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Oryzalin, a dinitroaniline herbicide, binds to plant tubulin and inhibits microtubule polymerization in vitro

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Abstract. The effects of oryzalin, a dinitroaniline herbicide, on chromosome behavior and on cellular microtubules (MTs) were examined by light microscopy and immunogold staining, respectively, in endosperm cells from *Haemanthus katherinae* Bak. Brief treatments with $1.0 \cdot 10^{-8}$ M oryzalin reduced markedly the migration rate of anaphase chromosomes and $1.0 \cdot 10^{-7}$ M oryzalin stopped migration abruptly. Oryzalin ($1.0 \cdot 10^{-7}$ M) depolymerized MTs and prevented the polymerization of new MTs at all stages of the mitotic cycle. The chromosome condensation cycle was unaffected by oryzalin. Endothelial cells from the heart of *Xenopus leavis* showed no chromosomal or microtubular rearrangements after oryzalin treatment. The inhibition by oryzalin of the polymerization of tubulin isolated from cultured cells of *Rosa* sp. cv. Paul's scarlet was examined in vitro by turbidimetry, electron microscopy and polymer sedimentation analysis. Oryzalin inhibited the rapid phase of taxol-induced polymerization of rose MTs at 24° C with an apparent inhibition constant (K_i) of $2.59 \cdot 10^6$ M. Shorter and fewer MTs were formed with increasing oryzalin concentrations, and maximum inhibition of taxol-induced polymerization occurred at approx. 1:1 molar ratios of oryzalin and tubulin. Oryzalin partially depolymerized taxol-stabilized rose MTs. Ligand-binding experiments with [^{14}C]oryzalin demonstrated the formation of a tubulin-oryzalin complex that was time- and pH-dependent. The tubulin-oryzalin interaction (24° C, pH 7.1) had an apparent affinity constant (K_{app}) of $1.19 \cdot 10^5$ M^{-1}. Oryzalin did not inhibit taxol-induced polymerization of bovine-brain MTs and no appreciable binding of oryzalin to brain tubulin or other proteins was detected. The results demonstrate pharmacological differences between plant and animal tubulins and indicate that the most sensitive mode of action of the dinitroaniline herbicides is the *direct* poisoning of MT dynamics in cells of higher plants.

Key words: Dinitroaniline – Rosa – Herbicide – Microtubule – Oryzalin – Haemanthus – Taxol – Tubulin.

Introduction

Microtubules (MTs) are filamentous, subcellular structures composed mainly of the heterodimeric protein, tubulin (MW = approx. 100000), and are organized as specific functional arrays in higher-plant cells. Microtubules are found in the mitotic and meiotic spindles, the preprophase band, the cytokinetic plate region of the phragmoplast and the cortical cytoskeleton (reviewed by Gunning and Hardham 1982). Microtubules participate in several motility-related processes during the mitotic cycle and differentiation, including chromosome migration, vesicle transport and the orientation of cellulose microfibril deposition in the developing cell wall. Because the activities of MT arrays determine the polarity of cell division and mediate cell differentiation during development, MTs have been implicated as subcellular effectors of plant morphogenesis (Gunning and Hardham 1982). The functions of MTs in arrays have been inferred, in large part, from studies that have observed the disruption of particular subcellular processes following treatment of cells with drugs that cause the disappearance of MTs. Among the various classes of anti-MT compounds that have been used in plant systems, members of the dinitroaniline herbicide class such as oryzalin (3,5-dinitro-N^4,N^4-

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Abbreviations: MT = microtubule; SIB = sucrose isolation buffer; TO = tubulin-oryzalin complex

dipropylsulfanilamide) and trifluralin (α,α,α-trifluoro-2-6-dinitro-N,N,-dipropyl-*p*-toluidine) have potent activities.

Dinitroanilines comprise a large class of preemergence herbicide producing gross morphological abnormalities in plants, particularly in regions having high meristematic activity such as root tips. Inhibition of the growth of primary and lateral roots is followed by excessive radial expansion and the formation of a bulbous root tip. Histological studies of dinitroaniline-treated roots have shown that cell division and mitosis are disrupted after brief exposure, and that cells in the meristematic tip become swollen subsequently. Affected cells typically contain "c-mitotic" chromosomes and restitution or polyploid nuclei, and do not form a cell plate following nuclear division (for review see Ashton and Crafts 1981, pp. 201–223). These gross morphological and cytological features resemble closely the effects of colchicine, an alkaloid that depolymerizes MTs (for review see Morejohn and Fosket 1986). Ultrastructural studies have shown that concentrations of dinitroanilines in the high nanomolar range cause the partial or complete disappearance of MTs in all subcellular arrays (Ashton and Crafts 1981).

Hess and Bayer (1977) were the first to report appreciable binding of a dinitroaniline herbicide to tubulin. They used gel-filtration chromatography to demonstrate the binding of [^{14}C]trifluralin to tubulin isolated from flagella of the unicellular alga *Chlamydomonas eugametos*. Strachen and Hess (1983) also performed a series of [^{14}C]oryzalin-binding experiments with *Chlamydomonas* tubulin and reported the rapid formation of a tubulin-oryzalin complex (TO) that was independent of pH and temperature. They reported a binding constant (K) of $2.08 \cdot 10^5\ M^{-1}$, and maximum binding stoichiometry of unity at 25° C, and suggested that TO was incapable of polymerizing into MTs. These studies were the first to demonstrate that tubulin from a plant, albeit an alga, was pharmacologically different from animal tubulin.

The development of a method for isolation of tubulin from cultured cells of higher plants (Morejohn and Fosket 1982) has facilitated studies on its pharmacological properties. Colchicine was found to inhibit the assembly of rose (*Rosa* sp. cv. Paul's scarlet) MTs half-maximally at much higher concentrations than that of bovine-brain MTs (Morejohn and Fosket 1984a; Morejohn et al. 1987). Colchicine-binding experiments demonstrated that plant tubulins have lower binding activities than brain tubulin (Morejohn et al. 1984) and that the affinity of colchicine for rose tubulin

