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POLYGODIAL, AN ANTIFUNGAL POTENTIATOR[1]

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ABSTRACT.—A series of sesquiterpene dialdehydes was isolated from the East African medicinal plants *Warburgia stuhlmannii* and *Warburgia ugandensis* (Canellaceae) as antibiotics, particularly against *Saccharomyces cerevisiae*, *Candida utilis*, and *Sclerotinia libertiana*. Among these sesquiterpene dialdehydes, polygodial [**1**] exhibited the most potent activity. When tested on *S. cerevisiae*, polygodial proved to be fungicidal rather than fungistatic. When the cells of *S. cerevisiae* are treated in vitro with polygodial for 10 min, the cell membrane becomes severely damaged, and many vesicles, possibly formed from the fragmented cell membrane, can be observed within the cytoplasm. The observation of cell membrane lesions led us to propose a rather innovative hypothesis: the use of polygodial to facilitate the transmembrane transport of exogenous chemicals into cells. For example, polygodial could be combined with an antibiotic having poor cell membrane permeability in an effort to increase its antibiotic activity by increasing its ability to gain entrance into the cell. We report here that a remarkably enhanced efficacy was obtained when actinomycin D was used in combination with polygodial. We believe polygodial may be acting as an "advance scout," punching holes in the plasma membrane and gaining an entrance into the cell for an antibiotic previously less effective because of problems with cell membrane permeability.

We live in a world where we can control many human and animal pathogens with the antibiotics presently available. However, it is not difficult to substantiate that the need for new antibiotics still exists. For example, systemic infections caused by filamentous fungal microorganisms have become increasingly serious, expecially when the host's defense mechanism is impaired. Many of the mechanisms that contribute to the development of opportunistic fungal infections remain an enigma to modern microbiology. While various empirically derived antifungal agents have been introduced, control of many of the fungal diseases has yet to be achieved.

It is highly desirable to develop new agents both to widen the spectrum of activities against harmful microorganisms and to combat any organisms expressing a resistance to presently available compounds. The majority of higher plant species have yet to be explored as potential sources of antimicrobial agents. Judging from past successes, it is likely that compounds isolated from these sources will exhibit the novel modes of action necessary for the development of future generations of antibiotics. It is for these reasons that we have begun an investigation of a number of plant species exhibiting antibiotic properties.

During a preliminary screening of extracts of 79 species of higher plants from East Africa, we found 40 extracts endowed with some activity (1). The choice of plants was based on information provided by the people native to the area, especially the "Bwana Mganga" (Swahili for medicine man). This seems to indicate that the information from a "Bwana Mganga" can sometimes be justified medicinally, because we found the probability of observing antimicrobial activity in "dawa ya miti" (Swahili for medicinal plants) extracts to be much higher than that in a random sampling. In particular, *Bala-*

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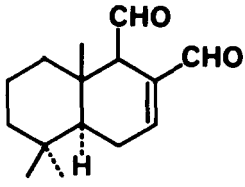
nites aegyptica, Erythrina abyssinica, Fagara chalybea, Warburgia stuhlmannii, and *Warburgia ugandensis* showed broad antibacterial and antifungal activities. The remaining 35 active extracts were only active against Gram-positive bacteria. Our emphasis has been placed upon *Warburgia* species because the extracts of these two trees were found to be the most potent.

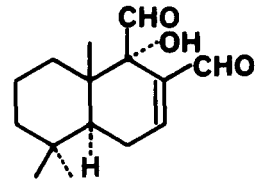
RESULTS AND DISCUSSION

WARBURGIA SESQUITERPENE DIALDEHYDE ANTIBIOTICS.—The genus *Warburgia* (Canellaceae) consists of two species distributed in East Africa, *W. stuhlmannii* Engl. and *W. ugandensis* Sprague. The two species are widely used in the local folk medicine to alleviate toothache, rheumatism, general body pains, diarrhea, and malaria (2). In addition, the leaves of *W. ugandensis* are sometimes used locally as a spice for food. The bark of *W. ugandensis* is commonly known by several different names, depending on the local tribe, such as "Apacha" (Kakamego), "Muthiga" (Kikuyu), "Olosogoni" (Masai), "Soget" (Kipsigis), "Soke" (Tugen), and "Sogo-Maitha" (Luo). The distribution of *W. stuhlmannii* is limited to the coastal areas, and it is known as "Mukaa" (Swahili). Both plants can be purchased in the open marketplace as well as through a "Bwana Mganga."

