Cytoplasmic Microtubules and Fungal Morphogenesis: Ultrastructural Effects of Methyl Benzimidazole-2-ylcarbamate Determined by Freeze-substitution of Hyphal Tip Cells

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ABSTRACT   The effects of methyl benzimidazole-2-ylcarbamate (MBC), one of only a few agents that are active against microtubules of fungi, were analyzed at the ultrastructural level in freeze-substituted hyphal tip cells of *Fusarium acuminatum*. Nontreated and control cells had numerous microtubules throughout. After just 10 min of exposure to MBC, almost no cytoplasmic microtubules were present, except near spindle pole bodies. After 45 min of exposure to MBC, no microtubules were present in hyphal tip cells, but they were present in the relatively quiescent subapical cells. These observations suggested that there are different rates of turnover for cytoplasmic microtubules in apical and subapical cells and for microtubules near spindle pole bodies and that MBC acts by inhibiting microtubule assembly. A statistical analysis of the distribution of intracytoplasmic vesicles in thick sections of cells treated with MBC, D_2O or MBC + D_2O was obtained by use of a high-voltage electron microscope. More than 50% of the vesicles in the apical 30 μm of control cells were found to lie within 2 μm of the tip cell apex. MBC treatment caused this vesicle distribution to become uniform, resulting in a substantial increase in the number of vesicles in subapical regions. The reduction in the number of cytoplasmic microtubules, induced by MBC, apparently inhibited intracellular transport of these vesicles and rendered random the longitudinal orientation of mitochondria. In most cases, D_2O appeared capable of preventing these MBC-effects through stabilization of microtubules. These observations support the "vesicle hypothesis" of tip growth and establish a transport role for cytoplasmic microtubules in fungal morphogenesis.

Recent employment of an alternate fixation procedure, freeze-substitution, has revealed that several components of hyphal tip cells of fungi are largely or totally destroyed by conventional protocols of specimen preparation (22, 24). Among these labile cellular components are numerous cytoplasmic microtubules that lie parallel to the direction of intracytoplasmic vesicle transport. Presumably, these vesicles contain the materials necessary for cell wall synthesis and are en route to the tip cell apex where cellular expansion occurs (i.e., tip growth, see reference 17). By using antimicrotubular agents, such as colchicine, Colcemid, Vinca alkaloids, etc. (see reference 10), a possible role of cytoplasmic microtubules has been established for similar exocytotic processes in a variety of eukaryotic cell types—except fungi. It appears that fungal tubulin has little or no affinity for these standard antimitotic agents (4, 9). Moreover, earlier reports of such binding activity in fungal extracts have been seriously questioned (4, 9). It is thus apparent why a role for cytoplasmic microtubules in the tip growth process has not previously been adequately investigated.

Methyl benzimidazole-2-ylcarbamate (MBC), the active moiety of widely used fungicides, is bound by tubulins of various fungi (4, 9). Relative MBC toxicities have been correlated with tubulin binding affinities (9). Furthermore, a mutation in the gene that codes for β-tubulin may result in resistance

to MBC (38).

In a previous study of living cells (23), we inferred that all of the cytological effects of MBC could have been attributable to an interference with the normal functioning of microtubules.

The MBC inhibition of normal growth of hyphal tip cells was postulated to result from the loss of microtubule-mediated intracellular transport. The protection of cells against MBC effects that was provided by heavy water (D_2O) was thought to
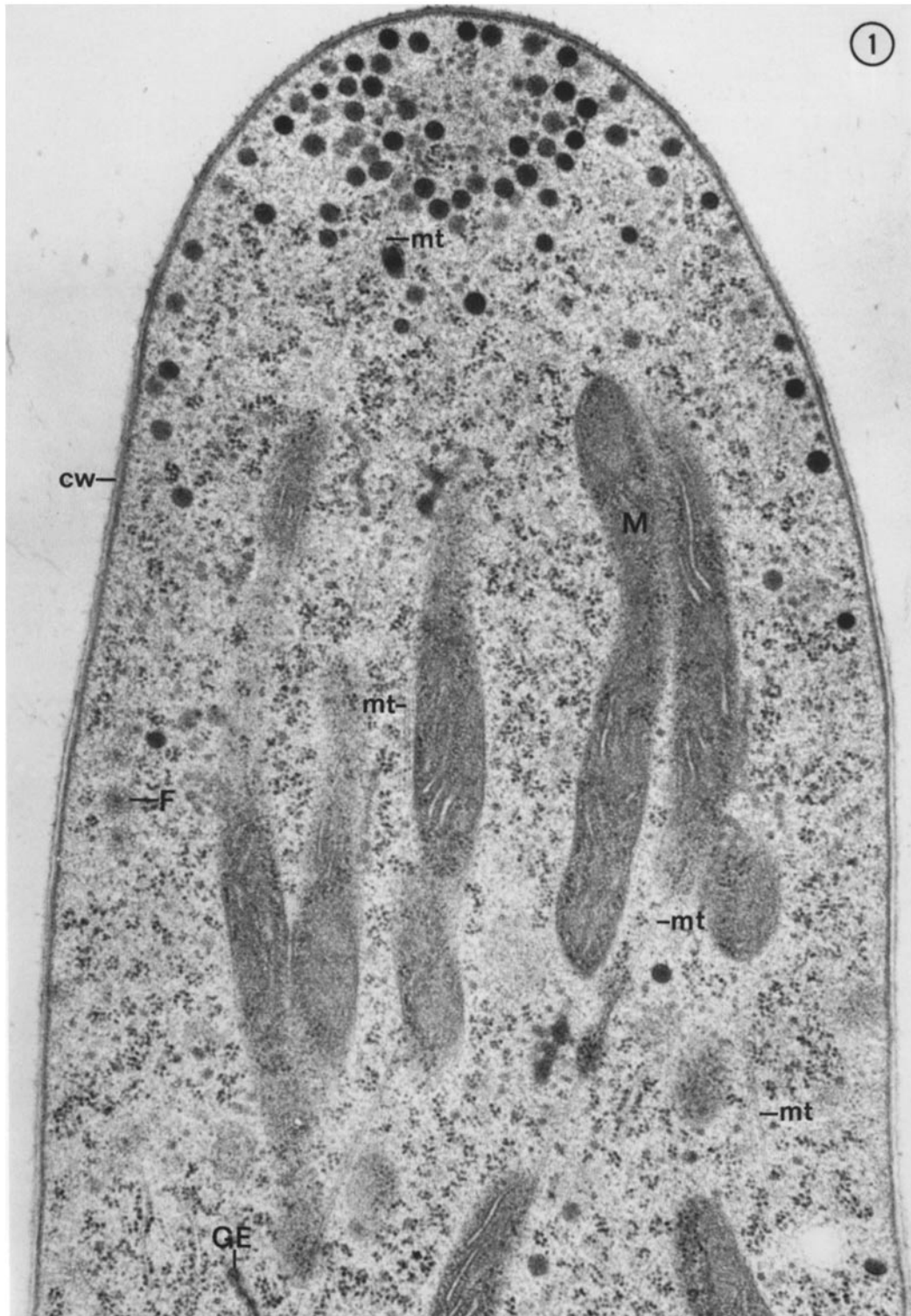
FIGURES 1 and 2   Median apical regions of hyphal tip cells treated with D_2O + DMSO (45 min) and MBC + DMSO (10 min), respectively. Fig. 1: no effects of D_2O + DMSO were recognized. Note the numerous microtubules (*mt*) and network of microfilaments within the apical cluster of vesicles. Fig. 2: 10 min after exposure to MBC + DMSO, few microtubules (*mt*) were present, the numbers of vesicles and microfilaments near the cell apex were reduced, and mitochondria were displaced from their normal subapical position. The outermost cell wall layer appears irregularly thickened, as compared with that in Fig. 1. Cell wall, *cw*; filasome, *F*; Golgi-like endomembrane cisterna, *GE*; mitochondrion, *M*. Bar, 1 μm. × 35,000.