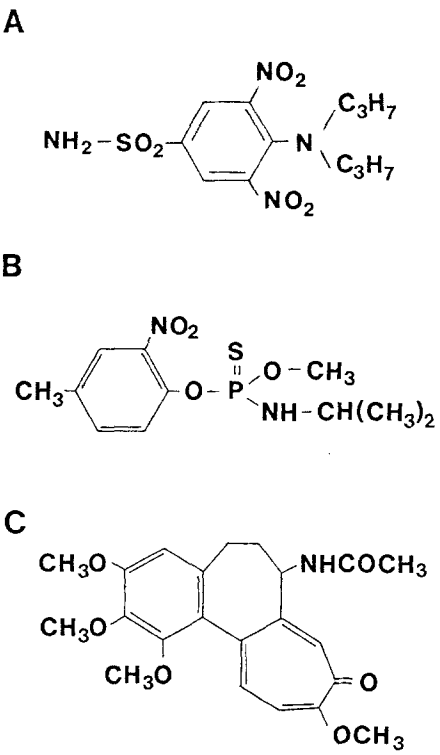


Fig. 1A–C. Structures of anti-MT compounds. **A** oryzalin; **B** amiprophos-methyl; **C** colchicine

is much lower than that for brain tubulin (Morejohn et al. 1987). On the other hand, the phosphoric amide herbicide amiprophos-methyl was found to be a potent anti-MT drug in the plant MT system and to inhibit MT polymerization half-maximally at herbicide concentrations in the low micromolar range (Morejohn and Fosket 1984b). Amiprophos-methyl has no effect on animal MT polymerization. These results demonstrated pronounced differences in the pharmacological properties of tubulins from higher plants and animals, and the results closely paralleled the relative effects that these anti-MT compounds have on MT-dependent processes in cells of these diverse organisms (reviewed by Morejohn and Fosket 1986). The chemical structures of oryzalin, amiprophos-methyl and colchicine are quite different from each other (see Fig. 1).

While the binding of oryzalin and trifluralin to *Chlamydomonas* tubulin (Hess and Bayer 1977; Strachen and Hess 1983) was suggestive evidence that the dinitroanilines inhibit directly the functions of MTs in cells, these herbicides are known to affect cellular activities in higher plants that apparently are unrelated to their anti-MT properties. For example, Moreland et al. (1972a, b) reported inhibition of photosynthesis and respiration by several dinitroanilines, and Ashton et al. (1977)

found that trifluralin inhibited photosynthesis and synthesis of protein, RNA and lipid. Dinitroanilines were reported to inhibit the uptake of calcium in vitro by plant mitochondria (Hertel et al. 1980; Hertel and Marmé' 1983). These later workers proposed that the effects of dinitroanilines on MTs in cells were indirect and that calcium from mitochondria caused MTs to depolymerize (reviewed by Robinson and Quader 1982). Thus, the role of dinitroanilines in the inhibition of MT-dependent processes has remained controversial.

To define more clearly the anti-MT activities of dinitroanilines we have used oryzalin to determine threshold parameters of herbicide-treatment time and herbicide concentration that inhibit mitosis and depolymerize MTs in endosperm cells from *Haemanthus katherinae* Bak. Direct microscopic observations of chromosome movements were correlated with the organization of immunogold-stained MTs in a large number of cells. The effects in vitro of oryzalin on the polymerization of tubulin from cultured cells of rose and the binding of [^{14}C]oryzalin to rose tubulin were examined. The data provide strong evidence that oryzalin inhibits *directly* the dynamics of MT assembly and disassembly, and further implicate MTs as the most sensitive target of dinitroaniline action in higher plants.

Material and methods

Chemicals. Piperazine-N,N′-bis(2-ethane-sulfonic acid) dipotassium salt (Pipes), ethyleneglycol-bis-(β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid (EGTA), dithiothreitol (DTT), sucrose, leupeptin hemisulfate, pepstatin A, diethyldithiocarbamic acid, glucose, sodium citrate, sodium azide, bovine β-lactoglobulin, bovine serum albumin (fraction V), and chicken-egg lysozyme were obtained from Sigma Chemical Co., St. Louis, Mo., USA. Guanosine triphosphate trilithium salt (GTP) was from Calbiochem, La Jolla, Cal., USA. Taxol was provided by Dr. Matthew Suffness, Natural Products Branch, Division of Cancer Treatment, National Cancer Institute, Bethesda, Md., USA.

Light microscopy and immunogold staining of MTs. Endosperm cells were gently extruded from ovules of *Haemanthus katherinae* Bak. and mounted in a gel on slides as described previously (Molè-Bajer and Bajer 1968; Bajer and Molè-Bajer 1986). Primary cultures of heart endothelial cells from *Xenopus leavis* were prepared as described by Rieder and Bajer (1978) for lung cells of newt. Analytical grade (98.3%) oryzalin was dissolved in a medium containing 3.5% glucose and 0.05 M sodium citrate, pH 5.2, for treatment of *Haemanthus* cells and in culture medium for *Xenopus* cells. Both solutions contained 1% ethanol. No effects of ethanol on mitosis in endosperm cells were observed at concentrations of $<5\%$. Mitosis in living cells was followed in the light microscope using differential interference phase optics and recorded with a video camera and recorder as described in detail in Molè-Bajer and Bajer (1968) and Bajer and Molè-Bajer (1986). At appropriate times after perfusion of cells with oryzalin, preparations were fixed and processed for immunogold staining as described by De Mey et al. (1982). Primary antibodies used in these studies were rabbit anti-rose β-tubulin immunoglobulin G (Morejohn et al. 1984) or affinity-purified rabbit anti-tubulin (dog brain) (De Mey et al. 1982). Immunogold-stained cells were photographed with Kodak Kodachrome slide film and color enlargements were made from slides.

Tubulin isolations. Tubulins from cultured cells of *Rosa* sp., cv. Paul's scarlet, and from bovine cerebrum were isolated by anion-exchange chromatography as described by Morejohn and Fosket (1982), Lee et al. (1973) and Morejohn et al. (1984). Ammonium-sulfate precipitates of rose tubulin were dialyzed against an isolation buffer (IB) consisting of 50 mM Pipes-KOH, pH 7.1, 1 mM EGTA, 0.5 mM $MgCl_2$, 0.1 mM GTP, 1 mM DTT and supplemented with 1 M sucrose, 1 μM leupeptin hemisulfate, 1 μM pepstatin A and 0.02% sodium azide (SIB). Ammonium-sulfate precipitates of brain tubulin were dialyzed against SIB without leupeptin and pepstatin. Dialysates were clarified by centrifugation and stored at −80° C as described in Morejohn et al. (1984). All other methods of protein analysis, including gel electrophoresis, densitometry, immunoblotting and protein determinations, were performed exactly as described previously by Morejohn et al. (1984, 1985, 1987). Concentrations of rose tubulin and brain tubulin were 11.4 mg/ml and 14.6 mg/ml, respectively.

