

# Taxol Binds to Cellular Microtubules

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**ABSTRACT** Taxol is a low molecular weight plant derivative which enhances microtubule assembly *in vitro* and has the unique ability to promote the formation of discrete microtubule bundles in cells. Tritium-labeled taxol binds directly to microtubules *in vitro* with a stoichiometry approaching one (Parnes, J., and S. B. Horwitz, 1981, *J. Cell Biol.* 91:479-487). We now report studies in cells on the binding of [<sup>3</sup>H]taxol and the formation of microtubule bundles.

[<sup>3</sup>H]Taxol binds to the macrophagelike cell line, J774.2, in a specific and saturable manner. Scatchard analysis of the specific binding data demonstrates a single set of high affinity binding sites. Maximal binding occurs at drug concentrations which produce maximal growth inhibition. Conditions which depolymerize microtubules in intact and extracted cells as determined by tubulin immunofluorescence inhibit the binding of [<sup>3</sup>H]taxol. This strongly suggests that taxol binds specifically to cellular microtubules. Extraction with 0.1% Nonidet P-40 or depletion of cellular ATP by treatment with 10 mM NaN<sub>3</sub> prevents the characteristic taxol-induced bundle formation. The binding of [<sup>3</sup>H]taxol, however, is retained under these conditions. Thus, there must be specific cellular mechanisms which are required for bundle formation, in addition to the direct binding of taxol to cytoplasmic microtubules.

Taxol, a novel diterpenoid, was originally isolated from the stem bark of the western yew, *Taxus brevifolia*, and has been found also in the leaves, stems and roots of a variety of other *Taxus* species (35, 63). The drug is a complex ester, shown to be a taxane derivative containing a rare oxetan ring, and is the first compound of this type to have antileukemic and tumor inhibitory properties. Early work in our laboratory demonstrated that the drug inhibits replication of HeLa cells (47). Studies with P-388 cells taken from taxol-treated mice identified taxol as a mitotic spindle poison (22).

Studies *in vitro* have shown that in marked contrast to other antimetabolic drugs, taxol enhances both the rate and yield of microtubule assembly. The critical concentration of microtubule protein required for assembly is reduced, and the microtubules formed are stable to depolymerization by calcium or cold (47). Taxol also assembles tubulin under conditions in which polymerization would not normally occur. These include the absence of microtubule-associated proteins, 36S tubulin-containing ring structures, exogenously added guanosine 5' triphosphate, or organic buffer (27, 50). Taxol induces microtubule assembly even at low temperatures (24, 47, 60). Microtubules assembled to steady state and then incubated with taxol become resistant to depolymerization by calcium, suggesting that there is a taxol-binding site on the microtubule. Maximal effects *in vitro* are seen at taxol concentrations stoichiometric

with the tubulin dimer concentration (49). Recent work with [<sup>3</sup>H]taxol confirms that the drug does bind to the microtubule, and that such binding occurs with a stoichiometry approaching one (41).

HeLa cells incubated with taxol accumulate in the G<sub>2</sub> and M phases of the cell cycle. In contrast to cells treated with colchicine or vinblastine, these taxol-treated cells exhibit an unusual microtubule cytoskeleton as seen by tubulin immunofluorescence and electron microscopy. These taxol-cytoskeletons are characterized by the presence of discrete bundles of microtubules (18, 48). Treatment with taxol stabilizes cellular microtubules to depolymerization by cold, steganacin (48, 49) and colchicine (12). The effects of taxol have been studied in a number of different systems including mouse dorsal root ganglion-spinal cord cultures (33), *Xenopus laevis* eggs (25), the trypanosome, *Trypanosoma cruzi* (5), and a variety of mammalian tissue culture systems (2, 3, 8, 14, 17, 18, 38, 46, 55, 61). In all cases examined, the effects of taxol appear to be related to the tubulin-microtubule system.

We report here that [<sup>3</sup>H]taxol binds to the macrophagelike cell line, J774.2, in a specific and saturable manner. Conditions which depolymerize microtubules in intact and extracted cells inhibit the binding of taxol; this suggests that taxol binds to cellular microtubules. Further, we are able to demonstrate binding of [<sup>3</sup>H]taxol under conditions in which taxol-induced

bundle formation is inhibited; this suggests that specific cellular conditions are necessary for bundle formation. A preliminary report of this work has been presented (32).

## MATERIALS AND METHODS

### Drugs

Taxol was obtained from the National Cancer Institute (Bethesda, MD), podophyllotoxin from Aldrich Chemical Co. (Milwaukee, WI), and VP-16-213 from Sandoz (Hanover, NJ). Unless otherwise noted, all other drugs and chemicals were obtained from Sigma Chemical Co. (St. Louis, MO). Lumicolchicine was prepared by the method of Wilson and Friedkin (65). 1 ml of  $10^{-2}$  M colchicine in 95% ethanol was placed in a quartz cuvette positioned 2 in from a long wave Model UVSL-58 ultraviolet light source (Ultraviolet Products, San Gabriel, CA) for 7 h. Completion of the reaction was verified by diluting samples to  $3 \times 10^{-5}$  M in 95% ethanol and determining ultraviolet spectra on a Carey Model I4R recording spectrophotometer (Applied Physics, Monrovia, CA) using cuvettes with a 1-cm light path.

