THE ANTIMITOTIC PROPERTIES OF THE BENZIMIDAZOLE FUNGICIDE CARBENDAZIM AND A MECHANISM OF RESISTANCE TO THIS COMPOUND IN ASPERGILLUS NIDULANS

Dit proefschrift met stellingen van

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landbouwkundig ingenieur, geboren te Sint Laurens op 23 januari 1947 is goedgekeurd door de promotor, dr. ir. J. Dekker, hoogleraar in de fytopathologie.

De Rector Magnificus van de Landbouwhogeschool,

J.P.H. van der Want

Wageningen, 18 augustus 1976

L.C. Davidse

The antimitotic properties of the benzimidazole fungicide carbendazim and a mechanism of resistance to this compound in Aspergillus nidulans

Proefschrift
ter verkrijging van de graad van
doctor in de landbouwwetenschappen,
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Aan mijn ouders

Aan Willy

Stellingen

I

Op grond van hun werkingsmechanisme dient de toepassing van benzimidazool verbindingen als fungiciden in land- en tuinbouw te worden beperkt.

II

De conclusie van Nachmias en Barash dat de resistentie van Sporobolomyces roseus mutanten tegen MBC berust op een verminderde permeabiliteit van de celmembraan, wordt onvoldoende door hun experimentele gegevens ondersteund.

Nachmias, A. en Barash, I., 1976.
J. Gen. Microbiol. 94: 167-172.

III

Het door Dassenoy en Meyer waargenomen mutagene effect van benomyl in Fusarium oxysporum hangt waarschijnlijk samen met het werkingsmechanisme van deze verbinding.

Dassenoy, B. en Meyer, J.A., 1973.
Mutation Res. 21: 119-120.

IV

De resultaten van de door Olson en Heath uitgevoerde experimenten betreffende het voorkomen van colchicine-bindende eiwitten in Saprolegnia ferax en Allomyces neo-moniliformis, wettigen niet de door hen getrokken conclusies.

Olson, L.W., 1973.
Arch. Mikrobiol. 91: 281-286.
Heath, I.B., 1975.
Protoplasma 85: 177-192.
Dit proefschrift.

V

De klassificering van het herbicide isopropyl-N-phenylcarbamaat als een verbinding welke zijn effect op de assemblage van microtubuli uitoefent via een niet directe interactie met tubuline, is gebaseerd op te weinig experimentele gegevens.

Coss, R.A., Bloodgood, R.A., Brower, D.L., Pickett-Heaps, J.D. en McIntosh, J.R., 1975.
Exptl. Cell Res. 92: 394-398.

VI

De opname van systemische fungiciden in planten is een passief proces en vindt plaats in zowel de apoplast als de symplast.

VII

Bij de bestudering van de biochemische basis van de waardplant-parasiet relatie dient meer gebruik te worden gemaakt van de mogelijkheden die het werken met cel- en protoplast cultures van planten biedt.

VIII

De studie van de moleculaire basis van de gen-om-gen relatie in waardplant-parasiet systemen kan leiden tot een dieper inzicht in de processen welke een rol spelen bij de genregulatie in eukaryoten.

IX

Het verdient aanbeveling een mutageniteitstest, welke is gebaseerd op inductie van mitotische recombinatie in diploiden van Aspergillus nidulans, op te nemen in het toxiciteitsonderzoek van chemische verbindingen.

Käfer, E., Marshall, P. en Cohen, G., 1976.
Mutation Res. 38: 141-146.

X

De juistheid van de endosymbiose theorie van Lynn Margulis ten aanzien van het ontstaan van microtubulaire systemen is niet erg waarschijnlijk.

Margulis, Lynn, 1976.
in "De Microbiologie drie eeuwen na Antoni van Leeuwenhoek", blz. 72-96.
Biologische Raad Reeks, Pudoc, Wageningen.

Proefschrift van L.C. Davidse
Wageningen, 12 november 1976.

Contents

Voorwoord

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Mijn ouders wil ik graag noemen omdat zij mijn studie mogelijk maakten.
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Jaap Visser heeft een tweetal manuskripten kritisch doorgelezen.
De dames van de administratie van de vakgroep fytopathologie en van de afdeling tekstverwerking hebben de manuskripten uitgetypt en bijgedragen aan de uiteindelijke vormgeving van het proefschrift.
De leden van de vakgroep fytopathologie zorgden voor een klimaat waarin het prettig werken was.
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De Centrale Organisatie T.N.O., Sectie Landbouwkundig Onderzoek maakte het mij mogelijk aan dit onderwerp te werken en enkele kongressen te bezoeken.
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Academic Press, Inc. and Elsevier/North-Holland Biomedical Press B.V. have given permission to reprint the articles incorporated in this dissertation.

De steun die ik thuis kreeg van Willy en de afleiding die Ellen en Pieter mij bezorgden, mogen ook niet onvermeld blijven.
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Introduction

Since the time man began to grow plants for food, he was confronted with the problem how to protect his crop against diseases and pests, which often caused a considerable reduction in yield and quality. The causes of diseases and pests were initially shrouded in mystery and methods to control them were rather imaginative. In the middle of the nineteenth century when fungi were recognized as the cause of a number of plant diseases the first scientific and systematic studies appeared in the field of plant disease control. From that time progress in crop protection is closely linked with the discovery and development of new chemical compounds. The first fungicides used as such were elementary sulphur, copper and mercury preparations. Increasing knowledge in chemistry made it possible to synthesize new organic compounds and in 1934 the fungitoxic properties of some organic sulphur compounds were reported.

In the last forty years an enormous number of compounds have been tested for fungicidal activity and several hundreds have found use in practice. Most of these compounds do not penetrate into plant tissues and, consequently, can only prevent or protect against infection. Absence of penetration is also the reason why these compounds which mostly appeared to be general cell poisons, are not harmful to the host plant.

In the last ten years several compounds have been discovered which can penetrate into the plant and are translocated within the plant tissues. These compounds, which are usually called systemic fungicides, have the advantage that they may eradicate established infections, may protect newly grown parts of the plant and are less subject to weathering. It is evident that systemic fungicides have to be selective in their action with respect to the plant and the parasite. In addition to this type of selectivity systemic fungicides are usually also selective within the group of fungal plant pathogens. Some fungi are very sensitive to a certain compound, whereas others are resistant.

Soon after the introduction of systemic fungicides in practice negative aspects became apparent. In some instances disease control was no longer successful, because fungicide-resistant strains developed in previously sensitive fungal populations. This phenomenon, well known in insect pest control, was until recently seldom encountered in controlling fungal diseases and might, therefore, be inherent to the use of the new systemic compounds.

Selectivity of the systemic fungicides and resistance of fungi to these

compounds may be due to a differential uptake of the compound, a differential interaction with target sites or absence of target sites, a different conversion into non-toxic or toxic derivatives, or differences in regulatory systems of the organisms. For a detailed account of the developments in the field of fungicide research the reader is referred to "Systemfungizide - Systemic fungicides" (eds. H. Lyr and C. Polter, Akademie Verlag, Berlin), to "Systemic fungicides", 2nd edition 1976 (eds. R.W. Marsh, Longman Group Limited, London) and to "Antifungal compounds", Vol. 2, 1976 (eds. H.D. Sisler and M.R. Siegel, Marcel Dekker, Inc. New York).

Fundamental research on fungitoxic compounds has several aspects. From the point of view of fungal disease control, knowledge of their mechanism of action and possible mechanisms of resistance might elucidate vulnerable sites in fungi. It may lead to the development of new compounds which specifically interfere with these sites. From the cell biological point of view research on systemic compounds which act specifically may contribute towards our insight in cell processes and their regulatory systems. From the toxicological point of view elucidation of target sites makes it possible to carry out additional specific toxicological tests. It might be possible that compounds which have passed the general tests may require further attention on account of the result of a specific test.

This thesis deals with the mechanism of action of carbendazim or methyl benzimidazol-2-yl carbamate (MBC), one of the systemic benzimidazole fungicides and a mechanism of resistance of fungi to this compound. Biocidal activity of synthetic benzimidazole compounds was first reported in 1961, when the anthelmintic properties of thiabendazole were described. The antifungal properties of this compound were discovered in 1964. The systemic benzimidazole fungicides benomyl and fuberidazole were introduced in 1968. After that time a number of other biologically active benzimidazole compounds were found and introduced as anthelmintics like mebendazole, parbendazole and fenbendazole, as fungicides like carbendazim, cypendazole and as antitumoral drug like oncodazole.

This thesis contains four articles. In the first paper the localization of the site of action is described. The second and third paper deal with the biochemical aspects of fungitoxicity and the mechanism of resistance in fungi. Since metabolic conversion might play a role in the mechanism of resistance, this aspect was also studied. The results of this study are given in the fourth paper.

PESTICIDE BIOCHEMISTRY AND PHYSIOLOGY 3, 317–325

Antimitotic Activity of Methyl Benzimidazol-2-yl Carbamate (MBC) in *Aspergillus nidulans*

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Mitosis in germ tubes of *Aspergillus nidulans* was inhibited directly upon addition of methyl benzimidazol-2-yl carbamate to liquid cultures, whereas dry weight increase and DNA and RNA synthesis were progressively inhibited only after a few hours. Microscopic observation of the organism, grown on malt extract agar containing MBC revealed abnormal chromatin configurations. The possible mode of action of MBC through interference with spindle formation is discussed.

INTRODUCTION

Methyl benzimidazol-2-yl carbamate (MBC), the conversion product of benomyl, thiophanate-methyl and 2-(3-methoxycarbonylthioureido)-aniline, is generally accepted as the actual fungitoxic principle of these systemic fungicides. Although MBC has somewhat inferior systemic fungicidal properties (1), this compound is at least as toxic as the parent compound benomyl to *Aspergillus nidulans* (2) and many other fungi (3–5). A difference in toxicity of benomyl and MBC has only been found toward *Saccharomyces pastorianus* (6), but this appears to be an exception to the rule. Hence MBC was the toxicant chosen in this study.

Information concerning the effect of MBC on various cellular processes is given by Clemons and Sisler (3) and Hammerschlag and Sisler (6). These authors found an inhibition of DNA, RNA, and protein synthesis, which was progressive with time and which they ascribed to a failure of normal mitosis or cytokinesis.

This study was undertaken as part of a research program concerning the mode of action of benomyl and the mechanism of resistance of fungi to this systemic fungicide.

MATERIALS AND METHODS

Organism. Aspergillus nidulans bi A-1 Acr A-1, requiring biotin and resistant to acriflavine, was used throughout this work. This strain was kindly provided by Ir. C. J. Bos (Department of Genetics, Agricultural University, Wageningen) and was originally obtained from the Department of Genetics, University of Glasgow.

Chemicals. MBC was generously supplied by E. I. Du Pont de Nemours and Co. (Inc.) (Wilmington, Del.). Calf thymus DNA and yeast RNA were purchased from Sigma (St. Louis, Mo.) and Fluka AG (Buchs, Switzerland), respectively.

Culture methods. The organism was grown on malt extract agar in Petri dishes. To prepare conidial suspensions, the conidia were washed off with sterile water containing one drop of Tween 20 per 20 ml and filtered through two layers of Kleenex tissue to remove most of the hyphal fragments. The suspension was washed twice with sterile water, and resuspended in

sterile water (final concn approx 10^9 conidia per ml). Spores were then added to 100 ml of glucose–nitrate medium (7), supplemented with 500 μg biotin per liter, in a 300-ml Erlenmeyer flask to give a concentration of 10^7 conidia per ml. Flasks were incubated at 37°C on a Gallenkamp orbital shaker at 150 rpm, for about 10 or 16 hr, depending on the method used in the inhibition experiments.

Inhibition experiments. MBC, dissolved in methanol, or methanol alone was added to 300-ml Erlenmeyer flasks containing either 10 hr old cultures or 100 ml of a mycelial suspension, which was obtained by filtration of 16 hr old cultures on a Büchner filter and resuspension of the mycelial cake in fresh medium. In the latter case also 50-ml Erlenmeyer flasks containing 10 ml mycelial suspensions were used. In all instances, the final MBC concentration in the medium was 4 μM; the methanol concentration never exceeded 0.5% (v/v) in treated and control media.

The suspensions were incubated as described above. Samples of treated and control suspensions, consisting of the whole contents of four 50-ml and two 300-ml flasks per treatment were taken at intervals and immediately cooled in an ice-bath to stop growth.

The contents of the 50-ml flasks were used for dry weight determinations. The contents of the two 300-ml flasks were added together and divided in 20-ml portions for dry weight, DNA and RNA determinations and, in the experiments with 10 hr old cultures, also for determining the average number of nuclei per germ tube.

Dry weight determinations. Growth was measured by determining increase in fungal dry weight of the cultures. Four 10- or 20-ml samples of treated and control suspensions were filtered using preweighed filter papers. After washing with distilled water the filter papers were dried overnight at 60°C and reweighed.

DNA and RNA analysis. DNA and RNA synthesis was measured as increase of DNA and RNA contents of the cultures. To this end, four 20-ml samples of treated and control suspensions were spun down in centrifuge tubes and washed twice with distilled water. To remove low molecular weight compounds, samples were incubated in 5 ml ice-cold 0.2 *N* perchloric acid (PCA) for 10 min in an ice-bath and centrifuged. This process was repeated twice. The pellet was suspended in 4 ml 0.3 *N* KOH and kept overnight at 37°C to hydrolyze RNA. The suspension was cooled in an ice-bath and 0.9 ml 2.5 *N* PCA was added to precipitate DNA. After centrifugation the supernatant was decanted and the residue was washed with 4 ml ice-cold 0.2 *N* PCA. The extract and the washing were combined and made up to 10 ml with 0.2 *N* PCA. This fraction was considered the RNA fraction and was assayed for RNA by the orcinol method (8) with yeast RNA as a standard.

The residue remaining after extracting RNA, was treated with 1 ml 1.5 *N* PCA for 20 min at 70°C to extract DNA. After centrifuging and decanting the supernatant, the residue was again extracted with 1 ml 1.5 *N* PCA in the same way. The supernatants were combined and made up to 2.5 ml with 1.5 *N* PCA. This fraction was considered the DNA fraction and was assayed for DNA by the diphenylamine method of Burton (9) as modified by Giles and Myers (10). Calf thymus DNA was used as a standard.

Determination of number of nuclei per germ tube. Two and a half milliliter samples of treated and control suspensions were pipetted in an equal volume of cold 10% trichloroacetic acid, kept at 0°C for 30 min and centrifuged (7). After washing with distilled water, the residue was taken up in 0.05 *M* phosphate buffer, pH 7.2 to which acridine orange was added, the final concentration being 100 μg per ml (11). Clumps of germ tubes were broken up by agitating the samples in a 50 ml beaker placed in a Metason 1500 ultrasonic cleaner (Struers Scientific Instruments,

Copenhagen). The material was not damaged by this treatment. The hyphae were viewed with a Zeiss microscope, equipped with an ultraviolet light source (barrier filters 50 and 41, exciter filters I and II). Using this technique the nuclei appear green in red cytoplasm. Nuclei were counted in at least 50 germ tubes (12).

Cytological observations. Conidia were allowed to germinate overnight on sheets of dialysis tubing, sterilized by boiling in water, and placed on the surface of malt extract agar (13, 14) with or without 4 μM MBC. After incubation the sheets were removed from the agar and fixed in modified Helly's solution (15) for 10 min and washed with 70% (v/v) ethanol in water. After hydrolysis for 12 min in 1 N HCl at 60°C (14) and after washing with distilled water, the material was stained for at least 1 hr with Giemsa (2 ml Giemsa's Lösung, Merck, in 100 ml 0.05 M phosphate buffer, pH 7.0). The colonies were viewed with a Wild photomicroscope.

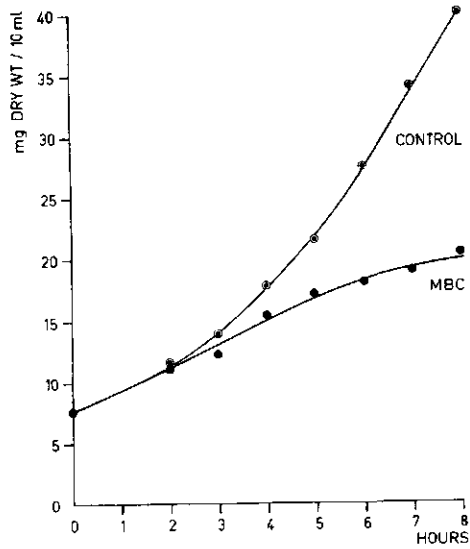


FIG. 1. *Effect of 4 μM MBC on increase of dry weight of mycelium of* Aspergillus nidulans. *MBC added at t = 0 to 16 hr old mycelium in fresh medium. The initial inoculum was 7.9 mg dry wt/10 ml mycelial suspension.*

RESULTS

MBC (4 μM) did not markedly inhibit the increase in dry weight of *A. nidulans* mycelium during the first 4 hr of the incubation period, when an initial inoculum of 7.6 mg dry wt per 10 ml suspension of 16 hr old cultures was used. Strong inhibition became evident after 4 hr (Fig. 1). Treatment of the mycelium with 1 μM MBC during the preexperimental period did not eliminate or reduce the delay in MBC inhibition of growth. As to increase in DNA and RNA content of the samples a similar pattern was observed (Fig. 2); however, in this case the inhibition became apparent already after 2 hr. In the latter experiment the inhibition of growth, measured as increase in dry weight during 8 hr of incubation, was about 13% (initial inoculum 26 mg dry wt/20 ml mycelial suspension of 16 hr old cultures). The inhibition of increase in DNA and RNA content was about 62% during the same period. This inhibition proved to be progressive with time so that after 8 hr of incubation with the toxicant synthesis of DNA and RNA was inhibited for almost 100%. At this time, the DNA content of treated samples was almost doubled, while in the controls the amount of DNA was more than three times the initial amount.

Inhibition of increase in number of nuclei per germ tube was much more pronounced (Fig. 3). At a concentration of 4 μM, MBC completely inhibited the increase in number of nuclei per germ tube, immediately after addition of the toxicant to 10 hr old cultures. Counting the nuclei revealed synchrony of mitosis in individual hyphae as has been reported by Rosenberger and Kessel (7). Under these circumstances, inhibition of increase in dry weight was not noted, while increase in DNA and RNA content was inhibited for 43 and 13%, respectively, during 4 hr of incubation (initial inoculum 16.4 mg dry wt/20 ml mycelial suspension of 10 hr old cultures).

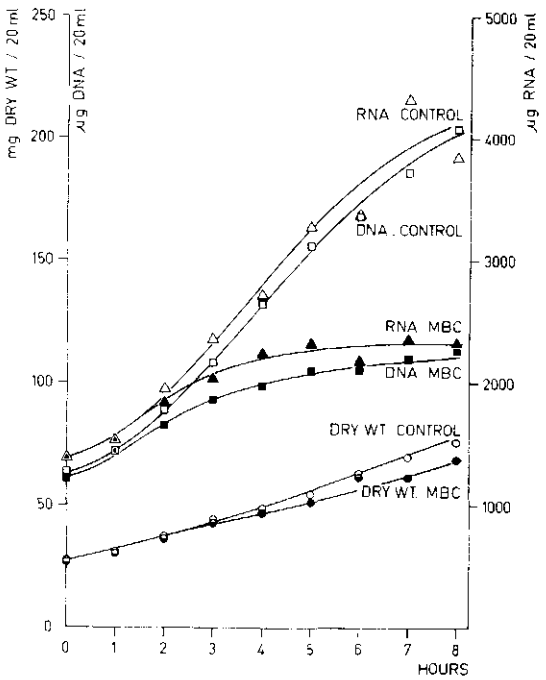


FIG. 2. *Effect of 4 µM MBC on increase of dry weight (○,●), DNA (□, ■) and RNA (△,▲) content of mycelial suspensions of* Aspergillus nidulans. *MBC added at t = 0 to 16 hr old mycelium in fresh medium. The initial inoculum was 26 mg dry wt 20 ml mycelial suspension.*

With regard to the last experiments, the decreasing RNA content of the germ tubes, viz, from 92 µg RNA mg dry wt at $t = 0$ to 69 µg RNA mg dry wt at $t = 4$, is worth noticing. This phenomenon was not observed in experiments with 16 hr old germ tubes. The RNA content here was about 55 µg RNA mg dry wt and remained the same during the incubation period.

Giemsa–HCl staining of MBC treated hyphae revealed a remarkable effect on the structure of the nuclei. While in control hyphae the chromatin of the interphase nuclei had a ring-shaped appearance, due to the size and form of the nucleolus (14, Fig. 4A), in treated hyphae the chromatin was much more contracted and did not appear in a ring shape (Fig. 4C, D). No separate nuclei could be observed; this observation and the uneven distribution of irregular masses of chromatin in the hyphae seems to indicate a disturbance of mitosis caused by MBC. Normal phases of mitosis, as were found in control hyphae (Fig. 4B), were not seen in treated hyphae.

DISCUSSION

The inhibition of growth of *A. nidulans* by MBC can be ascribed to an interference with mitosis by the fungicide. Upon addition of MBC to liquid cultures the average DNA content per nucleus increases till nearly twice the initial content (Fig. 5), since DNA synthesis is not directly inhibited. This means that the inhibition of DNA synthesis after 2 hr of incubation in the presence of MBC is the result of arrested mitotic activity. Due to the asynchronous character of the culture, nuclei will be arrested in increasing number in some stage of mitosis, resulting in a progressive inhibition of DNA synthesis.

