DIFFERENTIAL BINDING OF METHYL BENZIMIDAZOL-2-YL CARBAMATE TO FUNGAL TUBULIN AS A MECHANISM OF RESISTANCE TO THIS ANTIMITOTIC AGENT IN MUTANT STRAINS OF *ASPERGILLUS NIDULANS*

L. C. DAVIDSE and W. FLACH

From the Research Unit for Internal Therapy of Plants T. N. O. Laboratory of Phytopathology, Agricultural University, Wageningen, The Netherlands

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 oncodazole and colchicine. Other inhibitors of microtubule function 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 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 liters/mol. Mutants showing resistance and increased sensitivity to MBC are candidates to have alterations in tubulin structure. affinity of tubulin for MBC is probably a common mechanism of resistance to this to this compound in fungi.

Low affinity of tubulin for MBC is probably a common mechanism of resistance binding constant of 2.5×10^3 liters/mol.

In the last 15 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 thiaben-

[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, so-

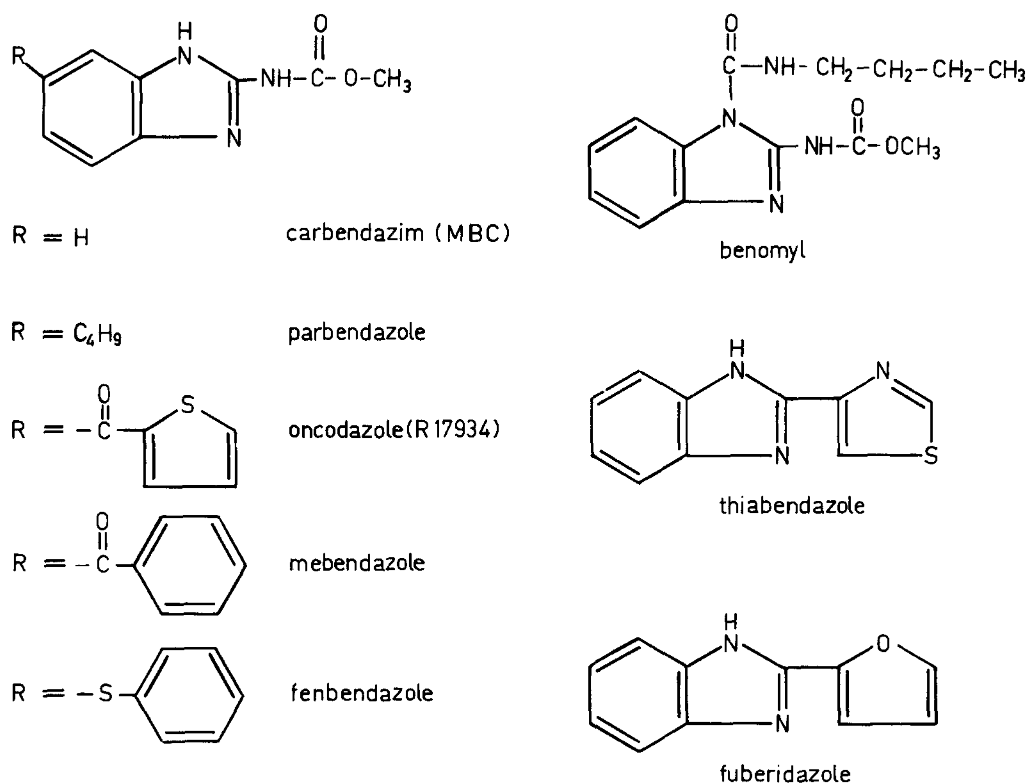

FIGURE 1 Structural formulas of biologically active benzimidazole compounds.

dazole (12), or as antitumoral drug, like oncodazole or R 17934 (3, 4). Structural formulas 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 methyl benzimidazol-2-yl carbamate (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 nondividing 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 nontoxicity 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).

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

dium dodecyl sulfate; 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)ethane sulfonic acid; EGTA, ethyleneglycol-bis-(β-aminoethylether)tetraacetic acid; TEMED, *N,N,N′,N′*,-tetramethylenediamine.

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 nondividing 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 nontoxicity 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 inhibitors of microtubule function 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 *A. nidulans biA1 AcrA1* (strain 003) and two mutant strains (strains 186 and R), which differ in MBC sensitivity. The sensitivity of these strains, in terms of inhibitory concentrations which cause a 50% reduction in growth (mean inhibitory dose, ID_{50}) on agar, is 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* as well as 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, Oxoid Ltd., London, England), supplemented with 0.5% (wt/vol) yeast extract powder (Oxoid). Cultures were incubated on a Gallenkamp orbital shaker (Gallenkamp & Co. Ltd., London) 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 Produkter, Stockholm, Sweden) with 0.5 ml of homogenization buffer per g 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 h 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 *g* for 10 min and the resultant supernate recentrifuged at 48,000 *g* for 30 min. The 48,000-*g* supernate 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 im-

mediately processed after arrival. Blood vessels and meninges were removed and the tissue was washed three times with ice cold 0.05 M potassium phosphate buffer, pH 6.8, and once with PKMg solution. 100 g of tissue was then homogenized with 100 ml of PKMg solution, containing 0.1 mM GTP, using a motor-driven glass homogenizer with Teflon pestle (B. Braun Apparatebau, Melsungen, Germany). The homogenate was centrifuged at 48,000 *g* for 60 min and the resulting supernate 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 to prevent photodecomposition of colchicine. Incubation was at 4° or 37°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% vol/vol.

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 × 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 (Nuclear-Chicago Corp., Des Plaines, Ill.) 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 A. G. Inc., Darmstadt, West Germany) 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 1,500 *g* for 5 min at 4°C. Aliquots of the supernate 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 disintegrations per minute 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-*g* supernatant mycelial extract was further centrifuged at 127,000 *g* for 60 min. The soluble proteins were fractionated with a neutralized (pH 6.8 after 20:1 dilution) saturated ammonium sulfate 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 × 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 (LKB Produkter). 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, dialyzed 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 dialyzed 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 2-(*N*-morpholino)ethane sulfonic acid (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 *g* for 10 min and the resulting supernate at 48,000 *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 *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 × 32 cm), according to the method of Andrews (2). The column was calibrated in two parallel runs with alcohol dehydrogenase (yeast), mol wt 150,000 and cytochrome *c* (horse heart), mol wt 12,400; and with lipoamide dehydrogenase (pig heart), mol wt 100,000 and α-chymotrypsin (beef pancreas), mol wt 24,500, respectively.

