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Rapid and Reversible High-Affinity Binding of the Dinitroaniline Herbicide Oryzalin to Tubulin from *Zea mays* L.[1]

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Oryzalin, a dinitroaniline herbicide, was previously reported to bind to plant tubulin with a moderate strength interaction (dissociation constant [K_d] = 8.4 μM) that appeared inconsistent with the nanomolar concentrations of drug that cause the loss of microtubules, inhibit mitosis, and produce herbicidal effects in plants (L.C. Morejohn, T.E. Bureau, J. Molé-Bajer, A.S. Bajer, D.E. Fosket [1987] Planta 172: 252–264). To characterize further the mechanism of action of oryzalin, both kinetic and quasi-equilibrium ligand-binding methods were used to examine the interaction of [^{14}C]-oryzalin with tubulin from cultured cells of maize (*Zea mays* L. cv Black Mexican Sweet). Oryzalin binds to maize tubulin dimer via a rapid and pH-dependent interaction to form a tubulin-oryzalin complex. Both the tubulin-oryzalin binding strength and stoichiometry are underestimated substantially when measured by kinetic binding methods, because the tubulin-oryzalin complex dissociates rapidly into unliganded tubulin and free oryzalin. Also, an uncharacterized factor(s) that is co-isolated with maize tubulin was found to noncompetitively inhibit oryzalin binding to the dimer. Quasi-equilibrium binding measurements of the tubulin-oryzalin complex using purified maize dimer afforded a K_d of 95 nM (pH 6.9; 23°C) and an estimated maximum molar binding stoichiometry of 0.5. No binding of oryzalin to pure bovine brain tubulin was detected by equilibrium dialysis, and oryzalin has no discernible effect on microtubules in mouse 3T3 fibroblasts, indicating an absence of the oryzalin-binding site on mammalian tubulin. Oryzalin binds to pure taxol-stabilized maize microtubules in a polymer mass- and number-dependent manner, although polymerized tubulin has a much lower oryzalin-binding capacity than unpolymerized tubulin. Much more oryzalin is incorporated into polymer during taxol-induced assembly of pure maize tubulin, and half-maximal inhibition of the rapid phase of taxol-induced polymerization of 5 μM tubulin is obtained with 700 nM oryzalin. The data are consistent with a molecular mechanism whereby oryzalin binds rapidly, reversibly, and with high affinity to the plant tubulin dimer to form a tubulin-oryzalin complex that, at concentrations substoichiometric to tubulin, copolymerizes with unliganded tubulin and slows further assembly. Because half-maximal inhibition of maize callus growth is produced by 37 nM oryzalin, the herbicidal effects of oryzalin appear to result from a substoichiometric poisoning of microtubules.

Microtubules are hollow, filamentous polymers composed principally of the globular protein tubulin, a 100-kD heterodimer having similar α- and β-subunits. Tubulin dimers polymerize in a head-to-tail manner to form 4- to 5-nm linear protofilaments, typically 13 of which are laterally associated in the wall of the 24-nm diameter microtubule (Amos, 1979; Fosket and Morejohn, 1992). During the plant cell cycle, microtubules are reversibly polymerized to form several functionally distinct arrays that mediate subcellular motility phenomena and provide cytosolic organization. The functions of plant microtubule arrays have been deduced in large part by observing particular subcellular processes after short-term treatments of plant cells with drugs causing the specific loss of microtubules (Lloyd, 1987). Among the various antimicrotubule compounds effective in plants, dinitroaniline herbicides are the most potent (Ashton and Crafts, 1981; Morejohn, 1991). Dinitroanilines are commercially important herbicides used to inhibit selectively the emergence of annual grass weeds from the soil surface in dicotyledonous crops such as soybean and cotton. These herbicides produce characteristic gross morphological effects in plants, including an inhibition of lateral root development and swollen root tips (Upadhyaya and Nooden, 1977; Ashton and Crafts, 1981). Herbicide-treated plants stop growing because cell division and elongation are inhibited, and cells take on isodiametric shapes in "tumor" roots because cellulose microfibril deposition becomes random. These cellular activities are affected when the normal microtubule-dependent polar distribution of subcellular components and biochemical processes is lost (Morejohn, 1991). For these reasons microtubules are considered to be essential subcellular determinants of plant morphogenesis (Lloyd, 1991).

The dinitroaniline herbicide oryzalin has been used as an antimicrotubule agent for studying microtubule-dependent processes not only in higher plants but also in mosses, algae, and a variety of protoctistan organisms (Morejohn, 1991). Oryzalin induces flagellar shortening, inhibits flagellar outgrowth, and binds to flagellar tubulin purified from the protoctistan alga *Chlamydomonas reinhardtii* (Quader and Filner, 1980; Strachen and Hess, 1983). Mutations resulting in single amino acid substitutions in the β-subunit of *Chlamydo-*

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Abbreviations: IB, isolation buffer; K_{app}, apparent association constant; K_d, dissociation constant; K_i, inhibition constant; TO, tubulin-oryzalin; r, molar binding ratio (moles oryzalin bound per mole tubulin).

monas tubulin confer complete resistance to several dinitroaniline herbicides (Schibler and Huang, 1991). Dinitroanilines inhibit growth and differentiation and bind to tubulin isolated from the parasitic protozoan *Leishmania mexicana* (Chan and Fong, 1990; Chan et al., 1991). Most recently, oryzalin was used to study the regulation of tubulin synthesis after depolymerization of microtubules in the ciliated protoctistan *Tetrahymena thermophila* (Stargell et al., 1992). In these different groups of organisms, millimolar colchicine concentrations are required to produce antimicrotubule effects similar to those obtained with low micromolar or nanomolar levels of oryzalin, apparently because their tubulins have a low affinity for colchicine (Morejohn and Fosket, 1986).

Studies of the molecular mechanism of dinitroaniline action provide important information concerning the structure of plant tubulin and the regulation of plant microtubule dynamics. Our previous work has shown that low micromolar concentrations of oryzalin inhibit the taxol-induced polymerization of tubulin isolated from cultured cells of rose (*Rosa* sp.) (Morejohn et al., 1987a). Ligand-binding experiments using the "kinetic" ligand-binding method of DEAE-cellulose disc filtration showed that oryzalin binds slowly to rose tubulin to form a TO complex with an apparent $K_d = 8.4$ μM ($K_a = 1.19 \times 10^5$ M^{-1}), and maximum r of 0.14. However, the relatively high K_d and low binding stoichiometry have appeared inconsistent with the observations that much lower oryzalin concentrations (≥100 nM) rapidly depolymerize microtubules and inhibit anaphase chromosome migration in isolated endosperm cells from *Hemanthus katherinae* Bak. (Morejohn et al., 1987a). Ligand-binding and polymerization experiments were performed with a buffer containing 1 M Suc, a tubulin-stabilizing component known to prevent the normal decay of colchicine binding to tubulin (Frigon and Lee, 1972). However, we have found recently that Suc inhibits both the rate and extent of taxol-induced plant microtubule polymerization (Bokros et al., 1993). Because Suc increases solution viscosity substantially, polymerization is inhibited, at least in part, by slowed diffusion rates of tubulin dimer and polymer, and the interaction of oryzalin and tubulin may be altered by Suc as well.

The purpose of this study was to reinvestigate in detail the interaction of oryzalin with plant tubulin and microtubules using solutions not containing Suc. We report here that in the absence of Suc, oryzalin binds rapidly, reversibly, and with high affinity and good capacity to tubulin from cultured cells of maize (*Zea mays* L. cv Black Mexican Sweet). Oryzalin interacts with maize tubulin polymer in a manner consistent with a previously proposed substoichiometric endwise binding mechanism that inhibits further tubulin polymerization. Concentrations of oryzalin producing herbicidal effects in maize cells are congruous with the idea that tubulin and microtubules are the primary targets of dinitroaniline action.

MATERIALS AND METHODS

Materials

Taxol was kindly provided by Dr. Nancita Lomax, Drug Synthesis and Chemistry Branch, Division of Cancer Treatment, National Cancer Institute (Bethesda, MD). Analytical grade (98.6%) unlabeled oryzalin (3,5-dinitro-N^4,N^4-dipropylsulfanilamide) and [ring^{14}C]oryzalin (specific activity 6.99 mCi mmol^{-1}) were gifts from Eli Lilly and Co. (Indianapolis, IN). Stock solutions of 10 mM taxol, 1 mM oryzalin, and 1 mM [^{14}C]oryzalin were prepared in 100% (v/v) DMSO and stored at −80°C. DEAE-cellulose filter discs (2.5-cm diameter) were from Whatman (Hillsboro, OR) and SpectraPor 7 mol wt cutoff 50000 dialysis tubing and ScintiVerse BioHP scintillation cocktail were from Fisher (Houston, TX). Gelrite was obtained from Scott Laboratories, Inc. (Carson, CA). Colchicine was obtained from Aldrich Chemical Co. (Milwaukee, WI). All other materials were purchased as described previously (Bokros et al., 1993).

Suspension Culture of Maize Cells

Suspension cultures of maize (*Zea mays* cv Black Mexican Sweet) were grown in the dark at 28°C on an orbital shaker as described previously (Bokros et al., 1993). At 7-d intervals, cells were subcultured by 10-fold dilution into fresh medium.

Isolation and Purification of Maize Tubulin

Maize tubulin was isolated from stationary phase (d 7–8) cells using DEAE-Sephadex A50 chromatography according to the method of Morejohn and Fosket (1982) with modifications as described by Bokros et al. (1993). Ammonium sulfate precipitates of DEAE-tubulin were resuspended in an IB consisting of 50 mM Pipes-KOH (pH 6.9), 1 mM EGTA, 0.5 mM $MgSO_4$, and 1 mM DTT and supplemented with 0.1 mM GTP, 50 μg mL^{-1} Na-*p*-tosyl-L-Arg methyl ester, and 5 μg mL^{-1} each of pepstatin A, leupeptin hemisulfate, and aprotinin. Small aliquots of tubulin were frozen in microfuge tubes by immersion in liquid N_2 and stored at −80°C until used.

For certain experiments, maize DEAE-tubulin was purified to homogeneity by a single cycle of taxol-induced microtubule polymerization and cold/Ca^{2+}-induced depolymerization (Bokros et al., 1993). Samples were thawed in an ice bath and clarified of particulates by centrifugation for 1 h at 100,000*g* (2°C) in a Beckman TL-100 ultracentrifuge (TLA-100 rotor). GTP was added to a final concentration of 1 mM, and tubulin (5 μM) was polymerized with 6 μM taxol (2% [v/v] DMSO) by gradual temperature ramping from 0 to 25°C. Polymer was collected by centrifugation through a cushion of 20% (w/v) Suc in IB containing 1 μM taxol for 40 min at 30,000*g* at 25°C. To prepare dimeric tubulin, taxol-stabilized polymer was depolymerized by resuspension to approximately 1 mg mL^{-1} in ice-cold IB containing 3 mM $CaCl_2$ for 30 min and clarified of residual stable polymer by centrifugation for 30 min at 30,000*g* (2°C). Taxol does not bind to unpolymerized tubulin and was separated from dimer by gel filtration chromatography on a Sephadex G10 column at 0°C as described previously (Collins and Vallee, 1987; Bokros et al., 1993). The resulting pure maize tubulin was diluted to appropriate concentrations for binding assays or polymerization experiments and was used immediately.

Protein Determinations

Protein was quantified by dye binding (Bradford, 1976) according to a standard assay procedure provided with the protein dye reagent from Bio-Rad (Richmond, CA). BSA from Sigma (St. Louis, MO) was used as a standard. Because BSA standard curves provide A values that are approximately 2-fold higher than those of several other gravimetrically determined proteins (Bio-Rad Instruction Manual) and bovine brain tubulin (Morejohn et al., 1984), the estimated concentrations of tubulin were adjusted upward by a factor of 2.