The doubling of the DNA content per

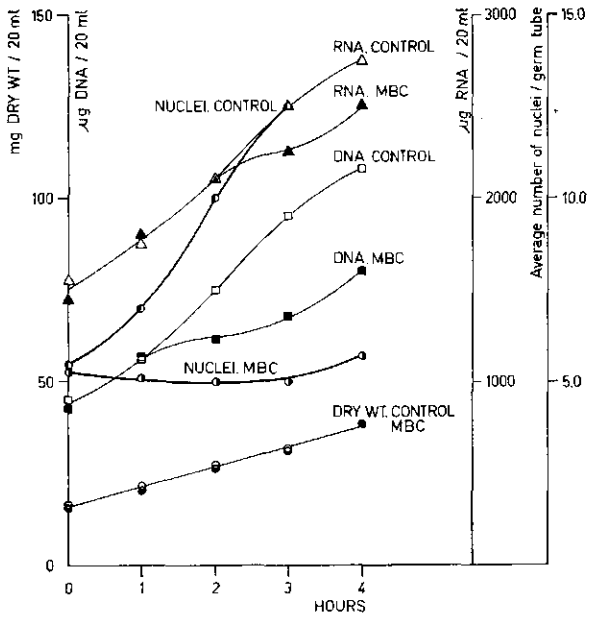


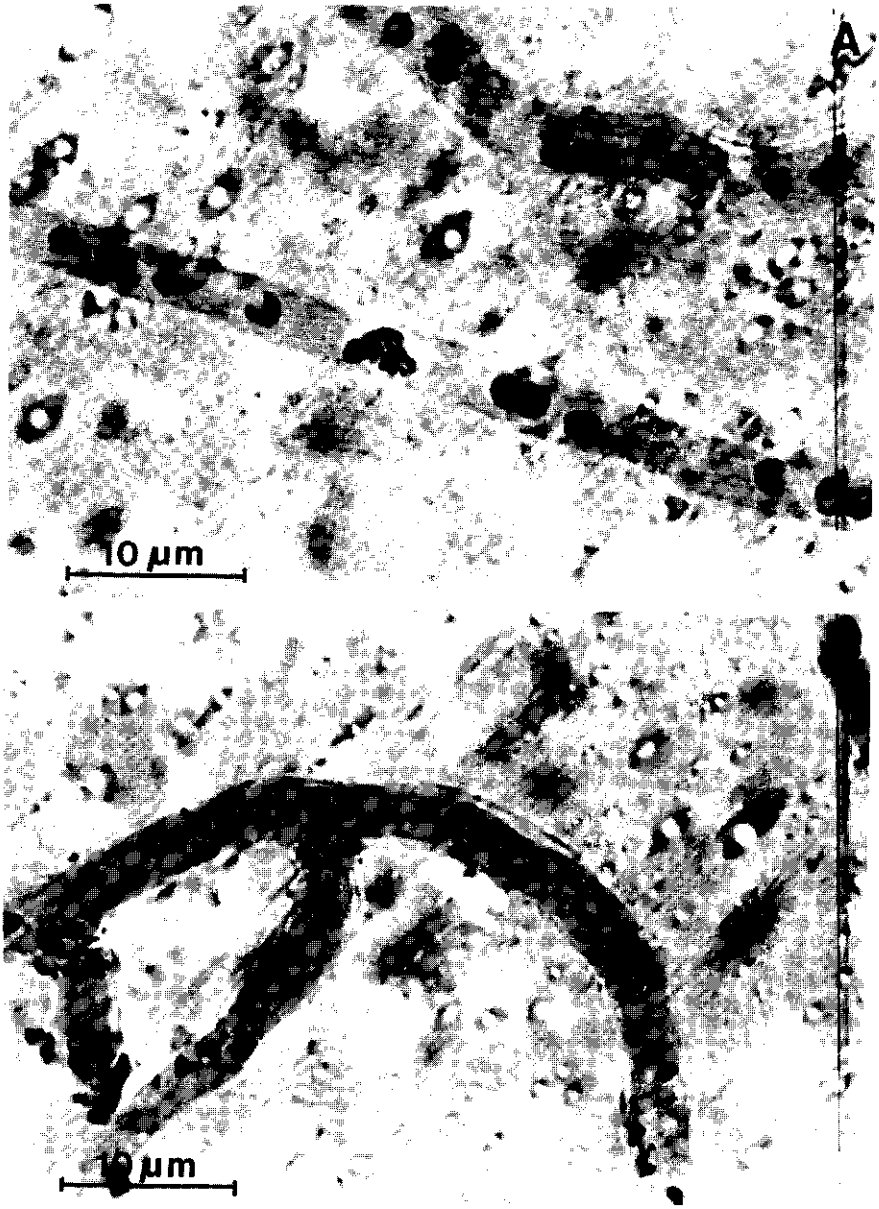
FIG. 3. *Effect of 4 µM MBC on increase in dry weight* (○,●), *DNA* (□, ■) *and RNA* (△, ▲) *content of mycelial suspensions and on average number of nuclei per germ tube* (◐,◑) *of mycelium of* A. nidulans. *MBC added at* t = *0 to 10 hr old cultures. Initial dry weight 16.0 mg/20 ml mycelial suspension.*

nucleus after addition of MBC to the culture also means that the G_1 period[1] should be long in comparison with the G_2 period of the division cycle. When phase G_1 is long and phase G_2 is short, most of the nuclei are in phase G_1 and upon addition of MBC to the culture, these nuclei can go through the S phase, and thus synthesize DNA, before the division cycle is blocked in mitosis. On the other hand, when phase G_1 is short and G_2 is long most of the nuclei are in the latter phase and addition of MBC will result in at most a slight increase of DNA content per nucleus. The result of this indirect measurement of the length of phase G_1 and G_2 agrees well with the results of Bainbridge (12).

Clemons and Sisler (3) reported a different response of *Neurospora crassa* conidia and *Ustilago maydis* sporidia to MBC treatment with respect to inhibition of DNA synthesis. In the light of our results the doubling of the DNA content of the culture of *N. crassa* conidia after addition of MBC is to be expected, since resting nuclei in spores have a minimal DNA content and can go through the G_1–S–G_2 phases of the division cycle before inhibition becomes apparent. With *U. maydis* sporidia, however, DNA content of cultures only slightly increased upon treatment with MBC. As this system is similar to the system we used to study the effect of MBC, the results of Clemons and Sisler would suggest that in *U. maydis* sporidia phase G_1 is short and phase G_2 is long.

The effect of MBC on RNA synthesis is dependent on the age of the germ tubes. In 10 hr old germ tubes, in which RNA content per mg dry weight is decreasing, RNA synthesis is less inhibited than DNA synthesis over a 4-hr incubation period. In 16 hr old germ tubes RNA synthesis is inhibited to the same extent as DNA syn-

[1] The (S–) phase of DNA synthesis during interphase is preceded and followed by two phases, $G(ap)_1$ and $G(ap)_2$, respectively, during which no DNA synthesis takes place.



thesis, indicating a close correlation between DNA and RNA content. In younger germ tubes this correlation is apparently much weaker and inhibition of DNA synthesis might not directly result in inhibition of RNA synthesis.

The antimitotic activity of MBC in *A. nidulans* shows a striking resemblance with the antimitotic activity of colchicine in human cell cultures (16). The mechanism of action is based on the complex forming ability of colchicine with a protein subunit of microtubules (17–19). Preliminary experiments showed that *A. nidulans* was not inhibited by colchicine at concentrations of this compound up to 100 µg/ml in malt

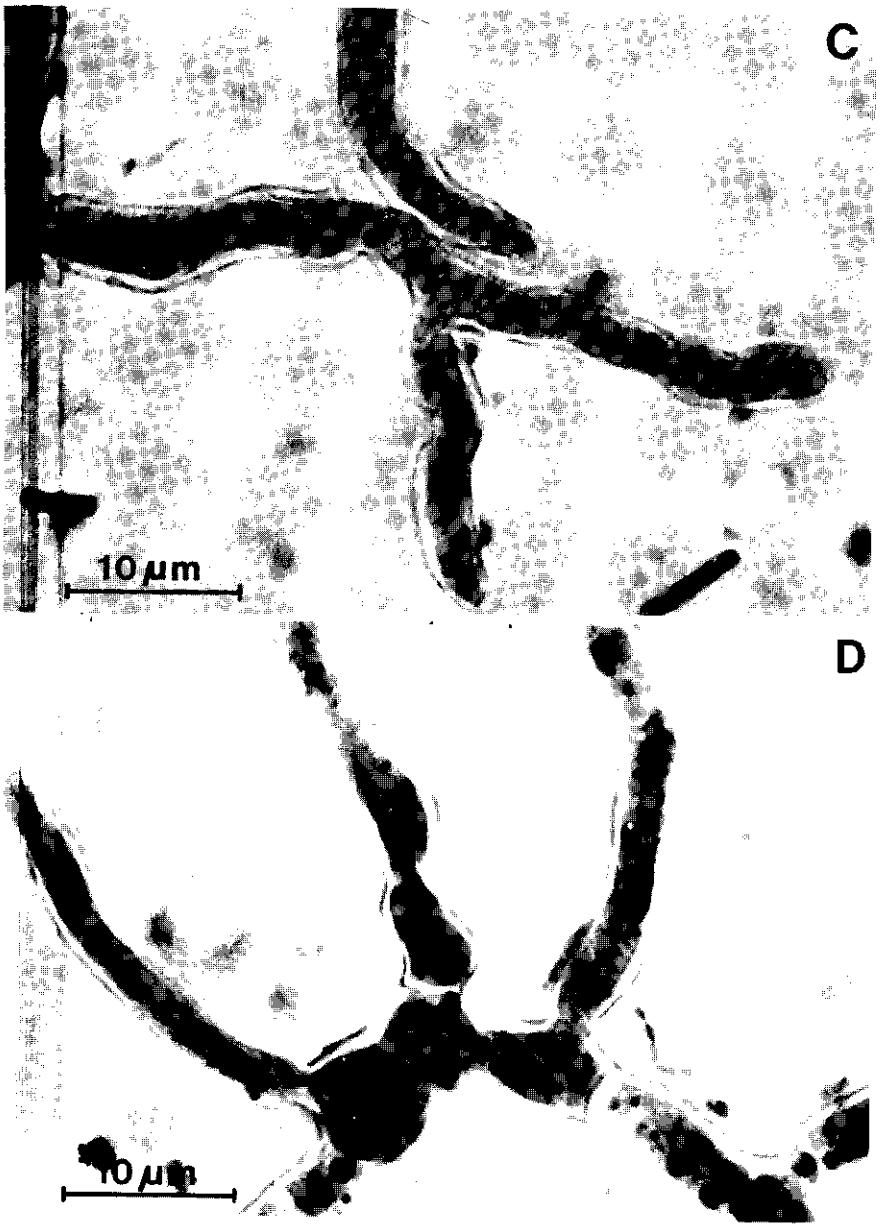


FIG. 4. *Photomicrographs of Giemsa–HCl stained hyphae of* Aspergillus nidulans. *A. Untreated; nuclei in interphase, chromatin ring-shaped. B. Untreated; nuclei in mitosis. C, D. Treated with 4 µM MBC. Abnormal configurations of dense masses of chromatin.*

extract agar. However, as has been shown by Haber *et al.* (20), colcemid, a colchicine derivative, inhibits growth and division of *Saccharomyces cerevisiae* under restricted culture conditions. Also here, colchicine itself was not inhibitory to the organism. This difference in activity could not be ascribed to a difference in ability of the two compounds to permeate the yeast cell, but to a difference in affinity of the two agents

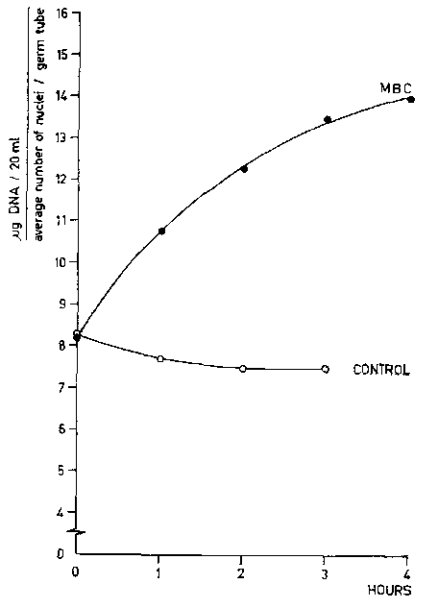


FIG. 5. *Effect of 4 µM MBC on relative DNA content per nucleus. Data are based on those in Fig. 3.*

for a cellular binding site, which was provisionally identified as a microtubule subunit. This difference in affinity to the binding site might be due to a minor modification of the structure of colchicine, for colchicine has a NH-CO-CH_3 group and colcemid a NH-CH_3 group.

The resemblance in the effect of MBC with that of colchicine and colcemid on mitosis and synthesis of macromolecules might suggest a similar mode of action, viz, the formation of a complex between MBC and a subunit of microtubuli, resulting in inhibition of spindle formation. The NH-CO-OCH_3 side chain of MBC might act in the same way as the active group of colchicine and colcemid in determining the binding properties between toxicant and protein. From this point of view the lower intrinsic fungicidal activity of ethyl benzimidazol-2-yl carbamate (EBC) (21–23) might be due to a lower affinity of this toxicant to a microtubule subunit.

This suggested mode of action of MBC could also account for the nuclear instability of *A. nidulans* diploids (24), when exposed to sublethal concentrations of benomyl.

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ANTIMITOTIC ACTIVITY OF METHYL BENZIMIDAZOL-2-YLCARBAMATE IN FUNGI AND ITS BINDING TO CELLULAR PROTEIN

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1. Summary

Several lines of evidence indicate that methyl benzimidazol-2-ylcarbamate (MBC or carbendazim)and some other benzimidazole derivatives may be considered as antimitotic agents, acting as spindle poisons. A macromolecular MBC receptor with tubulin-like properties appeared to be present in Aspergillus nidulans and other MBC-sensitive fungi.

The differences in response of fungi to MBC and the readiness with which changes in response in originally sensitive fungi can be induced may indicate a very specific action.

A high specificity of the mechanism of action may also account for the failure of MBC to inhibit both porcine brain tubulin polymerization in vitro and the copolymerization of fungal tubulin into microtubules in a heterologous assembly system with porcine brain tubulin.

In view of this, benzimidazole derivatives may prove to be valuable tools to compare biochemical and functional properties of tubulin from different sources.

Purification of tubulin in the heterologous assembly system did not involve copurification of the high molecular weight proteins as is normally observed. Incorporation of fungal tubulin into microtubules might prevent their association with structures, composed of these proteins.

The heterologous assembly system might provide a new approach by which the in vitro and in vivo control of microtubule assembly can be investigated.

2. Introduction

Methyl benzimidazole-2-ylcarbamate (MBC or carbendazim, fig. 1) is one of the benzimidazole derivatives which is highly toxic to some groups of fungi. Particularly fungi belonging to the Ascomycetes are very sensitive, whereas others belonging to the Oomycetes are resistant[1]. Other benzimidazole derivatives like 2-(thiazol-4-yl)benzimidazole (thiabendazole or TBZ, fig. 1) which is also known as an anthelminthic and 2-(2-furyl)benzimidazole (fuberidazole, fig. 1) have a similar spectrum of activity, although these compounds are less active than MBC.

In agricultural practice these compounds have become increasingly important for the control of plant diseases. In addition to their high toxicity their systemic behaviour within plants is an important property for effective disease control. Due to efficient translocation these compounds can eradicate established infections and cure the plant. In some cases protection of new growth may be achieved by these systemic compounds.

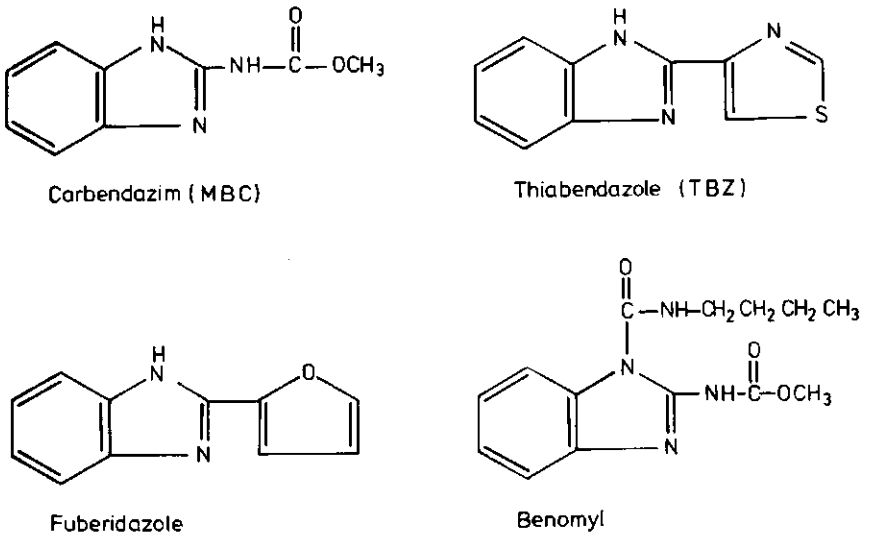

Fig. 1. Structural formulae of carbendazim (MBC), thiabendazole (TBZ), fuberidazole and benomyl.

Although MBC is used as such in spray formulations, methyl 1-(butylcarbamoyl) benzimidazol-2-ylcarbamate (benomyl, fig. 1) is more widely employed. The reason why benomyl which breaks down to MBC in aqueous solution, is used, is due to its slightly superior qualities in a number of applications[2,3].

However, soon after the introduction in practice of the benzimidazole derivatives which were occasionally considered as the final and decisive solution to the control of certain diseases, negative aspects became apparent. In some cases, disease control was no longer successful, because resistance developed in previously sensitive fungal populations. Also in laboratory experiments resistance could be induced by mutagenic agents. This phenomenon, well known in insect pest control, was seldom encountered in controlling fungal diseases until recently and might, therefore, be inherent to the use of the new systemic compounds[4].

Since the benzimidazole derivatives were assumed to be very specific in their action, scientists became interested in these compounds not only from the point of view of their fungicidal aspects, but also because of their possible use as investigative tools to study the physiology of fungi.

The purpose of this paper is to report recent findings on the mechanism of action of MBC and the identification of its site of action. Also the biochemical basis of resistance to this compound will be discussed.

3. Mechanism of action

The first report[5] concerning the mode of action of MBC drew attention to DNA and RNA synthesis as the possible targets of MBC action. However, inhibition of these processes became only evident several hours after addition of the compound to liquid cultures of the fungi studied, viz. Neurospora crassa and Ustilago maydis. The observed inhibition might therefore be considered as a secondary effect.

Since nuclear and cell division might be affected by MBC, these processes were studied separately from DNA synthesis using synchronous cultures of Saccharomyces cerevisiae and U. maydis[6] or by simply counting nuclei in growing germ tubes of Aspergillus nidulans[7]. These studies revealed that primarily mitosis was inhibited by MBC and that inhibition of DNA and RNA synthesis were secondary effects attributable to mitotic failure. In addition, light microscopic observations of MBC-treated hyphae revealed abnormal chromatin configurations.

The antimitotic activity of MBC in fungi resembles mitotic arrest caused by colchicine in mammalian cell cultures[8]. Because of this similarity MBC might be a member of a class of antimitotic agents commonly known as spindle poisons, of which colchicine can be considered as a prototype[9].

4. Induced resistance to MBC and TBZ in Aspergillus nidulans

In order to study more precisely the mechanism of action of the benzimidazole derivatives and the biochemical basis of resistance, resistance to MBC and TBZ was induced in A. nidulans[10]. It appeared that the TBZ-resistant strains could be distinguished into two categories. Strains belonging to the first category which comprised about 95 % of the total number of mutant strains examined, were also resistant to MBC. As distinct from these strains, strains belonging to the second category which comprised the remaining 5 %, showed an increase in sensitivity to MBC. Table 1 gives the inhibitory concentrations of TBZ or MBC causing a 50 % reduction in radial growth on agar (ED_{50} values) for one representative strain of each category, viz. strain R and strain 186, as well as those for the wild type strain 003.

Genetic analysis proved both resistance and increased sensitivity to MBC to be caused by mutation in the same gene, located on linkage group VIII[10]. In addition to this a lower resistance to MBC was found to be caused by mutation of two other genes[11,12].

For biochemical studies, strain R, strain 186 and strain 003 were chosen, because of their characteristic behaviour with respect to MBC.

Table 1

Response of Aspergillus nidulans strains R, 186 and 003 to MBC and TBZ. Effects were estimated by measuring radial growth on malt agar medium containing MBC or TBZ at different concentrations. Values given are inhibitory concentrations causing a 50 % reduction in growth (ED_{50} value)

	ED_{50} (10^{-6} M)	
Strain	TBZ	MBC
R	800	95
186	195	1.5
003	50	4.5

5. Binding studies with ^{14}C-MBC

The characteristic property of spindle poisons is their interference with the function of microtubules. Colchicine accomplishes this by binding tightly and very specifically to tubulin, the protein dimer of which the microtubule is composed.

In order to determine the involvement of a similar binding of MBC to fungal tubulin in the antimitotic action of this compound, cell-free extracts of mycelium of the three A. nidulans strains were incubated with ^{14}C-MBC. Binding was determined by the standard gel filtration procedure using Sephadex G-100 columns. Typical elution patterns are shown in fig. 2.

From this figure it is evident that radioactivity was present in macromolecular fractions, when extracts of sensitive mycelium were used. The MBC sensitivity of the strains was reflected in the amount of bound radioactivity. Mycelial extracts of the resistant strain did not show binding activity.

Thin layer chromatographic analysis of the bound radioactivity showed that this as still present in ^{14}C-MBC.

As distinct from colchicine binding, MBC binding did not require a high temperature and appeared to be rapidly reversible, as is suggested by the fact that the peak of bound radioactivity showed considerable tailing. In this respect the binding mechanism resembles that of the spindle poison podophyllotoxin[9].

The relation between binding of MBC and MBC sensitivity was further studied using normal sensitive strains of Penicillium species and resistant strains which emerged in benomyl-treated crops[14]. Also two naturally resistant fungi were included, viz. an Alternaria and a Pythium species. It appeared that only mycelial extracts of sensitive strains showed considerable MBC-binding activity, whereas mycelial extracts from resistant strains did not.

These data strongly suggest that MBC binding to fungal cellular material is essential in the antimitotic action of MBC.

According to the elution volume of the MBC complex it can be concluded that the molecular weight of the binding species is approximately 110,000 daltons. This

value has also been obtained in a more accurate study using a Sephadex G-200 column, which was calibrated with proteins of known molecular weight.

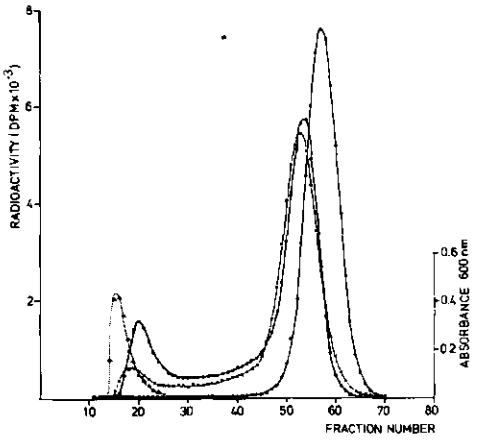

Fig. 2. Gel filtration of 40,000 x g supernatant mycelial extracts of Aspergillus nidulans strains containing approximately 40 mg protein per ml, incubated with 2.7×10^{-6} M 2-^{14}C-MBC (spec. act. 11.4 mCi/mmole) for 120 min. at 4°C. Extracts were prepared in 0.05 M potassiumphosphate buffer, pH 6.8, containing 0.1 M KCl, 5 mM $MgCl_2$, 0.1 mM GTP. Elution profiles of radioactivity of parallel experiments with extracts of strain 003 (x--x), strain 186 (o--o) and strain R (●--●) are shown. The elution profile of Blue Dextran is indicated by ▲...▲ (from Davidse[13]).

The MBC-binding substance was further characterized by chromatography on a DEAE-Sephadex A-50 anion exchanger. This method was employed because of the supposed identity of the MBC-binding substance with fungal tubulin. Elution profiles of radioactivity and protein in experiments with mycelial extracts of strain 186 and strain R, incubated with ^{14}C-MBC are shown in fig 3A and 3B, respectively. With extracts of strain 186 radioactivity was eluted in two peaks, one with the bed volume of the column and the other at 0.6 M KCl, whereas with extracts of strain R the activity was eluted in one single peak with the bed volume of the column.