result from a stabilization of microtubules, as has been reported in studies using other antimitotic compounds (see reference 23). Results of the present report support these interpretations. MBC apparently inhibits the long-distance intracellular transport of cell wall precursors by blocking the assembly of microtubules.

MATERIALS AND METHODS

Procedures for the storage, culture, and freeze-substitution of hyphal tip cells of *Giberella acuminata* Booth (*Fusarium acuminatum* Ellis and Everhart [ATCC

32965]) for electron microscopy were given elsewhere (22–24). Briefly, tip cells grown on cellulose membrane supports were frozen in molten fluoroform, substituted in acetone for 14 d at −85°C, and processed through chemical fixatives at ultralow temperatures according to reference 22. Individual hyphal tip cells were subsequently selected in flat Epon-Araldite embedments by the use of ×100 oil-immersion phase-contrast light optics and remounted for thick or thin sectioning (24). Sections were stained with uranyl acetate and lead citrate according to procedures already described (22).

Hyphal tip cells were exposed to the experimental reagents in the same manner as that used previously (23). Solutions were applied gently to colonies, flooding the entire surfaces, and were then partially drained off. Reagents, concentrations, and durations of exposure (i.e., minutes between first application and quenching)
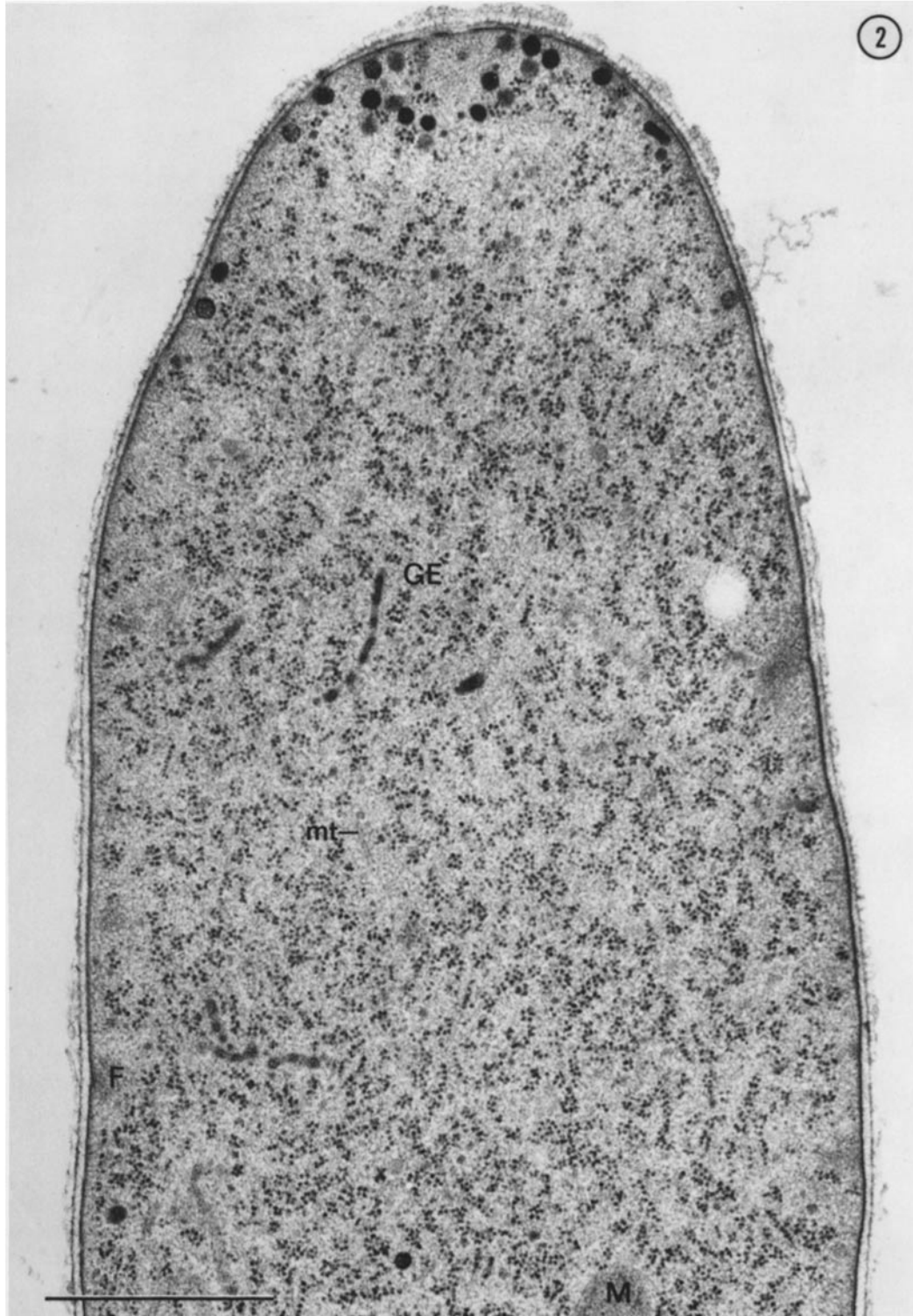
in each treatment were as follows: (A) 0.1% dimethyl sulfoxide (DMSO), 10 min; (B) 0.1% DMSO, 45 min; (C) 0.1% DMSO in 40% (vol/vol) deuterium oxide (D$_2$O), 45 min; (D) 1.0 μg methyl benzimidazole-2-ylcarbamate (MBC)/ml of 0.1% DMSO in 40% (vol/vol) D$_2$O, 45 min; (E) 1.0 μg MBC/ml of 0.1% DMSO, 10 min; (F) 1.0 μg MBC/ml of 0.1% DMSO, 45 min. MBC was the gift of E. I. du Pont de Nemours & Co., Wilmington, Delaware. All solutions were prepared with sterile, glass-distilled water and were used within 2 h of preparation. Treatments A-F were the same as those used in a previous light microscope study (23). Results of the present report, therefore, may be directly compared to our earlier findings.

