EFFECTS OF MALACHITE GREEN

ON ZOOSPORES OF THE

MARINE FUNGUS *LAGENIDIUM CALLINECTES* COUCH

by

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ABSTRACT

Michael T. Hall. THE EFFECTS OF MALACHITE GREEN ON ZOOSPORES OF THE MARINE FUNGUS, LAGENIDIUM CALLINECTES COUCH. (Under the direction of Dr. Charles E. Bland), Department of Biology, December 1978.

The fungicide, malachite green, has been found effective in inhibiting growth of the marine fungus, Lagenidium callinectes Couch at a concentration (0.015 ppm) which makes it of potential use in controlling this and other fungi-causing diseases of cultured marine crustacea. In spite of the use of malachite green in this and related areas, little is known of its site and mode of action as a fungicide. In an effort to gain information concerning both these factors, electron microscope observations were made of zoospores of L. callinectes which had been treated with malachite green for varying time intervals. When treated spores were compared with untreated, marked differences were noted in mitochondrial fine structure. A progressive degeneration of the mitochondria was observed in samples taken at 10-minute intervals for one hour. Mitochondria of 60-minute treated spores were swollen, spherical in shape, dense, had fragmented cristae and clumped matrices. Mitochondria of controls were elongate, had regular cristae, and a granular matrix. Other cell organelles appeared unaffected by treatment. Correspondingly, sublethal concentrations of malachite green were found to inhibit O_2 uptake by zoospores of L. callinectes in a manner like 2,4-dinitrophenol. Thus, the site of action of malachite green is probably the mitochondrion where it possibly acts by uncoupling respiratory oxidative processes from phosphorylation.

EFFECTS OF MALACHITE GREEN ON ZOOSPORES OF THE MARINE FUNGUS *LAGENIDIUM CALLINECTES* COUCH

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DEDICATION

This thesis is dedicated to my wife, Denise.

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ABBREVIATIONS

c - cyst

cV - coated vesicle

cwV - cell wall vesicle

Dy - dictyosome

ER - endoplasmic reticulum

F - flagellum

Ft - tinsel flagellum

Fw - whiplash flagellum

G - germ tube

GR - groove region

K - kinetosome

L - lipid

Lg - longitudinal groove

LC - minimum lethal concentration

M - mitochondrion

MB - microbody

MCV - multivesicular complex

N - nucleus

Nb - nuclear beak

NE - nuclear envelope

No - nucleolus

Pm - plasma membrane

Pb - phospholipid

RER - rough endoplasmic reticulum

Vcw - cell wall vesicle

Vp - peripheral vesicle

WEV - water expulsion vesicular apparatus

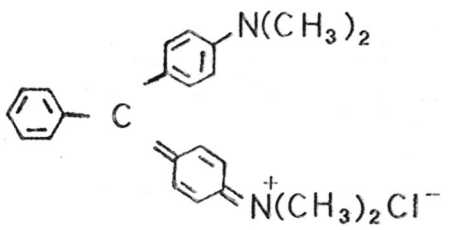
INTRODUCTION

Lagenidium callinectes Couch class Oomycetes, family Lagenidiaceae, was first isolated by Couch (1942) from ova of the blue crab, Callinectes sapidus, collected from Chesapeake Bay, Virginia by Dr. C. L. Newcombe, Director of the Virginia Fisheries Laboratory. Strains of Lagenidium have since been isolated and/or identified from marine crustacea (shrimp, crabs and lobster) being grown at mariculture projects throughout the world. Lightner and Fontaine (1973) isolated the strain L-3b from larvae of the white shrimp, Penaeus setiferus, which were being cultured in Texas. In California, a lagenidiaceous fungus appears to cause a serious disease of cultured eggs, larvae and, occasionally, post larvae of the American lobster, Homarus americanus (Wilson, Fisher, and Shleser, 1976). Armstrong (1976) isolated from larvae of the Dungeness Crab, Cancer magister, a fungus of the genus Lagenidium which caused a 40 percent mortality. Other strains have been isolated from larval shrimp being reared in Mexico, Tahiti, Honduras, and Florida. In many instances, the mortalities attributed to this fungus reached a level of 100 percent (Bland, 1976). In view of the severity and frequency of the epizootics caused by the fungus Lagenidium, questions arise concerning what can be done to prevent or control this infection in cultured marine animals.

As in the culture of terrestrial organisms, the use of fungitoxic chemicals which inhibit the growth of fungi but are not harmful to the cultured organism is one approach to the problem (Bland, 1976). At present the chemical, malachite green, is being used

effectively on an experimental basis in controlling or preventing infection of crustacea with Lagenidium.

Malachite green, 4- [p-(dimethylamino)-α-phenyl-benzylidene)] -2, 5-cyclohexadiene-1-ylidine dimethylammonium chloride, is a triphenyl methane dye that is structurally related to the quinones (Stecher, 1968). It is extremely soluble in water and aqueous solutions are relatively stable and blue-green in color above pH 2.0. The reduced, leuco (colorless) form of the chemical is non-toxic (Stecher, 1959; Weaver, 1959). The structural formula of zinc-free malachite green oxalate, the form most often used for disease control in aquaculture, is:



Currently, malachite green is used primarily as a dye, a biological tissue strain, or as an antispetic; however, it has been used since 1936 as a preventative and curative dip for fungal diseases in fish. In these treatments, the fish are dipped into a solution containing from 2-66 ppm malachite green (Foster and Woodburg, 1936; O'Donnel, 1941; Scott and Warren, 1964). Although effective in controlling fungi, sustained culture of fish would not be possible at these concentrations since it penetrates gill filaments and ultimately

results in death (Scott and Warren, 1964). However, it appears that crustacean culture in the presence of malachite green may be possible since fungitoxic concentrations (0.006-0.01 ppm) of this compound have been shown to have little or no effect on larval survival in the shrimp, Penaeus californiensis, P. stylirostris, P. vanammei or the hatching and development of brine shrimp, Artemia salina (Bland, et al, 1976). Similarly, Johnson (1974), reported malachite green to be non-toxic to larvae of P. duorarum when the compound was used at concentrations of 0.64 ppm and below for a 96-hour period. In several instances, larvae treated with malachite green exhibited higher survival percentages than the controls. Additionally, Fisher, Nilson, Follett and Schleser (1975), have had some success in fungal disease control by dipping larvae of the American lobster, Homarus americanus, into a malachite green solution followed by ultraviolet irradiation of culture water.

Although toxicity studies and bioassays for malachite green (Ruch, 1974) showed that the chemical is very effective at low concentrations, little is known about its site or mode of action or the degree to which it accumulates in the organisms being treated. Knowledge of the site of action of a fungicide in a fungal spore is essential to a proper understanding of its mode of action (Somers, 1966). Preliminary electron microscope studies by Werth and Boiteaux (1967a) on isolated rat liver mitochondria indicated that malachite green caused a structural modification of this organelle. In vitro studies by Werth and Boiteaux (1967b) using tumor cells of mice and yeast cells showed that large doses of malachite green decreased both respiration and glycolysis. The influence of malachite green on the respiratory chain was analogous to that of 2,4-dinitrophenol, which interferes with

electron transport. This indicates that malachite green inhibits respiration (oxidation and phosphorylation) rather than glycolysis (Werth and Boiteaux, 1967).

Malachite green has not been registered for aquatic use by either the Food and Drug Administration or the Environmental Protection Agency because information required for registration--toxicity, mode of action, efficacy, residues and metabolites--is incomplete (Bills, Marking, Chandler, 1977). Therefore, the primary objective of the proposed research is to further investigate the site and mechanism of action of this potentially useful chemical and to determine the degree to which it concentrates in the treated organisms. This information must be obtained before malachite green can be registered by the proper agencies and used to treat organisms commercially reared for human consumption.

MATERIALS AND METHODS

Organisms and Culture Methods

Lagenidium callinectes Couch (Strain L-3b)

The strain of *Lagenidium callinectes* (L-3b) used in the present study was isolated from larvae of the white shrimp, *Penaeus setiferus*, which were in culture at the Gulf Coast Fisheries Center, Galveston, Texas (Lightner and Fontaine, 1973). This and other strains of *L. callinectes* are maintained in the mycological culture collection of the Department of Biology, East Carolina University, Greenville, N.C. Stock cultures of strain L-3b were maintained on PYGS agar medium (Bacto-peptone-1.25 g, yeast extract-1.25 g, dextrose-3.0 g, agar-20.0 g, 1 liter of sea water at 33 ppm total salinity) which was sterilized for 20 minutes at 15 lbs. pressure and 125° C before use. Zoospores were obtained initially by a technique in which split, sterile hemp seeds were placed cut-side down at the perimeter of a 2-4-day-old culture on agar. After allowing about 24 hours for the hyphae to penetrate, the seeds were transferred to 10-15 ml sterile sea water in a Petri dish. Sporulation generally occurred about 48-72 hours after the seeds were transferred to sea water.

To obtain large numbers of zoospores for research, approximately 60,000 zoospores* were inoculated into 50 ml of sterile PYGS broth in a 125 ml Erlenmeyer flask. Spore number was determined via an AO Spencer Brightline hemocytometer. Inoculated flasks were then placed

*shown previously by Ruch (1974) to yield optimum growth and sporulation.

on a horizontal rotary shaker oscillating at 110 rpm. At the end of a 72-hour growth period, the PYGS medium was separated from the mycelia by filtration with a nylon filter. Fifty milliliters of sterile sea water were then added to the mycelia to initiate zoospore formation and discharge. Zoospore discharge occurred 4-18 hours later and continued for a 4-6-hour period.

Penaeus setiferus

To induce spawning and thereby obtain subsequent larval stages of the white shrimp, Penaeus setiferus, an ovigerous female (previously collected from the Gulf of California and held in an enclosed concrete raceway at the University of Arizona's Shrimp Culture Laboratory at Puerto Penasco, Mexico) was placed into a 20-gallon plastic container containing 15 gallons of aerated sea water. Spawning began after about 4 hours and was completed within eight hours. After spawning was completed, aeration was terminated, allowing the eggs to settle to the bottom. Eggs were then siphoned into a one-gallon jar where they were again aerated. After determining egg concentration, dilutions were made to yield a concentration of about 300 eggs/10 ml aliquot.

Procedure for Determination of Residual Malachite Green in Shrimp Eggs

At the University of Arizona Shrimp Culture facility, as a routine prophylactic treatment for Lagenidium callinectes infections, shrimp eggs, after spawned, are immediately dipped for 10 minutes into a 0.1 ppm solution of malachite green before being transferred to hatch tanks. In the present study, the degree of concentration or retention of malachite green in tissues after such a treatment was determined from

larvae which, after washing, were assayed for residual chemical over a 5-day period. Tritiated malachite green was substituted for unlabeled malachite green to enable assay.

Procedures for this experiment were designed by Lightner and Salser (personal communication) of the University of Arizona (Appendix A).

Eggs were collected by pipeting a 10 ml aliquot of eggs and water through a nylon mesh filter stretched tautly over the end of a 2.5 cm long piece of 12.5 cm diameter plastic pipe. The filter containing the eggs was then placed for 10 minutes into a 0.10 ppm carrier-free solution of tritiated malachite green (ICN Pharmaceuticals, Inc., Irvine, California) with a specific activity of 12 mCi/m mole. After dipping, the eggs were washed in four changes of fresh sea water to remove the excess chemical. The total wash time was 20 minutes.

After treatment and washing, the eggs were put into 500 ml beakers containing 400 ml of fresh sea water. The beakers were then placed in a water bath at 28°C. Airstones were put into all beakers for aeration. Hatching occurred about four hours after treatment and 18 hours after spawning. Twenty-four hours after hatching, the nauplii were fed the unicellular diatom, Skeletonema. Mysis and protozoeal stages were fed dinoflagellates of the genus Tetraselmis.

Larval and egg samples were collected at intervals by pouring the contents of a single 500 ml beaker (300 eggs or larvae) through a nylon mesh filter. Subsequently, samples were placed in 5-10 ml of sea water in a vial and frozen.

Sample Preparation for Liquid Scintillation Spectroscopy

Shrimp eggs or larvae were placed in a mortar containing 3 ml Beckman Ready-Solv pre-mixed scintillation counting solution and homogenized with a pestle. The shrimp suspension was quantitatively transferred to a 20 ml scintillation vial and the final volume adjusted to 15 ml. Standards were prepared by adding known amounts of ^{3}H-malachite green to 15 ml of Ready-Solv. Samples were analyzed with a Packard Tri-Carb Scintillation Spectrometer at a gain of 80 percent and window settings of 0.5 to 10.

Malachite Green Treatment of Zoospores

Concentrated stock solutions of zinc-free malachite green oxalate (Fisher Scientific Company, Fair Lawn, New Jersey) were prepared by mixing weighed portions with sea water. One milliliter of a stock solution (0.5 mg/100 ml) was pipeted into 50 ml of a zoospore suspension, yielding a final concentration of 0.01 ppm (LC_{100}). Zoospore suspensions were then placed on a horizontal rotary shaker for treatment times of 0, 10, 20, 30, 40, 50, and 60 minutes. At the end of exposure, zoospores were fixed for electron microscopy.

IN VITRO Fungicide Test

To determine the minimum lethal concentration (LC_{100}) of malachite green, a series of flasks containing 50 ml of PYGS and varying concentrations of malachite green were inoculated with 60,000 spores. The flasks were placed on a horizontal rotary shaker for 72 hours at 25°C. At the end of the 72-hour growth period, the hyphae from each flask were collected with a Buchner funnel on tared Whatman #1 quali-

tative 5.5 cm filter papers. Filter papers were placed in a 70°C drying oven for two days, allowed to cool in a dessicator, and weighed on an H16 Mettler balance.

Oxygen Consumption Determinations

The effect of malachite green on oxygen consumption by zoospores was determined with a Clark oxygen electrode (Yellow Springs Instrument Company). [Analysis of oxygen with this instrument is based on the electro-reduction of oxygen at a platinum cathode surface maintained at 0.8 volts. The reduction of oxygen results in a current flow proportional to the concentration of oxygen. The current is then amplified and recorded by a Servo-Ritter II recorder (Texas Instruments, Inc.).] Prior to the assay, zero and 100 percent saturation points were determined using fresh sea water and sea water with sodium hydrosulfate added to eliminate all free dissolved oxygen. For the assay, 10.0 ml of a zoospore suspension in sea water ($\sim 10^6$ spores) were added to a glass reaction vessel equipped with an O_2 electrode and a magnetic stirrer. Malachite green was added to the reaction vessel via a syringe to yield desired concentrations at specified times. The results were automatically recorded on chart paper.

Electron Microscopy

Zoospores were fixed by adding an equal volume of 6 percent (V/V) glutaraldehyde in sea water (pH 7.6) to a suspension of zoospores in sea water. The spores were fixed for 12-15 minutes after which they were pelleted in a clinical centrifuge ($\sim$1500 rpm). The spores were resuspended in 0.2 M sodium cacodylate buffer (pH 7.6) for one hour

(four changes of 15 minutes each). In all cases, spores were centrifuged after each solution change and resuspended in the next solution. Post-fixation was for 10 minutes in 2 percent (W/V) osmic acid in sodium cacodylate buffer. The spores were then washed for 10 minutes in buffer, dehydrated in a graded ethyl alcohol series and propylene oxide, and infiltrated and embedded in either Araldite 6005 or Epon 812 resin.

Sections were cut on a Reichert OmU2 ultramicrotome with a Dupont diamond knife at a thickness of 600-900 Å and collected on 200 mesh copper grids. Sections were stained with uranyl acetate and Reynold's lead citrate (Reynolds, 1963) for 5-10 minutes each to enhance contrast. Specimens were examined in an Hitachi HS-8 electron microscope operating at an accelerating voltage of 50 KV. Photomicrographs were taken on Kodak Kodalith LR film.

Light Microscopy

Photomicrographs of both living specimens and of specimens that had been fixed in the fumes of 4 percent osmic acid or by immersion in 3 percent glutaraldehyde were taken with a Nikon Microflex camera mounted on a Zeiss WL Research microscope equipped with phase contrast and Nomarski interference contrast optics. Kodak Panatomic-X film was used for all photographs.

Autoradiography

Prior to autoradiographic analysis with the electron microscope, zoospores were incubated for 30 minutes and one hour in sea water containing the LC_{100} (0.015 ppm) concentration of carrier-free tritiated

malachite green (ICN Pharmaceuticals, Inc., Irvine, California) having a specific activity of 12 mCi/m mole. The treated zoospores were fixed, embedded and sectioned as outlined previously. Specimens for analysis were prepared according to the flat substrate technique (Salpeter and Bachmann, 1964). A description of the various steps in specimen preparation is outlined below:

Procedure

1) Glass microscope slides were cleansed with 100% ethanol and wiped dry with Kleenex tissues to remove the oily film and debris. (Kimwipes were not acceptable as they left an oily residue.)

2) Cleansed slides were dipped into a freshly prepared and filtered solution of 0.5% collodion in amyl acetate. The slides were suspended in a vertical position after dipping and the excess collodion blotted off with filter paper. The collodion films showed a silver interference color when stripped off onto a water surface.

3) Ribbons of 600-900 Å sections were placed onto a drop of water on the collodion-coated slides with care taken not to tear the film, which happened if the collodion was touched while wet. Excess water was drained off with tissue paper and the sections allowed to dry on a 60°C slide warmer.

4) Sections were stained with Reynold's lead citrate (Reynolds, 1963) and 5% uranyl acetate for 5-10 minutes each. This was done by placing a few drops of stain over the sections in a covered dish. Excess stain was flushed off with water.

5) Stains were stabilized by vacuum evaporating a 50 Å carbon layer over the sections. The carbon film also provided a flat, even surface for emulsion coating and prevented latent image fading.

