig. 3 and 4); there were no motile organisms. These vegetative cells, 7-14 μ diameter, contained chromatophores, a nucleus, and various vacuoles and droplets. A remarkable feature of these vegetative cells is the toughness of their outer membrane, as indicated by the lack of distortion following centrifugation. The membrane's osmotic character is indicated by a tolerance of high antibiotic and nutrient concentrations, as well as of washings in freshwater. Further, cells on dry agar for as long as 15 days were viable when transferred to liquid media.

The next step was enrichment of seawater and synthetic media. Two different basal marine media, both known to support growth *in vitro* of a number of marine microorganisms (10), were selected as starting points.† In empirical concentrations ranging from 1 to 100 mg %, one, some, or all of the following supplements were added to further enrich the basal media: milk protein hydrolysate, serum extract, corpus luteum extract, yeast autolysate, Trypticase, brain-heart extract, desiccated bone marrow, and sterile Seitz-filtered juice from the macerated tissues of *Cassiopeia* or *Condylactis*. Our selection of organic supplements and variations of inorganic concentrations was based on earlier microbiological work at the Haskins Laboratories. By adding these supplements it was hoped to supply nutritional sources and physiological conditions necessary for proliferation of zooxanthellae *in vitro*. Such enriched media were made up in liquid, solid (1.5% agar,) and semi-solid (0.5% agar) form. For suppression of bacterial growth, all media received at least 1 ml % of the antibiotic mix.

Hundreds of media permutations were tested. There is no need to list all, for while good growth was observed in a number of the media, the combination that seemed to encourage proliferation most profusely and most consistently, is: *Basal Medium B*,† to which was added 1 ml % of amino acid mix,‡ 0.01 mg % yeast autolysate; 1.5% agar; at 24-26°C. Ten ml of agarized medium was slanted in screw-cap test tubes (20 X 125 mm), and 2-4 ml of liquid medium plus antibiotic were added to the slanted surface prior to inoculation with 1-3 drops (5-10,000 cells) of zooxanthellae suspension. The physical environment of the inoculated cells was thus biphasic (solid-liquid). An additional type of inoculum consisted of fragments of zooxanthellae-containing tissue cut directly from the living *Cassiopeia* or *Condylactis*. Such fragments, before being added to the nutrient tubes, were washed repeatedly in sterile seawater, then in sterile media-plus-antibiotics solution, as described above. The tissue fragments were allowed to stand in the last antibiotic wash for 1½ hours, then were washed repeatedly in sterile media, and transferred to the final culture plate or tube. The zooxanthellae of such tissue fragments showed growth characteristics and media preferences similar to those of zooxanthellae introduced as suspensions.

† A. *Enriched Seawater Medium* (ASW-III), containing: aged seawater, 100 ml; KNO_3, 20 mg; K_2HPO_4, 2 mg; Fe (as Cl), .01 mg; soil extract, 4 ml; Mn (as Cl), .04 mg; Na-H glutamate, 50 mg; glycine, 50 mg; vit. mix No. 8, .1 ml; liver OXOID, 1 mg; pH 7.4-7.6. B. *Artificial Seawater Enriched Medium,* containing: NaCl, 2.8%; KCl, .06%; $MgSO_4 \cdot 7H_2O$, 0.6%; Fe (as Cl), 0.13 mg%; Zn (as Cl), 15 μg%; Mn (as Cl), 0.12 mg%; Co (as Cl), 0.3 μg%; Cu (as Cl), 0.12 μg%; Na_2EDTA, 3 mg%; Na_2CO_3, 5 mg%; Ca (as Cl), 15 mg%; NTA, 10 mg%; $NaNO_3$, 1 mg%; K_2HPO_4, 0.1 mg %; vit. mix No. 8, .05 ml%; B_{12}, .01 μg%; thiamine HCl, .05%; Tris buffer, .05%; pH 8-8.3. For formulation details of these basal media, see (10).

‡ 1 ml of amino acid mix contained: Na-H glutamate, 10 mg%; DL-alanine, 10 mg%; DL-aspartic acid, 10 mg%; DL-asparagine, 10 mg%; DL-methionine, 1 mg%; L-histidine HCl, 1 mg%.

Results. After inoculation, some tubes and plates were kept in the dark, some in continuous light (5-7 inches from 40-watt, cool, white, fluorescent source), others in alternating light and dark (8-8, 12-12, and 24-24 hours), at 24-32°C. In some tubes, motile cells began to appear within 3 days, especially if subjected to alternate light and dark at 12-hour intervals. These motile forms (4-6 μ diameter) were unequivocally identified as dinoflagellates, both for the *Cassiopeia* and the *Condylactis* zooxanthellae (Plate II, Fig. 1 and 2). They showed: (*a*) a transverse girdle; (*b*) 2 flagella, one enclosed in the girdle and the other extending backwards; (*c*) a characteristic spiral motility. Identification of these zooxanthellae as dinoflagellates supports earlier conclusions of Hovasse(3) and Pringsheim(4) for corals based tentatively on the morphology of resting cells, and of Kawaguti(5) based on observation of motile cells. Whether zooxanthellae vary according to the host species, the season of the year, or local ecological conditions, is presently unknown.