Data on vesicle distributions in cells after the above treatments were derived as follows. Near-longitudinal, 0.5-μm-thick sections of hyphal tip cells were micrographed in stereo over the apical 30 μm, using a high voltage electron microscope (HVEM) operated at 900 or 1000 kV. This region was then divided into 30 portions of 1 μm each. The number of "apical vesicles" (~80 nm diameter) in each area was tabulated, resulting in 30 numerical observations on each section. Data from all sections within each treatment were then combined by calculating the average number of vesicles found in each successive micrometer from the cell apex. These average vesicle numbers (per micrometer), for each treatment, were then converted to the percentage of their total. This procedure yielded a numerical representation of vesicle distribution for each of the six different treatments analyzed. The vesicle distributions of all of the treatments were compared, two at a time, using the chi-square statistic. In addition, the vesicle distributions rendered by each treatment were compared with a random distribution to determine whether or not any particular treatment resulted in a uniform vesicle distribution. The numbers of cells, and sections of these cells, respectively, that were examined in the HVEM (comprising the data base for the above described analyses) after each of the treatments (A-F) follow: (A) 2, 8; (B) 4, 9; (C) 7, 27; (D) 4, 6; (E) 6, 18; (F) 12, 32. In addition, observations of serial thin sections from the following numbers of cells were used in determining the ultrastructural effects of each treatment: (A) 5; (B) 4; (C) 4; (D) 6; (E) 8; (F) 12.

RESULTS

No difference in the ultrastructure of hyphal tip cells was recognizable after a 10- or 45-min exposure to DMSO, nor after a 45-min exposure to D$_2$O + DMSO, compared with cells that received no treatment at all (22, 24). The "Spitzenkörper" region contained a large number of apical vesicles and microvesicles, lying within a network of microfilaments (Fig. 1). Microtubules and mitochondria were generally oriented parallel with the long axis of the cell. Mitochondria were usually located ~2 μm behind the apex, whereas microtubules were observed within the Spitzenkörper region, as well as all other areas of the cytoplasm. Filasomes (22) and Golgi-like endomembrane cisternae (GE) appeared unaffected by DMSO and/ or D$_2$O with respect to ultrastructure, distribution, and relative numbers (Fig. 1).

A 10-min exposure to MBC drastically altered tip cell ultrastructure. Microtubules were no longer observed as a ubiquitous component of the cytoplasm, but were only occasionally observed (Fig. 2). This low number of microtubules was apparently uniform throughout MBC-treated tip cells, except near spindle pole bodies (SPBs), where numerous microtubules were usually seen (Fig. 3). Mitochondria were no longer present in near-apical regions of hyphal tip cells (Fig. 2), as was also reported from light microscope observations (23).

After a 45-min exposure to MBC, no microtubules were present in hyphal tip cells, but some were present in subapical cells (Fig. 4). Mitochondria were no longer oriented parallel to the long axis of tip cells, having assumed random orientations.

In addition to affecting the number of cytoplasmic microtubules and the position and orientation of mitochondria, MBC had a marked effect on apical vesicle distribution (Table I and Fig. 5). Hyphae treated with DMSO or D$_2$O + DMSO represent control cells in which normal cytoplasmic distributions of 70- to 90-nm diameter vesicles were identified. More than 50% of the vesicles in the apical 30 μm of control cells were found

within 2.0 μm of the apex. After just 10 min of exposure to MBC, only ~30% of the vesicles in the apical 30 μm were within 2.0 μm of the apex. After 45 min of exposure to MBC, the percentage of these vesicles lying within 2.0 μm of the apex was reduced to ~12. Concomitantly, the percentage of vesicles in subapical regions increased markedly upon MBC treatment. Statistical comparisons showed that the vesicle distributions rendered by DMSO at 10 min and 45 min were significantly different ($P = .01$) from the distributions rendered by MBC + DMSO at 10 min and 45 min, respectively. The distribution of vesicles after MBC + D$_2$O + DMSO (45 min) was not significantly different from the distribution after D$_2$O + DMSO (45 min), even at $P = .10$, but was significantly different from the vesicle distribution rendered by MBC + DMSO (45 min). Vesicle distributions rendered by the various reagents were also compared with a uniform distribution. This analysis showed that MBC + DMSO (for 10 or 45 min) caused the vesicle distributions to become uniform, whereas the other four experimental treatments (including MBC + D$_2$O + DMSO) did not. These results indicate that MBC alters vesicle distribution significantly, and that D$_2$O prevents MBC from causing this effect.

The effects of MBC on the cytoplasmic distribution of microvesicles (~30 nm diameter) and filasomes were not analyzed statistically, but in no cell exposed to MBC was an apical cluster of microvesicles (as shown in Fig. 1) observed. No other effect on microvesicle or filasome distribution was detected.

The network of microfilaments near the apex of DMSO- and D$_2$O + DMSO-treated cells was visible in some MBC-treated cells after a 10-min exposure to MBC. After 45 min of exposure to MBC, filaments were sometimes present at the hyphal apex in association with the few remaining apical vesicles; however, a network of microfilaments was never observed. These remnants of the Spitzenkörper may explain why the hypha can still grow slowly in the presence of MBC (23; see also reference 22).

Hyphal tip cell wall that apparently formed in the presence of MBC appeared to differ from newly formed cell wall synthesized in the absence of MBC. This difference was identified in the outermost wall layer, previously designated as layer "4" (24), and consisted of abnormal, irregular thickening (Fig. 2) over the apical dome and along lateral walls. This effect was not evident in cells exposed to MBC + D$_2$O. In a few MBC-treated tip cells an additional effect on wall formation was evident: cell walls were about twice the thickness of control cells, the cell outline was extremely irregular, and a cytoplasmic layer of electron-dense material (containing apical vesicles, microvesicles, ribosomes, multivesicular bodies, and smooth membrane cisternae) was appressed to the plasma membrane.

Autolytic vacuoles and an abnormally large number of multivesicular bodies were evident throughout the cytoplasm of hyphal tip cells after 45-min exposure to MBC (Figs. 6, 7). These structures were rare in cells that had received the MBC + D$_2$O treatment (Fig. 8). These MBC + D$_2$O-treated cells usually exhibited many cytoplasmic microtubules (Fig. 8) and appeared identical to controls in every other respect. In some of these MBC + D$_2$O-treated cells, mitosis was evident (Fig. 9).