[ $^3$ H]Taxol was prepared as previously described (41). Aliquots of a  $10^{-3}$  M stock solution in methanol were dried under vacuum and dissolved in dimethyl sulfoxide (DMSO) to a specific activity of 0.25 Ci/mmol. Labeled drug was stored at  $-20^\circ\text{C}$  and used within 1 mo of dilution. Purity of [ $^3$ H]taxol was  $>95\%$  as determined by silica gel thin-layer chromatography (41).

Lumicolchicine was stored in 95% ethanol; other drugs were kept as stock solutions in DMSO. The final concentration of DMSO had no effect on cell morphology, binding of [ $^3$ H]taxol or the microtubule display seen by tubulin immunofluorescence in control cells. In general, we find that taxol is insoluble in aqueous solutions at concentrations  $>50 \mu\text{M}$ .

### Cells

The continuous macrophagelike cell line, J774.2 (37, 44), was grown in complete medium consisting of Dulbecco's modified Eagle's medium (DME) supplemented with penicillin, streptomycin, 1% glutamine, 1% nonessential amino acids, and 20% heat-inactivated horse serum. The cells were grown in monolayer at  $37^\circ\text{C}$  in a humid incubator (Forma Scientific, Div. of Mallinckrodt, Inc., Marietta, OH) with an air mixture containing 8%  $\text{CO}_2$ . All cell culture media, sera, and solutions described herein were obtained from Gibco Laboratories (Grand Island Biological Co., Grand Island, NY).

Cells for growth curves were plated at a density of  $1 \times 10^5$  cells/ml in 35-mm tissue culture dishes. 24 h later, fresh complete medium plus drug was added. After appropriate time intervals, the medium was aspirated. Cells were incubated in 1.0 ml of Trypsin/EDTA solution for 10 min at  $37^\circ\text{C}$ , 0.5 ml of a 0.4% Trypan blue solution was added, and samples counted in a Bright-Line hemacytometer (American Optical Corp., Buffalo, NY).

### Quantification of [ $^3$ H]Taxol Binding

The assay was generally performed on confluent 35-mm tissue culture dishes. For J774.2 this typically represented  $\sim 4 \times 10^6$  cells/dish or  $\sim 1$  mg protein. Before assay, 2 ml of fresh complete medium containing the appropriate drugs was added to each dish. Incubations were performed for the appropriate time intervals at  $37^\circ\text{C}$ . At the end of the assay, the medium was removed and each dish was washed five times with ice-cold Dulbecco's phosphate-buffered saline, pH 7.4, without calcium or magnesium (PBS) (20). There was no change in the amount of label remaining with further washes. Washed cells were lysed in 1 ml of 0.1 N NaOH at room temperature for 12 h. Protein determinations were made using the method of Lowry et al. (31). Samples for counting were neutralized with an equal volume of glacial acetic acid, added to 20 ml of ACS II (Amersham, Arlington Heights, Ill.), and radioactivity was determined in a Model SL4000 liquid scintillation counter (Intertechnique, Plaisir, France). The external standards ratio method was used to convert counts per minute to picomoles of taxol. Efficiency of counting averaged 35%.

Specific binding was calculated as the difference between binding of [ $^3$ H]taxol in the presence and absence of a 100-fold excess of unlabeled taxol. Nonspecific binding was determined as binding of [ $^3$ H]taxol in the presence of a 100-fold excess of unlabeled taxol.

### Cytoskeletons

Cytoskeletons were prepared by two sequential detergent extractions. Cells were plated in 35-mm tissue culture dishes and washed twice with 2 ml of stabilizing buffer (SB) (40), containing 0.1 M piperazine-*N,N'*-bis(2-ethane sulfonic acid) (PIPES), pH 6.9, 1 mM ethyleneglycol-*bis*-( $\beta$ -aminoethyl ether) *N,N'*-tetraacetic acid (EGTA), and 4% polyethylene glycol 6000 (Baker, Phillipsburg,

NJ). Cells were incubated for 5 min at  $37^\circ\text{C}$  with 1 ml of SB containing 0.1% Nonidet P-40 (NP-40), followed by washing twice with 2 ml of SB. The extraction and subsequent washings were repeated, [ $^3$ H]taxol plus any additions were made in SB and [ $^3$ H]taxol binding to cytoskeletons was done in the same manner as with intact cells, except that the final washes were done with SB at room temperature.

### Immunofluorescence

Goat antitubulin which had been purified on a tubulin affinity column was kindly provided by Dr. Felicia Gaskin (Albert Einstein College of Medicine). Rhodamine or fluorescein conjugated rabbit anti-goat antibody was obtained from N. L. Cappel Laboratories (Cochranville, PA). The fluorescein conjugate produced sharper images.