Reduction and Carboxymethylation of Proteins

Proteins were reduced by adding β-mercaptoethanol and sodium dodecyl sulfate (SDS) at final concentrations of 1% to the samples and heating for 2 min at 100°C. The reduced proteins were dialyzed 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 dialyzed 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 1 h in the dark, and the solution was then dialyzed against 0.01 M Tris-HCl, pH 8.0, for at least 6 h at room temperature also in the dark.

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% [wt/vol] acrylamide, 0.13% [wt/vol] bisacrylamide, 0.075% [wt/vol] ammonium persulfate, 0.075% [vol/vol] *N,N,N',N'*,-tetramethylenediamine [TEMED] in 0.1 M sodium phosphate buffer, pH 7.0) at 40 V for about 6 h. 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 h.

Gels were stained for 15 h 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]MBC (sp act 11.4 mCi/mmol) was purchased from International Chemical & Nuclear Corp. (Burbank, Calif.). Stock solutions were prepared in methanol at approximately 1,200 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, A. G., Inc.) 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 (Nuclear-Chicago Corp.) 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 (sp act 3.8 Ci/mmol) as a solution in benzene/ethanol (9/1) was obtained from New England Nuclear (Boston, Mass.). The preparation, diluted to a sp act of 20-200 mCi/mmol, was used either 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 A. G., Inc. Griseofulvin and vinblastine sulfate were obtained from Sigma Chemical Co., (St. Louis, Mo.). Melatonin and podophyllotoxin were purchased from Fluka A. G. (Basel, Switzerland) and Aldrich Chemical Co. (Milwaukee, Wis.), respectively.

Purification of [^{3}H]Colchicine

An aliquot of the [^{3}H]colchicine solution in benzene/ethanol was diluted with unlabeled colchicine in the same solution, giving a concentration of 10^{-4} M colchicine at approximately 10^6 dpm per 100 μl solution. 200 μl of this solution was applied as a small band to a silica gel plate (DC-Alufolie 60 F 254, Merck A. G., Inc.) and chromatographed in chloroform/acetone/diethylamine (5/4/1) over a distance of 17 cm with unlabeled colchicine as a reference. The center part of the [^{3}H]colchicine band (visible under UV at 254 nm) with an R_f value of 0.57 and a 1-cm broad band corresponding to the R_f value of an unknown radiolabeled compound X, which was found to be present in our colchicine preparation, with an R_f value of 0.46 was cut out and transferred to a centrifuge tube. The silica gel was scraped off and subsequently eluted four 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 the chromatography tank were wrapped in aluminum foil. Approximately 1.3×10^6 dpm were recovered in [^{3}H]colchicine and 1.4×10^5 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 R_f 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 an R_f value of 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.

RESULTS

Evaluation of the Charcoal Assay

In addition to the gel filtration method for measuring MBC binding, in this work a second method was used, which is based on separation of free MBC from bound MBC by adsorption to charcoal. This method was developed because it is rapid and suitable for multiple determinations.

Activated charcoal appeared to be very effective in adsorbing free MBC in solutions of bovine serum albumin at 40 mg/ml in PKMg buffer. More than 99.5% of the amount of MBC could be removed from the solution, when the compound was initially present in concentrations up to 40 μM. In mycelial extracts incubated with MBC, however, removal of free MBC may not involve removal of bound MBC. Therefore the charcoal assay was compared with the gel filtration method. The amount of bound MBC as determined with the charcoal assay was about one and one-half times as high as that found 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 this phenomenon.

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 equilibrium was minimum and removal of free MBC was maximum.

Selectivity of MBC Binding

MBC binding was found in 48,000-*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 correlated with the in vivo MBC sensitivity of the species or strain examined. Table I 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. Native porcine brain tubulin, partially purified by ammonium sulfate fractionation, did not bind MBC in significant amounts at 4 or 37°C, indicating that mammalian tubulin has no or at least a low affinity for MBC. This is in agreement with the observation that MBC at a concentration which is lethal

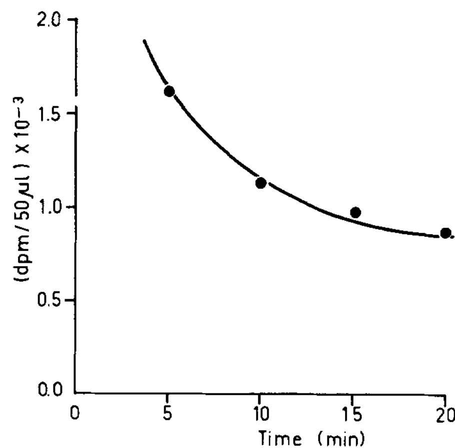

FIGURE 2 Effect of incubation time during the charcoal assay on the quantity of bound MBC. A mycelial extract of *A. nidulans* strain 003 at 38 mg protein per ml was incubated with [^{14}C]MBC at 21.2 μM (53.0×10^4 dpm/ml) for 2 h at 4°C. Binding was determined with the charcoal assay, in which the length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

TABLE I

MBC Binding in 48,000-g Supernatant Mycelial Extracts of Fungal Species and Strains, Which Differ in MBC Sensitivity

Species	Strains	ID_{50}*	Protein concn.	MBC concn. (dpm/ml) × 10^{-3}	MBC concn. μM	Bound MBC (dpm/ml) × 10^{-2}	Bound MBC pmol/mg protein
		μM	mg/ml				
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	>2,000‡	20	56.0	2.2	2.2	0.4
Penicillium corymbiferum	S	<2‡	24	66.0	2.6	20.8	3.4
	R	>2,000‡	38	56.8	2.3	1.4	0.1
Alternaria brassicae		>1,000§	37	64.0	2.6	3.7	0.4
Pythium irregulare		>1,000§	ND	65.0	2.6	2.5	ND

Mycelial extracts were incubated for 2 h 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 (6) for benomyl.

§ Data from Bollen and Fuchs (7) for benomyl.

ND, not determined.

for MBC-sensitive fungi does not interact with in vitro microtubule assembly in brain extracts (23, 27, 44).

For further work the three *A. 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 Sephadex G-200 column. The bulk of the 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 behavior of both complexes was compared on a 1.5 × 27-cm Sephadex G-200 column. The elution volume of the MBC complex appeared to be similar to that of the colchicine-tubulin complex.