Turbidimetry, sedimentation analysis and electron microscopy. Polymerization of tubulin at 24° C was measured by turbidimetry (Gaskin et al. 1974) at A_{400} as described by Morejohn and Fosket (1984a) and Morejohn et al. (1987). Tubulin in SIB was thawed in an ice bath and mixed with SIB (24° C) containing assembly-saturating concentrations of taxol and appropriate concentrations of oryzalin. All reactions contained 0.5% dimethyl sulfoxide (DMSO) and 2% ethanol. Materials and methods of sedimentation analysis (Johnson and Borisy 1975) and electron microscopy of polymers have also been described previously by Morejohn and Fosket (1984a) and Morejohn et al. (1987).

Oryzalin-binding measurements. Analytical grade (98.3%) oryzalin (3,5-dinitro-N^4,N^4-dipropylsulfanilamide) and [ring-^{14}C]oryzalin ($1.23 \cdot 10^8$ Bq·mmol^{-1}) were obtained from Eli Lilly & Co., Indianapolis, Ind., USA. Stock solutions of unlabeled 1.0 mM oryzalin and 0.3 mM [^{14}C]oryzalin were prepared in 100% and 50% ethanol, respectively, and aliquots were stored at −20° C. Radiochemical purity of [^{14}C]oryzalin was verified by thin-layer chromatography (TLC) using two different chromatographic supports, a 6060 Silica-gel sheet (Eastman Kodak Co., Rochester, N.Y., USA) and a Polygram Gel 300 PEI (polyethyleneimine-impregnated cellulose) sheet (Brinkmann Instruments Co., Westbury, N.Y., USA). Samples (10–30 μl) containing 10 nmol of stock solutions were spotted on chromatogram sheets, dried under N_2, and developed in a solvent composed of 95% toluene and 5% ethanol. Sheets were exposed to X-ray film (Kodak X-Omat RP) for 48 h. On both TLC supports a single fluorescent spot of yellow-orange color was resolved in both unlabeled oryzalin and [^{14}C]oryzalin samples. Autoradiographs showed a single radioactive species that comigrated with the pigmented material on both chromatograms, indicating $\geq 98\%$ radiochromatographically pure [^{14}C]oryzalin.

Using the methodology of Strachen and Hess (1982), the solubility of [^{14}C]oryzalin in SIB was examined prior to ligand-binding experiments. It was determined that concentrations of [^{14}C]oryzalin ≤ 12.5 μM remained constant when SIB was sup-

plemented with 2% ethanol. Thus, in all experiments involving oryzalin and protein-containing solutions, a final concentration of 2% was used, including the turbidimetric measurements of MT polymerization described above.

Several methods of ligand-binding were examined for suitability in this study, including equilibrium dialysis, gel filtration, equilibrium gel filtration and diethylaminoethyl (DEAE)-cellulose filtration. In every case the [^{14}C]oryzalin bound non-specifically to filtration media, even in the presence of 2% ethanol, making quantitation of binding difficult. However, it was determined that binding could be quantitated with the DEAE-cellulose filter-disc method (Borisy 1972) if tubulin-oryzalin samples were allowed to bind to the discs slowly (2.0–2.5 min) and subsequent washing of the discs was carefully controlled by monitoring the filtration-chamber vacuum with a gauge. Protein determinations on filtrates showed no loss of protein from washed filters when three discs were used in each port. For each concentration of [^{14}C]oryzalin assayed, samples containing ligand, but no tubulin were applied to filters and washed in the same way. These filters (blanks) were used for correction of non-specific binding of [^{14}C]oryzalin to filters.

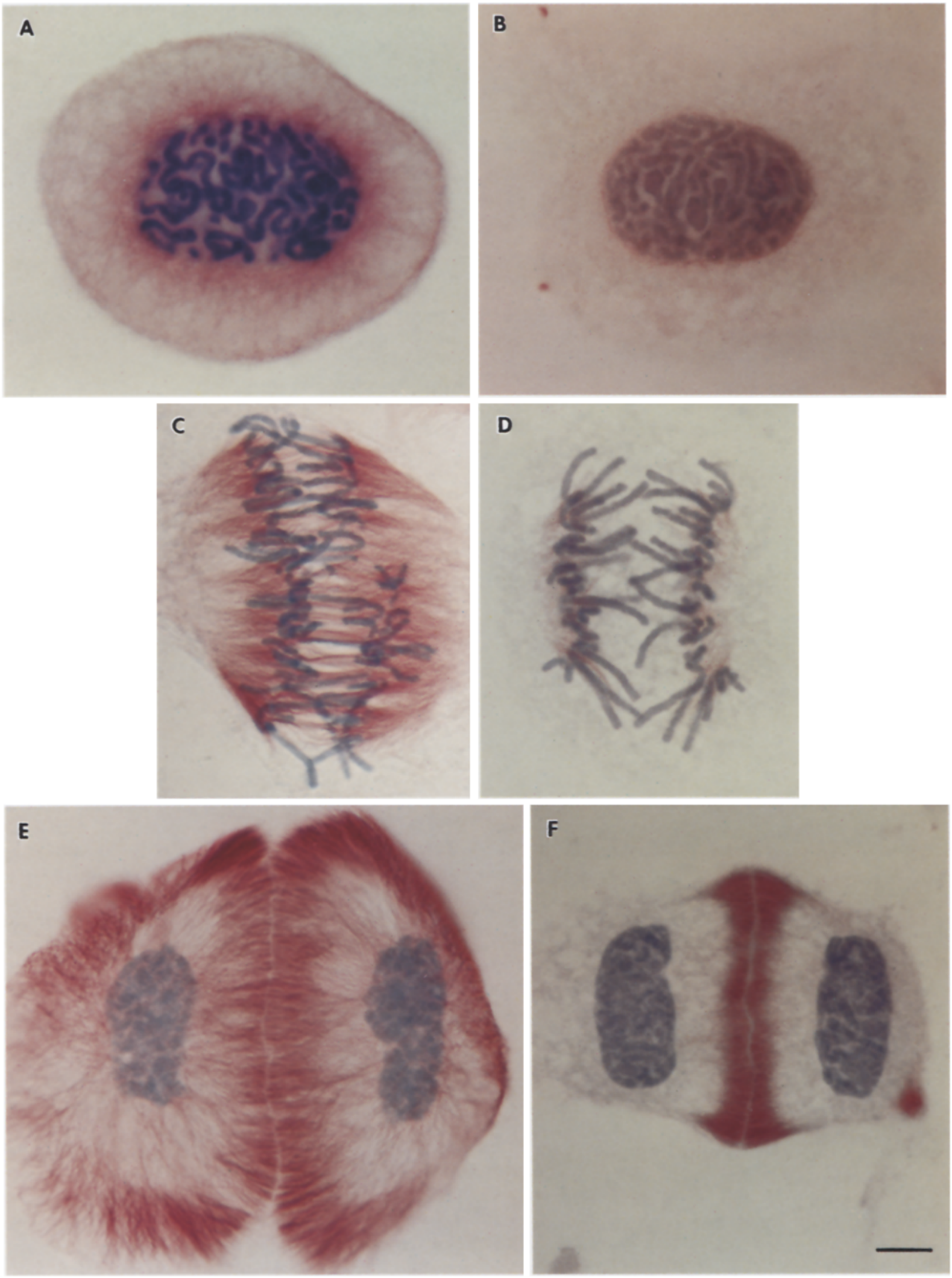
For all [^{14}C]oryzalin-binding experiments, the following protocol was used. Protein (in SIB) was thawed in an ice bath and was diluted to a concentration of 3.5 μM in SIB containing 2% ethanol and an appropriate concentration of [^{14}C]oryzalin and incubated in a water bath at 24° C. At desired times, 100 μl aliquots (0.35 nmol) were applied to DEAE-cellulose filter discs (2.5 cm diameter; Whatman, Hillsboro, Ore., USA) that had been previously moistened with 2 ml of IB in a Millipore filtration apparatus (12 ports). Samples were slowly adsorbed and washed with 10 ml of cold IB five times. Discs were air-dried, placed in 10 ml of Hydrofluor scintillation mixture (National Diagnostics, Somerville, N.J., USA), and each sample was counted for 50 min ($\leq 2\%$ counting error) in a Beckman LS-230 liquid scintillation spectrophotometer.