Cells were plated on sterile glass cover slips in 35-mm petri dishes, at a density of  $2.5 \times 10^6$  cells/ml, and used within 24 h. All operations unless otherwise noted were performed at room temperature. The cells were extracted twice as described above, and then fixed for 20 min in 3.7% formaldehyde (Mallinckrodt, Inc., Paris, KY) in PBS. Cover slips were washed twice in PBS, plunged into absolute acetone at  $-20^\circ\text{C}$  for 5 min, and subsequently washed in PBS. Incubation with 20  $\mu\text{l}$  of undiluted goat antitubulin antibody was for 45–60 min in a humid atmosphere at  $37^\circ\text{C}$ . Cover slips were washed extensively in PBS and then incubated with 20  $\mu\text{l}$  of the fluorescent conjugated rabbit anti-goat antibody (1:100 dilution) for 45–60 min at  $37^\circ\text{C}$ . Cover slips were again extensively washed in PBS and then mounted using Aqua-mount (Lerner Laboratories, New Haven, CT) on clean microscope slides. A Zeiss (Oberkochen, W. Germany) standard 14 microscope equipped with epifluorescent optics was used to view the preparations. The combination of a  $\times 10$  ocular lens and a  $\times 63$  oil immersion lens (numerical aperture, 1.4) gave a depth of field of 0.8  $\mu\text{m}$ . Photographs were then taken on Plus-X pan 35mm film (Kodak, Rochester, NY) using an ASA of 1,000; the meter on the microscope gave exposure times ranging from 15–30 s. Film was developed in Kodak HC-100 (Dilution B) and printed on Kodabromide F4 grade paper (Kodak, Rochester, NY).

### Determination of ATP Levels

Confluent 35-mm dishes of J774.2 were incubated for 60 min at  $37^\circ\text{C}$  in 2 ml of SB  $\pm 10$  mM  $\text{NaN}_3$ . The dishes were extracted and ATP levels determined according to Loike et al. (30). Luciferin-luciferase was obtained from Sigma Chemical Co. A standard curve in the range of  $10^{-7}$  to  $10^{-9}$  M ATP was used; dilutions were made from a  $10^{-5}$  M stock solution in 0.04 M Tris-borate, pH 9.2, and calibrated by absorption measurements ( $\epsilon_{259} = 1.54 \times 10^4 \text{ M}^{-1}/\text{cm}$ ).

## RESULTS

### Binding of [ $^3$ H]Taxol Is Specific and Saturable

Specific binding is defined as that fraction of the total binding which is inhibited by an excess of unlabeled ligand. The total, and therefore specific, binding of [ $^3$ H]taxol to the macrophagelike cell line, J774.2, saturates within 45 min at  $37^\circ\text{C}$ . The nonspecific component, which is binding that can not be diluted even with a 100-fold excess of unlabeled taxol, saturates sooner, generally within 15 min (Fig. 1a). Both total and nonspecific binding persist for at least 8 h (Fig. 1b). Unlabeled taxol specifically dilutes the binding of [ $^3$ H]taxol in a concentration-dependent manner to a level already defined as nonspecific binding. A 100-fold excess of unlabeled drug is used in all subsequent experiments to determine nonspecific binding. The presence of serum has no effect on the specific binding of [ $^3$ H]taxol; thus, experiments were performed in complete medium containing 20% horse serum. Nonspecific binding, on the other hand, is inhibited by increasing concentrations of horse serum; the nonspecific binding of [ $^3$ H]taxol will vary depending on the particular concentration and lot of serum used.

Specific binding of [ $^3$ H]taxol to J774.2 saturates in a concentration-dependent manner. Maximal binding occurs at 21 pmol bound/mg total cellular protein; half-maximal binding occurs at 0.08  $\mu\text{M}$ . As to be expected, nonspecific binding increases linearly in a concentration-dependent manner, ranging from

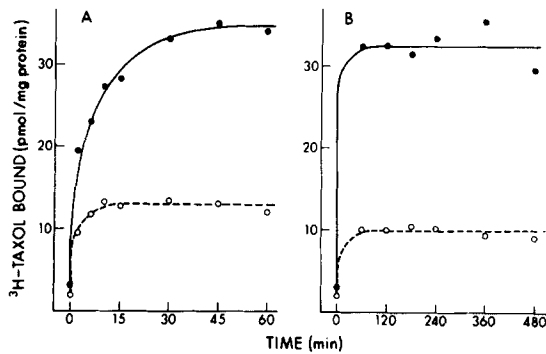


FIGURE 1 (A) Binding of [<sup>3</sup>H]taxol to J774.2 saturates within 60 min. Confluent 35-mm dishes of J774.2 were incubated for various time intervals at 37°C in 2 ml of complete medium containing 0.3 μM [<sup>3</sup>H]taxol ± 30 μM unlabeled taxol. Cells were washed five times with 2 ml of ice-cold PBS, and lysed in 1 ml of 0.1 N NaOH. Radioactivity and protein concentration were determined as described in Materials and Methods. Total binding was measured in the presence of 0.3 μM [<sup>3</sup>H]taxol (●). Nonspecific binding was measured by simultaneous addition of 0.3 μM [<sup>3</sup>H]taxol and 30 μM unlabeled taxol (○). (B) Binding of [<sup>3</sup>H]taxol to J774.2 persists for at least 8 h. The experiment was performed as in A, except for longer time intervals.