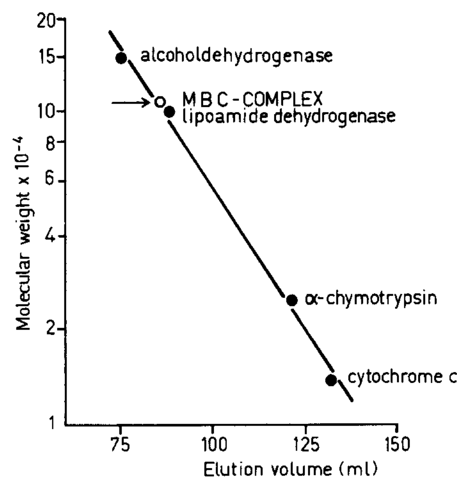

FIGURE 3 Molecular weight determination of the MBC-protein complex. 5 ml of an ammonium sulfate fractionated (35–50%) mycelial extract (48,000 *g*) at 38 mg protein per ml was incubated with [^{14}C]MBC at 8 μM for 2 h at 4°C and gel filtrated through a calibrated Sephadex G-200 column. The labeled complex was eluted as a single peak at an elution volume corresponding to a mol wt of ~110,000.

Factors which Influence MBC Binding

The binding of MBC to its receptor was rapid and increased to a plateau within 15 min of incubation at 4°C. Therefore an incubation period of 1 h 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. 4. Maximum binding appeared between pH 6.5 and 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 was not stable. At 4°C the ability of mycelial extracts to bind MBC decayed according to first order kinetics (Fig. 5). The half time for inactivation was 6.5 h. 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 decay of the binding activity was enhanced by high temperature. Binding activity of extracts, which had been heated for 1 h at 37°C, cooled in ice for another hour, and centrifuged to remove heat-denatured proteins, was 11% of that of control samples which were kept at 4°C during heating and centrifuging of the experimental sample. Since for unknown reasons the charcoal assay applied to heated extracts gave anomalous binding data, binding activity was measured with the gel filtration method. Binding activity in the control sample was determined with the charcoal assay.

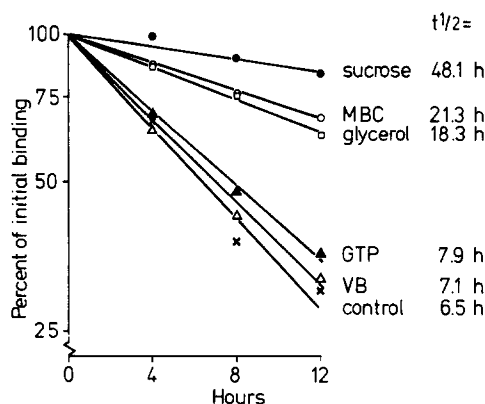

FIGURE 5 Stability of the MBC-binding activity under various conditions at 4°C. 3 ml of a mycelial extract of *A. nidulans* strain 186 at 24 mg protein per ml was mixed with an equal volume of a solution of respectively, 1 mM vinblastine sulfate (VB), 2 mM GTP, 8 M glycerol, 22.8 μM [^{14}C]MBC (56.8 × 10^4 dpm/ml), and 2 M sucrose in PKMg solution. As a control preparation a 3-ml extract was diluted with 3 ml of 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 ($t_{1/2}$) of the MBC-binding activity have been calculated from the slopes of the lines.

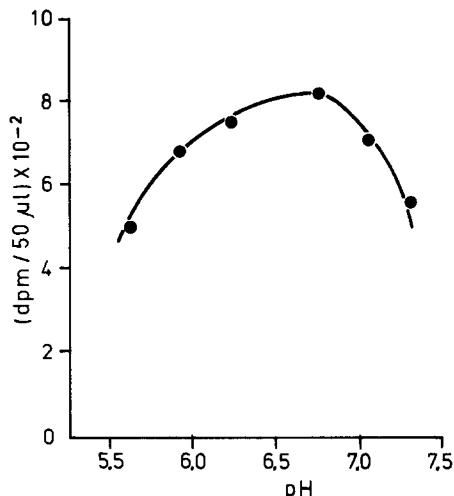

FIGURE 4 Effect of pH on MBC binding. 2-ml samples of a 127,000-*g* (1 h) supernatant mycelial extract of *A. nidulans* strain 186 at 24 mg protein per ml were adjusted to different pH values with 50 μl of a H_3PO_4 or KOH solution, respectively, of different strength. 1 ml of the resulting preparation 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 milliliter of the preparation.

Incubating mycelial extracts with 0.1% trypsin for 1 h at 4°C before incubation with [^{14}C]MBC resulted in a 55% loss of binding capacity.

Centrifuging at 127,000 *g* for 1 h at 4°C of the 48,000-*g* supernate lowered the binding activity of the resulting supernate 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 A. nidulans Strains 003, 186, and 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 the 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 (11).

Results of a number of experiments are summarized in Fig. 6. From the slopes of the lines and the intercepts, values of S_o, expressed as maximum binding capacity in picomoles MBC per milligram 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 strains 003, 186, and R, respectively. These values correspond with the inhibitory concentration of MBC causing a 50% reduction in growth (see Table I). 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

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 inhibitors of microtubule function (52), was also ineffective at 500 μM.

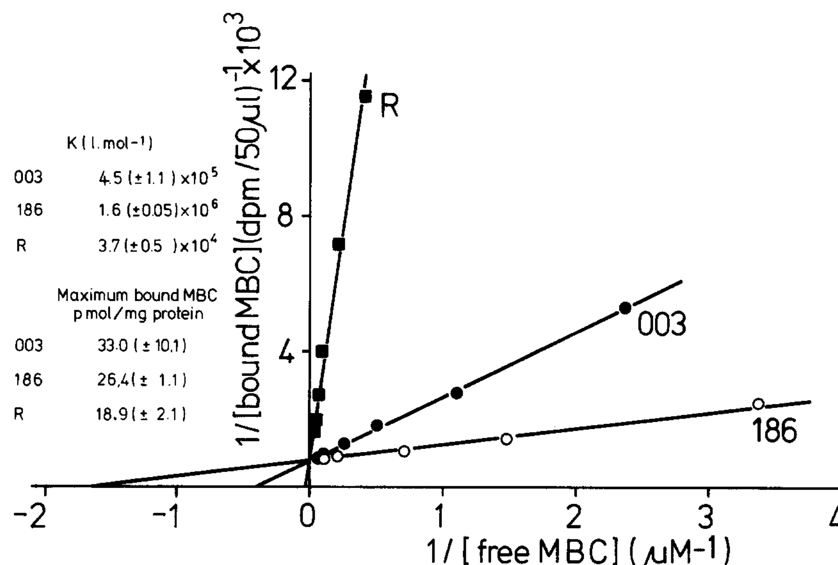

FIGURE 6 Binding of [^{14}C]MBC to mycelial extracts of *A. nidulans*, strains 003, 186, and R. Mycelial extracts of strains 003, 186, and R at 38, 39, and 40 mg protein per ml, respectively, were incubated with [^{14}C]MBC at increasing concentrations for 1 h at 4°C. The quantity of bound MBC was determined with the charcoal assay. The concentration of free MBC was calculated from the differences between total and bound MBC. Values of binding constants K (liters/mol) and maximum binding capacity (picomoles MBC/milligrams protein) with their respective standard deviations are given as determined in at least four experiments.

