0022-202X/81/7706-0444$02.00/0
THE JOURNAL OF INVESTIGATIVE DERMATOLOGY, 77:444–451, 1981
Vol. 77, No. 6
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Morphological Changes Induced by Naftifine, a New Antifungal Agent, in *Trichophyton mentagrophytes*

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The morphological changes in the hyphae of *Trichophyton mentagrophytes* treated with Naftifine (0.01–0.5 μg/ml) were studied by light and electron microscopy. The most striking changes observed following treatment with this new antimycotic agent were bulb-shaped thickenings at the hyphal tips and dose-dependent, spherical, or drop-shaped depositions of varying size within the cells. The abnormal formations were not only visible in the cytoplasm (discrete or aggregated in vacuoles), but also in the region of the cell membrane, in all layers of the cell wall and on the cell surface. Their lipid nature can be deduced from several significant characteristics including osmiophily, the conchoidal fracture surface observed in freeze-fracture replicas and their extractability with acetone.

This evidence suggests that the antimycotic action of Naftifine results from interaction of the agent with fungal lipid metabolism and possibly from alterations of the cell wall structure. The latter is also suggested by the changes observed in the hyphal tips.

Naftifine ((E)-N-Methyl-N-(1-naphthylmethyl)-3-phenyl-2-propen-1-amine-hydrochloride) is a new antimycotic agent with a broad spectrum of activity [1]. It is highly active against *Trichophyton*, *Epidermophyton*, and *Microsporum* species and it also shows activity against *Aspergillus* spp., *Sporothrix schenckii*, and *Candida* spp. The *in vivo* antifungal activity was confirmed after topical application in guinea-pig dermatophytosis model infections [2].

This report is concerned with light and electron microscopical observations on the morphology of *Trichophyton mentagrophytes* treated with various concentrations of Naftifine *in vitro*, in an attempt to elucidate a possible mechanism of action of this drug.

MATERIALS AND METHODS

Organism and Culture Conditions

T mentagrophytes, strain 56066, obtained from the Centraalbureau voor Schimmelcultures, Baarn, The Netherlands, was used in this study.

Stock cultures, stored in liquid nitrogen [3], were subcultured either into 250 ml flasks of Sabouraud broth (pH 6.5, containing 2% glucose) at 1×10^5 CFU per 100 ml medium or onto Sabouraud agar plates (pH 6.5, containing 4% glucose) on which were superimpsoed a polycarbonate membrane (0.4 μm pore size, 25 mm diameter, Nucleopore Corp.) at 200 CFU per plate. Both cultures were incubated at 30°C.

Incubation of T mentagrophytes *with Naftifine*

Two treatment schedules were used. Broth cultures were allowed to grow for 48 hr and then treated for 24 hr with concentrations of 0.01, 0.05, 0.1 and 0.5 μg/ml. Plate cultures were treated during the first 48 hr of incubation. The drug was added to agar in concentrations of 0.01, 0.05 and 0.1 μg/ml (0.1 μg/ml was the minimal inhibitory concentration determined in a serial dilution test [1]).

Broth cultures were used for light microscopy and scanning-, ultrathin-section-, and freeze-etch electron microscopy. Plate cultures were used only for thin sectioning.

Untreated cultures containing an equal amount of drug vehicle (0.1% dimethylsulfoxide) were used for comparison.

Light Microscopy

Hyphae of treated and untreated mycelia were observed with a Leitz Orthoplan microscope equipped with interference contrast optics. Lipid granules were demonstrated in frozen, 20 μm thick hyphal sections using Sudan III.

Electron Microscopy

(*I*) *Freeze fracture replication*: It was found to be of particular importance to use native material for freeze-etching experiments since both chemical fixation and cryoprotective treatments (glycerination) can mimic the drug-induced morphological changes of the cytoplasmic membrane. For this reason small portions (<1 μl) of unfixed, noncryoprotected fungal material were placed on gold discs and immediately frozen in Freon 22.

Freeze-etching was carried out in a Balzers BAF 300 freeze-etching unit, using an electron gun for platinum-carbon shadowing. Fracture faces obtained by freeze-cleaving at −100°C were etched for 20–30 seconds before replication. Replicas were cleaned with 35% chromic acid and finally washed in distilled water. Cleaned replicas were picked up on uncoated copper grids.

(*II*) *Ultrathin section*: The fixation method used was similar to that described by Grove and Bracker [4]. Plate cultures were flooded directly in the Petri dishes with 2% glutaraldehyde (GA) in 0,1 M sodium cacodylate containing 0,01 M $CaCl_2$ (pH 7.2). Broth cultures were fixed by immersion in the same fixative. Primary fixation was performed under light negative pressure for better wetting of the hyphae for 30 min at +4°C. Further processsing for both specimens was identical. They were washed in buffer at +4°C and postfixed with 1% OsO_4 in the above buffer for 3 hr at room temperature. Mycelia were subsequently washed in buffer, rinsed in distilled water, and soaked in 0.5% aqueous uranyl acetate for 3 hr. After dehydration through a graded ethanol series the samples were embedded in epoxy resin [5]. The blocks were trimmed with a Reichert TM 60 for specific orientation. Only portions from the mycelial periphery were used. Sections were cut with glass knives in a Reichert Ultracut, mounted on 300 mesh grids, stained for 20 min with lead citrate [6] and subsequently examined with a Zeiss EM 10 B electron microscope at 60 kv.

Some thin-sectioning experiments were conducted on specimens after lipid extraction. These were prefixed with GA for 1 hr at +4°C, washed in buffer and treated with 20, 50, 70, 90, 100, 50, and 20% acetone for 10 min. in each concentration. Further fixation was performed with 2% OsO_4 for 2 hr only; uranyl acetate was omitted in this procedure, which was in other respects identical with the method outlined above.