Oryzalin-Binding Measurements

Technical problems in handling dinitroaniline compounds for ligand-binding experiments have been documented (Strachen and Hess, 1982). One complication with oryzalin is its unavailability in a form that is labeled to high specific radioactivity. The relatively low specific radioactivity (6.99 mCi mmol^{-1}) of [^{14}C]oryzalin used in the present work limited our detection of oryzalin binding by scintillation counting to concentrations $\geq$0.1 μM when the ligand is dissolved in IB (containing 0.1 mM GTP and 2% [v/v] DMSO). Another complicating problem is that dinitroaniline compounds have very low solubility in aqueous solutions, although oryzalin is more soluble than most members of this herbicidal class of compounds and, thus, is regarded as a model dinitroaniline herbicide (Strachen and Hess, 1982, 1983). Although the maximum aqueous solubility of oryzalin is reported to be 7.2 μM (Fedtke, 1982), at concentrations substantially below this, we found that oryzalin is lost from solution by binding to glass or plastic container surfaces. Inclusion in the buffer of a low concentration of a polar solvent enhances slightly the solubility of oryzalin in aqueous solutions (Strachen and Hess, 1982; Morejohn et al., 1987a). For the current work, all solutions of IB contained a final concentration of 2% (v/v) DMSO.

The highly hydrophobic nature of oryzalin obviates the use of certain common methods of ligand binding, such as batch gel filtration, gel filtration column chromatography, and Hummel-Dreyer column gel permeation, because virtually all of the herbicide binds to the chromatographic matrix during separation (Strachen and Hess, 1983; Morejohn et al, 1987a). Nevertheless, two different methods of ligand binding, DEAE-cellulose disc filtration (Borisy, 1972) and equilibrium dialysis (Barnes et al., 1983), were used in this study, albeit with limitations, which are described below.

Kinetic Measurements of Oryzalin Binding to Tubulin

The DEAE-cellulose filter disc method (Borisy, 1972) was used to measure the time dependence and concentration dependence of oryzalin binding to tubulin. With this method [^{14}C]oryzalin is mixed with tubulin to allow binding, and the sample is applied to a DEAE-cellulose filter, to which the strongly polyanionic tubulin dimer (Fosket and Morejohn, 1992) adsorbs through electrostatic interactions. Unbound ligand is washed through the filter disc using a low ionic strength buffer such as IB, and the amount of ligand remaining bound to tubulin is determined by counting the radioactivity bound to tubulin adsorbed to filter discs (Borisy, 1972). This is considered a kinetic binding method because the measurement of bound ligand is made after washing away unbound ligand and, as such, constitutes a nonequilibrium situation.

With this method, tubulin samples isolated by DEAE-Sephadex chromatography were thawed in ice and clarified of particulates by centrifugation at 100,000g for 1 h at 2°C. Samples were diluted to 2.5 μM tubulin with IB containing 0.1 mM GTP, 2% (v/v) DMSO and desired concentrations of [^{14}C]oryzalin and were incubated at 23°C. At appropriate times, 100-μL aliquots (2.5 nmol) of tubulin (100 kD) (Fosket and Morejohn, 1992) were applied to a stack of three DEAE-cellulose filter discs that had been previously moistened with buffer in a Millipore filtration apparatus (12 ports). The disc-washing protocol was optimized as described previously (Morejohn et al., 1987b). Tubulin was slowly adsorbed (2 min) to the filter discs, and filters were washed four times with 5 mL of IB containing 2% (v/v) DMSO (23°C). Discs were air dried, placed in 8 mL of Scintiverse BioHP scintillation cocktail, and counted for radioactivity for 50 min per sample ($\leq$3% counting error) in a Beckman LS-133 liquid scintillation spectrophotometer. For each concentration of oryzalin assayed, samples containing [^{14}C]oryzalin and no tubulin (blanks) were used for correction of nonspecific binding of oryzalin to the DEAE-cellulose filters. Unless otherwise specified, all binding data were derived from the mean of triplicate binding assays.

Quasi-Equilibrium Measurements of Oryzalin Binding to Tubulin

The feasibility of using an equilibrium dialysis method (Barnes et al., 1983) for measurement of oryzalin binding to tubulin was tested in preliminary experiments. The solubility characteristics of oryzalin were examined by incubation of [^{14}C]oryzalin in a clear acrylic equilibrium dialysis apparatus (PGC Scientific Corp.) having five in-line cells composed of two 1-mL cavities. Cavities of each cell were separated by a cellulose SpectraPor 7 dialysis membrane (50,000 mol wt cutoff; PGC Scientific Corp.) (Barnes et al., 1983). Different concentrations of oryzalin (0.2–2 μM) dissolved in IB (containing 0.1 mM GTP and 2% [v/v] DMSO) were added to one cavity of each cell, buffer alone was added to the other cavity, and oryzalin was allowed to diffuse while the apparatus was gently agitated at 23°C on a vortex shaker. Triplicate samples (50 μL) of [^{14}C]oryzalin were taken from all cavities for periods up to 16.5 h and dissolved in scintillation cocktail (8 mL), and radioactivity was determined by scintillation counting (50 min per sample). The results showed a time-dependent diffusion of oryzalin across the membrane, with the accumulation of ligand in the receiving cavity and concomitant loss of ligand from the source cavity. Equal concentrations of oryzalin were obtained in both cavities of the cell within 3 h of dialysis. However, at all ligand concentrations tested, oryzalin bound to the acrylic walls of the chamber, resulting in a continuous and equal loss of total soluble ligand from both cavities of each cell.

The rate of oryzalin loss after 3 h was very slow and apparently linear, and no binding saturation to the chamber was found at any ligand concentration. [^{14}C]Oryzalin (2.5 μM)

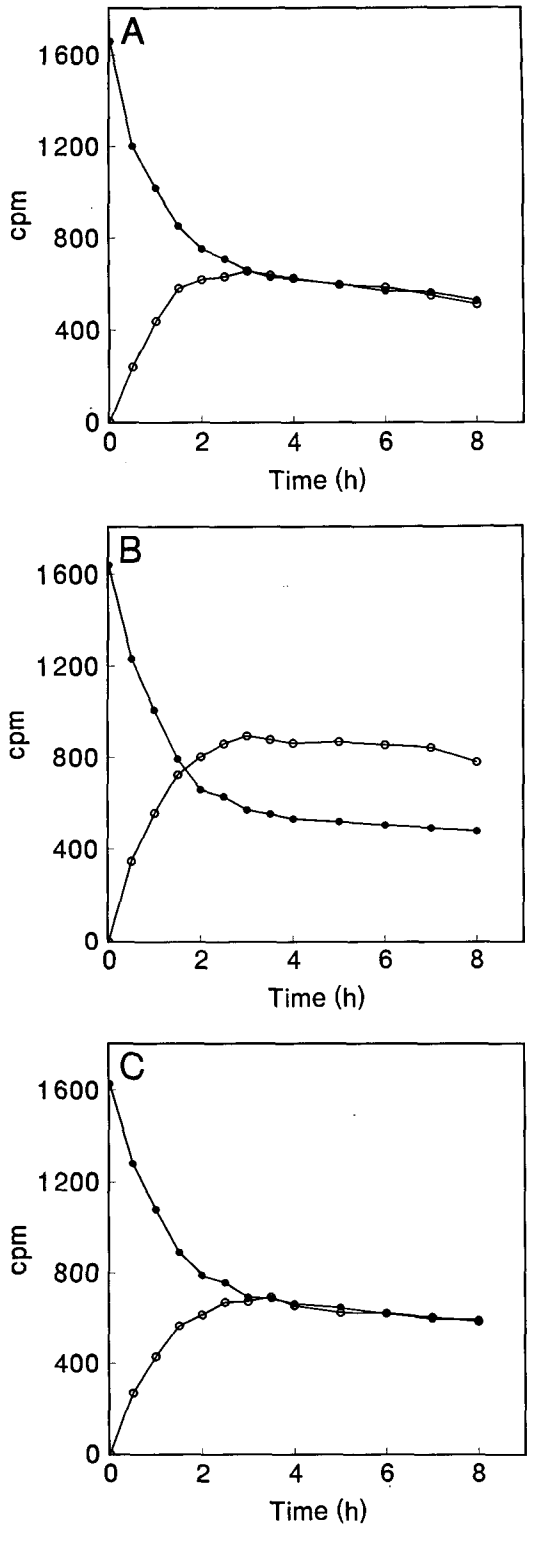

Figure 1. Time courses of oryzalin diffusion and binding during equilibrium dialysis. Amounts of radioactivity (cpm) in the cavities to which [^{14}C]oryzalin (●) or buffer/protein (○) was initially added are plotted versus time. Data are from experiments in which [^{14}C]-oryzalin was dialyzed against buffer (A), maize DEAE-tubulin (B), and bovine brain tubulin (C).

was dialyzed against buffer for 8 h at 23°C, samples (50 μL) were taken at 30- or 60-min intervals from both cavities of the cell, and radioactivity was measured by scintillation counting (Figure 1A). Although the theoretical oryzalin concentration in both cavities at equilibrium should have been 1.25 μM, the actual concentration determined by scintillation counting at 3 h was 0.99 μM, indicating a 21% loss of oryzalin. Thereafter, the oryzalin concentration continued to decrease at the slow, but constant, rate of 3% h^{-1}. At lower initial oryzalin concentrations, the extents and rates of oryzalin loss were lower. This pattern of oryzalin loss also occurred when [^{14}C]oryzalin was placed in both cavities of a cell.

When tubulin was placed in one cavity of each cell, different concentrations of [^{14}C]oryzalin were added to the other cavity, and dialysis was performed for 3 h at 23°C, oryzalin bound to tubulin in a ligand concentration-dependent fashion. Figure 1, B and C, shows examples of binding data obtained from the dialysis of 2.5 μM [^{14}C]oryzalin against maize DEAE-tubulin (2 μM) and bovine brain tubulin (2 μM), respectively. The data show maximum oryzalin binding to maize tubulin at 3 h and no binding to bovine brain tubulin. However, in the case of maize tubulin (Fig. 1B), the oryzalin concentration at 3 h was 1.13 μM, which is a 10% loss of oryzalin from solution and indicates that oryzalin binding to maize tubulin prevents the normal extent of oryzalin loss to the chamber walls. Figure 1B also shows that, subsequent to 3 h, oryzalin was lost at nearly the same rate (2% h^{-1}) as that seen in the absence of protein (Fig. 1A). Apparently, oryzalin continued to slowly adsorb to the chamber walls in the presence or absence of tubulin.

The possibility was examined that the chamber walls compete with tubulin for binding to soluble oryzalin. [^{14}C]Oryzalin was first placed in two cells and allowed to diffuse for 3 h, and maize tubulin was added to both cavities of one cell, and an equal volume of buffer not containing tubulin was added to both cavities of the other cell. After another 3 h of dialysis, the oryzalin concentrations in all cells were found to be the same, meaning that when oryzalin has bound nonspecifically to the chamber wall, it does not readily dissociate. This shows that the chamber wall does not compete substantially with tubulin for binding to oryzalin. Also, this finding indicates that, after 3 h of dialysis with tubulin in one cavity of a cell, the concentrations of free oryzalin on both sides of the chamber are the same and that the difference in radioactivity between the cavity containing both tubulin and oryzalin and the cavity containing only oryzalin represented the amount of oryzalin bound to tubulin. For these reasons, all data obtained in equilibrium dialysis experiments were derived from measurements taken from both cavities of each cell after 3 h, the earliest time that maximum oryzalin binding to tubulin may be observed under these solution conditions (Fig. 1B). Because after 3 h of dialysis the soluble ligand concentration continues to decrease very slowly and never stabilizes, a true binding equilibrium between tubulin and oryzalin is never achieved. Thus, apparent values of K_d for oryzalin binding to maize tubulin were derived from these quasi-equilibrium measurements.