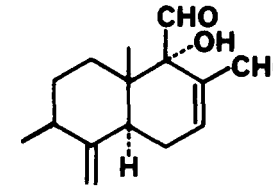
We previously reported that the aqueous MeOH extracts of the barks of *W. stuhlmannii* and *W. ugandensis* were active against Gram-positive bacteria, yeasts, and filamentous fungi (1). The extracts were fractionated and monitored with the previously described assay method. This led to the isolation of the antimicrobial principles as a series of unique sesquiterpene dialdehydes: polygodial [**1**], warburganal [**2**], and muzigadial [**3**]. All are considered to be oxidation products of the drimane skeleton. It is interesting that these sesquiterpene antibiotics, first isolated as insect antifeedants from the same sources (3–5), taste very hot to humans, and that the structural requirements for the hot taste have been described (6).



1 polygodial



2 warburganal



3 muzigadial

The minimum inhibitory concentrations (MIC) of these three compounds against a variety of microorganisms are presented in Table 1. Polygodial, warburganal, and muzigadial showed similar antimicrobial spectra and exhibited particularly potent activity against *Saccharomyces cerevisiae, Candida utilis,* and *Sclerotinia libertiana* (7). Among these three sesquiterpene dialdehydes, polygodial exhibited the most potent activity. It was 2–8 times more active than warburganal and muzigadial against all species of yeasts and filamentous fungi tested. Polygodial, structurally the simplest and also the most active antifungal of the three, was first isolated from the sprout of *Polygonum hydropiper* (Polygonaceae), a well-known relish for sashimi in Japan (8). Polygodial is slightly more active than the commercially available antifungal amphotericin B, which is one of several antifungal agents used to stop the severe evolution of numerous deep-seated mycoses (candidiasis, aspergillosis, cryptococcosis, histoplasmosis), although its high toxicity limits its wide use. These observations suggest polygodial itself may be a very promising antimicrobial agent because of its broad spectrum of effectiveness, particularly on yeasts and filamentous microorganisms. However, polygodial exhibits a far greater potential when combined with other antibiotics.

TABLE 1. Antimicrobial Activity of the *Warburgia* Sesquiterpene Dialdehydes.

Microorganisms Tested	MIC (μg/ml)		
	Polygodial [1]	Warburganal [2]	Muzigadial [3]
Staphylococcus aureus NCTC 8530	>100	>100	>100
Bacillus subtilis K-49	>100	>100	>100
Micrococcus lysodeikticus IFO 3333	>100	>100	>100
Escherichia coli IFO 3545	>100	>100	>100
Proteus vulgaris IAM 12003	>100	>100	>100
Pseudomonas aeruginosa IAM 1007	>100	>100	>100
Saccharomyces cerevisiae IFO 0203	0.78	3.13	1.56
Schizosaccharomyces pombe IFO 0342	6.25	12.5	25
Hansenula anomala IFO 0136	1.56	12.5	25
Candida utilis ATCC 42402	1.56	3.13	3.13
Sclerotinia libertiana SS	1.56	3.13	3.13
Mucor mucedo IFO 7684	6.25	25	25
Rhizopus chinensis IFO 4745	12.5	100	100
Aspergillus niger ATCC 6275	25	50	50
Penicillium crustosum Thom	25	50	50

COMBINATION EFFECTS OF POLYGODIAL WITH ACTINOMYCIN D.—Figure 1 shows the combined effect of polygodial with actinomycin D, an antitumor antibiotic isolated from *Streptomyces antibioticus*. Actinomycin D proved to be devoid of activity against *C. utilis* and was only weakly active against *S. cerevisiae* when used alone. However, a remarkably enhanced efficacy was obtained when it was applied in combination with polygodial. Even new antibiotic properties could be observed, such as its transformation into an antifungal agent against *C. utilis*. Against *S. cerevisiae*, a sixteenfold increase in the potency of actinomycin D was obtained when applied in combination with polygodial.