Scanning Preparation

Before fixation all samples were rinsed at + 4°C in 0,1 M cacodylate buffer (pH 7.2), which was used throughout the procedure. After 1 hr of fixation in 2% GA at +4°C the cells were washed in buffer, then postfixed in 1% OsO_4 for 3 hr. Dehydration in an acetone series followed. The material was subsequently subjected to critical point drying, using the acetone/CO_2-system, in a CE/PE/DE 8 instrument (Centre for electron microscopy, Graz, Austria), after which the hyphae were coated with gold to a thickness of about 70 nm using Balzers' sputtering device. The specimens were observed with a Cambridge Stereoscan S4

Manuscript received February 4, 1981; accepted for publication April 22, 1981.

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Abbreviations:
GA: 2% glutaraldehyde
EF: extracellular half
PF: plasmatic half

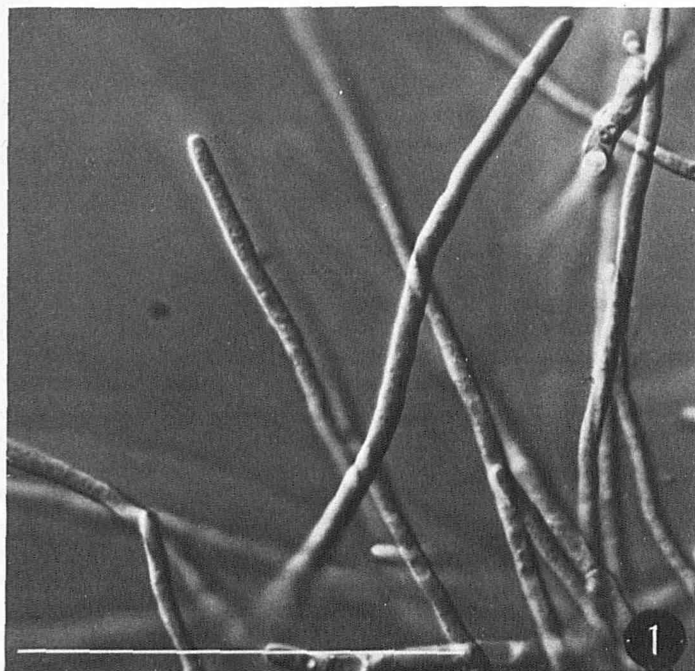
scanning electron microscope (SEM) at an accelerating voltage of 20 kv.

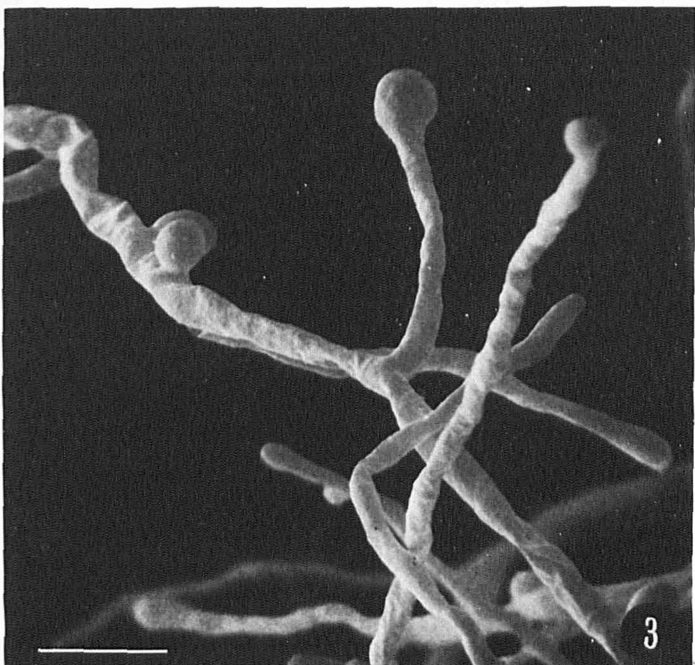
RESULTS

In comparison to untreated controls (Fig 1), mycelia of *T mentagrophytes* treated with 0.1 μg/ml of Naftifine and viewed under the light microscope, had swollen, club-shaped hyphal tips and hyphae with a rough and granulated cytoplasmic structure (Fig 2). Drug-induced changes in the morphology were also observed by scanning electron microscopy. Cultures treated with the 10-fold concentration (1.0 μg/ml) of Naftifine can be seen in Fig 3. Particularly noticeable in this preparation were the bulbiform blunt hyphal ends and the shrunken and distorted hyphal filaments, not seen in the untreated controls (Fig 4).

The normal morphology of untreated mycelia as revealed by freeze fracture replication and thin sectioning techniques are illustrated in Fig 5 to 10. The fibrillar network of the cell surface, the homogeneous granular structure of the cross-fractured cell wall and the fracture face of the cytoplasmic half of the cell membrane, with randomly distributed membrane-associated particles, can be clearly seen in deep etched preparations (Fig 5, 6). The morphology and the distribution of the cell organelles, as well as the cell compartments, are seen in both freeze-etching preparations (Fig 7–9) and thin sections (Fig 10).

