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CYTOLOGICAL INVESTIGATION OF DIGESTION AND RE-ESTABLISHMENT OF SYMBIOSIS IN THE LARGER BENTHIC FORAMINIFER AMPHISTEGINA LESSONII

City University of New York

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CYTOLOGICAL INVESTIGATION OF DIGESTION AND RE-ESTABLISHMENT OF SYMBIOSIS IN THE LARGER BENTHIC FORAMINIFER *AMPHISTEGINA LESSONII*

by

ROBERT J. KOESTLER

A dissertation submitted to the Graduate Faculty in Biology in partial fulfillment of the requirements for the degree of Doctor of Philosophy, The City University of New York.

1984

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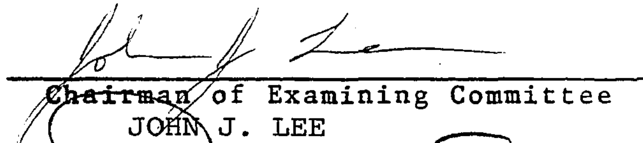

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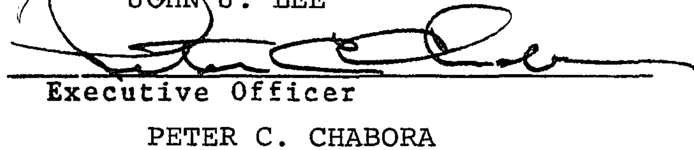

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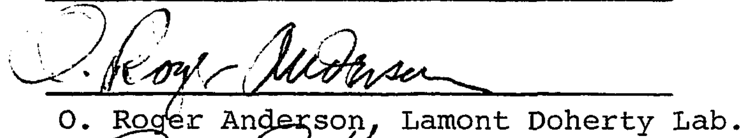

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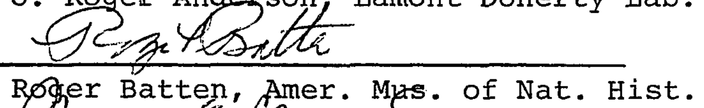

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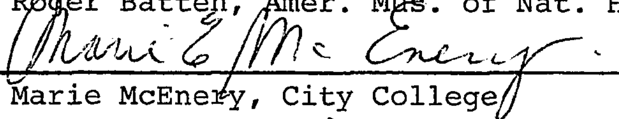

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ABSTRACT

CYTOLOGICAL INVESTIGATION OF DIGESTION AND RE-ESTABLISHMENT OF SYMBIOSIS IN THE LARGER BENTHIC FORAMINIFER *AMPHISTEGINA LESSONII*

by

ROBERT J. KOESTLER

Adviser: Professor John J. Lee

The fine structure of specimens of the larger foraminifera *Amphistegina lessonii* was studied with the aim of clarifying aspects of experimentally induced resynthesis of symbiosis, and comparing that to normal food uptake.

Normal healthy foraminifera had one to three layers of symbionts within the dorsal and dorsal-lateral regions of cytoplasm, inside the shell. The cytoplasm behind the endosymbionts was dense and filled with vacuoles of all sizes, food particles, and some apparently normal endosymbionts. Undigested algae ultrastructurally similar to the endosymbionts were found in all chambers.

Food organisms *Chlorella* sp (AT) and *Amphora* sp (BL 45), and previously isolated and cultured algal endosymbionts *Nitzschia laevis* and *Fragilaria shiloi* were all contacted and transported by *A. lessonii*

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into the aperture in a similar manner. Fine pseudopodia attached and pulled the captured particles close to the shell and eventually through the aperture into the animal. Digestion of Chlorella sp (AT) and Amphora sp (BL 45) began outside soon after contact. Not all individuals of the endosymbiont species tested were digested. N. laevis were much more resistant to digestion than were F. shiloi.

All of the endosymbionts harbored in A. lessonii had pyrenoids with simple internal lamellae identical to those from cultures of N. laevis, N. panduriformis, and F. shiloi.

Incubation with the herbicide DCMU for 120 h bleached most of A. lessonii's endosymbionts and reduced their total numbers in the cortical cytoplasm. The endosymbionts remaining after DCMU treatment characteristically had closely packed chloroplast lamellae and lacked stored starch.

After two weeks of starvation the numbers of endosymbionts in A. lessonii were reduced with some undergoing apparent autolysis or digestion. The remaining endosymbionts were more like normal endosymbionts than those incubated with DCMU. This suggests that even functionally active endosymbionts were digested by the host under nutritional stress.

A. lessonii rapidly recovered from DCMU treatment and within one week were close to normal in color. The endosymbionts divided to replenish their numbers. The rapidly re-browning endosymbionts had a characteristic "normal" appearance with internal vacuoles, widely separated chloroplast lamellar structure, and starch storage around the pyrenoid body. Cultured potential endosymbionts fed to A. lessonii during the experiment could have avoided digestion and become available

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to re-colonize the host during the re-browning process. Because of the similarity in fine structure of the various algal species involved, it was not possible to visualize in the experiment how one species of endosymbiont could have replaced another.

To explain why some diatoms persist while others are digested, evidence is presented for host selectivity and/or algal survival mechanisms of varying effectiveness. Whether or not the frustule-less diatoms which are found in the animals reproduce within the foraminifera in nature or if they are recruited continually from the environment is not known, but in culture it is clear that depleted populations repopulate their host without addition of new symbionts. Isolations from the field collections and the present experimental results suggest that there is an order of preference among potential endosymbionts. *Fragilaria shiloi* and *Nitzschia panduriformis* were low in the order of preference of these brief experiments, and yet they have been frequently isolated from the three collecting sites (Red Sea, Hawaii, and Great Barrier Reef). It is therefore obvious by analogy to the *Convoluta roscoffensis* system that time or other factors may be involved in selective algal persistence.

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INTRODUCTION

Symbiosis is a widespread phenomenon in invertebrates in general, e.g., radiolarians (Anderson, 1980; 1983), corals (Muscatine and Porter, 1977), sponges (Muscatine et al., 1967), flatworms (Provasoli et al., 1968), ciliates (Karakashian, 1975), hydras (Pardy, 1976; Jolly and Smith, 1980), and foraminifera (Lee, 1980, 1983; Lee and McEnery, 1983).

In foraminifera, symbiotic associations were described over 70 years ago (Butschli, 1886; Rhumbler, 1909; Winter, 1907), but it has only been within the past 15-20 years that much attention has been devoted to studying the relationships of foraminifera and their symbionts. The first of the modern reports was the description of an apparently symbiotic association between the planktonic foraminifer *Globigerinoides ruber* and a dinoflagellate similar to *Symbiodinium microadriaticum* (Lee et al., 1965).

Since 1965 numerous studies on foraminifera have been made (e.g., Anderson and Bé, 1976a, 1976b, 1977, 1978; Anderson and Faber, 1984; Anderson et al., 1977; Bé et al., 1982; Lee, 1974, 1980; Lee and Bock, 1976; Lee and Zucker, 1969; Lee et al., 1979a; Muller-Merz and Lee, 1976). Foraminifera have been shown to harbor a wide diversity of algal types. At present, representatives of four divisions of algae have been shown to be symbionts within foraminifera. They are: Pyrrhophycophyta, e.g., *Symbiodinium microadriaticum* in *Sorites marginalis* (Muller-Merz and Lee, 1976); Chlorophycophyta, e.g., *Chlamydomonas hedleyi* in *Archaias angulatus* (Lee et al., 1974); Bacillariophyta, e.g., *Nitzschia panduriformis* in *Amphistigina lessonii* (Lee et al., 1979), and

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Rhodophycophyta, e.g., Porphyridium sp in Peneropolis planatus (Leutenegger, 1977b).

It appears at present that many species of larger benthic foraminifera are the host of only a single unicellular algal species, e.g., Archaias angulatus, Cyclorbiculina compressa, Sorites marginalis, Amphisorus hemprichii (Lee et al., 1974, 1979a, 1980a; Muller-Merz and Lee, 1976, respectively). On the other hand, the diatom-bearing larger foraminifera seem to be capable of harboring more than one species of symbiont, although usually it is only one species at a time (reviewed in Lee, 1980, 1983; Lee and McEnery, 1983; Lee and Reimer, 1984). In Heterostegina depressa three different diatom species of algae have been isolated from specimens captured in two widely separated stations--off Elat, in the Red Sea, and off Hawaii, in the Pacific. The ability of larger foraminifera in general to harbor many different groups of algae, or of a single host species to harbor more than one algal species, seems to indicate a general adaptation of these animals for symbiosis (Lee et al., 1979b).

Foraminiferal/algal symbiotic associations have been postulated as major factors during the evolution of the larger foraminifera (Ross, 1972; Lee et al., 1974, 1979a), and are today significant sources for primary production in tropical and semi-tropical lagoonal sands (Sournia, 1976, 1977). Their rates of photosynthesis and their population densities suggest that foraminifera may well exceed the hermatypic corals in their contributions to reef biogenesis (Muller, 1974, 1976). The importance of symbiotic associations in the gross carbon budget of the animals seems to vary depending on the species being considered. H. depressa seems to receive 100% of its carbon budget

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from the primary production of its symbionts (Rottger, 1974), while A. lessonii receives only 10% (Lee et al., 1980a,b,c; Muller, 1974). Experiments on calcification and growth suggest that they are light-mediated processes, and symbiont-dependent (Lee and Zucker, 1969; Muller, 1978; Duguay and Taylor, 1978; Erez and Honjo, 1981; Hallock, 1981; Duguay, 1983; Anderson and Faber, 1984). Growth and calcification in both A. lessonii and H. depressa increase with light intensity from 180 to 800 lux (Rottger et al., 1980). Photoinhibition was observed at high light intensities. Duguay and Taylor (1978) also found photosynthesis and calcification in Archaias angulatus to be directly proportional to light intensity in the range 0-200 uEin. $in^{-2}sec^{-1}$, two to three times that in the dark. The herbicide 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) in concentrations of 1-100 uM completely inhibited photosynthesis (at photosystem II in the endosymbiotic chloroplasts [Vandermeulen et al., 1972]), and light-enhanced calcification. Methyl viologen inhibits the ferredoxin to NADP step in photosynthesis (Vandermeulen et al., 1972).

The importance of the symbiotic relationship for the algae has been proposed after nutritional studies of isolated algal symbionts. In culture the algal symbionts were stimulated by or required thiamine, biotin, vitamin B_{12}, nitrogen, and phosphates. In the host it is believed the symbionts obtain these nutrients from bacteria ingested by their host (Lee et al., 1981).

Fine structural studies of symbiont bearing foraminifera show that these animals are generally exceedingly complex and regionalized single-celled animals (e.g. Anderson and Bé, 1976; Berthold, 1978; Lee et al., 1965; Leutenegger, 1977a,b,c,d; McEnery and Lee, 1981; Muller-Merz and

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Lee, 1976; and Schmaljohann and Rottger, 1976, 1978; reviewed in Lee, 1983). Phagosomes and symbiotic vesicles were generally segregated to specific regions, e.g., in A. lessonii symbiotic vesicles were located dorsally and laterally in the pore rims on the inner surface of the test while phagosomes were located ventrally (McEnery and Lee, 1981). The mechanisms by which endosymbiontic algae are sequestered are unknown as are any recognition events that must occur for A. lessonii to differentiate organisms that are to be phagocytized from those that are retained as symbionts.

Cytochemical techniques are becoming increasingly important for elucidating the functions of intracellular components revealed by electron microscopy. Staining tests for digestive enzymes, particularly acid phosphatase (Gomori, 1952), have been applied to planktonic foraminifera (Anderson and Bé, 1976a), demonstrating the role of golgi bodies and lysosomes in digestion of prey in Hastigerina pelagica. Other protozoa have had the Gomori technique applied to them (e.g., ciliate Campanella umbellaria, Goldfischer et al., 1963; and radiolaria, Anderson, 1976a,b,c; 1983), but to my knowledge no benthic foraminifera have been studied using this technique.

The leucocyte acid phosphatase technique for cytochemical marking of golgi and lysosomes was developed for mammalian liver tissue (Smith and Fishman, 1969), and has not heretofore been applied to protozoan tissue.

Peroxisomes are other small, single membrane organelles that are found in planktonic and benthonic foraminifera, and whose function and structure can only be demonstrably linked by cytochemical techniques as

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shown by Anderson and Tuntivate-Choy (1984), in Hastigerina pelagica (d'Orbigny).

Ruthenium red (a vital stain used in light microscopy) and ferritin have been used as marker molecules in pinocytotic studies of amoeba. (e.g., Brandt and Pappas, 1962), but heretofore not in benthonic foraminifera.

The aims of the research reported here were: (1) to see if it was possible to obtain aposymbiotic host foraminifera A. lessonii; (2) to see if it was possible to replace the endosymbiont species in an adult host with another species of endosymbiont; (3) to determine if a preferental order of particular diatom species exists during resynthesis; and (4) to study food and symbiont ingestion, transport, and digestion or (re-)establishment of symbiosis. Parts 1, 2, and 3 have been reported on elsewhere (Lee et al., 1983), and will be treated only briefly here so that the fine structural study can be interpreted in the context of the experimental.

MATERIALS AND METHODS

Because this thesis was part of a larger collaborative project that has just been published (Lee et al., 1983), only the methodology peculiar to this subproject will be emphasized here.