Although the observations reported above represent the typical result of MBC + D$_2$O treatment, a few cells were affected as if D$_2$O were not present at all. Such cells appeared similar to those exposed to MBC alone, except that a few microtubules were present even after 45 min (Fig. 10).
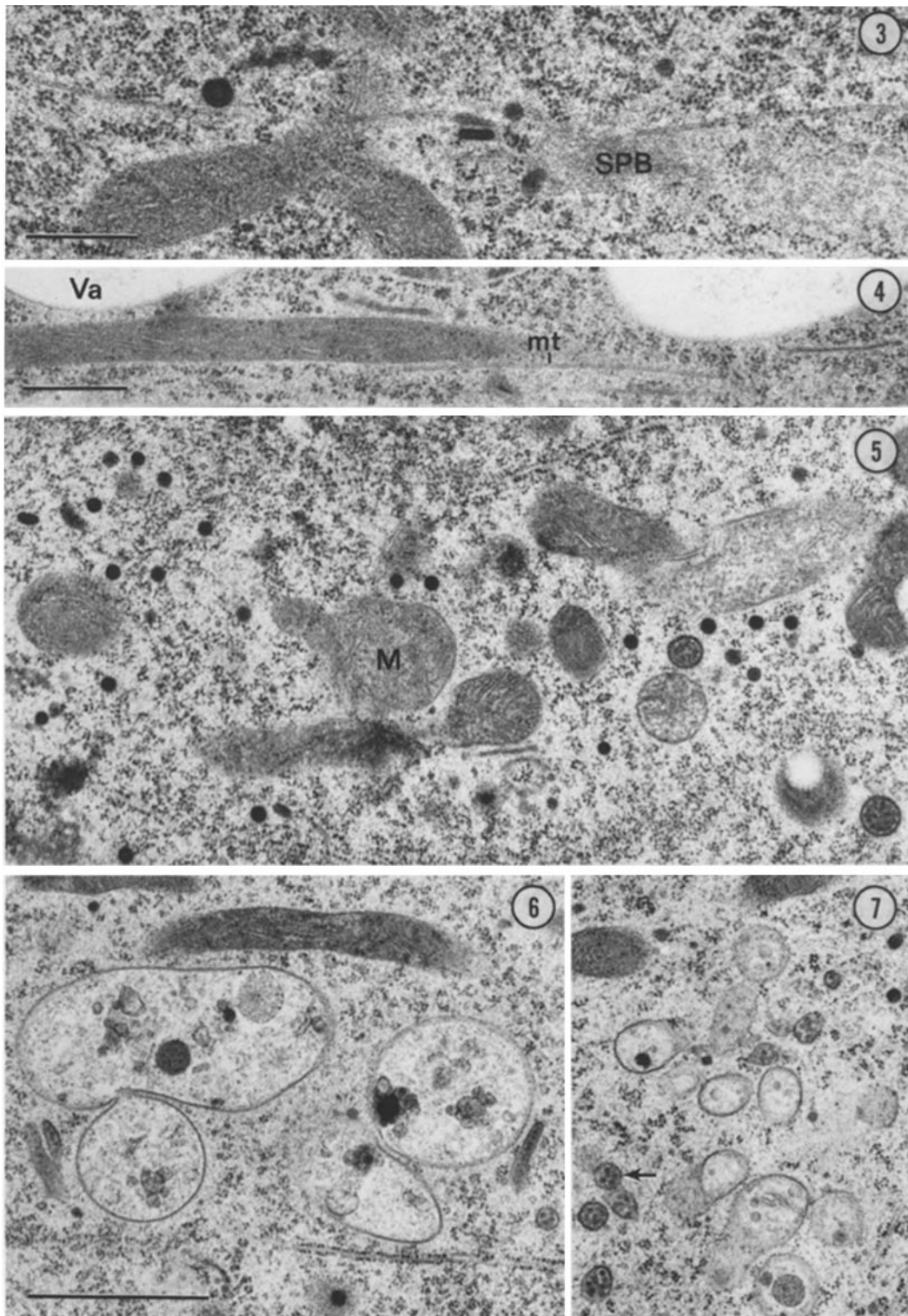
FIGURE 3 After a 10-min MBC + DMSO treatment, microtubules were observed infrequently throughout tip cells, except in association with spindle pole bodies (SPB). Bar, 500 nm. × 35,000.

FIGURE 4 After 45 min of exposure to MBC + DMSO, microtubules could be found in highly vacuolated, subapical cells. Microtubule, mt; vacuole, Va. Bar, 500 nm. × 31,000.

FIGURES 5–7 Subapical regions of tip cells after 45-min exposure to MBC + DMSO. Fig. 5: the cytoplasmic distribution of vesicles has become random such that the concentration of vesicles in subapical regions has increased significantly. In addition, mitochondria (M) are less uniform in shape, orientation and position than in controls. Fig. 6: autolytic vacuoles are abundant. Fig. 7: large numbers of autolytic vacuoles and multivesicular bodies (arrow) can be found throughout subapical areas of these tip cells. Bar, 1 μm. × 27,000.

Comparison of the Effects of Various Treatments on the Distributions of 70–90 nm Diameter Vesicles in Hyphal Tip Cells of Fusarium acuminatum

Distance from cell apex,	DMSO, 10*		DMSO, 45		D_2O + DMSO, 45		MBC + D_2O + DMSO, 45		MBC + DMSO, 10		MBC + DMSO, 45	
μm	no.	%‡	no.	%	no.	%	no.	%	no.	%	no.	%
1	132	48.5	95	37.4	91	45.7	67	37.6	52	20.8	22	7.6
2	22	8.1	37	14.6	14	7.0	24	13.5	24	9.6	14	4.8
3	16	5.9	21	8.3	9	4.5	11	6.2	15	6.0	14	4.8
4	12	4.4	13	5.1	2	1.0	5	2.8	10	4.0	13	4.5
5	8	2.9	8	3.1	2	1.0	6	3.4	9	3.6	13	4.5
6	4	1.5	7	2.8	7	3.5	5	2.8	9	3.6	13	4.5
7	8	2.9	5	2.0	2	1.0	6	3.4	8	3.2	13	4.5
8	8	2.9	4	1.6	5	2.5	4	2.3	9	3.6	11	3.8
9	4	1.5	4	1.6	5	2.5	3	1.7	8	3.2	10	3.4
10	4	1.5	3	1.2	5	2.5	3	1.7	7	2.8	11	3.8
11	2	0.7	3	1.2	5	2.5	3	1.7	8	3.2	13	4.5
12	2	0.7	4	1.6	5	2.5	3	1.7	7	2.8	9	3.1
13	2	0.7	5	2.0	7	3.5	2	1.1	8	3.2	10	3.4
14	4	1.5	2	0.8	5	2.5	2	1.1	9	3.6	7	2.4
15	4	1.5	3	1.2	2	1.0	3	1.7	6	2.4	8	2.8
16	2	0.7	3	1.2	2	1.0	2	1.1	6	2.4	7	2.4
17	2	0.7	6	2.4	2	1.0	3	1.7	5	2.0	8	2.8
18	2	0.7	3	1.2	2	1.0	2	1.1	7	2.8	7	2.4
19	2	0.7	3	1.2	2	1.0	3	1.7	5	2.0	6	2.1
20	4	1.5	1	0.4	2	1.0	2	1.1	8	3.2	6	2.1
21	4	1.5	1	0.4	2	1.0	2	1.1	2	0.8	6	2.1
22	6	2.2	1	0.4	2	1.0	2	1.1	4	1.6	8	2.8
23	4	1.5	2	0.8	2	1.0	2	1.1	3	1.2	6	2.1
24	0	0	4	1.6	2	1.0	2	1.1	4	1.6	7	2.4
25	4	1.5	2	0.8	2	1.0	3	1.7	1	0.4	8	2.8
26	2	0.7	1	0.4	2	1.0	1	0.6	4	1.6	7	2.4
27	2	0.7	5	2.0	5	2.5	2	1.1	3	1.2	8	2.8
28	2	0.7	4	1.6	2	1.0	2	1.1	4	1.6	7	2.4
29	2	0.7	2	0.8	2	1.0	2	1.1	3	1.2	7	2.4
30	2	0.7	2	0.8	2	1.0	1	0.6	2	0.8	11	3.8
Total no.	272		254		199		178		250		290	

