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Effects of Cytochalasins on *Neurospora crassa* I. Growth and Ultrastructure

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Summary

Growth of various strains of *Neurospora crassa* in the continued presence of cytochalasins A and B results in the following pattern. At low concentration the drugs cause an increase in branching which, depending on the strain, may also reveal an increase in dry weight. Increasing the concentration results in abnormal hyphae (swollen, irregular) and eventually in inhibition of growth. Most strains are inhibited at 10–20 μg/ml cytochalasin A. Ultrastructure of hyphae grown in the presence of cytochalasin A reveals the presence of abnormal wall deposits. These deposits are not observed in cultures grown with cytochalasin B which generally causes a lower growth response also.

Keywords: Cytochalasins; Hyphal ultrastructure; Mycel development; *Neurospora crassa.*

1. Introduction

The cytochalasins represent a group of fungal metabolites which affect a variety of cell functions. The first published report detailing the structure of a cytochalasin was that describing phomin (cytochalasin B) isolated from *Phoma exigua* in 1966 by ROTHWEILER and TAMM [20]. At about the same time ALDRIGE *et al.* [1] isolated cytochalasins A and B from *Helminthosporium dematioideum.* Since that time, several other cytochalasins and related compounds from various fungi have been isolated and described (see review by TAMM [23].

Although cytochalasins were shown first to have antifungal properties, cytochalasin B (CB) is now known to inhibit cytoplasmic cleavage and cell movements while inducing multinucleate and enucleate cells [8]. In addition, a wide variety of other responses are elicited in both higher plants [24] and animals [13], with CB as the substance used most extensively.

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In contrast to most other organisms, the fungi are less sensitive to CB than to some other cytochalasins [18, 24]. Thus, cytochalasin A (CA) which has been used only occasionally on higher plants and animals has been shown to decrease the distance between branches, increase beading (or periodic constrictions) along the hyphae and cause swollen or spatulate tips (see review by Thomas [24]. In *Achlya,* CA has been shown to inhibit cellulase secretion [26] as well as uridine and histidine transport and incorporation of uridine into RNA in cells pre-loaded with labelled uridine (Le John, H. B., personal communication cited by Thomas [24].
Our work describes the effects of CA and CB on the growth pattern and morphology of *Neurospora crassa.*

2. Materials and Methods

With the exception of *ipa* (S 12), the strains of *Neurospora crassa* used in this study (Tab. 1) were obtained from the Fungal Genetics Stock Center, California State University, Arcata, California. *Ipa* [21] was obtained from the collection of one of the authors (R.A.).
Conidia used in the various experiments were obtained by growing cultures in 25 ml of solidified Vogel's medium [31] (supplemented with 2% sucrose) in 125 ml Erlenmeyer flasks. A few of the strains with highly restricted growth were encouraged to conidiate by reducing the sucrose content to 0.1% and by adding a pleated disc of 9.9 cm No. 1 Whatman filter paper to the medium. Stocks were grown for 5–21 days at 22 °C.
Cytochalasin A (CA) and cytochalasin B (CB) (obtained from Aldrich Chemical Co., Milwaukee, Wisconsin) were dissolved in dimethyl sulfoxide (DMSO) and diluted to a final stock concentration of 1.0% DMSO. The concentrations of the cytochalasins used in this study varied from 0.001 to 20.0 µg/ml and were obtained by volumetric dilutions of the stock solution (200 µg/ml). The higher concentrations of CA ($>$ 10 µg/ml) are not soluble and are used as suspensions.
Preliminary experiments had indicated that the cytochalasins prevented germination of conidia from *Neurospora.* Therefore conidia were transferred to liquid culture and allowed to germinate and grow (3–5 hours) before being treated with the cytochalasins in 0.1% DMSO or in 0.1% DMSO alone (controls) [4].
Growth was studied by transferring 1.0 ml of germinated conidia and 1.0 ml of the cytochalasin stock solution to 25 or 50 ml, Erlenmeyer flasks containing 8.0 ml of Vogel's liquid medium (with 2% sucrose). The cultures were grown on a reciprocal shaker (120 rpm) at room temperature (22 °C). To obtain the dry weight of mycelia, the cultures were grown for 48 hours, harvested by vacuum filtration and dried (70 °C, 24 hours).
Cultures for examination by light or electron microscopy were grown in the

same manner except that they were agitated very gently (30 rpm), thereby permitting the location and examination of the growing mycelial fronts. Methods used to fix the samples and prepare them for electron microscopy have previously been described [3].