Results

Inhibition of chromosome migration by oryzalin. The effects of oryzalin on chromosome migration and arrangement were examined in living endosperm cells of *Haemanthus katherinae* (Molè-Bajer and Bajer 1968). This system of single, wall-less cells was used in order to minimize the potentially complicating effects of non-uniform herbicide uptake and translocation, and non-specific adsorption to cell wall that have been observed in intact organs (Bayer et al. 1967). Cell preparations were perfused with oryzalin-containing buffer (1% ethanol) and chromosomes were observed by differential interference phase microscopy. There was no apparent effect of 0.01–1.0 μM oryzalin treatment on the chromosome cycle. Cells treated with 0.01 μM oryzalin in interphase, prophase and metaphase proceeded to the next mitotic stage. Condensation of prophase chromatin proceeded normally and the nuclear envelope disappeared. Cells treated with 0.1 μM oryzalin in prophase or metaphase exhibited chromosomal arrangements typical of the classical c-mitotic figure. Analysis of 50 cells in anaphase showed that after treatment with 0.01 μM oryzalin, poleward migration of chromosomes was slowed to a rate of 0.4–0.8 $\mu m \cdot min^{-1}$ from the mean rate of 1.2 $\mu m \cdot min^{-1}$ observed in control cells treated with 1% ethanol alone. This effect was nearly immediate. Perfusion with 0.1 μM oryzalin halted migration of anaphase chromosomes immediately (≤ 30 s). These cells produced groups of chromosomes in nuclear membranes that sometimes appeared to fuse. Long-term observations (≥ 2 h) showed that numerous cells had c-mitotic arrangements of chromosomes and that cells having restitution nuclei produced no cell plate.

The inhibition of anaphase chromosome migration was not readily reversible. After a short treatment (5 min) with 0.1 μM oryzalin, followed by washing (up to 1 h) with oryzalin-free buffer, chromosomes did not resume poleward movements. Exposure of endothelial cells (primary cultures) from heart of *Xenopus leavis* to oryzalin (10 μM) for periods up to 10 h had no effect on chromosome movements and condensation.

Disruption of MTs in cells by oryzalin. The effects of 0.01–0.1 μM oryzalin on MTs in *Haemanthus* cells in different mitotic stages were examined with immunogold staining and correlated with the effects observed in vivo. Cells treated with 0.01 μM oryzalin at interphase and prophase contained the full complements of MTs that have been described previously (De Mey et al. 1982). After ≤ 2 min in 0.1 μM oryzalin, virtually all MTs in these cells were depolymerized. Only a few short MTs remained associated with the nucleus (Fig. 2A, B). Oryzalin (0.1 μM) completely inhibited the appearance of kinetochore MTs on chromosomes of cells treated prior to metaphase, a result consistent with the c-mitoses observed above. Metaphase and anaphase cells treated with 0.01 μM oryzalin showed no change in the number or arrangement of MTs, but at 0.1 μM oryzalin (≤ 2 min) non-kinetochore MTs were completely disassembled and kinetochore MTs depolymerized partially (Fig. 2C, D). Microtubule "branches" of kinetochore complexes were the most labile, while the centrally located MTs of complexes remained associated to form short bundles that are presumably stabilized by MT-associated proteins (Fig. 2D). Cells affected in these stages ultimately produced restitution nuclei. Cytoplasmic MTs in telophase cells (phragmoplasts) were depolymerized by oryzalin (0.1 μM), but many MTs in the region of the developing cell plate were not completely depolymerized (Fig. 2E, F). The ends of MTs nearest the plate were resistant to treatment even after 2 h at 1.0 μM oryzalin. Among 3153 mitotic cells treated with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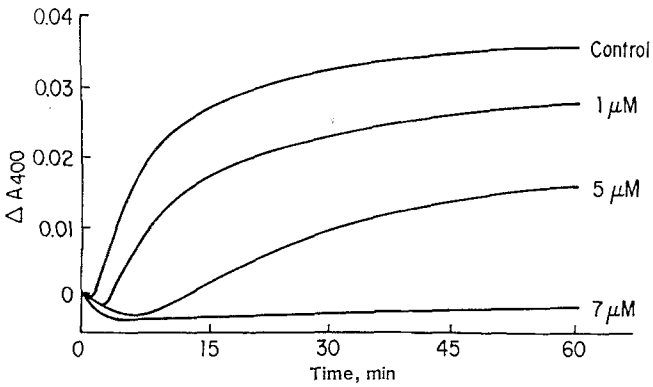

Fig. 3. Turbidity measurements of oryzalin inhibition of rose MT polymerization. Rose tubulin was polymerized with taxol in the absence (Control) or presence of oryzalin (1, 5 or 7 μM) and monitored turbidimetrically (A_{400}) for 1 h at 24° C

0.1 μM oryzalin only 50 (1.6%) apparently were unaffected and most of these cells were in late telophase.

Immunogold staining of cells treated with oryzalin and then washed showed that MTs did not repolymerize. This result correlated with the lack of resumption of anaphase chromosome migration observed in vivo. As expected, oryzalin-treated (10 μM, 10 h) *Xenopus* cells contained a normal distribution of cytoplasmic and mitotic MTs.