6) Slides with sections were emulsion-coated by dipping into Ilford L4 nuclear track emulsion with a silver halide size of 1200-1600 Å (Ilford Nuclear Research, Ilford, Limited, Ilford, Essex, England). The jelled strips of emulsion were dissolved in water in a warm water bath (30°C) and dilutions made until the desired thickness was attained (1500 Å), yielding a purple interference color when stripped off on a water surface. The purple interference color indicated a monolayer of silver halide particles. This step was carried out in total darkness.

7) After emulsion coating, the slides were placed in light-tight microscope boxes and stored in a dessicator at -5°C for the four-month exposure period.

8) After exposure in total darkness, specimens were developed with freshly prepared Kodak Microdol-X at 27°C in total darkness for 3 minutes, rinsed, dipped into Kodak stop bath for 30 seconds, rinsed, and fixed with non-hardening fixer for 3 minutes. Slides were placed in water for 10 minutes prior to stripping.

9) After development, the "sandwich" (consisting of collodion, stained section, carbon layer and emulsion) was stripped onto a water surface and picked up on 200 mesh copper grids.

Specimens were examined with an electron microscope as described previously.

RESULTS

IN VITRO Fungicide Studies

The effectiveness of malachite green in inhibiting growth of Lagenidium callinectes, L-3b, was determined by dry weight analysis of mycelia collected from cultures grown for 72 hours at various concentrations of the chemical. The assay allowed for determination of the lowest concentration required to inhibit growth (production of increased biomass) of spores inoculated into PYGS medium. This index, the LC_{100} or minimum lethal concentration for malachite green, was determined to be 0.015 ppm. The dosage response curve for malachite green is shown in Figure 1.

Although malachite green at 0.015 ppm inhibits growth of Lagenidium callinectes to levels undetectable by dry weight analysis, 0.015 ppm is ineffective in inhibiting spore germination, another commonly used assay for determining fungitoxicity. Zoospores inoculated into PYGS broth containing the dry weight-assay-determined LC_{100} of malachite green exhibit 100 percent germination. Germination of spores occurs also at concentrations of 0.025 ppm and 0.03 ppm. Although zoospore germination occurs at concentrations of 0.015 ppm and higher, germ tubes and hyphae are distorted and growth rates extremely reduced (Figures 2-6).

Even though germination and limited growth is observed at the dry weight-assay-determined LC_{100}, the fungus is unable to sporulate and thus unable to complete its life cycle at 0.015 ppm. When cultures grown for 72 hours at 0.015 ppm are transferred to sea water, they do

not sporulate, whereas control, untreated mycelia, sporulate liberally, often greater than 60,000 spores/ml.

Light and Scanning Electron Microscopy

As viewed with the light and scanning electron microscope, zoospores appear biflagellate, reniform, and measure 9 µm X 15 µm (Figures 7,8,&10). Scanning electron microscopy reveals zoospores to have highly irregular surfaces (Figures 10 & 12). The oppositely directed, heterokont flagella (anteriorly directed tinsel, posteriorly directed whiplash) arise from a raised portion in a longitudinal groove which runs along one side of the spore (Figures 9,10&11).

Transmission Electron Microscopy

Mature, swimming zoospores of L. callinectes are typically ovoid and have a deep, longitudinal groove, giving the spores a reniform appearance when viewed in cross-section (Figures 12). The internal fine structure of L. callinectes, L-3b, zoospores can be seen in figures 12-23. The plasma membrane-bound, ribosome-dense cytoplasm of zoospores contains typical eukaryotic organelles including nuclei, mitochondria, rough endoplasmic reticulum, lipid bodies, dictyosomes, and microbodies, as well as a variety of types of vesicles. The most prominent internal organelle is the 3-4 µm in diameter nucleus and its large, centrically located nucleolus (Figures 16 & 17). The nucleus of the swimming spore is typically beaked or pyriform in shape, the tip of which is squared off and extends toward the kinetosomes (Figures 12,13&21) which lie in the groove region. Microfibrills are often seen connecting the flattened portion of the nucleus to the

kinetosomes. The groove region consists of a reticulum of internal membranes, dictyosomes and dictyosome-derived vesicles (Figure 19). Dictyosomes appear as a stack of flat sacs with numerous vesicles at the periphery (Figure 21). The vesicles are generally of two types--coated and smooth (Figure 19). The reticulate membrane network, dictyosomes, and small vesicles are collectively referred to as the "water expulsion vesicular apparatus" or WEV (Figure 19). The WEV region is generally free of mitochondria, ER, and fibrous vesicles. Endoplasmic reticulum transverses much of the spore's cytoplasm and is often arranged in stacks of several lamella (Figure 22). Most of the ER is of the rough type.

Mitochondria are found throughout the cytoplasm of the zoospores, with the exception of the WEV area. Mitochondria of mature zoospores are 1-2 μm in length and have both filiform and concentric cristae (Figure 23). The electron opaque matrix areas of the mitochondria are homogenous, and often contain numerous cristae.

Peripheral vesicles are the most common of the vesicular inclusions. They are most often found at the periphery of the zoospore and are often seen with fibrous contents extruding to the extracellular environment (Figures 18&20). The number of these vesicles greatly increases during spore germination (Figure 15). Other vesicular inclusions found in zoospores include microbodies (Figure 20), and cell wall vesicles (Figure 17). Packets of presumptive mastigonemes are seen occasionally (Figure 18).

Under nutrient-rich environmental conditions or extreme physical conditions, most zoospores quickly encyst. Encystment is a 10-15

minute process by which flagellated swimming spores are transformed into round, nonflagellated, thick-walled resistant cysts. Encysted spores are ultrastructurally similar to swimming spores except that the cysts have a thick cell wall adjacent to the plasma membrane, are round, and lack flagella (Figure 14). Nuclei of encysted spores are spherical as opposed to the pyriform nuclei of swimming zoospores. Under favorable environmental conditions; i.e., nutrient-rich PYGS broth or agar, encysted spores germinate. Germination usually occurs within 30 minutes via a single germ tube (Figure 15). Other cell organelles of germinating spores are similar to encysted spores except that the number of peripheral vesicles appears to increase during germination.

Electron Microscopy of Malachite Green-Treated Zoospores

Zoospores of L. callinectes incubated in the LC_{100} concentration of malachite green in sea water swim before encysting for time periods equal to those of control, untreated spores. However, when treated, swimming zoospores are compared ultrastructurally to untreated con-controls, significant alterations of the internal fine structure of treated spores are evident.

After a 10-minute exposure to the LC_{100} of malachite green, the only observable alteration in zoospore fine structure is in the mitochondria where, although dimensions are the same as untreated mitochondria, a slight degeneration of cristae and matrix areas is evident. The matrix of treated spores appears clumped and granular (Figures 25-27), whereas that of untreated mitochondria is electron-dense and

homogenous (Figure 23). Nuclei, vesicles, endoplasmic reticulum, microbodies, and golgi appear unaffected by the 10-minute exposure (Figures 25,26,27&29). Encysted spores treated similarly show no detectable changes following treatment (Figure 24).

Zoospores exposed to the LC_{100} concentration of malachite green for 20 minutes retain a typical reniform shape (Figure 30) but show an increased degradation of mitochondrial matrix and cristae (Figures 21,32&34). Many of the mitochondria appear swollen and spherical after the 20-minute treatment; however, outer mitochondrial membranes are unaffected (Figures 30&32). Cytoplasm density after 20 minutes appears equal to controls. Also, plasma membranes and other organelles appear unaffected after a 20-minute treatment.

Zoospores treated for 30 minutes exhibit further degeneration of fine structure (Figures 35-38) with mitochondria again the organelles most obviously altered. A 30-minute exposure results also in swelling of the cisternae of the endoplasmic reticulum in some spores (Figure 38).

Mitochondria of spores exposed to malachite green for 40 minutes exhibit extreme structural modifications (Figures 39&41). After such treatment, mitochondrial matrices are completely "washed out," granular in appearance and electron-transparent. Additionally, only remnants of cristae remain. Nuclear materials, nuclear membranes, lipid bodies, vesicles, dictyosomes and the WEV areas are unaffected by a 40-minute exposure to a 0.01 ppm solution of malachite green (Figures 39-41).

By increasing the incubation time to 50 minutes and 60 minutes, pronounced alterations in zoospore internal fine structure are evident;

however, the spores retain their overall reniform shape (Figures 42-48). The cytoplasm of spores treated for 50 minutes or longer is highly vacuolate (Figures 42&43) and there is extensive swelling of the ER (Figures 42-48). After 50 minutes, all mitochondria are swollen and spherical in shape and many have broken outer membranes (Figures 43,46,47&48). Almost complete dissolution of the mitochondrial matrix and cristae occurs after a 60-minute exposure (Figures 44,46,47&48). However, encysted spores exposed for 60 minutes appear to be only slightly affected by the malachite green (Figure 45).

Autoradiography

EM-autoradiographic analysis of zoospores treated with malachite green (120-day exposure) show metallic silver grains to be free in the cytoplasm, in the nuclei, in the mitochondria, and in the proximity of the plasma membrane. Although the grains were not dense in the spores, none were found in the plastic embedding medium.

Effects of Malachite Green on O_2 Consumption

Zoospores were tested for their ability to consume O_2 in the presence of sublethal (0.005 ppm) and super-lethal (0.025 ppm) concentrations of malachite green. Rates of O_2 uptake by zoospores in the presence of malachite green, expressed graphically, were qualitatively compared to rates of uptake by spores in 33 ppt sea water (Figure 50). An assay of O_2 consumption of sea water with malachite green added at times (arrows) corresponding to additions to assays of O_2 with zoospores was designed to determine whether the chemical itself alters the dissolved O_2 content of the sea water. From this assay, it was

determined that the fungicide does not alter the O_2 concentration of sea water at 0.005 and 0.025 ppm.

Zoospores in sea water consume O_2 at a rate almost twice as fast as the same spores consume O_2 after malachite green is added, yielding a concentration of 0.005 ppm (m = -4.2 vs -2.5) (Figure 50). When malachite green is added (concentration of 0.025 ppm) to the reaction vessel containing spores, O_2 consumption ceases (m = 0). The broken line in Figure 50 indicates the rate of O_2 uptake by spores if not exposed to malachite green over a 25-minute period. Uptake versus time over this time period is linear (m = -4.2).

Retention of Malachite Green in Shrimp Larvae

Immediately after treatment with a 0.1 ppm solution of ^{3}H-malachite green for 10 minutes, and washing, each larva retains approximately 0.5 nanogram malachite green. The level of malachite green quickly decreases over a 24-hour period to about 0.1 ng where it becomes constant over a 5-day period (Figure 49). (It should be noted that, due to the limited quantity of labeled chemical, this was a preliminary experiment involving only one run.)

Figure 1. Dosage-response of *Lagenidium callinectes*, L-3b, to malachite green.

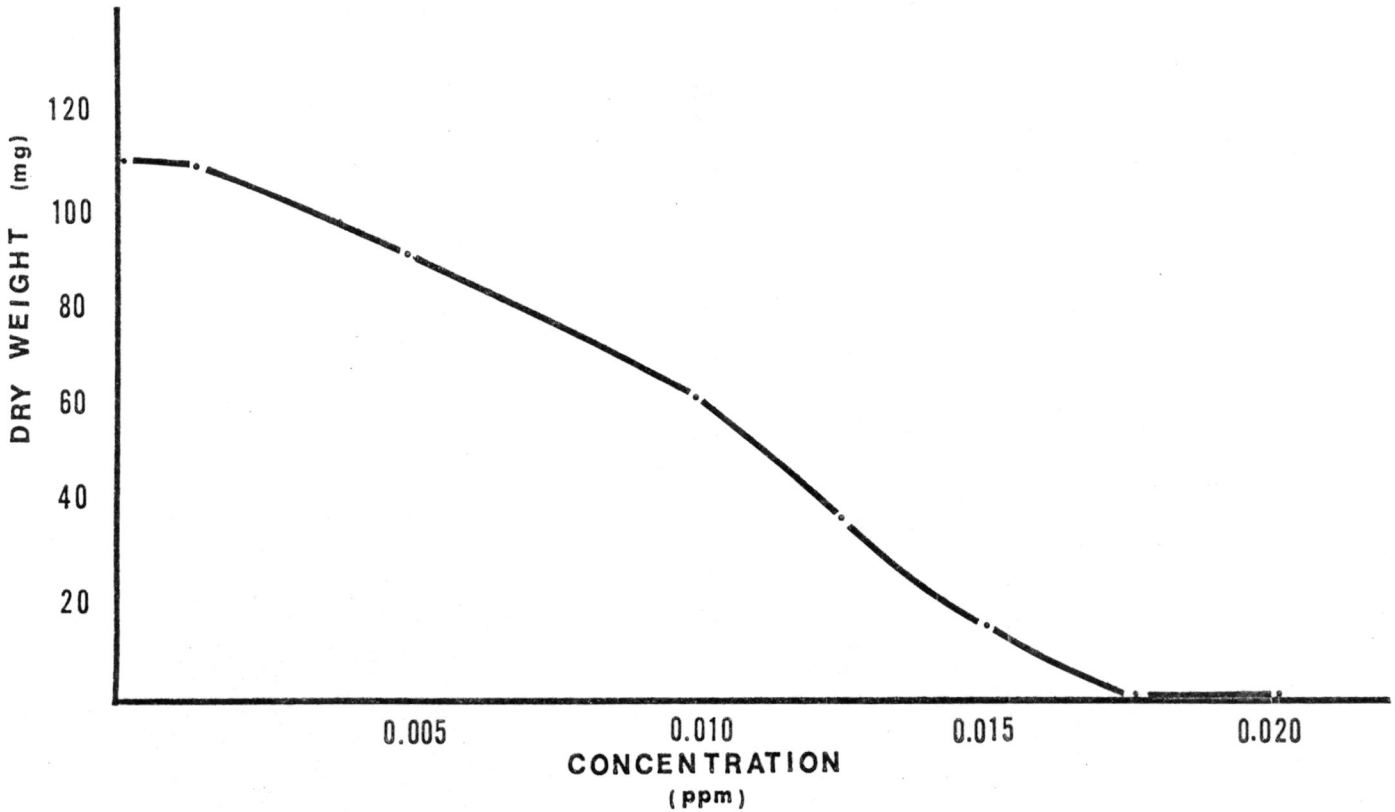

DRY WEIGHT (mg)
120
100
80
60
40
20
0.005
0.010
0.015
0.020
CONCENTRATION
(ppm)

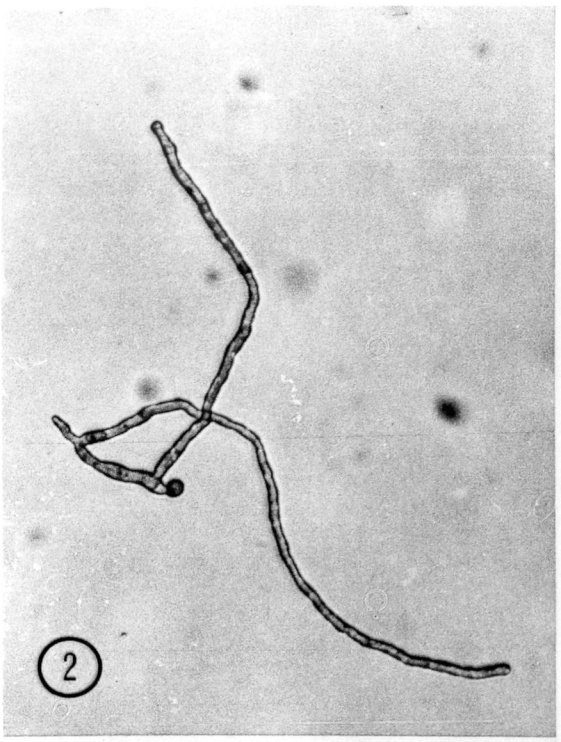
Figure 2. Germinating spore of Lagenidium callinectes 12 hours after inoculation into PYGS liquid broth.

Figure 3. Hyphal growth after 24 hours in PYGS liquid broth. Note cyst (C) and germ tube (Gt).

Figure 4. Germinating spore 24 hours after inoculation into PYGS liquid broth containing LC_{100} of malachite green (0.015 ppm).

Figure 5. Extent of hyphal growth after 72 hours in PYGS broth containing malachite green at a concentration of 0.025 ppm.