Oryzalin Binding to Polymerized Tubulin

To examine the binding of oryzalin to the polymerized form of tubulin, 1 μM [^{14}C]oryzalin was incubated either with

tubulin during taxol-induced polymerization or with preformed taxol-stabilized microtubules. Preliminary experiments showed that 1 μM oryzalin does not completely inhibit taxol-induced polymerization or cause complete depolymerization of taxol-stabilized tubulin polymer at ≥2 μM tubulin. After incubation for 30 min at 23°C, samples of microtubules were layered on 20% (w/v) Suc cushions (40 μL) and sedimented for 30 min at 30,000*g* (25°C). To prevent contamination of microtubule pellets by unbound [^{14}C]oryzalin, supernatants were carefully removed by slow pipetting from the top of each centrifuge tube. Polymer pellets were gently resuspended in 40 μL of 100% (v/v) ethanol, residual [^{14}C]-oryzalin in the pellet region of centrifuge tubes was extracted with another 40 μL of ethanol, and samples were combined for further analysis. Care was taken also not to wash nonspecifically adsorbed [^{14}C]oryzalin off the supernatant region of centrifuge tubes. Protein assays were performed on one set of polymer pellets, and the amount of oryzalin binding to polymer was determined by scintillation counting with the other set. Samples for control experiments containing [^{14}C]oryzalin and no microtubules were treated identically and were used to correct for oryzalin nonspecifically adsorbed to the pellet region of the centrifuge tubes.

EM

The morphology of tubulin polymers was examined by negative-stain EM. Polymer samples (4 μL) were placed on 200-mesh copper grids coated with Formvar and carbon (Ted Pella, Redding, CA) and allowed to adsorb to the grid surface for 2 min. The sample solution was removed by wicking with a filter paper, grids were rinsed for 10 s with 4 μL of water, and adsorbed polymer was negatively stained for 1 min with 4 μL of 2% (w/v) uranyl acetate (Polysciences, Inc., Warrington, PA). Polymer samples were observed and photographed on a Hitachi HE-11A electron microscope at 50 kV.

Indirect Immunofluorescence Microscopy

Mouse 3T3 fibroblasts were obtained from Dr. Ben A. Murray, Department of Developmental and Cell Biology, University of California at Irvine. Cells were grown on microscope coverslips in 35-mm Petri dishes containing Dulbecco's modified Eagle's medium supplemented with 10% (v/v) fetal calf serum and were used when 70 to 80% confluent. Fibroblasts were incubated at 37°C in the presence of either 100 μM oryzalin (0.5% [v/v] DMSO), 0.5% (v/v) DMSO alone, or 0.6 μM colchicine. At desired times, coverslips were removed from dishes, washed with PBS, and processed for indirect immunofluorescence microscopy according to the method of Schultz and Kirschner (1987). Primary antibody was a 1:500 dilution of mouse monoclonal DM1A raised against α-tubulin (Amersham Corp., Arlington Heights, IL), and secondary antibody was a 1:100 dilution of fluorescein isothiocyanate-conjugated, rabbit anti-mouse antibody (Pierce Chemical Co., Rockford, IL). Observations and photographs were made on an Olympus BH-2 fluorescence microscope.

Inhibition of Maize Cell Growth by Oryzalin

Preliminary experiments using siliconized glass flasks showed that [^{14}C]oryzalin binds rapidly to the flask surface and that it is not possible to maintain constant concentrations of oryzalin for a typical 7-d suspension culture period. However, loss of oryzalin could be controlled by solubilization in solidified medium. To test the effect of oryzalin on growth, maize cell suspensions were grown as a lawn of callus on plastic Petri plates (150 mm). Specifically, suspension cells were collected in stationary phase (7 d), and large cell clumps were removed by filtration over a 30-mesh screen. Cells in small clumps were grown in suspension for 2 to 3 d more and resuspended in distilled water at 150 mg mL^{-1}, and 1 mL of this suspension was evenly distributed on the surface of medium solidified with 3.5 mg mL^{-1} of Gelrite and containing different concentrations (0.01–10 μM) of oryzalin. All plates contained a final concentration of 0.5% (v/v) DMSO. Cells were grown for 30 d in the dark at 30°C, and at 6-d intervals the callus lawn was washed off plates with a stream of distilled water. Cells were collected on Miracloth filters, and the fresh weight was determined.

Miscellaneous Techniques

Other techniques including SDS-PAGE, quantitative densitometry of gels, turbidimetric measurements of polymerization, and tubulin polymer sedimentation assays were performed exactly as described previously (Bokros et al., 1993).

RESULTS

General Characteristics of Oryzalin Binding to Maize Tubulin

Tubulin was isolated from stationary phase cells of maize by DEAE-Sephadex A50 chromatography (Morejohn and Fosket, 1982), with certain modifications (Bokros et al., 1993). Quantitative densitometry of Coomassie blue-stained SDS-PAGE gels containing DEAE-tubulin fractions (Fig. 2) estimated their purity to be 70 to 80% in different batches. The time dependence of the binding of [^{14}C]oryzalin to maize DEAE-tubulin was examined with the DEAE-cellulose disc filtration (Borisy, 1972), a kinetic ligand-binding method with which free ligand is washed away before measurement of bound ligand. Details concerning this ligand-binding method and known technical limitations of dinitroaniline binding, including those related to low aqueous solubility and low specific radioactivity of [^{14}C]oryzalin, are discussed in "Materials and Methods." Binding experiments were restricted to a concentration range of 0.1 to 3.0 μM oryzalin. Tubulin (3 μM) and [^{14}C]oryzalin (5 μM) were incubated at 23°C, and aliquots were assayed for binding at 5, 15, 30, 60, and 120 min. A plot of the mean *r* of oryzalin and tubulin versus time shows that a maximum level of oryzalin binding is observed within 5 min and that no substantial change in binding occurs for periods up to 2 h (Fig. 3A). This result demonstrates the apparently instantaneous formation of a maize TO complex. However, the mean value of *r* (0.03) in this experiment is very low and suggested that the TO complex dissociates rapidly into unliganded tubulin and free oryzalin during the

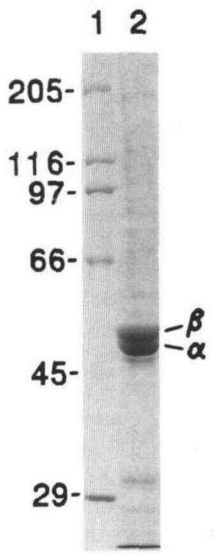

Figure 2. Coomassie blue-stained SDS-PAGE of DEAE-isolated maize tubulin. Lane 1 contains standard molecular mass markers (kD), and lane 2 contains 25 μg of the DEAE-tubulin fraction. Positions of tubulin α- and β-subunits are indicated.

filter washing protocol, which washes away and does not replace oryzalin.

The possibility of TO complex dissociation was tested with gel filtration column chromatography. We reasoned that, if TO complex readily dissociates into free oryzalin and unliganded tubulin under nonequilibrium conditions, then chromatographic separation of TO complex from free oryzalin would result in a substantial release of bound oryzalin from tubulin during the flow of TO complex through a gel filtration column. DEAE-tubulin (3 μM) was incubated with 3 μM [^{14}C]oryzalin for 5 min at 23°C to permit binding, and the sample (0.5 mL) was applied to a column (2-mL bed volume) of Sephadex G10 equilibrated with IB containing 0.1 mM GTP at 23°C, and the protein fraction was eluted within 3 min. The column was eluted further with 3 bed volumes of IB, and aliquots of the eluted fractions, along with samples of washed Sephadex G10 resin from the column, were counted for radioactivity. No radioactivity was detected in either the fraction containing tubulin or the fraction eluted subsequently, and >95% of the [^{14}C]oryzalin remained bound to the resin (data not shown). This observation not only confirmed that oryzalin binds to the Sephadex gel (Strachen and Hess, 1983; Morejohn et al., 1987a), but also showed that the TO complex rapidly dissociates into free oryzalin and unliganded tubulin upon separation of unbound oryzalin. The results also indicated that the extent of TO complex formation is underestimated substantially by the DEAE-cellulose filter disc method of ligand binding.

Further measurements of oryzalin-binding characteristics were made with equilibrium dialysis (Barnes et al., 1983), the limitations of which are described in detail in "Materials and Methods." To examine whether TO complex formation is affected by pH, DEAE-tubulin (2 μM) was incubated with [^{14}C]oryzalin (2 μM) in IB solutions adjusted to pH values in the range of 5.5 to 8.5, and after 3 h of dialysis at 23°C, samples were assayed for binding. A plot of *r* versus pH (Fig. 3B) demonstrates that substantial TO complex formation occurs across a wide pH range but that a sharp binding optimum exists at pH 6.9 (Fig. 3B). A comparison of these results with the kinetic binding measurements performed at pH 6.9 (Fig. 3A) shows that ≥5-fold more TO complex is detected with quasi-equilibrium binding measurements, although this level still appeared unreasonably low. When

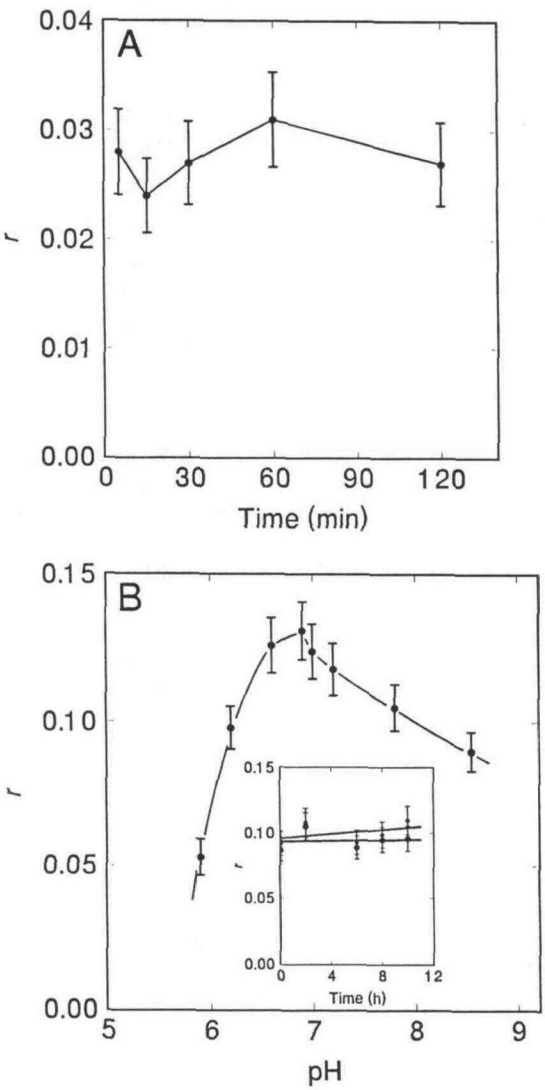

Figure 3. Characteristics of oryzalin binding to maize DEAE-isolated tubulin. A, Time dependence of oryzalin binding to DEAE-tubulin. Moles of oryzalin bound per mole of tubulin are expressed as the mean *r* and are plotted as a function of time. B, pH dependence and decay (inset) of oryzalin binding to DEAE-tubulin. Values of *r* were obtained by quasi-equilibrium measurements and are plotted versus pH or time of tubulin preincubation (inset). The inset shows *r* values obtained for DEAE-tubulin preincubated at either 0°C (●) or 23°C (○) for each time shown. Approximations of the decay rates of the oryzalin-binding site on tubulin are represented as lines derived by linear regression analysis of the data. All values of *r* are given ±SE from triplicate assays.

tubulin was boiled for 5 min before equilibrium dialysis, no binding of oryzalin was detected, indicating that the oryzalin-binding site is formed by tertiary or quaternary interactions of tubulin subunits.