Chi-Square§

	DMSO, 10*	DMSO, 45	D_2O + DMSO, 45	MBC + D_2O + DMSO, 45	MBC + DMSO, 10	MBC + DMSO, 45
Uniform distribution	$\chi^2 = 30.19\|$	$\chi^2 = 21.39\|$	$\chi^2 = 25.33\|$	$\chi^2 = 19.87\|$	$\chi^2 = 2.84**$	$\chi^2 = 7.08**$
DMSO, 10					$\chi^2 = 21.21\|$	
DMSO, 45			$\chi^2 = 7.33**$			$\chi^2 = 40.91\|$
D_2O + DMSO, 45				$\chi^2 = 5.17**$		
MBC + D_2O + DMSO, 45					$\chi^2 = 11.76¶$	$\chi^2 = 38.61\|$

* Treatments are designated by reagent and minutes of exposure.
‡ Data are given as the average number (no.), and that number's percent (%) of the total, of vesicles per 0.5-μm section per 1-μm unit.
§ Degrees of freedom = 6; $P = .10, \chi^2 = 10.64$; $P = .05, \chi^2 = 12.59$; $P = .01, \chi^2 = 16.81$.
‖ $P < .01$.
¶ $.10 \leq P \leq .05$.
** Not significant.

DISCUSSION

The evidence that demonstrates an interaction between MBC and fungal tubulin (4, 9, 23, 32, 38) is to us thoroughly convincing. However, the point that MBC-specificity remains unproven (18) is well taken. The same may be said of every other drug that has been used to investigate microtubule function. MBC exhibits, as do most of these antimicrotubule drugs, a high affinity for tubulin and appears to specifically affect microtubule integrity in vivo.

The idea that cell asymmetry and polarity are related to cytoplasmic microtubules (33) has been supported by a battery of subsequent reports (see reference 10). This apparent role of microtubules pertains to both (a) the maintenance of mature cell shape and (b) the determination of shape during cell morphogenesis. The possible role of microtubules in the former is irrelevant with respect to the walled cells of plants and fungi. However, it is clear that microtubules participate in the determination of plant cell shape in a variety of species (see reference 10) for none of which are the actual mechanisms of microtubule involvement known. In either case, microtubules have usually been thought to participate (a) as a cytoskeleton and/or (b) by mediating subcellular organization and intracellular transport.

In addition to plants, wall formation in cells of fungi is directly responsible for cell shape and it is the pattern of localized cell wall component deposition that is responsible for morphogenesis in many of these organisms. Among filamentous fungi, and mycelial states of dimorphic fungi, cell wall
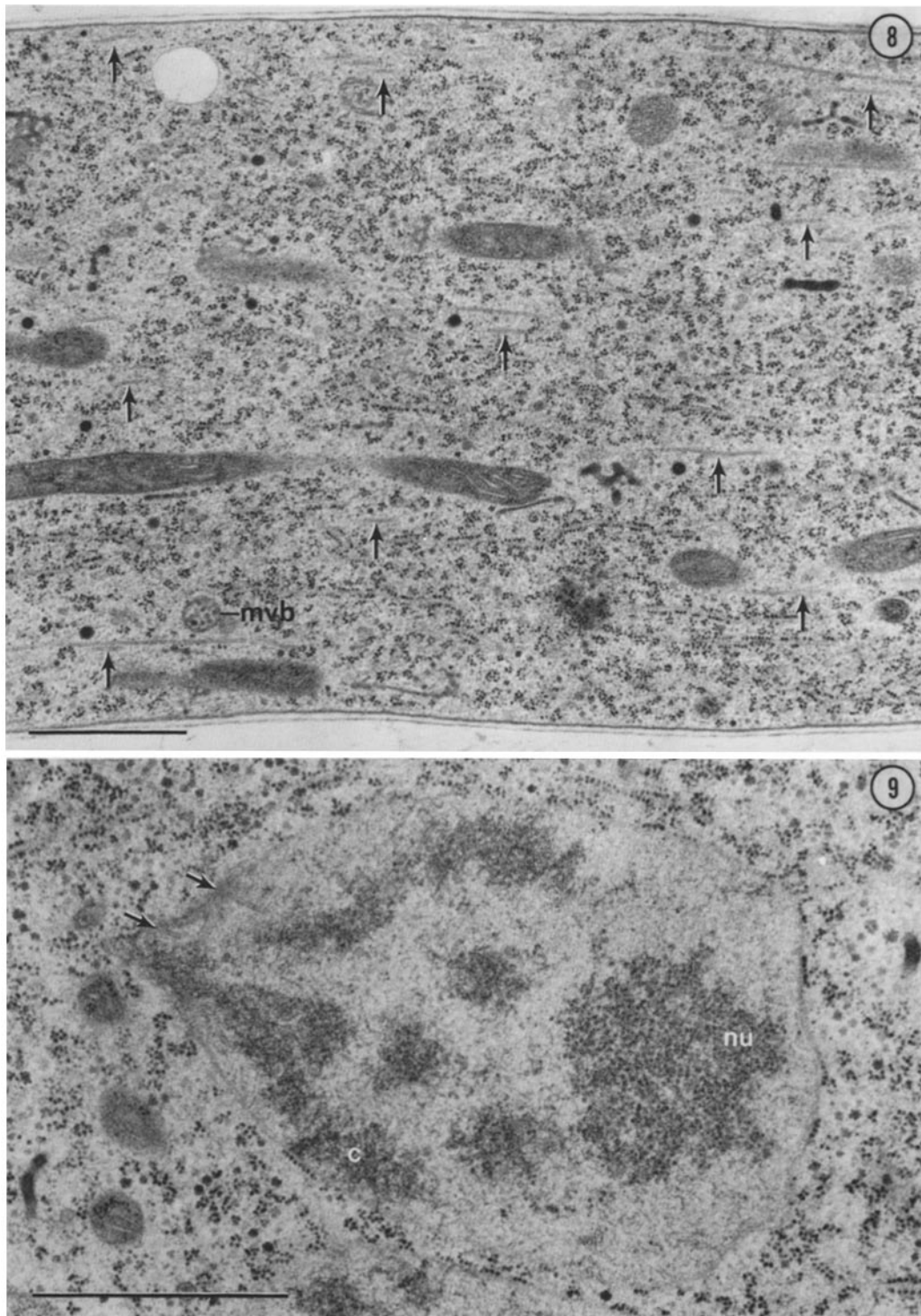
FIGURES 8 and 9 Subapical regions of hyphal tip cells treated with MBC + D₂O + DMSO for 45 min. Fig. 8: numerous cytoplasmic microtubules (arrows) lie parallel to the long axis of the cell. Mitochondria appear normal in every respect. Multivesicular body, mvb. Fig. 9: a mitotic prophase nucleus exhibiting microtubules (arrows), condensed chromosomes (c), and a nucleolus (nu). Bars, 1 μm. Fig. 8, × 29,000; Fig. 9, × 39,000.