3. Results

Dry weights of cultures grown for 48 hours in the presence of the various concentrations of CA or CB are shown in Tab. 1 and the percentage of growth of the various strains relative to controls is shown in Tab. 2. It can be seen

Table 1. *Dry Weights of Untreated and Cytochalasin-Treated Cultures of Strains of Neurospora crassa*

Strain	Cytochalasin	Average growth in dry weight (mg) at (μg/ml) [1]						
		0.0	0.001	0.01	0.1	1.0	10.0	20.0
Standard strain	CA	75.6	78.0	90.9	75.6	52.8	20.9	2.3
(74-OR 23-1 A)	CB	67.5	72.8	72.2	70.4	69.8	59.1	42.6
Snowflake	CA	38.4	36.8	33.4	35.1	6.8	3.5	0.0
(507 a)	CB	28.0	34.9	40.2	38.2	32.6	31.2	20.1
Crisp-1	CA	56.9	52.3	48.4	44.8	45.7	0.0	0.0
(B 123)	CB	56.6	57.1	57.8	60.7	54.3	41.8	18.1
Crisp; snowflake	CB	25.9	24.3	28.8	27.2	26.8	20.8	14.3
Ipa	CA	64.1	64.3	59.6	55.8	9.8	2.4	0.0
(S 12)	CB	57.4	63.1	57.1	50.9	55.1	41.5	21.7
Ipa; crisp	CB	59.7	67.9	68.4	62.1	69.2	51.8	36.4
Spray	CA	59.5	76.2	77.4	66.6	63.7	43.4	16.1
(B 132)	CB	59.5	88.1	78.5	75.6	66.6	54.1	36.3
Granular	CA	20.1	23.1	24.4	15.7	1.7	2.1	0.0
(B 42)	CB	25.8	44.6	32.4	36.0	28.2	25.2	9.0
Dot	CA	11.9	16.4	14.8	18.3	15.2	0.5	—
(1211)	CB	11.9	17.6	18.5	19.2	17.2	11.7	—

[1] Each value shown represents the average of 12 individual samples.

in Tab. 2 that the highest concentration of both cytochalasin used (20 μg/ml) results in a drastic inhibitory effect on the growth of all the strains examined. This is also the case with CA at a concentration of 10 μg/ml and in some of the strains (*snowflake, ipa, granular*) grown in 1.0 μg/ml. Of the strains examined in this study, most grow as well as the controls at the lower concentrations of either CA or CB used (0.001 to 0.1 μg/ml).

However, three strains, *dot*, *granular*, and *spray* show greater growth as measured by dry weight than their respective controls at these concentrations. This effect was observed with CA and CB in cultures of *dot* and *spray* and only with CB in cultures of *granular*.