Replicas of deep etched preparations of cells treated with 0.1 μg/ml Naftifine (Fig 11, 12) show, in contrast to untreated cells (Fig 5, 6), numerous globular structures with diameters from 30 to 120 nm lying at different levels within the fibrillar network of the cell wall. Cross-fractured globules occasionally appear to





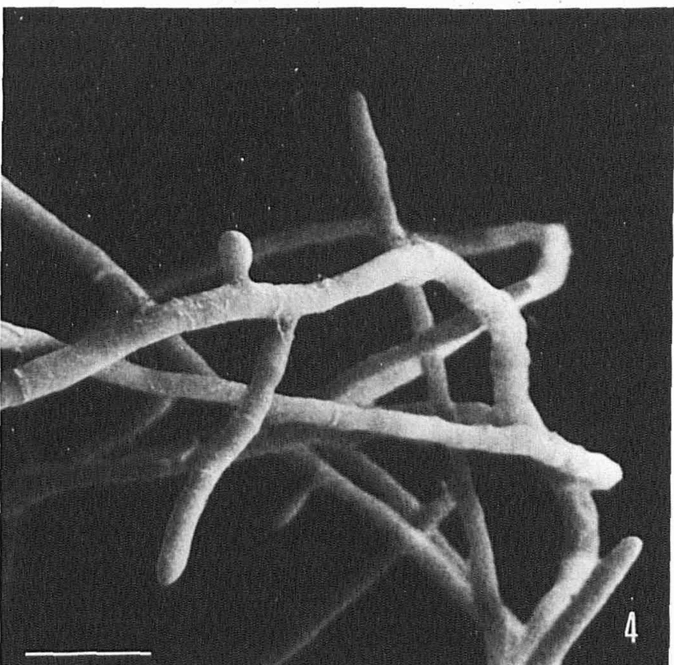


FIG 1 and 2. Interference contrast micrographs of *Trichophyton mentagrophytes* cultures, grown for 72 hr. Fig 1 Control culture. Fig 2 Culture grown for 48 hr and then exposed to 0.1 μg Naftifine/ml for 24 hr. Swelling of the tips and the granulous cytoplasm are shown (*Bars* = 100 μm).

FIG 3 and 4. Scanning electron micrographs of *T. mentagrophytes* cultures, grown for 72 hr. Fig 3 48 hr cultures exposed to 1.0 μg Naftifine/ml for 24 hr on day 3. Note the distorted filaments and the swelling of the tips. Fig 4 Control cultures (*Bars* = 10 μm).

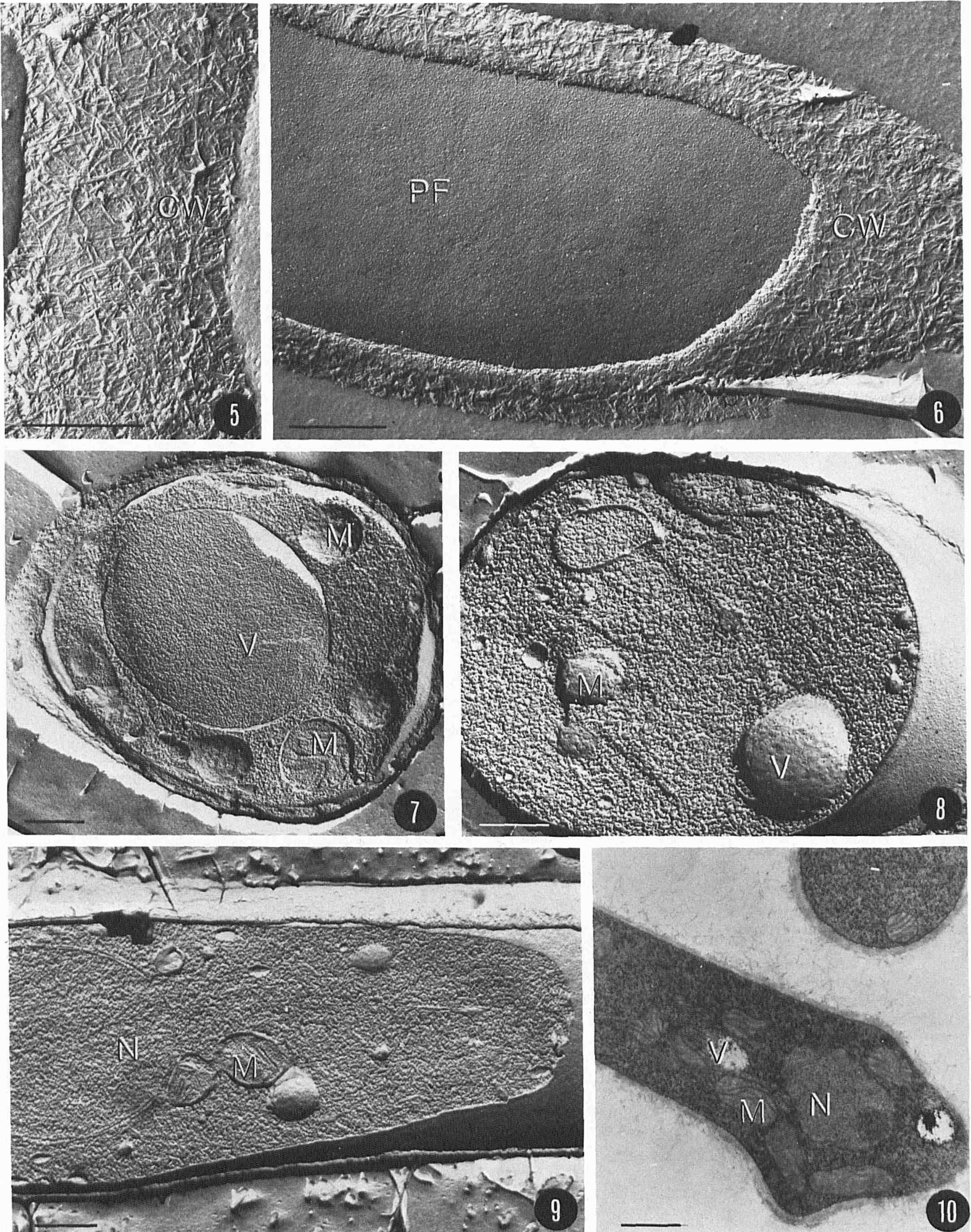
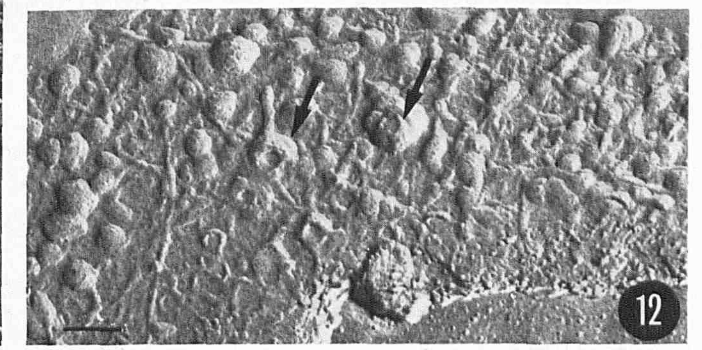