Inhibition of plant MT polymerization in vitro. The effects of oryzalin on taxol-induced MT polymerization in vitro were assessed by turbidimetric measurements (Gaskin et al. 1974), electron microscopy and sedimentation analysis (Johnson and Borisy 1975) as previously described by Morejohn and Fosket (1984a, b) and Morejohn et al. (1987). Rose tubulin (10 μM) was mixed with SIB containing taxol (40 μM) and an appropriate concentration of oryzalin (1, 5 or 7 μM) and reactions were monitored at 24° C by turbidimetry. The inclusion of low-micromolar concentrations of oryzalin in rose MT polymerization reactions produced increased lag-times and decreased rates and extents of turbidity development (Fig. 3). Nearly 1:1 molar ratios of oryzalin and tubulin produced maximum inhibition of turbidity. When bovine brain tubulin (10 μM) was polymerized in taxol (27 μM) at 24° C in the presence of 7 μM oryzalin, no change in the pattern of turbidity development from the control (without oryzalin) was observed. Inclusion of 10 μM colchicine with the brain-tubulin reaction completely inhibited turbidity development (Morejohn and Fosket 1984a). An inhibition constant (K_i) for the rapid phase of rose MT polymerization was calculated from the steepest portion of each turbidity measurement in the absence or presence of oryzalin (Wilson et al. 1976) as described for colchicine previously (Morejohn et al. 1987). This analysis gave $K_i = 2.59$ μM (correlation coefficient = 1.0). Thus, 2.59 μM oryzalin would inhibit half-maximally the rapid phase of taxol-induced rose MT polymerization.

To determine whether oryzalin depolymerizes preformed MTs, 10 μM rose tubulin was polymerized with 40 μM taxol for 1 h (steady state) and oryzalin was mixed with the reaction using a wide-bore pipet (to prevent shearing) to give a final oryzalin concentration of 12.5 μM. The turbidity level immediately decreased from the steady-state value ($A_{400} = 0.033$), and a new steady state ($A_{400} = 0.023$) was achieved within 3 min. This decrease resulted from a reduction in the mass of MTs (Gaskin et al. 1974) and not from the minor effect of dilution (Karr and Purich 1979).

The morphology of rose tubulin polymers formed in the presence of oryzalin after 1 h was examined by electron microscopy. Samples (2 μl) were taken from each reaction described above (Fig. 3 legend) and negatively stained. Numerous rose MTs were polymerized by taxol in the absence of oryzalin (Fig. 4A). Fewer and shorter MTs and more amorphous materials were seen at increasing oryzalin concentrations, and at 7 μM oryzalin only amorphous structures are observed (Fig. 4B). Brain MTs polymerized in the presence of 7 μM oryzalin were indistinguishable in number and morphology from those of control reactions without oryzalin.

The effect of oryzalin on the yield (mass) of taxol-induced polymer was quantitated by sedimentation analysis. Samples polymerized in the presence or absence of oryzalin for 1 h were centrifuged and pellets were assayed for protein. The data in Table 1 show that oryzalin decreased substantially the yield of rose MTs; approx. 50% inhibition occurred at 5 μM oryzalin. At an oryzalin concentration (7 μM) that inhibited by 94% the yield of rose polymer, the yield of brain MTs was inhibited by only 2% (Table 1). Colchicine

Fig. 2A–F. Effects of oryzalin on MTs in endosperm cells of *Haemanthus katherinae* Bak. (bar = 10 μm; ×1000). Control cells (untreated) and cells treated with 0.1 μM oryzalin for 5 min are shown on the left and the right, respectively. Microtubules detected by immunogold staining appear red and chromosomes are stained with toluidine blue. **A, B** prophase; **C, D** mid-anaphase; **E, F** telophase (phragmoplasts)

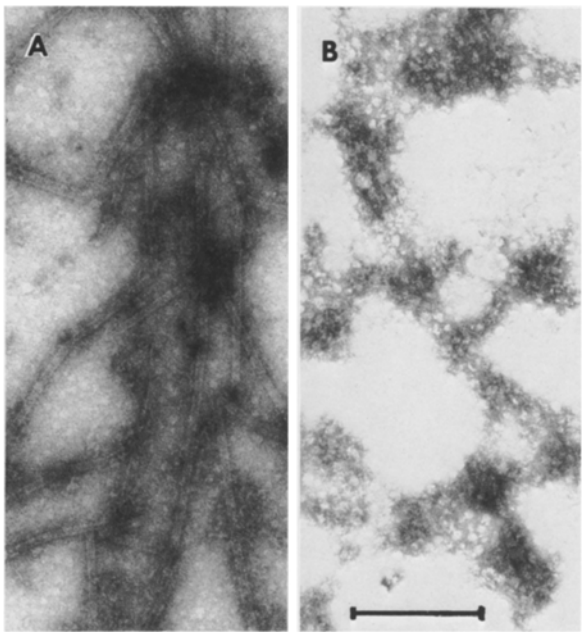

Fig. 4A, B. Electron micrographs of negatively stained polymerization products of rose tubulin (bar=0.5 μm; ×35000). Samples are **A** MTs polymerized with taxol alone, and **B** amorphous aggregates formed in the presence of 7 μM oryzalin

(10 μM) reduced the yield of brain MTs to 10% of the control reaction that contained no colchicine (Morejohn and Fosket 1984a). The sample in which 12.5 μM oryzalin was added to steady-state MTs contained 61% of the polymer mass of the the control reaction when polymer was assayed 15 min after the addition of oryzalin. These data demonstrate that oryzalin inhibits the taxol-induced polymerization of plant tubulin in a concentration-dependent fashion and also partially depolymerizes MTs previously assembled and stabilized with taxol.

Binding of oryzalin to plant tubulin. Technical problems associated with the quantitative use of dinitroanilines have been enumerated by Hess and Bayer (1977), Hess (1979) and Strachen and Hess (1982). These difficulties include the non-specific adsorption of dinitroanilines to container surfaces, presumably resulting from their low solubility in aqueous solutions, and the unavailability of dinitroanilines radiolabeled to high specific activity. In the present study, preliminary experiments showed that inclusion of 2% ethanol in the buffer (SIB) to be used for binding served to maintain the solubility of [^{14}C]oryzalin up to 12.5 μM. However, pilot experiments also demonstrated that although [^{14}C]oryzalin bound to rose tubulin, the reaction could not be detected reliably at <5 μM oryzalin because of the low-specific-activity ligand. These problems restricted the use of [^{14}C]oryzalin to a narrow concentration range (5.0–12.5 μM). Details on these experiments and the DEAE-filter disc method of binding are described in *Materials and methods*.