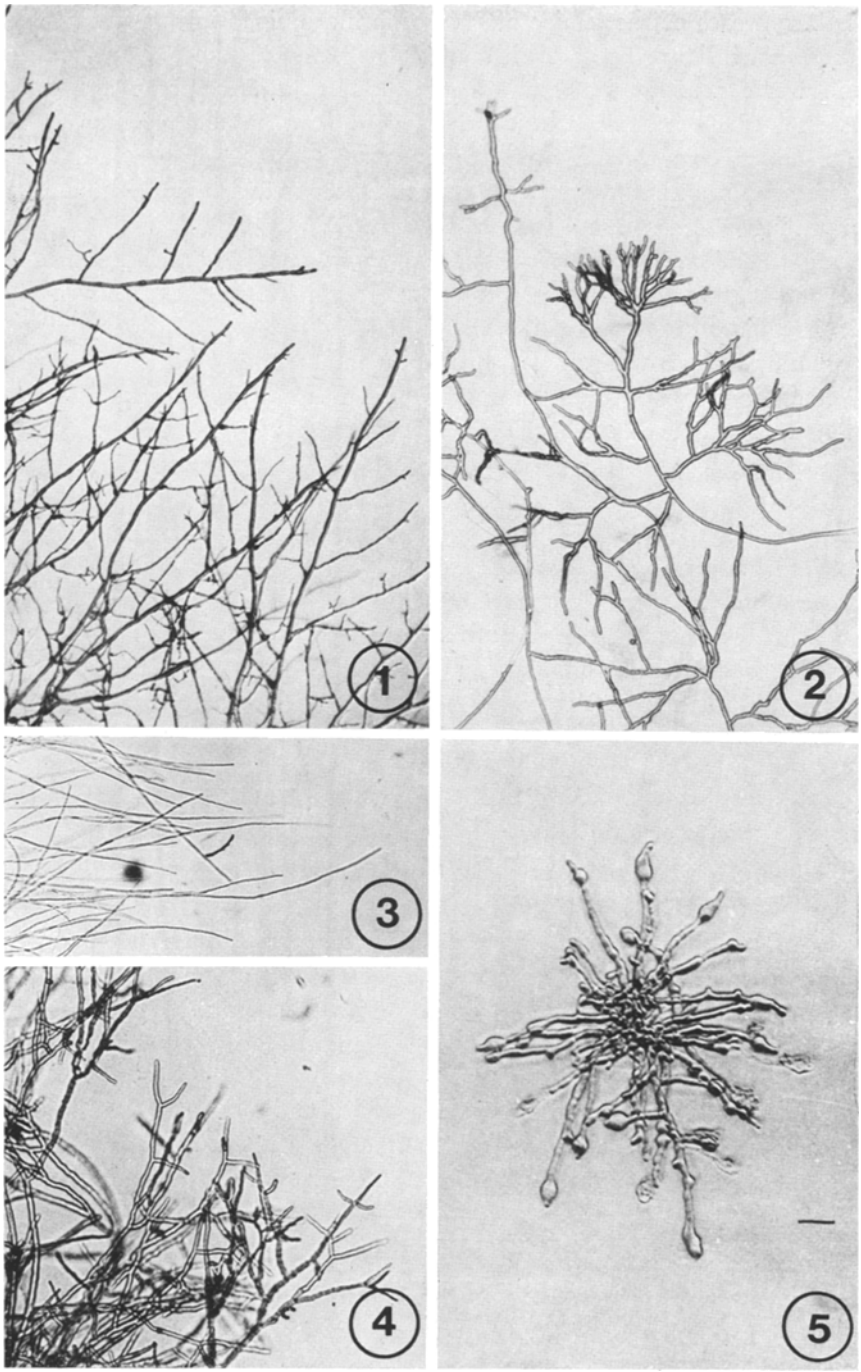
As mentioned in the section on Materials and Methods, light micrographs were obtained from cultures grown in liquid media. We had noted during earlier experiments that when cultures were grown on agar to study the growth habit, response to the cytochalasins occurred only when the hyphae were adjacent to the applied drug. If aerial hypha started growing, or the mycelial front advanced ahead of the applied drug, the strain exhibited its normal growth pattern. By contrast, the liquid cultures used in the present studies responded uniformly to the applied drugs.

Table 2. *Growth Determinations of Untreated and Cytochalasin-Treated Strains of Neurospora crassa* [1]

Strain	Cytochalasin	Average percentage growth of control at (μg/ml)						
		0.0	0.001	0.01	0.1	1.0	10.0	20.0
Standard strain	CA	100	114	124	96	67	20	3
(74-OR 23-1 A)	CB	100	111	110	103	107	92	60
Snowflake	CA	100	118	99	91	16	12	0
(507 a)	CB	100	139	144	137	120	112	73
Crisp-1	CA	100	92	85	81	74	0	0
(B 123)	CB	100	97	98	101	92	72	26
Crisp; snowflake	CB	100	94	111	105	103	80	55
Ipa	CA	100	100	93	87	14	3	0
(S 12)	CB	100	100	99	87	96	92	69
Ipa; crisp	CB	100	114	115	116	104	88	61
Spray	CA	100	128	130	112	107	73	27
(B 132)	CB	100	148	132	127	112	91	61
Granular	CA	100	136	146	43	7	10	0
(B 42)	CB	100	165	125	138	109	93	33

[1] Data from Table 1, controls set at 100.