The organization and steps involved in the resynthesis experiments are summarized in the flow chart (text fig. 1). The preliminary

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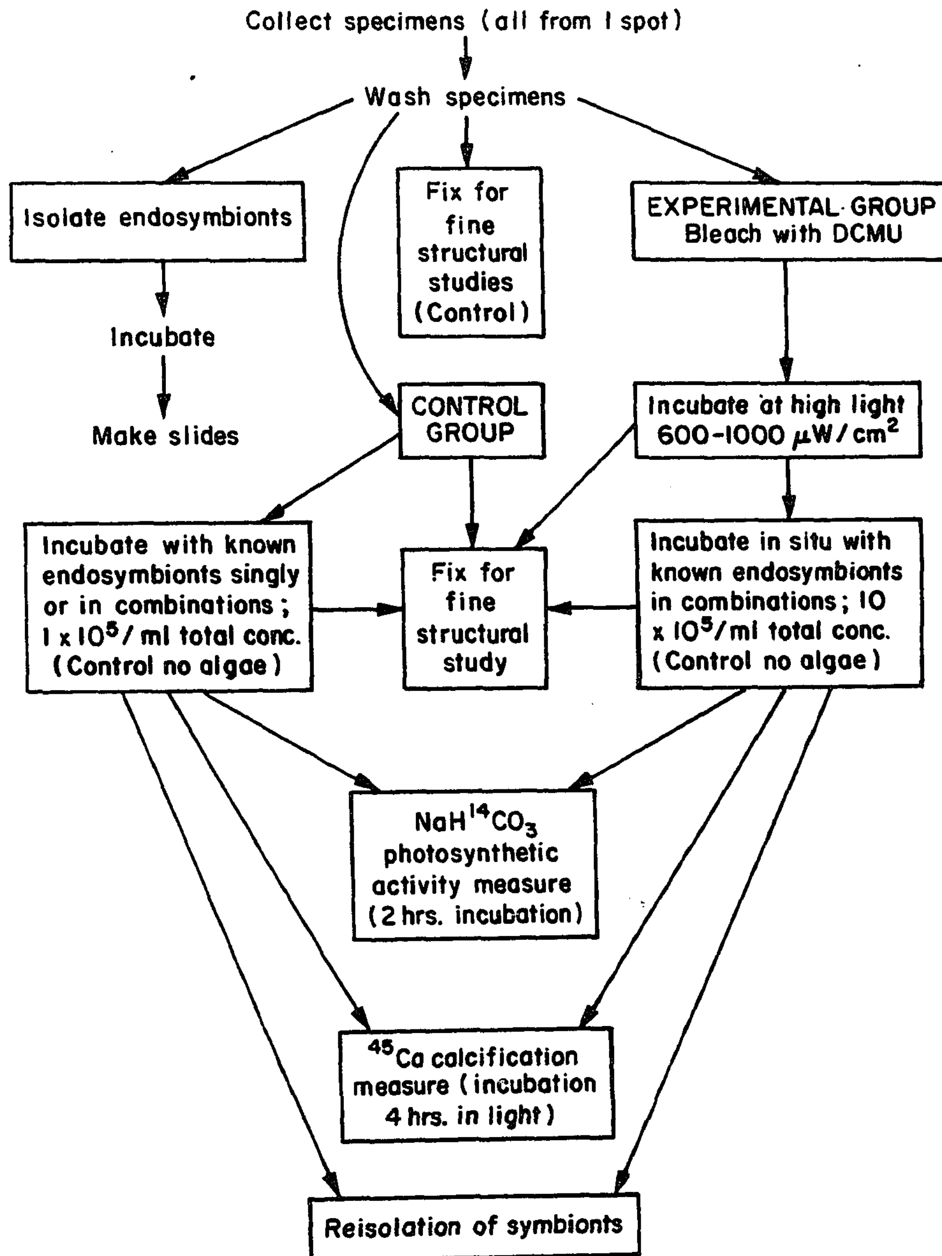
TEXT FIGURE 1



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experimental work, i.e., fixation regime, and the final set of feeding experiments were done at the Marine Microbial Laboratory of City College of New York (CCNY). The main sets of experiments were performed at the Heintz Steinitz Marine Biological Laboratory (HSMBL) of Hebrew University of Jeruselum in Elat by the Red Sea, on the Gulf of Elat, Israel, December 1981 to January 1982. The subsequent animals used at CCNY were hand-delivered from Elat and were generally collected immediately before delivery from Wadi Taba (generally 2-3 days from the sea to our lab).

Freshly collected organisms were brushed vigorously with 0000 sable paint brushes to remove all adhering organisms. Twenty organisms were removed from the rest and more carefully brushed, and their endosymbionts were isolated to give a reasonable sample of the initial population of endosymbiotic diatoms. The procedure for isolation and cultivation of the endosymbionts has been outlined previously (Lee et al., 1980b). The bulk of the experimental organisms were divided into two sets of closed flasks without food. The organisms in one set were incubated with DCMU ($1x10^{-5}M$); the other set served as the control. They were incubated at 2 m depth (high light for these animals). The flasks were removed from the sea each day to examine the condition of the animals and to change their media.

At the end of five days the starved animals were harvested from their respective flasks into petri dishes with fresh sea water. Animals that formed pseudopodial networks during the selection process were used in the experiments. The animals (100-125 for each flask) were divided into 28 groups (14 bleached and 14 controls) and placed into tissue culture flasks with membrane windows. The flasks were then inoculated

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with 3 x 10^5 cells of the mixture of algae indicated in tables 1 and 2. The algae were chosen from among the stock of previously isolated endosymbiotic diatoms (a-f), and easily identified (in TEM) endosymbiotic chlorophyte from Cycloribiculina compressa, Chlamydomonas provasolii (g), and 3 small (about 10-uM) free-living pennate diatoms from Sippewissett Marsh, Falmouth, Massachusetts (h,i,k). Because of the labor-intensive nature of the experiment and the limited duration of our stay at the Marine Station in Elat, not every combination of algae nor every species of endosymbiotic diatom previously isolated from larger foraminifera, could be tested. Algae used in the experiment were harvested from axenic cultures by centrifugation, enumerated by haemocytometer, and resuspended at the proper concentration in fresh sterile sea water. Depending upon the experiment, the flasks were incubated at either 10 or 20 m. Since we had not had experience with membranes at depths greater than 3 m, the flasks were brought down to incubation depths in 5-m stages to allow for pressure equalization on both sides of the membranes. Approximately 100-125 animals were involved in flasks of each variable.

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Table 1

Algae used in the resynthesis of symbiosis experiments

	ALGA	STRAIN
a	*Nitzschia laevis*	(HAL)
b	*Nitzschia frustulum*	(RSAL)
c	*Fragilaria shiloi*	(RS 13)
d	*Nitzschia valdestriata*	(HH)
e	*Amphora rottgerii*	(GBRH)
f	*Nitzschia gandersheimensis*	(RS 18)
g	*Chlamydomonas provasolii*	(CC)
h	*Amphora* sp.	(BL 45)
i	*Nitzschia* sp.	(WH 8022)
k	*Nitzschia ovalis*	(WH 80136)

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Table 2

Mixtures of algae* used in the resynthesis of symbiosis experiments

1	c,k,a	8	d,k,b
2	d,i,a	9	a,h,b
3	c,d,f	10	c,h,g
4	c,e,i	11	d,g,i
5	d,e,g	12	f,h,i
6	e,f,i	13	"re-brown" control "no add"
7	c,k,a	14	formalin killed control

* Species given in Table 1.

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Aliquots, each of 20-25 animals, were taken to measure comparative rates of photosynthetic activity and calcification and for the isolation of endosymbiotic algae. In addition, many organisms were fixed and embedded for later study with the TEM. The ^{45}Ca used in the experiment was diluted to an approximate specific activity of 5.2×10^{-4} Ci/mg calcium (based on average calcium concentration in the Gulf of Elat). Radioactivity was measured in a Packard Tri-Carb liquid scintillation counter.

Although it was not possible to follow the entire protocol, we included small numbers (about 20-25) of *Amphistigina lobifera* in many of our experimental flasks. As time permitted at the end of an experiment, we isolated the endosymbionts from these animals as well. We also incubated a few flasks of DCMU bleached animals with *Chlamydomonas hedleyi*, the endosymbiotic chlorophyte from *Archaias angulatus*.

Unfortunately, the necks of two flasks were smashed against coral heads in rough weather while we were towing the experiments back to the lab. The contents of these flasks were largely lost at sea.

Different concentrations of the prefixative, glutaraldehyde (3-6% in 40°/oo sea water), and different fixation durations were tested to develop optimal regimes for these animals. Fixation was begun at room temperature, and then the specimens were placed in an ice bath for the rest of the time they were treated. In the preliminary tests, unless another aspect was varied, all specimens were post-fixed in 2% OsO_4 in 40°/oo sterile sea water, decalcified with 10-15% acetic acid for 1 h, en bloc stained with 2% uranyl acetate in 10% ETOH for 10 min, and dehydrated through acetone and embedded in Epon 812 or its successor

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LX-112 (Ladd Research Ind., Inc., Burlington, VT). Sections were cut on an LKB ultramicrotome using glass or diamond knives, stained for 5 min with Reynold's lead citrate and examined in a Philips 300 TEM. This specimen preparation regimen was modeled after that of Anderson and Bé (1978).

The best TEM preparations were those fixed in 5% glutaraldehyde in sterilized sea water ($40^{o}/oo$) for 20 min, begun at room temperature and then cooled to 0°C in an ice bath, post-fixed in 2% OsO_4 for 2 h in an ice bath, decalcified in 10-15% acetic acid for 1-1½ h, stained for 10 min in 2% uranyl acetate, acetone dehydrated, and embedded in LX-112. This regimen was used in subsequent preparations. Specimens used in the ruthenium red, the Gomori test and control experiment, or the leucocyte acid phosphatase stain and control experiment were not stained with uranyl acetate or Reynolds lead citrate. The main set of feeding, bleaching, and resynthesis experiments was conducted in Israel at the HSMBL with freshly collected animals.

In the Red Sea, *A. lessonii* adhere to benthic plants, (e.g., *Halophila* sp), rocks, and sediment. Their range extends from 10 m to at least 40 m deep. The bulk of the samples used in this study were collected at Wadi Taba, Elat, Israel, in December 1981, from 23 m depth. Organisms directly from the sea were selected based on the presence of extensive pseudopodial activity. Only *A. lessonii* specimens that were darkly pigmented and showed extensive pseudopodial networks were selected for experimentation. Experimental vessels were tissue culture flasks with windows cut in them and covered with 1 um pore nylon filter membranes (Coccetti & Lee, 1979).

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During the bleaching process unmodified flasks were used. "Dark" flasks were wrapped in black plastic to achieve total darkness. All flasks were held in lucite racks designed to protect the flasks and filters. The racks were held at desired depths in the water column by suspending them between pairs of ropes with anchors on the bottom and floats on the surface.

A preliminary experiment designed to test the effectiveness of methyl viologen and DCMU (both at 10^{-5} M) to produce aposymbiosis was incubated in closed tissue culture flasks at 5 m. A 10^{-3} M solution of DCMU (3-(3,4-dichlorophenyl)-1,1 dimethyl urea) was freshly prepared in a 1-L flask fitted to a reflux condenser. The solution was boiled for 1/2 h and then simmered for 24 h while being constantly stirred with a magnetic bar. The solution was then diluted to the working concentration. Approximately 500 animals were involved in the experiment. The flasks were retrieved each day in the late afternoon and the organisms were examined with a dissecting microscope to observe pigmentation and pseudopodial activity. Each day the sea water was replaced with fresh sterile sea water containing the bleaching agent. Controls were incubated with sea water alone.

After 120 h incubation in DCMU or methyl viologen, the pigments in the treated animals were sufficiently bleached so as to suggest that the animals were partially or largely aposymbiotic. Pseudopodial activity seemed normal. Selected animals from each group were carefully brushed of all adhering algae and placed in sterile sea water containing 0.1 uCi $NaH^{14}CO_3$/ml; incubation was for 2 h. Half of the control group were killed with formalin to provide dead controls. DCMU was found to be more effective than methyl viologen in creating nearly aposymbiotic

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foraminifera and thus was used in subsequent experiments. Some of the organisms in each group were also fixed for fine structural studies in the transmission electron microscope (TEM). The fixation procedure was as detailed above.

At the end of five days (120 h), the starved animals were harvested from their respective flasks into petri dishes with fresh sea water. Aposymbiotic *A. lessonii*, after DCMU treatment, were divided into groups as follows:

A. Aposymbiotic forams fixed for TEM study.

B. Aposymbiotic forams allowed to re-brown seven days.

C. Aposymbiotic forams fed *F. shiloi* fixed after 0,1,2,4 h.

D. Aposymbiotic forams fed *Amphora* sp fixed after 0,1,2,4 h.

E. Gomori experiment and lead nitrate control for part C, at 0,1,2,4 h.

F. Gomori experiment and lead nitrate control for part D, at 0,1,2,4 h.

G. Specimens from experimental groups fixed for TEM after incubation with mixtures of algae (see Lee et al., 1983).

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Organisms directly from the sea were starved for three days, then treated as follows:

A. Fixed directly from the sea.

B. Fed *F. shiloi* for 0,1,2,4 h fixed for TEM.

C. Fed *Amphora* sp for 0,1,2,4 h fixed for TEM.

D. Fed ruthenium red and ferritin plus *F. shiloi* 0,1,2,4 h.

E. Fed ruthenium red and ferritin plus *Amphora* sp 0,1,2,4 h.

F. Gomori experiment and lead nitrate control for part B, at 0,1,2,4 h.

G. Gomori experiment and lead nitrate for part C,at 0,1,2,4 h.

Ruthenium red (estimated final concentration of 10^{-5} M), was used in an attempt to cytologically mark the invaginating membranes of food/symbiont vacuoles. Uptake of ruthenium red food and ruthenium red symbiont vacuoles could then be followed ultrastructurally for any differences in their membranes by examining for alterations in the stain. Ferritin (estimated final concentration of 10^{-5} M), was used as a marker molecule to test for leakiness of the food/symbiont vacuolar membranes to small molecules.

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A further set of feeding experiments were conducted at CCNY on two-week starved A. lessonii. They were as follows:

A. Fed N. laevis for 30 min.

1. Fixed for pseudopodial contact.

2. Leucocyte acid phosphatase staining experiment and tartrate control for TEM and LM.

B. Fed Chlorella sp for 30 min.

1. Fixed for pseudopodial contact.

2. Leucocyte acid phosphatase staining experiment and tartrate control for TEM and LM.

Amphora sp and Chlorella sp were chosen as food organisms because of their availability and their presumptive digestablity by the host foraminifera. F. shiloi and N. laevis were chosen as the resynthesis symbionts since they had been isolated and cultured from earlier collections of A. lessonii from the Red Sea.

As an aid in reconstructing and visualizing in three dimensions the location of various sections, samples of A. lessonii tests were vacuum impregnated with latex plastic, oven dried, decalcified in 15% acetic acid, coated with 15 nm of gold in a Polaron sputter coater, and viewed in an Amray 1600T scanning electron microscope (SEM). In addition, some

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samples of A. lessonii tests only were cleaned, mounted on aluminum stubs, coated with 15 nm gold, and viewed in the SEM.

RESULTS

Summary of the resynthesis experiment

After 5 days incubation in strong light, 1×10^{-5}M DCMU seemed more effective in bleaching and reducing photosynthetic activity of the endosymbionts than did 1×10^{-5}M methyl viologen (Table 3). Since the animals had normal pseudopodial activity, we judged the DCMU to be both effective and safe.