FIG 5–10. Control cultures of *T. mentagrophytes* grown for 72 hr. The morphology of the cell wall, the cytoplasmic membrane and the organelles of untreated cells are seen. Fig 5–9. Freeze-etched preparations. Fig 10 Thin section (*Bar* = 1 μm). *CW* = cell wall; *PF*, fracture face of the plasmatic half of the cytoplasmic membrane; *V*, vacuol; *M*, mitochondrium; *N*, nucleus.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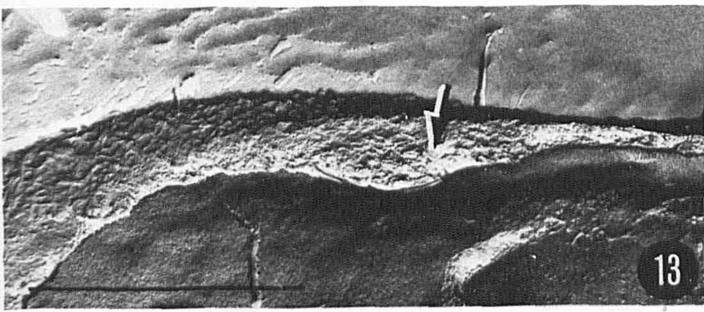
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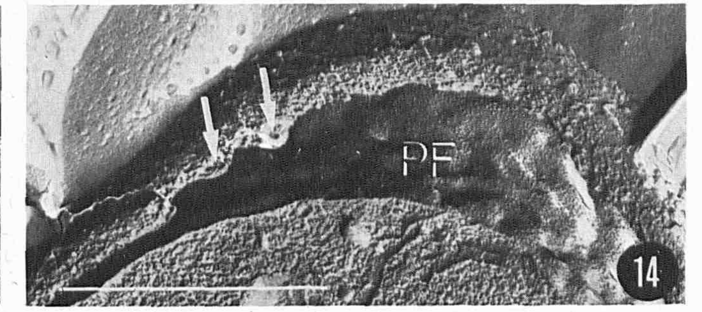
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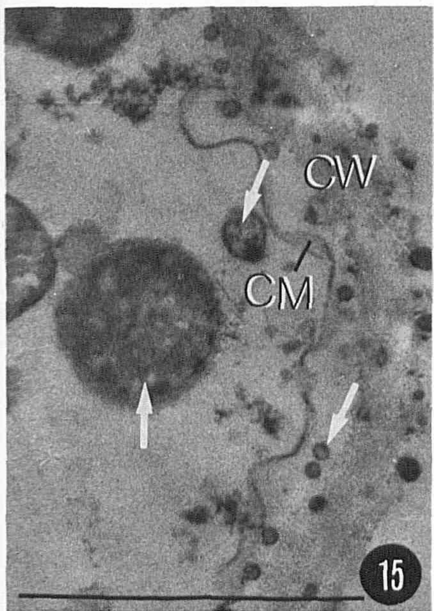
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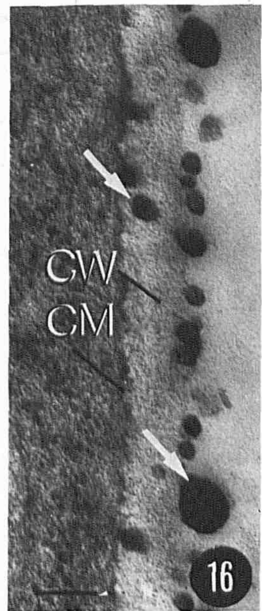
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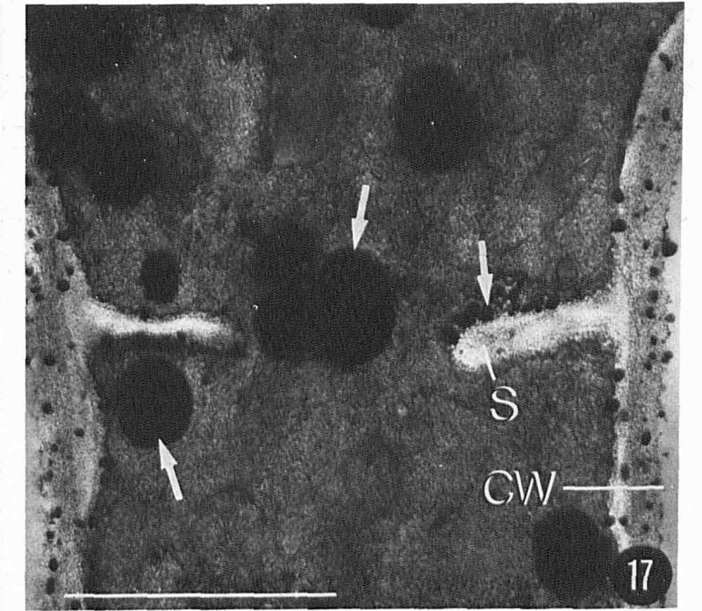
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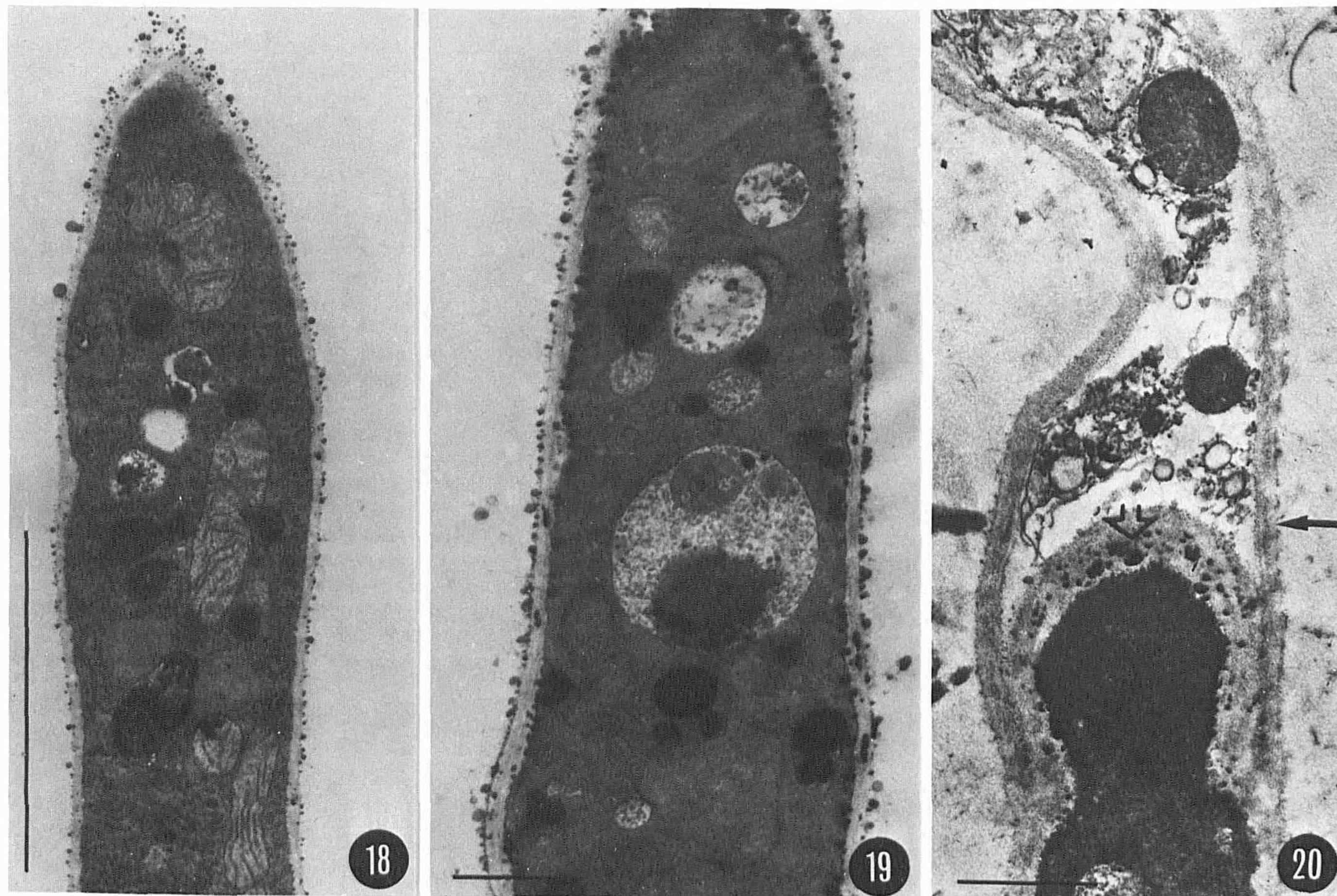