The dependence of the formation of rose tubulin-oryzalin complex (TO) upon conditions of time and pH were examined at 24° C. Tubulin (3.5 μM) was incubated with 10 μM [^{14}C]oryzalin and aliquots were assayed for binding at 10-min, 1-, 2- and 4-h time points. A plot of the mean molar binding ratio (r) of oryzalin and tubulin versus time (Fig. 5) shows that TO formation is slow, taking approx. 2 h to reach equilibrium. To determine whether TO formation is influenced by pH, tubulin (3.5 μM) was incubated at 24° C for 2 h with 5 μM [^{14}C]oryzalin in SIB previously adjusted to pH values in the range 6.3–7.7 and assayed for binding. A plot of r versus pH (Fig. 5, inset) shows that pH 7.1 is optimum for TO formation.

The dependence of TO formation on the concentration of oryzalin was tested by incubating tubulin (3.5 μM) at 24° C for 2 h with different concentrations of [^{14}C]oryzalin (5.0, 7.5, 10.0 and 12.5 μM). A plot of $r/[O_{free}]$ versus r (Scatchard

Table 1. Sedimentation analysis of oryzalin inhibition of polymer yield. Samples used in turbidimetric measurements (Fig. 3) were sedimented in a Beckman airfuge (Beckman Instruments, Palo Alto, Cal., USA) at 48000·g (23° C for 1 h) and polymer pellets were assayed for protein. Data are expressed as percent of control (100% = amount of polymer sedimented in the absence of oryzalin) after correction for aliquots used in electron microscopy

Source of tubulin	Oryzalin concn. (μM)	Amount of sedimented polymer (μg)	% of control
Rose cells	0	71	100
	1	77	108
	5	37	52
	7	4	6
Bovine brain[a]	0	84	100
	7	82	98

[a] Tubulin (10 μM) was polymerized with 27 μM taxol at 24° C for 1 h

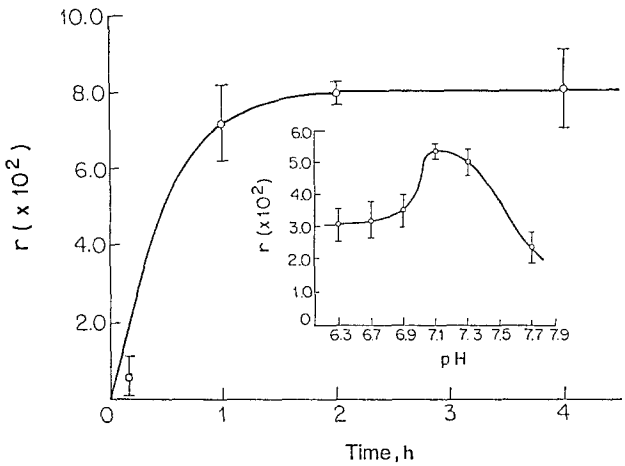

Fig. 5. Time- and pH-dependence of rose TO formation. Moles of oryzalin bound per mole of tubulin (r) is the mean molar binding ratio from triplicate 100-µl assays; r is expressed as a function of time. *Inset*: r expressed as a function of pH. Bars = SE of triplicate assays

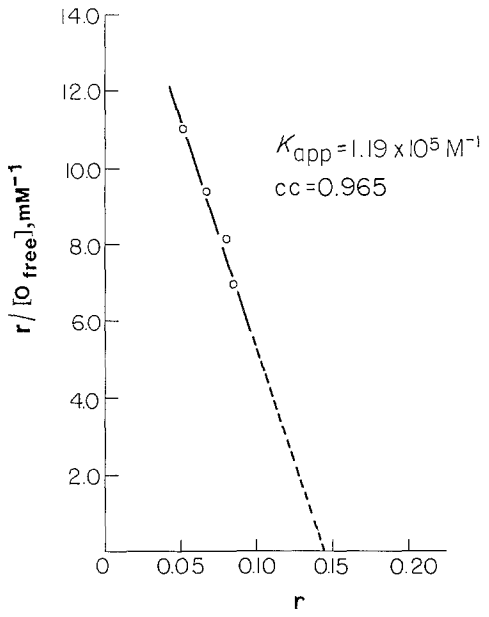

Fig. 6. Concentration-dependent binding of oryzalin to rose tubulin. The mean molar binding ratio (r) and the concentration of free oryzalin [O_{free}] were calculated from binding assays. Linear regression analysis of a plot of $r/[O_{free}]$ versus r yields a line with slope $= -1.19 \cdot 10^5\ M^{-1}$ (correlation coefficient = 0.965) and an extrapolated x-intercept (r) = 0.14. Each point represents the mean of triplicate 100-µl assays

1949) gave a line corresponding to a single class of binding sites (Fig. 6). The apparent binding constant (K_{app}) for TO formation is $1.19 \cdot 10^5\ M^{-1}$ and extrapolation to infinite concentrations of oryzalin (x-intercept) gave an estimated maximum binding stoichiometry of 0.14. To determine if the relatively low value of r may have resulted from an intrinsic

Table 2. Specificity of oryzalin binding to plant tubulin. [^{14}C]Oryzalin binding is expressed as the molar binding ratio (r) and is also shown as percent of the value obtained for rose tubulin (control)

Protein	r (10^{-2})	% of control
Rose tubulin (control)	8.00	100
Bovine brain tubulin	0.45	6
Bovine β-lactoglobulin	0.75	9
Bovine serum albumin	0.31	4
Chicken-egg lysozyme	0.00	0

rate of decay of the binding site on tubulin, 3.5 µM tubulin was incubated in SIB at 24° C and aliquots were taken at 2-h time points up to 10 h and examined for binding. No decrease in the extent of TO formation was found. However, the concentration range of these measurements does not satisfy certain requisite features of stoichiometry analysis outlined by Klotz (1982), a matter that is discussed below.

The specificity of [^{14}C]oryzalin binding to rose tubulin was surveyed by incubating various purified proteins (that bind to DEAE-cellulose) with 10 µM [^{14}C]oryzalin for 2 h at 24° C in SIB (pH 7.1) and assaying binding by the filter-disc method. The binding of [^{14}C]oryzalin to rose tubulin was approx. 17-fold greater than for bovine brain tubulin (Table 2). That the binding to brain tubulin may be non-specific was indicated by the observation that 3% more oryzalin bound to bovine β-lactoglobulin than to bovine brain tubulin. Bovine serum albumin and chicken-egg lysozyme had still lower binding of oryzalin. These results indicate that oryzalin binds selectively to plant tubulin. Because the critical concentration for taxol-induced rose-tubulin assembly (approx. 2.3 µM) (Morejohn and Fosket 1984a; Morejohn et al. 1987) is slightly lower than the concentration of tubulin (3.5 µM) applied to filter discs, the binding of [^{14}C]oryzalin to preformed taxol-MTs could not be determined sensitively with this method.