deposition related to cellular expansion is highly localized into one small area of the cell, the hyphal apex. The result of this localization is the phenomenon of tip growth, which is based on a polarity in the subcellular organization of the entire hyphal tip cell.

One obvious manifestation of polar organization in *F. acuminatum*, as well as all other adequately prepared species of

ascomycetous and basidiomycetous fungi (see reference 17), is a massive accumulation of intracytoplasmic vesicles at the hyphal apex (22, 24). Under phase-contrast light optics the position of this vesicle cluster, known as the Spitzenkörper, has been used as an indicator of the direction of future cell expansion: a slight change in the position of the Spitzenkörper within the apical dome results in a subsequent and corresponding
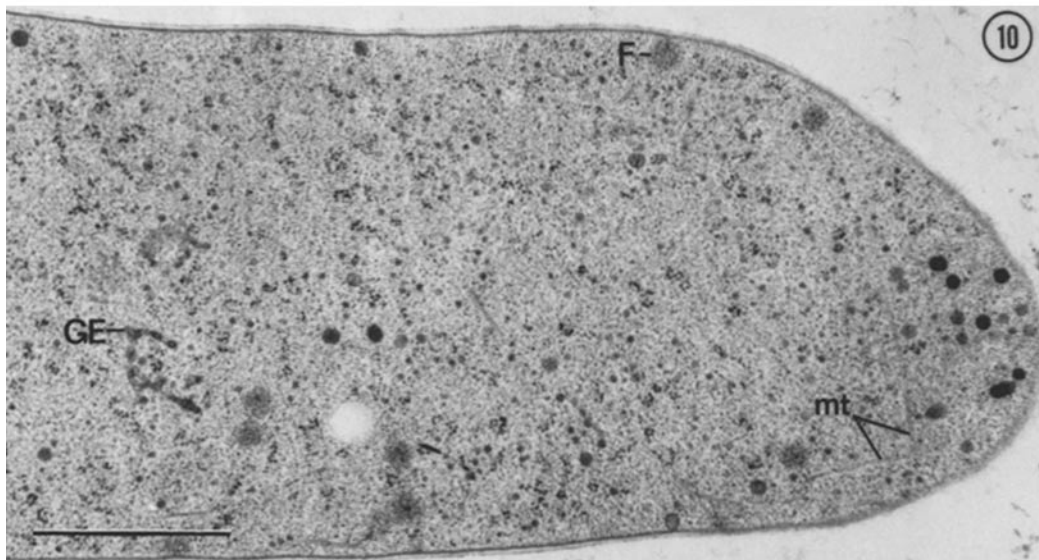
FIGURE 10 Apical region of a cell after 45-min exposure to MBC + D₂O + DMSO. Most cells treated in this manner appeared normal. However, a few hyphae, like this one, appeared similar to cells treated with MBC in the absence of D₂O, except that microtubules were present. Note the oblique orientation of one of the microtubules. Microtubules, mt; Golgi-like endomembrane cisterna, GE; filasome, F. Bar, 1 μm. × 27,000.

change in the direction of growth (see reference 17). It has been suggested that existence of the Spitzenkörper (i.e., an accumulation of hundreds of apical vesicles that exhibit movement en masse) may be attributable in part to a network of microfilaments among the component vesicles (22).

In a previous study (23) we reported, from observations of living cells, that MBC caused a gradual disappearance of the Spitzenkörper and a reduction in the rate of cell expansion. We interpreted these MBC effects "as resulting from disruption of microtubule-mediated transport of wall materials or Spitzenkörper integrity, or both" (23). This role for cytoplasmic microtubules in fungi has been postulated elsewhere (5, 40). In light of our present findings, we suggest the following conclusions. First, cytoplasmic microtubules do indeed mediate long-distance intracellular transport of cell wall precursors in hyphal tip cells. These microtubules are oriented parallel to the direction of growth (22, 24) and are associated closely with vesicles en route to the cell apex (22). MBC-induced loss of these microtubules inhibits vesicle transport, which results in randomization of their distribution. Second, the concept that the ultrastructural equivalent of the Spitzenkörper is an accumulation of apical vesicles (see reference 22) is supported by our present findings. Loss of microtubules after exposure to MBC results in a loss of efficient transport of Spitzenkörper component vesicles toward the tip cell apex. Because vesicles continue to fuse with the plasma membrane (as is indicated by continued, but gradually decreasing, cell expansion [23]), the apical accumulation (Spitzenkörper) is depleted of vesicles more rapidly than they are supplied. Hence, a gradual disappearance of the Spitzenkörper occurs. That some microfilaments remain in the apex of MBC-treated cells might explain why growth, albeit slow, continues in a polar fashion (see reference 22). Third, the scheme for tip growth first proposed by McClure et al. (29) is basically correct. Vesicles containing wall materials are produced in basipetal regions of the cell (22) and are transported to the apex where they fuse with the plasma membrane. Fourth, our previous suggestion that "one role of the Spitzenkörper might be to orient cell wall-component deposition at the hyphal

apex" (23) is also basically correct. Because several different components involved in cell wall formation (3, 15, 22, 24) are all apparently transported in the above manner, loss of efficient transport of any of these components would destroy the capacity of the Spitzenkörper for regulating each component's participation. Loss of efficient transport would disrupt any relative balance (2) between numbers of various participating precursors and enzymes that the Spitzenkörper might ordinarily maintain. The irregular thickening of the outermost cell wall layer seen after MBC treatment may be a manifestation of this effect. In addition, in the subsequent absence of a Spitzenkörper, precise control over the location of interactions between the plasma membrane and vesicles would be lost. The expected result would be uncontrolled fusions over the inner surface of the apical dome and alterations in the shape of the cell apex, as was previously reported to occur (23).