Figure 6. Extent of hyphal growth 72 hours after inoculation into PYGS containing LC_{100} of malachite green.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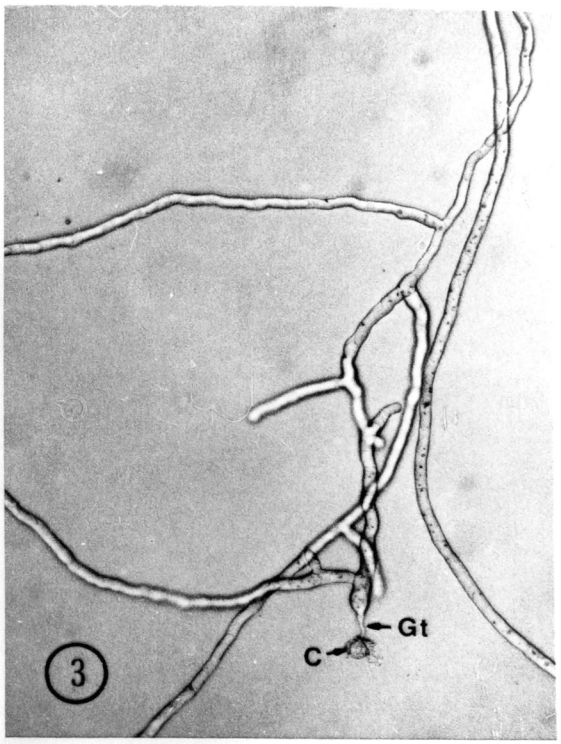
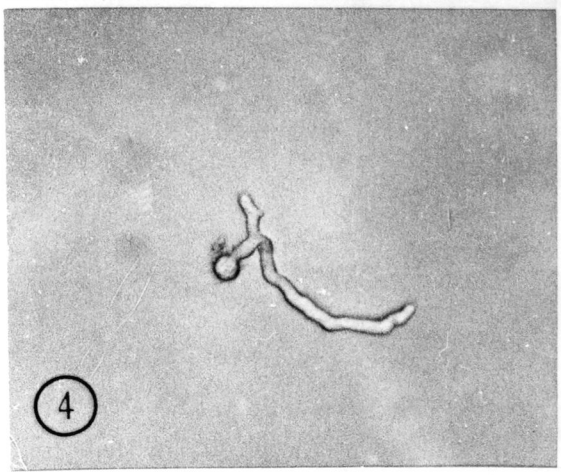
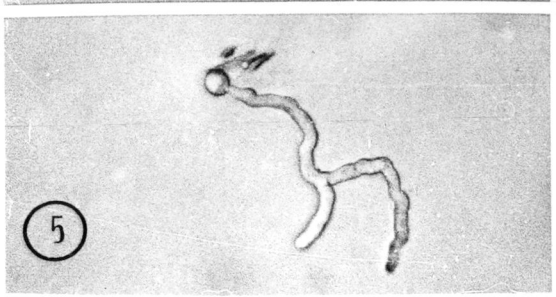

Gt
C
3


4


5

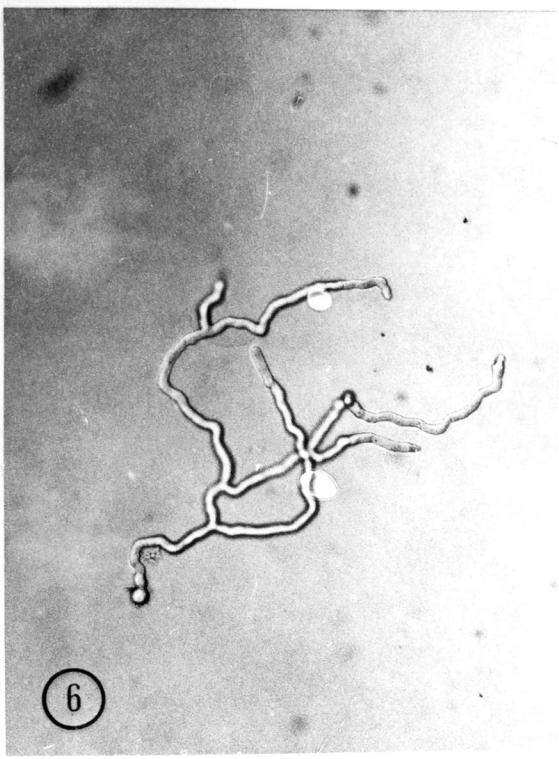

6

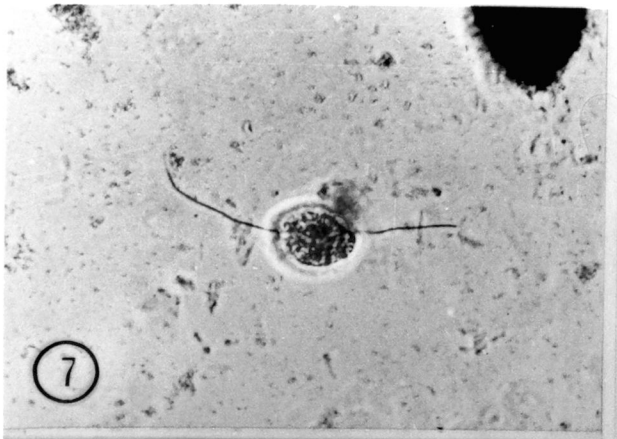
Figure 7. Light micrograph of a Lagenidium zoospore, illustrating typical oomycete flagellation (anterior tinsel, posterior whiplash). Courtesy of Dr. C. E. Bland. X 800.

Figure 8. Light micrograph of a Lagenidium zoospore exhibiting secondary reniform nature. X 2250.

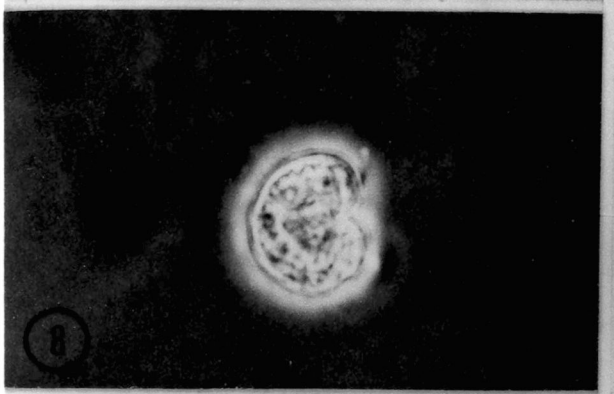
Figure 9. Negatively stained whole amount of a zoospore showing anterior tinsel flagellum (Ft) and posterior whiplash flagellum (Fw).

Figure 10. Scanning electron micrograph of a zoospore showing nature and surface morphology. Courtesy of Carol Z. Lunney. X 7200.

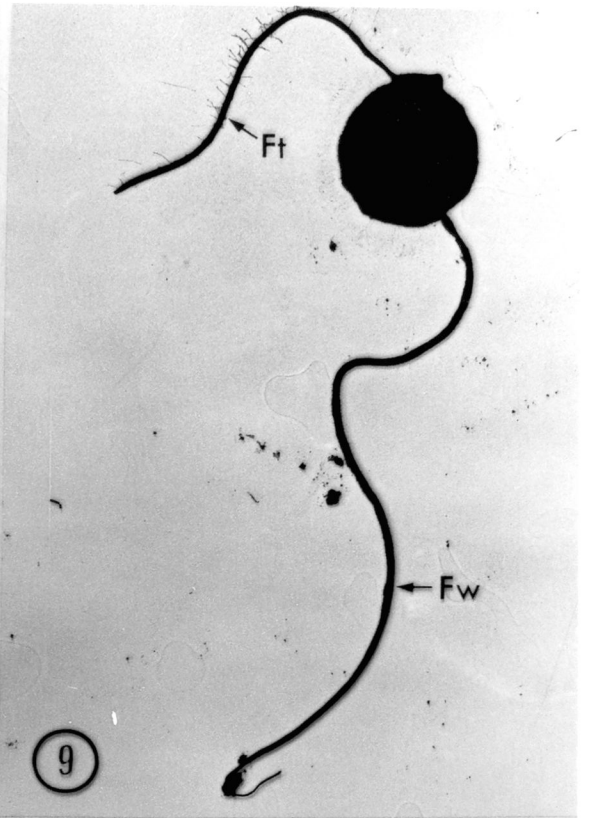
Figure 11. Scanning electron micrograph of a zoospore showing longitudinal groove region (Lg) and point of flagellar attachment. Courtesy of Carol Z. Lunney. X 7200.



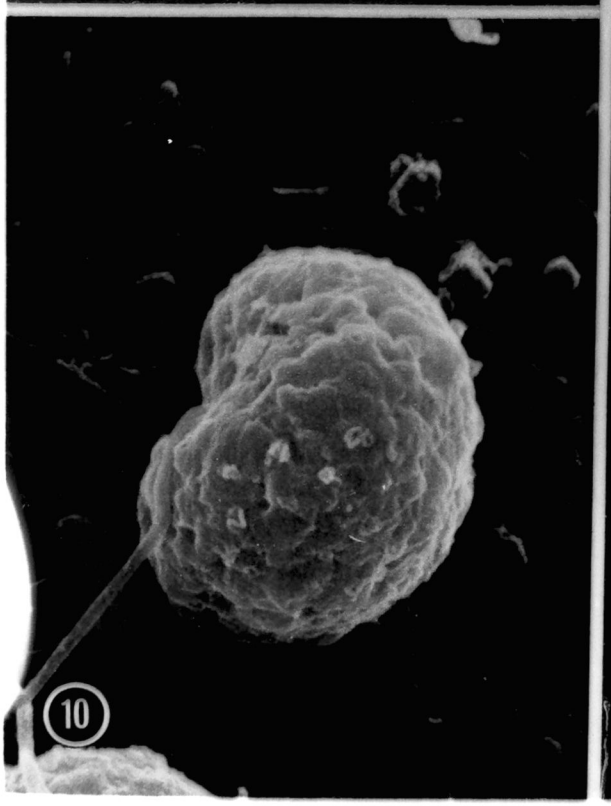
7



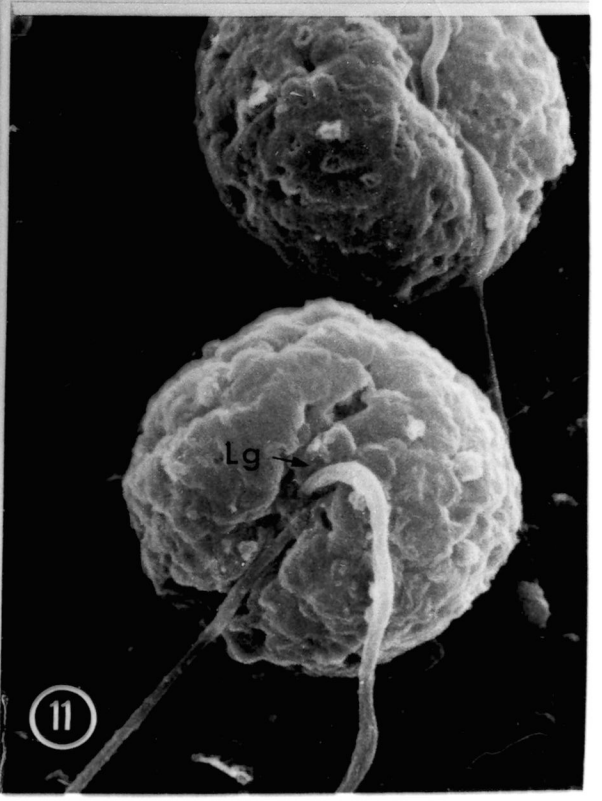
8



9



10



11

Figure 12. Median cross section of a typical secondary zoospore of Lagenidium, L-3b, showing reniform nature and normal internal fine structure. Note pyriform nucleus (N) extending toward kinetosomes (K), mitochondria (M), lipid (L), microbodies (MB), cell wall vesicles (Vcw), and groove region (GR). X 18,600.

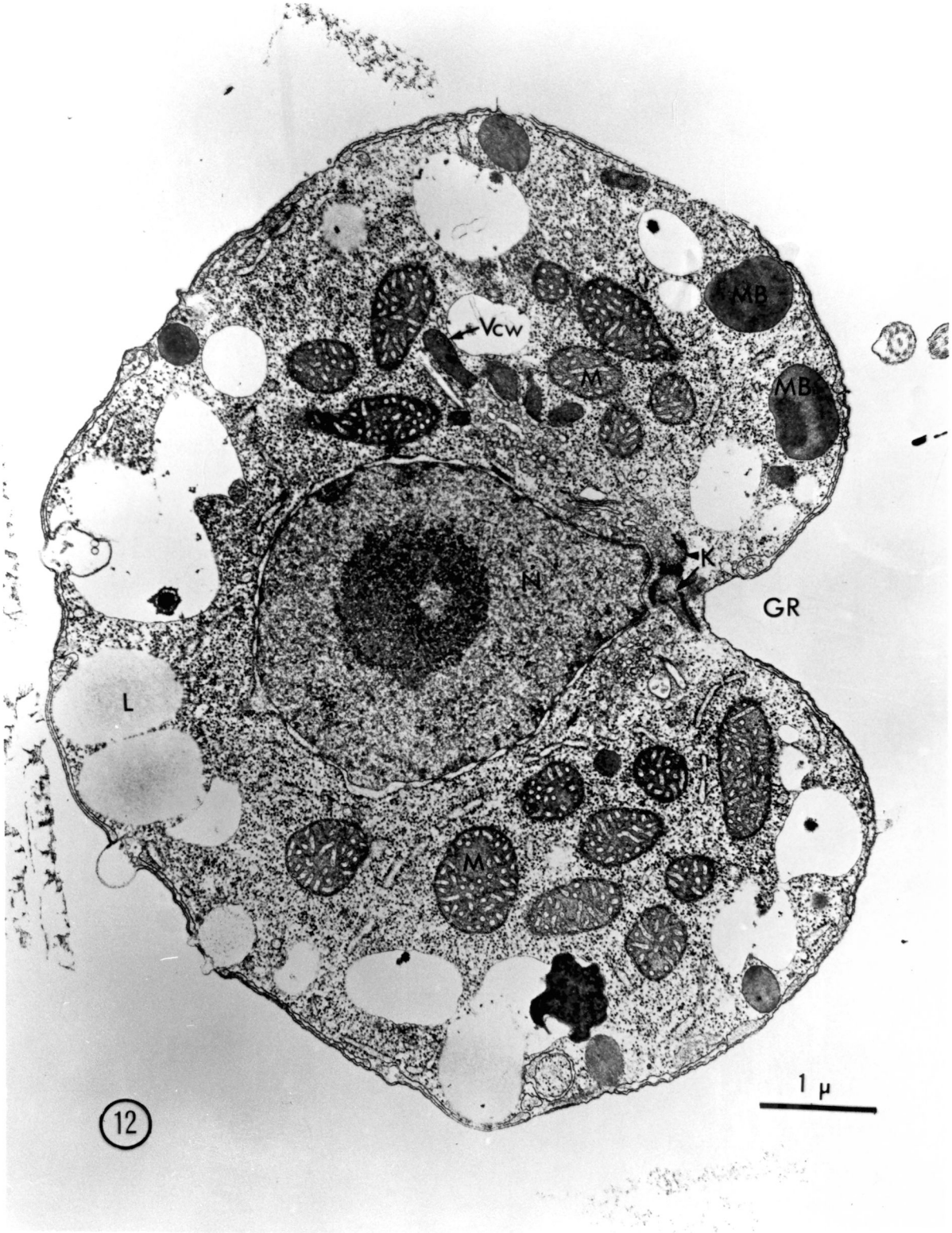
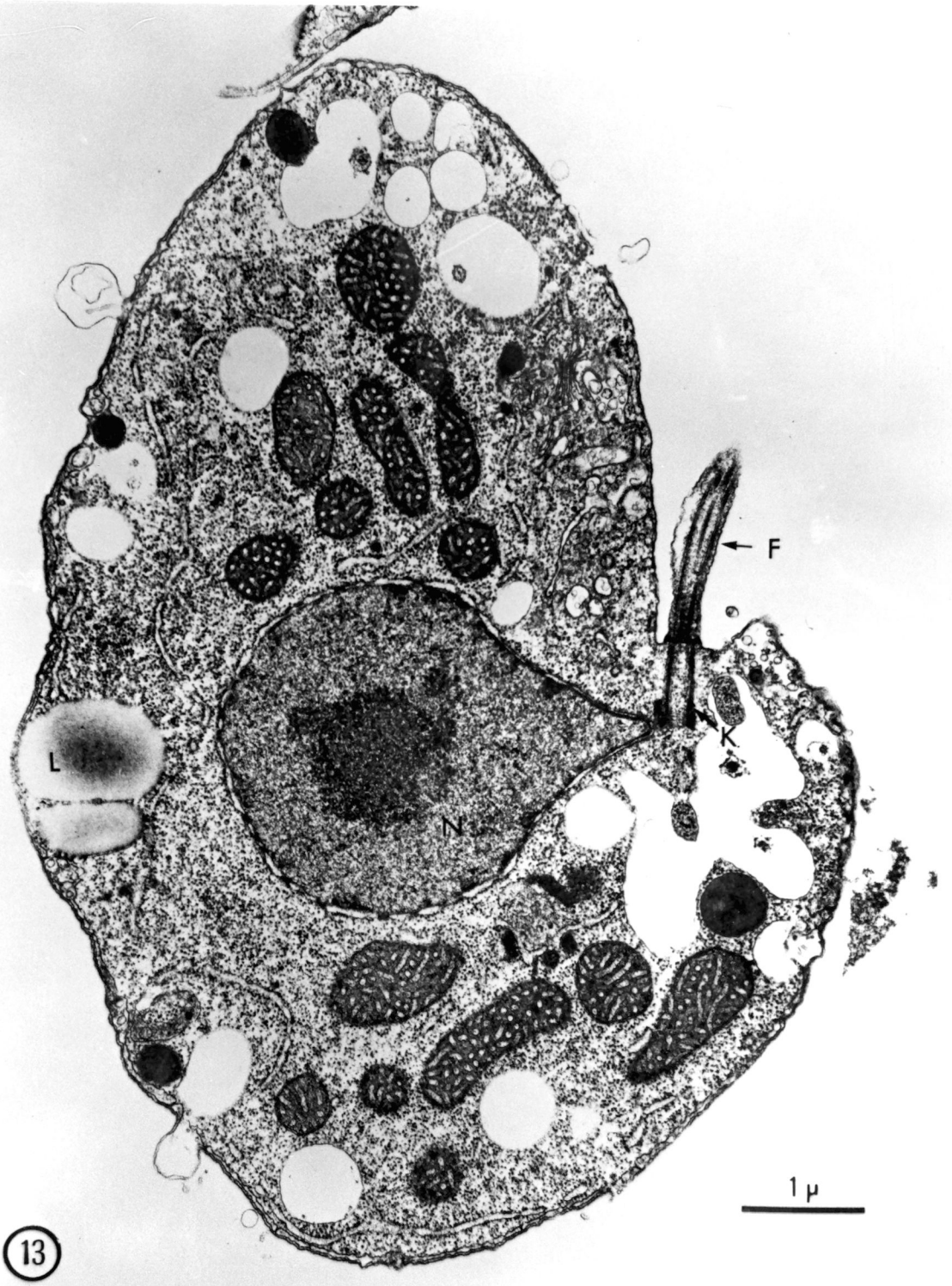
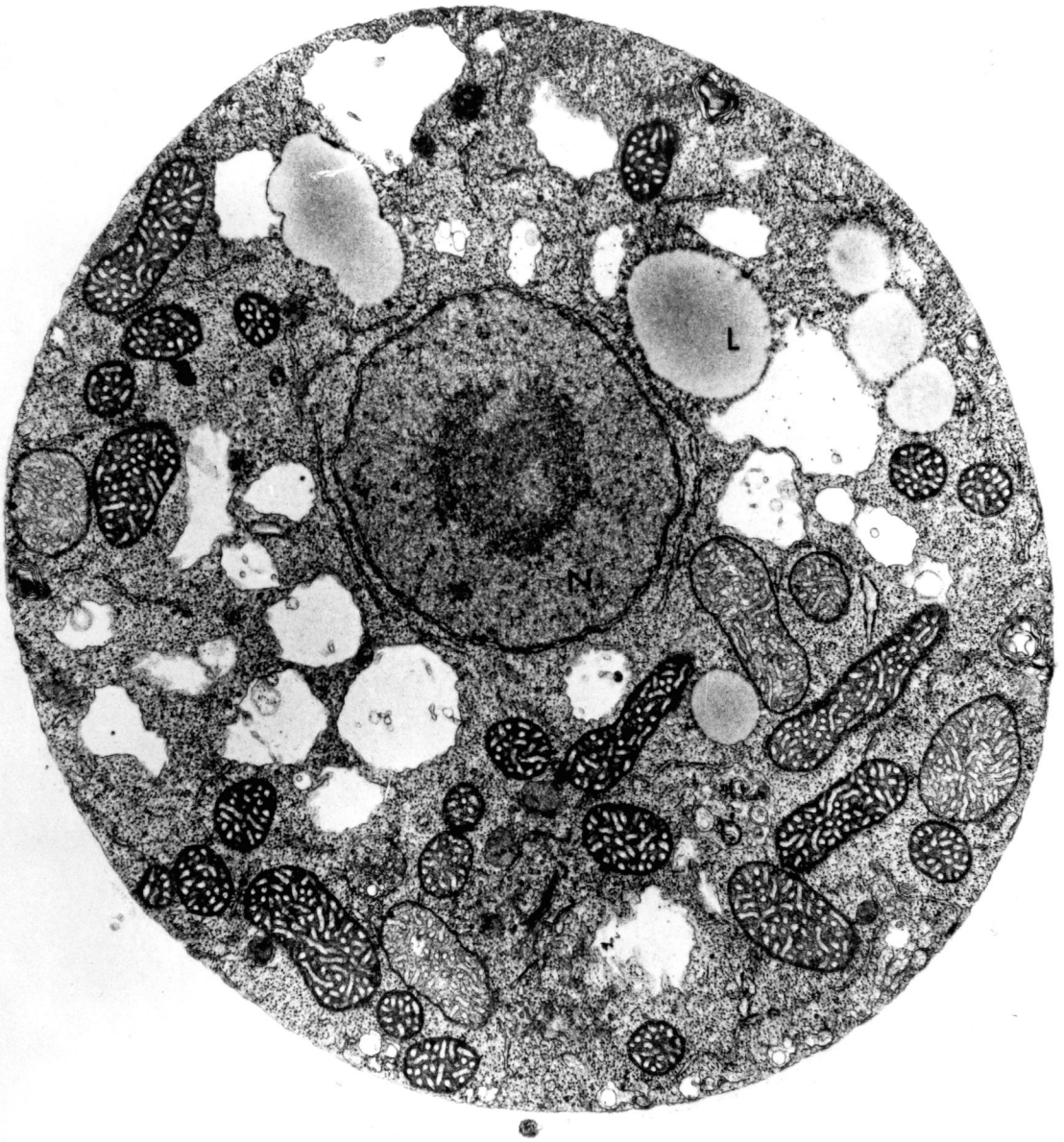