The results of the resynthesis experiments were quite interesting and replicable under the same environmental conditions and season of the year. The endosymbiotic diatoms in the animals in our first collection from Wadi Taba were principally Nitzschia panduriformis with some foraminifers in the sample having a Nitzschia frustulum var symbiotica, Amphora tenerrima or mixtures of the first two. Two and three weeks later the endosymbionts in the animals from the same general site were also mostly Nitzschia panduriformis; some had Fragilaria shiloi, Amphora tenerrima, Nitzschia frustulum var symbiotica, Nitzschia frustulum, Navacula sp, and Protokeelia hottingerii. The new species and variety have been studied and are described in separate papers (Lee and Reimer, 1984; Reimer and Lee, in press). We suspect that the greater variety of secondary symbionts in the second collection may have been due to the fact that we collected a larger number of animals and may have harvested

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them from a larger area without appreciating the fact at the time. Considering the small numbers of animals we used for isolation (25 each), there were no appreciable qualitative or quantitative differences in the endosymbionts recovered from *A. lessonii* and *A. lobifera* from the same collection.

Table 3

Relative photosynthetic activity of *Amphistegina lessonii* after exposure to DCMU and methyl viologen

Treatment	CPM/organsim
Control (untreated)	522
Control (formalin killed)	(18.5)
DCMU (1×10^{-5}M)	35
Methyl viologen	287

Average of 50 organisms in two experiments. Background and formalin-killed control value subtracted.

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DCMU (1×10^{-5}) inhibits the endosymbiotic diatoms and apparently makes them more vulnerable to replacement (compare Tables 4 and 5). Not all of the symbiotic diatoms lose viability during treatment, since after a week incubation in fresh sterile sea water many treated animals regained their brown color and their relative rates of photosynthetic activity; and calcification, on the average, reached half of the average rate of untreated controls. There was a great deal of variation in rates of photosynthetic activity and calcification among the various experimental groups. In general they ranged from 1/3 to 2/3 of live control groups. Under the microscope there was a wide range in the coloration of individual organisms which suggested that the rate of "rebrowning" was a variable and individual process which was incomplete in most animals at the time of harvest. About 20% of the individuals in the experimental flasks did not form pseudopodia during the picking and sorting process. Since they had had pseudopodia the previous week it is possible that these animals were moribund or had died during incubation.

The observations of the fine structure studies support the physiological measurements; the numbers of endosymbionts were considerably reduced even though many endosymbionts remained apparently intact in the dorsal cortical region of the animal after treatment with DCMU. Chloroplast thylakoids of many algae were more compact and some were disrupted. The pyrenoids of most but not all algae were collapsed or not observed. Some cells seemed shrunken and perhaps were undergoing autolysis. After one week in the light many of the algae in starved but illuminated animals (re-browned controls) were unchanged or moribund.

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Many other algae, however, were more normal in appearance with apparently functional chloroplasts.

Nitzschia panduriformis was the principal diatom isolated and cultured from both the treated (DCMU-bleached) and untreated but starved controls (Tables 4 and 5). We recovered a mixture, or a different species of diatom, in 21.6% of the cases of the controls from all experiments. This was not appreciably different from the variations (23.2%) found in the isolation made directly from the field collections. Apparent replacement of endosymbionts was noted in both DCMU-treated and untreated resynthesis experiments. Although we originally had planned to independently sample 10 colonies of diatoms from each animal, that goal turned out to be logistically impractical except when there were apparent mixtures of three colonial types. For most animals we picked four colonies from each plate and inoculated them individually into a single tube of media. As a check on the method we also took two brushed animals from each experimental flask and, after crushing them, incubated the entire homogenate from each animal separately into tubes of media. In general there was fairly good agreement in the results obtained by both methods. In a few cases there was greater diversity in the entire animal homogenate than there was in individual colony sampling. This was easy to understand because of the small sample sizes involved.

Chlamydomonas provasoli, *C. hedleyi*, and the three free-living species of small diatoms from the Greater Sippewissett Salt Marsh were not recovered from either the experimental or control animal populations (Tables 4 and 5). On the other hand, it was quite apparent that many of the diatom colonies previously isolated from the same hosts and placed

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Table 4

Symbionts recovered from resynthesis of symbiosis experiments.

Untreated controls. 10 m incubation.

Choice between***	Symbiont(s) recovered*
shiloi/laevis**	laevis (9) amph (1) Mix pand/laevis shiloi (1) pand (2) (12)
valde/laevis	laevis (4) Mix valde/laevis (2) valde (3) Mix valde/laevis/pand (13)
shiloi/valde/laevis	laevis (3) Mix valde/laevis (4) valde (5) Mix valde/laevis/pand (13)
shiloi/amph	amph (6) Mix pand/amph (19)
valde/amph	valde (3) pand(2) pand/laevis (16) pand (5) Mix valde/laevis/pand (16)
laevis/amph	laevis (3) pand (2) pand/laevis (16) pand/laevis/amph (5)
laevis/frust	pand (5) laevis (4) frust (1) Mix pand/frust (14)
valde	valde (8) pand (3) symb (1) Mix pand/laevis (1)

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Table 4 (cont.)

laevis pand/laevis(16)	pand (4)laevis (5) Mix
frust/valde	lost in sea
shiloi	pand (all)
starved control	pand (21) shiloi (2) symb (1) Mix pand/shiloi (1)

*All other diatoms and the chlorophyte tested were presumably digested since they were not recovered from hosts.

**Abbreviations:

shiloi--*Fragilaria shiloi*

valde---*Nitzschia valdestriata*

amph----*Amphora rottgeri* (GBR)

frust---*Nitzschia frustulum*

laevis--*Nitzschia laevis*

symb----*Nitzschia frustulum* var *symbiotica*

amph 2--*Amphora tennerema*

pand----*Nitzschia panduriformis* var *continua*

***25 organisms.

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Table 5

Symbionts recovered from resynthesis of
symbiosis experiments. 10 m incubation.

CHOICE BETWEEN**	SYMBIONT(S) RECOVERED*
shiloi/laevis	laevis (23)
valde/laevis	Mixture valde/laevis
shiloi/valde/laevis	Mixture valde/laevis (20) above + shiloi/pand/symb (5)
valde/amph	valde (22) amph (3)
laevis/amph	laevis (all)
laevis/frust	laevis (21) laevis/frust (4)
valde	valde (20) valde/pand (3) valde/symb (2)
laevis	laevis (all)

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Table 5 (cont.)

frust/valde	valde (18) valde/symb (18)
shiloi	pand (9) Mixture pand/shiloi (16)
no add control	pand (18) symb (4) amph (2) Mix pand/symb (2)

*All other diatoms and the chlorophyte tested were presumably digested since they were not recovered from hosts.

**Abbreviations: see Table 4.

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in the experimental flasks were eaten and retained alive within the animals (Tables 4 and 5). In retrospect it is regrettable that we chose not to quantitatively sample each experimental animal; nevertheless, the results obtained seem quite instructive. In most cases we isolated mixtures of algae from more than one-half of the untreated animals (Table 4). Of the endosymbiotic algae tested only, Fragilaria shiloi seemed not to be retained. The original endosymbionts in some (0-36%) of the animals in each population of untreated controls seemed to have been replaced with algae from the experimental mixtures (Table 4). The exception seemed to be F. shiloi. This alga was recovered as the sole endosymbiont from only one experimental animal.

The results of the isolations of endosymbionts from treated animals made it quite clear that some algal species were selected over, or were more competitive than others during the re-browning of Amphistegina lessonii (Table 5). By comparing the results of different pairs of incubation mixtures (Table 6), we found that Nitzschia valdestriata and N. laevis were the most successful endosymbionts and Fragilaria shiloi the least. Amphora rottgeri (GBR), Nitzschia frustulum, and N. panduriformis were intermediate. At 20 m the results showed that N. valdestriata was preferred over N. laevis but otherwise the relationships were identical to the situation at 10 m.

The results of isolations from the smaller numbers of A. lobifera incubated in the experimental flasks were identical with those of A. lessonii under test conditions. The same hierarchy of endosymbiont preference was obtained with N. laevis/valdestriata being the most preferred and F. shiloi the least.

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Table 6

Summary of resynthesis experiments, 10 m incubation

Dominant alga				Other alga
laevis		>		shiloi
valde		≅		laevis
amph		>		shiloi
valde		>		amph
laevis		>		amph
laevis		>		frust
pand		≅		shiloi
valde	>	amph	>	pand
laevis	>	frust	>	shiloi

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The experiments designed to achieve aposymbiotic hosts of *A. lessonii* and the results have just appeared in print (Lee et al., 1983). This same report details the preferential order of persistence of particular diatom species during resynthesis.

Symbiont structure: Observations of axenically cultured specimens

The results of the isolations from control specimens in the resynthesis experiment indicated that *N. panduriformis* was the most common original endosymbiotic diatom (Lee et al., 1983). Some specimens also harbored *F. shiloi*. *Nitzschia laevis* was the highest in the preferential order of persistence. All three species are very similar, if not identical, in their fine structure (fig. 1). All have a single cup-shaped chloroplast, which occupies almost 60% of the cell volume. Thylakoid lamellae are typically triple layered. Continuity between the chloroplast envelope and rough endoplasmic reticulum were observed (fig. 2). A prominent girdle lamella is observed in most sections of the chloroplast. Pyrenoids are generally lens-shaped and internal within the chloroplast (fig. 1). They are pierced by a single thylakoid sheet in the equatorial plane. As might be expected, pyrenoid morphology varies more widely in organisms isolated in culture. This presumably is a physiological response to different phases of the culture growth cycle. For example, some specimens of *Nitzschia panduriformis* from the same clone culture, just after the conclusion of log phase (fig. 2, 15 days)

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had associated pyrenoids on short stalks and in very old stationary phase they had invaginated pyrenoids (fig. 3, 14 weeks). On occasion sections of pyrenoids were obtained which seemed to be pierced by two thylakoid stacks (fig. 4), but most were single (fig. 1). We did not observe any F. shiloi or N. laevis with more than one triple stack per pyrenoid. In common with most diatoms, the cell nucleus and many mitochondria are in direct contact with the chloroplast membrane which generally cups very tightly around them (e.g., figs. 1 & 3). The cell periphery of even non-dividing diatoms seems to have active membrane (silicalemma; Reimann et al., 1966; Schmid, 1984), and silica deposition vesicles (S) (Drum and Pankrantz, 1964), just below the plasmalemma (e.g., figs. 1 & 5). The silicalemma and silica deposition vesicles were quite conspicuous even in the exceptionally old specimens (14 weeks) which no longer formed recognizable frustules. The food organism Amphora (SBL 39) (fig. 6) was easily distinguished from the other diatoms in TEM sections by its large internal lipid vacuole (V). Chlorella sp (AT) was likewise easily distinguished in TEM sections by its typical chlorophyte pyrenoid structure with a starch sheath and its chloroplast organization (fig. 29; compare with figs. 1-4).

Controls: General observations of fine structure

Healthy foraminifera, A. lessonii, directly from the sea were heavily pigmented orange-brown. They extended a dense pseudopodial net from their aperture that reached two-three times their body diameter. A.

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lessonii are plano-convex with a heavily calcified test (fig. 7). Decalcification of the test, after latex infiltration, revealed the chamber arrangement under the test (fig. 8). The individual chambers were not only connected ventrally through the foramina but had additional supplementary foramina or channels that communicated from one chamber to the next near the surface (fig. 11, arrow). Figure 11 also shows the impression of how the endoplasm fits inside the test pore rims. Endosymbionts were usually found in these endoplasmic protrusions under the test (also, e.g., figs. 9 and 31).

Semi-serial sections of the host from the dorsal to the ventral region revealed different degrees of cytoplasmic density. The dorsal cytoplasm exhibited a very dense architecture containing numerous vacuoles of a range of sizes, food particles, and apparent endosymbionts going to or away from the cell cortex (fig. 9). More ventral sections were filled with empty frustules, waste material, and algal cells, some of which were partially digested and others of which were undigested (figs. 10 & 12). Regions of the host near the aperture were generally filled with empty diatom frustules (fig. 16). Sections near the central axis of the foraminifer were filled with apparent unenvacuolated waste material and an occasional algal cell with attached pseudopods (fig. 15).

Freshly fixed organisms were generally densely packed with endosymbionts along their dorsal and dorso-lateral surfaces. There was generally one densely packed row of endosymbionts just inside the cell membrane along the dorsal and lateral surfaces of each chamber. Frequently one or two more loosely organized rows were found just below the most peripheral row. The cytoplasm around and more central to the

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endosymbiont regions was dense and filled with vacuoles of all sizes. Most distinctive of all the vacuolar types are the fibrous vacuoles (Fv), which are similar if not identical to those Anderson and Be (1976b) suggested contain adhesive substances (fig. 23, arrow). These fibrous vacuoles are not common in or near normal appearing dorsal or laterally peripheral endosymbiotic diatoms, but are common in ventral regions near sites of active digestive activity (fig. 17, arrows) and near endosymbiotic algae which by other morphological criteria (e.g., fig. 32) seem to be undergoing digestion or autolysis. A few of the fibrous vacuoles (Fv) are found even near normal appearing symbionts (fig. 13).

Conspicuous throughout the cytoplasm are electron translucent roughly spherical vacuoles (Tv, figs. 9 & 10). These vacuoles range in size from about 0.05 to 5 um. In control specimens fixed directly from the sea, the larger vacuoles tend to be concentrated in the ventral and outermost chambers. Digestive residua and scattered electron dense material are found in many vacuoles that are otherwise similar. Lipid bodies (Lb) are uniformly electron translucent (grey) spherical and subspherical vacuoles. Lipid bodies are fairly common throughout the cytoplasm of organisms taken directly from the sea but are more concentrated in the ventral cytoplasm (fig. 21). Smaller (about 0.5-1.0 um) spherical electron opaque osmiophilic vacuoles (O) are also scattered throughout the cytoplasm (e.g., fig. 9). Test precursor material vacuoles (T) are common in sections of the outermost chamber (fig. 14). Golgi bodies have been found in the dorsal cortical cytoplasm but they are more common in meduliary and more ventral cytoplasm. Microbodies are also common in the deep ventral cytoplasm but they are

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occasionally seenat the cell periphery near perfectly normal appearing endosymbionts (fig. 13). Ribosome studded endoplasmic reticulum and mitochondria are most dense in the cortical regions near the endosymbionts and are scattered in patches in the cytoplasm elsewhere. Mitochondria are particularly abundant in the penultimate chamber, the pseudopods, and the internal pseudopodial stream extensions.