We tested the notion that the relatively low values of r result from an intrinsic decay of the oryzalin-binding site on maize tubulin. Samples of DEAE-tubulin (1 μM) were incubated at 0 or 23°C, and at different times aliquots were mixed with 2 μM [^{14}C]oryzalin and assayed for binding at 23°C with equilibrium dialysis. A plot of r versus time of tubulin preincubation at each temperature is given in the inset to Figure 3B. The data show no decay of oryzalin binding on maize tubulin after preincubation at either temperature for periods up to 10 h. This result shows that the low values of r obtained with DEAE-tubulin do not result from a decay of the oryzalin-binding site on tubulin.

High-Affinity Oryzalin Binding to Maize Tubulin

The dependence of TO formation on the concentration of oryzalin was examined. DEAE-tubulin (2 μM) was incubated with different concentrations of [^{14}C]oryzalin (0.1–3 μM) for 3 h at 23°C, and binding was assayed with equilibrium dialysis (Barnes et al., 1983).

Figure 4A illustrates a plot of bound oryzalin versus free oryzalin at each ligand concentration and shows specific and saturable oryzalin binding to maize DEAE-tubulin. A semi-logarithmic plot of the concentration of free oryzalin versus r according to the method of Klotz (1982) produces a sigmoidal curve, with estimated half-maximal binding to tubulin (inflection point) at approximately 0.1 μM free oryzalin (Fig. 4A, inset). A plot of $r/[\text{Ory}_{\text{free}}]$ (μM^{-1}) versus r according to the method of Scatchard (1949) provides a line corresponding to a single affinity-class of oryzalin-binding sites having a K_d of 97 nM and a maximum estimated r of 0.2 (Fig. 3B). When the DEAE-cellulose filter disc method (Borisy, 1972) was used to measure the concentration dependence of oryzalin binding to maize DEAE-tubulin, Scatchard analysis provides an apparent K_d = 1.3 μM and r = 0.07 (Fig. 4B, inset). Thus, quasi-equilibrium binding measurements provide a 13.4-fold lower K_d and a 2.9-fold greater binding stoichiometry than obtained with kinetic binding measurements. These results confirm that substantial TO complex is lost during kinetic binding measurements and demonstrate that quasi-equilibrium measurements resolve higher levels of oryzalin-binding affinity and stoichiometry to maize tubulin. Nevertheless, the r value (0.2) obtained by equilibrium dialysis seemed unexpectedly low, because most maize tubulin dimers were anticipated to have at least one oryzalin-binding site, in which case the value of r should approach unity. Because the DEAE-protein fraction contains small amounts of various nontubulin polypeptides (Fig. 2, lane 2) that may interfere with oryzalin binding, maize tubulin was purified for subsequent ligand-binding experiments.

DEAE-tubulin was polymerized with taxol, polymer was sedimented through a Suc cushion as previously described (Bokros et al., 1993), and the fractions were analyzed by SDS-PAGE (Fig. 5A). Duplicate gels stained with Coomassie blue or silver show that all polypeptides contaminating the DEAE-tubulin fraction are retained in the supernatant frac-

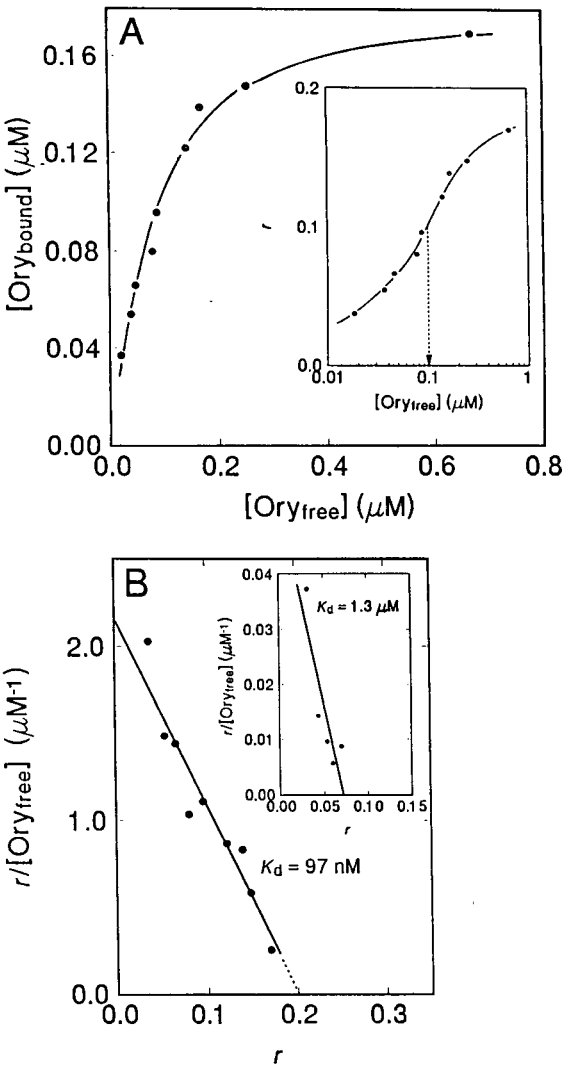

Figure 4. Oryzalin concentration-dependent binding to maize DEAE-tubulin. A, Saturation binding of oryzalin to DEAE-tubulin. The mean of triplicate binding assays at each concentration of oryzalin was used to determine the concentrations of bound oryzalin ([$\text{Ory}_{\text{bound}}$]) and free oryzalin ([$\text{Ory}_{\text{free}}$]). The inset shows the binding isotherm of TO formation at 23°C (Klotz, 1982). Values of r are plotted versus [Ory_{free}], and the arrow denotes the estimated concentration of oryzalin (1 μM) that coincides with the inflection point of the sigmoidal curve. B, Apparent dissociation constants for oryzalin binding to maize DEAE-tubulin. Binding data were analyzed by plotting $r/[\text{Ory}_{\text{free}}]$ (μM^{-1}) versus r according to Scatchard (1949). The slope of the linear regression represents the negative reciprocal of the K_d. The estimated maximum r is obtained by extrapolation of each regression line to the x intercept. Quasi-equilibrium binding measurements with DEAE-tubulin gives K_d = 97 nM (correlation coefficient = 0.95) and r = 0.2. The inset presents data obtained from kinetic binding measurements with DEAE-isolated tubulin, which provide K_d = 1.3 μM (correlation coefficient = 0.73) and r = 0.07.

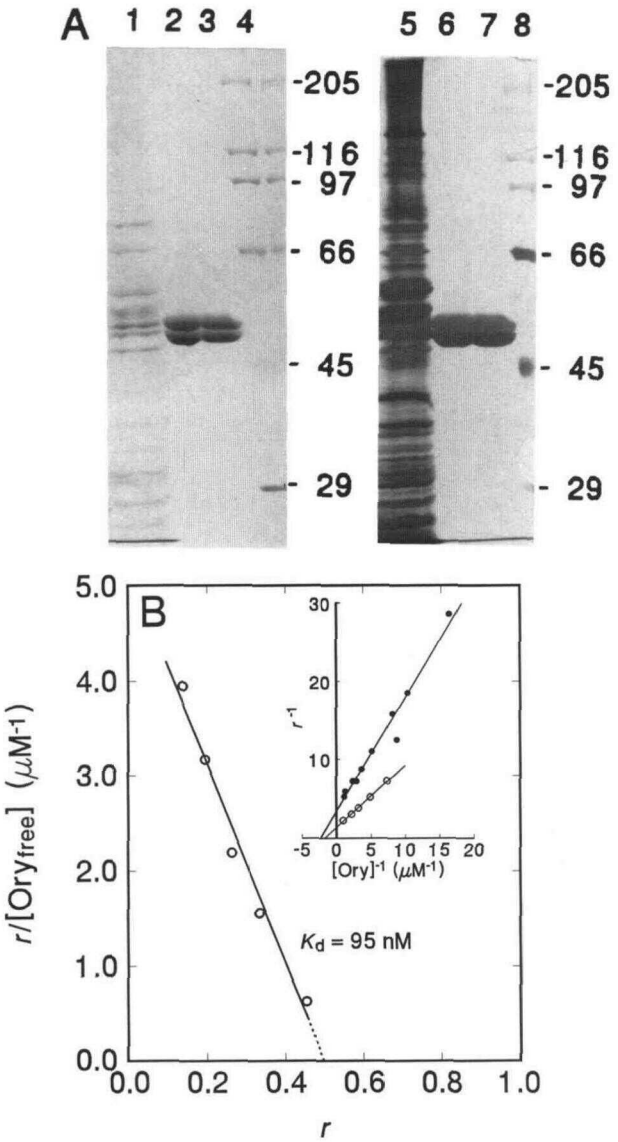

Figure 5. Oryzalin concentration-dependent binding to pure maize tubulin. A, SDS-PAGE analysis of maize tubulin purification. Gels were stained with Coomassie blue (lanes 1–4) and silver (lanes 5–8). Lanes 1 and 5 contain supernatant proteins (13 μg). Lanes 2 and 6 contain sedimented tubulin polymer (28 μg). Lanes 3 and 7 contain supernatant tubulin (26 μg) after cold/Ca^{2+} depolymerization and centrifugation. Lanes 4 and 8 contain standard molecular mass markers (kD). B, K_d for oryzalin binding to pure maize tubulin. Quasi-equilibrium binding data were plotted as described in the legend to Figure 4B and afford an apparent K_d = 95 nM (correlation coefficient = 0.98) and r = 0.5. The inset presents a Lineweaver-Burk transformation of quasi-equilibrium binding data derived from oryzalin (Ory) binding to pure maize tubulin (○) (correlation coefficient = 0.966), and to maize DEAE-tubulin (●) (correlation coefficient = 0.998) (shown in Fig. 4).

tion (Fig. 5A, lanes 1 and 5) and that polymerized tubulin is electrophoretically pure (Fig. 5A, lanes 2 and 6). Dimeric maize tubulin prepared by cold/Ca^{2+}-induced depolymerization of microtubules and sedimentation of residual polymer (discarded) is also electrophoretically pure (Fig. 5A, lanes 3 and 7). Taxol does not bind to unpolymerized tubulin (Collins and Vallee, 1987) and was removed from depolymerized maize tubulin by Sephadex G10 gel filtration chromatography at 0°C as described previously (Bokros et al., 1993).

The concentration dependence of oryzalin binding to pure maize tubulin was examined with equilibrium dialysis (Barnes et al., 1972), which provides quasi-equilibrium measurements as described in "Materials and Methods." Tubulin (1.5 μM) was mixed with different concentrations of [^{14}C]oryzalin (0.3–3.0 μM) and was assayed for binding after 3 h of dialysis at 23°C. Scatchard analyses of the data (Fig. 5B) afford an apparent K_d = 95 nM, which is nearly identical with that of DEAE-tubulin (K_d = 97 nM) (Fig. 4B) but also provides an estimated maximum molar binding stoichiometry (r = 0.5), which is 2.5-fold greater than that of DEAE-tubulin (r = 0.2) (Fig. 4B). When these quasi-equilibrium binding measurements for DEAE-tubulin and pure tubulin are treated by Lineweaver-Burk analysis, a pattern of noncompetitive inhibition is obtained (Fig. 5B, inset). Because no binding of [^{14}C]-oryzalin to the tubulin-depleted supernatant protein fraction (Fig. 5A, lanes 1 and 5) was detected by equilibrium dialysis experiments (data not shown), these results indicate the presence of a factor(s) in the DEAE-tubulin fraction that is co-isolated with maize tubulin during anion-exchange chromatography and is separated from tubulin by a single cycle of polymerization and depolymerization. Thus, both the oryzalin-binding strength and -binding capacity of maize tubulin are underestimated through the combined effects of the dissociation of the TO complex during kinetic binding measurements and an inhibitory factor(s) present in the DEAE-tubulin fraction.