These suggested conclusions are based on a working hypothesis that hyphal tip growth is the result of a highly ordered system of exocytosis (see reference 22). In many other cell types, secretory processes have been assumed to be dependent on cytoplasmic microtubules because of the observed inhibition by drugs that disrupt microtubules (see references 8, 10, 11, 23, 26, 27, 35, 41, and references therein), and because of the close association between intracytoplasmic vesicles and microtubules (1, 16, 31, 39). However, these facts do not provide unequivocal evidence for the direct participation of microtubules in intracellular transport phenomena. The observed association between (apical) vesicles and cytoplasmic microtubules may or may not represent an interaction relevant to the flow of these vesicles (see reference 12; cf. reference 7).

The effect of MBC on intracellular transport of vesicles in hyphal tip cells cannot be separated from the effects on subcellular organization. MBC-induced loss of cytoplasmic microtubules drastically altered the usual alignment of mitochondria parallel with the long axis of the cell, for example. That microtubules are directly or indirectly associated with mitochondria has been presumed (19, 24; see also reference 10), and it has been demonstrated that elimination of microtubules can

randomize mitochondrial orientation (20; see also reference 23, and Results above). One might ascribe to microtubules the function of maintenance of internal organization and cell polarization (14, 21, 23, 36; see also reference 10), the loss of which might interfere with efficient transport phenomena. This possibility would appear especially relevant to the hyphal tip cell (and cells of similar shape [e.g., see references 34 and 37]) in which efficient transport would be impossible if internal organization were not strictly maintained parallel to the direction of transport.

In addition to causing mitochondrial displacement, disappearance of the Spitzenkörper, and reduction of linear growth rate, treatment of living cells with MBC also caused a metaphase arrest of all mitoses (23). Because all of these responses were apparently attributable to an effect on microtubules, one might expect that heavy water (D_2O), in an appropriate concentration, would protect microtubules against the action of MBC. (Examples of this protective effect of D_2O were previously reviewed [23]). Following this line of reasoning, we reported earlier that simultaneous treatment of hyphal tip cells with MBC + D_2O caused responses that were intermediate between those caused by MBC and those caused by D_2O when applied separately, except in the case of nuclear division (23). Our present results support the conclusion that this D_2O protection against MBC ultimately resulted from the opposing effects of these two agents upon microtubules. Even though the results of MBC + D_2O treatments were somewhat variable, such variation could again be ascribed to effects on microtubules. After a 45-min exposure to MBC, no microtubules were observed in hyphal tip cells, and mitochondrial orientation and vesicle distribution were random. After a 45-min exposure to MBC + D_2O, cells usually appeared unaffected, but some of them were similar to cells treated with MBC alone, except that a few microtubules were present. In the latter case, microtubule orientation appeared random, which might partially explain why MBC effects were still exhibited by these few cells. Why this concomitant treatment did not affect all cells uniformly is not known, but this result is consistent with our previous observations (23). Whether or not D_2O interferes with the activity of MBC through some effect on tubulin (e.g., inducing a conformation rearrangement (42) that alters affinity for MBC), on microtubules (e.g., by slowing the rate of metabolic turnover), or at some other level (see references 13 and 30) should be assessed in vitro and in vivo.

Our observation of a differential sensitivity of SPB-associated and -nonassociated microtubules to MBC may represent an important clue to the mechanism of action of this compound. The in vitro capacity of SPBs of *Saccharomyces cerevisiae* to act as microtubule-organizing centers (MTOCs) has been independently reported from two different laboratories (6, 25). The fact that SPB-associated microtubules of *F. acuminatum* were more resistant to MBC treatment may indicate that they are more stable than those microtubules not associated with an SPB (viz., the nonassociated microtubules may have a faster rate of metabolic turnover, as was suggested by Dustin [10], and would therefore be more rapidly affected by MBC). Accordingly, the fact that MBC apparently did not affect cytoplasmic microtubules in subapical cells would indicate that metabolic turnover of these microtubules is relatively slow. From this argument it would appear that the mechanism of action of MBC may be defined in terms of its ability to prevent the assembly of microtubule subunits. This was the same conclusion reached by Künkel and Hädrich (28). Alter-

natively, if MBC also disassembles microtubules, one would expect to see only microtubules associated with MTOCs until the unbound pool of tubulin was depleted. According to this interpretation, the relative insensitivity of microtubules in subapical cells may indicate that these cells took up too little MBC for the disassembly effect to be noticed.