Griseofulvin, which affects spindle functioning in *A. nidulans* (19) and selectively interferes with in vitro microtubule assembly (73), did not affect MBC binding at 1,000 μM, either when added simultaneously with [^{14}C]MBC or after extracts had been incubated for 1 h before incubation with [^{14}C]MBC for another hour.

Oncodazole, which is more toxic to the *A. nidulans* strains than MBC and to which compound MBC-resistant strains are cross-resistant (23), appeared to be a very effective inhibitor of MBC binding. At 10 μM, oncodazole inhibited MBC binding for 92%, if it was added to extracts 1 h before these were incubated with [^{14}C]MBC at 5.5 μM for another hour.

Colchicine, which did not inhibit mycelial growth on agar of any of the three *A. nidulans* strains at concentrations up to 10 mM, slightly inhibited MBC binding at 500 μM, when added simultaneously with MBC. At 10 mM and if added to extracts 1 h before addition of [^{14}C]MBC at 11.6 μM, this compound inhibited binding for 40%.

Both compounds inhibited MBC binding in a competitive manner as is illustrated in Figs. 7 and 8. The long incubation period used here was found necessary to obtain equilibrium in the binding reactions. To reduce the decay of binding activity during this period sucrose was added to the extracts.

The inhibition constant (K_i) for oncodazole

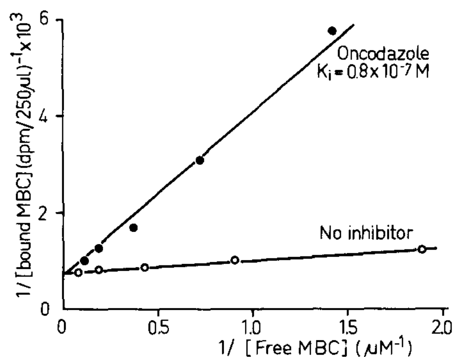

FIGURE 7 Inhibition of MBC binding by oncodazole. A mycelial extract of *A. nidulans* strain 186 at 33 mg protein per ml was diluted 1:1 with a solution of 2 M sucrose in PKMg buffer. Aliquots of the solution were incubated with increasing concentrations of [^{14}C]MBC with (●) and without (○) 1 μM oncodazole 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.

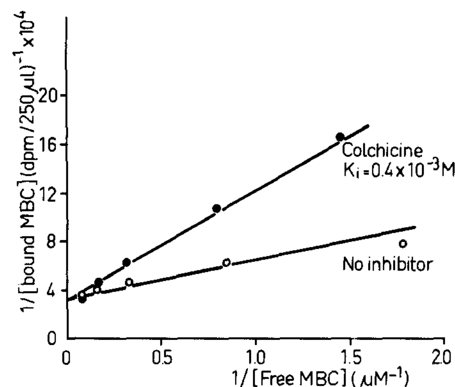

FIGURE 8 Inhibition of MBC binding by colchicine. A mycelial extract of *A. nidulans* strain 186 at 31 mg protein per ml was diluted 1:1 with a solution of 2 M sucrose in PKMg buffer. Aliquots of the solution were incubated with increasing concentrations of [^{14}C]MBC with (●) and without (○) 1 mM colchicine 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.

was 0.8×10^{-7} M. This value indicates a higher affinity of the MBC-binding protein for oncodazole than for MBC, which is compatible with the observation that oncodazole showed a higher fungitoxicity than MBC. Oncodazole inhibited the growth of strain 186 for 50% at a concentration of 0.23 μM. MBC achieved this at 1.5 μM. The inhibition constant for colchicine was 0.4×10^{-3} M indicating a low affinity of the MBC-binding protein for this compound.

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 II, exp. 1). Since this amount was unexpectedly high, the nature of this bound radioactivity was investigated. Thin-layer chromatographic (TLC) analysis, with chloroform/acetone/diethylamine (5/4/1) as solvent, 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, because 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 was identified as compound X with TLC analysis. 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 Table II, exps. 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 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 anion exchanger has been used. Mycelial extracts were incubated with [^{3}H]colchicine at 500 μM (approximately 19.6×10^6 dpm/ml) for 1 h at 4°C and run onto DEAE-Sephadex columns. The elution pattern of radioactivity, which is shown in Fig. 9*a* shows two high peaks and one very small peak. TLC analysis revealed that radioactivity in fraction 4, which contained the bulk 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 (1.5×20 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 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. 9*b*). 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 tempera-

TABLE II

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 protein per ml (exp. 1) or strain R at 42 mg protein per ml (exps. 2 and 3) had been incubated with the radiochemicals for 2 h at 4°C.

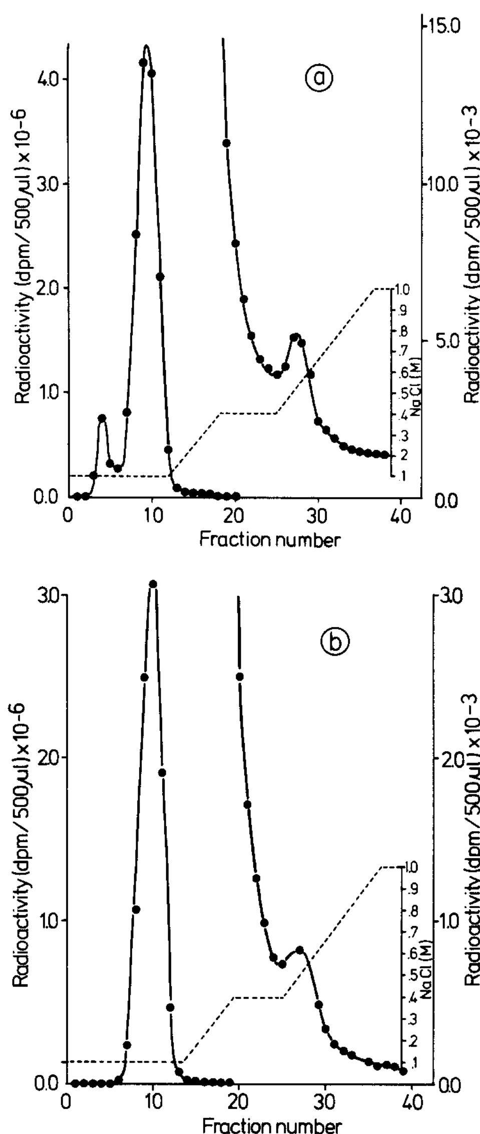

FIGURE 9 DEAE-Sephadex A-50 column chromatography of mycelial extracts of *A. nidulans* strain 186 incubated with [^{3}H]colchicine at 4°C (*a*) and 37°C (*b*) for 1 h. 48,000-*g* supernatant mycelial extracts at 32 (*a*) and 29 (*b*) mg protein per ml were incubated with 500 μM [^{3}H]colchicine (19 × 10^6 dpm/ml (*a*) and 12.2 × 10^6 dpm/ml (*b*)). 4 ml of the incubation mixture (*a*) or supernate of the centrifuged incubation mixture (*b*) was loaded onto DEAE-Sephadex columns and eluted as described under Materials and Methods. The graphs show the elution pattern of radioactivity. The dashed line indicates the NaCl concentration.

ture 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 preceding 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, analogous 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-*g* supernatant mycelial extracts could be selectively precipitated with ammonium sulfate between 35 and 50% saturation. 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 twofold.