On the basis of the retention properties on DEAE-Sephadex ion exchangers and the estimated molecular weight of 110,000 daltons there is good reason to believe, that the MBC-binding macromolecule is identical with fungal tubulin. Assuming that binding prevents assembly of tubulin into functional microtubules, the mechanism of action of MBC might be similar with that of colchicine and MBC might be considered a spindle poison, especially active in certain fungi. Induced resistance and extra-sensitivity of A. nidulans strains might then be based on changes in affinity of the receptor site to MBC, caused by mutation of the coding gene. Mutations involving resistance and mutations involving increased sensitivity were shown to be about 16 nucleotides apart from each other[12], corresponding with a distance of about 5 amino acids in the protein. These data are consistent with

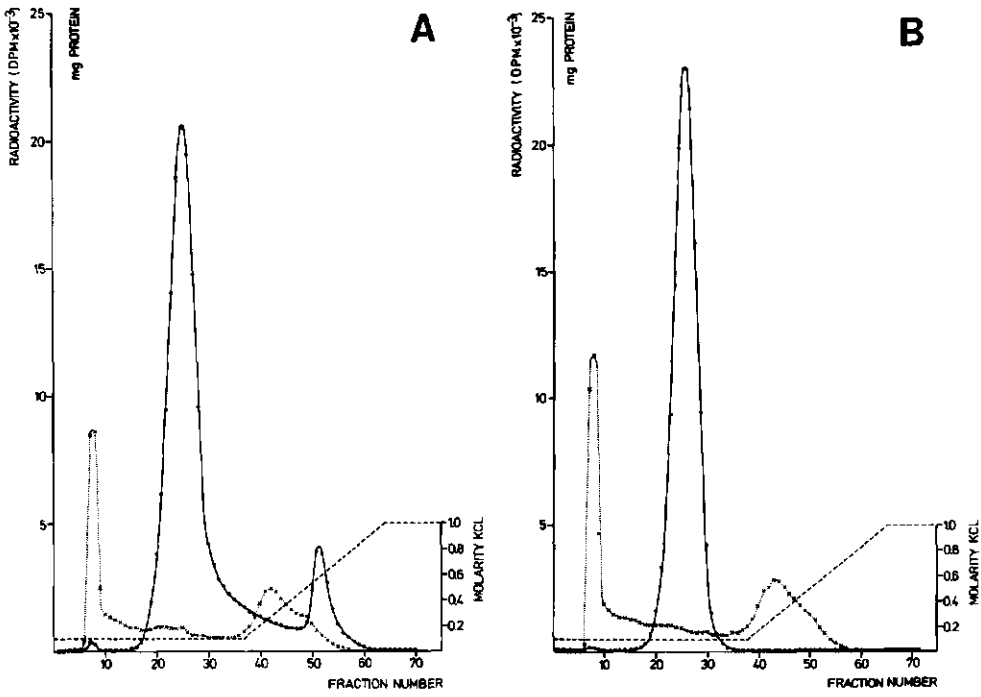

Fig. 3. DEAE-Sephadex A-50 column chromatography of 40,000 x g supernatant mycelial extracts of strain 186 (A) and strain R (B) of Aspergillus nidulans. A 1.5 x 23 column was loaded with 4 ml of an incubation mixture (for details see text fig. 2) and was eluted first with buffer containing 0.1 M KCl, followed by a linear gradient of 0.1 - 1.0 M KCl. Radioactivity (●--●) and protein (x--x) was determined in 1 ml of each 2 ml fraction (----) KCl concentration. (From Davidse[13]).

the hypothesis that changes in affinity might be associated with changes in amino acid composition of the MBC-binding site on tubulin, without interfering with its functional properties.

6. Partial purification of the MBC-binding protein

In order to know more about its biochemical properties an attempt was made to purify the MBC-binding protein, using ^{14}C-MBC as a marker. Since the binding protein was assumed to be identical with fungal tubulin, a standard tubulin purification procedure[15] was employed. A 127,000 x g (60 min) supernatant mycelial extract was fractionated with a saturated ammonium sulphate solution in water. The fraction precipitating between 35 and 50 % saturation was taken up in buffer and incubated with ^{14}C-MBC. The incubation mixture was run onto a DEAE-Sephadex A-50 column and the eluted fractions were assayed for radioactivity. Fractions eluted at c. 0.6 M KCl which contained bound MBC and, therefore, the binding protein were

subjected to SDS-polyacrylamide electrophoresis (fig. 4). From this figure it is evident that in these fractions a protein is present with similar electrophoretic properties as brain tubulin. According to the specific tubulin purification procedure used, this protein might be considered as fungal tubulin. However, in addition to this protein several other proteins are present, so that from this experiment it can not be definitely concluded that fungal tubulin is responsible for MBC-binding.

Further purification of the MBC-binding protein was greatly hindered by the instability of the binding activity. In 40,000 x g supernatant mycelial extracts binding activity rapidly decayed in a first order manner with a half time of 280 min. at $4^{o}C$. Guanosine triphosphate (GTP), which is known to stabilize colchicine--binding activity of tubulin, did not affect the rate of decay. However, in these crude extracts, GTP might be rapidly hydrolyzed, so that no effect can be anticipated. MBC itself, at 10^{-5} M, stabilized the binding activity resulting in a half time of decay of 920 min.

Possible due to this rapid decay in the absence of MBC hardly any binding activity could be detected in combined and concentrated (vacuum dialysis or ammonium sulphate precipitation) DEAE-Sephadex A-50 fractions, eluted at c. 0.6 M KCl, when these were incubated with ^{14}C-MBC and analyzed for bound radioactivity by gel filtration on Sephadex G-100 columns. However, the possibility that passage of an extract through this type of column might remove a factor required for the formation of the MBC-protein complex, can not be ruled out.

In mycelial extracts showing MBC-binding activity no ^{3}H-colchicine-binding activity could be demonstrated. Although normally colchicine binding can be seen as an indication of the presence of tubulin, in fungi colchicine probably can not be used as a diagnostic tool. Fungal mitosis appeared to be insensitive to colchicine[18,19] presumably due to low affinity of fungal tubulin to this compound.

Although the definite proof has not yet been given the data presented support the idea that MBC is bound to fungal tubulin and that MBC might be used as a diagnostic tool for fungal tubulin.

7. Toxicity of MBC in mammalian cell cultures

As has been shown[20,21] MBC exhibits antimitotic activity in mammalian cell cultures. Studies of the mechanism of action in mammalian cell extracts might give more information about the character of MBC as a spindle poison. Binding studies with ^{14}C-MBC and porcine brain extracts revealed that no binding occurred after incubation at $4^{o}C$ nor at $37^{o}C$, although the preparations showed considerable ^{3}H-colchicine-binding activity.

In vitro polymerization of porcine brain tubulin into neurotubules was not affected by MBC. Another benzimidazole derivative, methyl 5-(2-thenoyl)-

benzimidazol-2-ylcarbamate, (R 17934), however, proved to be very effective[22].

8. Effects of R 17934 on fungi

In preliminary studies with R 17934 it was shown that this compound could effectively inhibit the growth of fungi. It was found active against A. nidulans even at lower concentrations than MBC. The response of the three above-mentioned strains was similar to that towards MBC, viz. strain R was resistant compared with strain 003, whereas strain 186 appeared to be most sensitive.

In binding experiments with ^{14}C-MBC, R 17934 could effectively reduce the amount of MBC bound to the protein. These facts indicate that at least in fungi R 17934 and MBC have a similar mechanism of action.

9. Heterologous assembly of fungal and porcine brain tubulin

Assuming interference of MBC with microtubule formation during mitosis in fungi, a study of its effect on fungal tubulin polymerization in vitro might add to our understanding of the ability of MBC to act as a spindle poison.

Preliminary polymerization experiments with fungal tubulin preparations, purified by DEAE-Sephadex A-50 chromatography were not successful. This could be due to the low amount of protein which could be purified and which was probably not enough to reach the critical concentration essential for tubulin polymerization[23]. On the other hand also cofactors essential for polymerization might have been removed during the purification procedure[24,25].

Possible interference of MBC with the assembly of fungal tubulin might also be studied in a heterologous assembly system. Literature data[23,26] report the successful assembly of brain tubulin onto flagellar microtubules from Chlamydomonas and sea urchin sperm. However, tubulin from the cystoplasmatic pool of Chlamydomonas did not copolymerize with brain tubulin, but prevented assembly[27].

In preliminary experiments using 40,000 x g (60 min) supernatant porcine brain extracts and 127,000 x g (120 min) supernatant mycelial extracts of A. nidulans no polymerization could be obtained, as was shown by analysis of the various fractions by SDS-polyacrylamide gel electrophoresis. Partial purification of the mycelial extract by 35 - 50 % ammonium sulphate fractionation and passage of the preparation over a Sephadex G-25 column, equilibrated with reassembly buffer was found necessary to obtain polymerization equal to that in the porcine brain extract, as was revealed by protein determinations in corresponding fractions. That indeed microtubule assembly occurred in the mixture was established by electron microscopy. Microtubules being assembled in the mixture appeared to be similar to those in porcine brain extracts. Fig. 5 shows the results of electro-

phoretic analysis of the tubulin preparations after one cycle of assembly and disassembly.

It is evident that the assembly product, obtained in the mixture does not contain the two high molecular weight proteins which were found to be always present in tubulin preparations[23,28,29,30], purified by the assembly procedure. These proteins were reported to be the components of filaments attached to in vitro assembled brain microtubules (neurotubules) and neurotubules in situ[31].

Besides the tubulin band several other minor bands were visible on the gels loaded with the assembly product of the mixture which did not appear on the gels of the control preparation. These components persisted through a further second assembly and disassembly step.

These results prompted us to carry out a heterologous assembly experiment, in which the proteins of the fungal extract were labeled. Proteins were labeled by growing mycelium during the last three hours of the growing period in the presence of 25 µCi ^{14}C-phenylalanine per 1 of medium. About 90 % of the label was taken up and proteins were labeled at about 44,850 dpm/mg protein as was determined in the 35 - 50 % ammonium sulphate preparation after gel filtration on the Sephadex G-25 column.

After one cycle of assembly and disassembly MBC (10^{-5} M) was added to a sample of the preparation, whereas a similar sample served as control, and a second cycle of assembly was performed.

The various fractions were analyzed by SDS-polyacrylamide gel electrophoresis. Gels were sliced and the amount of radioactivity was determined in each segment.

Fig. 4. SDS-polyacrylamide electrophoretic analysis[16] of DEAE-Sephadex A-50 fractions containing MBC-binding protein. Five ml of a 35 - 50 % ammonium sulphate preparation in extraction buffer (see text fig. 1) of a 127,000 x g (60 min) supernatant mycelial extract were incubated with ^{14}C-MBC (final concentration 9 x 10^{-6} M; 226,000 dpm/ml) for 120 min. at 4°C. The incubation mixture was loaded onto a 20 ml DEAE-Sephadex column, which was subsequently eluted with 15 ml of a linear gradient of 0.1 - 0.4 M KCl, 15 ml of 0.4 M KCl, 30 ml of a linear gradient of 0.4 - 1.0 M KCl and 20 ml 1.0 M KCl, respectively, all made up in buffer. Fractions of 2.5 ml were collected and assayed for radioactivity. Fractions from the 0.6 M region which contained resp. 1670, 2160, 4170, 7230, 5570, 2590 and 1070 dpm bound ^{14}C-MBC per ml were subjected to electrophoresis (gel A - G). As reference tubulin purified by two cycles of assembly[17] from a porcine brain extract was run on a separate gel (T). Densitometer tracings were made of gel T and gel D (peak fraction of MBC-binding protein). Samples were prepared for electrophoresis as described[16]. Gels consisted of 7.5 % acrylamide, 0.13 % bisacrylamide, 0.1 % SDS in 0.1 M sodium phosphate buffer (pH 7.0). Gels were stained for 60 min. in 0.1 % Coomassie Brilliant Blue in a methanol, water, acetic acid mixture (227:227:46). Destaining was performed by diffusion in 7 % acetic acid in water.

Fig. 5. SDS-polyacrylamide gel electrophoresis of tubulin, partially purified by one cycle of assembly[17] in porcine brain extract (1) and in a mixture of porcine brain and fungal extract (2). For electrophoretic and staining conditions see fig. 4.

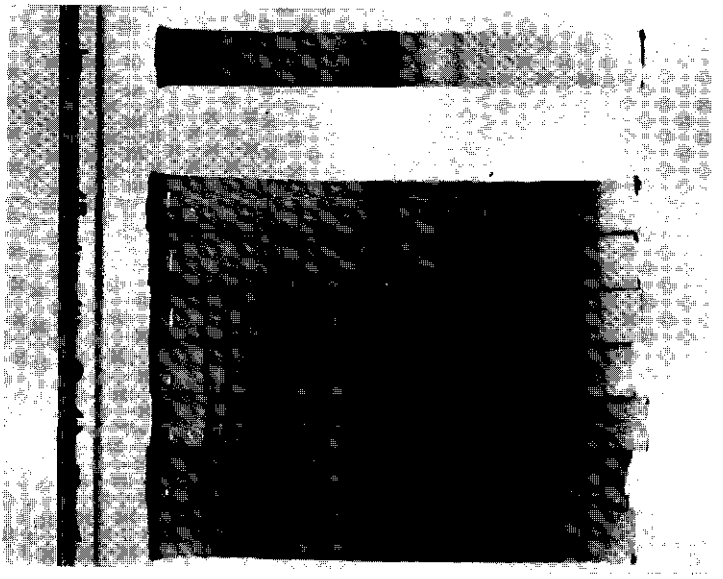

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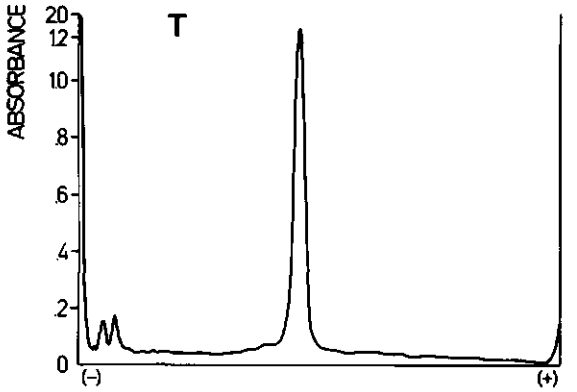
Fig. 5



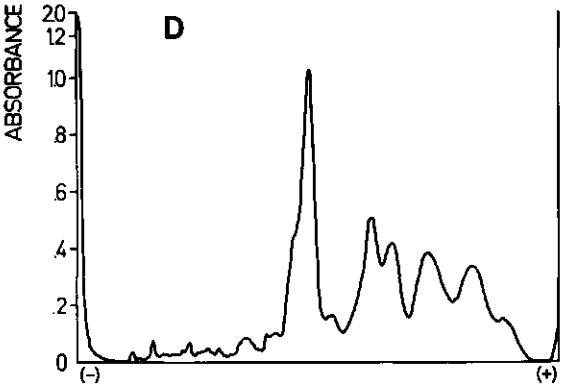


Fig. 4

The results of this experiment are given in table 2.

Table 2

Heterologous assembly of fungal and porcine brain tubulin. Two ml of a partially purified mycelial extract (13 mg protein per ml; 44,850 dpm/mg protein) of Aspergillus nidulans strain 003 were mixed with 8 ml of a porcine brain extract (5.5 mg protein per ml). Two cycles of assembly and disassembly were performed[17]. In cyle II the effect of MBC (10^{-5} M) was studied. Total radioactivity (dpm) and its distribution (%) among the various fractions are given. Also the percentages of radioactivity actually present in tubulin subunits as revealed by SDS-polyacrylamide gel electrophoretic analysis are given.

Assembly		Radioactivity of the fractions (dpm)		
		mixture	H_1S	C_1S
cycle I		1,166,000 (100 %)	1,150,000 (98.6 %)	15,750 (1.4 %)
		C_1S	H_2S	H_2P
cycle II	control	10,500 (100 %)	7,680 (73 %)	2,410 (23 %)
	MBC	10,500 (100 %)	8,000 (76 %)	2,390 (23 %)

SDS-polyacrylamide gel electrophoresis		Radioactivity of tubulin (% of total radioactivity of the fraction)		
		C_1S	H_2S	H_2P
cycle II	control	49	42	73
	MBC	49	47	72

H_1S : 45,000 x g (30 min. 25°C) supernatant from first assembly
C_1S : 45,000 x g (30 min. 4°C) supernatant from resuspended pellet (30 min. 0°C) from first assembly
H_2S : 45,000 x g (30 min. 25°C) supernatant from second assembly
H_2P : resuspended pellet (30 min. 0°C) from second assembly

As is evident from this table, copolymerization of radiolabeled fungal protein with porcine brain tubulin under microtubule assembly conditions strongly suggests that heterologous assembly has occurred in a mixture of brain and mycelial extract. Although in the first cycle only 1.4 % of the radioactivity could be recovered in the tubulin preparation, in the second cycle this percentage increased to 23 % indicating that actual temperature dependent polymerization of labeled protein, has occurred. Gel electrophoresis proved that tubulin was mainly responsible for this phenomenon, because the amount of radioactivity present in tubulin increased from 49 % in C_1S to 73 % in H_2P.

At the concentration used MBC did not have any effect on polymerization of radioactive protein, although the fungal preparation used did bind MBC, as was determined in similar preparations.

An explanation for the inability of MBC to inhibit the assembly of fungal tubulin in a heterologous system can only be tentative, as long as the processes of MBC binding and fungal and procine brain tubulin copolymerization are not

completely clear. The inability of MBC to interfere with the heterologous assembly might be explained by supposing that an MBC-fungal tubulin complex is the result of interaction of MBC with the suggested Y-state of the tubulin molecule[25,32] and that Y-state fungal tubulin molecules do not take part in the heterologous assembly process.

At present experiments along these lines are in progress.

10. Discussion

Although the exact mechanism of action of MBC has not yet been elucidated, several lines of evidence indicate that it acts as a spindle poison in fungi. Its similarity with colchicine in binding to a protein with several biochemical characteristics of tubulin is one of these. The structural relationship of MBC with R 17934, a compound which certainly belongs to this class of antimitotic agents[22] and which probably displays a similar mechanism of action in fungi, also supports this idea.

Whether MBC binding to fungal tubulin is comparable with colchicine binding to mammalian tubulin or whether MBC binding requires a specific state of the tubulin molecule is not yet clear.

Changes in amino acid composition of the MBC-binding site which might result in affinity changes might explain the phenomena of resistance and increased sensitivity to the benzimidazole derivatives. However, mechanism underlying these phenomena might be more complicated and might also involve changes in amounts of tubulin molecules being able to bind MBC.

Although it has been shown that part of the primary structure of the tubulin molecule is strongly conserved during evolution[33], the colchicine-binding site might have evolved along different lines according to the differential colchicine--binding properties of fungal and mammalian tubulin. In a comparable way, a binding site for benzimidazole derivatives might have been evolved leading to a differential response of mammalian and fungal cells to MBC. However, modification of the molecule might strongly influence its interaction with tubulin. Benzimidazole derivatives, therefore, might be valuable tools to study differences in biochemical and functional properties of tubulin from different sources.

Purification of tubulin in a mixture of porcine brain and mycelial extract did not involve copurification of high molecular weight proteins as is normally observed. Supposing polymerization of these proteins being independent of that of microtubules, this phenomenon might be due to an inhibitory action of components of the mycelial extract. However, according to literature data[29,31] it is more likely that presence of the high molecular weight proteins in microtubule preparations is caused by a structural linkage of these proteins to microtubules.

Their absence in tubulin preparations purified from mixtures of brain and mycelial extracts, might, therefore, indicate that in this case linkage did not occur. This might be due to incorporation of fungal tubulin into microtubules making these less suitable for association with high molecular weight proteins.

The incorporation of radioactive fungal protein in microtubule preparations in the heterologous assembly system suggests copolymerization of fungal and brain tubulin. This means that despite the above mentioned differences between the two tubulin species, binding sites involved in polymerization should be highly conserved during evolution.

The heterologous assembly system might provide a new approach by which the in vitro and in vivo control of microtubule assembly can be investigated.

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The mechanism of resistance to the antitubulin methyl benzimidazole-2-yl carbamate in the fungus Aspergillus nidulans

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ABSTRACT

The antimitotic compound methyl benzimidazol-2-yl carbamate (MBC) formed a complex in vitro with a protein present in mycelial extracts of fungi. The binding protein of Aspergillus nidulans showed a set of properties which is unique for tubulin. Binding occurred rapidly at 4°C and was competitively inhibited by the antitubulins oncodazole and colchicine. Other antitubulins such as podophyllotoxin, vinblastine sulfate, melatonin and griseofulvin did not interfere with binding of MBC. Electrophoretic analysis of partially purified preparations of the binding protein revealed the presence of proteins with similar mobilities as mammalian tubulin monomers. Hence it is concluded that the binding protein is identical with fungal tubulin.

The effect of MBC on mycelial growth of mutant strains of A. nidulans was positively correlated with the affinity of the binding sites for this compound. The apparent binding constant for MBC and tubulin from a wild-type strain was estimated at 4.5×10^5, from a resistant strain at 3.7×10^4, and from a strain with increased sensitivity to MBC at 1.6×10^6 l. mole^{-1}. Mutants showing resistance and increased sensitivity to MBC are candidates to have alterations in tubulin structure. Low affinity of tubulin for MBC is probably a common mechanism of resistance to this compound in fungi.

Binding of colchicine to A. nidulans tubulin occurred at 4°C with an apparant binding constant of 2.5×10^3 l. mole^{-1}.

In the last fifteen years several benzimidazole compounds have been introduced as fungicides, like benomyl[1] (29), fuberidazole (58), and thiabendazole (66), as anthelmintics, like fenbendazole (5), mebendazole (68), parbendazole (1), and thiabendazole (12), or as antitumoral drug, like oncodazole or R 17934 (3,4). Structural formulae of these compounds are shown in Fig. 1.

In recent years considerable attention has been given to the mechanism of action of these compounds. Benomyl and its conversion product, carbendazim or MBC, interfere with mitosis in fungi (21,39,56), plants (56) and mammalian cells in vivo(59,67) and in vitro(27,59,67). Mebendazole induces degenerative changes in intestinal cells of parasites after treatment of their hosts (8). These effects are probably caused by the interaction of this drug with cytoplasmic microtubules (9,10). Oncodazole interferes with the structure and function of microtubules both in interphase and mitotic mammalian cells cultured in vitro (25,26). Microtubules of dividing and non-dividing malignant cells in vivo are similarly affected (26).

Although the biological activity of these benzimidazole compounds is probably based on interference with the formation or functioning of microtubules, which are present in all eukaryotic cells, eukaryotes are not equally sensitive to each benzimidazole compound. Benomyl and MBC partly owe their success as systemic fungicides to a relative non-toxicity to plants and animals. But also within fungi, there are differences in sensitivity to these compounds. For instance, fungi belonging to the Ascomycetes are sensitive, whereas others belonging to the Oomycetes are resistant (7). In addition to natural resistance to benomyl and MBC, resistant strains of naturally sensitive species are frequently found in sprayed crops. In the laboratory such strains can be readily obtained by mutagenic treatment (28).

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[1] The following common names and abbreviations are used: benomyl, methyl 1-(butylcarbamoyl)benzimidazol-2-yl carbamate; fuberidazole, 2-(2'-furyl)benzimidazole; thiabendazole, 2-(4'-thiazolyl) benzimidazole; fenbendazole, methyl 5-phenylthiobenzimidazol-2-yl carbamate; mebendazole, methyl 5-benzoylbenzimidazol-2-yl carbamate; parbendazole, methyl 5-butylbenzimidazol-2-yl carbamate; oncodazole or R 17934, methyl 5-(2-thienylcarbonyl) benzimidazol-2-yl carbamate; carbendazim or MBC, methyl benzimidazol-2-yl carbamate; SDS, sodium dodecylsulfate; PKMg buffer, 0.05 M potassium phosphate buffer, pH 6.8, containing 0.1 M KCl and 0.005 M $MgCl_2$; DMSO, dimethylsulfoxide; PNaMg buffer, 0.05 M sodium phosphate buffer, pH 6.8, containing 0.1 M NaCl, and 0.005 M $MgCl_2$; MES, 2-(N-morpholino)ethanesulfonic acid; EGTA, ethyleneglycol-bis-(ß-aminoethylether)tetraacetic acid; TEMED, N,N,N',N',-tetramethylenediamine; VB, vinblastine sulfate.