25% of total binding at 0.01 μM to 45% at 0.4 μM (Fig. 2 a). Scatchard analysis of the specific binding data produces a linear plot, indicating a single set of binding sites (Fig. 2 b). Taking into consideration the limitations discussed by Weisiger et al. (64) in applying such analysis to binding in cells, we calculate  $4.8 \times 10^6$  sites/cell and a dissociation constant of 0.2 μM. Binding is unaffected by varying cell density in the range of  $1 \times 10^5$  cells/ml to  $2 \times 10^6$  cells/ml.

### Binding of [<sup>3</sup>H]Taxol Correlates with Growth Inhibitory Effects

The 50% effective lethal dose (LD<sub>50</sub>) for taxol in the J774.2 cell line at 48 h is  $\sim 5 \times 10^{-8}$  M; this correlates well with the half-maximal binding concentration of  $8 \times 10^{-8}$  M (Fig. 2 a). Further, maximal growth inhibition of J774.2 occurs at taxol concentrations which produce maximal binding (Fig. 3).

### Effect of Simultaneous Addition of Mitotic Inhibitors on Binding of [<sup>3</sup>H]Taxol

Simultaneous addition of [<sup>3</sup>H]taxol and a 100-fold excess of either colchicine, podophyllotoxin, vinblastine, nocodazole, or unlabeled taxol completely inhibits specific binding (Fig. 4). Griseofulvin, an antimetabolic agent whose mechanism of action is still controversial, but whose cellular activity may not be mediated by microtubule depolymerization (11, 23), does not inhibit the binding of [<sup>3</sup>H]taxol. Lumicolchicine and VP-16-213, congeners of colchicine and podophyllotoxin, respectively, which do not interact with tubulin (29, 66) are also without effect.

Mitotic inhibitors such as colchicine and podophyllotoxin at concentrations greater than those that block cells in mitosis have been shown to also inhibit the transport of nucleosides across the cell membrane. The tubulin-inactive congeners, lumicolchicine and VP-16-213, retain the transport inhibitory properties of their parent compounds (29, 67), but nevertheless do not inhibit the binding of [<sup>3</sup>H]taxol. This suggests that inhibition of [<sup>3</sup>H]taxol binding by colchicine and podophyllo-

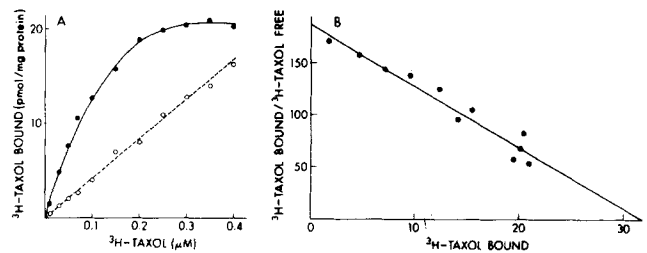


FIGURE 2 (A) [<sup>3</sup>H]Taxol binds J774.2 in a saturable manner. Confluent 35-mm dishes of J774.2 were incubated for 60 min at 37°C in 2 ml of complete medium containing various concentrations of [<sup>3</sup>H]taxol. Cells were washed five times with 2 ml of ice-cold PBS, and lysed in 1 ml of 0.1 N NaOH. Radioactivity and protein concentration were determined as described in Materials and Methods. Specific binding (●) is calculated as the difference between binding of [<sup>3</sup>H]taxol in the presence and absence of a 100-fold excess of unlabeled taxol. Nonspecific binding (○) is binding of [<sup>3</sup>H]taxol in the presence of a 100-fold excess of unlabeled taxol. (B) Scatchard analysis reveals a single class of specific binding sites for [<sup>3</sup>H]taxol in J774.2. The specific binding data from A have been plotted in the appropriate Scatchard analysis form (45). The line was determined by a least squares linear regression. Units on the abscissa are pmol [<sup>3</sup>H]taxol/mg protein/μM. Units on the ordinate are pmol [<sup>3</sup>H]taxol/mg protein/μM.

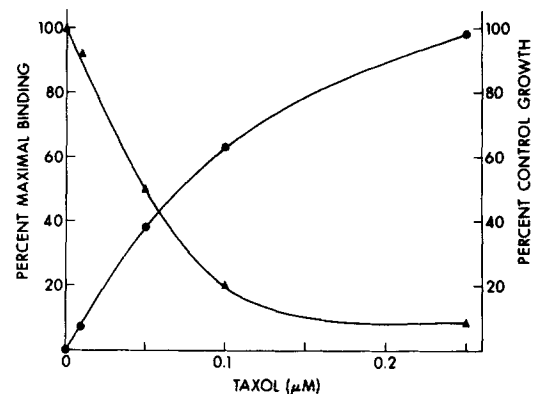


FIGURE 3 Maximal specific binding to J774.2 occurs at taxol concentrations which produce maximal growth inhibition. A growth curve of J774.2 incubated for 48 h with various concentrations of taxol was performed as described in Materials and Methods. Percent control growth (▲) is plotted. The specific binding data from Fig. 2 A, presented as percent maximal binding (●) is also shown. A maximal binding of 0.4 μM [<sup>3</sup>H]taxol was used.

toxin is microtubule-specific and is not related to the ability of these drugs to inhibit nucleoside transport.