MB
Vcw
M
MB
K
N
GR
L
M
1 μ
12

Figure 13. Median longitudinal section of a typical Lagenidium zoospore showing association of pyriform nucleus (N), kinetosome (K), and flagellum (F). Note general density of cytoplasm. X 17,800.



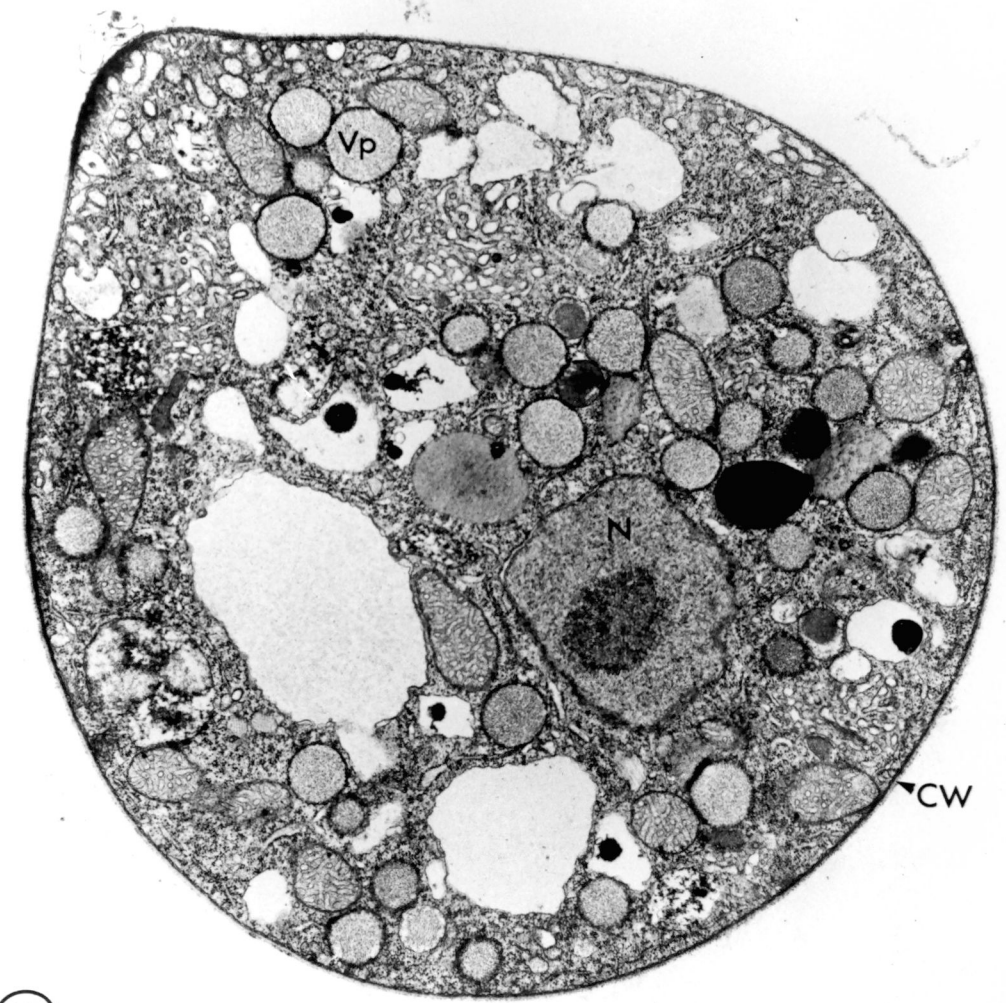
13

Figure 14. Median section of an encysting zoospore. Note spherical nature and lack of cell wall. X 19,000.



14

Figure 15. Electron micrograph of a spore in early stage of germination. Note germ tube and large number of peripheral vesicles (Vp). X 20,500.

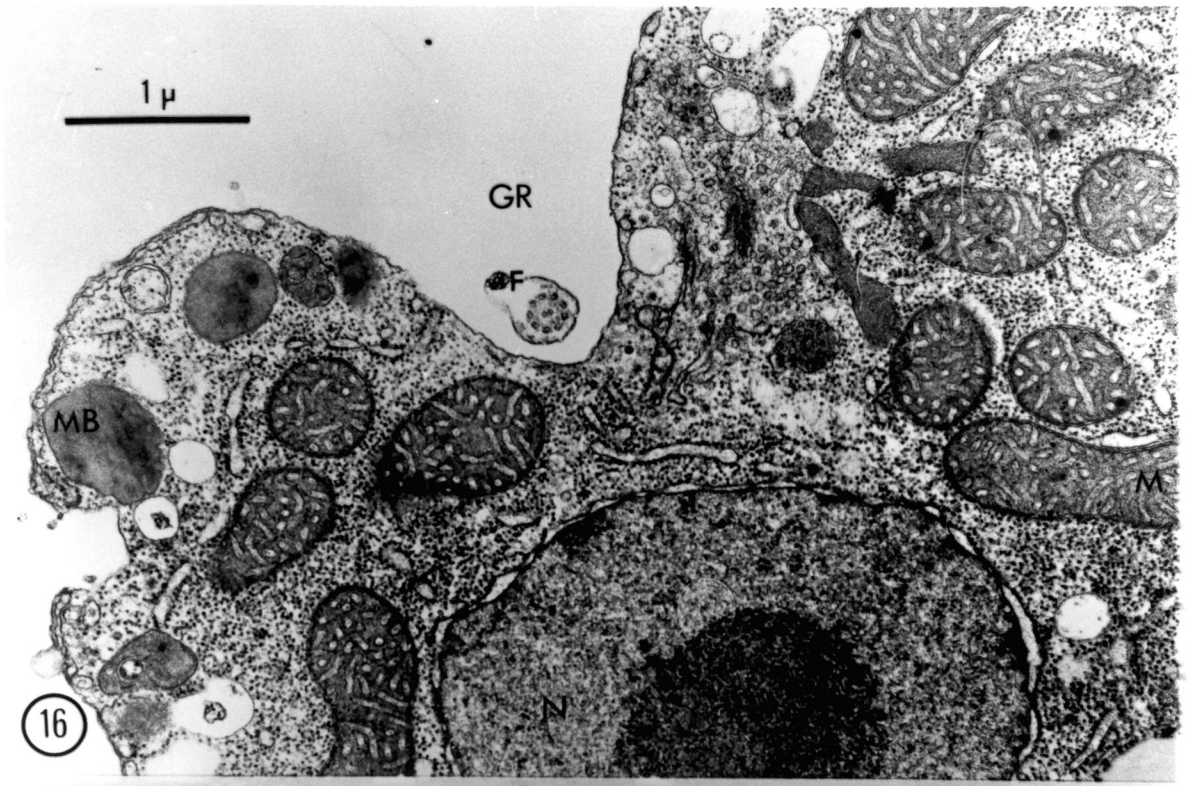


15

1.0μ

Figure 16. Electron micrograph of a cross section of a zoospore showing groove region (GR), and cross section of a flagellum (F). X 23,200.

Figure 17. Typical nucleus with centrically located nucleolus (No). Note presence of bar body (arrow) associated with nuclear envelope. X 27,500.


1 μ
GR
F
MB
M
N
16

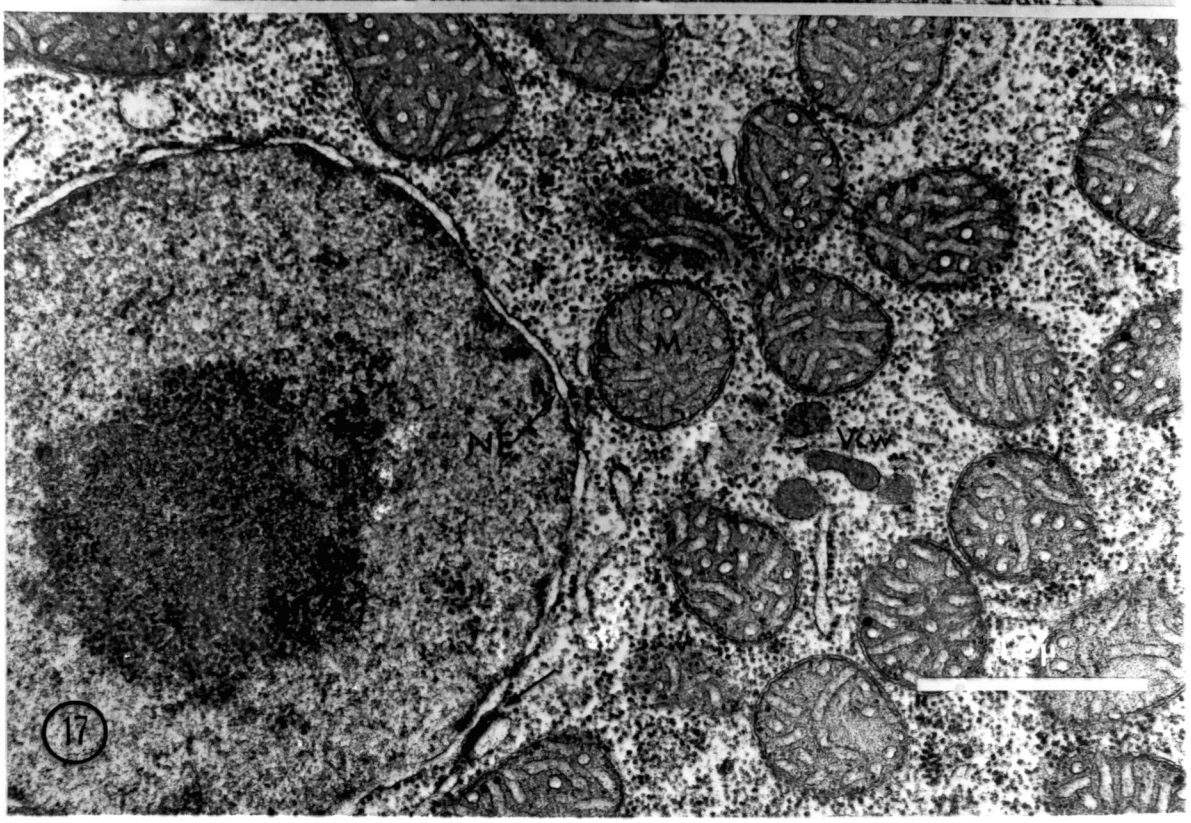
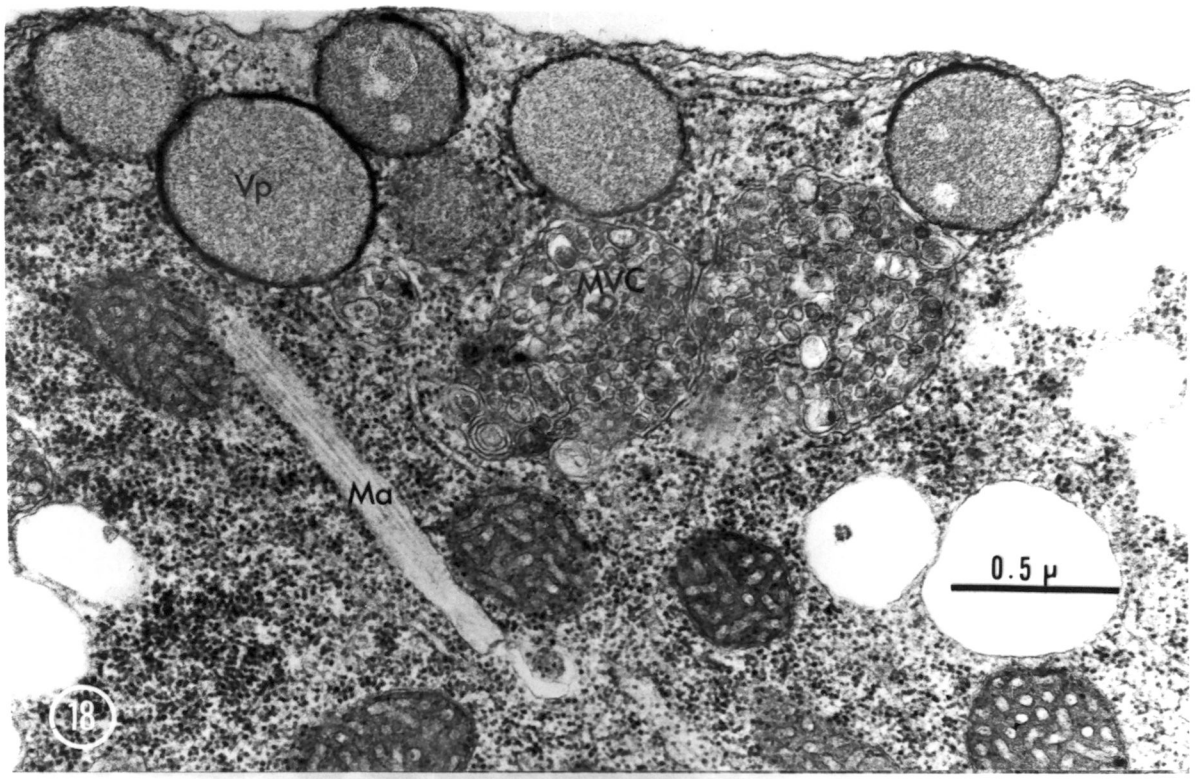

M
NE
17

Figure 18. Common vesicles found at periphery of a zoospore include peripheral vesicles (Vp) and multivesicular complexes (MCV). Note also the presence of a mastigoneme or tinsel packet (Ma). X36,000.

Figure 19. Groove region of a zoospore with associated water expulsion vesicular apparatus (WEV), associated dictyosomes (Dy) and dictyosome-derived coated vesicles (cV). X40,000.