The effect of CA on the external appearance of the growing mycelium of *Neurospora crassa* is shown in Figs. 1–5. The *initial* response of CA is an increase in branch frequency per unit length of hyphae. This can be seen by comparing Fig. 1 which shows the standard strain (74 a) grown with DMSO alone, with this strain grown in the presence of 10 μg/ml CA (Fig. 2). A similar increase in branching is observed with *ipa* (S 12) grown in the presence of 0.1 μg/ml CA (Fig. 4). The normal growth pattern of *ipa* results in a mycelial front in which the hyphae contain few branches (Fig. 3). Fig. 5 shows the growth patterns of *snowflake* in the presence of 10 μg/ml CA. The abnormal hyphae with swollen tips, outgrowths, etc. are seen at concentrations greater than those which results in increased branching. *Snowflake*, for example, reveals an increase in branching at the lowest concentration examined in this study (0.001 μg/ml CA).



Figs. 1–5. Light micrographs indicating appearance of mycelia grown in the presence of cytochalasin A. 1. *74 a* Control, ×150; 2. *74 a* 10 µg/ml CA, ×185; 3. *Ipa* (S 12) Control, ×150; 4. *Ipa* 0.1 µg/ml CA, ×185; 5. *507* 10 µg/ml CA, ×300

Although each of the strains of *Neurospora* examined in this study may vary somewhat in its response to the cytochalasin, all are affected by the drug and at lower concentrations of CA than CB. The *initial* response to CA appears as an increase in branching which may or may not be accompanied by an increase in dry weight. The concentrations of the drug which causes an increase in branching varies with the particular strain. For example, increased branching is seen in *snowflake* (507) with 0.001 μg/ml CA while the standard strain (74 a) requires 1.0 μg/ml CA. Increasing the concentration of CA beyond that which causes an increase in branching results in increased numbers of irregular, swollen hyphae (Fig. 5). This is usually accompanied by a reduction in growth so its probably indicates growth inhibition.

Electron microscopy of hyphae grown in the presence of various concentrations of CA reveals unusual wall formations. These usually are seen as deposits adjacent to the existing cell wall and separated from the cytoplasm by the plasma membrane (Figs. 6 and 9). This material may extend some distance into the cell and, depending on the orientation of this material during sectioning, may appear isolated in the cell cytoplasm (Figs. 7 and 10).

Some of the material appears similar in electron density to the existing cell wall but regions of different electron density are also found (Fig. 7). At times vesicles containing material similar in electron density to the latter are in close proximity to the deposit (Figs. 7 and 10). However, we have not seen any direct contact between the vesicle membrane and those adjacent to the wall deposits.

On occasion, an additional cell wall adjacent to the existing one is induced. This additional wall layer may extend for a short distance (Fig. 8) or completely surround the cell (not shown). That the several aberration described can coexist in the same hypha (cell) is shown in Fig. 11.

The controls grown in DMSO possess no wall aberrations. As noted, differences exist among the various strains with respect to sensitivity to the cytochalasins. For example, *snowflake* (507) contains deposits in cells grown in the presence of 0.01 μg/ml whereas *ipa* (S 12) shows them only at concentrations of 1.0 μg/ml and greater.