Discussion

Despite the generally accepted idea that dinitroaniline herbicides have anti-MT activities, it has been difficult to establish a direct temporal correspondence between the inhibition of MT-dependent processes and the depolymerization of MTs in intact plant tissues (Ashton and Crafts 1981). For example, ultrastructural studies have demonstrated that the dinitroaniline herbicides arrest mitosis and cause the disappearance of MTs in cells of roots (Bartels and Hilton 1973; Hess and Bayer 1974),

but Bayer et al. (1967) found evidence for non-uniform uptake and translocation in trifluralin-treated roots of several plants, making difficult the analysis of primary versus secondary effects of the herbicide on mitosis. Jackson and Stetler (1973) studied with light and electron microscopy the effects of trifluralin on mitosis in individual endosperm cells isolated from *Haemanthus katherinae* and found it to be inhibitory at very low concentrations ($\geq 3 \cdot 10^{-10}$ M). This plant tissue is quite appropriate for such a study, not only because it obviates considerations of differential herbicide translocation, but also because these cells possess no cellulosic wall that may non-specifically adsorb herbicide. Moreover, the mitotic apparatus of *Haemanthus* is enormous (70–100 μm) and contains large chromosomes whose condensation and movements can be viewed directly at low magnification on the light microscope (Molè-Bajer and Bajer 1968). However, Jackson and Stetler (1973) did not address fully the issue of threshold values of herbicide-treatment time, and in-vivo observations of the inhibition of chromosome movements were not closely correlated with the organization of MTs in a large number of cells because electron-microscope studies have the inherent limitation of small sample size. Because trifluralin binds to glass and is very insoluble in aqueous solutions (Strachen and Hess 1982), oryzalin was used as a model dinitroaniline in the current study on *Haemanthus* mitosis and MTs. Our observations demonstrate that the complete inhibition of chromosome migration by ≥ 0.1 μM oryzalin is correlated with the immediate (≤ 2 min) depolymerization of anaphase spindle MTs.This result indicates the direct binding of oryzalin to MT protein, a possibility that we have confirmed.

The value of K_{app} ($1.19 \cdot 10^5$ M^{-1}) for the oryzalin-rose tubulin interaction is remarkably similar to that ($2.08 \cdot 10^5$ M^{-1}) reported for the binding of oryzalin to *Chlamydomonas* flagellar tubulin (Strachen and Hess 1983). However, the different values of r for rose (0.14) and *Chlamydomonas* (1.0) tubulins, indicate some decay of the site on rose tubulin as is known to occur for the colchicine-binding site on animal tubulin (Wilson et al. 1976). While no decay of oryzalin binding to rose tubulin was detected after preincubation of tubulin in sucrose isolation buffer (SIB) for periods up to 10 h (24° C), some decay may have occurred during the isolation procedure prior to dialysis of tubulin into SIB. This possibility is indicated by the observations that *Chlamydomonas* TO formation is virtually instantaneous (Strachen and Hess 1983), but maximum rose TO formation is slow (2 h). In this regard, it is important to consider the limitations of the ligand-binding experiments reported by Strachen and Hess (1983) and herein. Because the oryzalin-tubulin binding reactions were observed by necessity within a narrow concentration range of ligand, it was not possible to determine reliably whether half the available oryzalin-binding sites were saturated (Klotz 1982). Furthermore, the low specific activity of [^{14}C]oryzalin has precluded an examination of the rate of TO dissociation (into dimer and free oryzalin) by dilution with excess unlabeled oryzalin. If TOs dissociated during measurement by "kinetic" methods (DEAE-cellulose filtration), the extent of TO formation will have been underestimated. Indeed, the complete inhibition of rose-tubulin polymerization by a stoichiometric concentration of oryzalin indicates that many, if not most, of the assembly-competent dimers do possess an active oryzalin-binding site. Thus, the values of K_{app} and r for rose tubulin must be regarded as approximations and are likely to represent minimum values for these binding parameters. Notwithstanding these qualifications, the similar binding constants derived for rose and *Chlamydomonas* tubulins when using kinetic methods indicate that the oryzalin-binding site has been conserved during evolution of the plant kingdom.

Upadhyaya and Noodén (1980) followed the kinetics of [^{14}C]oryzalin uptake and accumulation in maize root apices. They found the rates of both processes to be consistent with the binding of oryzalin to a single site; half-maximal values of oryzalin uptake ($K_m = 2.1 \cdot 10^{-5}$ M) and accumulation ($K_m = 3.7 \cdot 10^{-5}$ M) were similar. These rates indicate a moderate affinity binding of oryzalin to an intracellular site, but they are somewhat lower than the value of half-maximum binding derived from the reciprocal of K_{app} for rose TO formation. This disparity may result from the partitioning of ligand in root-tip cells between the aqueous cytoplasm and the lipid phase of membranes, inasmuch as oryzalin is very lipophilic and was used at concentrations near its aqueous solubility limit (Ashton and Crafts 1981; Fedtke 1982, pp. 123–141). Given the technical problems that we have found associated with measurements of [^{14}C]oryzalin binding to isolated plant tubulin, it is not surprising that Upadhyaya and Noodén (1980) did not detect [^{14}C]oryzalin binding to tubulin in extracts of maize roots. The detection of tubulin diluted in protein supernatants of root extracts seems impossible with the combined limitations of a low-specific-activity ligand and a binding constant of moderate strength.

Previous studies of the in-vitro effects of oryza-

Table 3. Comparison of properties of anti-MT compounds. Values of K_i (taxol-induced polymerization) and K_{app} were derived from in-vitro studies on *Rosa* tubulin, while minimum effective concentrations for inhibition of mitosis were obtained from in-vivo studies on endosperm cells from *Haemanthus*

Drug	K_i (μM)	K_{app} (M^{-1})	Effective concn. (μM)
Oryzalin	2.59	$1.19 \cdot 10^5$	0.01–0.10
Amiprophos-methyl	1.57[a]	not determined	0.01–0.10[b]
Colchicine	140.00[c]	$9.7 \cdot 10^2$[c]	125[d]

[a] Calculated from the data in Morejohn and Fosket (1984b)
[b] Bajer and Molè-Bajer (1986)
[c] Morejohn et al. 1987
[d] Molè-Bajer (1958)

lin and trifluralin on mammalian MTs (porcine brain) have yielded contradictory results. Bartels and Hilton (1973) found no effect on polymerization, Hertel and Marmé (1983) reported a partial inhibition of polymerization rates, and Robinson and Herzog (1977) observed complete inhibition of polymerization and nearly total depolymerization of previously assembled MTs. The meaning of these results is complicated further by the results of studies of dinitroaniline binding to mammalian tubulin. Bartels and Hilton (1973) found low levels of [^{14}C]trifluralin binding to MT proteins from porcine brain. No binding of [^{14}C]oryzalin to rat brain extract and no inhibition by oryzalin of [^{3}H]colchicine binding to rat tubulin was observed by Upadhyaya and Noodén (1980). Regardless of the interpretation of the preceeding experiments, the results are necessarily equivocal because of the presupposition that tubulins from plants and animals are pharmacologically similar. In these studies, protein from mammalian brain was utilized because this organ is a rich source of MT proteins and because a method for the isolation of cytoplasmic tubulin from higher plants had not been developed.