REFERENCES

1. Allen, R. D. 1975. Evidence for firm linkages between microtubules and membrane-bounded vesicles. *J. Cell Biol.* 64:497–503.
2. Bartnicki-Garcia, S. 1973. Fundamental aspects of hyphal morphogenesis. *In* Microbial Differentiation. J. M. Ashworth and J. E. Smith, editors. Cambridge University Press, England. 245–267.
3. Bartnicki-Garcia, S., C. E. Bracker, E. Reyes, and J. Ruiz-Herrera. 1978. Isolation of chitosomes from taxonomically diverse fungi and synthesis of chitin microfibrils in vitro. *Experimental Mycology.* 2:173–192.
4. Baum, P., J. Thorner, and L. Honig. 1978. Identification of tubulin from the yeast *Saccharomyces cerevisiae.* Proc. Natl. Acad. Sci. U. S. A. 75:4962–4966.
5. Byers, B., and L. Goetsch. 1975. Behavior of spindles and spindle plaques in the cell cycle and conjugation of *Saccharomyces cerevisiae. J. Bacteriol.* 124:511–523.
6. Byers, B., K. Shriver, and L. Goetsch. 1978. The role of spindle pole bodies and modified microtubule ends in the initiation of microtubule assembly in *Saccharomyces cerevisiae. J. Cell Sci.* 30:331–352.
7. Caron, J. M., and R. D. Berlin. 1979. Interaction of microtubule proteins with phospholipid vesicles. *J. Cell Biol.* 81:665–671.
8. Chaldakov, G. N., S. Nikolov, and V. Vancov. 1977. Fine morphological aspects of the secretory process in arterial smooth muscle cells. II. Role of microtubules. *Acta Morphol. Acad. Sci. Hung.* 25:167–174.
9. Davidse, L. C., and W. Flach. 1977. Differential binding of methyl benzimidazole-2-ylcarbamate to fungal tubulin as a mechanism of resistance to this antimitotic agent in mutant strains of *Aspergillus nidulans. J. Cell Biol.* 72:174–193.
10. Dustin, P. 1978. Microtubules. Springer-Verlag, New York.
11. Eichhorn, J. H., and B. Peterkofsky. 1979. Local anesthetic-induced inhibition of collagen secretion in cultured cells under conditions where microtubules are not depolymerized by these agents. *J. Cell Biol.* 81:26–42.
12. Fitzsimons, J. T. R., G. A. Kerkut, and G. A. Sharp. 1978. Localization of microtubular ATPase in rat, snail and crab nerves. *J. Physiol. (Lond.).* 278:6P–7P.
13. Flaumenhaft, E., S. Bose, H. L. Crespi, and J. J. Katz. 1965. Deuterium isotope effects in cytology. *Int. Rev. Cytol.* 18:313–361.
14. Galatis, B., P. Apostolakos, and Chr. Katsoros. 1978. Ultrastructural studies on the oil bodies of *Marchantia paleacea* Bert. I. Early stages of oil-body cell differentiation: origination of the oil body. *Can. J. Bot.* 56:2252–2267.
15. Girbardt, M. 1969. Die Ultrastruktur der Apikalregion von Pilzhyphen. *Protoplasma.* 67:413–441.
16. Gray, E. G. 1976. Microtubules and synapses of the retina. *J. Neurocytol.* 5:361–370.
17. Grove, S. N. 1978. The cytology of hyphal tip growth. *In* The Filamentous Fungi. Vol 3. J. E. Smith and D. R. Berry, editors. John Wiley & Sons, New York. 28–50.
18. Heath, I. B. 1978. Experimental studies of mitosis in the fungi. *In* Nuclear Division in the Fungi. I. B. Heath, editor. Academic Press, Inc. New York. 89–176.
19. Heath, I. B., and M. C. Heath. 1978. Microtubules and organelle movements in the rust fungus *Uromyces phaseoli* var *vignae. Cytobiologie.* 16:393–411.
20. Heggeness, M. H., M. Simon, and S. J. Singer. 1978. Association of mitochondria with microtubules in cultured cells. *Proc. Natl. Acad. Sci. U. S. A.* 75:3863–3866.
21. Hoffstein, S., I. M. Goldstein, and G. Weissmann. 1977. Role of microtubule assembly in lysosomal enzyme secretion from human polymorphonuclear leukocytes. *J. Cell Biol.* 73:243–256.
22. Howard, R. J. 1980. Ultrastructural analysis of hyphal tip cell growth in fungi: Spitzenkörper, cytoskeleton and endomembranes after freeze-substitution. *J. Cell Sci.* In press.
23. Howard, R. J., and J. R. Aist. 1977. Effects of MBC on hyphal tip organization, growth and mitosis of *Fusarium acuminatum,* and their antagonism by D_2O. *Protoplasma.* 92:195–210.
24. Howard, R. J. and J. R. Aist. 1979. Hyphal tip cell ultrastructure of the fungus *Fusarium:* improved preservation by freeze-substitution. *J. Ultrastruct. Res.* 66:224–234.
25. Hyams, J. S., and G. G. Borisy. 1978. Nucleation of microtubules in vitro by isolated spindle pole bodies of the yeast *Saccharomyces cerevisiae. J. Cell Biol.* 78:401–414.
26. Hyams, J. S., and H. Stebbings. 1979. The formation and breakdown of nutritive tubes—massive microtubular organelles associated with cytoplasmic transport. *J. Ultrastruct. Res.* 68:46–57.
27. Knudson, C. M., B. H. Stemberger, and S. Patton. 1978. Effects of colchicine on ultrastructure of the lactating mammary cell: membrane involvement and stress on the Golgi apparatus. *Cell Tissue Res.* 195:169–181.
28. Künkel, W., and H. Hädrich. 1977. Ultrastruckturelle Untersuchungen zur antimitotischen Aktivität von Methylbenzimidazol-2-ylcarbamate (MBC) und seinen Einfluß auf die Replikation des Kern-Assoziierten Organells ("centriolar plaque", "MTOC", "KCE") bei *Aspergillus nidulans. Protoplasma.* 92:311–323.
29. McClure, W. K., D. Park, and P. M. Robinson. 1968. Apical organization in the somatic

hyphae of fungi. *J. Gen. Microbiol.* 50:177-182.

30. Middaugh, C. R., and G. W. Litman. 1977. Effect of D₂O on the temperature-dependent solubility of cryoglobulin and noncryoglobulin IgM. *FEBS (Fed. Eur. Soc. Biochem.) Lett.* 79:200-202.

31. Mollenhauer, H. H. 1974. Distribution of microtubules in the Golgi apparatus of *Euglena gracilis. J. Cell Sci.* 15:89-98.

32. Morris, N. R., M. H. Lai, and C. E. Oakley. 1979. Identification of a gene for α-tubulin in *Aspergillus nidulans. Cell.* 16:437-442.

33. Porter, K. R. 1966. Cytoplasmic microtubules and their functions. *In* CIBA Foundation Symposium. Principles of Biomolecular Organization. G. E. W. Wolstenholme and M. O'Connor, editors. Little, Brown & Co., Boston, Mass. 308-345.

34. Raine, C. S., B. Ghetti, and M. L. Shelanski. 1971. On the association between microtubules and mitochondria within axons. *Brain Res.* 34:389-393.

35. Reaven, E. P., and G. M. Reaven. 1977. Distribution and content of microtubules in relation to the transport of lipid. *J. Cell Biol.* 75:559-572.

36. Roos, U. P., and G. Turian. 1977. Hyphal tip organization in *Allomyces arbuscula.*

Protoplasma. 93:231-247.

37. Sasaki, S., and Y. Tashiro. 1976. Studies on the posterior silk gland of the silkworm *Bombyx mori.* VI. Distribution of microtubules in the posterior silk gland cells. *J. Cell Biol.* 71:565-574.

38. Sheir-Neiss, G., M. H. Lai, and N. R. Morris. 1978. Identification of a gene for β-tubulin in *Aspergillus nidulans. Cell.* 15:639-647.

39. Smith, D. S., U. Jarlfors, and B. F. Cameron. 1975. Morphological evidence for the participation of microtubules in axonal transport. *Ann. N. Y. Acad. Sci.* 253:472-506.

40. Stewart, P. R., and P. J. Rogers. 1978. Fungal dimorphism: A particular expression of cell wall morphogenesis. *In* The Filamentous Fungi. Vol. 3. J. E. Smith and D. R. Berry, editors. John Wiley & Sons, New York. 164-196.

41. Thyberg, J., S. Moskalewski, and U. Friberg. 1978. Effects of antimicrotubular agents on the fine structure of the Golgi complex in embryonic chick osteoblasts. *Cell Tissue Res.* 193:247-257.

42. Volotovskii, I. D., L. A. Boranova, and S. V. Konev. 1976. Influence of heavy water (D₂O) on the conformation and UV sensitivity of proteins. *Mol. Biol. (Mosc.).* 10:1077-1083.