The binding results are compatible with observations made with tubulin immunofluorescence. Although they are a cloned line, J774.2 cells exist in culture in a wide variety of shapes and sizes. Its tubulin cytoskeleton (34), similar to that of the primary macrophages (21, 42), is characterized by a single distinct microtubule organizing center (MTOC) from which all microtubules in the cell appear to emanate. Treatment with 0.3 μM [<sup>3</sup>H]taxol for 60 min does not affect this organization (Fig. 5 a); this display is identical to that of a control cell.

Simultaneous addition of 30 μM podophyllotoxin and 0.3 μM [<sup>3</sup>H]taxol, however, results in a complete loss of this characteristic microtubule display (Fig. 5 c); specific binding of [<sup>3</sup>H]taxol is inhibited. Its congener, VP-16-213, does not affect binding nor the cytoskeletal display. Griseofulvin, likewise, affects neither microtubule immunofluorescence, nor binding

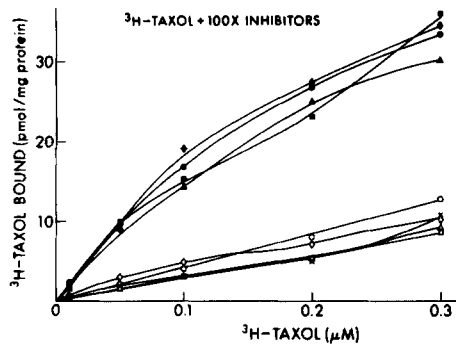


FIGURE 4 Effect of simultaneous addition of mitotic inhibitors on binding of [ $^3$ H]taxol to J774.2. Confluent 35-mm dishes of J774.2 were incubated for 60 min at 37°C in 2 ml of complete medium containing various concentrations of [ $^3$ H]taxol and a 100-fold excess of a mitotic inhibitor. Cells were washed five times with 2 ml of ice-cold PBS, and lysed in 1 ml of 0.1 N NaOH. Radioactivity and protein concentrations were determined as described in Materials and Methods. (■) Lumicolchicine. (◆) Griseofulvin. (●) Control. (▲) VP-16-213. (○) Taxol. (×) Nocodazole. (◇) Vinblastine. (△) Podophyllotoxin. (□) Colchicine.

of [ $^3$ H]taxol. Simultaneous treatment with 30  $\mu$ M vinblastine and 0.3  $\mu$ M [ $^3$ H]taxol results in a disruption of cytoskeletal structure and the formation of numerous well-defined paracrystals (Fig. 5 *d*). Since the binding of [ $^3$ H]taxol is inhibited in these preparations, vinblastine-induced paracrystals must not bind taxol. Hence, the taxol-binding site present on one polymeric form, the microtubule, is absent from another, the vinblastine-induced paracrystal.

Microtubule disassembly causes a rapid, characteristic change in the cell shape of a variety of leukocytes, including this particular cell line, J774.2. Treatment with agents such as colchicine or podophyllotoxin induces the formation of a large bulge or protuberance in the cytoplasm of these cells (34). These can readily be seen here (Fig. 5 *c* and *d*). Vinblastine-induced paracrystals appear to be excluded from the protuberance. This is presumably due to their displacement by actin filaments which are concentrated in these regions (1).

Unlabeled taxol, as expected, does not destroy the microtubule cytoskeleton (Fig. 5 *b*), but completely inhibits the binding of [ $^3$ H]taxol. It should be noted, however, that a 30  $\mu$ M incubation with taxol for 60 min does induce the formation of