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 an 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 strains 003, 186, and R are shown in Fig. 10. 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 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 III) that depending on the strain used, considerable amounts

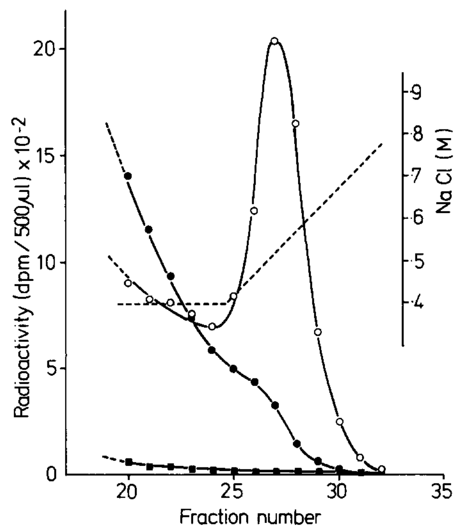

FIGURE 10 DEAE-Sephadex A-50 column chromatography of partially purified mycelial extracts of *A. nidulans* strains 003, 186, and R. 127,000-*g* supernatant mycelial extracts were fractionated with a saturated ammonium sulfate solution (Materials and Methods). 5 ml of the preparations containing 15 mg (strain 003), 12 mg (strain 186), and 19 mg (strain R) protein per ml was 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; (○) strain 186; (■) strain R. The dashed line indicates the NaCl concentration.

TABLE III
Gel Filtration of DEAE-Purified MBC-Protein Complex on a Sephadex G-100 Column

	Strains	
	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-*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.

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 (Sartorius MembranFilter GmbH, Göttingen, West Germany), 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 analyzed with the continuous SDS system. Gels loaded with reduced protein samples of fractions 24–30 (Fig. 10) from the experiment with strains 186 and R are shown in Figs. 11 and 12, 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 pres-

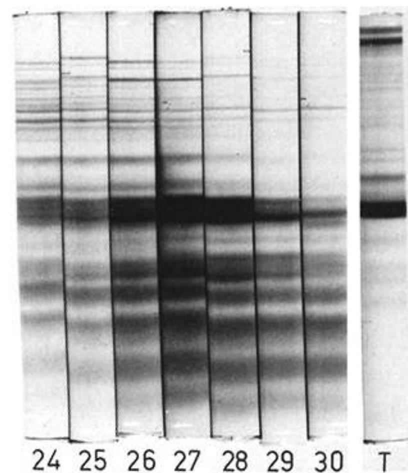

FIGURE 11 Electrophoretic analysis of proteins isolated from *A. nidulans* strain 186 by DEAE-Sephadex column chromatography. Samples of fractions 24–30 (see Fig. 10, strain 186) were reduced and analyzed on the continuous SDS system. Reduced porcine brain tubulin (*T*) purified by one polymerization cycle was run as a reference.

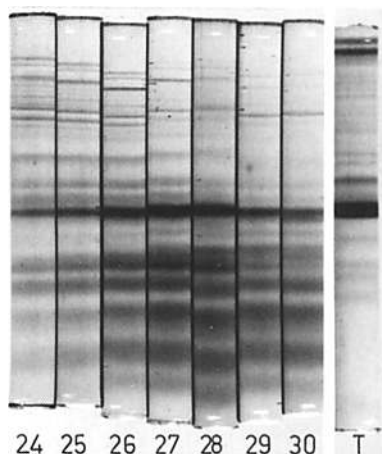

FIGURE 12 Electrophoretic analysis of proteins isolated from *A. nidulans* strain R by DEAE-Sephadex column chromatography. Samples of fractions 24–30 (see Fig 10, strain R) were reduced and analyzed on the continuous SDS system. Reduced porcine brain tubulin (*T*) purified by one polymerization cycle was run as a reference.

ent 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 strains 003, 186, and R are shown in Fig. 13*a*. Carboxymethylated DEAE-purified porcine brain tubulin was run as reference protein. It is evident that the preparation of strain R differed from those of strains 003 and 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. 13*b*). 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.

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 supernate in which, upon electrophoresis in the continuous SDS system, the two slower moving bands were not present (Fig. 14*a, 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

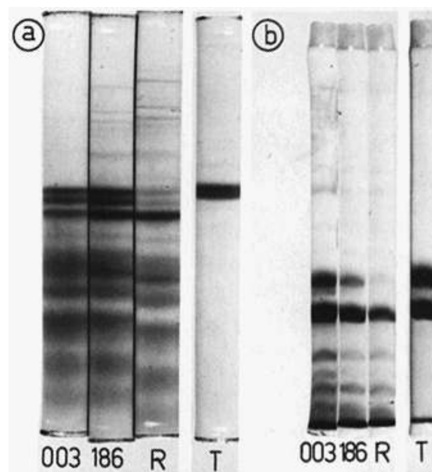

FIGURE 13 Electrophoretic analysis of proteins isolated from *A. nidulans* strains 003, 186, or R, by DEAE-Sephadex column chromatography. Lyophilized proteins from pooled fractions eluted at 0.45–0.90 M NaCl were carboxymethylated and run in the continuous SDS system (*a*) and the discontinuous system (*b*). Carboxymethylated DEAE-purified porcine brain tubulin was run as a reference.