Vp
MVC
Ma
0.5 μ
18

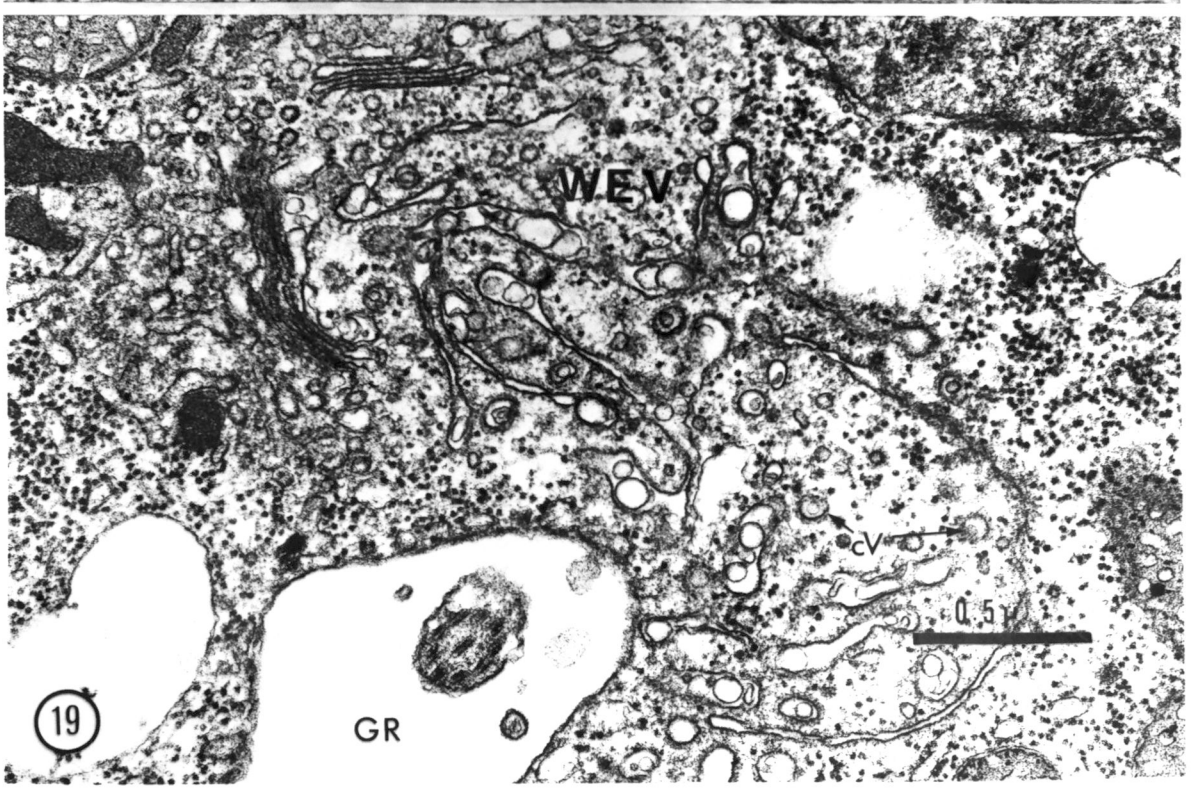

WEV
cV
0.5 μ
GR
19

Figure 20. Periphery of a zoospore with lipid globules (L), microbodies (MB), and peripheral vesicles (Vp). Note double plasma membrane (PM) and lack of cell wall. X 40,000.

Figure 21. Association between nucleus (N), kinetosome (K), and flagellum (F). X 21,000.

Figure 22. Layers of rough endoplasmic reticulum (RER) in peripheral cytoplasm of a zoospore. X 36,000.

Figure 23. Longitudinal sections of typical mitochondria with both concentric and filiform cristae. Note density of matrix areas. X 39,600.

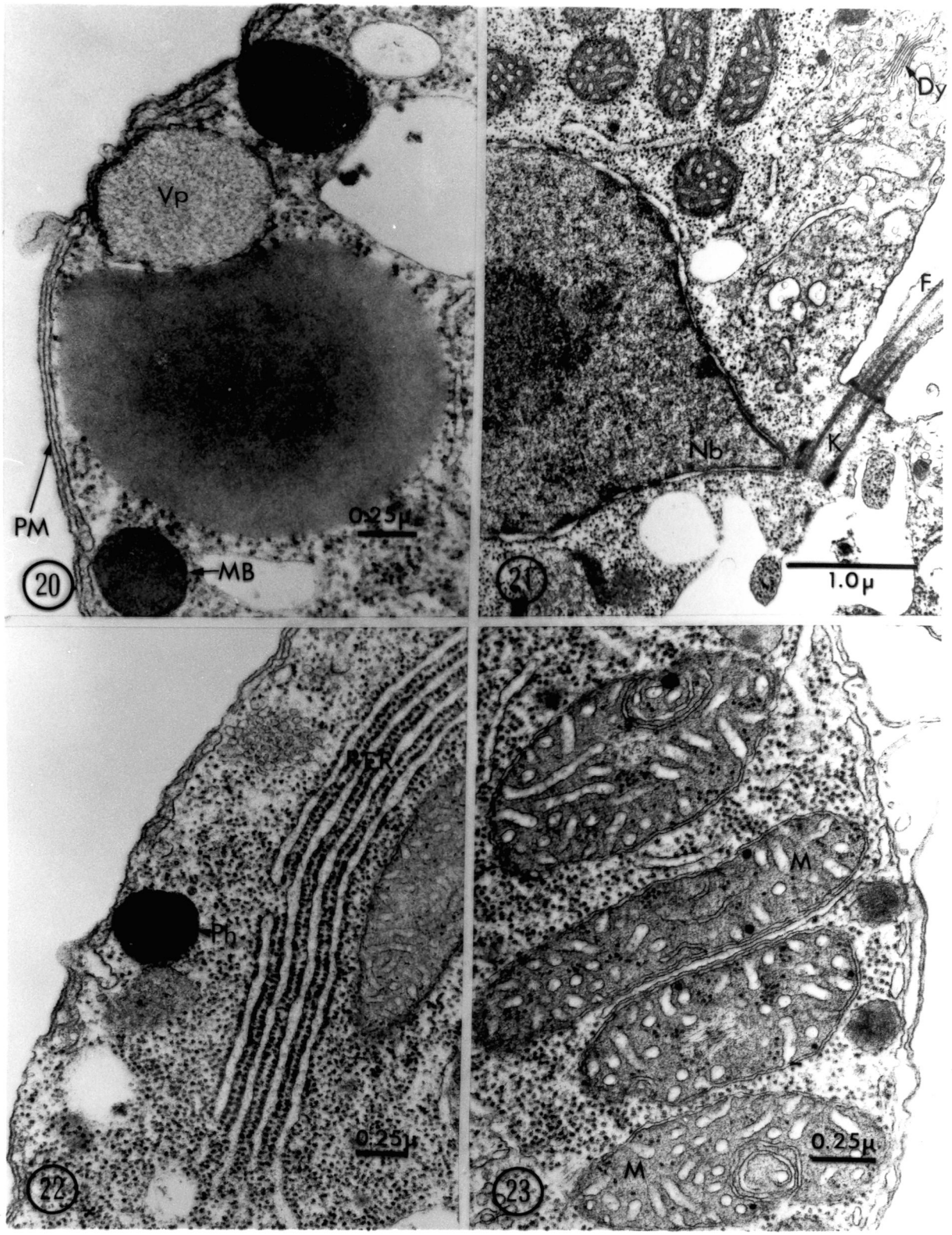
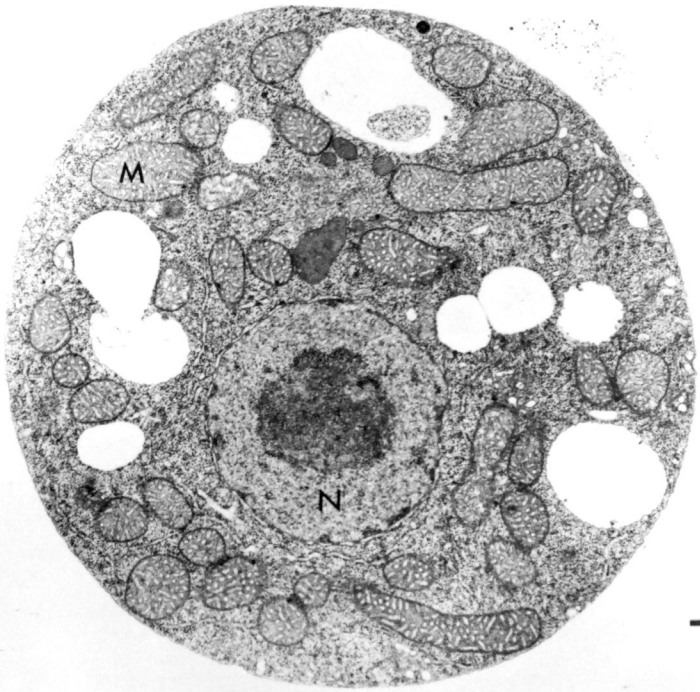
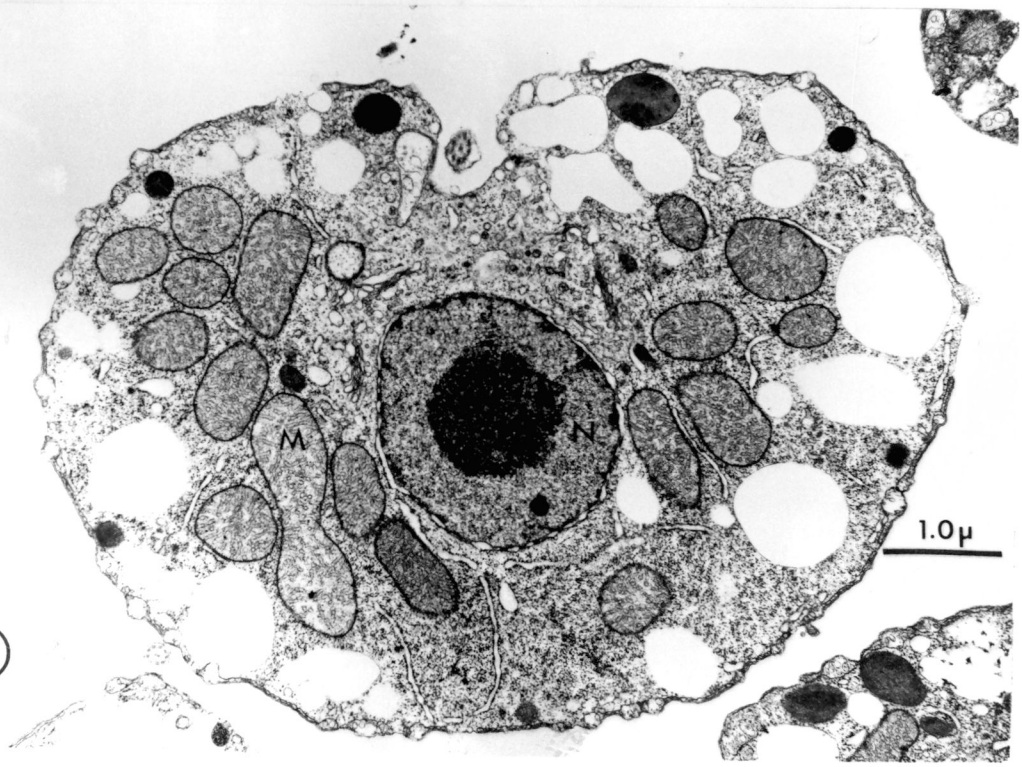

Vp
PM
MB
0.25μ
20
Dy
F
Nb
K
1.0μ
21
RER
Pb
0.25μ
22
M
M
0.25μ
23

Figure 24. Electron micrograph of a spore after ten-minute exposure to malachite green at LC_{100} (0.01 ppm) in early state of encystment. X 11,800.

Figure 25. Median cross section of a zoospore treated for ten minutes at the LC_{100} of malachite green. Note "washed out" appearance of mitochondrial matrix areas. X 015,800.



24



25

Figure 26. Electron micrograph showing organelles after 10-minute exposure to malachite green. X 25,000.

Figure 27. Appearance of nucleus (N) and mitochondria (M) after 10-minute exposure. X 22,000.

Figure 28. Appearance of mitochondria (M) after 10-minute exposure. Note "washed out" appearance of matrix areas. X 35,000.

Figure 29. Dictyosome (Dy) after 10-minute treatment. X 36,000.

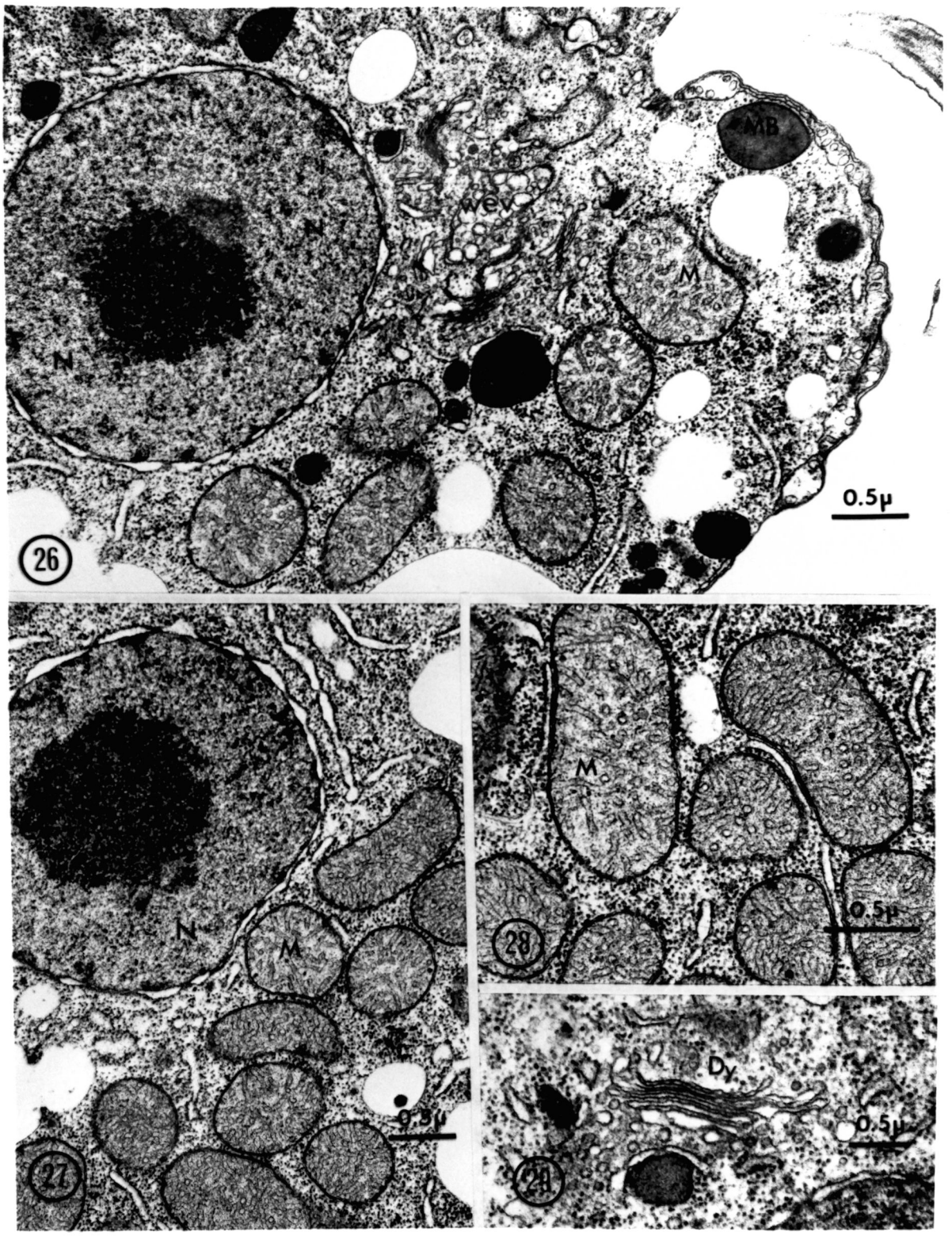

MB
wev
M
N
0.5μ
26
N
M
0.5μ
27
M
0.5μ
28
Dy
0.5μ
29

Figure 30. Zoospore of Lagenidium callinectes, L-3b, after 20-minute exposure to malachite green at LC_{100}. Note similarity to untreated spores except for changes in mitochondrial structure. X 13,500.

figure 31. Electron micrograph showing the internal structure of a Lagenidium zoospore exposed to malachite green for 20 minutes.