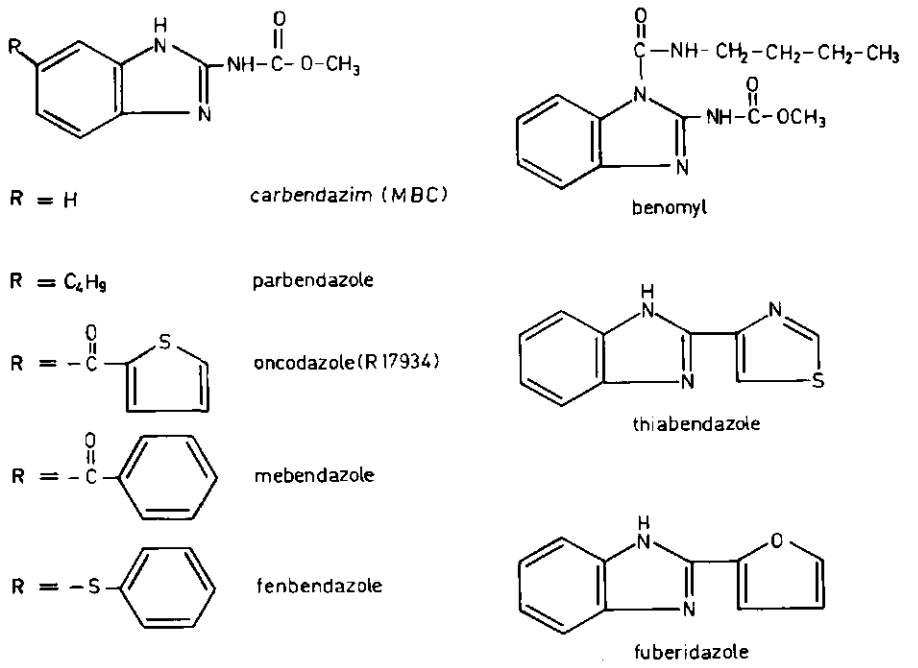


Fig. 1. Structural formulae of biologically active benzimidazole compounds.

The curative action of mebendazole in animal as well as in human helminthiases implies selectivity with respect to host and parasite. Ultrastructural studies have shown that upon treatment of the host microtubules in cells of the parasite are completely destroyed, whereas cytoplasmic and spindle microtubules of the host cells remain unaffected, even though both types of cells have been exposed to identical drug concentrations (9,10).

In vivo experiments on the effect of oncodazole on experimental and human neoplasms have shown that this compound specifically eliminates microtubules in dividing and non-dividing neoplastic cells, whereas microtubules of interphase normal cells are apparently intact. Microtubules in mitotic cells, however, are seriously affected (26).

Biochemical studies on the mechanism of action of benzimidazole compounds have shown that the antimitotic action of MBC in fungi is probably mediated via binding to fungal tubulin (22,23). Oncodazole is bound to mammalian brain tubulin at the colchicine binding site (45) and is a potent inhibitor of microtubule assembly in vitro (26,27,45). Benomyl and MBC only slightly affect this process (27,44).

The molecular basis of selectivity of these benzimidazole compounds is yet unknown. Differential uptake or metabolism may be responsible for the relative non-toxicity of benomyl and MBC to animals and plants (36,65). For natural and induced resistance in fungi, it has been found that no differences in metabolism exist between MBC-resistant and MBC-sensitive strains (24). Here a differential binding of MBC to tubulin in strains differing in MBC sensitivity might underly the selective action of this compound (22,23). The selective action of mebendazole does not appear to be related to a differential drug-uptake between host and parasite (9,10). A differential interaction of the drug with the target inside the cells is assumed to be responsible (9,10).

In this study the interaction of MBC with its receptor site in MBC-sensitive and MBC-resistant strains of the fungus Aspergillus nidulans has been investigated in detail. Since it has been assumed that MBC binds to fungal tubulin the effect of oncodazole and of other antitubulins on MBC binding has been studied. With [^{14}C]MBC as affinity label the binding protein was partially purified and electrophoretically characterized.

MATERIALS AND METHODS

Organisms. Most of the experiments were carried out with Aspergillus nidulans biA1 AcrA1 (Strain 003) and two mutant strains (Strain 186 and Strain R), which differ in MBC sensitivity. Sensitivity of these strains in terms of inhibitory concentrations, which cause a 50% reduction in growth (ED_{50}) on agar, are 4.5 µM MBC for Strain 003, 1.5 µM for Strain 186 and 95 µM for strain R. Both strains were selected in the laboratory after UV treatment of conidia of Strain 003 (69). Genetic analysis has shown that both increased sensitivity in Strain 186 and resistance to MBC in Strain R had been caused by a mutation in one single gene, located on linkage group VIII (69,70).

We also used MBC-sensitive strains of Penicillium brevicompactum and P. corymbiferum and resistant strains of these fungi, which emerged in MBC-treated crops (6). Alternaria brassicae and Pythium irregulare represented the naturally MBC-resistant fungi.

Culture methods. Conidia of A. nidulans strains were grown on a 2% malt extract, 0.1% bacto-peptone, 2% glucose, 1.5% agar medium. Conidial suspensions were prepared as described previously (21). Conidia of Penicillia strains and A. brassicae were harvested from potato-dextrose agar and oatmeal agar, respectively. P. irregulare was maintained on potato-dextrose agar. Mycelium of A. nidulans strains was grown in a glucose-nitrate medium (57), supplemented

with 1 µg biotin per ml. Mycelium of the other fungi were cultured in Czapek-Dox liquid medium (Oxoid), supplemented with 0.5% (w/v) yeast extract powder (Oxoid). Cultures were incubated on a Gallenkamp orbital shaker at 200 rpm at 37°C for A. nidulans and at 25°C for the other fungi.

Preparation of mycelial extracts. Exponentially growing mycelium was harvested by filtering on a Büchner filter. The mycelium was washed three times in cold 0.05 M potassium phosphate buffer, pH 6.8 and frozen at -22°C in a previously cooled X-Press Cell Disintegrator (LKB-Biotec, Sweden) with 0.5 ml of homogenization buffer per gram wet weight of mycelium. The homogenization buffer consisted of 0.05 M potassium phosphate buffer, pH 6.8, containing 0.1 M KCl and 0.005 M $MgCl_2$ (PKMg solution). After 1 hour at -22°C the mycelium was homogenized by passing it five times through the press. The homogenate was thawed and then guanosine triphosphate (GTP) was added to a final concentration of 0.1 mM. The suspension was centrifuged at 40,000 x g for 10 min. and the resultant supernatant recentrifuged at 48,000 x g for 30 min. The 48,000 x g supernatant was immediately used in binding studies. All steps were done at 4°C

Preparation of porcine brain extracts. Fresh porcine brains were obtained from Stroomberg's Exportslachterij (Ede, The Netherlands) and immediately processed after arrival. Blood vessels and meninges were removed and the tissue was washed three thimes with ice cold 0.05 M potassium phosphate buffer, pH 6.8 and once with PKMg solution. Hundred grams of tissue were then homogenized with 100 ml of PKMg solution, containing 0.1 mM GTP, using a motor-driven glass homogenizer with Teflon pestle (Braun, Melsungen, GDR). The homogenate was centrifuged at 48,000 x g for 60 min and the resulting supernatant was fractionated with a neutralized saturated ammonium sulfate solution. The fraction precipitating between 35 and 50% saturation was taken up in 10 ml PKMg solution containing 1 mM GTP. This preparation was used in binding assays either directly or after storage at -22°C.

Binding assays. Varying amounts of a methanolic solution of [^{14}C]MBC were added to mycelial or brain extracts and incubated either at 4° or 37°C for various periods as specified in the legend of Figures and Tables. Small samples of a [^{3}H]colchicine solution in benzene/ethanol (9/1) were placed in empty scintillation vials or centrifuge tubes and the solvent was evaporated to dryness in a stream of nitrogen. The dried compound was dissolved directly in mycelial or porcine brain extract. Vials and tubes were wrapped in aluminum foil in order to prevent photodecomposition of colchicine. Incubation was at

4^{o} or 37^{o}C for various periods as specified. Potential inhibitors of MBC binding were added as solutions in dimethylsulfoxide (DMSO). The DMSO concentration of treated and control samples never exceeded 0.1% v/v.

Binding of [^{14}C]MBC or [^{3}H]colchicine was measured by gel filtration of 1 ml of the incubation mixture on a Sephadex G-100 column (28 x 1.5 cm) with PKMg solution as elution buffer. Fractions of 16 drops (approximately 1 ml) were collected with a LKB UltroRac fraction collector and radioactivity in each fraction was measured in a Nuclear Chicago Mark I Liquid Scintillation Spectrometer with Bruno and Christian's (13) scintillation liquid. Counting efficiency was determined by external standardization procedures. Radioactivity present in protein fractions was considered to represent bound ligand.

Binding of [^{14}C]MBC was also measured with a second method, which has been introduced recently to measure colchicine binding to tubulin (62,63). Aliquots of the incubation mixture were placed in centrifuge tubes containing an equal volume of a charcoal suspension (Merck, Darmstadt, GDR) at 6 mg/ml in PKMg solution. The tubes were placed in a shaker and the mixture was heavily agitated for 10 min and then centrifuged at 1500 x g for 5 min at 4^{o}C. Aliquots of the supernatant were assayed for radioactivity. Blanks, which contained 40 mg/ml bovine serum albumin in PKMg solution incubated with [^{14}C]MBC were handled in the same way. The difference in amount of radioactivity found in supernatant aliquots of sample and blank was assumed to represent bound [^{14}C]MBC. Bound MBC was expressed as dpm per unit of volume of the original extract.

Purification of fungal tubulin. Mycelial extracts were prepared as described above, but with 0.05 M sodium phosphate, pH 6.8, containing 0.1 M NaCl and 0.005 $MgCl_2$ (PNaMg solution) as homogenization buffer. The 48,000 x g supernatant mycelial extract was further centrifuged at 127,000 x g for 60 min. The soluble proteins were fractionated with a neutralized (pH 6.8 after 20 : 1 dilution) saturated ammonium sulphate solution. The fraction precipitating between 35 and 50% saturation was taken up in PNaMg solution containing 1 mM GTP.

The resulting preparations were incubated with [^{14}C]MBC and run onto a DEAE-Sephadex A-50 column (10 x 1,5 cm), which had been previously equilibrated with PNaMg solution. The column was subsequently eluted with 15 ml of a linear gradient of 0.1-0.4 M NaCl, 15 ml of 0.4 M NaCl, 30 ml of a linear gradient of 0.4-1.0 M NaCl and 20 ml of 1.0 M NaCl, respectively, all made up in buffer. Gradients were produced by a LKB Ultrograd Gradient Mixer. The eluate was continuously monitored at 254 nm and fractions of constant volume were collected. Radioactivity was measured in each fraction.

Once the elution properties of the MBC complex were established, the incubation step was omitted and the 35-50% ammonium sulfate preparation was applied directly to the DEAE-Sephadex column. Fractions which were eluted at 0.45-0.90 M NaCl, were combined, dialysed against bidistilled water, and lyophilized. Dry samples were stored at -22°C above silica gel until analysis.

Purification of porcine brain tubulin. Two purification methods were used. With the first method, a tubulin preparation prepared as described above, but with PNaMg solution as extraction buffer, was chromatographed onto a DEAE-Sephadex A-50 column in the same way as described above for mycelial extracts. Fractions containing tubulin were dialysed against bidistilled water and lyophilized. The second method used was a slightly modified assembly-disassembly procedure according to Shelanski et al. (61). Fresh porcine brains were washed and homogenized in 0.1 M MES buffer, pH 6.90, containing 1 mM EDTA, 0.1 mM GTP, and 0.5 mM $MgCl_2$ (MES buffer). The homogenate was centrifuged at 40,000 x g for 10 min and the resulting supernatant at 48,000 x g for 60 min at 4°C. To achieve assembly of microtubules extracts were mixed with an equal volume of MES buffer containing 8 M glycerol and 2 mM GTP and incubated for 30 min. at 37°C. Assembled microtubules were pelleted by centrifuging at 48,000 x g at 25°C. After resuspension and depolymerization of microtubules in MES buffer at 4°C and centrifuging, a second assembly cycle was performed.

Molecular weight determination of the MBC-protein complex. The molecular weight of the MBC-protein complex was determined by gel filtration of an ammonium sulfate fractionated mycelial extract, which had been incubated with [^{14}C]MBC on a Sephadex G-200 column (2.5 x 32 cm), according to the method of Andrews (2). The column was calibrated in two parallel runs with alcohol dehydrogenase (yeast), MW 150,000 and cytochrome C (horse heart), MW 12,400; and with lipoamide dehydrogenase (pig heart), MW 100,000 and α-chymotrypsin (beef pancreas) MW 24,500, respectively.

Reduction and carboxymethylation of proteins. Proteins were reduced by adding β-mercaptoethanol and sodium dodecylsulfate (SDS) at final concentrations of 1% to the samples and heating for 2 min at 100°C. The reduced proteins were dialysed overnight at room temperature against 0.01 M sodium phosphate buffer, pH 7.0, containing 0.1% β-mercaptoethanol and 0.1% SDS.

Protein samples were reduced and carboxymethylated according to the method of Crestfield et al. (20) as modified by Renaud et al. (55). Protein solutions were made 1% in β-mercaptoethanol and mixed with an equal volume

of reducing solution (0.35 M Tris-HCl, pH 8.8, containing 0.12 M β-mercaptoethanol, 8 M urea and 0.1% EDTA). The mixture was dialysed overnight at room temperature against this solution. Lyophilized proteins were directly dissolved in reducing solution and incubated overnight at room temperature under nitrogen. The reduced proteins were carboxymethylated by adding one volume of a 1.1 M iodoacetic acid - 8 M urea solution in 2.5 N NaOH to nine volumes of protein solution. The reaction was allowed to proceed for one hour in the dark, and the solution was then dialysed against 0.01 M Tris-HCl, pH 8.0 for at least 6 hours at room temperature also in the dark.

Sodium dodecylsulfate (SDS) polyacrylamide gel electrophoresis. Two SDS systems were employed. The first one was based on that of Weber and Osborn (71). Reduced or carboxymethylated protein samples were run on 6 cm gels (7.5% (w/v) acrylamide, 0.13% (w/v) bisacrylamide, 0.075% (w/v) ammonium persulfate, 0.075% (v/v) TEMED in 0.1 M sodium phosphate buffer, pH 7.0) at 40 Volt for about 6 hours. The second system was similar to the discontinuous SDS system as has been described by Luduena and Woodward (51). Carboxymethylated protein samples were prepared for electrophoresis as described and run on 9 cm gels at 4 mA per gel for approximately 2.5 hours.

Gels were stained for 15 hours in 0.015% Coomassie Brilliant Blue in methanol/acetic acid/water (45/9/46). Destaining was performed by diffusion in methanol/acetic acid/water (2/3/35).

Protein determination. Protein was determined according to the method of Lowry et al. (50) as modified by Hartree (40) with bovine serum albumin as a standard.

Chemicals. [2-^{14}C]Methyl benzimidazol-2-yl carbamate (spec. act. 11.4 mCi/mmole) was purchased from International Chemical & Nuclear Corporation (Irvine, Cal., USA). Stock solutions were prepared in methanol at approximately 1200 μM [^{14}C]MBC. Radiochemical purity of the preparation was checked at intervals by thin-layer silica gel chromatography on DC-Alufolie 60 F 254 (Merck, Darmstadt, GDR) with ethylacetate saturated with 0.05 M potassium phosphate buffer, pH 6.8, as solvent. Chromatograms were scanned with a Nuclear Chicago Actigraph III radiochromatography system and then cut transversely into 1 cm sections, which were placed in scintillation vials containing Bruno and Christian's scintillation liquid and counted. Purity was always found higher than 97%.

[*Ring C-methoxyl*-^{3}H]colchicine (spec. act. 3.8 Ci/mmole) as a solution

in benzene/ethanol (9/1) was obtained from New England Nuclear (Boston, Mass., USA). The preparation diluted to a specific activity of 20-200 mCi/mmole was either used as such or after being purified according to the procedure described below.

Methyl 5-(2-thienyl carbonyl)-benzimidazol-2-yl carbamate (oncodazole or R 17934) was a gift of Dr. M. De Brabander (Janssen Pharmaceutica, Beerse, Belgium). Colchicine was purchased from Merck (Darmstadt, GDR). Griseofulvin and vinblastine sulfate were obtained from Sigma (St. Louis, Mo., USA). Melatonin and podophyllotoxin were purchased from Fluka (Buchs, Switzerland) and Aldrich Chemical Co. (Milwaukee, Wisc., USA), respectively.

Purification of [^{3}H]colchicine. An aliquot of the [^{3}H]colchicine solution in benzene/ethanol was diluted with unlabelled colchicine in the same solution, giving a concentration of 10^{-4} M colchicine at approximately 10^6 dpm per 100 µl solution. Two hundred µl of this solution were applied as a small band to a silica gel plate (DC-Alufolie 60 F 254, Merck, Darmstadt, GDR) and chromatographed in chloroform/aceton/diethylamine (5/4/1) over a distance of 17 cm with unlabelled colchicine as a reference. The centre part of the [^{3}H] colchicine band (visible under UV at 254 nm) with Rf-value 0.57 and a 1-cm-broad band corresponding to the Rf-value of an unknown radiolabelled compound X, which was found to be present in our colchicine preparation, with Rf-value 0.46 were cut out and transferred to a centrifuge tube. The silica gel was scraped off and subsequently eluted 4 times with 1 ml methanol. To avoid photodecomposition of the compounds, exposure of samples to light was kept to a minimum during the procedure. Tubes and chromatography tank were wrapped in aluminum foil. Approximately 1.3 x 10^6 dpm were recovered in [^{3}H]colchicine and 1.4 x 10^5 dpm in compound X. To analyze the purified preparations aliquots were spotted onto a chromatogram and developed with the same solvent as used before. Chromatograms were cut transversely into 1 cm sections which were placed in scintillation vials containing Bruno and Christian's scintillation liquid and counted. Radioactivity present in sections corresponding to the Rf-values of colchicine and compound X was 86 and 84%, respectively of the total radioactivity recovered. In the case of [^{3}H]colchicine 11% of the radioactivity was present in a third compound with Rf-value 0.83. This compound is probably identical to that found by Borisy and Taylor (11), which was shown to be formed during storage of [^{3}H]colchicine preparations purified by silica gel chromatography.

Evaluation of charcoal assay. The gel filtration method for measuring MBC-binding activity in mycelial extracts, as used in previous work (22,23) gives reproducible results. However the assay is laborious and not suitable for multiple determinations. Therefore, in the present investigation a second method was used in addition to the Sephadex G-100 assay. The usefulness of this method, which is based on separation of free MBC from bound MBC by adsorption to charcoal, will be discussed first.

The effectiveness of charcoal in adsorbing free MBC was determined in solutions of BSA at 40 mg/ml in PKMg buffer. More than 99.5% of the total radioactivity present in the incubation mixture could be removed at [^{14}C]MBC concentrations, ranging from 1-40 µM, which correspond with 2.5-100 x 10^4 dpm/ml. Removal of free MBC, however, may not involve removal of bound MBC which is present in mycelial extracts incubated with MBC. Therefore the charcoal assay was compared with the gel filtration method. As is evident from Table I even higher values for bound MBC were found with the charcoal assay than with the gel filtration method. Since incubation of mycelial extracts with [^{14}C]MBC in the presence of a specific inhibitor of MBC binding (see below) resulted in complete adsorption of radioactivity to charcoal, the higher degree of binding found with the charcoal assay, is not due to incomplete adsorption of free MBC to charcoal in mycelial extracts. Hence, less dissociation of MBC from the complex during the charcoal assay than during the gel filtration procedure is probably responsible for the higher degree of binding found.

Due to dissociation the value of bound MBC decreases with the length of the incubation period with charcoal (Fig. 2). In the standard procedure mixtures were shaken for 10 min with charcoal, so that disturbance of

Table I Comparison of charcoal and Sephadex G-100 assays for MBC binding

Assay	bound dpm/ml extract	pmole bound MBC/mg protein
charcoal	31.4×10^3	30
Sephadex G-100	22.3×10^3	22

Freshly prepared mycelial extract of A. nidulans Strain 186 at 41 mg protein per ml was incubated with [^{14}C]MBC at 5.7 µM (14.2×10^4 dpm/ml) for 2 h at 4°C. Binding assays were carried out as described under Materials and Methods.

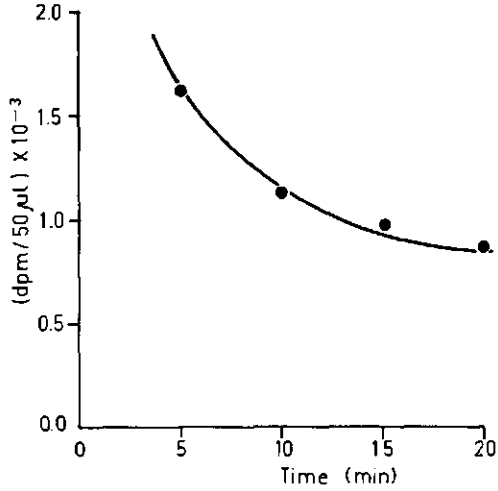


Fig. 2. Effect of incubation time during the charcoal assay on the quantity of bound MBC.
A mycelial extract of A. nidulans, Strain 003 at 38 mg protein per ml was incubated with [^{14}C]MBC at 21.2 µM (53.0×10^4 dpm/ml) for 2 h at 4°C. Binding was determined with the charcoal assay, in which the length of the incubation period was varied.

equilibrium was minimum and removal of free MBC was maximum.

Selectivity of MBC binding. MBC binding was found in 48,000 x g supernatant extracts of mycelial homogenates in PKMg solution after the extracts were incubated with [^{14}C]MBC at 4°C (22,23). Binding activity appeared to be correlate with the in vivo MBC sensitivity of the species or strain examined. Table II gives detailed binding data obtained with the Sephadex G-100 assay for various MBC-sensitive and MBC-resistant fungal species or strains. Whereas extracts of sensitive strains did bind MBC in quantities between 3 and 18% of the quantity added, resistant strains did not bind more than 0.6% of this amount which is hardly above background.

Since it has been suggested (22,23) that the MBC-binding substance is identical with fungal tubulin, the MBC-binding properties of mammalian brain tubulin were investigated. As is evident from Table III porcine brain tubulin did not bind MBC in significant amounts at 4°C nor at 37°C. As expected the preparation showed [^{3}H]colchicine binding activity which implies the presence of native tubulin in the preparation used. Therefore, it can be concluded that

Table II. MBC binding in 48,000 x g supernatant mycelial extracts of fungal species and strains, which differ in MBC sensitivity

Species	Strain	ED_{50} * µM	protein conc. mg/ml	MBC conc. (dpm/ml)x10^{-3}	µM	bound MBC (dpm/ml)x10^{-2}	pmole/mg protein
Aspergillus nidulans	003	4.5	38	64.0	2.6	56.4	6.0
	186	1.5	42	68.2	2.7	122.0	11.5
	R	95 ‡	40	68.2	2.7	3.2	0.3
Penicillium brevicompactum	S	< 2 ‡	22	67.0	2.7	41.4	7.6
	R	> 2000 ‡	20	56.0	2.2	2.2	0.4
Penicillium corymbiferum	S	< 2 ‡	24	66.0	2.6	20.8	3.4
	R	> 2000 ‡	38	56.8	2.3	1.4	0.1
Alternaria brassicae		> 1000 §	37	64.0	2.6	3.7	0.4
Pythium irregulare		> 1000 §	-"	65.0	2.6	2.5	-"

Mycelial extracts were incubated for 2 hours at 4°C with [^{14}C]MBC. Binding was determined by gel filtration of 1 ml of the incubation mixture through a Sephadex G-100 column.

* Inhibitory concentration causing a 50% reduction in growth on agar medium.

‡ Data from Bollen (28) for benomyl.

§ Data from Bollen and Fuchs (21) for benomyl.

" Not determined.

Table III. MBC-binding and colchicine-binding activity of partially purified porcine brain tubulin

Exp.	Binding agent	Temp.	Concentration dpm/ml	µM	Binding activity dpm/ml	pmole/mg protein
1	[^{14}C]MBC	4°C	15.3x10^4	6.1	2.1x10^2	0.3
2	[^{14}C]MBC	37°C	19.5x10^4	7.8	4.1x10^2	0.6
3	[^{3}H]colchicine	37°C	1.2x10^6	3.0	48.6x10^4	45.0

A porcine brain extract purified by ammonium sulfate fractionation (35-50%) at 27 mg protein per ml was incubated with [^{14}C]MBC and [^{3}H]colchicine, respectively, for 2 h at the temperatures indicated. The tubulin preparation had been stored for 0 (exp. 1), 11 (exp. 2) and 13 days (exp. 3) at -21°C, respectively.

mammalian tubulin has no or at least a low affinity for MBC. These findings are in agreement with the observation that MBC at a concentration which is lethal for MBC-sensitive fungi does not interact with in vitro microtubule assembly in brain extracts (23,27,44).