We reported earlier (Morejohn et al., 1987a) that no binding of oryzalin to bovine brain tubulin could be detected with kinetic measurements made with the DEAE-cellulose filter disc method. This possibility was reinvestigated with equilibrium dialysis binding, and again no binding of 2.5 μM [^{14}C]-oryzalin to pure bovine brain tubulin (2 μM) was detected (Fig. 1C). This result confirms the selective binding of oryzalin to plant tubulin.

Oryzalin Inhibition of Taxol-Induced Maize Tubulin Polymerization

The effects of oryzalin on taxol-induced polymerization of maize tubulin were investigated with the same Suc-free buffer (IB) used in ligand-binding experiments. Purified maize tubulin dimer (5 μM) was mixed with IB containing taxol (5 μM) and different oryzalin concentrations (0.3, 0.9, 2.0, and 4.0 μM), and samples were shifted rapidly from 0 to 25°C. The polymerization kinetics were monitored turbidimetrically at 25°C by recording the continuous change in A_{350} for 1 h. Tubulin polymerized in the absence of oryzalin exhibits hyperbolic turbidity kinetics composed of a rapid early phase followed by a slow phase that approaches steady state at 1 h (Fig. 6). Oryzalin produces decreased rates and

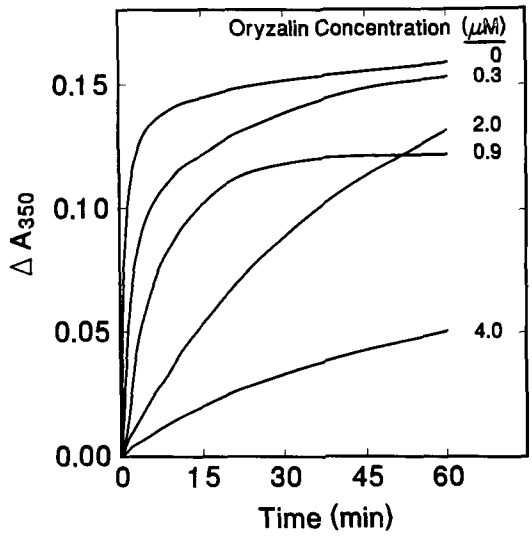

Figure 6. Oryzalin inhibition of taxol-induced maize tubulin polymerization. Continuous turbidity measurements (A_{350}) of oryzalin inhibition of taxol-induced polymerization of pure maize tubulin (5 μM) are presented as a function of time (min).

extents of tubulin polymerization, and maximum inhibition of polymerization is obtained at approximately 1:1 molar ratios of oryzalin and tubulin dimer (Fig. 6). A K_i for the rapid phase of taxol-induced polymerization was determined by calculation of the difference between the maximum rate of turbidity increase (slope derived from the steepest portion of the curve) for polymerization at each concentration of oryzalin (Morejohn et al., 1987b). A double-reciprocal plot of the fraction inhibition versus the oryzalin concentration gave a linear relationship, with an apparent K_i = 700 nM (analysis not shown). Thus, half-maximal inhibition of the rapid phase of taxol-induced maize tubulin (5 μM) polymerization is predicted to occur at a substoichiometric concentration of oryzalin (700 nM).

The morphology of maize tubulin polymers formed in the presence or absence of oryzalin after 1 h was examined by negative-stain EM. Numerous short microtubules (0.8 ± 0.1 μm) and a variety of polymorphic structures are formed by taxol in the absence of oryzalin (Fig. 7A). Some of these taxol-induced polymorphic structures appear as hoops and helical ribbon polymers composed of three to eight protofilaments (Fig. 7B), which we have observed previously to result from aberrant polymerization events during taxol-induced assembly (Bokros et al., 1993). Fewer polymorphic structures and fewer but longer microtubules are formed with increasing oryzalin concentrations. At the highest oryzalin concentration (4 μM), fewer polymorphic structures are formed and the microtubules (3.3 ± 0.5 μm) are very long (Fig. 7, C and D). These observations demonstrate that oryzalin inhibits the nucleation phase of taxol-induced polymerization more efficiently than the elongation phase and suppresses the formation of aberrant tubulin structures. The lengths of microtubules formed at each concentration of oryzalin are summarized in Table I.

The effect of oryzalin on the yield (mass) of taxol-induced polymer was determined after 1 h by sedimentation analysis (Morejohn et al., 1987a). Polymerized samples were centrifuged and polymer pellets were assayed for protein. The data in Table I show an oryzalin concentration-dependent decrease in the mass of taxol-induced polymer. A plot of polymer yield versus oryzalin concentration showed that half-maximal inhibition of the extent of taxol-induced polymerization of 5 μM tubulin is obtained with approximately 1.7 μM oryzalin (analysis not shown).

Oryzalin Depolymerization of Taxol-Stabilized Maize Microtubules

Gradual temperature ramping from 0 to 25°C yields normal appearing, long taxol-stabilized microtubules rather than short microtubules and polymorphic structures (Bokros et al., 1993). The ability of oryzalin to depolymerize taxol-stabilized microtubules assembled by gradual temperature ramping was investigated with polymer sedimentation analysis. Microtubules were purified by sedimentation through a Suc cushion and resuspended to 5 μM tubulin in IB containing different concentrations of oryzalin (0.1–50 μM) at 23°C. After 30 min of depolymerization, samples were sedimented and protein assays were performed on polymer pellets. The data in Figure 8 show an oryzalin concentration-dependent depolymerization of microtubules, with an estimated half-maximal depolymerization occurring at approximately 4 μM oryzalin. Nearly complete depolymerization of taxol-stabilized microtubules requires >50 μM oryzalin (Fig. 8). These results, along with those obtained regarding assembly inhibition above, indicate that oryzalin is more effective at inhibiting taxol-induced polymerization than in depolymerizing taxol-stabilized microtubules.

Interaction of Oryzalin with Maize Tubulin Polymer

The possibility that oryzalin binds to maize microtubules was examined. For this experiment, long maize microtubules were assembled with taxol by the gradual temperature-ramping protocol. Preliminary turbidity experiments demonstrated that oryzalin concentrations substoichiometric to tubulin cause only partial depolymerization of taxol-stabilized maize microtubules and that a new, lower steady-state level of polymer mass is achieved within 30 min. Thus, taxol-stabilized microtubules were divided into two equal samples, and aliquots of each were diluted to final concentrations of 2.5, 5.0, 7.5, and 10 μM tubulin in 75 μL of IB containing 1 μM [^{14}C]oryzalin. After 30 min of depolymerization at 23°C, polymer was sedimented through Suc cushions and pellets were assayed for protein and radioactivity. Protein assays demonstrated limited depolymerization of taxol-stabilized microtubules by 1 μM oryzalin, and a plot of moles of bound oryzalin versus moles of tubulin in microtubules shows a polymer mass-dependent increase in oryzalin binding (Fig. 9, closed circles). However, the mean molar binding stoichiometry (r = 0.003) derived from the slope of the line is much lower than stoichiometries obtained with unpolymerized tubulin in either kinetic or quasi-equilibrium binding measurements described above (Figs. 4B and 5B). This result demonstrates that polymerized tubulin has little capacity to bind

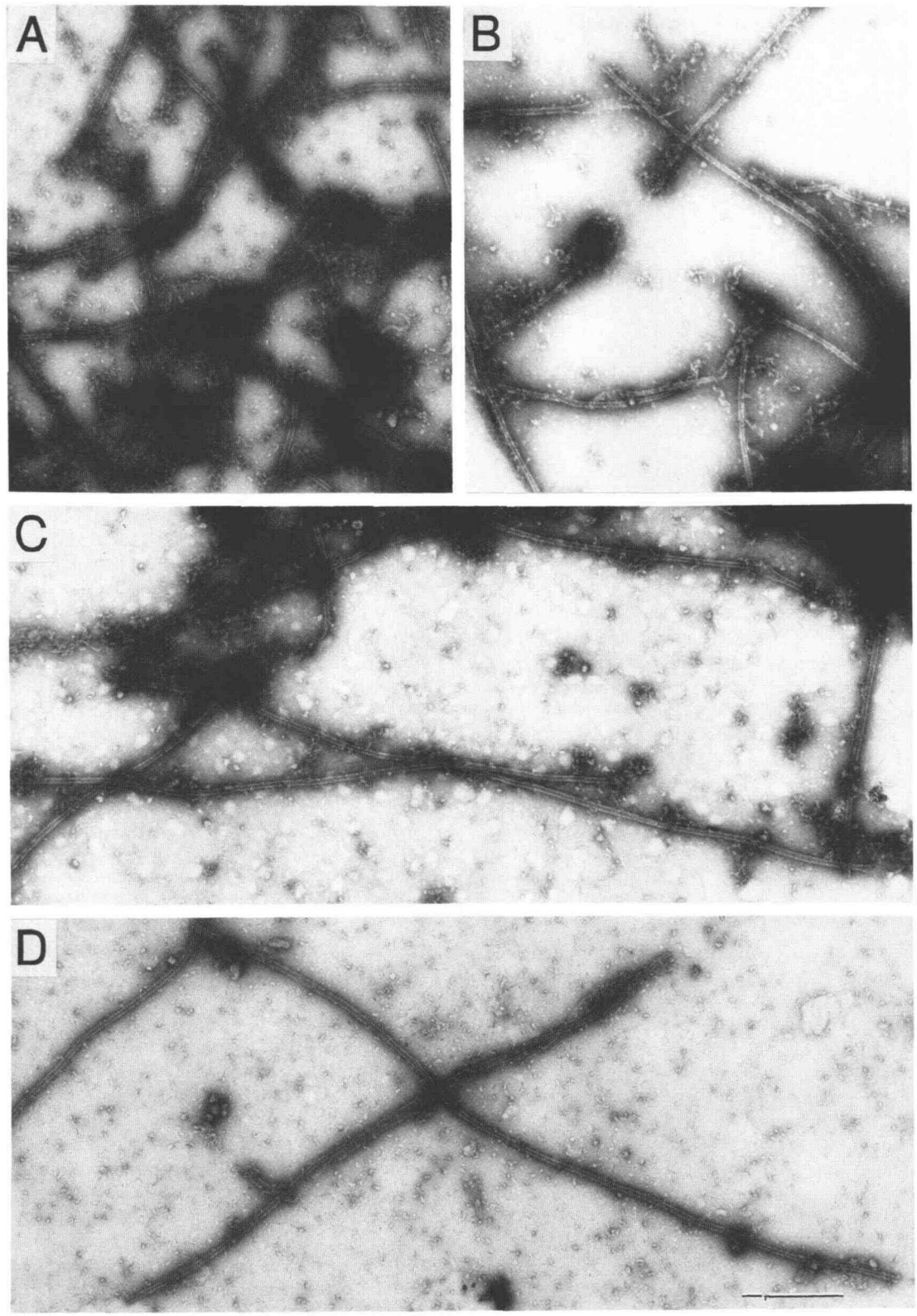

Figure 7. Electron micrographs of negatively stained taxol-induced tubulin polymers formed in the presence of 2% (v/v) DMSO (control) (A), 0.9 μM oryzalin (B), 2 μM oryzalin (C), and 4 μM oryzalin (D). Bar = 0.5 μm.

Table I. *Effects of oryzalin on taxol-induced microtubule length and polymer yield*

Microtubule length was measured directly from electron microscope negatives of negatively stained polymer samples, and protein assays were performed on polymer pellets described in Figure 7. The polymer yield for each concentration of oryzalin is expressed as a percentage of the control sample, which was taxol assembled in the presence of 1% (v/v) DMSO.