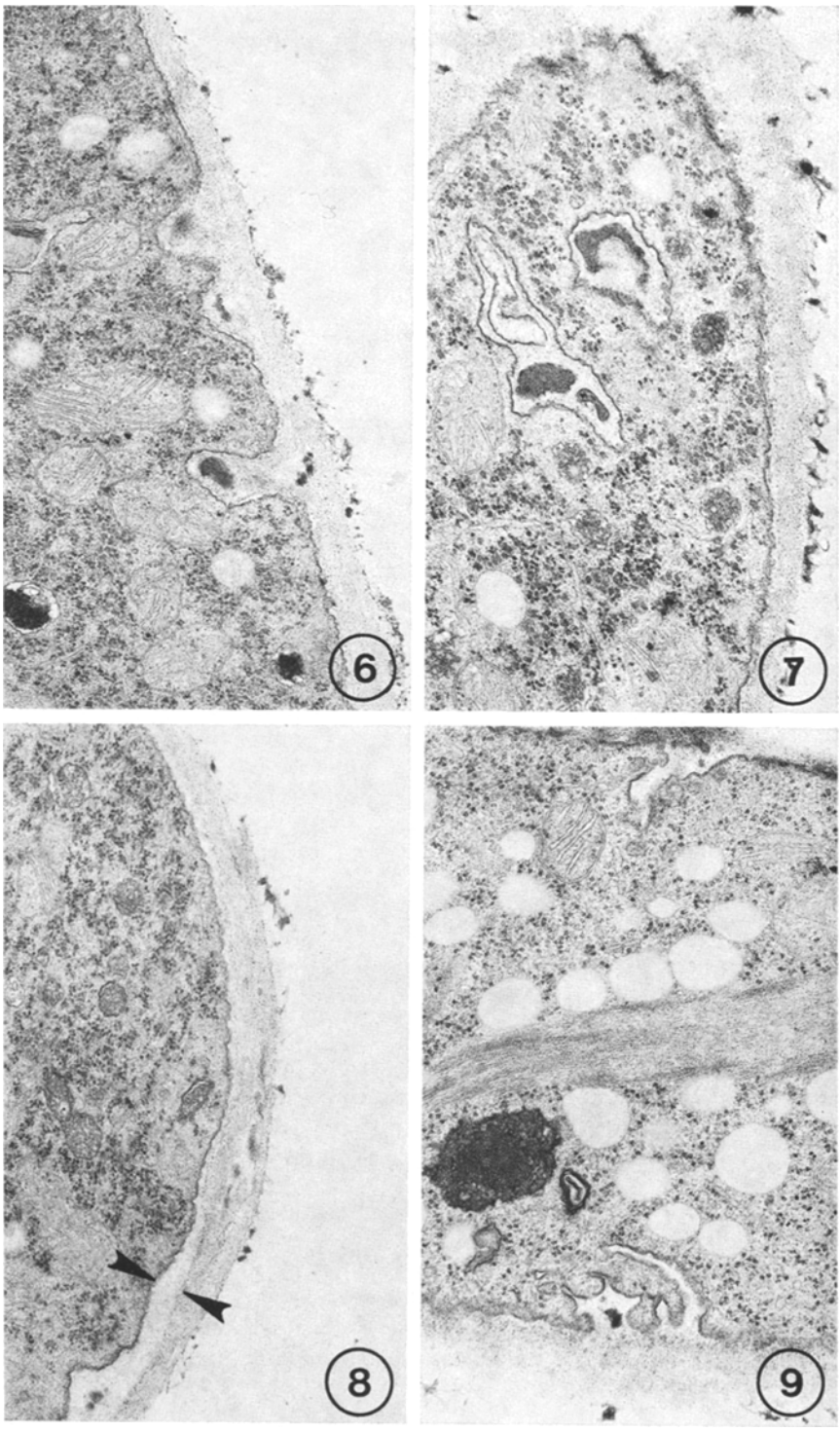
By contrast, CB appears not to affect the ultrastructure of the strains used. The lack of wall deposits and other effects is interesting since CB causes an increase in branching similar to that caused by CA, although at higher concentrations.

Fig. 6. Region of cell wall showing three wall deposits extending into the cytoplasm; *507* 1.0 μg/ml CA, ×28,000

Fig. 7. Presence of wall deposits in the cytoplasm. Deposits contain electron dense material similar in density to vesicles seen in cytoplasm; *Ipa* 10 μg/ml CA, ×32,200

Fig. 8. Additional wall layer (indicated between arrows) present within the existing cell wall, *507* 0.1 μg/ml CA, ×24,900

Fig. 9. Irregular outgrowths of wall deposits extending into cytoplasm. Also shown is a group of microfilaments. *74 a* 10 μg/ml CA, ×34,500



Figs. 6–9

Although microfilaments are seen occasionally in most strains of *Neurospora* we have examined, *snowflake* in particular, possesses large amounts [2]. We observed no difference in the appearance or frequency of these filaments in the presence of CB. On the other hand, although wild-type controls rarely show filaments, these organelles are seen on occasion in CA-treated cultures (Fig. 9).