For further work the three Aspergillus nidulans Strains 003, 186 and R were selected, merely because of the fact that their genetic background is known (69,70).

Molecular weight of the MBC complex. To determine the molecular weight of the MBC complex a partially purified mycelial extract, which had been incubated with [^{14}C]MBC, was gel filtrated through a calibrated Spehadex G-200 column. Bound radioactivity was eluted in a single peak and as can be seen in Fig. 3 the elution volume corresponds with a molecular weight of the MBC complex of approximately 110,000. Since this value is very similar to that found for the colchicine-tubulin complex (17), the elution behaviour of both complexes was compared on a Sephadex G-200 column (1.5 x 27 cm). The elution volume of the MBC complex appeared to be similar to that of the colchicine-tubulin complex.

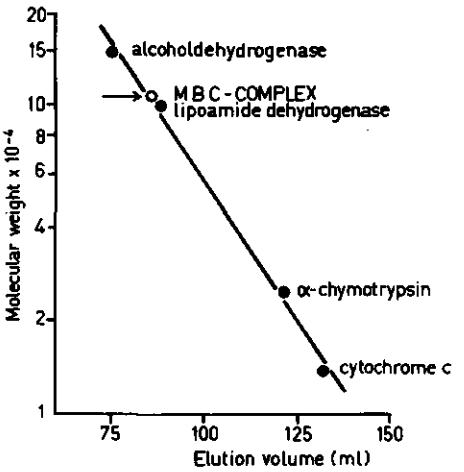


Fig. 3. Molecular weight determination of the MBC-protein complex. 5 ml of an ammoniumsulfate fractionated (35-50%) mycelial extract (40,000 x g) at 38 mg protein per ml was incubated with [^{14}C]MBC at 8 µM for 2 h at 4 °C and gel filtrated through a calibrated Sephadex G-200 column. The labelled complex was eluted as a single peak at an elution volume corresponding to a molecular weight of approximately 110,000.

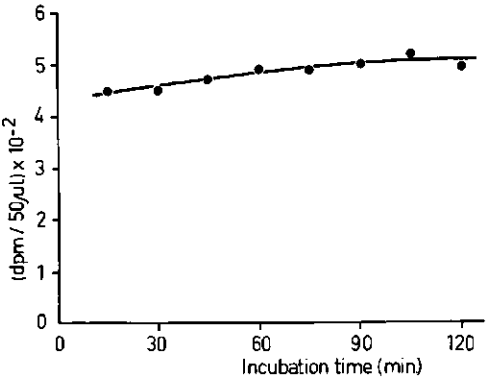


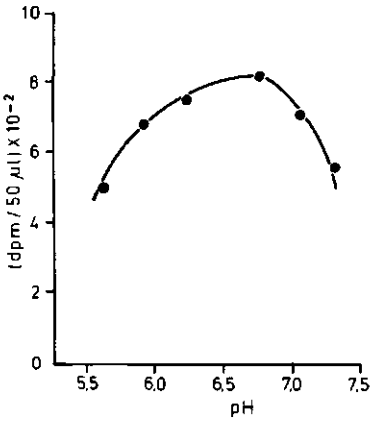
Fig. 4. Time course of MBC binding.
A mycelial extract of A. nidulans, Strain 003, at 38 mg protein per ml was incubated with [^{14}C]MBC at 1.1 µM (2.7×10^4 dpm/ml) for varying periods of time at 4°C. The quantity of bound MBC was determined with the charcoal assay.

Factors which influence MBC binding. Binding of MBC to its receptor was nearly complete within 15 minutes of incubation of the mycelial extract with [^{14}C]MBC (Fig. 4). Only a slight increase in bound radioactivity was found thereafter. Therefore an incubation period of 1 hour or more with [^{14}C]MBC, as routinely used in our experiments, can be considered to be long enough for maximum binding.

MBC-binding activity was influenced by pH as is illustrated in Fig. 5. Maximum binding appeared between pH 6.5-6.8. Whether pH affected binding of MBC indirectly through an effect on stability of the receptor or directly has not been investigated.

MBC-binding activity of mycelial extracts was not stable. At 4°C the ability to bind MBC decayed according to first order kinetics (Fig. 6). The half-time for inactivation was 6.5 hours. Sucrose, MBC and glycerol stabilized the binding activity. Known stabilizers of colchicine-binding activity of tubulin like vinblastine sulfate and GTP did not significantly increase the stability.

The influence of temperature on MBC-binding activity was also examined. When 48,000 x g supernatant mycelial extracts were incubated for 1 h at 37°C a considerable denaturation of proteins took place. Therefore, these extracts were cooled in ice for about one hour and centrifuged before binding activity was measured. Since for unknown reasons the charcoal assay applied to heated



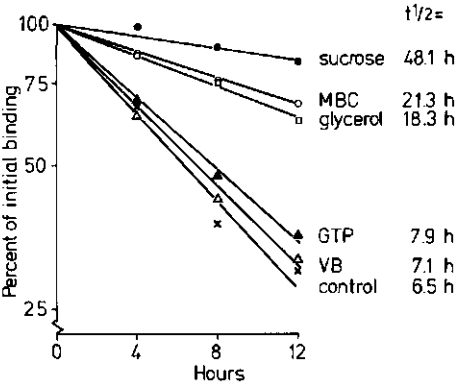


Fig. 5.(left) Effect of pH on MBC binding.
Two ml samples of a 127,000 x g (1 h) supernatant mycelial extract of A. nidulans, Strain 186, at 24 mg protein per ml, were adjusted to different pH-values with 50 µl of a H_3PO_4 or KOH solution, respectively, of different strenght. One ml of the resulting preparation was incubated with [^{14}C] MBC at 11 µM (27.5×10^4 dpm/ml) for 1 h at 4°C. The quantity of bound MBC was determined with the charcoal assay. The pH (22°C) was determined in the other ml of the preparation.

Fig. 6. (right). Stability of the MBC-binding activity under various conditions at 4°C. Three ml of a mycelial extract of A. nidulans, Strain 186, at 24 mg protein per ml were mixed with an equal volume of a solution of respectively, 1 mM vinblastine sulfate (VB), 2 mM GTP, 8 M glycerol, 22.8 µM [^{14}C] MBC (56.8×10^4 dpm/ml), and 2 M sucrose in PKMg solution. As control preparation 3 ml extract was diluted with 3 ml PKMg solution. At the times indicated [^{14}C] MBC was added (28.4×10^4 dpm/ml) to aliquots of the mixtures except to that which already contained [^{14}C] MBC. After incubation for 15 min at 4°C the quantity of bound MBC was determined with the charcoal assay using appropriate blanks. Binding activity was expressed as percent of initial binding directly after mixing the solutions (510 dpm/50 µl of 1 : 1 diluted extract). The half-times of decay ($t_{\frac{1}{2}}$) of the MBC-binding activity have been calculated from the slopes of the lines.

extracts gave anomalous binding data, binding activity was determined with the gel filtration method. Binding activity of the supernatant was 11% of that of control samples which were kept at 4°C during incubation and centrifuging of the experimental sample. Binding activity in the control sample was determined with the charcoal assay. This result indicates that loss of binding activity is enhanced by high temperature.

Incubating mycelial extracts with 0.1% trypsin for one hour at 4°C before incubation with [^{14}C] MBC resulted in a 55% loss of binding capacity.

Centrifuging at 127,000 x g for 1 hour at 4°C of the 48,000 x g supernatant lowered the binding activity of the resulting supernatant to 70-80% of that of control samples which were not centrifuged and were stored at 4°C for the duration of the run. This result suggests that the MBC-binding activity might be partially associated with some particulate fraction. The nature of this binding activity has not been studied further.

Affinity for MBC in extracts of Aspergillus nidulans Strain 003, Strain 186 and Strain R. Since it has been suggested (22,23) that differences in affinity of MBC binding sites for MBC might be the biochemical basis of the selectivity of MBC, we determined apparent binding constants (K) and number of binding sites (S_o) in mycelial extracts of the three A. nidulans strains. Total MBC binding was measured as a function of free MBC concentration and the data were plotted in double reciprocal form, according to the equation:

$$1/[\text{bound MBC}] = 1/S_o + 1/K.S_o \cdot 1/[\text{free MBC}] \qquad (11)$$

The concentration of bound MBC has been expressed as bound dpm per volume of mycelial extract and the concentration of free MBC in µM. Results of a number of experiments are summarized in Fig. 7. From the slopes of the lines

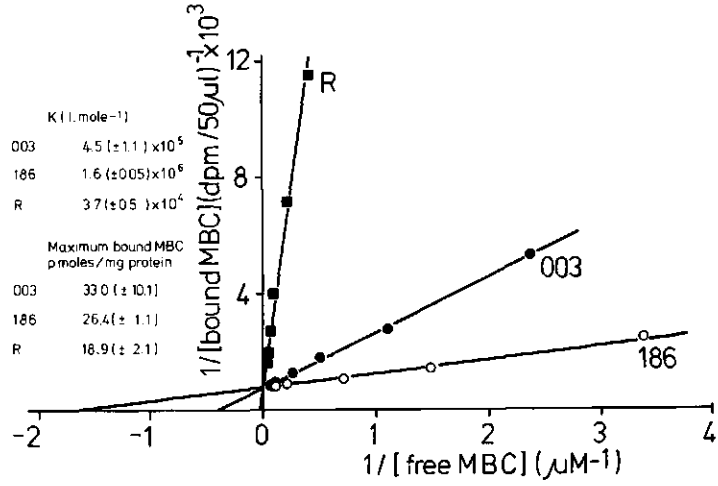


Fig. 7. Binding of [^{14}C] MBC to mycelial extracts of A. nidulans, Strain 003, Strain 186 and Strain R.
Mycelial extracts of Strain 003, Strain 186, and Strain R at 38, 39 and 40 mg protein per ml, respectively, were incubated with [^{14}C] MBC at increasing concentrations for 1 h at 4°C. The quantity of bound MBC was determined with the charcoal assay. The concentration of free MBC was calculated from the differences between total and bound MBC. Values of binding constants K ($l.mole^{-1}$) and maximum binding capacity (pmoles/mg protein) with their respective standard deviations are given as determined in at least four experiments.

and the intercepts, values of S_o, expressed as maximum binding capacity in pmoles per mg protein and the apparent binding constants were calculated. Evidently the number of binding sites in extracts of the three strains are about equal, but the respective binding constants differ considerably. The reciprocals of the binding constants, which are identical with the MBC concentration which will half-maximally saturate the MBC-binding site, are 2.2, 0.6 and 27 µM for extracts of Strain 003, Strain 186 and Strain R, respectively. These values correspond with the inhibitory concentration of MBC causing a 50% reduction in growth (see Table 2). This strongly suggests that the response of the three A. nidulans strains to MBC is governed by the affinity of the receptor site for MBC.

Inhibition of MBC binding by antimitotic agents. In order to characterize the MBC-receptor site in fungi, several compounds which interact in some way with tubulin, were tested for inhibitory effects on MBC binding. A first screening was carried out by adding these compounds simultaneously with [^{14}C]MBC, at varying concentrations to mycelial extracts of A. nidulans Strain 186. Podophyllotoxin and vinblastine sulfate, which compounds are known to bind to tubulin (74,75) did not reduce MBC binding at a concentration of 500 µM. Melatonin which has been shown to interfere with colchicine binding to mammalian brain tubulin (76) and which was active in the stentor oral migration assay for antitubulin drugs (52), was also ineffective at 500 µM. Griseofulvin, which affects spindle functioning in A. nidulans (19) and selectively interferes with in vitro microtubule assembly (73), did not affect MBC binding at 1000 µM, either when added simultaneously with [^{14}C]MBC or after extracts had been incubated for 1 hour before addition of [^{14}C]MBC.

Oncodazole was included in the inhibition experiments because in fungi the action of this compound has been suggested to be similar to that of MBC (23). This assumption was based on a similar response of the three strains to the growth-inhibitory action of oncodazole. ED_{50} values for mycelial growth on agar were 0.55 µM for Strain 003, 0.23 µM for Strain 186 and 20 µM oncodazole for Strain R.

Oncodazole appeared to be a very effective inhibitor of binding in the primary test. At 50 µM, this compound inhibited MBC binding for 90%, if assayed at 6.0 µM with the gel filtration method.

Although the standard antitubulin colchicine did not inhibit mycelial growth on agar of any of the three A. nidulans strains at concentrations up to 10 mM, a slight inhibition of MBC binding in mycelial extracts was noticed at 500 µM.

The ability of the latter two compounds to inhibit MBC binding was investigated in further experiments, in which mycelial extracts were preincubated for one hour either with oncodazole or with colchicine at increasing concentrations. After this period [^{14}C]MBC was added and the mixtures were incubated for another hour. Figs. 8 and 9 show the result of this experiment. It is evident that oncodazole is a much more potent inhibitor of MBC binding than colchicine. Whereas oncodazole gave a 50% inhibition of MBC binding at a molar ratio of oncodazole to MBC of 0.18 : 1, colchicine could not even achieve this at a molar ratio of 830 : 1. Inhibition of MBC binding became apparent in extracts of Strain 186 at lower colchicine concentrations than in extracts of Strain 003.

The effect of oncodazole on the preformed MBC complex was also studied. [^{14}C]MBC was added to a mycelial extract and after 1 hour of incubation, oncodazole was added and the mixtures were incubated for another hour. In this case inhibition by oncodazole was much lower than that found when extracts were preincubated with the inhibitor (Table IV exp. 1). However, after 20 hours of incubation with both compounds, inhibition values were similar (Table IV, exp. 2). Evidently after this period equilibrium had been established, which confirms the reversible character of MBC binding.

These results suggest that oncodazole may competitively inhibit the

Table IV. Effect of oncodazole on MBC binding

Time at which compounds were added: t = 0 h	t = 1 h	% inhibition of MBC binding exp. 1 2 h after initiation of experiment	exp. 2 20 h after initiation of experiment
[^{14}C] MBC	-	0	0
oncodazole	[^{14}C] MBC	94	52
[^{14}C] MBC	oncodazole	41	54
[^{14}C] MBC + oncodazole	-	62	54

Mycelial extracts of A. nidulans Strain 186 were incubated with oncodazole at 10 µM, and [^{14}C]MBC at 2.7 µM (6.8×10^4 dpm/ml) in exp. 1 and 5.4 µM (13.6×10^4 dpm/ml) in exp. 2, respectively, in the order as indicated. Bound MBC was assayed 2 h (exp. 1) and 20 h (exp. 2) after initiation of the experiment by the charcoal method. The quantity of bound MBC in the absence of oncodazole (0% inhibition) was 1010 dpm/50 µl extract (exp. 1) and 540 dpm/50 µl extract (exp. 2), respectively.

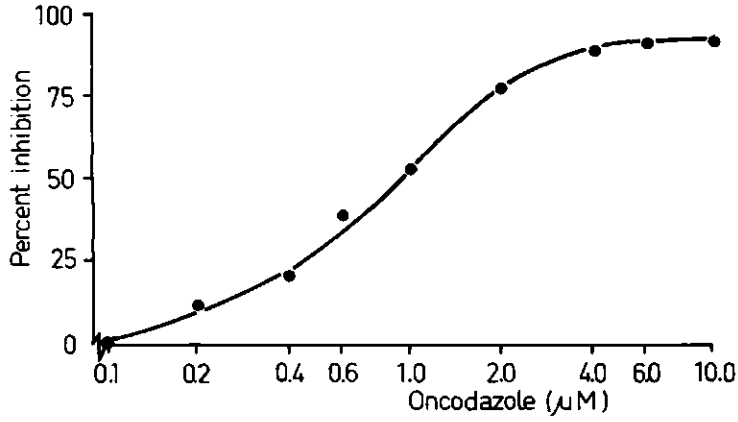


Fig. 8. Inhibition of MBC binding by oncodazole.
Aliquots of a mycelial extract of A. nidulans, Strain 186 at 30 mg protein per ml were incubated with increasing concentrations of oncodazole for 1 h and then for an additional hour with [^{14}C]MBC at 5.5 μM (13.8×10^4 dpm/ml) at 4°C. Bound MBC was assayed by the charcoal method. The quantity of bound MBC in the absence of oncodazole (0% inhibition) was 1000 dpm/50 μl extract.

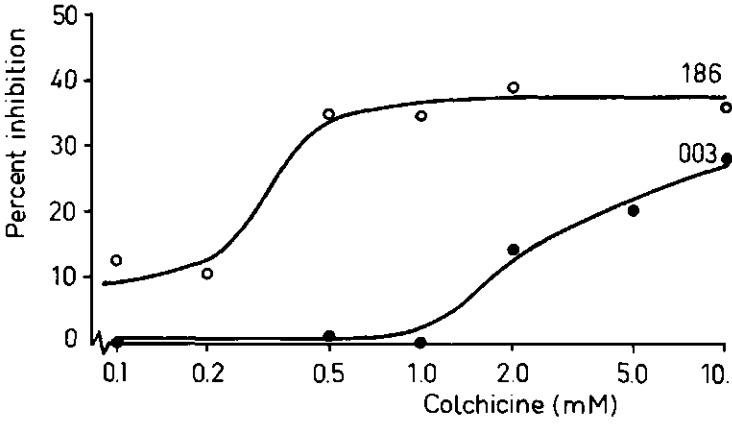


Fig. 9. Inhibition of MBC binding by colchicine.
Aliquots of mycelial extracts of A. nidulans, Strain 003 and Strain 186 at 30 mg and 29 mg protein per ml, respectively, were incubated with increasing concentrations of colchicine for 1 h at 4°C and then for an additional hour with [^{14}C]MBC at 11.6 μM (29.0×10^4 dpm/ml) at 4°C. Bound MBC was assayed by the charcoal method. The quantity of bound MBC in the absence of colchicine (0% inhibition) was 980 dpm (Strain 003) and 950 dpm (Strain 186) per 50 μl extract.

binding of MBC. Since this can only be established at equilibrium, mycelial extracts diluted 1 : 1 with a solution of 2 M sucrose in PKMg buffer to stabilize MBC-binding activity were incubated for 20 hours with [^{14}C]MBC at increasing concentrations with or without inhibitor. Data from an experiment with oncodazole as inhibitor are plotted in double reciprocal form in Fig. 10 and those with colchicine in Fig. 11. It is evident from these figures that both compounds inhibit MBC binding in a competitive manner, since only the slope of the line has been changed but not the intercept. Inhibition constants (K_i) can be calculated using the equation:

$$1/[\text{bound MBC}] = 1/S_o + 1/K.S_o \cdot (1 + [I]/K_i) \cdot 1/[\text{free MBC}]$$

where I is the concentration of free inhibitor. Since, however, the concentration of free inhibitor cannot be readily determined, the value of the total inhibitor concentration has been used to estimate the inhibiton constant. For oncodazole this estimation probably results in a value which is higher than the real value, because the concentration of binding sites is of the same order as that of the oncodazole added, resulting in a high proportion of bound oncodazole. For colchicine, which has been used at higher concentrations, the value calculated can be considered a real estimation of the inhibition constant.

The lower potency of colchicine to inhibit MBC binding in extracts of Strain 003, compared with that observed in extracts of Strain 186, suggests a differential affinity of the binding site for colchicine.

Since it has been shown that oncodazole and colchicine bind to mammalian tubulin at the same site (45), competitive inhibition of MBC binding in mycelial extracts by these two compounds indicates that the MBC-binding site is located on fungal tubulin.

Colchicine-binding activity of mycelial extracts. Competitive inhibition of MBC binding to fungal tubulin implies binding of colchicine to fungal tubulin. Since reports on colchicine binding in fungi are rather scarce and somewhat controversial (18,38,43,46,54), the colchicine-binding activity of mycelial extracts of A. nidulans was investigated.

Preliminary experiments in which mycelial extracts were incubated with [^{3}H]colchicine at 10 µM, showed that up to 6% of total radioactivity was bound to macromolecules (Table V, exp. 1). Since this amount was unexpectedly high, the nature of this bound radioactivity was investigated. Thinlayer chromatographic (TLC) analysis, with chloroform/aceton/diethylamine (5/4/1) as solvent,

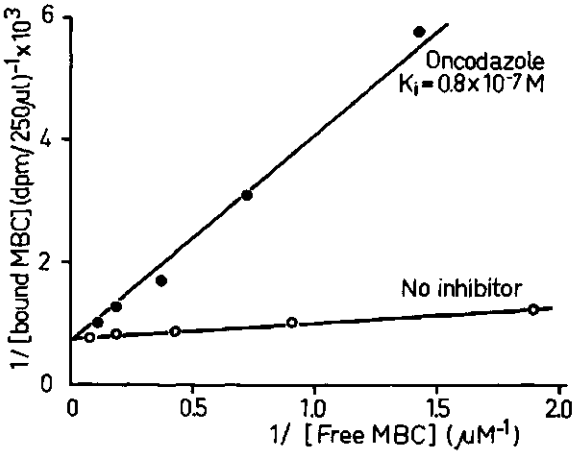


Fig. 10. Inhibition of MBC binding by oncodazole.
A mycelial extract of A. nidulans, Strain 186 at 33 mg protein per ml was diluted 1 : 1 with a solution of 2 M sucrose in PKMg buffer. Aliquots of the solution were incubated with increasing concentrations of [^{14}C]MBC with (●-●) and without (o-o) 1 µM oncodazole for 20 h at 4°C. Bound MBC was assayed by the charcoal method. The concentration of free MBC was calculated from the difference between total and bound MBC.

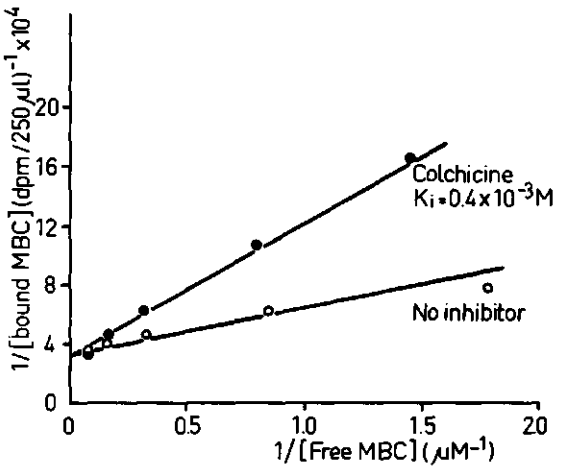


Fig. 11. Inhibition of MBC binding by colchicine.
A mycelial extract of A. nidulans, Strain 186 at 31 mg protein per ml was diluted 1 : 1 with a solution of 2 M sucrose in PKMg buffer. Aliquots of the solution were incubated with increasing concentrations of [^{14}C]MBC with (●-●) and without (o-o) 1 mM colchicine for 20 h at 4°C. Bound MBC was assayed by the charcoal method. The concentration of free MBC was calculated from the difference between total and bound MBC.

Table V. Binding of [^{3}H] colchicine in mycelial extracts

Exp.	Binding agent	Concentration µM	Concentration dpm/ml	Bound radioactivity ‡‡ dpm/ml	% bound
1	[^{3}H] colchicine from stock	10	3.7×10^6	2.1×10^5	5.7
2	[^{3}H] colchicine purified*	?	1.2×10^6	1.3×10^4	1.1
3	[^{3}H] X purified*	?	1.3×10^5	6.7×10^4	52.0

* [^{3}H] colchicine and [^{3}H] X were purified according to the method described under Materials and Methods.