Controls: Digestion

Food vacuoles with recognizable food in various stages of degradation are found in most chambers but tend to be more concentrated ventrally. Few food vacuoles are found in the youngest chambers and in the dorsal and lateral cortical regions of the cytoplasm. Food particles were enveloped in single-walled membrane sacs (fig. 18). Vacuoles containing algae in all stages of digestion were present in *A. lessonii*. The most common vacuoles, which presumably are the longest persisting stage in the food vacuolar cycle, contained diatom frustules with some granular material around the more electron dense frustules (fig. 12). Perhaps these are the precursors of the reddish-brown and dark brown residual matter (fecal matter) observed left behind the animal in culture. With the exception of the youngest chamber, food vacuoles are densest in the ventral regions of the chambers of the outer whorl. Electron translucent vacuoles with densely staining granules are abundant in the same region. The close juxtaposition of the vacuoles (V) with dense granules and food vacuoles (F, e.g., fig. 12) might suggest that the former contain products from the latter. Some pictures suggest budding of the vacuoles (figs. 12 & 18). Many food vacuoles in the

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ventral area are attached to profiles of internal extensions of pseudopodial streams with prominent mitochondria and fibrous vacuoles (fig. 17). Golgi and lysosomes are abundant as well in this region. Occasionally food vacuoles are found in the dorsal and lateral peripheral cytoplasm. Digestion seems more complete in these vacuoles which do not seem to be associated with internal pseudopodial streams. Gomori staining experiments gave some indication of digestive activity in the ventral region, but due to the extensive decalcification with acetic acid needed to dissolve the heavy test before sectioning, much of the lead stain leached out (figs. 16, 18, & 19). The aperture region was characterized by well defined pseudopodia (fig. 20). Vacuoles were abundant and generally partially surrounded by pseudopodia (figs. 20, 23, & 24). In animals fed *Amphora* sp or *F. shiloi* in the presence of ferritin and ruthenium red, the ruthenium red (the dark stained regions within the vacuoles, figs. 20 & 23) was picked up and envacuolated. The ruthenium red stain was transported, inside vacuoles, through the aperture and into the ventral chambers. There was no discernable difference between ruthenium red stained *Amphora* sp vacuoles and *F. shiloi* stained vacuoles. Ferritin was not seen in any vacuole. The initial concentration (10^{-5}) may have been too low. Algal cells and occasionally apparent micro-crustacean muscle tissue were found in the aperture region (figs. 24 & 22, respectively), near or attached to pseudopodia. Particles of food/endosymbiont were not fully envacuolated in the aperture region. Figure 25 shows what may be the beginning of envacuolation of an alga inside the aperture region.

The leucocyte acid phosphatase staining procedure was very successful. At the light microscope level (figs. 1a-6a), the red color

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and the black granules indicated the presence of digestive activity. Digestion began outside the organism soon after pseudopodia contacted the food. The enzymatic reaction products surrounded the food, in this case Chlorella sp, which had been pulled close to the outer shell surface (fig. 3a). Also visible were some dorsal chambers with well-vacuolated endosymbionts (fig. 3a). The tartrate control of the leucocyte acid phosphatase staining experiment indicated that the digestive enzymes were inactivated in the control (fig. 5a). TEM observations corroborated the LM observation. Chlorella sp were contacted by pseudopodia (fig. 29) and digestion of the particles began outside the test (figs. 26 & 28). At the initial contact stage, cell walls were intact. Black reaction product was visible in the pseudopodia near the algae. Several different types of reaction product were noted (figs. 26, 28, & 30). The intensity of the stain in the light microscope suggested that digestion occurred on the outside (figs. 1a-6a). TEM evidence indicated that some of the Chlorella sp entered the aperture in an undigested or a partially digested state (fig. 27). Ingestion and isolation of individual algae into single-cell vacuoles were apparently not necessary for digestion to proceed, as many partially or wholly digested food algae were found as groups outside the test attached to pseudopodia. Presumably these membrane bounded enclosures permit the pH to be regulated so that acid hydrolases can commence digestion of the algae.

The sequence of contact, transport, engulfment, and dipostion of food particle is illustrated in text figure 2.

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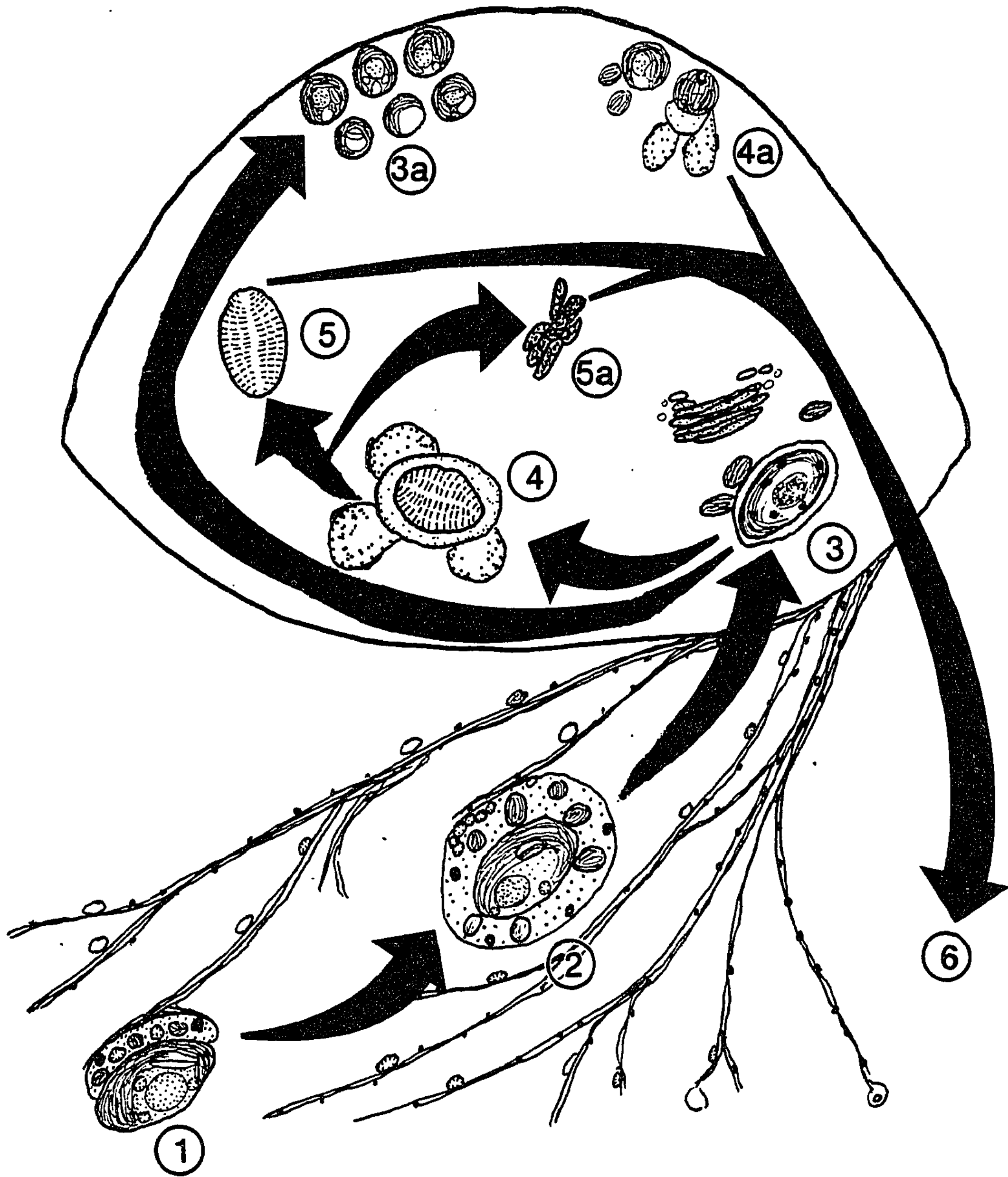
TEXT FIGURE 2



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Controls: symbiont fine structure

The endosymbionts were surrounded by two double-layered membranes and a "host" membrane (fig. 13). The rough endoplasmic reticulum of the host is very closely applied to the host epi-symbiotic vacuole. In some sections, clear vacuoles, which might be silica deposition vacuole vestiges (S) were present between the two symbiont membranes (fig. 13). The vacuole vestiges were not uniform in size or distribution. The fine structure of the chloroplast, mitochondria, and nucleus of the endosymbionts was quite similar to that observed in early stationary phase in the same diatoms grown in culture. Typically the chloroplast is tightly cupped around the nucleus and a single large mitochondrion. Ribosomes are squeezed into spaces between organelles. There is generally a single lens-shaped pyrenoid which is traversed by a single thylakoid stack. Other chloroplast thylakoid lamellae are in close juxtaposition around the pyrenoid. A single vacuole at the open end of the chloroplast cup contains an electron translucent-transparent presumably reserve product. Occasionally the vacuole had accumulations of granular material. Sometimes the vacuole appeared to be traversed by one or more membranes. The endosymbionts in most specimens collected and fixed directly from the sea have vacuoles that occupy one-fifth to one-half their total volume. (fig. 13). Sections of an occasional specimen will lack a vacuole (fig. 13). Osmophilic lipid droplets are sometimes found scattered between chloroplast lamellae.

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DCMU-treated specimens' fine structure

Compared to the controls from the sea, fewer algae were observed in the cortex of DCMU treated animals. The number of endosymbionts was reduced to a single layer or parts of a layer. A very thin cytoplasmic layer in close juxtaposition to the layer of endosymbionts contained mitochondria, ribosomes, fibrous vacuoles, osmiophilic vacuoles, and microbodies. Most of the cytoplasm was filled with more and larger (greater than 15 um) electron transparent vacuoles than in controls. Occasional patches of denser cytoplasm squeezed in between the electron transparent vacuoles (fig. 31). Some of the electron transparent vacuoles near the cortical endosymbionts had granular inclusions which made them resemble the vacuoles that we associated with digestive vacuoles (fig. 12).

The individual endosymbiotic algae in DCMU-treated animals were about 20% smaller in overall diameter than those in the controls. This may be attributed to the absence of electron transparent reserve product vacuoles in most symbionts. The intra-thylakoid distance of the endosymbionts' chloroplasts was considerably reduced (by about 30-40% to 22 nm) in the DCMU treated animals (fig. 32). In addition, many of the thylakoids were breaking down, as evidenced by the presence of large numbers of osmiophilic lipid globules (fig. 32; Dodge, 1973). The mitochondria of the DCMU-treated algae were contracted with well-defined tubular cristae (fig. 32). Pyrenoids, on the other hand, were similar to those in the controls (fig 32).

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Starved specimens' fine structure

Foraminifera starved for two weeks but incubated in the light were pigmented less deeply than controls but more than those treated with DCMU. Most of them still had some pseudopodial activity, albeit less extensive than that in the controls directly from the Red Sea. The endosymbionts, when present, were generally still vacuolated (figs. 33 & 34). As was the case for the DCMU-treated animals, the total number of cortical endosymbionts was greatly diminished; complete layers were rarely found. The majority of the endosymbionts in starved animals seemed abnormal. Most noticeable were interruptions in chloroplast lamellar structure. Within one host some endosymbionts' thylakoid stacks were short and interrupted by less electron-dense regions (fig. 33). In other endosymbionts in the same host, the inter-thylakoid spaces in the algal chloroplast were diminished and the thylakoids were almost indistinct from each other (fig. 33). Lipid vacuoles were also prominent in these chloroplasts. In some animals less than a third of the endosymbionts found in controls remained in the cortex. The cortical endosymbionts were remarkably distinct when compared to those in either the controls or the DCMU-treated animals. The most numerous organelles in close association to the endosymbionts were the fibrous vacuoles and microbodies. Clusters of mitochondria in thin protoplasmic streams were attached to many algae. The general appearance of the sub-cortical cytoplasm of the starved animals was similar to the ventral medial cytoplasm of the penultimate chamber in controls (fig. 21), giving the general impression of a region of active digestion. Unlike the DCMU-treated animals, which were filled with electron transparent vacuoles

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(figs. 31 & 34), most of the sub-cortical region of cytoplasm of starved animals was not compartmentalized into distinctive vacuoles.

Re-browned experimental controls' fine structure

The majority of the endosymbionts in experimental control specimens (bleached with DCMU and then fed and incubated in the light for 1 week) were similar to those in the control organisms. More than one row of endosymbionts was found in low-power micrographs (e.g., fig. 35). The general appearance of the cytoplasm closest to the endosymbionts was less dense than in organisms harvested and fixed directly from the sea (fig. 9), but much more dense than in either DCMU-treated or starved animals (figs. 31 & 34). This appearance was due to the presence of more electron transparant vacuoles in the cortex than in controls. Evidence of endosymbiont division was also seen (fig. 38). The chloroplast lamellar stacks were more widely separated than in DCMU-treated animals (compare figs. 32 & 37). There was a lot of granularity between stacks of thylakoids. Few or no osmiophilic lipid bodies were present within the chloroplast. The pyrenoid body was quite visible and was separated from the chloroplast lamellae by an open space presumably filled with starch storage products (fig. 37).

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Treatment of potential endosymbionts

Although the initial contact of *Chlorella* sp, *F. shiloi*, or *N. laevis* by the host was identical, the subsequent digestive treatment was different. The leucocyte staining procedure demonstrated, at the LM level, that digestive enzymes were not released or activated in vacuoles containing *N. laevis*, even when in close proximity to or inside the foraminifera (figs. 1a, 2a & 4a). An occasional *N. laevis* was digested while outside the foraminifera. It was clear that there were digestive enzymes surrounding the potential endosymbionts, but that most of them were undigested. Fine pseudopodia attached to *N. laevis* and *F. shiloi* in a manner similar to those attaching to *Chlorella* sp (figs. 29 & 36), and pulled them into the aperture. Once the food/symbiont was inside the aperture, it usually became separated into unialgal vacuoles (e.g., fig. 15). *F. shiloi* seemed to be more readily digested (fig. 36) by the foraminifera than was *N. laevis*, since far fewer healthy looking *F. shiloi* than *N. laevis* were found around the test, or inside the aperture.