4. Discussion

The strains examined in this study include *spray*, *granular*, *dot*, *crisp*, and *snowflake*, all of which are colonial mutants. Such mutants usually grow slowly and show an increased frequency of branching when compared to wild-type strains. The biochemical lesion(s) that cause these differences is not known so the strains have been classified by the appearance of their growing mycelia [12].

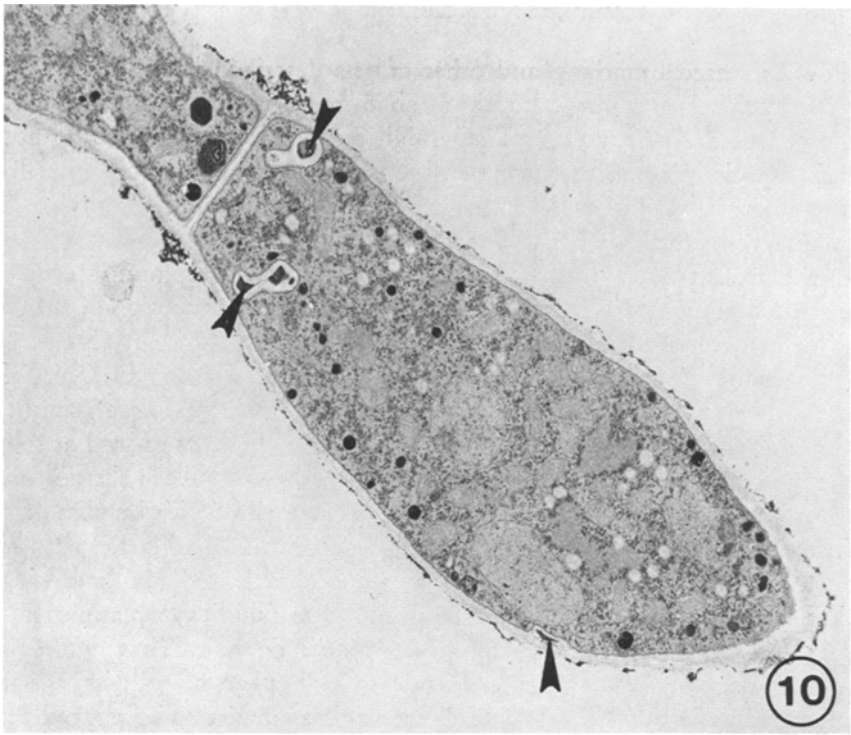
The effect of the cytochalasins on *Neurospora crassa* is to alter the usual growth pattern. The initial response at low concentrations of the drug is an increase in branching which may be accompanied by an increase in total growth (dry weight). As the concentration is increased, the amount of growth decreases and abnormal hyphae (swollen, irregular) are regularly seen. Higher concentrations of CA result in inhibition of growth. The effect on growth are similar for CB except that higher concentrations are needed.