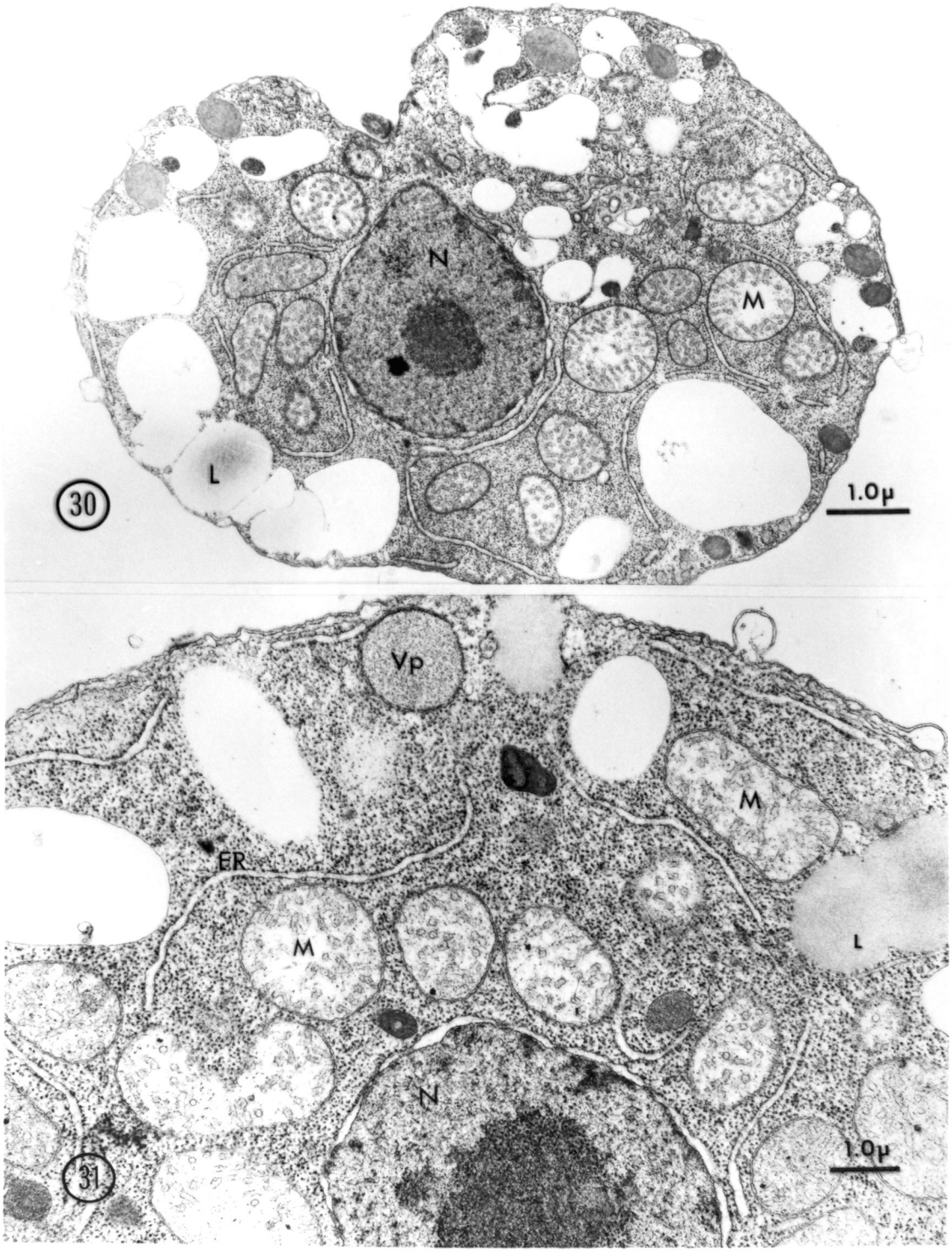
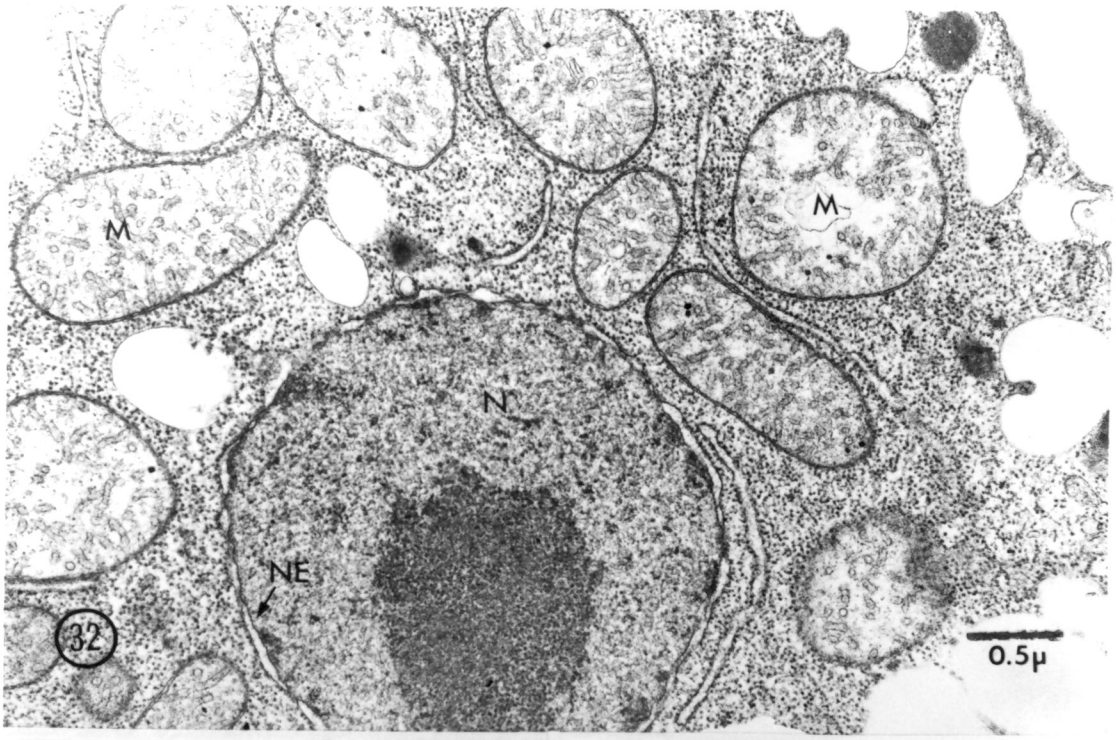
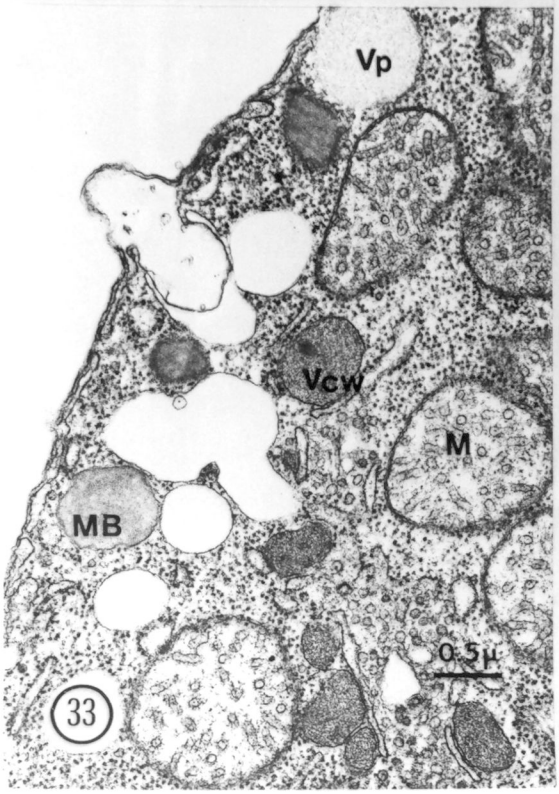

N
M
L
1.0μ
30
Vp
M
ER
M
L
N
1.0μ
31

Figure 32. Appearance of nucleus (N) and mitochondria (M) after a 20-minute exposure to malachite green at 0.01 ppm. X 24,500.

Figure 33. Periphery of a 20-minute treated zoospore with microbodies (MB), cell wall vesicles Vcw) and peripheral vesicles (Vp). X 30,000.

Figure 34. Mitochondria (M) after a 20-minute exposure to malachite green. Note increasing "washed out" appearance of matrices. X 28,800.


M
M
N
NE
32
0.5μ


Vp
Vcw
M
MB
0.5μ
33

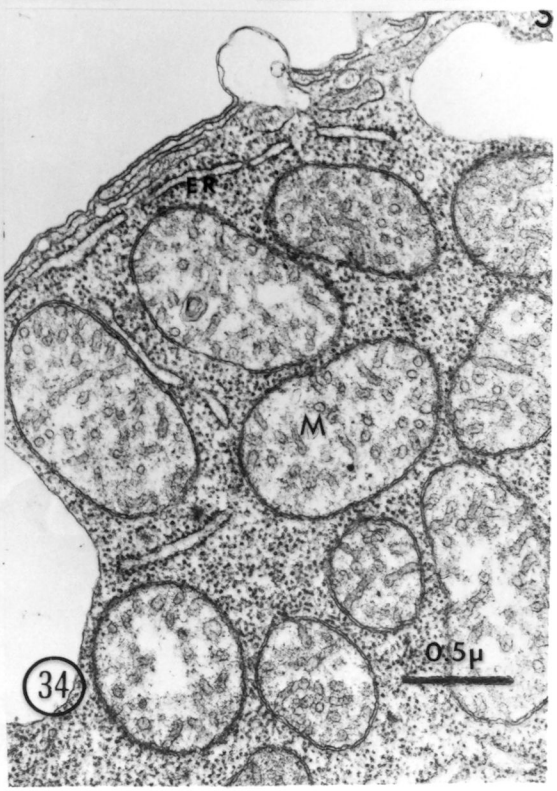

ER
M
0.5μ
34

Figure 35. Groove region of a 30-minute treated zoospore of Lagenidium showing nuclear beak (Nb) and water expulsion vesicular apparatus (WEV). X 28,000.

Figure 36. Dictyosome (Dy) and microbodies (Mb) after 30-minute exposure to malachite green at LC_{100}. X 35,100.

Figure 37. Internal fine structure of a zoospore after a 30-minute exposure to the LC_{100} of malachite green. X 14,000.

Figure 38. Appearance of mitochondria (M) and endoplasmic reticulum (ER) after a 30-minute exposure. Note spherical, swollen appearance of mitochondria and degeneration of the matrix and cristae. X 26,100.

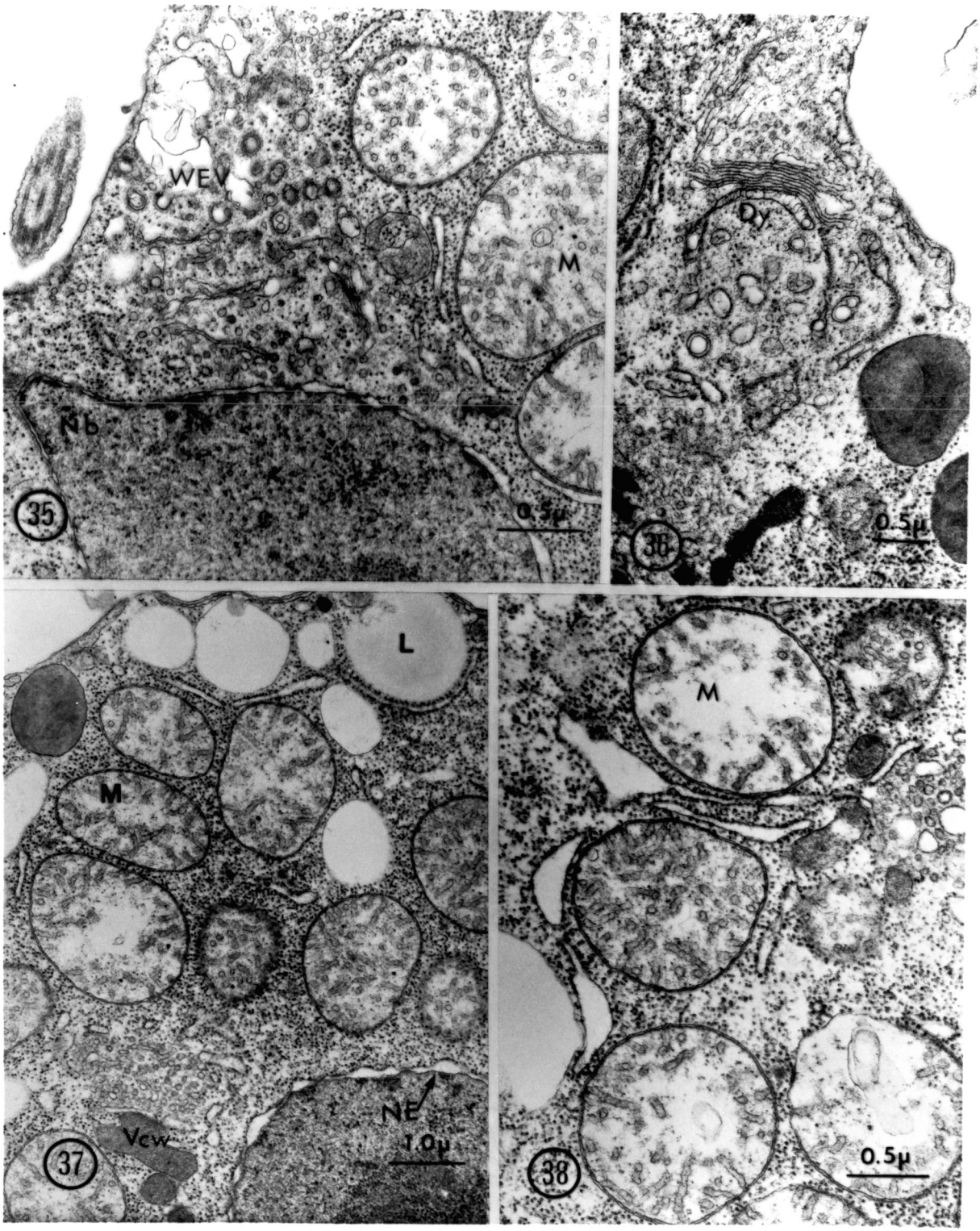

WEV
M
Nb
35
0.5μ
Dy
36
0.5μ
L
M
NE
Vcw
37
1.0μ
M
38
0.5μ

Figure 39. Electron micrograph of a portion of a zoospore exposed to the LC_{100} of malachite green for 40 minutes. Note "normal" appearance of nucleus and endoplasmic reticulum and swollen, degenerated mitochondria. X 22,000.

Figure 40. Periphery of a 40-minute treated zoospore with lipid globule (L) and peripheral vesicles (Vp). X 22,400.

Figure 41. Mitochondria after a 40-minute exposure. X 24,000.

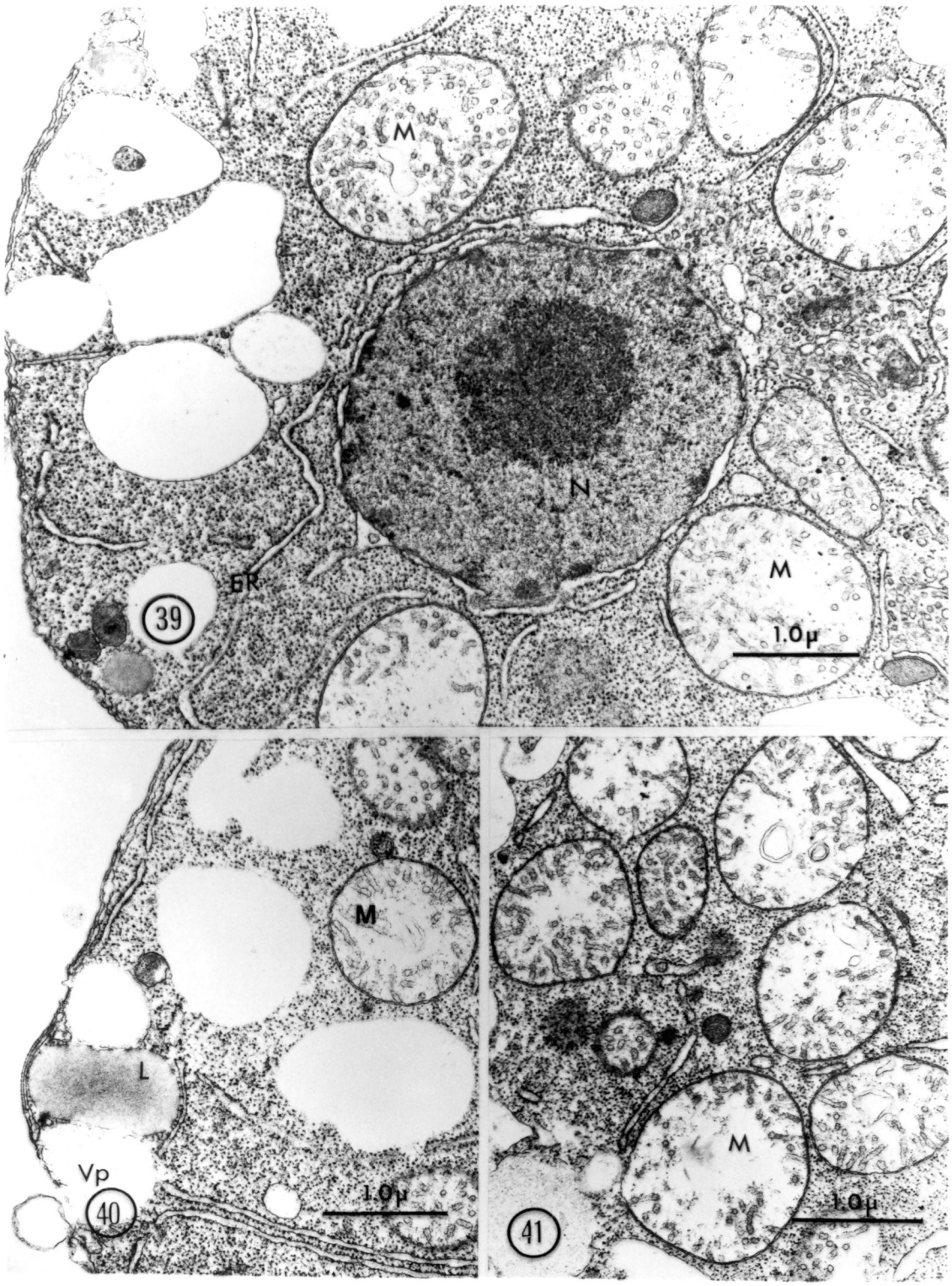
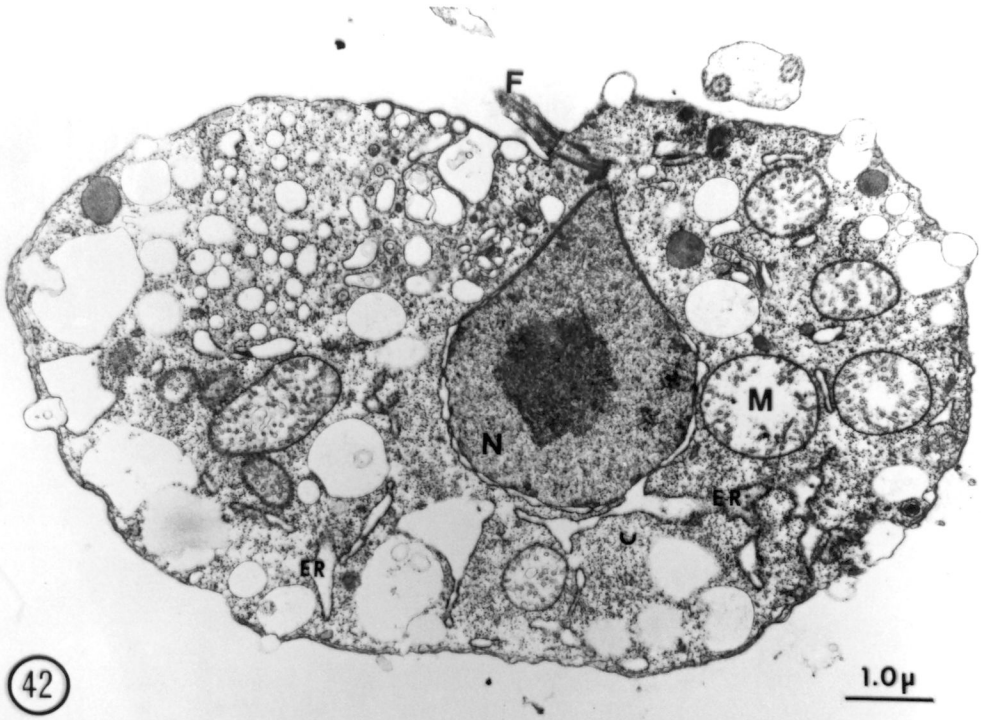
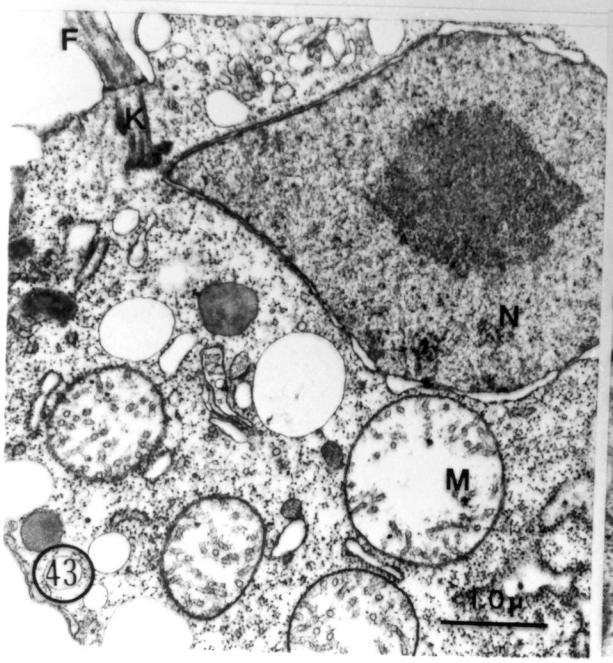