‡‡ Bound radioactivity was determined with the Sephadex G-100 assay after mycelial extracts of A. nidulans Strain 003 at 41 mg/ml (exp. 1) or Strain R at 42 mg protein per ml (exp. 2, 3) had been incubated with the radiochemicals for 2 h at 4° C.

of Sephadex G-100 column fractions which contained bound radioactivity revealed that the radiolabel was present in a Compound X, which was not identical with colchicine. Furthermore, in contrast with known colchicine-tubulin complexes, the complex was perchloric acid (PCA) stable, since after adding PCA at a final concentration of 0.5 N to the fractions mentioned above and centrifuging the mixture about 70% of the radioactivity remained associated with the pellet. Washing the pellet with ethanol/ether (3/1), removed most of the radiochemical, which with TLC analysis was identified as Compound X. Since it was presumed that this radiochemical was present as an impurity in our [^{3}H]-colchicine preparation, the latter was purified (for details see Materials and Methods) and binding experiments were performed with the purified compounds. Results of these experiments are given in Tabel V, exp. 2 and 3. As is evident about 50% of the radioactivity present in Compound X was bound to components of the mycelial extract. With purified [^{3}H]colchicine less radioactivity was bound than with the nonpurified preparation. These results indicate that binding activity of mycelial extracts upon incubation with unpurified [^{3}H]-colchicine is mainly due to association of the radiochemical impurity X with macromolecular components.

Since purification of [^{3}H] colchicine preparations is laborious and 100% purity can never be achieved, in further experiments a more specific binding

assay with DEAE-Sephadex A-50 ion exchanger has been used. Mycelial extracts were incubated with [^{3}H] colchicine at 500 µM (approximately 19.6 x 10^6 dpm/ml) for 1 hour at 4°C and run onto DEAE-Sephadex columns. The elution pattern of radioactivity, which is shown in Fig. 12a shows two high peaks and one very small peak. TLC analysis revealed that radioactivity in fraction 4, which contained the majority of the protein which was not adsorbed to the ion exchanger, was present in the form of Compound X, whereas radioactivity eluted at the two other peaks represented colchicine. The high peak is due to free colchicine since it is eluted at the bed volume of the column. The second [^{3}H]colchicine peak was eluted at approximately 0.52 M NaCl. Since colchicine-tubulin complexes are characteristically eluted at this ionic strength (see also next sections) this result suggests the formation of a colchicine-fungal tubulin complex in the incubation mixture. That indeed colchicine was eluted as bound to protein and not in a free form was determined by gel filtration of an aliquot of fraction 27 on a Sephadex G-25 column (1.5 x 20 cm), immediately upon elution of this fraction. About 37% of the radioactivity recovered in the eluate, eluted associated with protein in the void volume of

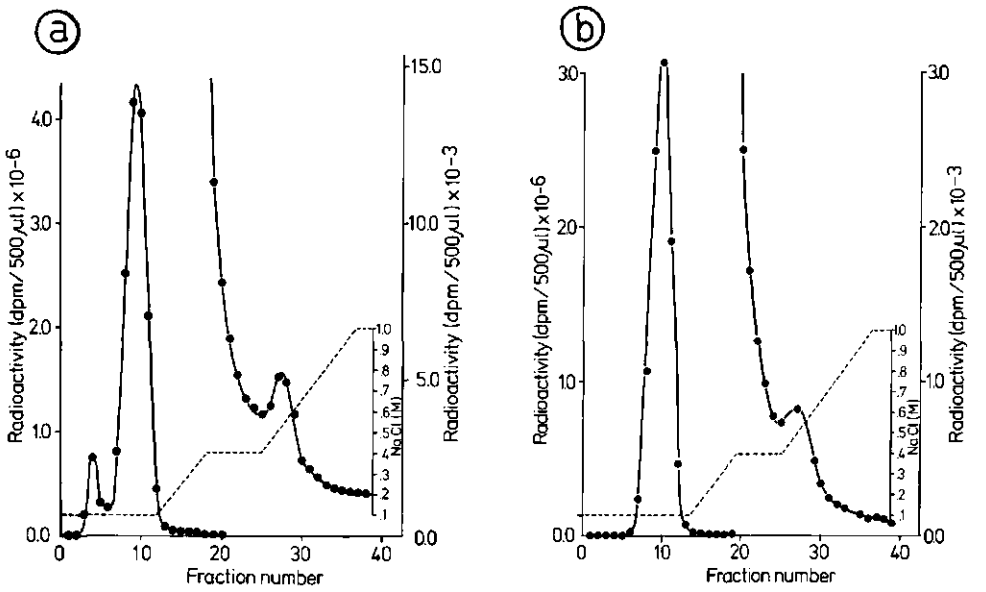


Fig. 12. DEAE-Sephadex A-50 column chromatography of mycelial extracts of A. nidulans, Strain 186 incubated with [^{3}H] colchicine at 4°C (a) and 37°C (b) for 1 hour. 48,000 x g supernatant mycelial extracts at 32 (a) and 29 (b) mg protein per ml were incubated with 500 µM [^{3}H] colchicine (19x10^6 dpm/ml (a) and 12.2x10^6 dpm/ml (b)). 4 ml of the incubation mixture (a) or supernatant of the centrifuged incubation mixture (b) were loaded onto DEAE-Sephadex columns and eluted as described under Materials and Methods. The graphs show the elution pattern of radioactivity. ---- NACl concentration.

the column.

When mycelial extracts were incubated at 37°C for 1 h with [^{3}H]colchicine and after centrifuging bound colchicine was assayed with the DEAE-Sephadex method, the quantity of bound colchicine seemed to be lower than that after incubation at 4°C (Fig. 12b). Since the binding site for colchicine is evidently identical with that of MBC, which appeared to be denatured under these conditions, this result would be expected. It should be further noticed that no significant amount of radioactivity was eluted associated with the bulk of the protein. This suggests that Compound X either did not bind at this temperature or was associated with components, which were removed from the extract by centrifuging. These possibilities were not studied further.

Since the DEAE-Sephadex binding assay is not suitable for quantitative measurements due to dissociation of the colchicine-tubulin complex during the procedure, no attempts were made to determine in this way the binding constant for colchicine and fungal tubulin.

Purification of fungal tubulin. As has been shown in the preceeding sections, the MBC-binding protein can be assumed to be identical with fungal tubulin. This makes MBC a suitable tool for the purification of fungal tubulin, analoguous to the use of colchicine in purification methods for mammalian tubulin. With a standard purification procedure for tubulin the following results were obtained. MBC-binding activity in 48,000 x g supernatant mycelial extracts could be selectively precipitated with ammonium sulfate between 35 and 50% saturation (Table VI). High speed centrifuging and ammonium sulfate fractionation together resulted in a preparation which contained approximately 50% of the initial binding activity. The specific activity of this preparation was increased about two-fold.

In previous work (22,23) it has been shown that the MBC complex was retained on a DEAE-Sephadex A-50 anion exchanger and could be selectively eluted at a ionic strength of 0.6 M KCl. Therefore, DEAE-Sephadex column chromatography which has proven to be useful in purification of tubulin from other organisms (cf. 74), was included as a final purification step for fungal tubulin. This step was first standardized with bound [^{14}C]MBC. A partially purified preparation was incubated with [^{14}C]MBC and applied to the column. Part of the elution patterns of radioactivity obtained with preparations of Strain 003, Strain 186, and Strain R are shown in Fig. 13. For Strain 186, part of the radioactivity was eluted as a distinct peak at approximately 0.52 M NaCl, whereas with Strain 003 only a small shoulder was observed in the elution

Table VI. Purification of MBC-binding protein from mycelial extract

Ammonium sulfate fractionation range	Total protein (mg)	% of total binding activity recovered	Specific binding activity (pmole/mg protein)
0 - 20	5	0.4	3.7
20 - 35	72	12.0	7.7
35 - 50	92	74.1	37.5
> 50	173	13.6	3.6

Mycelial extract (11.5 ml) of A. nidulans, Strain 003 at 29 mg protein per ml was fractionated with a saturated ammonium sulfate solution in water between limits indicated. The preparations were incubated with [^{14}C]MBC at 10.9 µM and bound MBC was assayed by the charcoal method.

Table VII. Gel filtration of DEAE purified MBC-protein complex on a Sephadex G-100 column

Strain	003	186
Radioactivity of DEAE-purified [^{14}C]MBC-protein complex (dpm/ml)	3,140	15,220
Recovery of bound [^{14}C]MBC after gel filtration on Sephadex G-100 (dpm/ml)	701	8,410
% recovery of bound [^{14}C]MBC	22	55

48,000 x g supernatant mycelial extracts of A. nidulans Strain 003 at 38 mg protein per ml and of Strain 186 at 37 mg protein per ml in PKMg buffer were incubated with [^{14}C]MBC at 7.8 µM (19.4×10^4 dpm/ml) and 8.1 µM (20.2×10^4 dpm/ml), respectively, for 2 h at 4°C. 10 ml of the incubation mixture was run onto a DEAE-Sephadex A-50 column equilibrated with PKMg buffer, as described under Materials and Methods, but using potassium buffers instead of sodium buffers. Radioactivity of the fractions was determined immediately after they had been eluted. 1 ml of the fraction containing the peak of bound MBC was filtered immediately through a Sephadex G-100 column.

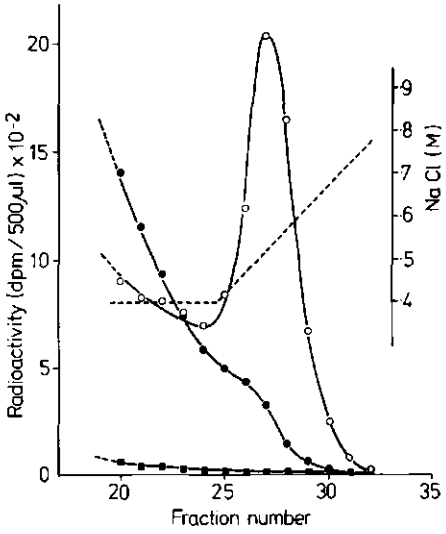


Fig. 13. DEAE-Sephadex A-50 column chromatography of partially purified mycelial extracts of A. nidulans, Strain 003, Strain 186 and Strain R. 127,000 x g supernatant mycelial extracts were fractionated with a saturated ammonium sulfate solution (see Materials and Methods). 5 ml of the preparations containing 15 mg (Strain 003), 12 mg (Strain 186) and 19 mg (Strain R) protein per ml were incubated with [^{14}C]MBC at 5.1 µM (12.8×10^4 dpm/ml) and run onto DEAE-Sephadex columns as described under Materials and Methods. Graphs show part of the elution pattern of radioactivity. ●-●, Strain 003; o-o, Strain 186; ■-■, Strain R. --- NaCl concentration.

pattern at this salt concentration. As expected with Strain R no significant amounts of radioactivity were eluted at NaCl concentrations of 0.4 M and higher.

Whether elution of radioactivity at 0.52 M NaCl was caused by elution of the MBC-protein complex itself or by dissociation of MBC from the DEAE-bound complex, due to increasing salt concentration, was determined by rechromatography of the fractions concerned on Sephadex G-100 columns. It appeared (Table VII) that depending on the strain used considerable amounts of radioactivity were bound to protein, which indicates the elution of purified MBC-protein complex from the column.

In subsequent experiments no [^{14}C]MBC was present, and fractions eluted at 0.45-0.90 M NaCl were considered to contain the purified binding protein. The MBC-binding activity of DEAE-purified protein has not yet been investigated in detail. In a preliminary experiment, in which a crude mycelial extract fractionated with ammonium sulfate was passed through the DEAE-Sephadex column and the resulting purified protein solution was concentrated with a Sartorius

ultrafiltration device, approximately 5% of the initial binding capacity of the crude extract was recovered. The specific activity of this preparation was raised to about 13-fold of that of the crude extract. This rather low yield indicates a considerable loss of binding activity during the procedure, which was probably due to the instability of the binding protein.

Proteins isolated from the three strains of A. nidulans by DEAE-Sephadex column chromatography were analysed with the continuous SDS system. Gels loaded with reduced protein samples of fraction 24-30 (Fig. 13) from the experiment with Strain 186 and Strain R are shown in Fig. 14 and 15, respectively. As reference, reduced porcine brain tubulin purified by one polymerization cycle was run on a separate gel. On gels loaded with proteins from Strain 186 three closely-spaced bands, among others, were present which have a comparable mobility as the porcine brain monomers which move together in this system. The banding patterns of gels loaded with reduced proteins from corresponding fractions derived from Strain 003 were very similar (not shown). In gels loaded with proteins from strain R, however, only one band was present at a migration distance which is comparable with that of brain tubulin.

The difference was more pronounced when proteins were reduced and carboxymethylated before analysis with the same system. Gels loaded with DEAE-purified proteins, eluted at 0.45-0.90 M NaCl, from Strain 003, Strain 186 and Strain R are shown in Fig. 16a. Carboxymethylated DEAE-purified porcine brain tubulin was run as reference protein. It is evident that the preparation of Strain R differed from those of Strain 003 and Strain 186. In gels loaded with proteins from the latter two strains, two distinct bands are present with a comparable mobility as the band representing porcine brain tubulin and one band which had moved slightly faster. With Strain R two weak bands are present at this migration distance whereas other bands showed about equal staining as corresponding bands present in the other two gels.

When identical protein preparations of the three strains were run on discontinuous SDS gels, two major bands of unequal staining density were found with similar mobility as the bands on the reference gel (Fig. 16b). The faster-moving band on the reference gel is due to β-tubulin and the slower-moving one to α-tubulin (51).

When gels from both SDS systems are compared it is evident that two bands, which are found to be separated in the continuous system, move together in the discontinuous system. With respect to the staining densities of the bands it seems that one of the two slower-moving bands of the continuous system moves together with the faster moving band in the discontinuous system.

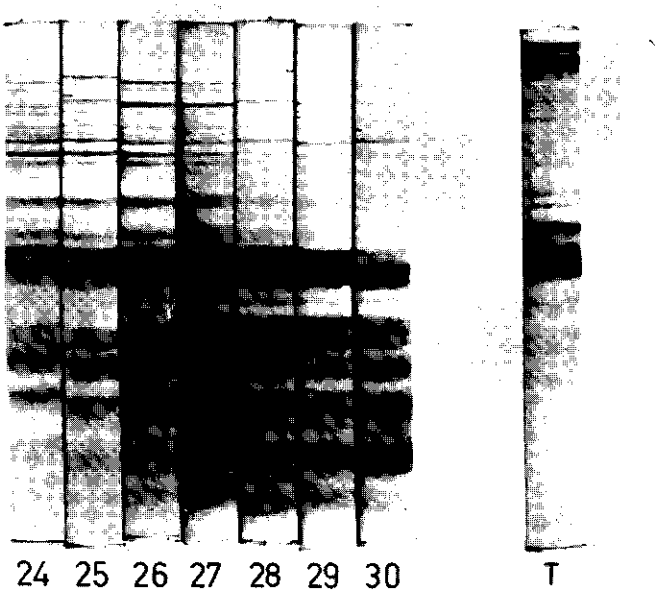


Fig. 14. Electrophoretic analysis of proteins isolated from A. nidulans, Strain 186 by DEAE-Sephadex column chromatography.
Samples of fractions 24-30 (see Fig. 13, Strain 186) were reduced and analysed on the continuous SDS system. Reduced porcine brain tubulin (T) purified by one polymerization cycle was run as a reference.

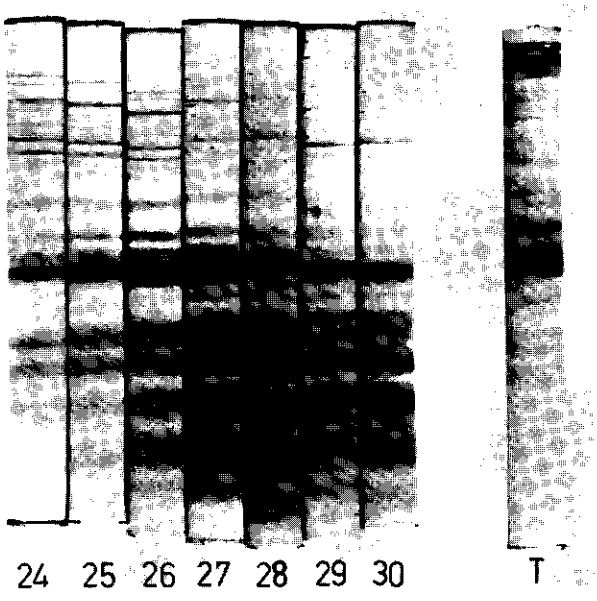


Fig. 15. Electrophoretic analysis of proteins isolated from A. nidulans, Strain R by DEAE-Sephadex column chromatography.
Samples of fractions 24-30 (see Fig. 13, Strain R) were reduced and analysed on the continuous SDS system. Reduced porcine brain tubulin (T) purified by one polymerization cycle was run as a reference.

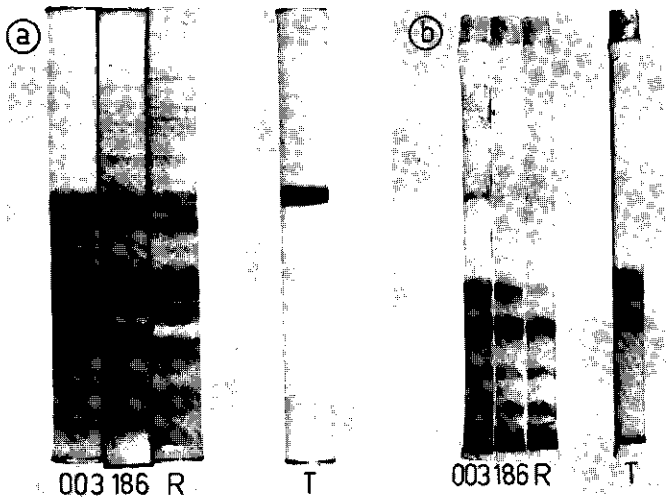


Fig. 16. Electrophoretic analysis of proteins isolated from A. nidulans, Strain 003, Strain 186 or Strain R, by DEAE-Sephadex column chromatography. Lyophilized proteins from pooled fractions eluted at 0.45-0.90 M NaCl were carboxymethylated and run in the continuous SDS system (a) and the discontinuous system (b). Carboxymethylated DEAE-purified porcine brain tubulin was run as a reference.

Both gel systems could be correlated in the following manner. It was found that heating a DEAE-purified preparation for 1 h at 37°C, cooling in ice and centrifuging, resulted in a supernatant in which upon electrophoresis in the continuous SDS system the two slower moving bands were not present (Fig. 17a,b). These bands appeared to be present in gels loaded with the pelleted material. Heat-dependent precipitation of proteins was not reversible by cold, nor was the process inhibited by Ca^{2+}, colchicine or MBC, which eliminates the possibility that microtubule assembly is responsible. Apparently denaturation by heat of the proteins concerned causes precipitation which is in agreement with the observation that mycelial extracts which are heated at 37°C and centrifuged, show rather low MBC-binding activity.

Upon electrophoresis in the discontinuous system the precipitated material runs mainly as two bands, with similar mobility as α- and β-tubulin from porcine brain (Fig. 17c). Because of this behaviour it can be concluded that these bands represent both tubulin monomers of A. nidulans. The third faster-moving band in continuous SDS gels which showed a similar mobility as β-tubulin in the discontinuous system, apparently represents a protein which copurified with fungal tubulin in this purification procedure.

It is evident that the purification procedure applied to extracts of

Strain R resulted in significantly less α- and β-tubulin, than was obtained with a similar procedure applied to extracts of Strains 003 and 186. Although a somewhat lower initial tubulin concentration might be partly responsible (see Fig. 7), it is certainly not the only factor involved. A reason for the failure to purify tubulin of Strain R might be a low affinity of this tubulin for DEAE-Sephadex. A low affinity of brain tubulin for DEAE-Sephadex has been reported (47,53) and seems to be related with phosphorylation of the protein. At any rate, the different behaviour of tubulin of Strain R in comparison with that of both other strains, suggests chemical differences of the protein itself or of factors associated with tubulin, an idea which is compatible with the observation that differences exist in affinity of the tubulins of the three strains to MBC.

The results of the electrophoretic analysis indicate that MBC might be used as an affinity label to purify fungal tubulin from MBC-sensitive strains of A. nidulans. It supports the idea that this protein is the primary target of MBC action.

DISCUSSION

The MBC-binding protein in A. nidulans is characterized by a set of properties which is unique for tubulin (cf. 14). Binding activity could be selectively fractionated with ammonium sulfate between 35 and 50% saturation. The MBC-protein complex was retained on DEAE-Sephadex columns and its molecular weight was estimated at 110,000. Binding activity was labile and could be stabilized by sucrose, glycerol and MBC itself. Binding was competitively inhibited by known antitubulins such as oncodazole and colchicine. Electrophoretic analysis of partially purified preparations of the MBC-protein complex, showed the presence of proteins with similar mobilities as mammalian tubulin monomers. On the basis of these results it can be concluded that the MBC-binding protein is identical with fungal tubulin.

MBC binding to fungal tubulin was rapid, reversible and dit not require a high temperature. As has been reported recently (45), binding of oncodazole to rat brain tubulin shows similar features. In this respect MBC and oncodazole resemble podophyllotoxin, a compound which competes with colchicine for the colchicine binding site on tubulin from different sources (75). However, podophyllotoxin did not inhibit MBC binding which suggest that fungal tubulin has no or at least a low affinity for this compound.

From the data given in Fig. 7 the number of MBC binding sites can be determined. Assuming that one molecule of MBC is bound per molecule of tubulin,

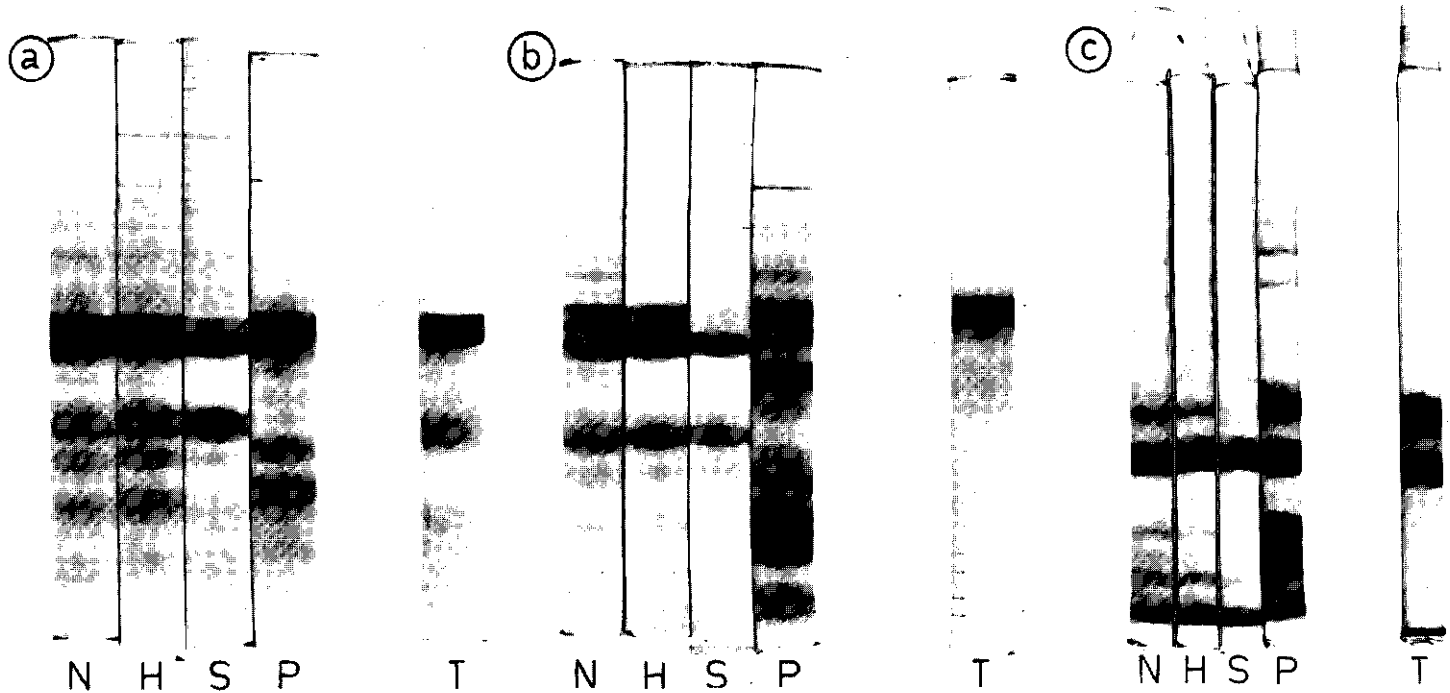


Fig. 17. Electrophoretic analysis of proteins isolated from A. nidulans, Strain 186 by DEAE-Sephadex column chromatography.
Pooled fractions eluted at 0.45-0.60 M NaCl were incubated for 1 h at 37°C, cooled in ice and centrifuged at 40,000 x g for 10 min. Aliquots of the non-heated (N), heated (H) and supernatant (S) protein solution and the pellet (P) were either reduced and run in the continuous SDS system (a) or carboxymethylated and run in both SDS systems (b,c). DEAE-purified porcine brain tubulin (T), either reduced (a) or carboxymethylated (b,c) was run as a reference.

one can calculate that tubulin is 0.2-0.4% by weight of total protein present in mycelial extracts. This value resembles the value of 0.6% found for the colcemid-binding protein in Saccharomyces cerevisiae (38) which proved to be in good correspondence with the figure of 0.36% calculated from the estimate of the number of microtubules per nucleus.