Unfortunately fine structural sections through DCMU-treated animals which were fed potential endosymbionts did not appear too much different than those animals allowed to re-brown without their addition (figs. 35. 37, & 38). Those foraminifera fed potential endosymbionts higher in the persistence order seemed to have more filled pore rims and second layer cortical symbionts than did DCMU re-browned controls. Lead precipitate following the Gomori technique was not observed in cortical regions of rebrowning experimentally fed or control cultures, indicating that digestive activity was not taking place there. The leucocyte acid

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phosphatase technique confirmed the fine structural observation (figs. 1a-6a).

DISCUSSION

It was clear from experimental manipulation that the diatom endosymbionts of Amphistegina lessonii are vulnerable to changes in host physiology due to host starvation, to drugs, and presumably to conditions (e.g., dark) that might affect their own photosynthetic activities. In both the starved animals and those exposed to DCMU, the endosymbionts as a population were not equally affected by the particular stress. Some endosymbionts were eliminated from the population before others, so that recovery and repopulation of the host was still possible after 120 h in DCMU or after starving the host for two weeks. The herbicide treatment gives us some insight into the normal host-symbiont relationship. It suggests that even when the endosymbionts are photosynthezising actively (a mechanism that protects the symbionts of P. bursaria; Reisser 1980, Weiss, 1980) the host can, and does, crop the endosymbionts when there is physiological need to do so.

The length of treatment needed for attaining nearly aposymbiotic A. lessonii was much longer than that needed to attain the same in Globigerinoides sacculifer. Endosymbionts in the planktonic foraminifera G. sacculifer were eliminated after a 72-h treatment (Bé et al., 1982). The difference between G. sacculifer and A. lessonii in length of time required to eliminate their endosymbionts was attributed to the more

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protected and sequestered environment for the endosymbionts within A. lessonii. In G. sacculifer the endosymbionts "migrate" along the test spines during the day where they would come into direct contact with the treated environment. In A. lessonii the endosymbionts remain within the test far from the aperture through which the herbicide presumably enters.

After starvation for two weeks, A. lessonii retained more endosymbionts and were much more pigmented than five-day DCMU treated foraminifera. The endosymbionts in the starved animals had a characteristically functional appearance to their chloroplast. Some were indeed being digested. DCMU shuts down photosystem II of the chloroplasts (Vandermeulen et al., 1972), which may then have made the endosymbionts more susceptible to host digestion than active algae.

Although it was not a variable tested in the present study, it would be quite instructive in terms of control mechanisms to study progressive fine structural changes in A. lessonii: (1) starved and placed in the dark; and (2) fed but placed in the dark. If the diatom symbionts in A. lessonii have a mechanism for repression of host digestion similar to that suggested for the Chlorella endosymbionts in P. bursaria, we would anticipate that in the dark the symbionts would be more rapidily eliminated than those incubated in the light. Perhaps the A. lessonii endosymbionts would be even more rapidly eliminated in starved organisms incubated in the dark.

Another line of future investigation might be a study of length of survival of starved and fed A. lessonii in the light and dark. The interpretaion of similar experiments done with P. bursaria (Karakashian, 1975) and Hydra viridis (Muscatine and Lenhoff, 1965b) is that the

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endosymbionts have adaptive survival value to their host in times of nutritional need.

There were technical problems in studying the digestive activities of A. lessonii. Even with a diamond knife as a cutting edge it was necessary to completely decalcify the test of the foraminifera. Of the methods considered to decalcify the tests, 0.5 M ethylene diamine tetra acetic acid (EDTA) and 0,15 M acetic acid, the acetic acid treatment seemed least destructive to the fine structural cytological picture, and it was the fastest. Unfortunately, acetic acid decalcification interfered with the Gomori staining technique causing a leaching out of some of the reaction products. Others have noted similar problems (eg. Smith and Fishman, 1969). A new method, 2% L-ascorbic acid, used to decalcify mammalian tooth germs and bone after fixation in a glutaraldehyde-paraformaldehyde mixture (Wakita et al., 1983), may prove to be a useful replacement for acetic acid decalcification in foraminifera. The procedure will probably have to be done in the dark to prevent oxidation of the ascorbic acid (Dietrich and Fontaine, 1975). The leucocyte acid phosphatase staining technique, on the other hand, proved to be highly successful in cytochemically demonstrating the presence and location of digestion enzymes.

Normal digestion in Amphistegina lessonii begins outside soon after food organisms are contacted and enveloped by host pseudopods (see text fig. 2 for a generalized scheme of digestion). The Gomori procedure suggested that at least part of the enzymes responsible for digestion are produced in the many golgi complexes found in the cytoplasm. Two types of vacuoles seemed involved in the process: microbodies and fibrous vacuoles.

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Contact of the food or symbionts were identical at the LM and EM level. Fine pseudopodia attached to the particles. Adhesive-type bodies similar to those described by Anderson and Bé (1976b) were found in the pseudopodia and also occasionally near the dorsal endosymbionts. As suggested by Anderson and Bé (1976b), if the adhesive material contained acid mucopolysaccharides, it may function to lower the pH of the surrounding sea water so that external digestive enzyme activity would be possible. Digestive activity clearly began shortly after contact. Certainly by the time the particles were adjacent to the test, digestion activity was well defined. Digestion continued inside the organism, as demonstrated by the TEM cytochemical tests. The digestive vacuoles are rapidly brought through the aperture and the youngest chambers by cytoplasmic pseudopodial streams and their roots, to the ventral-medial cytoplasm of the penultimate and adjacent chambers. There many of the vacuoles may be broken into smaller units since we encountered very few food vacuoles inside the animal with more than one alga in them. Vacuoles with small groups of algae occasionally occur because they have been figured in light micrographs of the same species (McEnery and Lee, 1981). After a time some digestive vacuoles seem to migrate more peripherally. From their appearance, with mostly frustule remains, most are more mature than those in the ventral region.

Fairly frequently vacuoles with frustuleless algae are seen in the same subcortical cytoplasm. Some of the algae appeared perfectly healthly and as normal as those in the sub-pore cortical layers of algae. Others seemed as if they were in stages of digestion. It is not reasonable to infer dynamic processes from static pictures; however, one wonders if some of the algae in the sub-pore cortical layers are not

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occasionally digested. If so, how frequently does this occur? How does the host regulate the numbers of algae in its cortex? Or does it? Perhaps some algae divide and others become mature and are digested. In the present study it was clear that the hosts' fibrous vacuoles are in close juxtaposition to the cortical endosymbionts and potentially could digest them. An experiment in which these host/symbiont systems are being incubated in the presence of tritiated thymidine with pulses of colchicine, presently in progress in the lab, has the potential for giving some insight into the dynamic aspects of the cortical endosymbiont diatom population.

The results of the present study and the one just published (Lee et al., 1983) clearly demonstrated that the endosymbionts, which remain viable after DCMU or starvation, divide and repopulate their hosts. Although it is a tenuous finding derived from static pictures of a dynamic process, the evidence obtained here corroborates the published study in that it suggests that ingested, but not digested, endosymbionts repopulated hosts when the ingested symbionts were higher on the persistence order. This inference is made because the sub-pore cortical layers in the experimental animals fed certain symbionts were filled faster than in re-browned controls.

The LM and TEM cytochemical experiments clearly demonstrated that the potential endosymbionts *N. laevis* and *F. shiloi* were not as frequently digested as were the food organsisms *Chlorella* sp and *Amphora* sp. Since the foraminifera in the experiments were all treated similarly, there was a real difference in resistance of endosymbionts to being digested. Indeed, it appeared that *N. laevis* was more resistant to digestion than was *F. shiloi*. The differences in the resistance of *N.*

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laevis and F. shiloi would lead one to expect that N. laevis would selectively survive over F. shiloi in A. lessonii. Indeed in the short-term experiments, in which mixtures of three different symbionts were given to aposymbiotic A. lessonii and allowed to competitively re-brown for seven days, N. laevis seemed to be preferentially retained over F. shiloi (Lee et al., 1983). Much more than survival at the initial step must be involved in the persistence of endosymbionts in A. lessonii because F. shiloi is a very common symbiont in the worldwide distribution of its host (reviewed by Lee, 1983; Lee and Reimer, 1984).

The contact through sequestration phase of food/symbiont by the host foraminifera is a dynamic process. In Cassiopia xamachana, the process can be parsed into four phases for convenience: (1) coming together; (2) uptake (endocytosis); (3) resistance to or avoidance of host cellular defenses; and (4) sequestration in the host (Fitt and Trench, 1983). The coming together and resistance to or avoidance of cellular digestion defenses in A. lessonii occur close in time and space and are probably not two processes. As the undigested symbiotic algal vacuoles are swept dorsally or laterally, they would avoid digestion.

The food/symbiont particles were envacuolated individually or in small groups in A. lessonii. The groups are probably further sequestered into individual vacuoles, as in the Chlorella-Paramecium bursaria system (Karakashian and Karakashian, 1973). No difference was noted in the engulfment process between food and symbionts as was apparent in the Hydra-Chlorella system (Cook, 1980).

Two resistance mechanisms that have been postulated for other algal/invertebrate systems are: (1) The active alga inhibits fusion of lysosomes by alteration or blocking of membrane binding sites; and/or

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(2) there is a neutralization of digestive enzymes once they have entered the peri-algal sac (reviewed by Jolly and Smith, 1980). In A. lessonii there was an algal species-dependant resistivity to host digestion. The resistance ranged from near total for healthy N. laevis to low for healthy F. shiloi and to near zero for healthy nonsymbionts Chlorella and Amphora. The evidence here was not sufficient to eliminate either mechanism; however it seems quite feasible to obtain additional evidence along these lines. If the feeding experiment were repeated using N. laevis, F. shiloi, and Chlorella sp(AT) as controls but incubated in the dark, one might anticipate by analogy to Paramecium bursaria that many more of the endosymbionts wold be digested.

In the present study no sections were obtained that presented evidence useful in interpreting the morphogenetic steps necessary for an ingested potential endosymbiont to lose or have its envelope digested. The ingested potential endosymbionts retain their frustules during envacuolation. In a recent in vitro study, Lee and co-workers (1984; in press) found that a homogenate from the host suppressed shell formation in logarithmically growing cultures of endosymbionts. The suppressed or abnormal shell formation ranged from a high of 70% of the organisms in culture for F. shiloi to 40-60% for N. laevis. Possibly this occurs as the potential endosymbionts repopulate a host, but more study is needed to confirm this within the foraminifera. Shell suppression may be a factor that helps explain F. shiloi's high frequency in long-term populations of A. lessonii. Algal division occurs rapidly and is difficult to catch with static TEM techniques.

All endosymbionts found in the dorsal region of the foraminifera lacked their tests. Some evidence of algal division was found in the

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mid-regions of the host. Presumably after division the algae would then migrate to the dorsal areas, probably as a result of cytoplasmic streaming although an active process cannot be ruled out.

It is interesting to note that while F. shiloi was a common isolate from A. lessonii directly from the Red Sea, it is less resistant to digestion than the less common isolate N. laevis. This may represent a short-term competitive advantage of N. laevis over F. shiloi. The factors that control the population mix of endosymbionts within the host are unknown but could include light level, division rate, or active secretion of a growth inhibitor by an alga or enhancers or inhibitors produced by the host that preferentially affect the algal species.

Endosymbiont structure was remarkably consistent. In the approximately one hundred A. lessonii examined with the TEM, all of the endosymbionts that had a visible internal pyrenoid structure had a simple internal pyrenoid with a single triple-thylakoid stack. According to Dodge (1973), the pyrenoid may be the only distinguishing species characteristic for algae at the ultrastructural level. Recently Leutenegger (1983,1984) has suggested that diatom species could be identified at the TEM level, but this remains to be demonstrated and indeed these results appear to contradict such a suggestion. In this study, three diatom species, representing two genera, were involved, and all had a simple internal pyrenoid type when in the host and were otherwise indistinguishable. One, N. panduriformis, occasionally had two separate internal thylakoid stacks in aged cultures.

Leutenegger's (1983, 1984) chief argument is that since all other algal/invertebrate symbiotic relationships involve specific pairs of algae, the larger foraminifera/diatom relationship should also be

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specific. The evidence she has used to support her argument is weak. In many cases she has examined only one or two specimens: in the case of *A. lessonii* she examined five specimens. She has no evidence on the fine structure of most of the organisms isolated as endosymbionts. As mentioned above, the fine structure of the few species examined in this study is identical. The only methods to date that permit positive identification of the endosymbionts are the isolation and culturing techniques of Lee (1980). To corroborate the endosymbiont isolation techniques, our laboratory is developing diatom species-specific fluorescent labeled antibodies which should prove to be a powerful and rapid technique for endosymbiont identification in freshly squashed hosts.

SUMMARY

1) The ability to obtain nearly aposymbiotic *A. lessonii* with the herbicides DCMU and methyl viologen was clearly demonstrated.

2) Short-term competitive re-synthesis experiments demonstrated that *N. laevis* was more successful in avoiding digestion than *F. shiloi*; based on isolation and culturing of the symbionts after one-week re-browning, and qualitative TEM investigations. No food organisms were recoverd in the isolation experiments. The fact that no food organisms were recovered indicated that: (1) all food organisms were digested; and (2) the isolation techniques did not select any external adhering algae,

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this implies that the isolation and culturing techniques reflect a true endosymbiotic population.

3) Short-term competitive resynthesis experiments revealed a definite order of preference for symbionts, i.e., Nitzschia valdestriata, Amphora rottgeri, Nitzschia panduriformis, Nitzschia laevis, Nitzschia frustulum, Fragilaria shiloi. This varies from isolates of A. lessonii from the Red Sea, Hawaii, and The Great Barrier Reef, Australia. In these, F. shiloi was much more prevalent than in the short-term experiments reported here. It is apparent that different factors must determine long-term endosymbiotic populations than short-term ones.

4) Symbiont ultrastructure of N. laevis, N. panduriformis, and F. shiloi, as viewed with the TEM, were identical.

5) The ultrastructure of normal healthy A. lessonii revealed: (1) dorsal cytoplasm was very dense with numerous vacuoles, food particles, and two to three rows of endosymbionts; (2) ventral sections were filled with digestion vacuoles, waste material, and undigested algal cells; (3) golgi bodies, fibrous vacuoles, and food vacuoles are more common in ventral chambers than dorsal; and (4) ribosome studded endoplasmic reticulum and mitochondria are concentrated in dorsal chambers, particularly near endosymbionts.

6) The ultrastructure of DCMU-treated A. lessonii revealed: (1) dorsal cytoplasm was filled with many more, and larger vacuoles than in controls, with only an occassional patch of dense cytoplasm apparent;

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(2) Endosymbionts were considerably reduced in number to less than one row per dorsal chamber, and their appearance was much more condensed and compressed than in controls; and (3) much more residua and empty shell material was apparent throughout the animal than in controls.