M
N
M
GR
39
1.0μ
M
L
Vp
40
1.0μ
M
41
1.0μ

Figure 42. Longitudinal section of a zoospore which has been exposed to minimum LC_{100} of malachite green for 50 minutes. Note increased number of vacuoles, swollen endoplasmic reticulum and degenerated mitochondria. X 13,000.

Figure 43. Nuclear beak and flagellar attachment region of a zoospore exposed for 50 minutes to LC_{100}. X 18,000.

Figure 44. Periphery of a 50-minute treated zoospore with peripheral vesicles (Vp), mitochondria (M), and microbodies (MB). X 24,000.


F
M
N
ER
ER
42
1.0μ


F
K
N
M
43
1.0μ

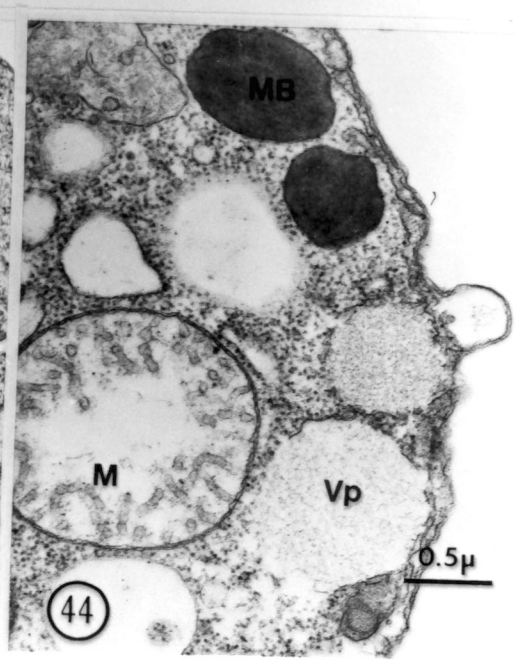
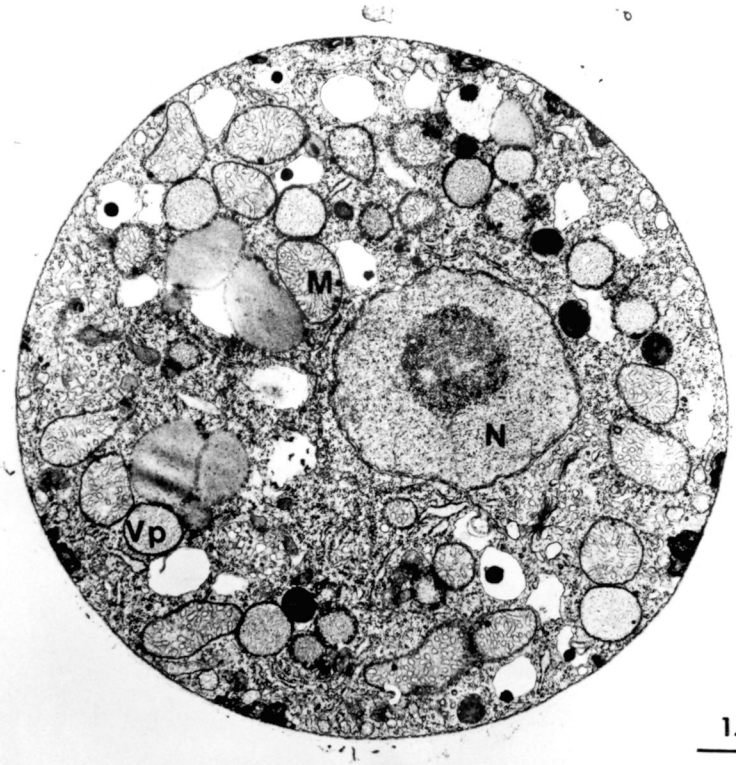

MB
M
Vp
0.5μ
44

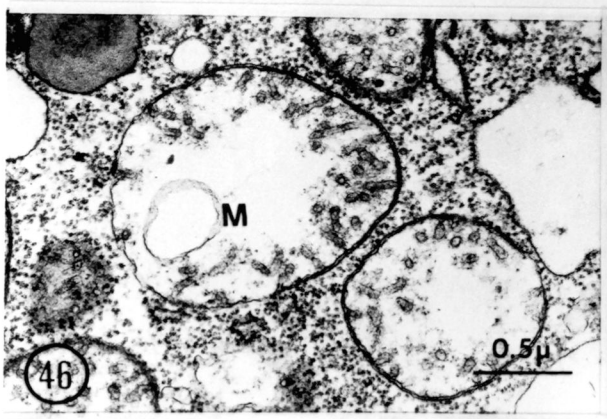
Figure 45. Encysted spore of Lagenidium callinectes, L-3b, after 60-minute exposure to malachite green at LC_{100}. Note that mitochondria of encysted spores are not as degenerated as those of swimming spores. X 12,100.

Figures 46-48. Electron micrographs showing highly degenerated mitochondria of zoospores after a 60-minute exposure to LC_{100} of malachite green. X 25,200.



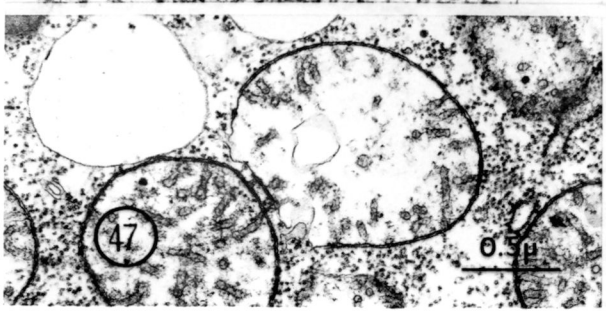
45

1.0μ



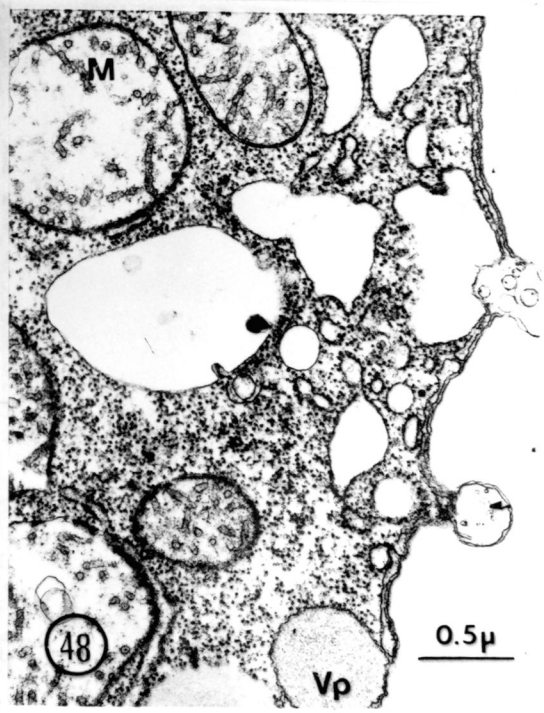
46

0.5μ



47

0.5μ



48

0.5μ

Figure 49. Effect of time on amount of malachite green remaining in shrimp larvae after a 10-minute dip into a 0.1 ppm solution.

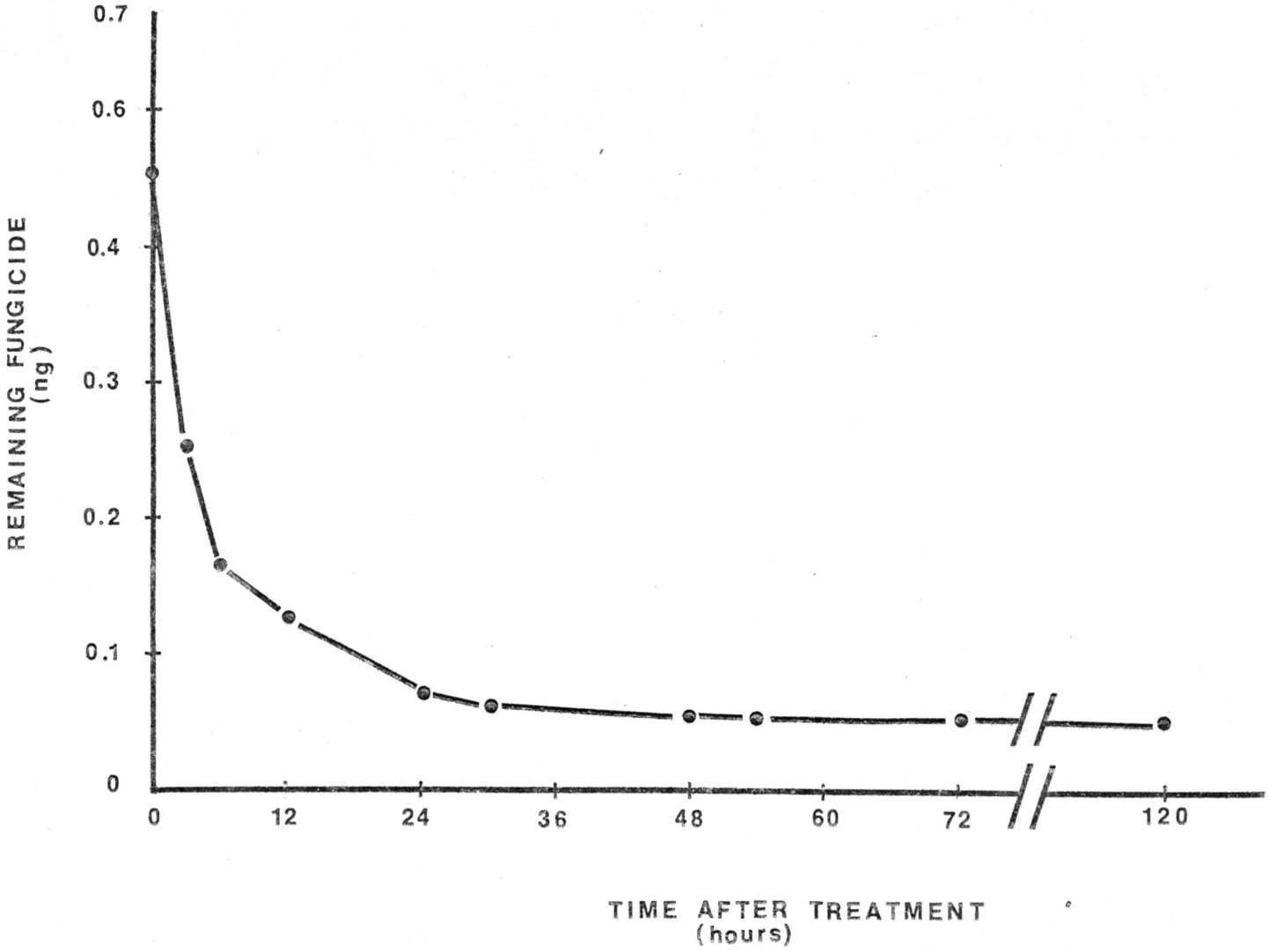
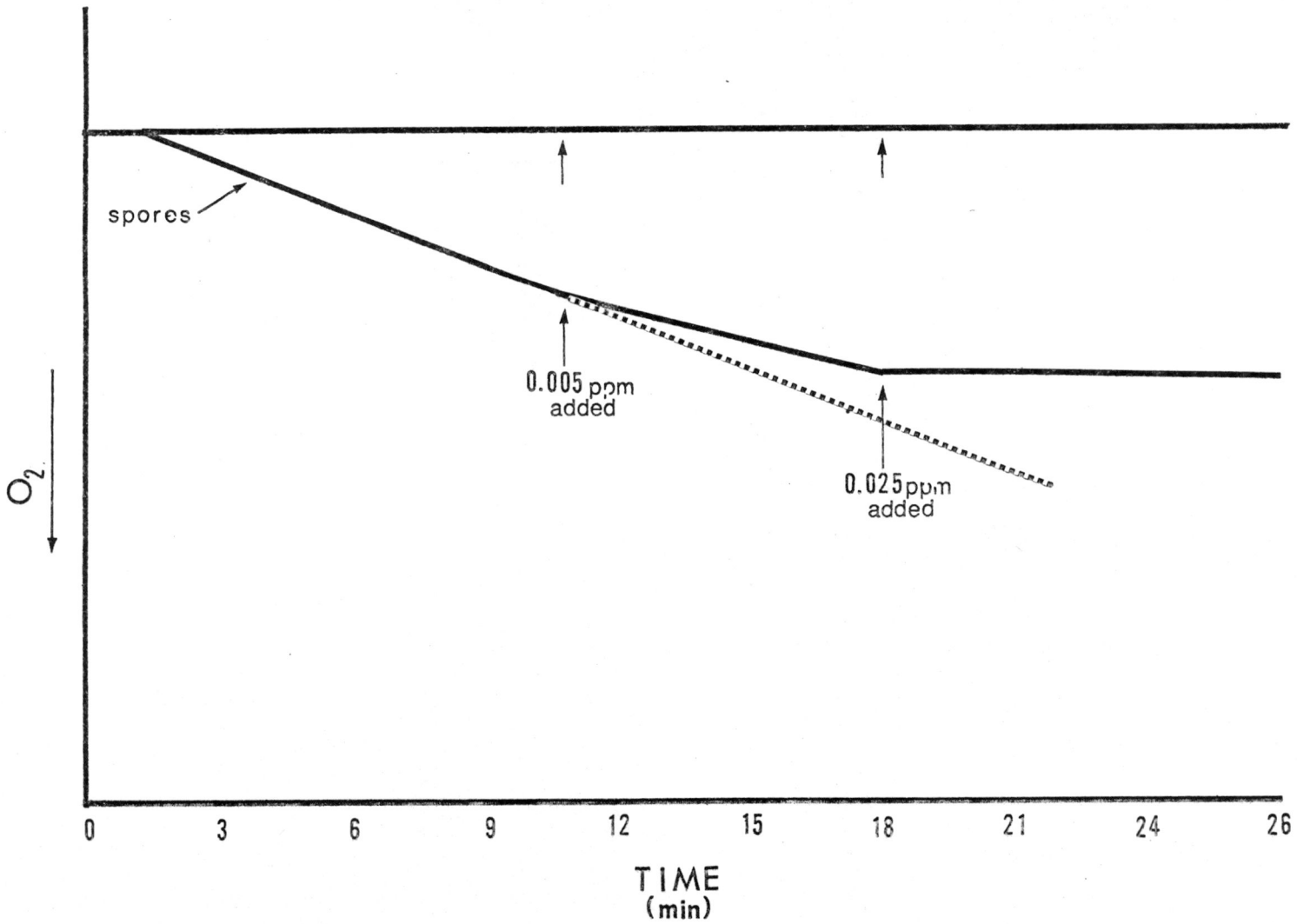

REMAINING FUNGICIDE (ng)
0.7
0.6
0.4
0.3
0.2
0.1
0
0
12
24
36
48
60
72
120
TIME AFTER TREATMENT (hours)

Figure 50. Effect of malachite green on O_2 uptake by Lagenidium callinectes, L-3b, zoospores.


spores
0.005 ppm added
0.025 ppm added
O_2
0
3
6
9
12
15
18
21
24
26
TIME (min)

DISCUSSION

Currently, fungicides play an essential role in terrestrial agriculture in that they are used to control fungal diseases of over one-half of the world's crops (Ordish and Mitchell, 1967). Culture of marine crustacea is similar to terrestrial agriculture in that in both cases organisms are monocultured under high density situations. Due to the frequency of contact between pathogens and cultured organisms, pathogens may spread rapidly when introduced in such systems. To date, the successful culture of crustacea has been severely hampered by outbreaks of the fungal diseases. It appears, therefore, that just as in agriculture, fungicides may play an important role in the success of crustacean culture.