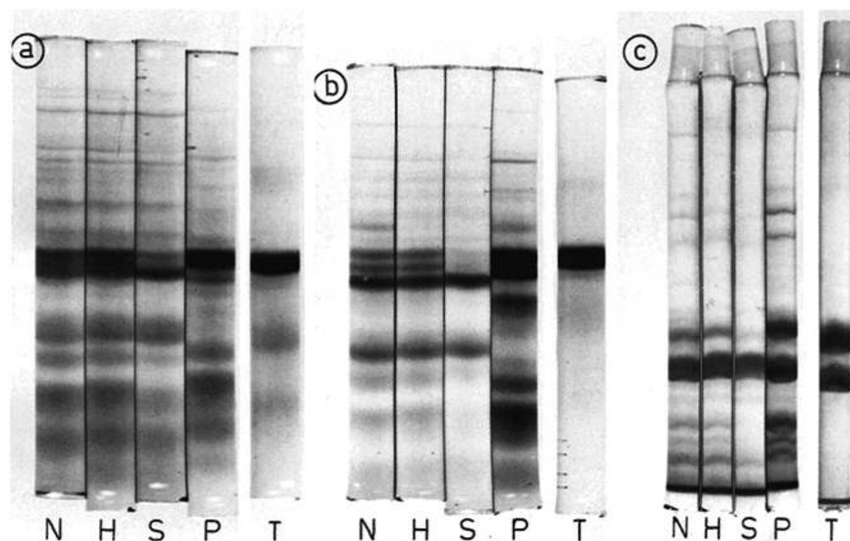

FIGURE 14 Electrophoretic analysis of proteins isolated from *A. nidulans* strain 186 by DEAE-Sephadex column chromatography. Pooled fractions eluted at 0.45-0.60 M NaCl were heated for 1 h at 37°C, cooled in ice, and centrifuged at 40,000 *g* for 10 min. Aliquots of the nonheated (*N*), heated (*H*) 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.

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. 14*c*). Because of this behavior 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 (Fig. 6), 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 behavior 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 inhibitors of microtubule function 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 mono-

mers. 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 did 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 suggests that fungal tubulin has no or at least a low affinity for this compound.

From the data given in Fig. 6 the number of MBC-binding sites can be determined. Assuming that one molecule of MBC is bound per molecule of tubulin, 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-labeled 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 two to three 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 (references 22 [Fig. 1] and 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 nondisjunction 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.

Until now no evidence has been presented that resistance to other agents which bind to tubulin might be caused by a similar mechanism. Resistance to compounds of this type 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 noteworthy 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.

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

We are grateful to Prof. Dr. Ir. J. Dekker, Dr. A. Fuchs, and Dr. Ir. J. Visser (Department of Genetics, Agricultural University, Wageningen, The Netherlands) for comments on the manuscript and to Mrs. Brouns-Murray (Pudoc, Wageningen, The Netherlands) for correcting the English text.

Received for publication 12 July 1976, and in revised form 27 September 1976.

REFERENCES

1. ACTOR, P., E. L. ANDERSON, D. J. DICUOLLO, R. J. FERLAUTO, J. R. E. HOOVER, J. F. PAGANO, L. R. RAVIN, S. F. SCHEIDY, R. J. STEDMAN, and V. J. THEODORIDES. 1967. A new broad spectrum anthelmintic, methyl 5(6)-butyl-2-benzimidazole carbamate. *Nature (Lond.).* **215**:321.
2. ANDREWS, P. 1964. Estimation of the molecular weights of proteins by Sephadex Gel-Filtration. *Biochem. J.* **91**:222.
3. ATASSI, G., C. SCHAUS, and H. J. TAGNON. 1975. R 17934-NSC 238159: A new antitumor drug. II. Effect on mitotic cycle of L1210 leukemia cells *in vivo* and synergism with cytosine arabinoside (NSC 63878). *Eur. J. Cancer.* **11**:609.
4. ATASSI, G., and H. J. TAGNON. 1975. R 17934-NSC 238159: A new antitumor drug. I. Effect on experimental tumors and factors influencing effectiveness. *Eur. J. Cancer.* **11**:599.
5. BAEDER, C., H. BÄHR, O. CHRIST, D. DÜWEL, H. M. KELLNER, R. KIRSCH, H. LOEWE, E. SCHULTES, E. SCHUTZ, and H. WESTEN. 1974. Fenbendazole: a new highly effective anthelmintic. *Experientia (Basel).* **30**:753.
6. BOLLEN, G. J. 1971. Resistance to benomyl and some chemically related compounds in strains of

Penicillium species. *Neth. J. Plant Pathol.* **77:**187.