FIG 18–20. Longitudinal section through cells exposed to Naftifine, showing dose-dependent formation of electron-dense globular particles in the hyphae of *T. mentagrophytes*. Fig 18. Cells grown on plates containing 0.1 μg Naftifine/ml (*Bar* = 10 μm). Fig 19 and 20. Cells harvested from both cultures after a 24 hr treatment with 0.5 μg Naftifine/ml. (*Bars* = 1 μm) Note the absence of electron-dense inclusions in the wall of a lysed cell (Fig 20, *arrow*). Depositions are only evident in the intrahyphal growing cell (Fig 20), *open arrow*).

be vesicular in structure (Fig 12). Due to their strong electron scattering power these globular structures are also clearly recognizable in thin sections (Fig 15–17). From both cross-fractured (Fig 13, 14) and sectioned (Fig 15–17) preparations it can be seen that the same particles are present within the cell wall, leading in some areas to considerable bulges (Fig 13). Some of the osmiophilic granules seem to be in close association with the cytoplasmic membrane, since in autolyzed cells (Fig 15) few of them remain attached to the outside of the plasmalemma remnants. In some hyphal sections the electron dense particles seem to accumulate both inside and outside of the cytoplasmic membrane, as can be seen at the septation site (Fig 17) and over the whole area of the plasma membrane in Fig 19. The number and the size of the cell wall and membrane-associated electron dense particles correlate with the concentration of Naftifine (Fig 18, 19). They are only present in metabolically active cells. This is shown in Fig 20. Drug-induced alteration is only present in the intact intrahyphal growing cell but not in the autolyzed one. Similarly, in Fig 24 electron dense bodies are only found in the intact part of the hypha but not in the adjoining dead cell. The freeze-etching image of the fracture face of the plasmatic half (PF) of the cytoplasmic membrane (Fig 11, 14) shows indentations of varying diameters which correspond to the complementary elevations seen on the fracture face of the extracellular half (EF) (Fig 22). Some of these depressions of the cell membrane are in close association with cytoplasmic vesicles (Fig 21, 23). From cross-fractured cells it can be seen that the contours of the plasma membrane closely follow the inner surface of the cell wall (Fig 13, 14, 23).