7) The ultrastructure of starved *A. lessonii* revealed: (1) in dorsal sections cytoplasm and vacuoles were scarce; and (2) few endosymbionts remained; of those that remained most were like control endosymbionts while a few appeared to be undergoing cell lysis.

8) The ultrastructure of re-browned *A. lessonii* revealed a cytological picture similar to that in controls. Their cytoplasm was well vacuolated and dense, one to three rows of symbionts were present, and food vacuoles were abundant.

9) Cytochemical tests indicated that: (1) significant food digestion occurs outside the foraminifera test, and is continued inside the aperture and ventral chambers; (2) some digestion is evident in the dorsal chambers; (3) potential symbionts are far more resistant to digestion than food organisms; and (4) there is a notable difference in digestion resistance between the potential endosymbionts *N. laevis* and *F. shiloi*, with the former being more resistant. This difference in digestion resistance supports the results of the short-term competitive re-synthesis experiments.

10) The herbicides DCMU and methyl viologen apparently interfered with the protective mechanisms of the endosymbionts to host digestion.

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11) Starved A. lessonii were able to eventually overcome the defense mechanisms of some of their endosymbionts and digest them. Starvation of the host was not nearly as effective a means of eliminating endosymbionts as was herbicide treatment.

12) Re-population of the host can occur within one to two weeks of release from the herbicide-stressed condition, without the addition of new algae. This is believed to result from cell division of the surviving endosymbiont population.

13) There were no discernible differences in contact, transport, or engulfment of food and symbionts by A. lessonii. Fine pseudopodia contact, adhere to, and transport both types of particles to the test exterior and eventually into the aperture. Digestion of non-resistant particles commences outside the test and continues inside the aperture and ventral chambers.

14) How new symbionts lose their tests was not clear from observations made in this thesis. No sections were obtained showing release of a frustuleless symbiont from a digestive vacuole, nor was division of algae observed within vacuoles. Evidence points to algal division with either active suppression of new shell formation by the host and/or symbiont, or passive suppression due to a lack of silicon in the host cytoplasm.

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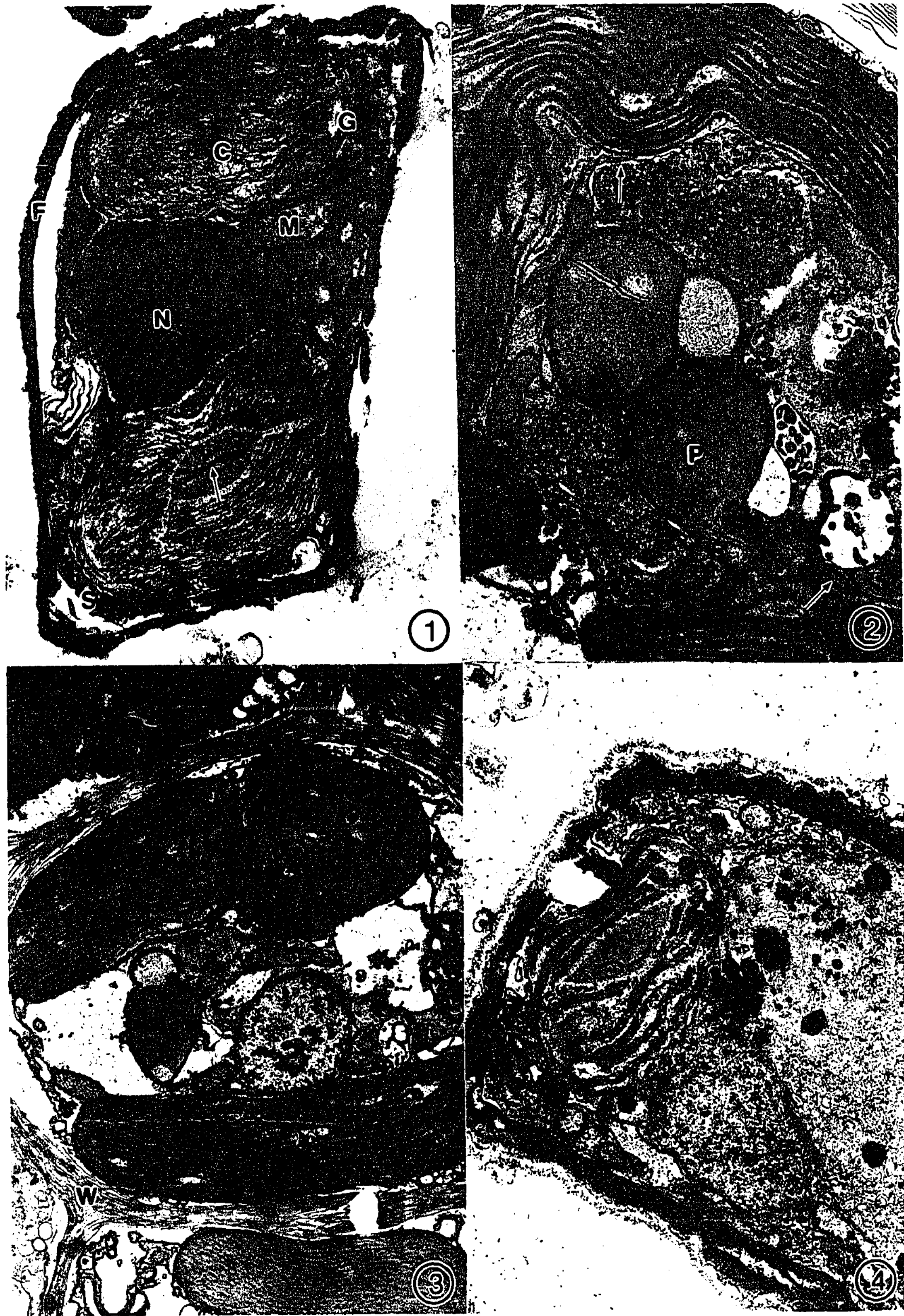

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Fig. 1. TEM micrograph of F. shiloi taken from 15-day culture. Note the thick outer cell wall, the interphase nucleus with nucleolus and condensed chromatin. Also present is a pyrenoid with simple internal thylakoid arrangement (arrow). The mitochondria (M) are the usual tubular eucaryotic type. C-chloroplast, F-frustule, G-girdle lamellae, S-silica deposition vacuoles. Mag. 26.5 KX.

Fig. 2. TEM micrograph of N. panduriformis from 15-day culture. P-pyrenoid, arrows show continuity between chloroplast envelope and rough endoplasmic reticulum. Mag. 23 KX.

Fig. 3. TEM micrograph of N. panduriformis from 14-week culture. W-fibrous wall laid down in many older cells in culture perhaps as a result of silica depletion in culture. Mag. 10.5 KX.

Fig. 4. TEM micrograph of N. panduriformis from 15-day culture. Note the presence of two lamellae stacks in the pyrenoid. Mag 23 KX.

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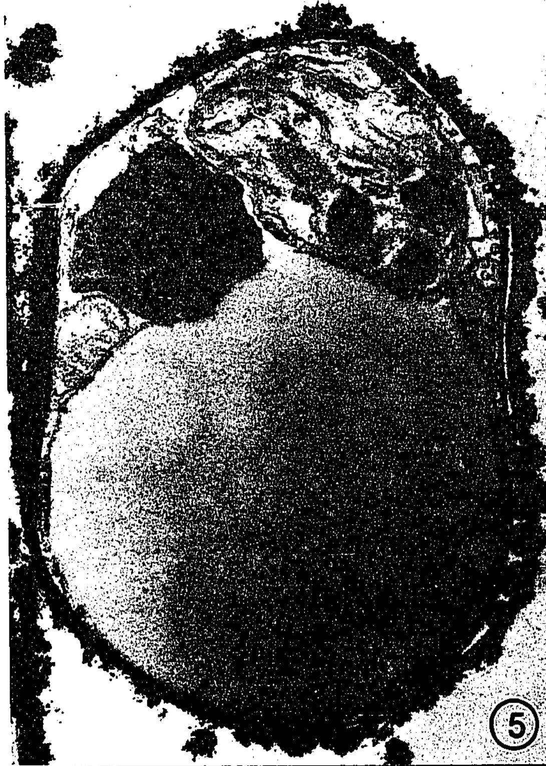

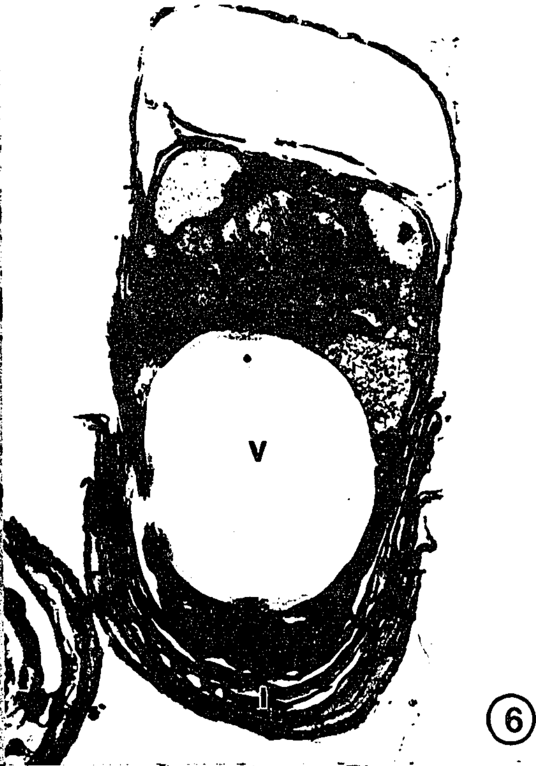

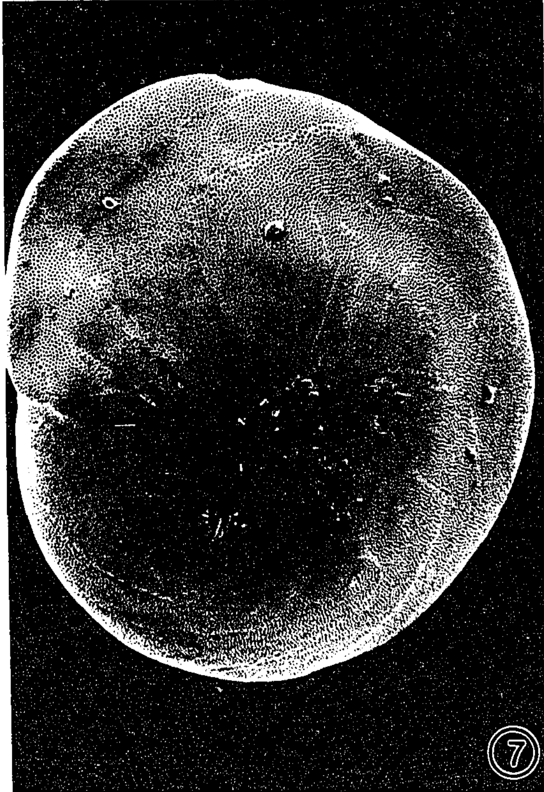

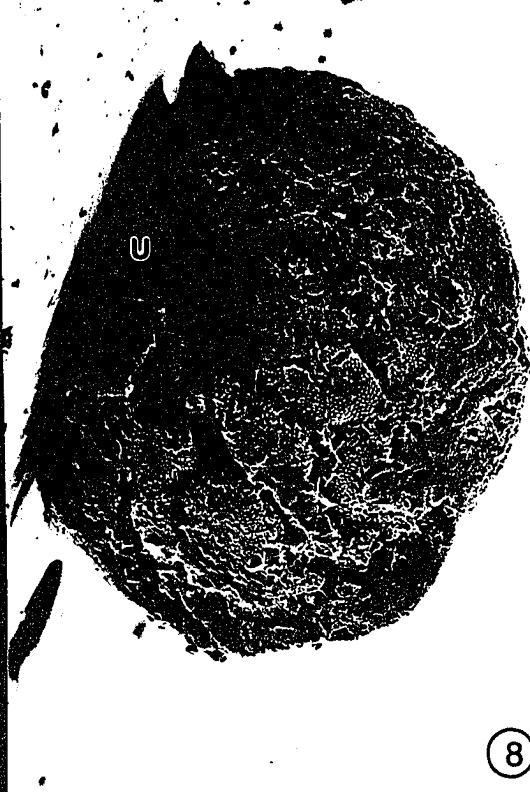

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Fig. 5. TEM micrograph of N. panduriformis from 34-week culture. Note the large "starch" storage vacuole, the distended lamellae in the chloroplast. Also present are large lipid bodies which may indicate incipient disintegration of the lamellae. A small pyrenoid with simple internal lamellae is also visible. Mag. 18.5 KX.

Fig. 6. TEM micrograph of Amphora sp. from 14-week culture. Note multiple layered cell wall, small chloroplast, large centralized "starch" storage vacuole (V), and intercallary bands (I). Mag. 14.1 KX.

Fig. 7. SEM micrograph of A. lessonii with the calcium carbonate test intact. Dorsal view. The chambers are obscured by the test. Mag 150X.

Fig. 8. SEM micrograph of A. lessonii after latex impregnation and test decalcification. U-umbilical region. Mag. 150X.