With respect to the above observation, an extensive study of the effects of polygodial has been carried out together with the elucidation of its mechanism. Figure 2 shows

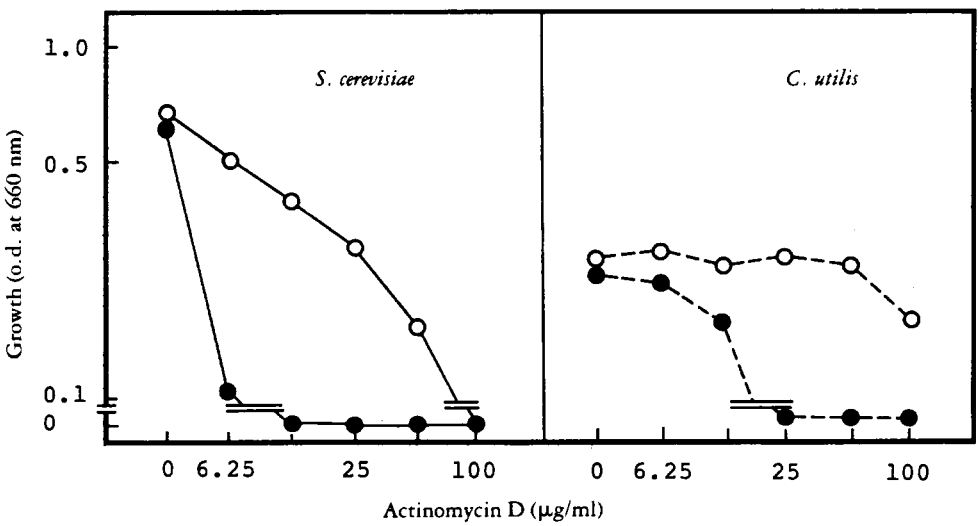


FIGURE 1. Inhibition of growth of *Saccharomyces cerevisiae* and *Candida utilis* by actinomycin D in combination with polygodial [0.39 μg/ml (½MIC)] (●---●). Control (○---○).

that polygodial is fungicidal rather than fungistatic when tested on *S. cerevisiae*. Although some loss of antifungal activity was observed in basic media, much better activity was observed under acidic conditions as shown in Table 2. Polygodial exhibited no

TABLE 2. Minimum Inhibitory Concentrations of Polygodial against Some Yeasts in Several Media.

Yeasts Tested	Polygodial (μg/ml)			
	Malt Wort	Henneberg Medium		
	pH 5.0	pH 5.2	pH 7.0	pH 3.1
Saccharomyces cerevisiae	0.78	1.56	>25	0.20
Candida utilis	1.56	3.13	>25	0.78
Schizosaccharomyces pombe	6.25	25	>25	6.25
Hansenula anomala	1.56	6.25	>25	1.56

inhibition in pH 7 media on *S. cerevisiae, C. utilis, Schizosaccharomyces pombe,* and *Hansenula anomala* even at 25 μg/ml. In contrast, the potency of polygodial's antifungal activity was increased 2–4 times when assayed in media with a pH of 5.0 than in neutral