Although colchicine was evidently bound to tubulin of A. nidulans the binding reaction showed some unusual features. The rate of complex formation seemed to be rapid and binding was not temperature dependent, which properties are in contrast with those of colchicine binding to mammalian tubulin (cf. 74). Moreover, affinity of A. nidulans tubulin to colchicine was rather low compared with that of mammalian tubulin. A low affinity of, presumably, tubulin from S. cerevisiae for colchicine has been reported by Haber et al. (38). Burns (18) was not able to demonstrate colchicine binding in Schizosaccharomyces pombe and Jockusch (46) did not find colchicine-binding proteins in Physarum polycephalum. Heath (43) reported the presence of two binding components in Saprolegnia ferax with a low affinity for colchicine, one of which was trichloroacetic acid (TCA) stable. A TCA-stable binding protein was also found by Olson (54) in Allomyces moniliformis. However, the last two authors did not investigate the identity of the bound radiolabel. Since in our colchicine-binding experiments the formation of a PCA-stable labelled complex could be ascribed to the presence of an impurity in our [^{3}H]colchicine preparation, the results of these authors should be interpreted with caution.

Low affinity of A. nidulans tubulin for colchicine is probably partly responsible for the failure of this compound to inhibit mycelial growth. Cell membrane permeability might also play a role since no effect on growth was noticed at concentrations 25-times higher than those which are needed to half-maximally saturate the binding sites in vitro. In comparison with MBC and oncodazole this ratio is rather high, since these compounds gave a 50% reduction in growth already at concentrations 2-3 times higher than the estimated value of their respective dissociation constants.

To our knowledge data concerning affinity of fungal tubulin for colchicine are restricted to those already mentioned. We think that low affinity is a characteristic property of fungal tubulin. This assumption is based on the fact that fungi are commonly resistant to the antimitotic action of colchicine (cf. 42), which seems only partially caused by a low permeability of the cell membrane (38,64) and on the fact that microtubules are supposed to play an essential role in fungal nuclear division (34,35). Until now no suitable agent has been found which specifically disrupts microtubules in

fungi (42). Because of their high fungitoxicity, MBC and oncodazole are potential candidates, since from both compounds it is known, that they induce disappearance of microtubules in mammalian cells (25-27).

The differential growth response of the two mutant Strains R and 186, in comparison with that of the wild type Strain 003, is probably based on a difference in affinity of their tubulins for MBC. This idea is also supported by the fact that no differences are found in uptake or detoxication of MBC between the various strains (24).

Genetic analysis has shown that mutations to resistance and to increased sensitivity took place in the same gene (69,70). Although it can be assumed that this gene codes for tubulin, this can only be definitely concluded when differences are found between the primary structures of tubulin of the three strains. A difference in affinity for MBC might also be caused by differences in post-translational modifications of tubulin, such as phosphorylation (30,31), glycosylation (32) or association with tau-like factors (15,16,72). A different modification, rather than a single amino acid substitution might also explain the failure to purify tubulin from Strain R.

Mutation to resistance or increased sensitivity did not affect growth rate nor sporulation of the strains (ref. 22, 69, Fig. 1), which indicates normal assembly and functioning of microtubules. However, diploids carrying both mutations were not stable as was evident from increased sectoring of diploid colonies[2]. Since non-disjunction of chromosomes is thought to be mainly responsible for sectoring, increased sectoring indicates improper functioning of microtubules. This might be caused by the presence of two incompatible types of tubulins in these diploids. Increased sectoring has also been found in ordinary A. nidulans diploids when they are exposed to MBC at sublethal concentrations (37,41,49). Griseofulvin induces a similar effect (37,48). These observations are compatible with the idea that binding of MBC to tubulin interferes with the assembly of tubulin into microtubules.

The action of MBC and the mechanism of resistance was only studied in detail in A. nidulans, but the data in Table I suggest that the mechanism of resistance found here might be a general type of resistance in fungi. Since in fungi various types of mitosis are found (35) it would be interesting to know whether resistance and sensitivity to MBC is related to a certain type of mitosis.

[2]Van Tuyl, J.M., personal communication and own observation.

Until now no evidence has been presented that resistance to other antitubulins might be caused by a similar mechanism. Resistance to antitubulins has recently been discussed by Freed and Ohlsson-Wilhelm (33). A drug exclusion mechanism was found to operate in several instances. The biochemical basis of resistance which appeared to be specific to the selecting agent, has not yet been studied.

Several possibilities exist to explain the selectivity of these benzimidazole compounds in general. In addition to a differential uptake or metabolism, a different affinity of tubulin from various sources for a certain benzimidazole compound might play a role. This is illustrated by the fact that no binding was found between porcine brain tubulin and MBC, although at identical concentrations of MBC considerable binding to fungal tubulin was found. A difference in affinity between tubulins from different sources for a certain benzimidazole compound might be caused by similar factors already discussed above.

It is note worthy here that despite differences in affinity between porcine and A. nidulans tubulin to MBC, the two types are able to copolymerize in vitro (23,60). Apparently, binding sites involved in polymerization have been highly conserved during evolution in contrast with the colchicine binding site.

Undoubtedly, these benzimidazole compounds will become useful as experimental tools in the study of microtubule structure and function in cells. Their use, however, in agriculture as fungicides and, quantitatively on a minor scale, in veterinary medicine, should be reconsidered from the point of view of their mechanism of action. Interference of MBC with nuclear division in mammalian cells has been found to occur in vitro (27,59,67) and in vivo (59,67). This implies a potential genetic risk for man. The toxicology and genetic effects of benzimidazole compounds have recently been reviewed by Seiler (59). We agree with him that the use of pesticides with this type of action should be restricted.

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Metabolic conversion of methyl benzimidazol-2-yl carbamate (MBC) in Aspergillus nidulans

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ABSTRACT

Methyl benzimidazol-2-yl carbamate was metabolized by Aspergillus nidulans mycelium to two metabolites, one of which was identified as methyl-5-hydroxybenzimidazol-2-yl carbamate. This compound was further converted to a second metabolite which was not identified. Conversion rate was highest when the culture medium was depleted of nutrients. Especially in aged concentrated mycelial suspensions conversion was rapid and complete. Since strains differing in MBC sensitivity metabolized MBC at equal rates, conversion of MBC to the non-fungitoxic metabolites has no bearing on the mechanism of resistance to this compound.

INTRODUCTION

Since the introduction of the systemic fungicides methyl 1-(butylcarbamoyl) benzimidazol-2-yl carbamate (benomyl) and 1,2-bis-(3-methoxycarbonyl-2-thioureido)benzene (thiophanate-methyl) several reports have been published about their non-biological and biological conversion in plants, animals and microorganisms.

In aqueous and non-aqueous media both compounds are readily converted to methyl benzimidazol-2-yl carbamate (MBC)[1] (1-3), which is generally

[1] The following abbreviations are used: MBC, methyl benzimidazol-2-yl carbamate; 2-AB, 2-aminobenzimidazole; 5-OH-MBC, methyl 5-hydroxybenzimidazol-2-yl carbamate.

regarded as the actual fungitoxic compound. Conversion of thiophanate-methyl is pH-sensitive (4) and the conversion rate is increased by plant extracts (5,6).

In plants, MBC is the only fungitoxic compound recovered after root treatment with benomyl or thiophanate-methyl (7-10). Metabolism studies in plants with radiolabelled compounds showed that MBC was further decomposed via 2-aminobenzimidazole (2-AB) (11-13) into benzimidazole, o-aminobenzonitrile and aniline (14). β-Glycosides of MBC and 2-AB are also found.

In animals benomyl and thiophanate-methyl are metabolized via MBC to methyl 5-hydroxybenzimidazol-2-yl carbamate (5-OH-MBC) and its 4-hydroxy-analog which compounds are eliminated from the organism as glucuronide and/or sulphate conjugates (9,15-17).

Microorganisms can degrade benomyl (18,19). This process involves the formation of 2-AB as an intermediate and the liberation of $^{14}CO_2$ from [2-^{14}C] MBC, which indicates that ring cleavage occurs (20,21). In fungi, thiophanate-methyl is converted to MBC at a rate which suggests the participation of fungal metabolic activity in this process (4,22). As minor metabolites 5-OH-MBC and an unknown substance are also found.

Since 5-OH-MBC is less fungitoxic than MBC (22,23), MBC metabolism in fungi might be regarded as a detoxication mechanism. Detoxication might play a role in the resistance of fungi to this compound.

The purpose of this study was to determine the metabolic fate of MBC in Aspergillus nidulans and its possible relation to mutationally induced resistance in this fungus.

MATERIALS AND METHODS

Organism. Aspergillus nidulans biA1 AcrA1, requiring biotin, resistant to acriflavine and with wild-type sensitivity to MBC (Strain 003) (24) was used in most experiments. Strain R, resistant to MBC and Strain 186, highly sensitive to MBC (25), were used to determine whether mutant strains with a differential response to MBC also differed in rate of metabolism.

Chemicals. Methyl benzimidazol-2-yl carbamate (MBC) and methyl 5-hydroxy-benzimidazol-2-yl carbamate (5-OH-MBC) were gifts of E.I. Du Pont de Nemours and Co. (Inc.) (Wilmington, Del. USA). Benomyl was purchased from AAgrunol (Groningen, The Netherlands) as a 50% wettable powder (Benlate). 2-Amino-benzimidazole (2-AB) and benzimidazole were purchased from K & K Laboratories (Inc.) (Plainview, N.Y., USA) and Koch-Light Laboratories (Ltd.) (Colnbrook,

England), respectively. [2-^{14}C]MBC (spec. act. 11.4 mCi/mmole) was purchased from International Chemical and Nuclear Corporation (Irvine, Cal., USA).

Culture methods. Conidia of Aspergillus nidulans strains were grown on a 2% malt extract, 0.1% Bacto-Peptone, 2% glucose, 1.5% agar medium. Conidial suspensions were prepared as described previously (24).

In metabolism experiments mycelium was either grown in Czapek-Dox liquid medium (Oxoid) or glucose-nitrate medium (26). Both media were supplemented with 1 µg biotin per ml. Incubation was at 37°C on a Gallenkamp orbital shaker at 200 rpm. With Czapek-Dox medium, benomyl (50% WP) or MBC was added as a sterile aqueous suspension or solution in methanol, respectively, either simultaneously with spores or after an initial growth period. Sterility of the aqueous benomyl suspension was achieved by first dissolving the material in ethanol, after which sterile water was added. Methanol concentration in the media never exceeded 1% (v/v).

Mycelium grown for 15 hours in glucose-nitrate medium was harvested by filtration on a Büchner filter and washed three times by resuspension in fresh medium and filtration. The mycelial cake was resuspended in glucose-nitrate medium or in 0.05 M potassium phosphate buffer, pH 6.8 at a concentration of 1 g wet weight of mycelium per 25 ml medium. MBC or [^{14}C] MBC was added to the mycelial suspensions either directly or after an initial incubation period.

Dry weight determinations. Dry weight of mycelium was determined as described previously (24).

Determination of MBC. MBC concentration in culture filtrates was measured by a thin-layer chromatographic bioassay (TLC-bioassay) (27). A 50 or 100 µl aliquot of the culture filtrate was spotted on a silica gel plate (DC Alufolie Kieselgel F 254, Merck, Darmstadt, W. Germany). Chromatograms were developed either with chloroform/methanol (97/3) (Solvent A), ethyl acetate (Solvent B) or ethyl acetate, saturated with 0.05 M potassium phosphate buffer, pH 6.8 (Solvent C). After drying, chromatograms were sprayed with a spore suspension of Penicillium brevicompactum in nutrient solution (27) and incubated in a humid chamber at 25°C. After 3 days of incubation, inhibition zones were replicated on tracing-paper (Schoellershammer Hochtransparent, Nr 205 glatt; Düren W. Germany), cut out and weighed in triplicate. The values obtained are an accurate estimation of the size of the inhibition zones. Amounts of MBC present in the aliquots were calculated

with the regression equation $y = a + b \ln x$, where a and b are constants, y is the relative size of the inhibition zone (mg) and x is the amount of MBC (ng). The regression constant a and the regression coefficient b of this equation were calculated by non-linear regression analysis of the relation between size of inhibition zones and known amount of MBC, with a Diehl Algotronic calculator. The non-linear correlation coefficient and t value ($n = 14$) were mostly higher than 0.99 and 30, respectively. With Solvent C, which proved to give the best correlation, quantities of MBC in the range of 3 - 60 ng could accurately be determined. This corresponds to a lowest detection level of 0.15 µM MBC, when 100 µl aliquots are spotted.

The MBC content of the mycelium was also estimated by the TLC-bioassay. Mycelium, that had been exposed to MBC was harvested and dried. Usually 300 mg of the dry material was suspended in 3 ml 50% methanol in water and allowed to stand for 24 hours at room temperature. The mycelial residue was then spun down and aliquots of the supernatant were analysed by the TLC-bioassay.

Analysis and identification of radiolabelled metabolites. Radioactivity in culture filtrates of mycelial suspensions exposed to [^{14}C]MBC was measured by liquid scintillation counting of aliquots in a Nuclear Chicago Mark I Liquid Scintillation Spectrometer, with Bruno and Christian's (28) scintillation fluid. Counting efficiency was determined by the external standardization procedure.

^{14}C-Metabolites in culture filtrates of mycelial suspensions exposed to [^{14}C]MBC were tentatively identified with thin-layer cochromatography using MBC, 2-AB, benzimidazole and 5-OH-MBC as references. As solvents, Solvent C and ethylacetate/acetic acid/chloroform (55 / 5 / 1) (Solvent D) were used. R_f values were determined from radioscans of the chromatograms with a Nuclear Chicago Actigraph III radiochromatography system. Reference compounds were localized under UV-light.

To determine the proportion of radioactivity present as MBC, chromatograms were cut into segments, and placed in scintillation vials containing the same scintillation liquid as mentioned above. Radioactivity present in segments corresponding with the R_f value of MBC has been expressed as percentage of total radioactivity recovered.

RESULTS

Analysis of the fate of MBC in growing cultures of MBC-sensitive fungi,

such as A. nidulans implies the need of a very sensitive method of MBC estimation. As such, the TLC-bioassay is highly suitable, because it can detect MBC in nanogram quantities (29), even in complex media. Culture filtrates containing MBC at concentrations which were sublethal to A. nidulans, Strain 003, could readily be analysed as is shown in Fig. 1. In fact, in the experiment concerned, cultures were incubated with benomyl at 2 µM but it can be assumed (1) that under the experimental conditions used, this compound was completely converted to MBC. Although growth was not affected, cultures treated with benomyl showed a yellow-brown discoloration after 16 hours of incubation. Upon further incubation the cultures became more darkly coloured and after 40 hours the mycelium was nearly black, whereas the culture filtrate was light brown. Non-treated cultures did not show any discoloration.

Analysis of the culture filtrate revealed that the MBC concentration was initially constant but decreased below the detection level after 32 hours of incubation. When MBC was added to non-inoculated medium adjusted to different pH values no decrease in MBC concentration was observed. Therefore it can be concluded that fungal metabolic activity was responsible for the elimination of MBC in inoculated cultures.

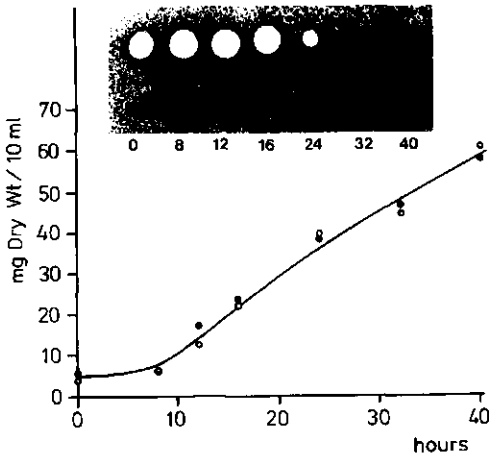

Fig. 1. Growth of A. nidulans Strain 003 in liquid Czapek-Dox medium, containing benomyl at 2 µM, ● - ●; control, o - o. 10 ml medium in 25 ml Erlenmeyer flasks were inoculated with spores at a final concentration of 5.10^7 spores per ml. Inset shows the inhibition zones caused by MBC on thin-layer chromatograms (Solvent A) of aliquots of the culture filtrate after different times of incubation.

To investigate whether the MBC elimination process had been induced by MBC an experiment was carried out in which cultures were incubated with MBC at 2 and 4 µM, respectively, for different times. MBC was added to the cultures simultaneously with inoculation or after 30 hours when growth was exponential.

Fig. 2 shows the decrease in MBC concentration under the various conditions. Comparison of curve A and C with curve B and D, respectively, indicates that MBC elimination from the culture filtrate started immediately upon addition of MBC to 30-hour-old cultures at a rate comparable with that of MBC elimination in cultures which had been continuously exposed to MBC. When cultures were initially incubated with 2 µM MBC and after 30 hours a

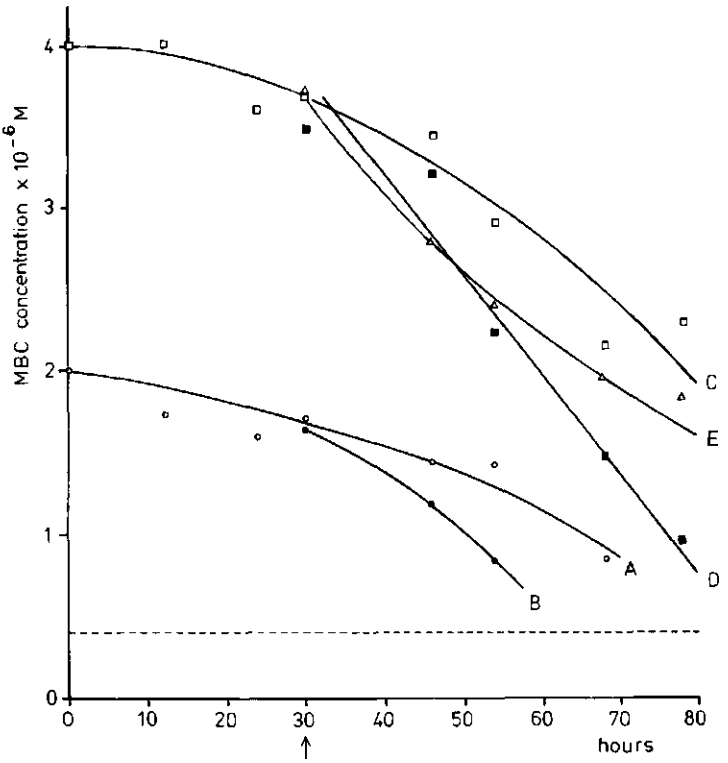

Fig. 2. Elimination of MBC from culture filtrates of A. nidulans Strain 003, growing in liquid Czapek-Dox medium. 100 ml medium in 300 ml Erlenmeyer flasks were inoculated with spores at a final concentration of 7.10^5 spores per ml. MBC was added either simultaneously with spores (2 and 4 µM, respectively) or after 30 hours of incubation. Part of the cultures to which MBC (2 µM) was added at t = 0 h received an additional amount of MBC (2 µM) at t = 30 h. MBC concentrations in culture filtrates were determined with the TLC-bioassay (Solvent B). o - o A, 2 µM at t = 0 h; ● - ● B, 2 µM at t = 30 h; □ - □ C, 4 µM at t = 0 h; ■ - ■ D, 4 µM at t = 30 h; Δ - Δ E, 2 µM at t = 0 h and 2 µM at t = 30 h. -----, minimal detectable concentration.

second portion of MBC was added, the rate of elimination (curve E) was found similar to that when MBC was added only once to the cultures growing exponentially 30 hours after inoculation (curve D).

Also in this experiment brownish discoloration of mycelium and culture filtrate was noticed. The colour was darker in cultures at higher MBC concentrations and after incubation for longer periods.

The results of this experiments suggest that MBC elimination was not induced by MBC, but was determined by certain properties of the culture.

To determine whether differences exist between A. nidulans strains in eliminating MBC from the culture medium, Strain 003 with wild-type sensitivity to MBC, and Strain R which is resistant, were compared. Results of this experiment are given in Table I. From the amounts of MBC recovered after different times of incubation, it is evident that elimination of MBC followed a similar pattern in cultures of both strains. These results indicate that here at least, elimination of MBC is not related to the mechanism of resistance to this compound.

MBC could not be detected in the mycelial fractions of the cultures, although the mycelium was processed in such a way that even the presence of 1% of the amount initially added to the cultures should have been readily

ble I. Elimination of MBC from culture filtrates of A. nidulans Strain 003 nd Strain R.

Incubation time (h)	Recovery of MBC in culture filtrates (% of the amount initially added)			
	Strain 003		Strain R	
	1 µM	2 µM	1 µM	2 µM
21	90	103	104	84
29	48	49	54	72
45	-[a]	-	-	-
69[b]	-	-	-	-

100 ml liquid Czapek-Dox medium in 300 ml Erlenmeyer flasks were inoculated with spores giving a final concentration of 10^7 spores per ml. MBC was added simultaneously with spores. Solvent B was used in the TLC-bioassay.

[a] amount of MBC below lower limit of detection.

[b] at this time MBC could not be detected in the mycelium (lower limit of detection 1% of the amount initially added).

demonstrated.

It should be noted that inoculum densities used in this experiment were higher than those used in the experiment described in Fig. 2. That elimination of MBC at this higher inoculum density became apparent after a shorter incubation period suggested that MBC was eliminated at maximum rates at the end of the exponential growth phase. Therefore, the process was further studied with concentrated mycelial suspensions (1 g of wet weight mycelium per 25 ml medium) in glucose-nitrate medium. Since this medium promotes rapid growth, nutrients are rapidly consumed and the stationary growth phase is readily attained. Table II gives the results of this experiment. As is evident from the dry weight determinations, growth of the cultures was rapid and sometime between 5 and 10 hours after initiation of the experiment the stationary phase was attained. After 15 hours dry weight decreased, indicating that lysis occurred. Rates of MBC elimination from the culture filtrates was highest during the stationary phase. Within 5 hours cultures in this phase eliminated over 90% of the amount of MBC initially added.