The cross morphology of cell organelles shows no obvious changes when hyphae are incubated with Naftifine in concentrations up to 0.1 μg/ml. Nevertheless, a remarkable accumulation of globular bodies with a smooth structure in cross fracture similar to lipid bodies, can be observed in treated cells (Fig 22, 23). The equivalent structures seen in thin sections have a strong electron scattering power (Fig 18, 19), identical to the osmiophilic globules seen in association with the plasma membrane and the cell wall. The number of these cytoplasmic structures increases with increasing concentrations of Naftifine.

As another characteristic change in treated cells large vacu-

FIG 11–14. Freeze-etched preparations of *T. mentagrophytes* grown for 48 hr and then exposed to 0.1 μg Naftifine/ml for 24 hr. Fig 11 Globular structures on the surface of the cell wall are seen. The P-face of the cytoplasmic membrane shows irregular indentations (*Bar* = 1 μm). Fig 12 Note the vesiculous structure of cross-fractured globules (*arrows*) (*Bar* = 100 nm). Fig 13 and 14 Cross-fractured faces showing globules within the cell wall and local centripetal bulgings of the cell wall (*arrows*) (*Bars* = 1 μm). *PF* = fracture face of the plasmatic half of the cytoplasmic membrane; *CW*, cell wall.

FIG 15–17. Micrographs of thin-sectioned cells derived from cultures exposed to Naftifine. Fig 15 and 16. Cultures grown for 72 hr on agar containing 0.1 μg Naftifine/ml. (*Bars* = 1 μm and 100 nm respectively) Fig 17. Specimen of 72 hr broth cultures exposed to 0.5 μg Naftifine/ml for the last 24 hr. (*Bar* = 1 μm) Fig 15. Partially autolyzed cell. Electron dense particles of various size (*arrows*) are seen in the layers of the cell wall (Fig 15, 16, 17), in association with the septum (Fig 17), the plasma membrane (Fig 15 and 16), and within the cytoplasm (Fig 15, 17). *CW* = cell wall; *CM*, cytoplasmic membrane; *S*, septum.