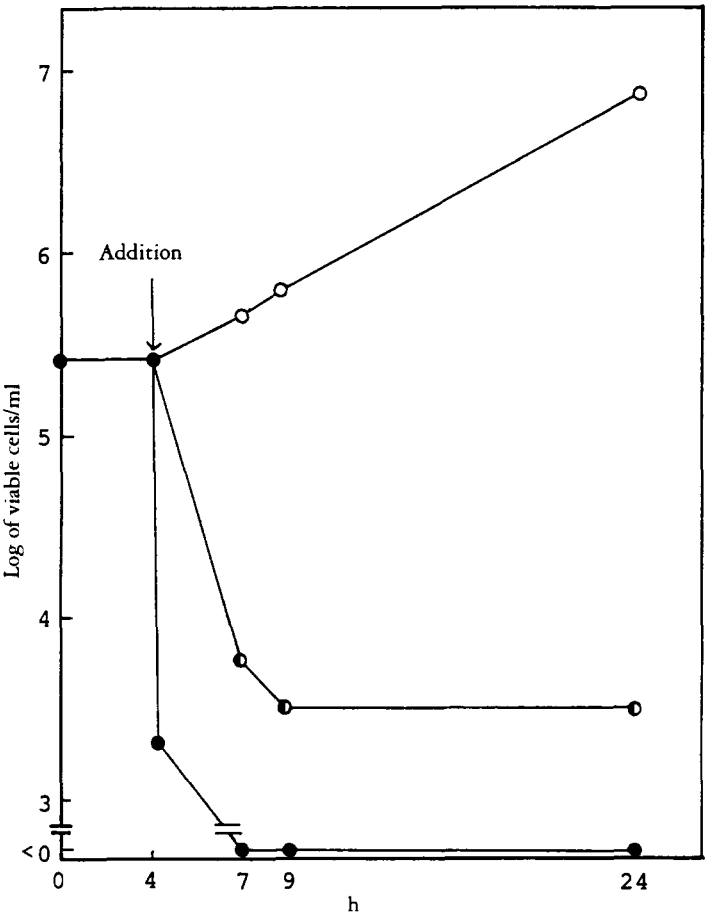


FIGURE 2. Effect on viability of *Saccharomyces cerevisiae* of 0.78 μg/ml polygodial (○---○) and 25 μg/ml polygodial (●---●). Control (○---○).

media. It was observed in media with a pH of 3.1 to have activity at concentrations as low as 0.20 μg/ml and 0.78 μg/ml against *S. cerevisiae* and *C. utilis*, respectively.

Biochemical studies have revealed that polygodial profoundly affects primary functions of the cell membrane of *S. cerevisiae* by causing a leakage of various cellular constituents. Figures 3 and 4 show the leakage of Folin reagent and phenol-H_2SO_4 positive

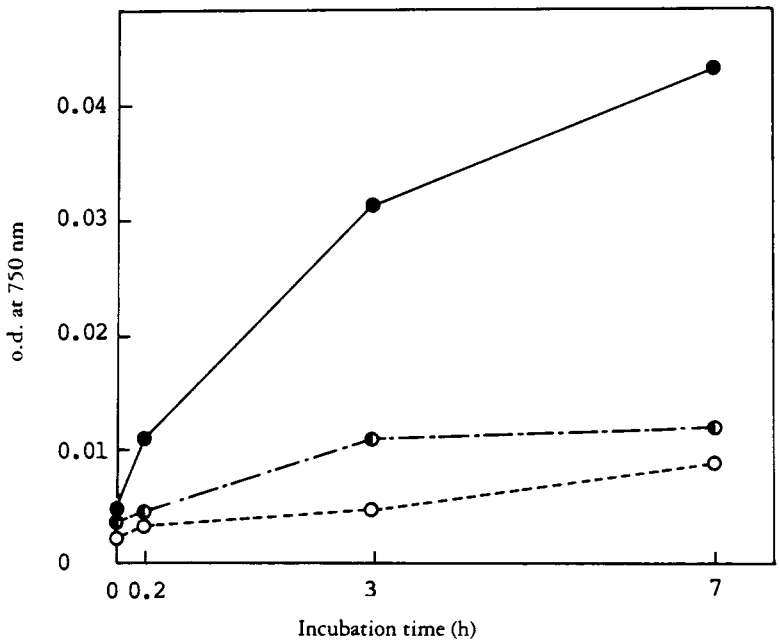


FIGURE 3. Leakage of folin reagent positive substances from yeast cells during incubation with 1 μg/ml polygodial (○---○) and 10 μg/ml polygodial (●---●). Control (○---○).

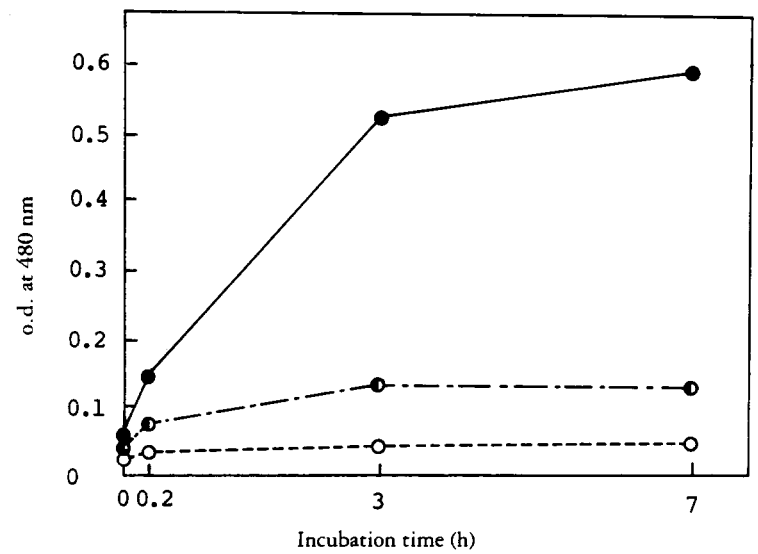


FIGURE 4. Leakage of phenol-H_2SO_4 test positive substances from yeast cells during incubation with 1 μg/ml polygodial (○---○) and 10 μg/ml polygodial (●---●). Control (○--- ○).