Oryzalin Concentration	Microtubule Length ± SE (*n*)	Sedimented Polymer	Polymer Yield
μM	μm	μg	% control
0	0.8 ± 0.1 (43)	196	100
0.3	Not done	200	102
0.9	1.3 ± 0.1 (27)	176	90
2.0	2.7 ± 0.1 (12)	72	37
4.0	3.3 ± 0.5 (7)	25	25

oryzalin and suggests that microtubule depolymerization results from the binding of oryzalin or the TO complex to microtubule ends.

The idea was tested that the antimicrotubule effects of oryzalin result from the incorporation of oryzalin into polymer during assembly. Duplicate samples of different concentrations of pure maize tubulin (3, 5, 7, and 10 μM) were assembled in 100 μL of IB containing taxol (10 μM) using a rapid temperature increase (0–25°C) in the presence of 1 μM [^{14}C]oryzalin. After 30 min the polymer samples were sedimented through Suc cushions, and polymer pellets were assayed for protein and radioactivity. A plot of bound oryzalin versus tubulin polymer mass (Fig. 9, open circles) shows that oryzalin is incorporated into polymer in a polymer mass-dependent manner. The mean molar binding stoichiometry (r = 0.016) is 5-fold greater than that found with depolymerized taxol-stabilized microtubules (r = 0.003), although the final masses of polymer in both experiments are similar (Fig. 9). However, the number of polymers generated during the rapid temperature increase assembly is much greater, because more nucleations occur than with gradual temperature ramping (Bokros et al., 1993). Thus, the greater oryzalin-binding stoichiometry occurring during polymerization results from the combined effects of more TO complex formation and its potential incorporation into a larger number of polymers. The molar binding stoichiometry obtained during polymerization of 1 μM [^{14}C]oryzalin and 5 μM tubulin (Fig. 9) shows that approximately 12 oryzalin molecules are incorporated per 1000 tubulin dimers in polymer. Because this concentration of oryzalin is similar to that producing half-maximal inhibition of the extent of 5 μM tubulin polymerization (1.7 μM) (Table I), these findings demonstrate that polymerization is inhibited by substoichiometric incorporation of oryzalin or TO complex into microtubules, presumably at their ends.

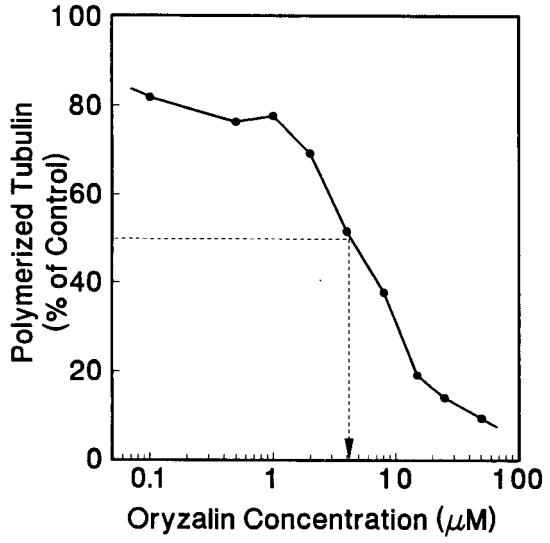

Figure 8. Oryzalin-induced depolymerization of taxol-stabilized maize microtubules. The amount of tubulin polymer obtained at each oryzalin concentration is expressed as a percentage of the control microtubule sample having no oryzalin. The dashed line and arrowhead indicate the oryzalin concentration (4 μM) at which half-maximal depolymerization of microtubules is obtained.

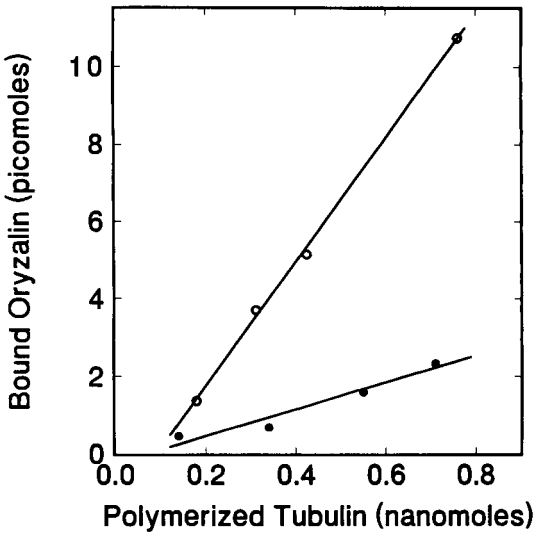

Figure 9. Interaction of oryzalin with tubulin polymer. Linear regression analysis of the data provides r = 0.003 (correlation coefficient = 0.94) for [^{14}C]oryzalin binding to taxol-stabilized microtubules (●) and r = 0.016 (correlation coefficient = 1.0) for [^{14}C]-oryzalin incorporation into taxol-induced tubulin polymer (○).

Oryzalin Inhibition of Maize Cell Growth

The K_d for oryzalin binding to pure maize tubulin (95 nM) is the concentration of herbicide at which half of the available oryzalin-binding sites on maize tubulin are saturated. The K_d value is also the oryzalin concentration anticipated to saturate half of the available dimers in the soluble tubulin pool in cells (Wilson et al., 1976; Hulme and Birdsall, 1992). If a substoichiometric mechanism of antimicrotubule action is operative in cells, then oryzalin concentrations that inhibit growth are predicted to be lower than the apparent K_d. To explore this hypothesis, the effect of oryzalin on the growth

of cultured maize cells was examined. Suspension cultures were transferred to Petri dishes and grown as a lawn at 30°C on solidified culture medium containing different concentrations of oryzalin (0.02–10 μM). At 6-d intervals, callus was rinsed off Petri dishes and collected on Miracloth filters, and fresh weights of cells were measured. The inhibition of growth by each concentration of oryzalin was calculated as a percentage of the control cells treated with 0.5% (v/v) DMSO and is plotted versus time in Figure 10. Cells treated with 0.5% (v/v) DMSO have growth characteristics that are indistinguishable from the untreated control cells (data not shown). Cells grown with oryzalin show a herbicide concentration-dependent inhibition of growth, although cultures treated with 20 to 80 nM oryzalin continue to grow slowly after 12 d. At ≥100 nM oryzalin, a complete inhibition of growth occurs (Fig. 10). A plot of the fraction growth inhibition versus oryzalin concentration at both 18 and 30 d provides an estimated half-maximal inhibition of growth at 37 nM oryzalin, a value that is 2.6-fold lower than the K_d (analysis not shown).

Resistance of Mammalian Cell Microtubules to Oryzalin

Microtubules in cells of monocotyledonous grasses show the greatest sensitivity to dinitroanilines, and microtubule depolymerization may be achieved after treatment with low micromolar or nanomolar concentrations of herbicide (Ashton and Crafts, 1981). We reported previously that 10 nM oryzalin slows the rate of chromosome migration during anaphase in isolated endosperm cells of the monocot *H. katherinae* Bak. and that after 2 min of treatment with ≥100 nM oryzalin, anaphase chromosome migration is completely inhibited, and interphase and mitotic microtubules are lost (Morejohn et al., 1987a).

Although we found that oryzalin has no effect on taxol-induced polymerization of bovine brain tubulin in vitro (Morejohn et al., 1987a), the effects of oryzalin on microtubules in mammalian cells have not been reported. Oryzalin effects were examined using mouse 3T3 fibroblasts, a model cell line used commonly to study the dynamics of the microtubule cytoskeleton (Schultz and Kirschner, 1987). Fibroblasts were incubated at 37°C in culture medium containing either 100 μM oryzalin (0.5% [v/v] DMSO), 0.5% (v/v) DMSO alone (negative control), or 0.6 μM colchicine (positive control). Although 100 μM oryzalin is not completely soluble in aqueous solutions, this concentration provides ≥7 μM free ligand over that which may adsorb nonspecifically to protein or lipid components in the culture medium (Fedtke, 1982; Strachen and Hess, 1982). At different times (0.5, 1, 2, and 4 h), cells were fixed and microtubules were visualized by indirect immunofluorescence microcopy (Schultz and Kirschner, 1987). Fibroblasts treated with oryzalin have abundant, normal-appearing microtubules during interphase and brightly staining spindles during mitosis, even after 4 h of treatment (Fig. 11A). The apparent number, length, and placement of microtubules in oryzalin-treated fibroblasts are indistinguishable from those observed in cells treated with DMSO alone (Fig. 11B). In contrast, after a 0.5-h treatment with 0.6 μM colchicine, many microtubules are lost, and only a few interphase microtubule bundles remain. After a 1-h treatment with colchicine, virtually all interphase and mitotic microtubules are lost, and only centrioles within centrosomes and short microtubules emerging from centrosomes are stained (Fig. 11C). These results confirm that microtubules in vertebrate cells are resistant to the depolymerizing effects of oryzalin.

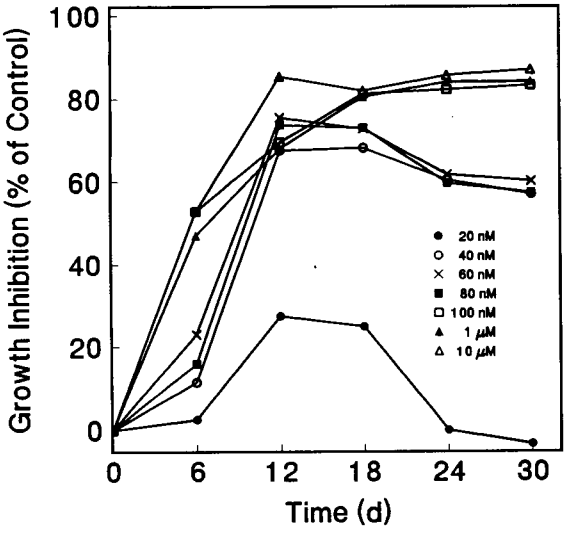

Figure 10. Oryzalin inhibition of maize cell growth. The percentage of increase in fresh weight of maize cells is shown as a function of time. All cultures treated with oryzalin had 0.5% (v/v) DMSO. Concentrations of oryzalin are indicated with symbols.

DISCUSSION

Our ligand-binding experiments demonstrate for the first time a high-affinity interaction of a dinitroaniline herbicide with plant tubulin. Oryzalin binds rapidly, reversibly, and in a pH-dependent manner to maize tubulin to form a TO complex having a very low apparent K_d (95 nM). The equilibrium dialysis method minimizes the dissociation of maize TO complex and provides a reasonable value for the maximum molar binding stoichiometry of oryzalin to tubulin (r = 0.5). Kinetic binding methods cause the dissociation of the maize TO complex, such that the affinity and molar binding stoichiometry are underestimated significantly. Because no decay of the oryzalin-binding site on DEAE-tubulin was observed, the binding stoichiometry of 0.5 suggests pharmacological heterogeneity among maize tubulin dimers. This possibility was recently demonstrated with bovine brain tubulin, in which dimers having different isotypic forms of β-tubulin were shown to bind colchicine differentially (Banerjee and Luduena, 1992).