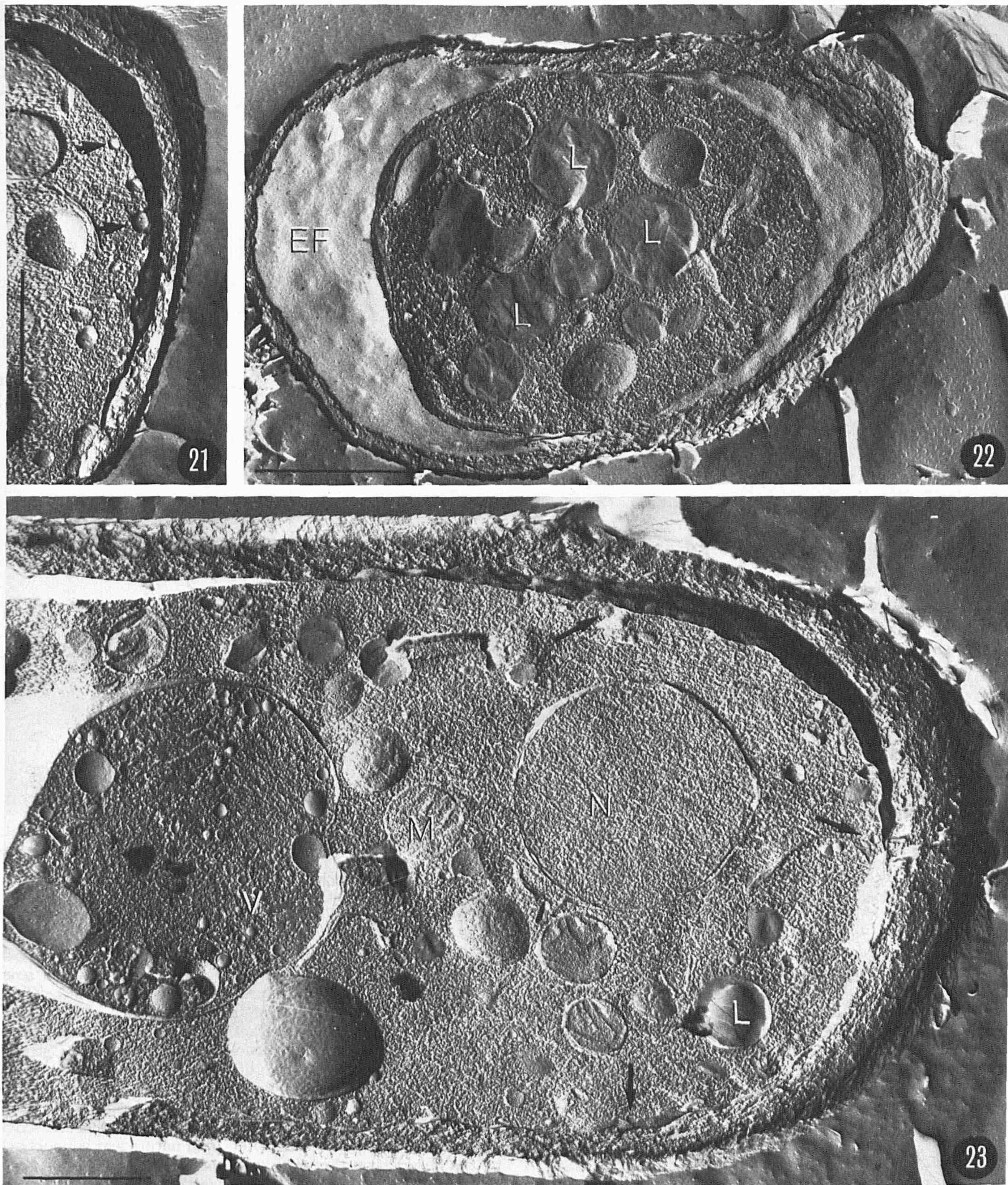
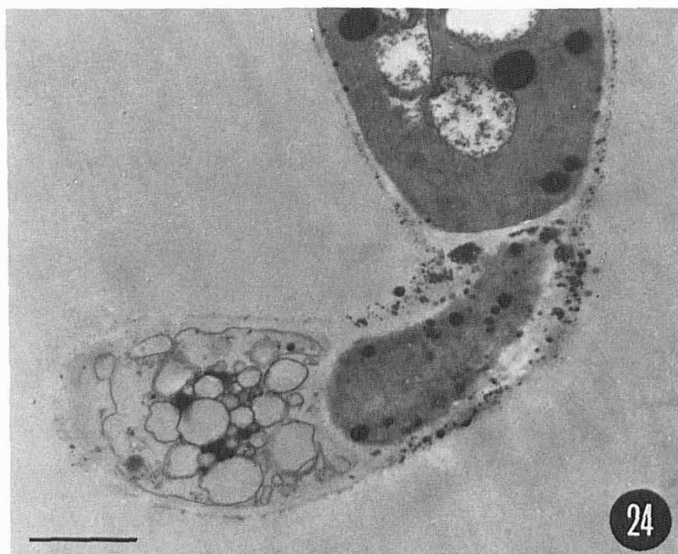
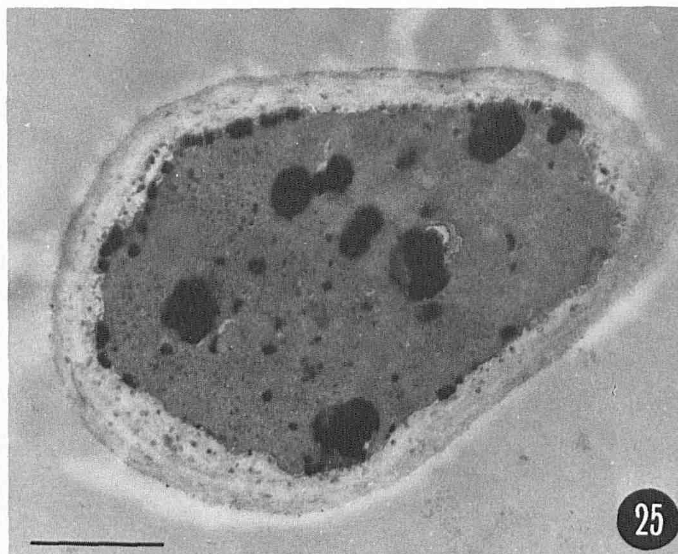
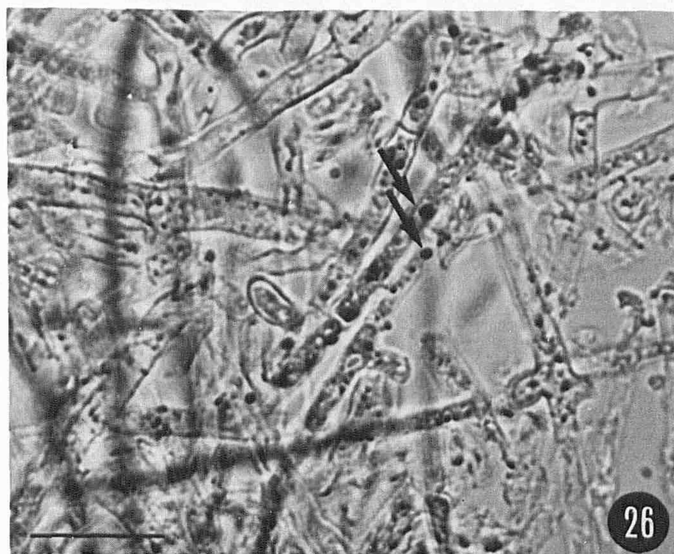


FIG 21-23. Cross-fractured faces of the cytoplasm of *T. mentagrophytes* cells exposed to 0.1 μg Naftifine/ml for the last 24 hr of a 72 hr incubation period. Note the accumulation of globular bodies with a smooth fracture-structure which are presumed to be lipid depositions (Fig 22) and a vacuole containing differently sized vesicles (Fig 23). Several vesicles are closely associated with the cell membrane (Fig 21, 23, *arrows*) (*Bars* = 1 μm). *EF* = fracture face of the extracellular half of the cytoplasmic membrane; *V*, vacuol; *L*, lipid granula.