7. Bollen, G. J., and A. Fuchs. 1970. On the specificity of the *in vitro* and *in vivo* antifungal activity of benomyl. *Neth. J. Plant Pathol.* **76:**299.
8. Borgers, M., and S. De Nollin. 1975. The ultrastructural changes in *Ascaris suum* intestine after mebendazole treatment *in vivo*. *J. Parasitol.* **61:**110.
9. Borgers, M., S. De Nollin, M. De Brabander, and D. Thienpont. 1975. Microtubules and intracellular organelle-movement in nematode intestinal cells. The influence of the anthelmintic mebendazole. *Am. J. Vet. Res.* **36:**1153.
10. Borgers, M., S. De Nollin, A. Verheyen, M. De Brabander, and D. Thienpont. 1975. Effects of new anthelmintics on the microtubular system of parasites. *In* Microtubules and Microtubule Inhibitors. M. Borgers and M. De Brabander, editors. North-Holland Publishing Company, Amsterdam. 497.
11. Borisy, G. G., and E. W. Taylor. 1967. The mechanism of action of colchicine. Binding of colchicine-^{3}H to cellular protein. *J. Cell Biol.* **34:**525.
12. Brown, H. D., A. R. Matzuk, I. R. Ilvues, L. H. Peterson, S. A. Harris, L. H. Sarett, J. R. Edgerton, J. J. Yaktis, W. C. Campbell, and A. C. Cuckler. 1961. Antiparasitic drugs IV. 2-(4'-thiazolyl) benzimidazole, a new anthelmintic. *J. Am. Chem. Soc.* **83:**1764.
13. Bruno, G. A., and J. E. Christian. 1961. Determination of carbon-14 in aqueous bicarbonate solutions by liquid scintillation counting techniques. *Anal. Chem.* **33:**1216.
14. Bryan, J. 1974. Biochemical properties of microtubules. *Fed. Proc.* **33:**152.
15. Bryan, J., and B. W. Nagle. 1975. Inhibition of tubulin assembly by RNA and other polyanions. A mechanism. *In* Microtubules and Microtubule Inhibitors. M. Borgers and M. De Brabander, editors. North-Holland Publishing Company, Amsterdam. 91.
16. Bryan, J., B. W. Nagle, and K. H. Doenges. 1975. Inhibition of tubulin assembly by RNA and other polyanions: Evidence for a required protein. *Proc. Natl. Acad. Sci. U. S. A.* **72:**3570.
17. Bryan, J., and L. Wilson. 1971. Are cytoplasmic microtubules heteropolymers? *Proc. Natl. Acad. Sci. U. S. A.* **8:**1762.
18. Burns, R. G. 1973. ^{3}H-Colchicine binding. Failure to detect any binding to soluble proteins from various lower organisms. *Exp. Cell Res.* **81:**285.
19. Crackower, S. H. B. 1972. The effects of griseofulvin on mitosis in *Aspergillus nidulans*. *Can. J. Microbiol.* **18:**683.
20. Crestfield, A. M., S. Moore, and W. H. Stein. 1963. The preparation and enzymatic hydrolysis of reduced and S-carboxymethylated proteins. *J. Biol. Chem.* **238:**622.
21. Davidse, L. C. 1973. Antimitotic activity of methyl benzimidazol-2-yl carbamate in *Aspergillus nidulans*. *Pest. Biochem. Physiol.* **3:**317.
22. Davidse, L. C. 1975. Mode of action of methyl benzimidazol-2-yl carbamate (MBC) and some biochemical aspects of acquired resistance against this fungicide in *Aspergillus nidulans*. *In* Systemfungizide-Systemic Fungicides. H. Lyr and C. Polter, editors. Akademie Verlag, Berlin. 137.
23. Davidse, L. C. 1975. Antimitotic activity of methyl benzimidazol-2-yl carbamate in fungi and its binding to cellular protein. *In* Microtubules and Microtubule Inhibitors. M. Borgers and M. De Brabander, editors. North-Holland Publishing Company, Amsterdam. 483.
24. Davidse, L. C. 1976. Metabolic conversion of methyl benzimidazol-2-yl carbamate in *Aspergillus nidulans*. *Pest. Biochem. Physiol.* **6** In press.
25. De Brabander, M., R. Van de Veire, F. Aerts, M. Borgers, and P. A. J. Janssen. 1976. The effects of methyl [5-(2-thienylcarbonyl)-1*H*-benzimidazol-2-yl] carbamate, (R 17934; NSC 238159), a new synthetic antitumoral drug interfering with microtubules, on mammalian cells cultured *in vitro*. *Cancer Res.* **36:**905.
26. De Brabander, M., R. Van de Veire, F. Aerts, G. Geuens, M. Borgers, and L. Desplenter. 1975. Oncodazole (R 17934): A new anti-cancer drug interfering with microtubules. Effects on neoplastic cells *in vitro* and *in vivo*. *In* Microtubules and Microtubule Inhibitors. M. Borgers and M. De Brabander, editors. North-Holland Publishing Company, Amsterdam. 509.
27. De Brabander, M., R. Van de Veire, F. Aerts, S. Geuens, and J. Hoebeke. 1976. A new culture model facilitating rapid quantitive testing of mitotic spindle inhibition in mammalian cells. *J. Natl. Cancer Inst.* **56:**357.
28. Dekker, J. 1973. Selectivity of and resistance against systemic fungicides. *Organ. Eur. Mediterr. Prot. Plant. Publ. Ser. A.* **10:**47.
29. Delp, C. J., and H. L. Klöpping. 1968. Performance attributes of a new fungicide and mite ovicide candidate. *Plant Dis. Rep.* **52:**95.
30. Eipper, B. A. 1972. Rat brain microtubule protein: Purification and determination of covalently bound phosphate and carbohydrate. *Proc. Natl. Acad. Sci. U. S. A.* **69:**2283.
31. Eipper, B. A. 1974. Properties of rat brain tubulin. *J. Biol. Chem.* **249:**1407.
32. Feit, H., and M. L. Shelanski. 1975. Is tubulin a glycoprotein? *Biochem. Biophys. Res. Commun.* **66:**920.
33. Freed, J. J., and B. M. Ohlsson-Wilhelm. 1975. Cultured haploid cells resistant to antitubulins. *In* Microtubules and Microtubule Inhibitors. M. Borgers and M. De Brabander, editors. North-Holland Publishing Company, Amsterdam. 365.