Mariculture of shrimp, crabs, and lobster, under dense, less than optimal environmental conditions, depends partly on the culturist's ability to control fungal pathogens in hatching and rearing systems. Although many possibilities for control exist, the most practical and economical approach appears to be the use of fungicides (Bland, et al, 1976). Many terrestrial fungicides have been tested (Ruch, 1974); however, one that has been used for many years in fisheries--malachite green--has been found in the present study and by Ruch (1974) to be effective at approximately 0.01 ppm in controlling the growth and development of one of the most common pathogens, Lagenidium callinectes. However, before this chemical or other fungicides can be used commercially, a proper knowledge and understanding of how they function is necessary for the development

of safety regulations and licensing. Studies of malachite green's effects on O_2 uptake and ultrastructure of L. callinectes spores have been completed in order to determine its site and mode of action. The following is a discussion of results of these studies in relation to similar work by other investigations.

IN VITRO Studies

Although the bacteriostatic properties of malachite green were known earlier (Stecher, 1968), this chemical was first used to control fungal diseases of fish in 1933 by Foster and Woodbury (1936). Since then, due to widespread usage and effectiveness, this compound has become known as the fish culturist's "holy water" (Wood, 1968; Warren, 1971). In early tests, a 3-minute dip was used (100 μg/ml) to treat phycomycetous fungal infections of green and eyed trout eggs and large mouth bass fry. The dip treatment proved effective in controlling fungal diseases; however, it was thought that the dip was more injurious to the fish than the diseases being treated (Fish, 1938). Sustained culture was tried at much lower concentrations; however, even at those concentrations ($\sim$ 2 ppm) which were needed to control certain fungal parasites, the chemical penetrated gill filaments and resulted in death (Scott and Warren, 1964). Most hatcheries today that use malachite green as a fungicide or bacteriocide, employ a constant-flow siphon to maintain a uniform malachite green concentration of 0.25 ppm to 0.33 ppm (Burrows, 1949; Davis, 1963; Nelson, 1974).

Malachite green has proved to be effective at very low concentrations (0.01-0.006 ppm) in present studies and by Ruch (1974)

in controlling growth and development *in vitro* of the fungus, *L. callinectes*. Sustained culture of shrimp larvae may be possible at low concentrations of the chemical since it apparently has no effect on shrimp larval survival at concentrations required to control the fungus (Bland, et al, 1976). In agreement with this, malachite green has been found effective in controlling *L. callinectes* infections in shrimp cultured at the University of Arizona (Lightner, personal communication). In these treatments, eggs, immediately after being spawned, were dipped into a 0.01 ppm solution of malachite green for 10 minutes. This treatment had no effect on the hatching and development of the eggs and was successful in preventing infections with the fungus, *L. callinectes*. Thus, it appears that malachite green, due to its low LC_{100} , ease of application, solubility, and minimal effect on crustacean development, would be the fungicide of choice in controlling or preventing infections of eggs and larvae of aquatic crustaceans with *L. callinectes*. However, the Fish and Wildlife Service of the U.S. Department of the Interior has stopped efforts to try to register the chemical [Mayo Martin, personal communication (Appendix B)] as recent research has produced increasing evidence that the chemical has potential teratogenic, mutagenic, oncogenic and carcinogenic properties [Director, FWS, personal communication (Appendix A)] . Thus, malachite green is not presently suitable for use as a fungicide in aquaculture except on an experimental basis.

Site of Action of Malachite Green

Knowledge of the cellular site of action of a fungicide is essential to a proper understanding of the mode of action of the chemical (Somers, 1966). Alterations of fine structure produced by a fungicide provide information from which probable sites of action can be proposed. Cellular changes produced by a fungicide are easily observed via an electron microscope. This technique was adapted to determine the cellular changes in L. callinectes zoospores induced by treatment with malachite green at the LC_{100}.

This technique has been used to study the effects of thiocyanatopyrazole derivatives on a dermatophytic fungus, Trichophyton mentagrophytes (Vanning, Dall'Olio, and Giori, 1974), in an attempt to single out the mechanism of action of the chemical. The effect of antibiotics on the ultrastructure of hyphae has been observed also by Gale (1963a) and Bent and Moore (1966), as has the effect of antifungal agents on Candida albicans (Gale, 1963b; Gale and McClain, 1964). Richmond, Somers and Millington (1966) examined the effect of the fungicide, Captan, on conidia of Neurospora crassa to confirm hypotheses concerning the mode of action and site of action of the chemical. Ruch (1974) used the electron microscope to study the effect of Captan on zoospores of Lagenidium callinectes.

The fine structure of malachite green-treated zoospores of L. callinectes indicates that the probable sites of toxic reaction are the mitochondria. Werth and Boiteux (1967c) observed rat liver mitochondria after they were incubated in malachite green and found that they were swollen, had granular, clumped matrices, and fragmented

cristae. Outer membranes were intact. Similar results are noted from observations of mitochondria of L. callinectes zoospores that have been exposed to the LC_{100} concentration of malachite green for more than 10 minutes. Mitochondria are the first organelles to be affected by the malachite green treatment and the only ones to be extensively modified. From this and the observation that the chemical reduced rates of O_2 uptake, it is concluded that the mitochondria are the primary cellular sites of action of malachite green. Since swelling and loss of normal structural integrity were noted, the dye may affect permeability of outer membranes or cause degeneration of structural elements. Malachite green probably inhibits growth of fungal spores by inhibiting mitochondrial function; more specifically, by uncoupling oxidation and phosphorylation.

Autoradiography

Zoospores treated with ^{3}H-malachite green were prepared for autoradiographic analysis and examined with the electron microscope in an attempt to determine the chemical's site of action and to determine if the chemical concentrated in any particular cytoplasmic subunit. However, silver grains (indicating the presence of a radioactive malachite green molecule) are found randomly distributed throughout the cytoplasm in nuclei, mitochondria, over membranes and microbodies. Thus, the chemical probably does not concentrate in any particular organelle. Although the developed grains were not dense, none were found in the plastic embedding medium, indicating that the grains resulted from ^{3}H-malachite green disintegrations rather than from background sources. It is also possible that the exposure time

was not long enough or that the chemical was modified during radioactive preparation.

Mode of Action

The exact mode of action of malachite green is unknown (Anonymous, 1973). Fisher (1967) proposed that malachite green and other triphenylmethane dyes (crystal violet, parafuscin, and new magenta) were fungistatic because of the planer structure of their three six-membered (phenyl) rings and substituted amino groups. Werth (1967a, 1967b); Werth and Boiteux (1967a, 1967b, 1967c); Werth and Bjerstedt (1967); and Werth and Boiteux (1968), in a series of seven papers on the toxicity and biological effects of malachite green, proposed mechanisms of action of this chemical as well as other triphenylmethane derivatives. Most of this data concerning the mode of action has come from studies using animal models; however, similar results can be expected from plant systems.

In vitro studies with rat tumor cells and yeast cells indicate that small doses of malachite green stimulate respiration and glycolysis, medium doses increase glycolysis and inhibit respiration, and high doses decreases rates of both respiration and glycolysis (Werth, 1967b). These results indicate that the fungicide in some way influences mitochondrial function. However, this is not the first information supporting the suppostiion that the chemical is a respiratory "poison." McCallan, Miller, and Weeds (1954) examined the effect of 17 fungicides including malachite green on respiration of conidia of five fungi. In these studies, malachite green was a strong inhibitor of O_2 uptake and respiration. My present studies

on the effect of malachite green on O_2 uptake by zoospores of L. callinectes indicate also that this chemical in some way inhibits the respiratory process, although the actual structural entity or enzymatic reaction has not been elucidated.

From results of previous studies and present studies indicating that malachite green affects mitochondrial function, and a knowledge of malachite green's structure, it is possible to further theorize and pinpoint the chemical's mode of action. The quinoid structure of malachite green makes it a likely candidate as an inhibitor of oxidative phosphorylation by two methods. Quinones are capable of robbing protons from NADH (Lukens, 1971). Thus, the chemical could interfere with the flow of electrons along the NAD-cytochrome system. Quinones can also be reduced to phenols by cellular thiols (Lukens, 1971). Enzyme studies have shown that malachite green causes inhibition of thiol enzymes (Fizhenko and Braun, 1967), indicating that there may be a binding complex between the malachite green and the thiol groups. The inhibition of mitochondrial thiol containing enzymes might be the means of poisoning cellular function; however, the reduction of the chemical to a phenol is a more likely cause. Phenols are uncouplers of oxidation and phosphorylation. This uncoupling is accompanied by an increase in O_2 uptake (Lukens, 1971). Werth (1967a) reported that small doses of malachite green stimulated respiration rates of tumor cells and larger doses decreased rates of respiration as measured by O_2 uptake. This phenomenon could have been due to the uncoupling induced by phenols that resulted from malachite green degradation. Although my studies did not indicate

an increase in O_2 uptake at low concentrations, inhibition of O_2 uptake by the chemical was confirmed at slightly below lethal concentrations. From this, it can be concluded that malachite green probably acts on L. callinectes by uncoupling oxidation from phosphorylation.

Toxicity, Residues, Precautionary Measures and Safety in Use of Malachite Green as a Fungicide

Stephens (1961) stated that the application and feasibility of use of malachite green in aquatic systems seems problematical. Although malachite green has been proven to be an effective fungal control agent, it has not been registered for use in aquatic systems by the FDA or the EPA (Anonymous, 1973; Bills, et al, 1977). The Fish and Wildlife Service of the U.S. Department of Interior stopped their efforts to register malachite green, since it has been implicated in teratogenicity, mutagenicity, and carcinogenicity. Lieder (1961) reported that trout eggs treated with malachite green at therapeutic levels had numerous chromosome defects. Rejniak and Piotrowska (1966) reported malachite green solutions (at 10^{-3} and 10^{-6} percent) to cause abnormal divisions in root tip cells in onions. Injections of the dye into the salivary gland of Drosophila melanogaster resulted in chromosome defects (Pheiffer, 1961). Many of the chromosome defects are similar to ones produced by x-rays.

The dye is also very toxic to mammals (Gleason, Marion, Gosselin, Hodge, and Smith, 1969). The lethal dose in rabbits is 75 mg/Kg (Christenson, 1972). This is also probably a lethal dose for humans (Anonymous, 1973). The dye can be made non-toxic by the addition of sodium sulfite (35 mg/mg of malachite green) (Wood, 1968).

Although lethal doses for mammals are relatively high (approx. 4.5 g/60 Kg man) considering the levels at which fish and crustaceans are treated, no data is available concerning levels that induce carcinogenesis. Shrimp larvae, five days after treatment of the eggs at 0.1 ppm for 10 minutes, retained approximately 0.1 ng of the chemical as detected by radioassay techniques. This concentration probably decreases with time as the larvae mature, thus the 0.1 ng is insignificant. Death or illness of individuals after consuming crustaceans treated with the chemical due to toxic effects are extremely remote since it would take about 5 g of the chemical to kill a 60 Kg man. However, the levels required to induce carcinogenesis in man may be extremely low. In spite of this, levels retained by adult shrimp are probably still not significant nor unsafe.

Since this is a preliminary experiment and limited literature is available, further safety research--levels including carcinogenesis, residues in adult shrimp, assay techniques--is needed before statements concerning the safe use of malachite green can be made with confidence.

APPENDIX A

Design and Writeup by Donald Lightner and Bill Salser.

Title: Egg Disinfectants
Starting Date: Open
Termination: Terminate when larvae reach PI

Objectives: To evaluate "egg disinfectants" for efficacy in preventing larval diseases, and for their effect on hatch success.

Statement of Need: Lagenidium callinectes, Sirolpidium sp. and Vibrio sp. are known potential pathogens that can infect and kill larval penaeid shrimp. These pathogens can only be introduced to the larval rearing tank via four routes:

1. by the transovarian route from the parent stock,
2. from the water carrying the spawned eggs and spawner,
3. by algae fed to the protozeal stages,
4. by Artemia fed to the mysis stages.

Dipping the gravid females and/or their spawn in disinfectant "dips" could reduce or eliminate introduction of these pathogens with the spawn or water carrying the spawning products. Further studies with Artemia, in which nauplii are dipped in a nontoxic disinfectant solution prior to being fed to the larval shrimp, may be necessary in the future.

Methods:

A. Eggs:
Spawned eggs of individual females to be collected as soon after being spawned as possible.

B. Sample Size:
Controlled aliquot samples giving known numbers (minimum 100 to max of 500) of egg to be transferred to 500 ml of sea water in 500 ml beakers held at a constant temperature by immersion in a water bath.

C. These egg and/or test containers will be treated with chemical disinfectants using two management approaches:

1. A 15-minute "dip" exposure to the disinfectant chemical at relatively high concentration.

2. A continuous exposure to the disinfectant chemical at low concentration.

D. Treatment:

1. Dips

a) Beginning with controlled aliquot sample, collect eggs in screen-bottomed Petri dish.

b) Immerse eggs and dish in larger Petri dish containing "dip reagent" made with sea water from "make up" tank.

c) Time dip and remove eggs and screen dish after 15 minutes.

d) Rinse dish and eggs through 3-to-5 changes of fresh sea water from "make up" tank.

e) Transfer eggs to hatching container (500 ml beaker).

f) Evaluate hatching success and survival to PI stage and compare to untreated control.

2. Continuous Exposure

a) Beginning with controlled aliquot sample, transfer eggs directly to hatch container (500 ml beaker).

b) Add reagent to bring total volume to desired test concentration.

c) Evaluate hatching success and survival to PI stage and compare to untreated control.

APPENDIX B

United States Department of the Interior

FISH AND WILDLIFE SERVICE
Fish Farming Experimental Station
Post Office Box 860
Stuttgart, AR 72160

August 14, 1978

Dr. Charles E. Bland
Professor
East Carolina University
Dept. of Biology
Greenville, North Carolina 27834

Dear Dr. Bland:

The Fish & Wildlife Service quit in its' efforts to try and register malachite green. Also the Fish and Wildlife Service quit using this chemical at its' fish hatcheries.

See the enclosures for further details.

Sincerely,

MAYO MARTIN,
Extension Biologist

Enclosures

UNITED STATES GOVERNMENT

Memorandum

: All Directors, Fishery Research Laboratories DATE:

: Assistant Deputy Associate Director-Research

...T: Disposal of Malachite Green Stocks

In view of the recent decision by the Fish and Wildlife Service to abandon its registration efforts for the use of malachite green, any supplies of this chemical on hand at your laboratory (and field stations) are to be disposed of immediately. To make certain that disposal is in accordance with EPA guidelines, you should contact the nearest Regional Office of the Environmental Protection Agency. Attached is a listing of their regional offices.

Richard B. Smith

Attachment

United States Department of the Interior

FISH AND WILDLIFE SERVICE
Fish Farming Experimental Station
Post Office Box 860
Stuttgart, AR 72160

April 11, 1978

MALACHITE GREEN

The Fish and Wildlife Service has abandoned its efforts to register malachite green in fish use. Some results with the mammal studies (rats) brings about very serious doubt that the registration attempt would be successful.

The Service is searching for a chemical that will control fungus on fish eggs. At this time a satisfactory substitute is lacking.

FISH FARMING EXPERIMENTAL STATION
Post Office Box 860
Stuttgart, AR 72160

UNITED STATES GOVERNMENT

memorandum

DATE: MAR 29 1978

LY TO TN OF: Acting Regional Director, FWS, Atlanta, Georgia

Date Rec'd 4-3-78
Disposition: File
Staff Review
Retain Until
Discard

JECT: Malachite Green Registration

TO: All Project Leaders, Region 4

A recent memorandum from Acting Deputy Associate Director - Fisheries Thomas J. Parisot states in part that the Service is abandoning efforts to register malachite green. Research has produced increasing evidence that the compound has potential teratogenic, mutagenic, oncogenic, and carcinogenic properties.

In view of the above, effective immediately, you are directed to cease use of this compound. Existing stocks should be stored in a safe place until further instructions are received. Since fish hatcheries likely have the largest inventories, Hatchery Biologists Camper and Carlson are specifically alerted to be prepared to survey amounts being held.

Boyce N. Brackin

cc:
Director, FWS, Washington, D. C. (AFW/F)

Director H
Asst. Dir. D
Admin. Clerk VB 18

Vic: Please post for all To See.

Be Safe Today

H: Asked Glenn To Store our Stock.

D.
4/5/78

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