substances from *S. cerevisiae* cells upon incubation with polygodial which eventually led to cell death. The leakage of these substances was not observed from dead yeast cells that were killed by uv light (Figure 5).

The leakage of cellular constituents is also supported by microscopic evidence. Cells of *S. cerevisiae* were treated with a fungicidal concentration (50 μg/ml) of polygodial so that almost all cells were killed within a short time (10 min); ultrastructural changes in

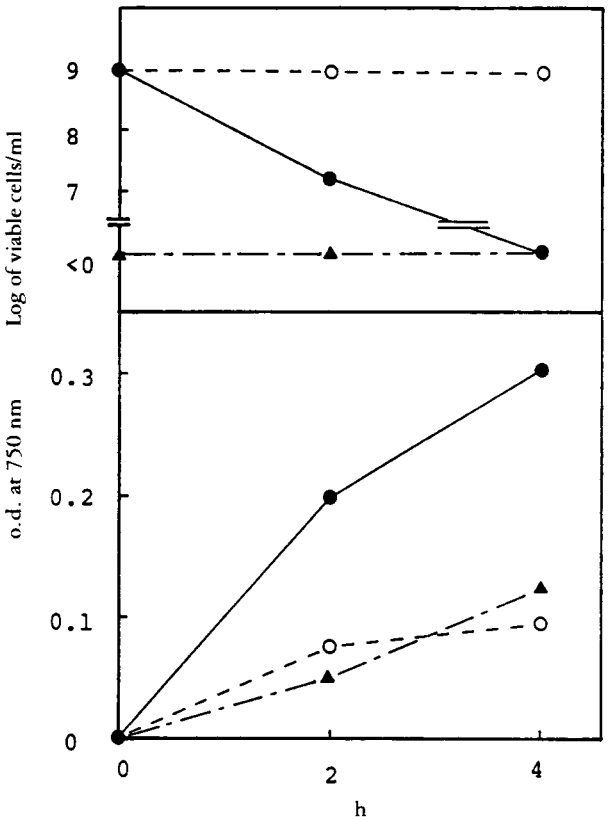


FIGURE 5. Effect of 10 μg/ml polygodial (●---●) and uv irradiation (▲---▲) on viability and leakage of Folin reagent positive substances from yeast cells. Control (○---○).

the cell membrane followed, as can be seen in Figure 6. These morphological data suggest that the primary site of action of polygodial is the plasma membrane, with the simultaneous involvement of organelle disorganization followed by the fatal loss of cellular constituents such as proteins and saccharides.

The above findings led us to propose a rather innovative hypothesis: the use of polygodial to facilitate the transmembrane transport of exogenous chemicals into cells. The increase in the activity of actinomycin D when combined with polygodial may be explained through the suggestion that polygodial is acting as an "advance scout," punching holes in the plasma membrane and thereby gaining an entrance into the cells of *S. cerevisiae* and *C. utilis* for actinomycin D. This results in an increase in activity for an antibiotic previously less effective because of lack of ability to penetrate the cell membrane.