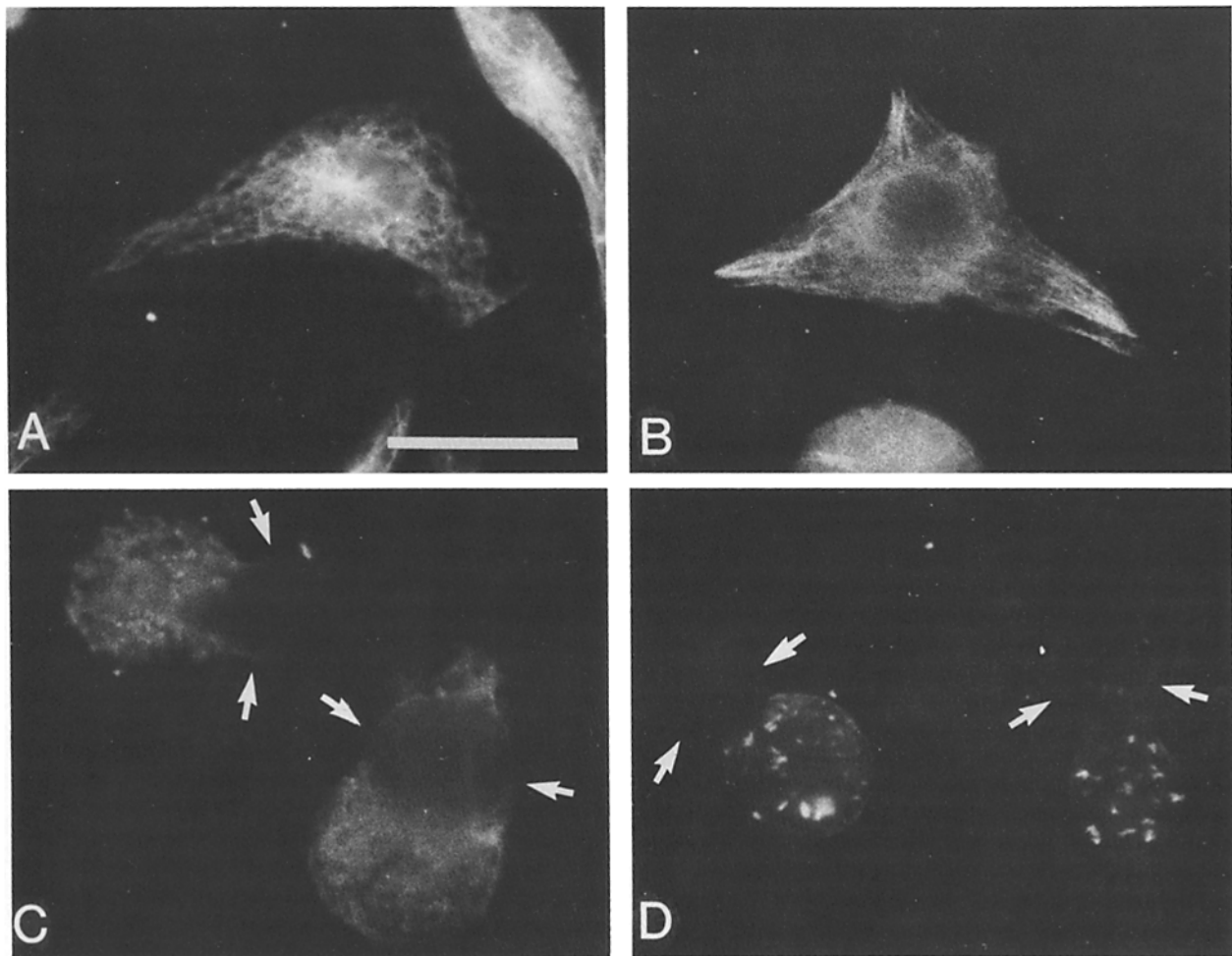


FIGURE 5 Tubulin immunofluorescence of J774.2 cells which have been treated with [ $^3$ H]taxol and a 100-fold excess of various inhibitors. Cells were incubated for 60 min at 37°C in complete medium containing 0.3  $\mu$ M [ $^3$ H]taxol alone (A) or labeled drug plus 30  $\mu$ M unlabeled taxol (B), plus 30  $\mu$ M podophyllotoxin (C), or plus 30  $\mu$ M vinblastine (D). Cells were processed for immunofluorescence as described in Materials and Methods. The arrows in C and D point to the limits of the protuberance seen after the appropriate treatment. Bar, 20  $\mu$ m.

microtubule bundles. These do not appear to be associated with a microtubule organizing center, which can easily be identified in control cells (see Fig. 5a). Taxol-induced bundle formation independent of an MTOC has been observed in other systems (18, 55).

#### Effect of Pretreatment with Mitotic Inhibitors on Binding of [<sup>3</sup>H]Taxol

Pretreatment with 3.0  $\mu$ M nocodazole for 2 h followed by washing and incubation with 0.3  $\mu$ M [<sup>3</sup>H]taxol results in binding similar to that of control cells (Table I). When these cells are examined by tubulin immunofluorescence, a microtubule cytoskeleton is observed (Fig. 6b). This is probably because the effects of nocodazole are readily reversible (15, 56). The effects of other antimetabolic drugs, however, are less so; for example, the binding of colchicine to tubulin *in vitro* is essentially irreversible (4, 66). Pretreatment with colchicine, colcemid or vinblastine, followed by washing, results in inhibition of [<sup>3</sup>H]taxol binding (Table I). Immunofluorescence of colchicine-reversed cells reveals the complete loss of all microtubule structure, and formation of the characteristic protuberance (Fig. 6a). Similar observations can be made with vinblastine-reversed cells; treatment with 3  $\mu$ M vinblastine does not cause paracrystal formation under these conditions. The microtubule-depolymerizing effects of 3  $\mu$ M colcemid are not reversible in J774.2 cells as seen by tubulin immunofluorescence and binding of [<sup>3</sup>H]taxol. Other laboratories have used colcemid as a reversible antimetabolic agent, but those experiments were performed at lower drug concentrations with other cell types (7, 39, 58).

As seen in Fig. 6b, cells pretreated with nocodazole, washed, and then incubated with 0.3  $\mu$ M [<sup>3</sup>H]taxol for 60 min form bundles of microtubules which are not associated with an organizing center. This differs from what is seen when 0.3  $\mu$ M taxol is added directly to a control cell, where at 60 min no alteration in the tubulin cytoskeleton is seen (Fig. 5a). Similar results have been reported by others (18, 55).