Our motile cells stayed in this condition for a few minutes to 6 hours. Increased light intensity regularly shortened the motility period. In losing motility, the cells cast off their flagella, became nearly spherical, and assumed a quiescent form resembling the vegetative cells (Plate II, Fig. 1, lower specimen). That these motile cells were actually being derived from the original vegetative form of the zooxanthellae of the inoculum type, and not from any contaminant organisms, was indicated by the nature of the reversion and by the observation of motile forms breaking out of dividing cells within the vegetative population. Further, vegetative cells (from 15-day-old dry agar plates which in daily microscopic observation showed no motile cells) when introduced to liquid medium, produced a profusion of flagellated cells within 1-3 days. Our original cultures of zooxanthellae have now undergone eight serial passages with no loss of integrity or virulence; inocula of 5-10,000 cells show an absolute increment within 15 days to about 30 million. Serial transfers are now routine in our laboratory. Single-cell transfers have not been attempted, pending completion of further nutritional studies to determine optimal growth conditions.

Proliferation of the zooxanthellae *in vitro* appears to be of 3 types: (*a*) vegetative cells giving rise to vegetative cells; (*b*) vegetative cells giving rise to motile cells; and (*c*) motile cells reverting to the vegetative form (Plate II, Fig. 1, 3, 4). The mechanics of such transitions awaits further study.

Motile forms were observed only in liquid or semi-solid media. No motile forms were ever observed in the coelenterate tissue either before or after maceration, or in samples of enteric or tentacle fluid taken by hypodermic puncture.

What factors control transition from the nonmotile to motile state of these organisms are not yet clear; light is certainly a factor, as is, of course, the inorganic and organic makeup of the nutrient medium. Further analysis of growth requirements continues.

Summary. 1. Single-cell algae (the so-called symbiotic zooxanthellae) living interstitially in a marine jellyfish and a sea anemone were isolated from the host tissue and cultured *in vitro*. 2. In culture the zooxanthellae gave rise to motile forms whose morphology clearly marked them as dinoflagellates. 3. Isolation procedures and some nutrient requirements for the *in vitro* culture of such zooxanthellae are described, as well as methods used for serial passage.

1. Buchner, P., *Endosymbiose der Tiere mit pflanzlichen Mikroorganismen,* Basel und Stuttgart (1953). Caullery, M., *Le Parasitisme et La Symbiose,* Paris (1950).

2. Keeble, F., and Gamble, F. W., *Quart. J. Micro. Sci.,* 1907, v51.

3. Hovasse, R., *C. R. Acad. Sci. Paris,* 1937, v205, 1015.

4. Pringsheim, E. G., *Publ. Staz. Zool. Napoli,* 1955, v27, 5.

5. Kawaguti, S., *Palao Trop. Biol. Stat. Studies,* 1944, v2(4), 675.

6. Yonge, C. M., Yonge, M. J., and Nicholls, A. G., *Sci. Rpts. Great Barrier Reef Expd.,* 1931, v1, 135.

7. Smith, H. G., *Carnegie Inst. Wash. Publ. #475,* 1936, v31, 17.

8. Sargent, M. S., and Austin, T. S., *Geol. Surv. Prof. Paper #260-E,* U. S. Gov. Printing Office,

Wash., 1954, 293.

9. Odum, H. T., and Odum, E. P., *Ecol. Mono.*, 1955, v25, 291.

10. Provasoli, L., McLaughlin, J. J. A., and Droop, M. R., *Arch. Mikrobiol.*, 1957, v25, 392.

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Cultivation of Measles Virus in Human Amnion Cells and in Developing Chick Embryo.* (23140)

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Enders and Peebles(1) presented evidence indicating that the virus of measles can be isolated in cultures of human and monkey renal epithelial cells from the blood and throat washings of patients in the early stage of the disease. Capable of continuous multiplication in such cultures, the agent produces a characteristic cytopathogenic effect which is specifically prevented by sera of man or monkey taken during the convalescent phase of measles. Moreover, an antigen appears in the fluid of infected tissue cultures that fixes complement specifically in the presence of convalescent phase measles sera(1,2,3). These findings have made it possible to investigate more conveniently and precisely the behavior of the virus in other systems. In this communication we shall describe the propagation of the virus and the cytopathic changes it induces in cultures of human amnion cells. In addition we shall record details of the procedures by which evidence of its multiplication in chick embryos was obtained. A summary account of these experiments has recently been published(2).

Methods and materials. Viruses. The "Edmonston" strain(1) of measles virus was most extensively employed. This agent was isolated in February, 1954, from the blood of a typical case of measles on the first day of the rash. From that time until January, 1955, it was subjected to a total of 24 passages in roller tube or trypsinized monolayer cultures of human postnatal renal cells maintained in bovine amniotic fluid medium. To a less extent certain other strains isolated in this laboratory were studied in respect to their capacity to multiply in human amnion cells and in chick embryos.

Tissue cultures. Suspensions of amnion cells were prepared according to the method of Zitcer and her coworkers(4). Approximately 300,000-400,000 cells in a volume of 1 ml of medium were allowed to settle on the wall of a tube 15 x 150 mm held at an angle of about 5° from the horizontal. During the period of cell outgrowth the medium consisted of the following constituents:

Normal horse serum (inactivated)	20 %
Bovine embryonic extract	5 %
Bovine amniotic fluid	37.5%
Hanks' balanced salt solution	37.5%
Streptomycin	100 mg/ml
Penicillin	100 units/ml
Mycostatin	100 "

In the most recent experiments the following medium, which gives equally satisfactory results, has been used to promote cell outgrowth:

	%
Normal horse serum (inactivated)	20
Hanks' balanced salt solution	70
L-Glutamine (2.9 mg/ml H_2O)	10

Antibiotics in quantities indicated in formula above.

When the monolayer was established and at the time the virus was introduced the medium used to promote cell outgrowth was replaced

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