34. FULLER, M. S. 1975. Mitosis in fungi. *Biosystems.* **7**:360.
35. FULLER, M. S. 1976. Mitosis in fungi. *Int. Rev. Cytol.* **46**:112.
36. GARDINER, J. A., J. J. KIRKLAND, H. L. KLÖPPING, and H. SHERMAN. 1974. Fate of benomyl in animals. *J. Agric. Food Chem.* **22**:419.
37. GEORGOPOULOS, S. G., A. KAPPAS, and A. C. HASTIE. 1976. Induced sectoring in diploid *Aspergillus nidulans* as a criterion of fungitoxicity by interference with hereditary processes. *Phytopathology.* **66**:217.
38. HABER, J. E., J. G. PELOQUIN, H. O. HALVORSON, and G. G. BORISY. 1972. Colcemid inhibition of cell growth and the characterization of a colcemid-binding activity in *Saccharomyces cerevisiae. J. Cell Biol.* **55**:355.
39. HAMMERSCHLAG, R. S., and H. D. SISLER. 1973. Benomyl and methyl-2-benzimidazole carbamate (MBC): Biochemical, cytological and chemical aspects of toxicity to *Ustilago maydis* and *Saccharomyces cerevisiae. Pest. Biochem. Physiol.* **3**:42.
40. HARTREE, E. F. 1972. Determination of protein: A modification of the Lowry method that gives a linear photometric response. *Anal. Biochem.* **48**:422.
41. HASTIE, A. C. 1970. Benlate-induced instability of *Aspergillus diploids. Nature (Lond.).* **226**:771.
42. HEATH, I. B. 1975. The effect of antimicrotubule agents on the growth and ultrastructure of the fungus *Saprolegnia ferax* and their ineffectiveness in disrupting hyphal microtubules. *Protoplasma.* **85**:147.
43. HEATH, I. B. 1975. Colchicine and colcemid binding components of the fungus *Saprolegnia ferax. Protoplasma.* **85**:177.
44. HOEBEKE, J., and C. VAN NIJEN. 1975. Quantitative turbidimetric assay for potency evaluation of colchicine-like drugs. *Life Sci.* **17**:591.
45. HOEBEKE, J., G. VAN NIJEN, and M. DE BRABANDER. 1976. Interaction of oncodazole (R 17934), a new antitumoral drug, with rat brain tubulin. *Biochem. Biophys. Res. Commun.* **69**:319.
46. JOCKUSCH, B. M. 1973. Nuclear proteins in *Physarum polycephalum. Ber. Dtsch. Bot. Ges.* **86**:39.
47. JORGENSEN, A. O., and S. M. HEYWOOD. 1974. Tubulin synthesis on polysomes isolated from brain and leg muscle of embryonic chick. *Proc. Natl. Acad. Sci. U. S. A.* **71**:4278.
48. KAPPAS, A., and S. G. GEORGOPOULOS. 1974. Interference of griseofulvin with the segregation of chromosomes at mitosis in diploid *Aspergillus nidulans. J. Bacteriol.* **119**:334.
49. KAPPAS, A., S. G. GEORGOPOULOS, and A. C. HASTIE. 1974. On the genetic activity of benzimidazole and thiophanate fungicides on diploid *Aspergillus nidulans. Mutat. Res.* **26**:17.
50. LOWRY, O. H., N. J. ROSEBROUGH, A. L. FARR, and R. J. RANDALL. 1953. Protein measurement with the Folin-phenol reagent. *J. Biol. Chem.* **193**:265.
51. LUDUENA, R. F., and D. O. WOODWARD. 1975. α- and β-Tubulin: Separation and partial sequence analysis. *Ann. N. Y. Acad. Sci.* **253**:272.
52. MARGULIS, L., S. BANERJEE, and J. K. KELLEHER. 1975. Assay for antitubulin drugs in live cells: oral regeneration in *Stentor coeruleus. In* Microtubules and Microtubule Inhibitors. M. Borgers and M. De Brabander, editors. North-Holland Publishing Company, Amsterdam. 453.
53. MURRAY, A. W., and M. FROSCIO. 1971. Cyclic adenosine 3′:5′-monophosphate and microtubule function: specific interaction of the phosphorylated protein subunits with a soluble brain component. *Biochem. Biophys. Res. Commun.* **44**:1089.
54. OLSON, L. W. 1973. A low molecular weight colchicine binding protein from the aquatic phycomycete *Allomyces neo-moniliformis. Arch. Mikrobiol.* **91**:281.
55. RENAUD, F. L., A. J. ROWE, and I. R. GIBBONS. 1968. Some properties of the protein forming the outer fibers of cilia. *J. Cell Biol.* **36**:79.
56. RICHMOND, D. V., and A. PHILLIPS. 1975. The effect of benomyl and carbendazim on mitosis in hyphae of *Botrytis cinerea Pers. ex. Fr.* and roots of *Allium cepa L. Pest. Biochem. Physiol.* **5**:367.
57. ROSENBERGER, R. F., and M. KESSEL. 1967. Synchrony of nuclear replication in individual hyphae of *Aspergillus nidulans. J. Bacteriol.* **94**:1464.
58. SCHUMANN, G. 1968. Beitrag zur systemischen Wirkung fungizider Benzimidazole-Derivate als Getreidebeizmittel. *Nachrichtenbl. Dtsch. Pflanzenschutzdienstes. (Braunschw.).* **20**:1.
59. SEILER, J. P. 1975. Toxicology and genetic effects of benzimidazole compounds. *Mutat. Res.* **32**:151.
60. SHEIR-NEISS, G., R. V. NARDI, M. A. GEALT, and N. R. MORRIS. 1976. Tubulin-like protein from *Aspergillus nidulans. Biochem. Biophys. Res. Commun.* **69**:285.
61. SHELANSKI, M. L., F. GASKIN, and C. R. CANTOR. 1973. Microtubule assembly in the absence of added nucleotides. *Proc. Natl. Acad. Sci. U. S. A.* **70**:765.
62. SHERLINE, P., C. K. BODWIN, and D. M. KIPNIS. 1974. A new colchicine binding assay for tubulin. *Anal. Biochem.* **62**:400.
63. SHERLINE, P., J. T. LEUNG, and D. M. KIPNIS. 1975. Binding of colchicine to purified microtubule protein. *J. Biol. Chem.* **250**:5481.
64. SLIFKIN, M. K. 1968. The effect of colchicine on *Olpidiopsis incrassata* and its host, *Saprolegnia delica. J. Elisha Mitchell Sci. Soc.* **84**:184.
65. SOLEL, Z., J. M. SCHOOLEY, and L. V. EDGINGTON, 1973. Uptake and translocation of benomyl and carbendazim (Methyl benzimidazol-2-yl carbamate) in the symplast. *Pestic. Sci.* **4**:713.
66. STARON, T., and C. ALLARD. 1964. Propriétés anti-

fongiques du 2-(4′-thiazolyl) benzimidazole ou thiabendazole. *Phytiatr.-Phytopharm. Rev. Fr. Med. Pharm. Veg.* **13:**163.

67. STYLES, J. A., and R. GARNER. 1974. Benzimidazolecarbamate methyl ester; evaluation of its effects *in vivo* and *in vitro*. *Mutat. Res.* **26:**177.
68. VAN DEN BOSSCHE, H. 1972. Biochemical effects of the anthelmintic drug mebendazole. *In* Comparative Biochemistry of Parasites. H. Van den Bossche, editor. Academic Press, Inc., New York. 139.
69. VAN TUYL, J. M., L. C. DAVIDSE, and J. DEKKER. 1974. Lack of cross resistance to benomyl and thiabendazole in some strains of *Aspergillus nidulans*. *Neth. J. Plant Pathol.* **80:**165.
70. VAN TUYL, J. M. 1975. Genetic aspects of acquired resistance to benomyl and thiabendazole in a number of fungi. *Meded. Fac. Landbouwwet. Rijksuniv. Gent.* **40:**1691.
71. WEBER, K., and M. OSBORN. 1969. The reliability of molecular weight determinations by dodecyl sulfate polyacrylamide gel electrophoresis. *Biol. Chem.* **244:**4406.
72. WEINGARTEN, M. D., A. H. LOCKWOOD, S. HWO, and M. W. KIRSCHNER. 1975. A protein factor essential for microtubule assembly. *Proc. Natl. Acad. Sci. U. S. A.* **72:**1858.
73. WILSON, L., K. ANDERSON, L. GRISHAM, and D. CHIN. 1975. Biochemical mechanisms of action of microtubule inhibitors. *In* Microtubules and Microtubule Inhibitors. M. Borgers and M. De Brabander, editors. North-Holland Publishing Company, Amsterdam. 103.
74. WILSON, L., J. R. BAMBURG, S. M. MIZEL, L. M. GRISHAM, and K. M. CRESSWELL. 1974. Interaction of drugs with microtubule proteins. *Fed. Proc.* **33:**158.
75. WILSON, L., and J. BRYAN. 1974. Biochemical and pharmacological properties of microtubules. *Adv. Cell Mol. Biol.* **3:**21.
76. WINSTON, M., E. JOHNSON, J. K. KELLEHER, S. BANERJEE, and L. MARGULIS. 1974. Melatonin: cellular effects on live *Stentors* correlated with the inhibition of colchicine-binding to microtubule protein. *Cytobios.* **9:**237.