#### Effect of Sodium Azide Treatment on Binding of [<sup>3</sup>H]Taxol

Pretreatment of cells with 10 mM NaN<sub>3</sub> for 60 min at 37°C depletes cellular ATP levels but has no effect on either the specific or nonspecific binding of [<sup>3</sup>H]taxol (Table II). Such azide treatment likewise has no effect on the organization of

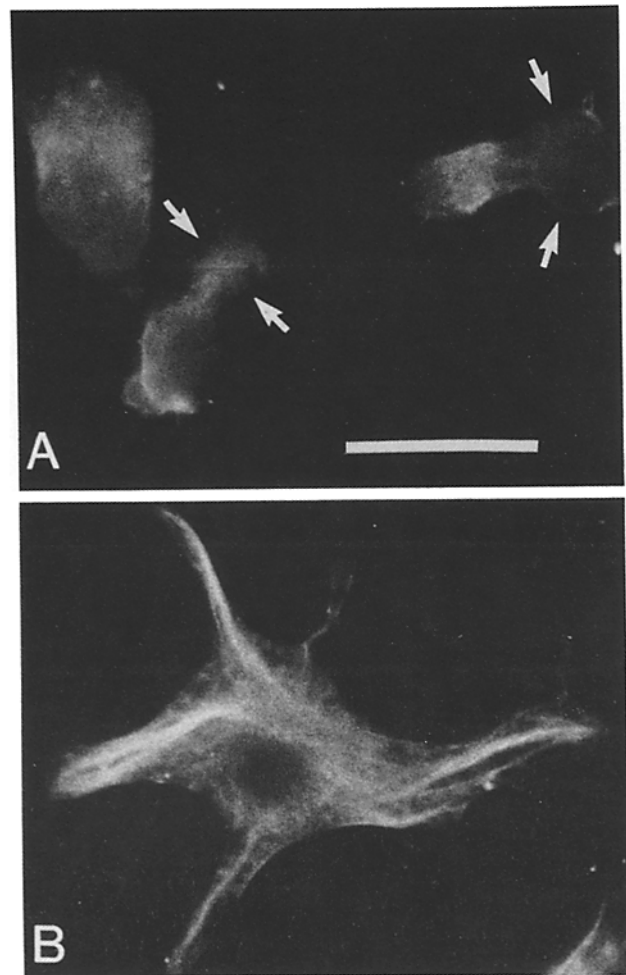


FIGURE 6 Tubulin immunofluorescence of J774.2 cells which have received various pretreatments before [<sup>3</sup>H]taxol incubation. Cells were pretreated in complete medium containing 3  $\mu$ M colchicine (A) or 3  $\mu$ M nocodazole (B) for 120 min at 37°C. This medium was aspirated; the dishes were then washed five times with DME at room temperature and subsequently incubated with 0.3  $\mu$ M [<sup>3</sup>H]taxol in complete medium for 60 min at 37°C. Cells were processed for immunofluorescence, as described in Materials and Methods. The arrows in A point to the limits of the protuberance seen after colchicine pretreatment. Bar, 20  $\mu$ m.

TABLE I  
Effect of Various Pretreatments on Binding of [<sup>3</sup>H]Taxol to J774.2 Cells

| Pretreatment          | Nonspecific<br><i>pmol/mg protein</i> | Specific<br><i>pmol/mg protein</i> |
|-----------------------|---------------------------------------|------------------------------------|
| 0.3% DMSO             | 21.4 $\pm$ 1.1                        | 26.2 $\pm$ 3.6                     |
| 3 $\mu$ M Colchicine  | 18.0 $\pm$ 1.7                        | <1.0                               |
| 3 $\mu$ M Colcemid    | 22.4 $\pm$ 1.9                        | <1.0                               |
| 3 $\mu$ M Vinblastine | 19.6 $\pm$ 2.0                        | <1.0                               |
| 3 $\mu$ M Nocodazole  | 19.3 $\pm$ 1.1                        | 20.4 $\pm$ 4.7                     |

Confluent 35-mm dishes of J774.2 were pretreated as indicated for 120 min at 37°C in 2 ml of complete medium. Cells were then washed five times with 2 ml of DME at room temperature and subsequently incubated with 0.3  $\mu$ M [<sup>3</sup>H]taxol  $\pm$  30  $\mu$ M unlabeled taxol in 2 ml of complete medium for 60 min at 37°C. Cells were washed five times with 2 ml of ice-cold PBS, and lysed in 1 ml of 0.1 N NaOH. Radioactivity and protein concentration were determined as described in Materials and Methods. Values are the average of triplicate determinations  $\pm$  one standard deviation.

TABLE II  
Effect of Sodium Azide Treatment on Binding of [<sup>3</sup>H]Taxol to J774.2 Cells

|                     | Nonspecific<br><i>pmol/mg protein</i> | Specific<br><i>pmol/mg protein</i> | ATP level<br><i>pmol/plate</i> |
|---------------------|---------------------------------------|------------------------------------|--------------------------------|
| -10 mM Sodium azide | 21.2 $\pm$ 1.1                        | 24.8 $\pm$ 2.9                     | 48.4 $\pm$ 0.2                 |
| +10 mM Sodium azide | 24.7 $\pm$ 2.6                        | 20.3 $\pm$ 2.2                     | 2.0 $\pm$ 0.1                  |

Confluent 35-mm dishes of J774.2 were incubated for 60 min at 37°C in 2 ml of stabilization buffer (SB)  $\pm$  10 mM NaN<sub>3</sub>. This buffer was then removed and 2 ml of fresh SB  $\pm$  10 mM NaN<sub>3</sub> containing 0.3  $\mu$ M [<sup>3</sup>H]taxol  $\pm$  30  $\mu$ M unlabeled taxol was added. After a further 60-min incubation at 37°C, cells were washed five times with 2 ml of ice-cold PBS, and lysed in 1 ml of 0.1 N NaOH. Radioactivity and protein concentration were determined as described in Materials and Methods. Values are the average of six determinations  $\pm$  one standard deviation. ATP levels were determined for confluent 35-mm dishes as described in Materials and Methods.