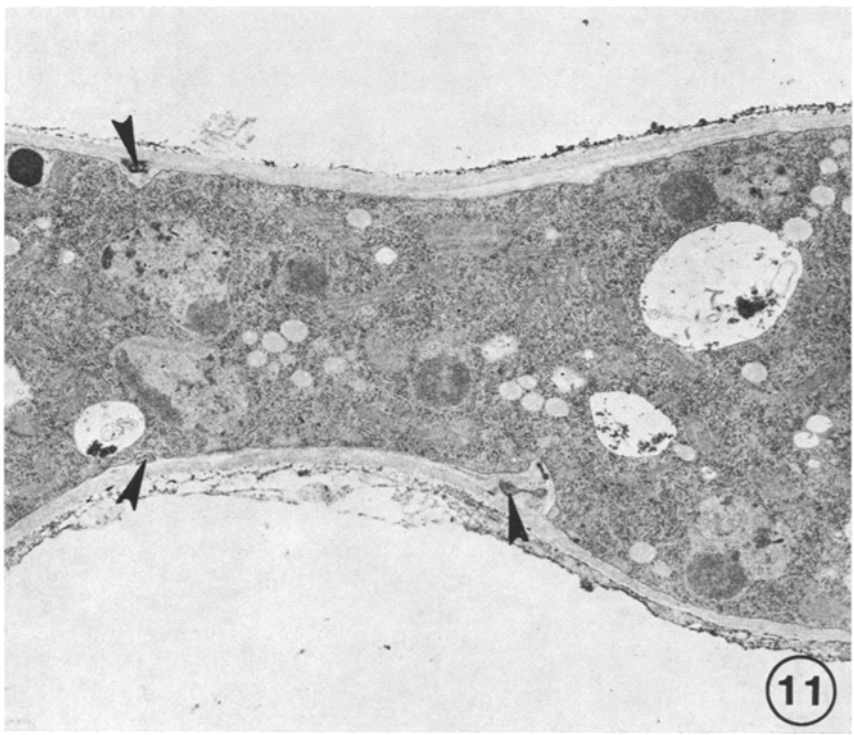
This response of *Neurospora* to the cytochalasins is similar to that found in other fungi by others (see THOMAS [24] for a review). As noted by Thomas others have found that CA is more potent than CB in causing a response in fungi. The response is generally one of inhibition of growth at higher concentrations and decreased internodal distances and increased branching at lower concentrations. Individual hyphae become stunted and may show periodic constrictions (beading) along their length and swollen or spatulate tips. As noted in this study the cytochalasins exhibit similar responses in *Neurospora*.

The most striking ultrastructural detail of cells grown in the presence of CA is the unusual deposits of wall material. Although some deposits appear "isolated" in the cytoplasm, we believe that attachment to the existing cell wall occurs at a point out of the plane of the sections, although we have not confirmed this with serial sections. As noted in the section on Results, deposits of wall material are not observed in controls which contain the same amount of DMSO (0.1%).

Although the presence of similar deposits have not, to our knowledge, been described by others, it is possible that fungi other than *Neurospora* which

Figs. 10–11. Lower magnifications of hyphae indicating the presence of the wall deposits (arrows) in the cells of *507*. An additional cell wall layer may also be seen in the top of Fig. 11. Fig. 10, 1.0 μg/ml CA, ×8,300. Fig. 11, 0.1 μg/ml CA, ×10,800





Figs. 10 and 11

show increased branching and other effects described herein after treatment with cytochalasins may also show abnormal deposition of wall materials. However, in *Neurospora,* even the higher concentrations of CB do not elicit such deposits, despite the increased branching and other effects upon hyphae. Therefore, it would be of interest to treat *these fungi* with cytochalasins and to observe them under the electron microscope.

In the present study, the general appearance of the cytoplasm in cytochalasin B-treated and untreated cultures is the same, with the exception of the deposits of wall material. The presence of microfilaments in some strains of *Neurospora* is noted both in untreated and drug-treated cultures. This includes *snowflake,* a strain which possesses large amounts of microfilaments [2]. Cytochalasin B has been shown to disrupt microfilaments as well as to inhibit the motility of animal systems [12]. In plants, CB has been shown to inhibit cytoplasmic streaming [6]. This includes *Nitella* in which bundles of microfilaments have been implicated in such streaming. Whether CB acts directly on the microfilaments however remains unresolved [13]. It should be noted that although untreated standard strains and *ipa* exhibit cytoplasmic streaming, some of the colonial mutants like *snowflake* do not. That cytochalasin-A induces the formation of microfilaments in hyphae of wild-type strains is suggested by our observations, but a mechanism remains to be worked out.

The results of our study suggest that the cytochalasins interfere with normal growth patterns, including the external appearance of individual hyphae (and the mycelia) and that CA also affects the intracellular organization (cell wall deposits). It is generally believed that hyphal growth requires the presence of vesicles that are commonly seen in the cytoplasm concentrated near the tip of growing hyphae. These vesicles are believed to contain various enzymes and wall precursors. According to this view, normal growth would require the action of these enzymes to allow loosening of existing wall material and the incorporation of the required wall precursors [5, 14].