Our previous work with oryzalin and rose tubulin relied solely on DEAE-cellulose disc filtration, and we observed a slow binding rate, a moderate affinity interaction (K_d = 8.4 μM), and a low binding stoichiometry (r = 0.14) (Morejohn et al., 1987a). The slow rate of binding was probably a trivial result of 1 M Suc in the binding buffer, because this concentration of Suc produces a high solution viscosity that may slow the diffusion rates of tubulin and oryzalin. Our present

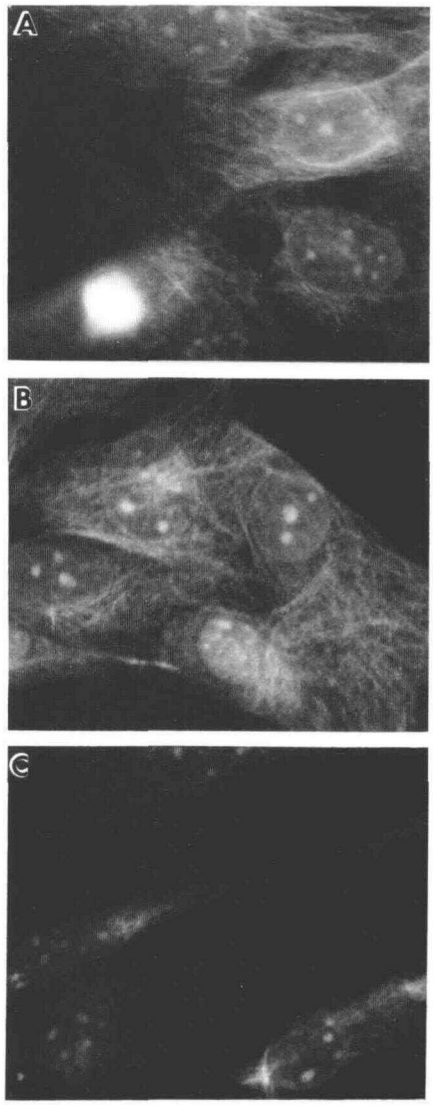

Figure 11. Effects of oryzalin on microtubules in mouse 3T3 fibroblasts. Microtubules in drug-treated cells were visualized with indirect immunofluorescence microscopy using anti-tubulin antibody. A, Control cells treated with 0.5% (v/v) DMSO for 4 h. B, Cells treated with 100 μM oryzalin for 4 h. C, Cells treated with 0.6 μM colchicine for 1 h.

results suggest that values of K_d and r for oryzalin binding to rose tubulin were underestimations resulting from the dissociation of the TO complex during washing steps of the kinetic binding method. However, it is also possible that DEAE-tubulin from rose cells contained a factor(s) that binds to the dimer and inhibits oryzalin binding, as we have found in the present study of DEAE-tubulin from maize cells. The factor(s) from maize cells apparently binds directly to the tubulin dimer and noncompetitively inhibits oryzalin binding. Because microtubule dynamics may be regulated, in part, by molecules that bind to and sequester the tubulin dimer (Kotani et al., 1990; Surridge and Burns, 1991), the identity of this factor(s) and the nature of its interaction with maize tubulin are currently under investigation.

At the present time, plant microtubule-associated proteins that stimulate tubulin polymerization have not been identified unequivocally; therefore, taxol was used to induce maize tubulin assembly. Although high nanomolar to low micromolar concentrations of oryzalin produce antimicrotubule effects in vitro, the antimicrotubule efficacy of oryzalin is masked substantially by the use of taxol, because its polymerization-promoting and -stabilizing effects are much stronger than those of microtubule-associated proteins (Schiff et al., 1979; Kumar, 1981). Taxol reduces the maize tubulin critical concentration nearly 14-fold, from 0.8 to 0.9 mg mL^{-1} to 0.06 mg mL^{-1}, and provides much more polymer from the available tubulin (Bokros et al., 1993). Taxol is known to slow or inhibit microtubule kinetics such as dynamic instability and treadmilling (Schiff and Horwitz, 1980; Kumar, 1981; Caplow and Zeeberg, 1982; Molé-Bajer and Bajer, 1983), such that in the absence of taxol 10- to 100-fold lower oryzalin concentrations will produce antimicrotubule effects in plant cells (Morejohn et al., 1987a).

Taxol-induced assembly of plant tubulin occurs so rapidly during a 0 to 25°C temperature increase that short microtubules and a variety of aberrant polymorphic structures such as hoops and helical ribbons are formed. Analysis of microtubule polymerization by time-resolved synchrotron x-ray scattering has shown these aberrant structures to result from the premature elongation of incompletely formed nuclei (Bordas et al., 1983; Mandelkow et al., 1983). Apparently, oryzalin suppresses the formation of aberrant nuclei and/or inhibits their elongation into structures such as helical ribbons while permitting elongation of fewer, but longer, morphologically normal microtubules. These results suggest that oryzalin inhibits the nucleation phase more effectively than the elongation phase of taxol-induced maize tubulin polymerization (Oosawa and Asakura, 1975).

Our ligand-binding results support a relatively simple mechanism involving a rapid bimolecular interaction between tubulin (T) and oryzalin (O) to form a high-affinity TO complex that is completely reversible according to the scheme:

$$T + O \rightleftharpoons TO.$$

The properties of oryzalin binding to tubulin are very different from those of colchicine binding, because oryzalin binding is not time dependent, and the TO complex dissociates completely into unliganded tubulin and free oryzalin. Colchicine binds slowly to tubulin, taking 2 to 4 h to reach equilibrium, and the tubulin-colchicine complex does not readily dissociate. The reaction proceeds via a two-step mechanism. First, tubulin binds to colchicine via a bimolecular interaction in which a rapid and relatively low-affinity preequilibrium tubulin-colchicine (TC) complex is formed and is completely reversible. This is followed by the slow unimolecular formation of a stable tubulin-colchicine (TC^*) complex having high affinity and essentially no reversibility. This two-step interaction is represented in the scheme:

$$T + C \rightleftharpoons TC \rightleftharpoons TC^*.$$

The second step is the result of a slow conformational change in tubulin and possibly in the colchicine molecule as

well (Hastie, 1991). The property of TC* nondissociation appears to be highly conserved among tubulins from diverse organisms such as plants and mammals. For example, tight binding of colchicine has been reported with tubulins isolated from cultured cells of carrot (*Daucus carota* L. cv Kintoki), rose (*Rosa* sp. cv Paul's scarlet), and hibiscus (*Hibiscus rosa-sinenis*) (Okamura, 1983; Morejohn et al., 1984, 1987b) and a wide array of animal species (reviewed by Hastie, 1991). The nondissociation of TC* permits the use of kinetic ligand-binding methods for comparative estimations of colchicine-binding affinity and stoichiometry. When measured in identical solutions by the DEAE-cellulose filter disc method, the maximum estimated binding stoichiometry of colchicine and rose tubulin ($r = 0.47$) and bovine brain tubulin ($r = 0.45$) are very similar (Morejohn et al., 1987b). Colchicine binding to animal tubulin is readily detected at colchicine concentrations substoichiometric to the dimer, because colchicine has a high affinity for brain tubulin ($K_{app} = 2.46 \times 10^6$ M^{-1}) (Morejohn et al., 1987b). However, colchicine binding to rose tubulin is detectable only when a vast stoichiometric excess of colchicine is used, because it has a very low affinity for rose tubulin ($K_{app} = 9.7 \times 10^2$ M^{-1}) (Morejohn and Fosket, 1984; Morejohn et al., 1987b). Thus, the presumptive rapid bimolecular interaction of plant tubulin and colchicine to form the preequilibrium TC complex must have an extremely low affinity and rapid reversibility, requiring excess colchicine to drive the binding reaction. The subsequent unimolecular formation of a nondissociating plant TC* is apparently slow, like that of mammalian tubulin (Okamura, 1983; Morejohn et al., 1987b). These results indicate that the property of tight binding per se does not necessarily confer a high-affinity interaction of a ligand with tubulin. In the case of oryzalin binding to maize tubulin, we document a rapidly formed high-affinity TO complex having complete reversibility, a finding that demonstrates that tight binding is not essential for the productive interaction of this herbicide with plant tubulin.

Our studies show that unpolymerized tubulin has a much higher oryzalin-binding capacity than polymerized tubulin and that the primary effect of oryzalin is to bind to dimer to form a TO complex. The precise molecular mechanism by which the TO complex may inhibit microtubule polymerization and depolymerize microtubules is more elusive. To examine the specific effects of TO complex on microtubules, it must first be separated from free oryzalin, but this has not been possible because the TO complex dissociates rapidly into unliganded tubulin and free oryzalin. Nevertheless, our data are consistent with the hypothesis that antimicrotubule effects are caused by copolymerization of the TO complex with tubulin. If the binding of only a few TO complexes to the ends of microtubules is sufficient to slow or inhibit subsequent addition of unliganded dimer, the polymerization process would become substoichiometrically poisoned. This mechanism is similar to that proposed for the inhibition of animal tubulin assembly by TC* complex (Wilson et al., 1976; Margolis and Wilson, 1977). A more complete understanding of the mechanism of oryzalin binding to tubulin requires a determination of whether TO exists as a conformationally distinct species, a possibility that may require circular dichroic spectra analysis.

Tubulin was identified originally in mammalian brain extracts by following the colchicine-binding activity of chromatographically separated proteins (Weisenberg et al., 1968). Later, the colchicine-binding assay was used also to isolate tubulin from extracts of cultured carrot cells (*D. carota*) (Okamura, 1983). However, when [^{14}C]oryzalin binding was used as an assay during the chromatographic separation of maize root proteins, no tubulin-like proteins were found to co-chromatograph with oryzalin (Upadhyaya and Nooden, 1980). The results of our oryzalin-binding experiments explain why these negative results were obtained; any oryzalin bound initially to tubulin in protein extracts would have dissociated rapidly and bound irreversibly to the chromatographic matrix. The use of an equilibrium binding method may be sensitive enough to detect tubulin in crude plant protein extracts, but the presence of factors that bind to tubulin may reduce the binding activity, as we have found with maize DEAE-tubulin.

Although it is generally accepted that dinitroanilines cause the disappearance of microtubules in cells of plants, algae, and certain protoctists, the primary mode of action of these herbicides has remained controversial (Morejohn, 1991). Dinitroanilines have been reported to inhibit RNA, lipid, and protein synthesis and photosynthesis and respiration (Ashton and Crafts, 1981). Inhibition of macromolecular synthesis may occur as a long-term indirect effect of the loss of the microtubule cytoskeleton and its motility and organizational functions in the cytosolic compartment (Lloyd, 1987, 1991). Most of these physiological processes probably do not depend immediately on microtubule function. The effects on photosynthesis and respiration most likely result from the demonstrated nonspecific intercalation of lipophilic dinitroanilines into organellar and plasma membranes, particularly at oryzalin concentrations that approach or exceed their aqueous saturation levels (Hertel et al., 1980; Upadhyaya and Nooden, 1980; Hertel and Marmé, 1983). In any case, the dinitroaniline levels necessary to produce these effects usually far exceed minimum herbicidal concentrations (Ashton and Crafts, 1981).

Middle micromolar dinitroaniline concentrations were reported to inhibit the in vitro uptake of calcium by isolated plant mitochondria (Hertel et al., 1980; Hertel and Marmé, 1983). Because mitochondria are involved in sequestration of calcium from the cytosolic compartment, and calcium inhibits the in vitro polymerization of microtubules, dinitroanilines were proposed to indirectly depolymerize microtubules in plant cells by release of mitochondrial calcium into the cytosolic compartment (Hertel et al., 1980; Hertel and Marmé, 1983). Recently, however, this idea was tested in vivo by examining calcium levels in oryzalin-treated stamen-hair cells of *Tradescantia virginiana* L. (Keifer et al., 1992). Concentrations of oryzalin (1–3 μM) that inhibit mitosis in 50% of cells have no effect on intracellular calcium levels as monitored by very sensitive fluorescence measurements with the calcium-binding indicator indo-1 (Keifer et al., 1992). Thus, although calcium depolymerizes plant microtubules in vitro (Bokros et al., 1993), and calcium flux may regulate microtubule dynamics during plant mitosis (Hepler, 1989), there is no evidence that oryzalin deregulates calcium in vivo.