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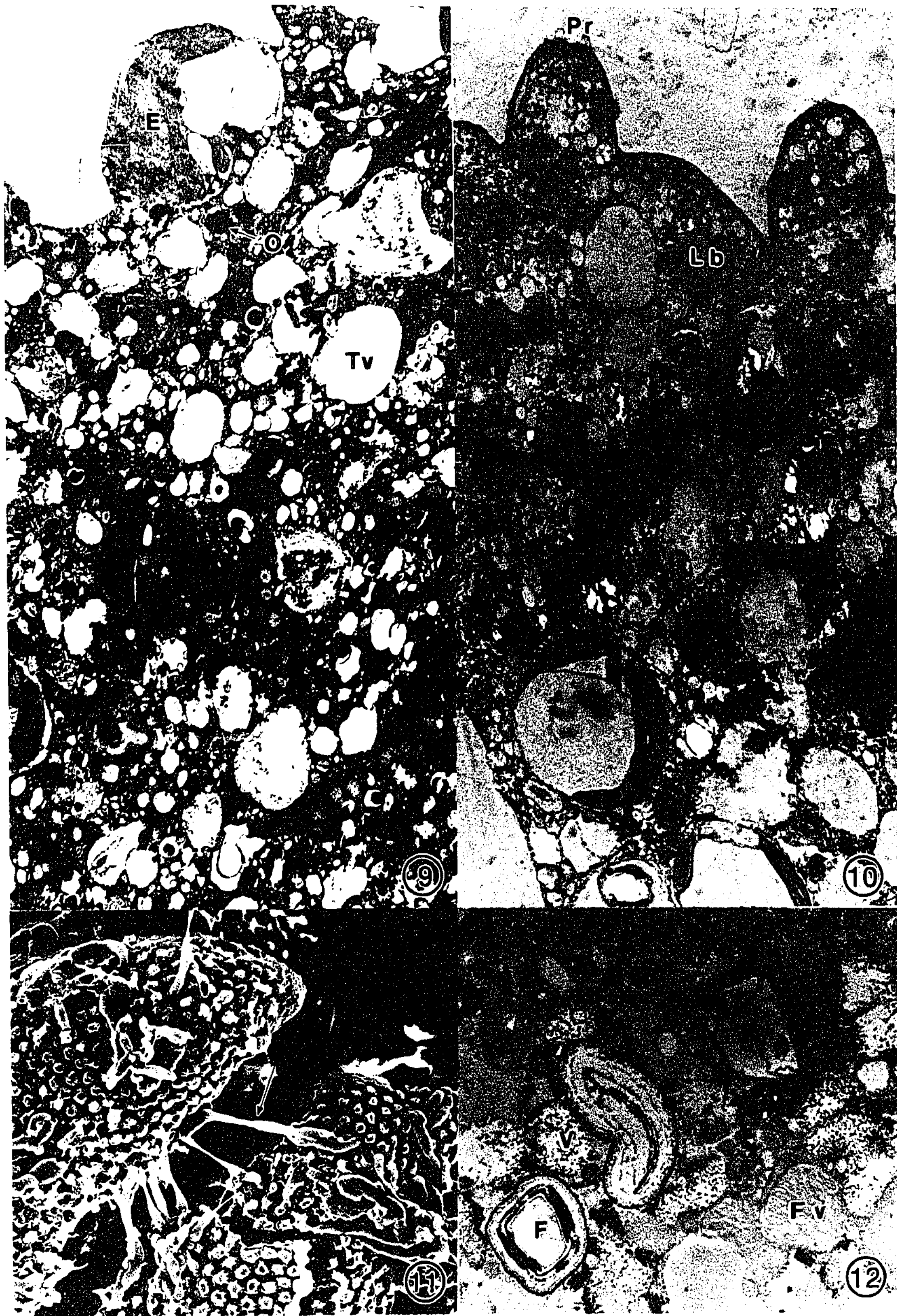

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Fig. 9. TEM micrograph of A. lessonii directly from the Red Sea. Dorsal section. The symbionts are socketed into the vaults of pore rims. The cytoplasm behind the symbionts is dense and filled with vacuoles of different diameters; some are apparent food vacuoles and some apparent symbionts. Tv-electron translucent vacuoles, O-osmiophilic vacuoles, Lb-lipid bodies, and E-endosymbiont. Mag. 6.6 KX.

Fig. 10. TEM micrograph of A. lessonii fixed directly from the Red Sea. Ventral section. Three healthy-looking algae clearly envacuolated are visible. The cytoplasm is also very dense. Pr-pore rim, Lb-lipid body. Mag. 5.5 KX.

Fig. 11. SEM micrograph of latex impregnated chamber region of A. lessonii after test decalcification. Arrow-supplemental channels. Mag. 350 X.

Fig. 12. TEM micrograph of A. lessonii fixed directly from the Red Sea. Ventral section of new chamber. V-vacuoles with dense granules, F-food vacuoles, Fv-food vacuoles with apparent chloroplast remnant. Mag. 6.6 KX.

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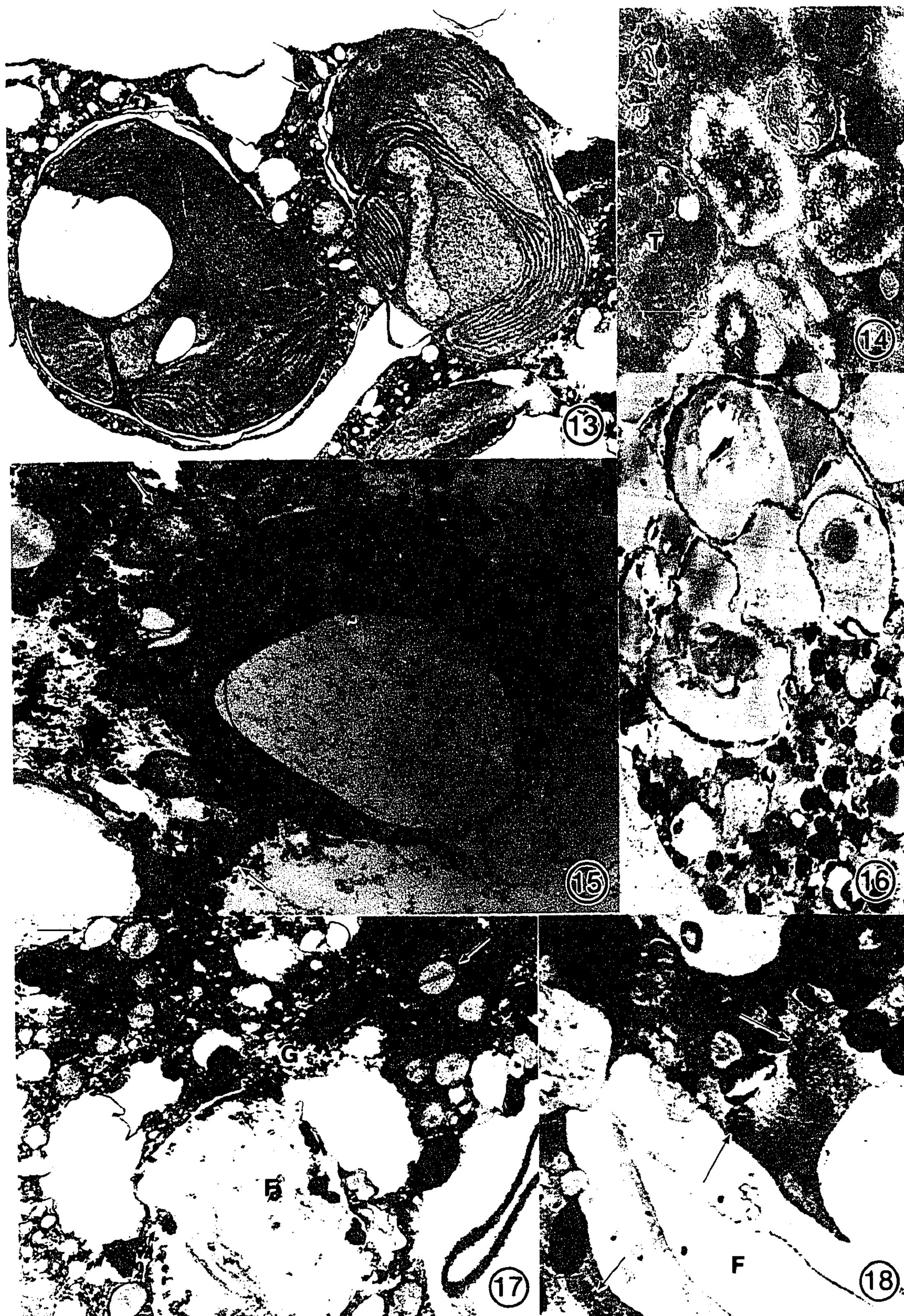

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Fig. 13. TEM micrograph of endosymbionts in A. lessonii. The endosymbionts have a cup-shaped chloroplast, tubular mitochondria, eucaryotic nucleus, and a pyrenoid with simple internal lamellae. A few lipid bodies are present between the chloroplast lamellae. Arrow-fibrous vacuole. Mag. 13 KX.

Fig. 14. TEM micrograph of new chamber forming region of A. lessonii. T-test precursor material vacuole. Mag. 21 KX.

Fig. 15. TEM micrograph of dorsal-lateral region of A. lessonii showing apparent transport of an alga cell to or away from the dorsal region. Arrows show attached pseudopodia. Mag. 23 KX.

Fig. 16. TEM micrograph of A. lessonii fixed directly from the Red Sea. Ventral section behind aperture. Note Amphora-like food envacuolated and largely digested. Gomori treated animal. Note the weak Gomori staining which probably resulted from the long acetic acid treatment used to remove the heavily calcified foraminifera test. Mag. 6 KX.

Fig. 17. TEM micrograph of ventral region of A. lessonii. G-golgi body, F-food vacuole, arrows-fibrous vacuoles. Mag. 15 KX.

Fig. 18. TEM micrograph of ventral region with Gomori staining experiment with A. lessonii. The golgi apparati are faintly stained and are juxtaposed near an apparent food vacuole. The faint Gomori lead staining is probably the result of leaching out of the lead by acetic acid during decalcification of the test. F-food vacuole, arrows-golgi and vesicles with Gomori lead precipate. Mag. 18 KX.

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Fig. 19. TEM micrographs of DCMU-treated A. lessonii, dorsal view. Gomori stained and acetic acid treated. G-golgi, O-osmiophilic granule, arrows-Gomori reaction product. Mag. 18 KX.

Fig. 20. TEM micrograph of the aperture region of A. lessonii. The dark staining resulted from pickup of ruthenium red. Note the large number of pseudopodia adhering to vacuoles containing ruthenium red indicating vacuolar formation and adherence to materials in the pseudopodia. Ps-pseudopodia, Rr-ruthenium red vacuole. Mag. 6.6 KX.

Fig. 21. TEM micrograph of ventral region of A. lessonii. Lb-lipid bodies, F-food vacuoles, V-vacuoles with dense granular material. 4 KX.

Fig. 22. TEM micrograph of micro-crustacean muscle (arrow) found in the ventral region of A. lessonii. Mag. 24 KX.

Fig. 23. TEM micrograph of the aperture region of A. lessonii fed food and ruthenium red. No lead citrate or uranyl acetate staining was used. Note the fibrous adhesive bodies (arrows) near the vacuole, similar to those found by Anderson and Be (1976b). Rr-ruthenium red vacuole. Mag. 26 KX.

Fig. 24. TEM micrograph of food particles in the aperture region of A. lessonii. F-food vacuole. Mag. 8.5 KX.

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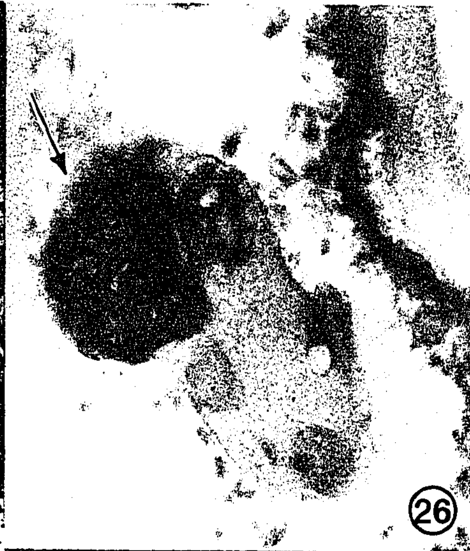

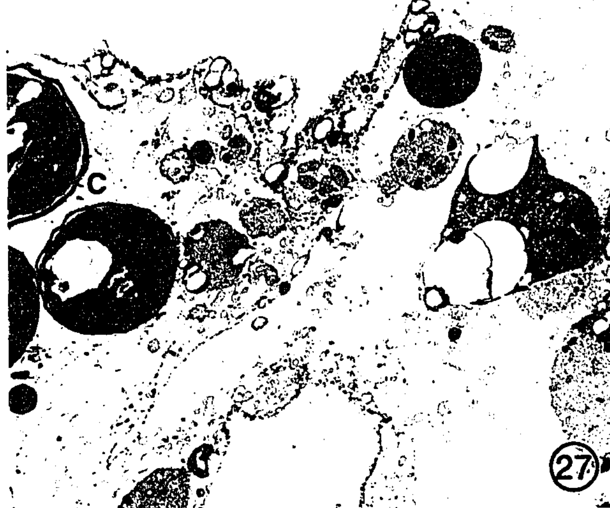

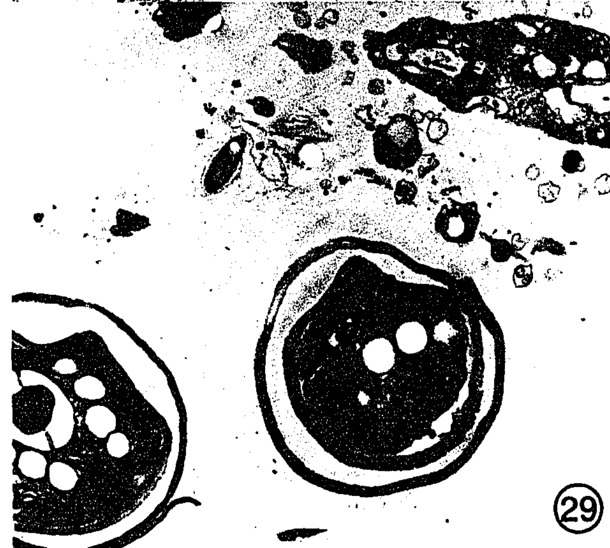

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Fig. 25. TEM micrograph of a food particle surrounded by pseudopods inside the aperture of *A. lessonii*. This is probably an early phase of engulfment. Unlike the ruthenium red vacuoles in Figs. 20 and 23, this particle is not envacuolated. Arrows-pseudopodia beginning to engulf an alga, ruthenium red stained also. Mag. 12 KX.

Fig. 26. TEM micrograph of leucocyte acid phosphatase experimentally treated animal. Psuedopodia with reaction product near a food particle (arrow). Mag. 42 KX.

Fig. 27. TEM micrograph of pseudopodial region of *A. lessonii*. *Chlorella* (C) are in close proximity to fine pseudopodia which eventually contact them. This is a leucocyte acid phosphatase experimentally treated animal. No lead citrate or uranyl acetate staining was used. The dense staining in the pseudopodia is the reaction product. Mag. 16 KX.

Fig. 28. TEM micrograph of leucocyte acid phosphatase experimentally treated animal. Food particle being digested outside the foraminfera, without apparent engulfment. Mag. 15 KX.

Fig. 29. TEM micrograph outside aperture showing pseudopodial contact of food organisms *Chlorella* sp. Mag. 13 KX.