In a recent review, CLUTTERBUCK [9] discusses in depth the colonial mutants of *Neurospora crassa. He* suggests that the increased branching characteristic of colonial mutants of *Neurospora crassa* may be a result of a defect in hyphal extension. This he believes would lead to a combination of extra branching and extra growth (of the branches) to compensate for the reduced growth of the hyphae tips.

Differences in the amounts of carbohydrates present in colonial mutants and wild-type strains of *Neurospora* have been noted. These include an increase in the ratio of glucosamine to glucose in a number of mutants (see CLUTTERBUCK [9]) and differences in a cell wall fraction which contains polypeptide, glucan, and galactosamine [17]. Certain morphological mutants have been identified with specific enzyme deficiencies (phosphoglucomutase, glucose-6-phosphate dehydrogenase, 6-phosphogluconic acid dehydrogenase and glucose phosphate isomerose) (reviewed by CLUTTERBUCK [9]). It is conceivable that

the increased branching of *Neurospora* in the presence of the cytochalasins could be caused by a defect in the incorporation of carbohydrate into the wall.

This explanation of growth which applies to hyphae tips would also explain growth of branch points. A difficulty, however, in discussing branching is that the location of branches along a hyphae or the factors which control their initiation is not well understood.

The swollen or spatulate tips seen in some of the cytochalasin-treated cultures (see Fig. 5) are similar to the ballooning of hyphae observed in mutants of *Aspergillus*. These mutants were found to be defective in glucosamine [10] and mannose [30] metabolism but grew normally when the respective sugar was present.

Given effects on branching such as those seen in Fig. 2, it is interesting to note that there is a strong resemblance between the *fan*-like assemblage of branches in cytochalasin-treated cultures and those reported by SUSSMAN *et al.* [22] depicting the normal pattern of growth of the *clock* mutant of *Neurospora*. One explanation of these mutations might be the overproduction of cytochalasin-like compounds. At the ultrastructural level, our unpublished data show that some double mutants show wall deposits similar to those seen after cytochalasin treatment, although they are less extensive.

Osmotic shock caused by flooding a growing hyphae of *Neurospora* with water can cause multilateral branching between septa during recovery of shock [29]. TRINCI believes this branching may be a result of vesicles fusing with the lateral hyphal wall along its length because normal polarity of vesicle transport to the hyphae tip is disrupted [11, 28]. Thus, the carbohydrate variations noted above may be related to transport of vesicles to growth sites.

As noted earlier, the cells we examined in this study are young cells at hyphal tips. Such cells do contain numerous vesicles in both controls and cytochalasin treated cultures.

As indicated, the cytochalasins show effects on fungal cells which are similar to those caused by deficiencies etc. in carbohydrates. The action of the drug could of course act at any level with respect to carbohydrate utilization. This could include carbohydrate metabolism, its transport across membranes and/or packaging into vesicles, and transport of the vesicle to the site of incorporation.

Although the literature on cytochalasin is somewhat limited with respect to a role related to carbohydrate utilization etc., some work has been described. These include inhibition of cellulase secretion in *Achyla* by CA [26] and decreased ATP levels in *Saccharomyces* during growth inhibition also by CA [15].

Although not observed in fungi, CB has caused responses in other plant systems which are related to wall formation (and thus carbohydrate utilization

etc.). For example, Palevitz [19] reported that CB disrupts orientation of the cell plate after mitosis in guard mother cells of *Allium cepa.* In the alga, *Micrasterias,* a desmid, regeneration of a new semi-cell in the presence of CB is inhibited so that a miniature half-cell is produced (and attached to a semi-cell of normal size) [27]. In *Avena* and *Pisum,* CB inhibits sugar uptake and incorporation into the wall [6]. CB has also been shown to cause altered cells in *Allium* [25].

Although the cytochalasins have been implicated in several functions in animal systems, the binding of CB to the cell membrane and its ability to inhibit glucose transport [16] could also affect cell wall metabolism.

We are currently attempting to determine the relationship of the vesicles noted in the cytoplasm to the wall deposits.

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