Early work showed that dinitroanilines neither inhibit the

polymerization of animal tubulins nor depolymerize animal microtubules in vitro (Bartels and Hilton, 1973; Hess and Bayer, 1977; Hertel and Marmé, 1983). Having made the reasonable assumption that tubulin is a highly conserved protein, Bartels and Hilton (1973) and Hertel and Marmé (1983) further presupposed that animal and plant tubulins are similar pharmacologically and, therefore, that dinitroanilines do not bind to plant tubulin. It is clear now from sequencing studies that plant and mammalian tubulins are highly conserved (Fosket and Morejohn, 1992) but that they are also pharmacologically distinct (Morejohn, 1991).

Oryzalin is ineffective as an antimicrotubule drug in animal cells. Oryzalin was previously reported to have no effect on microtubules in amphibian (*Xenopus leavis*) cells (Morejohn et al., 1987a), and our current finding that oryzalin has no effect on microtubules in mouse 3T3 fibroblasts extends this property of resistance to include mammals. Trifluralin, a much more hydrophobic dinitroaniline, was reported to have no effect on the cleavage of fertilized sea urchin (*Lytechinus*) eggs or on the division of normal and transformed cell lines of sheep lung (Hess and Bayer, 1977). Oryzalin has no effect on mammalian brain tubulin polymerization in vitro (Bartels and Hilton, 1973; Morejohn et al., 1987a), and in the present study no binding of oryzalin to brain tubulin was detected with sensitive equilibrium dialysis experiments. Thus, it appears that both invertebrate and vertebrate tubulins lack a dinitroaniline herbicide-binding site.

Both oryzalin-sensitive and oryzalin-resistant plant species have been documented (Ashton and Crafts, 1981; Cleary and Hardham, 1988) and may have different mechanisms of herbicide uptake and accumulation (Upadhyaya and Nooden, 1987) and/or differences in their tubulin-binding affinity for oryzalin. Because maize and most cereal grasses are dinitroaniline-sensitive species (Ashton and Crafts, 1981), it will be interesting to determine in future studies whether tubulin from these organisms has distinct oryzalin-binding properties from that of resistant species.

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LITERATURE CITED

Amos LA (1979) Structure of microtubules. *In* K Roberts, JS Hyams, eds, Microtubules. Academic Press, London, pp 1–64

Ashton FM, Crafts AS (1981) Dinitroanilines. *In* FM Ashton, AS Crafts, Mode of Action of Herbicides. John Wiley, New York, pp 180–223

Barnes LD, Robinson AK, Williams RF, Horowitz PM (1983) Binding of colchicine to renal tubulin at 5°C. Biochem Biophys Res Commun **116:** 866–872

Banerjee A, Luduena RF (1992) Kinetics of colchicine binding to purified β-tubulin isotypes from bovine brain. J Biol Chem **267:** 13335–13339

Bartels PG, Hilton JL (1973) Comparison of trifluralin, oryzalin, pronamide, propham, and colchicine treatments on microtubules. Pestic Biochem Physiol **3:** 462–472

Bokros CL, Hugdahl JD, Hanesworth VR, Murthy JV, Morejohn LC (1993) Characterization of the reversible taxol-induced polymerization of plant tubulin into microtubules. Biochemistry **32:** 3437–3447

Bordas J, Mandelkow E-M, Mandelkow E (1983) Stages of tubulin assembly and disassembly studied by time-resolved synchrotron X-ray scattering. J Mol Biol **164:** 89–135

Borisy GG (1972) A rapid method for quantitative determination of microtubule protein using DEAE-cellulose filters. Anal Biochem **50:** 373–385

Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem **72:** 248–254

Caplow M, Zeeberg B (1982) Dynamic properties of microtubules at steady state in the presence of taxol. Eur J Biochem **127:** 319–324

Chan MM, Fong D (1990) Inhibition of leishmanias but not host macrophages by the antitubulin herbicide trifluralin. Science **249:** 924–926

Chan MM-Y, Triemer RE, Fong D (1991) Effect of the anti-microtubule drug oryzalin on growth and differentiation of the parasitic protozoan *Leishmania mexicana*. Differentiation **46:** 15–21

Cleary AL, Hardham AR (1988) Depolymerization of microtubule arrays in root tip cells by oryzalin and their recovery with modified nucleation patterns. Can J Bot **66:** 2353–2366

Collins CA, Vallee RB (1987) Temperature-dependent reversible assembly of taxol-treated microtubules. J Cell Biol **105:** 2847–2854

Fedtke C (1982) Microtubules. *In* C Fedtke, ed, Biochemistry and Physiology of Herbicide Action. Springer-Verlag, Berlin, Germany, pp 123–141

Fosket DE, Morejohn LC (1992) Structural and functional organization of tubulin. Annu Rev Plant Physiol Plant Mol Biol **43:** 201–240

Frigon RP, Lee JC (1972) The stabilization of calf-brain microtubule protein by sucrose. Arch Biochem Biophys **153:** 587–589

Hastie SB (1991) Interactions of colchicine with tubulin. Pharmacol Ther **51:** 377–401

Hepler PK (1989) Calcium transients during mitosis: observations in flux. J Cell Biol **109:** 2567–2573

Hertel C, Marmé D (1983) Herbicides and fungicides inhibit Ca^{2+} uptake by plant mitochondria: a possible mechanism of action. Pestic Biochem Physiol **19:** 282–290

Hertel C, Quader H, Robinson DG, Marmé D (1980) Anti-microtubular herbicides and fungicides affect Ca^{2+} transport in plant mitochondria. Planta **149:** 336–340

Hess FD, Bayer DE (1977) Binding of the herbicide trifluralin to *Chlamydomonas* flagellar tubulin. J Cell Sci **24:** 351–360

Hulme EC, Birdsall NJM (1992) Strategy and tactics in receptor-binding studies. *In* EC Hulme, ed, Receptor-Ligand Interactions. Oxford University Press, New York, pp 63–76

Keifer AQ, Calaham DA, Hepler PK (1992) Inhibitors of cell division and protoplasmic streaming fail to cause a detectable effect on intracellular calcium levels in stamen-hair cells of *Tradescantia virginiana* L. Planta **186:** 361–366

Klotz IM (1982) Number of receptor sites from Scatchard graphs: facts and fantasies. Science **217:** 1247–1249

Kotani S, Kawai G, Aizawa H, Yokoyama S, Sakai H (1990) Microtubule assembly inhibitor protein consists of a rigid globule essential for its activity and highly mobile coils. J Biol Chem **265:** 1286–1292

Kumar N (1981) Taxol-induced polymerization of purified tubulin. J Biol Chem **256:** 10435–10441

Lloyd C (1987) The plant cytoskeleton: the impact of fluorescence microscopy. Annu Rev Plant Physiol **38:** 119–139

Lloyd C (1991) The Cytoskeletal Basis of Plant Growth and Form. Academic Press, London, UK

Mandelkow E, Mandelkow E-M, Bordas J (1983) Synchrotron radiation as a tool for studying microtubule self-assembly. Trends Biochem **8:** 374–377

Margolis RL, Wilson L (1977) Addition of colchicine-tubulin complex to microtubule ends: the mechanism of substoichiometric colchicine poisoning. Proc Natl Acad Sci USA **74:** 3466–3470

Molé-Bajer J, Bajer AS (1983) Action of taxol on mitosis: modification of microtubule arrangements and function of the mitotic spindle in *Haemanthus* endosperm. J Cell Biol **96:** 527–540

Morejohn LC (1991) The molecular pharmacology of plant tubulin and microtubules. *In* C Lloyd, ed, The Cytoskeletal Basis of Plant Growth and Form. Academic Press, London, UK, pp 29–43

Morejohn LC, Bureau TE, Molé-Bajer J, Bajer AS, Fosket DE (1987a) Oryzalin, a dinitroaniline herbicide, binds to plant tubulin and inhibits microtubule polymerization in vitro. Planta **172:** 252–264

Morejohn LC, Bureau TE, Tocchi LP, Fosket DE (1984) Tubulins from different higher plant species are immunologically nonidentical and bind colchicine differentially. Proc Natl Acad Sci USA **81:** 1440–1444

Morejohn LC, Bureau TE, Tocchi LP, Fosket DE (1987b) Resistance of *Rosa* microtubule polymerization to colchicine results from a low-affinity interaction of colchicine and tubulin. Planta **170:** 230–241

Morejohn LC, Fosket DE (1982) Higher plant tubulin identified by self-assembly of microtubules in vitro. Nature **297:** 426–428

Morejohn LC, Fosket DE (1984) Taxol-induced rose microtubule polymerization and its inhibition by colchicine. J Cell Biol **99:** 141–147

Morejohn LC, Fosket DE (1986) Tubulins from plants, fungi and protists: a review. *In* JW Shay, ed, Cell and Molecular Biology of the Cytoskeleton. Plenum, New York, pp 257–329

Okamura S (1983) Effect of tartrate on the colchicine-binding activity in cultured carrot cell extract. Protoplasma **118:** 199–205

Oosawa F, Asakura S (1975) Thermodynamics of Polymerization of Protein. Academic Press, London, UK

Quader H, Filner P (1980) The action of antimitotic herbicides on flagellar regeneration in *Chlamydomonas reinhardtii*: a comparison with the action of colchicine. Eur J Cell Biol **21:** 301–304

Scatchard G (1949) The interactions of proteins for small molecules and ions. Ann NY Acad Sci **51:** 660–672

Schibler MJ, Huang B (1991) The col^R_4 and col^R_{15} β-tubulin mutations in *Chlamydomonas reinhardtii* confer altered sensitivities to microtubule inhibitors and herbicides by enhancing microtubule stability. J Cell Biol **113:** 605–614

Schiff PB, Fant J, Horwitz SB (1979) Promotion of microtubule assembly in vitro by taxol. Nature **277:** 665–667

Schiff PB, Horwitz SB (1980) Taxol stabilizes microtubules in mouse fibroblast cells. Proc Natl Acad Sci USA **77:** 1561–1565

Schultz E, Kirschner M (1987) Dynamic and stable populations of microtubules in cells. J Cell Biol **104:** 277–288

Stargell LA, Heruth DP, Gaertig J, Gorovsky M (1992) Drugs affecting microtubule dynamics increase α-tubulin mRNA accumulation via transcription in *Tetrahymena thermophila*. Mol Cell Biol **12:** 1443–1450

Strachen SD, Hess FD (1982) Dinitroaniline herbicides bind to glass. J Agric Food Chem **30:** 389–391

Strachan SD, Hess FD (1983) The biochemical mechanism of action of the dinitroaniline herbicide oryzalin. Pestic Biochem Physiol **20:** 1–10

Surridge CD, Burns RG (1991) Identification of an inhibitor of microtubule assembly present in juvenile brain which displays a novel mechanism of action involving suppression of self-nucleation. Biochemistry **30:** 10813–10817

Upadhyaya MK, Nooden LD (1977) Mode of dinitroaniline herbicide action. I. Analysis of colchicine-like effects of dinitroaniline herbicides. Plant Cell Physiol **18:** 1319–1330

Upadhyaya MK, Nooden LD (1980) Mode of dinitroaniline herbicide action. II. Characterization of [^{14}C]oryzalin uptake and binding. Plant Physiol **66:** 1048–1052

Upadhyaya MK, Nooden LD (1987) Comparison of [^{14}C]oryzalin uptake in root segments of a sensitive and a resistant species. Ann Bot **59:** 483–485

Weisenberg RC, Borisy GG, Taylor EW (1968) The colchicine-binding protein of mammalian brain and its relation to microtubules. Biochemistry **7:** 4466–4479

Wilson L, Anderson K, Chin D (1976) Nonstoichiometric poisoning of microtubule polymerization; a model for the mechanism of action of the vinca alkaloids, podophyllotoxin and colchicine. *In* R Goldman, T Pollard, J Rosenbaum, eds, Cold Spring Harbor Conference on Cell Proliferation, 3 (Book C). Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, pp 1051–1064