oles become stuffed with differently sized (65–565 nm) vesicles (Fig 23). Similarly sized vesicles may be also present in close association with the cytoplasmic side of the vacuoles, and at concentrations of 0.5 μg/ml even within the matrix of mitochondria (not illustrated). The lipid nature of both the large electron dense bodies seen in the cytoplasma and the small bodies in contact with the cytoplasmic membrane and the cell wall, was demonstrated by extraction experiments with acetone







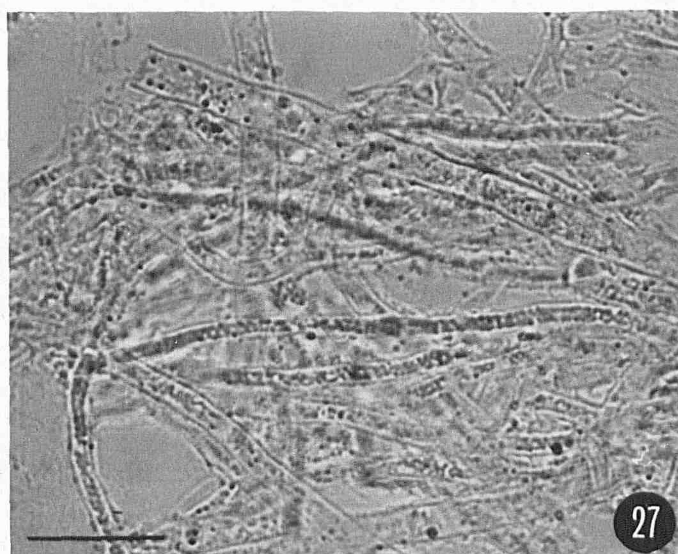


FIG 24 and 25. Section of *T. mentagrophytes* cells exposed to Naftifine as stated for Fig 21–23 in broth culture. Fig 24 Controls. Fig 25. A cell stained after lipid extraction with acetone. Note the absence of electron-dense particles at the surface of the cell wall after washing in acetone (*Bars* = 1 μm).

FIG 26 and 27. Sudan III-stained freeze cut cells. Fig 26. Shows cells exposed to 0.5 μg Naftifine/ml in broth culture, in Fig 27 the corresponding controls are seen. Note the abundant Sudan III-positive material in exposed cells (*arrows*) (*Bars* = 100 μm).

and Sudan III staining respectively. The granules clearly seen in the drug-treated cells (Fig 24) disappear from the cell surface and the superficial wall layers when GA-fixed cells are washed with acetone before the second fixation step in OsO_4 (Fig 25). Lipid bodies inside the cell wall and in the cytoplasma were not extracted by this procedure. The accumulation of lipid in the treated cells could be confirmed by light microscopy. In Sudan III-stained, freeze-cut sections a considerable increase in positively stained granules can be seen in Naftifine-treated cells when compared with untreated controls (Fig 26).

DISCUSSION

For the examination of morphological changes induced by Naftifine in *Trichophyton mentagrophytes*, a range of concentrations of this antimycotic was used throughout the experiments. Results have shown that the drug in concentrations of 0.1 to 0.5 μg/ml causes changes in the general cellular morphology and in the fine structure of hyphal cells *in vitro*. The most prominent change seen in the light and scanning electron microscope was swelling of the hyphal tips, showing that newly synthesized wall areas follow a different morphogenesis. The swelling of hyphal tips, seen in treated cells, may possibly be explained by the deposition of cell wall components which are less resistant to the osmotic pressure of the protoplast. The partially collapsed structure of the hyphal cells, seen in the SEM, might represent an artefact caused during critical point drying, due to the increased vacuolisation of treated cells, since similar changes are not seen in untreated controls.

The most significant drug-induced change at the ultrastructural level was the concentration-dependent formation of homogeneous electron dense bodies, seen in the cytoplasma and associated with the cell wall. Their lipid nature was supported by the observation that they are osmiophilic, leading to their electron dense appearance in thin sections, by their smooth conchoidal cross fracture faces, their staining properties with Sudan III and finally by their solubility in acetone after GA-fixation, as GA does not react with lipids. The solubility in acetone which was used for the scanning preparation explains also the absence of associated particles in the scanning electron micrographs.

From examining a large number of micrographs we conclude that the pathophysiologically synthesized lipid is either trans-

ported centripetally into vacuoles or alternatively to the cell surface by exocytosis. This process would explain the distortion of both fracture faces as well as the aggregation of vesicles on both sides of the plasma membrane. It remains questionable whether the structural changes seen in the cell wall are due to the described secretion process or to changes in the pattern of the cell wall synthesis.

The structural changes observed in *T mentagrophytes* are not artefacts since exclusively unfixed and uncryoprotected material was used in our study for freeze fracture replication procedures. This was of particular importance since we were able to show that structural changes in the cytoplasmic membrane, similar to the alterations induced by Naftifine, can be induced by commonly used methods for fixation and glycerination.

In summary it appears that Naftifine interferes with the lipid metabolism of the fungal cell. This tentative mode of action deduced from morphological observations is supported by biochemical data indicating that considerable amounts of squalene are accumulated in cells treated with Naftifine (Paltauf F, Daum G, Zuder G, Högenauer G, Schulz G, Seidl G: Squalene and ergosterole biosynthesis in fungi treated with Naftifine, a new antimycotic agent, in preparation).

The authors thank Dr. H. Müller for his help in scanning electron microscopy and Mrs. E. Kovacic and Ms. E. Pohoralek for their valuable technical assistance.

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Announcement

The Fourth UCLA Annual Review of Dermatology will take place on Saturday, January 23, 1982, in Los Angeles, CA. This course will review selected areas of significant new information in Dermatology. For further information contact Arnold W. Gurevitch, M.D., 1000 W. Carson St., Torrance, CA 90509, (213) 533-2465.

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