For the otherwise healthy person, fungal infections are more a nuisance than a health threat and are normally kept in check by a strong immune system and by innocu-

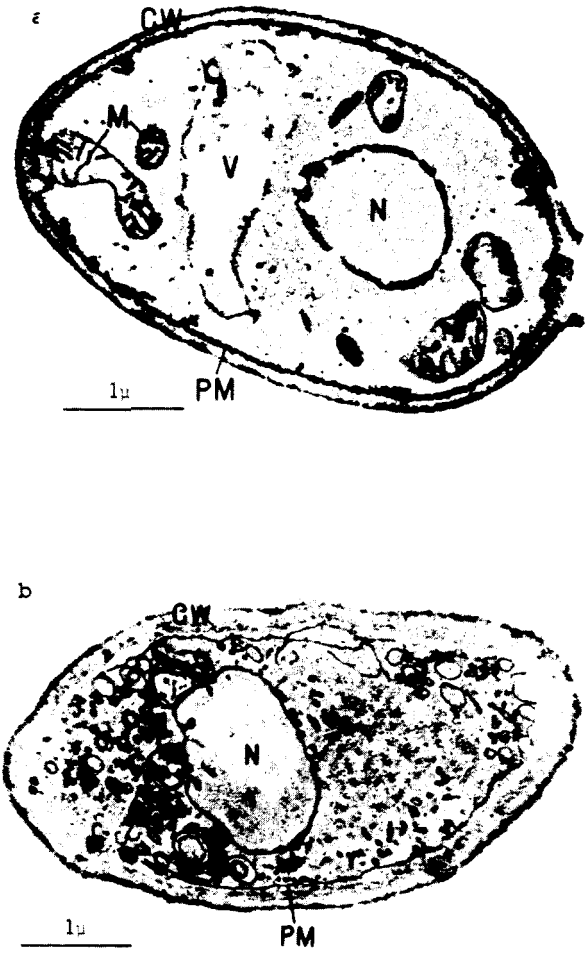


FIGURE 6. a. Section of untreated control cell of *Saccharomyces cerevisiae*.
b. Section of *S. cerevisiae* cell treated with 50 μg/ml polygodial for 10 min. CW, cell wall; PM, plasma membrane (cell membrane); N, nucleus; M, mitochondrin; V, vacuole.

ous bacteria of the throat and gut. However, when outside forces such as cancer chemotherapy or heavy doses of antibiotics derange the body's natural defenses, yeasts and other fungal populations can sharply increase and cause serious health problems. Substances that enable physicians to use lower, safer antibiotic dosages to kill the fungi would be a useful addition to the therapeutic arsenal. Further studies of increased potency due to polygodial may pave the way for the development of new and extraordinarily powerful antifungal agents.

EXPERIMENTAL

CHEMICALS.—The *Warburgia* sesquiterpene dialdehyde antibiotics were previously isolated (3–5). All common chemical reagents were of commercial grade.

CULTURE CONDITIONS.—*S. cerevisiae* was cultured with shaking at 25° in 2.5% malt extract medium, unless otherwise indicated.

GROWTH STUDIES.—A 10-h culture was diluted with the same fresh medium to give approximately 10^5 colony forming units (cfu) per ml. A 10-ml portion of this cell suspension was dispensed into each L-

tube. After a 3-h incubation, known concentrations of polygodial were added to these tubes, which were then shaken again. Portions of the culture were withdrawn at intervals to determine the optical density (660 nm) and cfu. The cfu was counted by plating dilutions of the culture in saline on malt agar plates and incubating at 25° for 2 days.

In examining the influence of inoculum size on the MIC of polygodial, an overnight culture was inoculated to give cfu values of 10^5, 10^6, and 10^7 per ml of the assay medium. After 2 days, growth was measured by optical density at 660 nm.

CELL PERMEABILITY STUDIES.—Exponentially growing cells were harvested, washed, and resuspended in saline to give approximately 5×10^7 cfu/ml. The suspension was shaken in the presence or absence of polygodial at 25°. At various times, portions were withdrawn and centrifuged. The cell exudates obtained were then analyzed for materials with absorption at 260 nm, Folin reagent-positive substances by the method of Lowry *et al.* (9). Phenol-H_2SO_4-positive substances were analyzed by the method of Dubois *et al.* (10).

ELECTRON MICROSCOPIC STUDIES.—Both polygodial-treated and untreated cells, prepared as described above, were washed with cold saline and fixed in 1% aqueous $KMnO_4$ overnight at 4°. After centrifugation, the cells were washed twice with distilled H_2O and postfixed in 1% aqueous uranyl acetate for 1 h at 4°. The fixed cells were dehydrated in graded series of EtOH and embedded in Spurr's epoxy resin (11). Thin sections were cut with a Porter-Blum MT 2B ultramicrotome (12), stained with lead citrate, and examined in a Hitachi H-300 electron microscope.

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