Fig. 30. TEM micrograph of uptake and digestion of some *N. laevis*. Leucocyte acid phosphatase staining technique. Arrow-reaction product. Mag. 16 KX.

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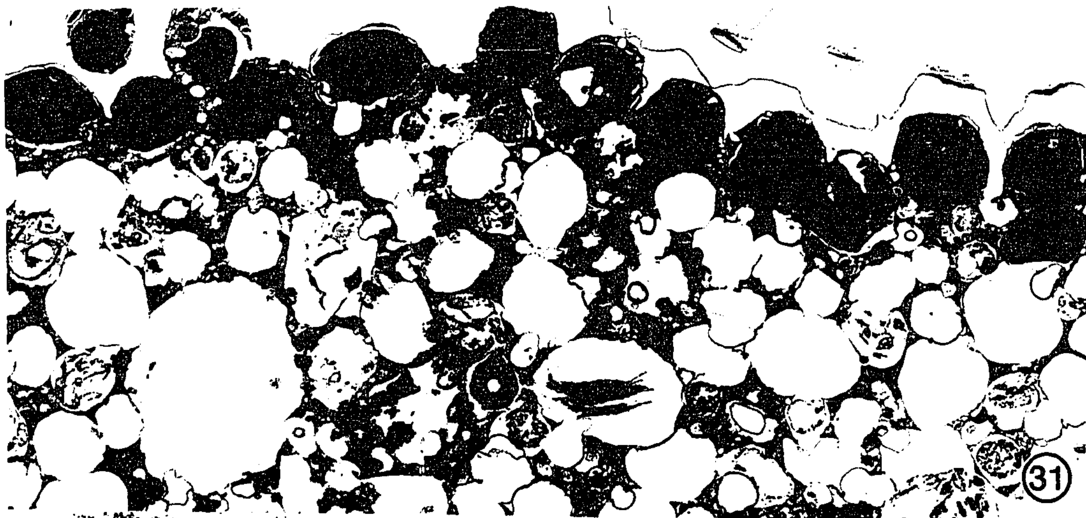

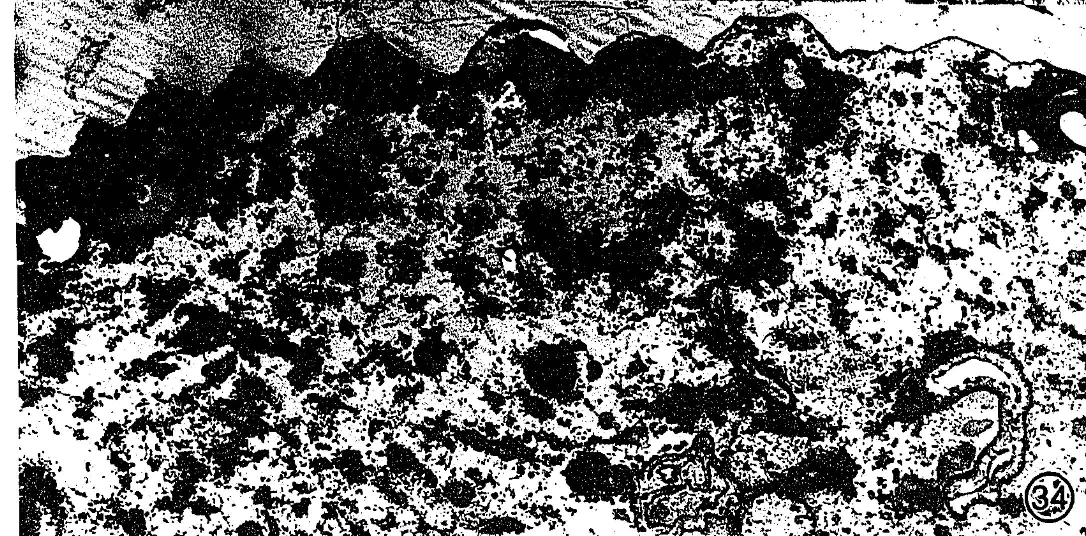

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Fig. 31. TEM micrograph of an animal treated for five days with DCMU. Mag. 10 KX.

Fig. 32. TEM micrograph of a DCMU treated animal. m-mitochondrion, arrow-lipid droplet. Mag. 25 KX.

Fig. 33. TEM micrograph of starved _A. lessonii_. Mag. 5.6 KX.

Fig. 34. TEM micrograph of doral-lateral section of _A. lessonii_ that had been starved for two weeks. Note the scarcity of symbionts, but those that are remaining are still well vacuolated. Also note the degenerative looking cytoplasm behind the symbionts. Mag. 1 KX.

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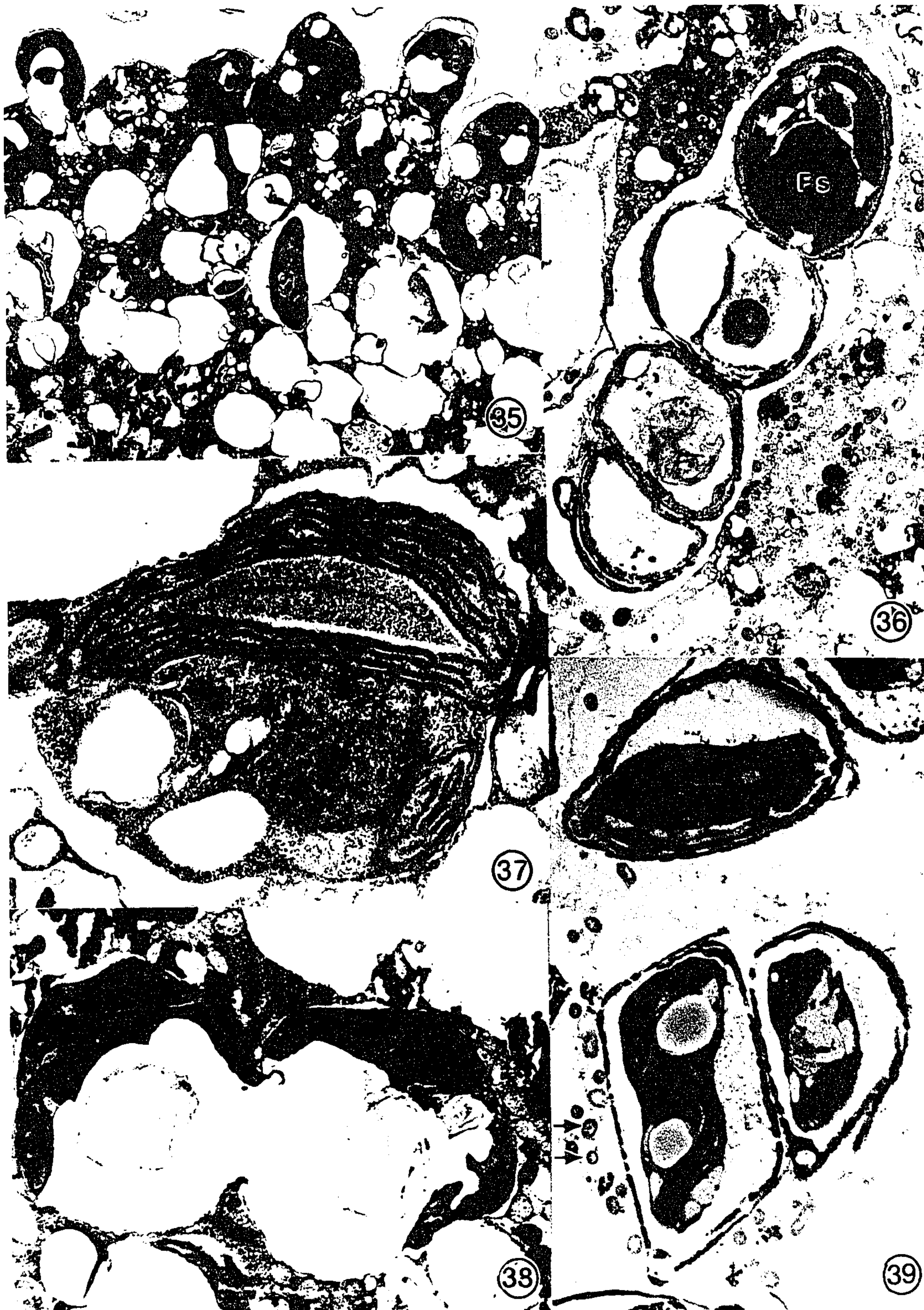

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Fig. 35. TEM micrograph of re-browned A. lessonii. Five day treatment with DCMU, then one week recovery. The symbionts in the dorsal region are generally well vacuolated and packed two rows deep. The pyrenoid bodies are clearly delimited by a space, probably filled with starch storage products; the lamellae are more separated than in DCMU stressed animals. The cytoplasm is also filled with multi-sized vacuoles. Mag. 3.5 KX.

Fig. 36. TEM micrograph of F. shiloi (Fs) surrounded by host membranes. Inside the aperture region. Mag. 6 KX.

Fig. 37. TEM micrograph of re-browned A. lessonii. This is a typical endosymbiont that would appear to be very active. Starch vacuoles are present surrounding the pyrenoid; the lamellae are separated in a presumed active state. Mag. 38.4 KX.

Fig. 38. TEM micrograph of apparently dividing endosymbiont in A. lessonii after one week recovery from DCMU treatment. Mag. 10 KX.

Fig. 39. TEM micrograph of N. laevis being contacted by pseudopodia (arrows) from A. lessonii. Mag. 7 KX.

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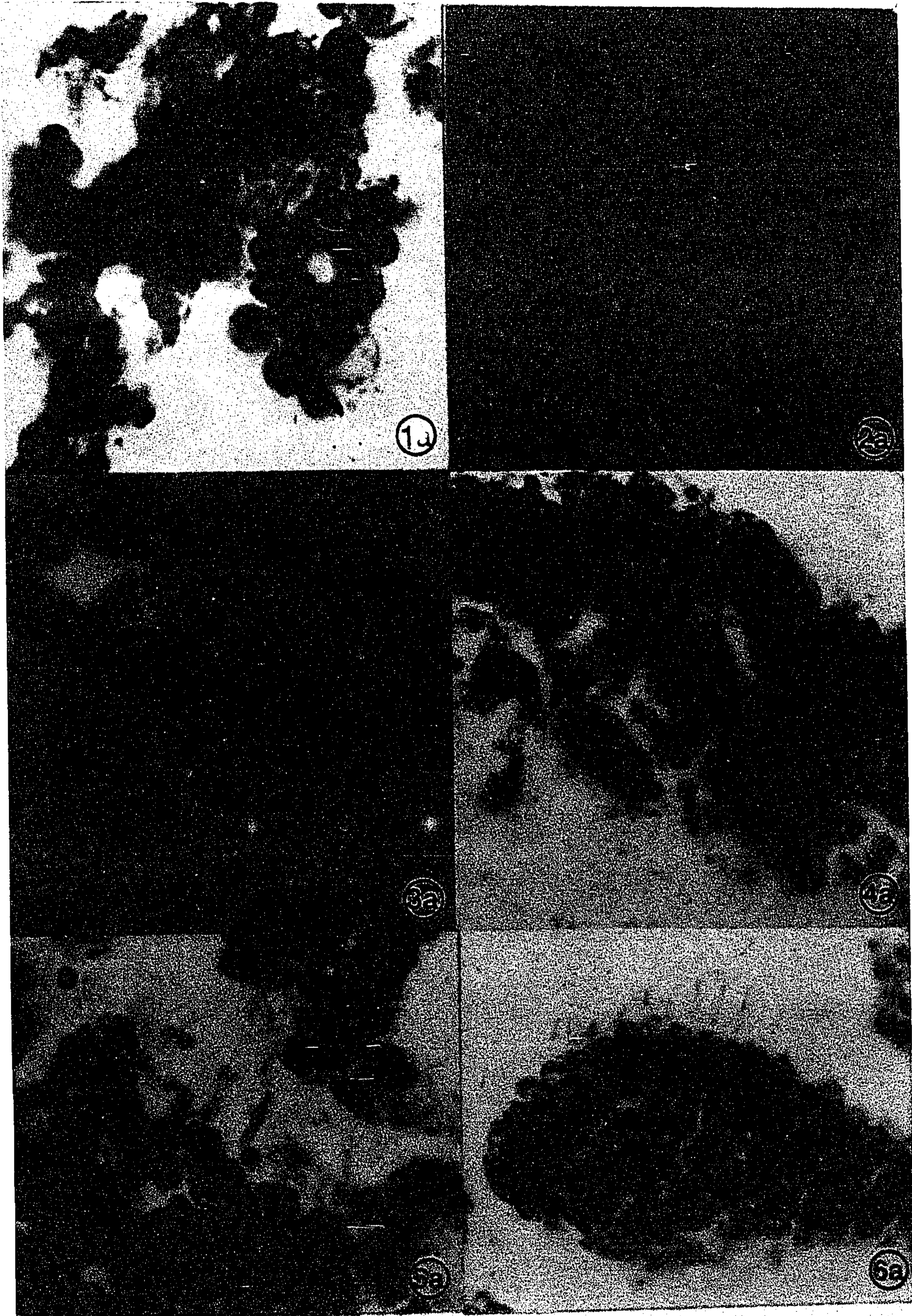

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Fig. 1a. Fed *N. laevis*, tartrate control for the leucocyte acid phosphatase experiment. LM micrograph of pseudopodial region surrounding *A. lessonii*. Note that many *N. laevis* are visible with chloroplasts clearly defined. Mag. 125 X.

Fig. 2a and 4a. Fed *N. laevis*, leucocyte acid phosphatase experiment. LM micrographs of *A. lessonii* showing capture of a potential endosymbiont. The red color and dense spots indicate the presence of digestive enzymes. Only rarely are the potential endosymbionts actually digested. Mags. Fig. 2a, 125 X. Fig. 4a, 79 X.

Fig. 3a. Leucocyte acid phosphatase experiment. LM of *Chlorella* sp fed to *A. lessonii*. Note the dark red color and granulation surrounding most of the algae. The chlorophytes are being digested outside of the shell. Mag. 79 X.

Fig. 5a. Leucocyte acid phosphatase experiment. LM of *A. lessonii* fed *Chlorella* sp. Tartrate control. Mag. 79 X.

Fig. 6a. Fed *N. laevis*, leucocyte acid phosphatase tartrate control. LM micrograph of *A. lessonii* dorsal chamber. Note that the color is similar to the control Fig. 1a, and the potential endosymbionts are vacuolated and greenish. Mag. 79 X.

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