This system seemed to be most suitable for studying the elimination

Table II. Effect of age of culture on MBC elimination from culture filtrates of concentrated mycelial suspensions of A. nidulans Strain 003.

Time after initiation of experiment (h)	Dry weight (mg/25 ml)	Recovery of MBC after 5 h of incubation (%)
0	146	30
5	224	< 10[a]
10	234	< 10
15	210	< 10
20	169	25
25	143	57
30	123	70

Mycelium grown for 15 hours in glucose-nitrate medium was harvested, washed and resuspended in fresh medium at a concentration of 1 g wet weight of mycelium per 25 ml medium. At different times during incubation MBC was added (4µM) and the amount of MBC recovered in culture filtrates after 5 hours of incubation was determined by the TLC-bioassay (Solvent B). Dry weight determinations were made every 5 hours.
[a] lower limit of detection.

process in more detail with [^{14}C]MBC. When [^{14}C]MBC was added to aged concentrated mycelial suspensions in the stationary phase, MBC was rapidly eliminated from the medium, as shown by the TLC-bioassay, but the amount of radioactivity in the medium remained constant (Fig. 3). Thus metabolism of MBC is responsible for the elimination phenomenon. The time required to metabolize 50% of the MBC was about 2.5 hours for both strains. Similar results were also obtained with Strain 186. Since ^{14}C-metabolites appeared to be present in the culture filtrates, aliquots of these were analysed by TLC. Radioscans (Fig. 4) showed that during incubation a Metabolite (I) was formed, which was converted into a second Metabolite (II) upon prolonged incubation. Tentative identification of the metabolites was performed by cochromatography of aliquots of culture filtrates with authentic samples of MBC, 2-AB, benzimidazole and 5-OH-MBC. From Table III it is clear that

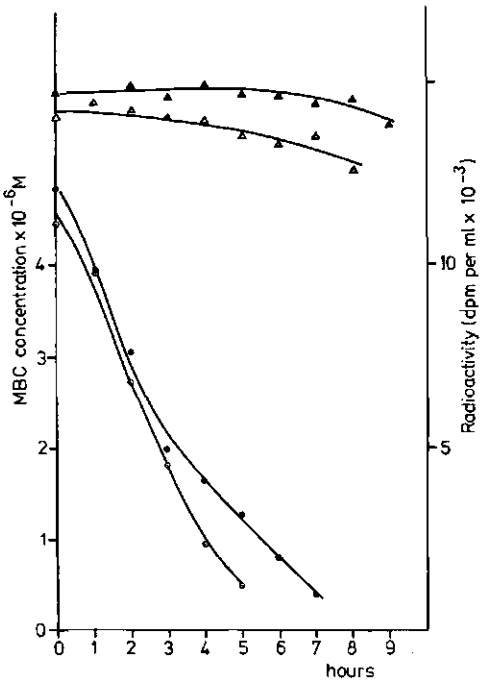

Fig. 3. Metabolic conversion of [^{14}C]MBC by aged concentrated mycelial suspensions of A. nidulans Strain 003 and Strain R. Mycelium grown for 15 hours in glucose-nitrate medium was harvested, washed and resuspended in fresh medium. After 15 hours of incubation [^{14}C]MBC (spec. act. 1.6 mCi/mmole) was added giving a final concentration of approximately 4 µM. After different times of incubation the MBC concentration of filtrates (o - o, Strain 003; • - •, Strain R) was determined with the TLC-bioassay (Solvent C). Radioactivity (Δ - Δ, Strain 003; ▲ - ▲, Strain R) was measured in 1 ml of the culture filtrates.

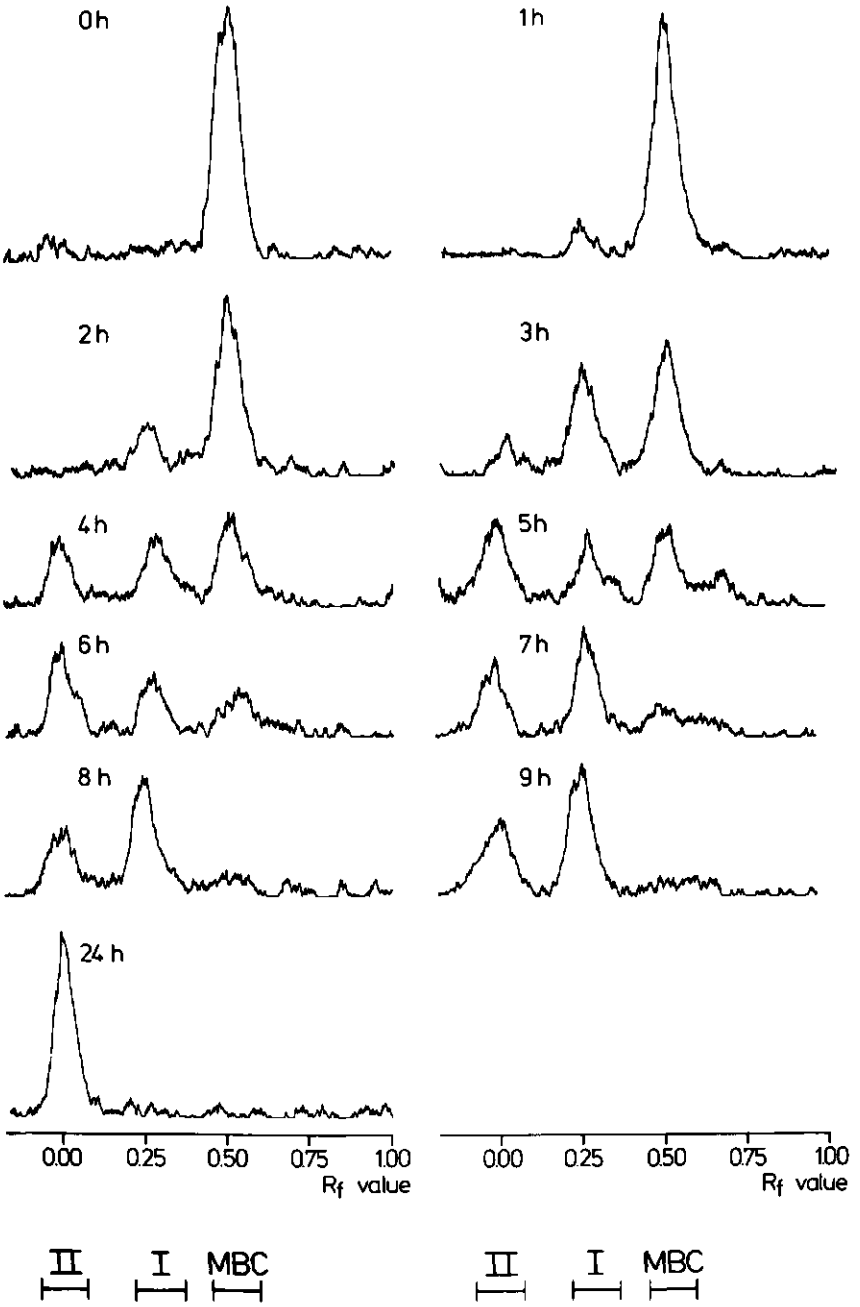

Fig. 4. Radioscans of chromatograms of culture filtrates of concentrated mycelial suspensions of A. nidulans Strain R exposed to [^{14}C]MBC. For details of incubation see Fig. 3.

Metabolite I cochromatographed with 5-OH-MBC in the two solvent systems used (C and D). Metabolite II remained on the origin in each of the solvent systems A and D, and in several other systems tested (ethanol/ammonia (70/30); hexane/ethyl acetate/methanol (10/10/1); ethylacetate/dioxane/methanol/ammonia (160/20/5/0.5); ethyl acetate/chloroform/acetic acid (15/85/2); butanol/ethanol/water (4/1/1); butanol/ammonia (4/1); ethylacetate/isopropanol/ammonia (35/45/20); and chloroform/methanol (90/10), No further attempt was, therefore, made to identify Metabolite II.

MBC was not metabolized in culture filtrates, obtained by centrifuging or filtrating mycelial suspensions after different times of incubation. Crude supernatant mycelial extracts prepared by homogenizing frozen mycelium with an X-Press (LKB-Biotec, Sweden) in 0.05 M potassium phosphate buffer, did not metabolize MBC nor did the mycelial debris.

Since metabolism was at its highest rate in the stationary growth phase, MBC conversion was studied in concentrated mycelial suspensions either made up in glucose-nitrate medium or in 0.05 M potassium phosphate buffer, pH 6.8. [^{14}C]MBC was added immediately after resuspension of the mycelium. Aliquots of the medium were analysed by TLC and the proportion of radioactivity present in MBC was determined. Fig. 5 shows the results. In the buffer in which no growth occurred, the initial rate of metabolism was rapid but slowed down upon further incubation. After 3.5 hours 50% of MBC was

Table III. Chromatographic characteristics of benzimidazole derivatives and of ^{14}C-metabolites found in culture filtrates of concentrated mycelial suspensions of A. nidulans Strain R exposed to [^{14}C]MBC.

Compound	R_f value	
	Solvent C	Solvent D
MBC	0.56	0.73
2-AB	0.00	0.45
Benzimidazole	0.23	0.17
5-OH-MBC	0.29	0.63
Metabolite I	0.29	0.64
Metabolite II	0.00	0.00

For details of incubation see Fig. 3.

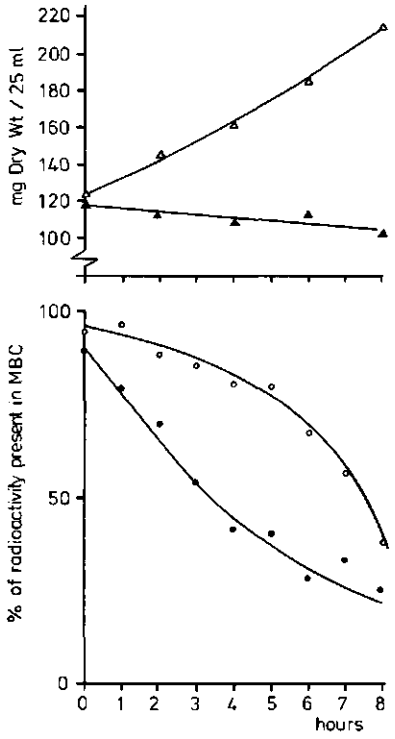

Fig. 5. Effect of incubation medium on metabolism of [^{14}C]MBC by concentrated mycelial suspensions of A. nidulans Strain 003. Mycelium grown for 15 hours in glucose-nitrate medium was harvested, washed and resuspended in fresh glucose-nitrate medium or 0.05 M potassium phosphate buffer, pH 6.8 [^{14}C] MBC (spec. act. 11.4 mCi/mmole) was added giving a final concentration of approximately 4 µM. After different times of incubation dry weight (Δ - Δ, glucose-nitrate medium; ▲ - ▲, buffer) and percentages of radioactivity present in MBC (o - o, glucose-nitrate medium; • - •, buffer) were determined.

metabolized. After 24 hours (not shown in Fig. 5) there was still some radioactivity in MBC, whereas most of the radioactivity was present in Metabolite I and Metabolite II. In glucose-nitrate medium, in which mycelial growth occurred, the rate of metabolism was rather low, but increased with time. After 7.5 hours 50% of MBC was metabolized. After 24 hours all of the radioactivity present in the medium was present in Metabolite II. During the first 8 hours of incubation the total amount of radioactivity in the media was unaffected, indicating that no fixation of radiolabelled compounds in mycelium occurred or no volatile degradation products were formed. After 24

hours of incubation, however, in suspensions in glucose-nitrate medium, the amount of radioactivity in the medium had decreased to approximately 70% of the amount initially added. With mycelial suspensions in buffer no decrease in amount of radioactivity in the filtrates was observed.

DISCUSSION

In mycelium of A. nidulans MBC was metabolized to 5-OH-MBC which in its turn was converted to a compound (Metabolite II) which could not be identified. Both compounds are less fungitoxic than MBC, since otherwise they would have been detected in the TLC-bioassay. The metabolic conversion of MBC in A. nidulans is similar to that reported by Yasuda et al. (22) in Pellicularia sasakii and Alternaria mali. These authors identified 5-OH-MBC as a minor metabolite along with MBC as the major metabolite when cultures of these fungi were exposed to radiolabelled thiophanate-methyl. In addition, they found two other labelled compounds, which were not identified. One of these which remained at the origin, upon TLC-chromatography, might be identical with Metabolite II. The other one, Metabolite X, was not detected in culture filtrates of A. nidulans. Since radiolabelled thiophanate-methyl was used, this compound might be a conversion product of thiophanate-methyl other than MBC. Formation of an intermediate fungitoxic conversion product in the in vitro conversion of thiophanate-methyl has been reported (30).

The rate of metabolism was maximum under conditions, at which the culture medium becomes depleted of nutrients. Normally, these conditions are attained after a certain incubation period, depending on the initial amount of inoculum. In concentrated mycelial suspensions in glucose-nitrate medium in which a rapid depletion of the medium occurs, the time required to metabolize 50 % of the MBC was 7.5 hours, when MBC was initially present at a concentration of 4 µM. Aged concentrated mycelial suspensions, incubated for 15 hours prior to adding MBC metabolized 50% of this amount in 2.5 hours. Mycelium in buffer and hence under starvation conditions, initially metabolized MBC at a high rate but this slowed down upon prolonged incubation. After 24 hours of incubation the amount metabolized was less than that by a similar curve in glucose-nitrate medium.

The observation that cultures in the presence of MBC at sublethal concentrations rapidly turned dark-brown suggests that melanin was synthesized. Synthesis of melanin is favoured under conditions of autolysis (31) and is mediated by the action of a phenol oxidase (32). Since similar cultural conditions promote hydroxylation of MBC to 5-OH-MBC and conversion of this

product to Metabolite II, the system responsible for MBC metabolism might parallel the system involved in melanogenesis. Hence, the final Metabolite II might be an oxidation and condensation product of 5-OH-MBC.

Strains with altered sensitivity to MBC did not show any difference in rate of MBC metabolism. Therefore, differences in metabolic activity cannot account for the different behaviour of these strains towards MBC. Since MBC is evidently metabolized within the cells, and because no differences exist in the rate of metabolism in sensitive and resistant strains, resistance to MBC does not seem to be related to differences in uptake of the compound.

Recently it has been shown that differences in sensitivity are in fact related to differences in interaction between MBC and its receptor site, of which the presence and significance in the mechanism of action of MBC has been demonstrated (33-34).

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Summary and general discussion

Systemic benzimidazole fungicides are well-known for their pronounced ability to control a large number of fungal plant diseases. On the other hand development of resistance in fungi to these compounds is as well widely known.

Biochemical aspects of both fungitoxic action and resistance in fungi are the subject of this thesis. It contains four articles describing a) the mechanism of action of carbendazim or methyl benzimidazol-2-yl carbamate, b) a mechanism of resistance to this compound and c) its metabolic conversion. Most of the work was done with Aspergillus nidulans, because this fungus is genetically well defined and new mutants can be readily characterized. Three strains were used, one strain with wild type sensitivity, one with an increased sensitivity and one with a decreased sensitivity to MBC. The behaviour of both mutant strains was due to a mutation in the benA locus.

In the first paper it has been shown that MBC is an effective inhibitor of mitosis in Aspergillus nidulans. Synthesis of DNA and RNA appeared also to be affected but this could be ascribed to the disturbance of the cell cycle, due to inhibition of mitosis. Hence, mitosis can be considered the process first affected by this compound.

In mammalian cells potent inhibitors of mitosis like colchicine, podophyllotoxin and vinblastine sulphate exert their action via interference with functioning and assembly of microtubules, the elements of the spindle. These compounds bind to tubulin, the dimeric subunit of microtubules, preventing in this way microtubule formation.

On the assumption that the antimitotic activity of MBC might be based on a similar binding, the MBC-binding properties of mycelial extracts were investigated. The results of these studies are presented in the second and third paper. An MBC-binding protein was found to be present in mycelial extracts which showed characteristic properties of tubulin. MBC binding was competitively inhibited by colchicine and oncodazole, a benzimidazole compound which binds to mammalian tubulin at the colchicine binding site. Partial purification of the binding activity resulted in a protein preparation in which the two tubulin monomers predominated. These results indicate that MBC is bound to fungal tubulin. Binding probably prevents microtubule formation or functioning which leads to disturbance of mitosis.

Study of the action of MBC at the molecular level also led to the elucidation of a mechanism of resistance of fungi to this compound. Results of

binding experiments with a number of MBC-sensitive and MBC-resistant fungal species and strains suggested that the affinity of the binding protein for MBC determined the fungal response to the action of MBC. Evidence in favour of this hypothesis was obtained from detailed binding experiments with three A. nidulans strains. Results clearly indicated the relation between degree of sensitivity for MBC and magnitude of the binding constant for MBC and tubulin.

In the fourth paper the conversion of MBC to a non-toxic metabolite has been described. Conversion was found to occur in three strains of A. nidulans examined and in non of these cases it had any bearing on the mechanism of resistance to this compound. Wheather conversion of MBC plays any role in the mechanism of resistance in other fungi remains to be elucidated.

The identification of MBC as an antitubulin, a compound which binds to tubulin, indicates that antitubulins can be successful fungicides. However, a wide spread and frequent use of such compounds does not seem advisable for a number of reasons. Their action based on the specific interference with one target site is possibly subjected to any change in this site. A mutation leading to a decreased affinity can finally result in the development of a resistant fungal population which cannot be controlled effectively.

The use of these compounds which are selective with respect to plant pathogenic fungi may also have ecological implications. A shift in the dynamic equilibrium between the various components of the microflora in the soil and on the surface of the plant is conceivable. Naturally resistant, as well as resistant strains of normally sensitive plant pathogens might be favoured in their development, giving rise to increased disease incidence.

The potential hazard of large scale use of compounds with this type of action should also be kept in mind. Mitosis in all eukaryotes follows a basic pattern, and in nearly all eukaryotes microtubules play an essential role in the separation of the chromosomes. The structure of microtubules seems to be highly conserved during evolution and, therefore, agents interfering with microtubules might be active in a wide range of organisms. Although MBC is relatively non-toxic to mammals, which is probably due to a rapid metabolic conversion and excretion, as well as to low affinity of mammalian tubulin for this compound, its ability to induce abnormalities in nuclear division in some cells of these organisms has been demonstrated. Such an effect in reproductive cells may have severe consequences. The use of compounds which act on mitosis through interference with microtubules, therefore, implies a potential genetic risk for men.

Whatever may be the future of practical use of benzimidazole compounds in agriculture or medicine, they will undoubtedly continue to be valuable tools in the study of the structure and functioning of microtubules.

Samenvatting

Systemische benzimidazool fungiciden genieten een algemene bekendheid in land- en tuinbouw vanwege hun uitgesproken kwaliteiten ten aanzien van de bestrijding van een groot aantal schimmelziekten. Anderzijds is evenwel het vermogen van schimmels om resistent te worden tegen deze middelen ook zeer bekend. De biochemische aspekten van fungitoxiciteit van deze middelen en resistentie van schimmels ertegen zijn het onderwerp van dit proefschrift. In een viertal artikelen wordt aandacht besteed aan a) het werkingsmechanisme van carbendazim of methyl benzimidazol-2-yl carbamaat (MBC), b) een resistentiemechanisme tegen dit middel en c) de omzetting van deze verbinding. De meeste experimenten werden uitgevoerd met Aspergillus nidulans omdat deze schimmel genetisch goed gedefinieerd is en nieuwe mutanten gemakkelijk kunnen worden gekarakteriseerd. Drie stammen werden gebruikt, waarvan één stam de normale gevoeligheid had voor MBC en de andere twee een toegenomen, respektievelijk een afgenomen gevoeligheid vertoonden, tengevolge van mutatie in de benA locus.

Het eerste artikel is gewijd aan de lokalisering van de plaats van werking van MBC in de gebruikte toetsschimmel Aspergillus nidulans. MBC bleek een effektieve remmer van de kerndeling te zijn, terwijl daarnaast effekten werden gevonden op DNA en RNA synthese. Deze laatste effekten konden worden toegeschreven aan de ontregeling van de celcyclus tengevolge van de geremde kerndeling. De kerndeling kon derhalve beschouwd worden als het eerste proces dat door MBC werd beïnvloed.

Remmers van de kerndeling van dierlijke cellen zoals colchicine, podophyllotoxine en vinblastine sulfaat oefenen hun werking uit door interaktie met het funktioneren of de assemblage van microtubuli, de elementen van de kernspoel. Deze stoffen worden gebonden aan tubuline, een dimeer eiwit dat beschouwd mag worden als de bouwsteen van de microtubuli. Deze binding belemmert de assemblage van tubuli tot microtubuli.

Met dit voorbeeld voor ogen hebben we het MBC bindende vermogen van mycelium extrakten onderzocht. De resultaten van dit onderzoek zijn beschreven in de tweede en de derde publikatie. Het bleek dat in mycelium extrakten een MBC-bindend eiwit aanwezig was met een aantal eigenschappen karakteristiek voor tubuline. MBC binding kon kompetitief worden geremd door colchicine en de benzimidazool verbinding oncodazole. Van oncodazole is bekend dat het wordt gebonden aan tubuline op de colchicine bindingsplaats. Na ge-

deeltelijke zuivering van de bindingsaktiviteit uit mycelium extrakt kon met behulp van elektroforese worden vastgesteld dat in de preparaten de beide tubuline monomeren aanwezig waren. Uit deze resultaten kon worden gekonkludeerd dat MBC bindt aan schimmeltubuline en dat binding de funktionering of vorming van microtubuli belemmert. Dit leidt waarschijnlijk tot een verstoring van de kerndeling.

De bestudering van het werkingsmechanisme van MBC verschafte tegelijkertijd inzicht in het mechanisme van de resistentie van schimmels tegen deze verbinding. Uit bindingsexperimenten met een aantal MBC-gevoelige en resistente schimmelsoorten en stammen werd de aanwijzing verkregen dat de affiniteit van de bindingsplaats voor MBC het gedrag van de schimmel ten opzichte van MBC zou kunnen bepalen. Bindingsexperimenten met drie stammen van A. nidulans toonden inderdaad aan dat de mate van gevoeligheid voor MBC en de grootte van de bindingskonstante voor MBC en tubuline een nauwe samenhang vertoonden.

In de vierde publikatie is de omzetting van MBC in een niet-fungitoxische verbinding beschreven. Omzetting kon worden aangetoond in kultures van een drietal A. nidulans stammen. Er kon geen relatie worden vastgesteld tussen mate van gevoeligheid voor MBC en mate van omzetting. In hoeverre dit ook voor andere schimmels geldt is nog een open vraag.

De aan de engelse samenvatting toegevoegde algemene diskussie is gewijd aan de implikaties van het praktische gebruik van deze middelen in land- en tuinbouw.

Curriculum vitae

Leendert Cornelis Davidse werd op 23 januari 1947 te Sint Laurens geboren. Hij behaalde in 1964 het einddiploma HBS-B aan de Christelijke HBS voor Walcheren te Middelburg. Hetzelfde jaar liet hij zich als student inschrijven aan de Landbouwhogeschool te Wageningen, waar hij in juni 1965 het propaedeutisch examen, in maart 1969 het kandidaatsexamen richting Plantenziektenkunde en in januari 1972 het doctoraalexamen richting Plantenziektenkunde met als hoofdvak Fytopathologie en als keuzevakken de Biochemie en de Fysiologie der Planten, met lof aflegde.

Van 1 januari 1972 tot 1 mei 1972 was hij werkzaam als wetenschappelijk assistent, en van 1 mei 1972 tot heden als gastmedewerker, in dienst van de Centrale Organisatie TNO, Sectie Landbouwkundig Onderzoek, op het Laboratorium voor Fytopathologie, Binnenhaven 9 te Wageningen.