The inhibition by oryzalin of mitosis in *Haemanthus* cells and of rose MT polymerization in vitro is similar to that caused by the phosphoric amide herbicide amiprophos-methyl, but much more dramatic than that caused by colchicine (Molè-Bajer 1958; Morejohn and Fosket 1984a, b; Bajer and Molè-Bajer 1986; Morejohn et al. 1987). A summary of the inhibition constants (K_i) for polymerization, apparent affinity constants (K_{app}), and effective concentrations in cells for oryzalin, amiprophos-methyl and colchicine is given in Table 3. The sensitivity of MTs in vitro to these drugs is reduced from that seen in vivo because of the polymer-stabilizing effects of taxol. Nevertheless, these data demonstrate anti-MT activities of herbicides at concentrations that are considerably lower than those affecting either calcium uptake by mitochondria or other physiological processes (Ashton and Crafts 1981). The concentration of oryzalin (0.1 μM) that completely depolymerizes *Haemanthus* MTs within 2 min is 300-fold lower than that (30 μM) causing half-maximum inhibition of calcium uptake by plant mitochondria after 15 min (Hertel and Marmé 1983). Thus, an indirect depolymerization of MTs in cells by calcium (Hertel et al. 1980; Hertel and Marmé 1983) seems untenable given this disparity in effective concentrations of oryzalin.

We do not know the molecular mechanism by which oryzalin (or amiprophos-methyl) inhibits the polymerization of plant MTs. Interestingly, the effects of these herbicides on MTs in plant cells and on taxol-induced plant MT polymerization parallel closely the effects of colchicine and podophyllotoxin on animal MTs. Both colchicine and podophyllotoxin inhibit mitosis and depolymerize MTs in cultured animal cells at 0.1–1.0 μM (Dustin 1984). Both compounds inhibit the rate and extent of taxol-induced polymerization of brain tubulin at high nanomolar to low micromolar concentrations, and partially depolymerize previously assembled taxol-MTs. Maximum inhibition of taxol-induced polymerization of brain MTs is obtained at approx. 1:1 molar ratios of colchicine or podophyllotoxin and tubulin (Schiff and Horwitz 1981; Kumar 1981). Colchicine and podophyllotoxin are mutually competitive in binding to a single overlapping site on the animal-tubulin dimer, yet these drugs depolymerize MTs in cells and inhibit GTP-induced polymerization of brain MTs (without taxol) at concentrations *substoichiometric* to the tubulin-dimer concentration (Olmsted and Borisy 1973; Wilson et al. 1976). That is, only a small fraction of the total tubulin need be complexed with the drug for maximum anti-MT effects to occur. This poisoning mechanism involves the formation of tubulin-drug complexes that bind to MT

ends and prevent further assembly of unliganded tubulin (Margolis and Wilson 1977; Sternlicht and Ringel 1979). Because plant tubulin devoid of MT-associated proteins does not polymerize readily (Morejohn and Fosket 1982), we have relied upon the strong polymerization-promoting activity of taxol for these studies, and we have not been able to determine whether anti-MT herbicides act through a substoichiometric mechanism. The similar effects of taxol on the polymerization of plant and animal tubulins in vivo (Schiff and Horwitz 1980; Bajer et al. 1982) and in vitro (Schiff and Horwitz 1981; Kumar 1981; Morejohn and Fosket 1984a) indicate that under more physiological conditions of polymerization, oryzalin and amiprophos-methyl would inhibit plant MT assembly at much lower concentrations. Although we have demonstrated the binding of oryzalin to tubulin to form TOs, the effect of TO on plant MT assembly or disassembly could not be examined because of technical problems. The stability of TOs to dissociation following the separation of free oryzalin from liganded tubulin must be determined as well, and this problem awaits the development of more sensitive methods of measurement of herbicide binding to MT protein.

The moderate affinity binding of an anti-MT ligand to tubulin, as we have found for oryzalin and rose tubulin, is not necessarily inconsistent with very low effective concentrations of ligand in vivo. For example, the colchicine analogue 2-methyl-5-(2′,3′,4′-trimethoxyphenyl)tropone binds to animal tubulin with moderate strength ($K = 3.5 \cdot 10^5\ M^{-1}$) (Bane et al. 1984), inhibits mitosis maximally in HeLa cells at 0.1 μM (Fitzgerald 1976), and inhibits substoichiometrically the polymerization of brain MTs in vitro (Ray et al. 1981). Because this analogue is a competitive inhibitor of colchicine binding ($K_i = 1.6 \cdot 10^{-5}$ M) (Ray et al. 1981), its anti-mitotic activity probably results from a substoichiometric mechanism as does that of colchicine (Wilson et al. 1976). Thus, the moderate value of K_{app} for TO formation and the very low effective concentrations of oryzalin (0.1 μM) in vivo indicate an analogous (substoichiometric) mechanism in plant cells.

Our findings that oryzalin binds to plant tubulin and inhibits the polymerization of MTs strongly indicate that the dinitroanilines *directly* disrupt MTs in cells. Further corroboration of the direct interaction of dinitroanilines with plant MTs has come from recent studies on a dinitroaniline-resistant biotype of goosegrass (*Eleusine indica*). Mudge et al. (1984) reported that the resistant biotype arose spontaneously in cotton fields where trifluralin had been used for weed control. Vaughn (1986a) and Vaughn et al. (1987) have shown that MTs in the resistant biotype of *Eleusine* are cross-resistant not only to several members of the dinitroaniline class, but also to amiprophos-methyl. The resistant and sensitive biotypes are equally resistant to colchicine-treatment; MTs are depolymerized at 0.1–1 mM colchicine concentrations. Preliminary results (Vaughn 1986b) indicate that the β-subunit of the tubulin of the resistant biotype is modified. The results of our studies and those of Vaughn and co-workers are consistent with the notions that the dinitroaniline and phosphoric amide herbicides are more potent inhibitors of plant MT function than is colchicine, and that MTs are the most sensitive target of dinitroaniline action in higher plants.

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