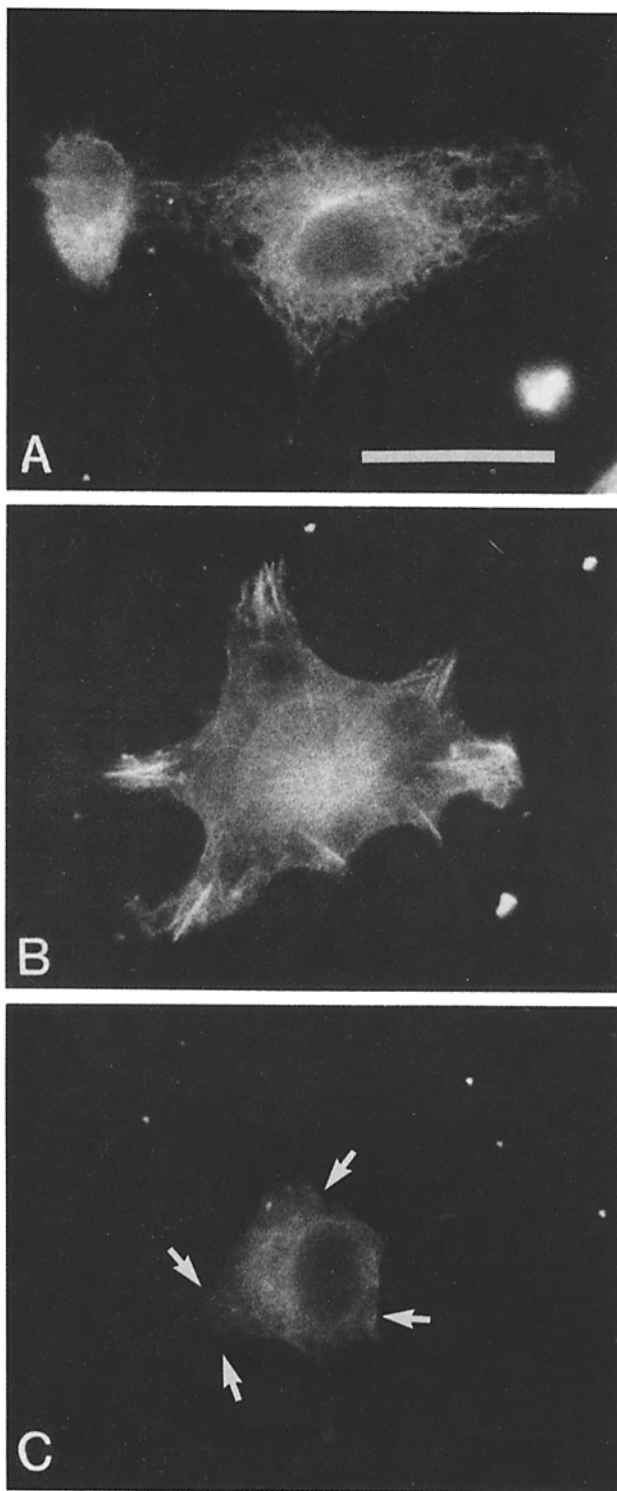


FIGURE 7 Tubulin immunofluorescence of J774.2 cells after treatment with sodium azide, or extraction with calcium. (A) Cells were incubated for 60 min at 37°C in SB containing 10 mM  $\text{NaN}_3$ , this buffer was removed, and fresh SB containing 10 mM  $\text{NaN}_3$  and 30  $\mu\text{M}$  unlabeled taxol was added for a further 60-min incubation at 37°C. (B) Cells were incubated for 60 min at 37°C in SB alone, this buffer was removed, and fresh SB containing 30  $\mu\text{M}$  unlabeled taxol was added for a further 60-min incubation at 37°C. Cells were processed for immunofluorescence as described in Materials and Methods. (C) Cells were washed twice at room temperature with SB and then extracted for 5 min at 37°C with SB containing 0.1% NP-40 and 5 mM  $\text{CaCl}_2$ . Cells were subsequently washed twice at room temperature with SB and the extraction procedure was re-

cellular microtubules as seen by tubulin immunofluorescence (data not shown; 16). However, under conditions in which taxol induces microtubule bundles that are independent of an organizing center in control cells (Fig. 7b), no such alteration is seen in the tubulin cytoskeleton of azide-treated cells (Fig. 7a). Similar observations have been made by De Brabander and associates in the PtK<sub>2</sub> cell line (M. De Brabander, personal communication).

#### Effect of 0.1% NP-40 Extraction and Calcium Treatment on [<sup>3</sup>H]Taxol Binding

Extraction of cells with nonionic detergents such as NP-40 or Triton X-100 releases most cellular lipid and soluble protein (28), including unassembled tubulin (19). The remaining cytoskeletal structure consists mainly of various filaments and microtubules, and their associated proteins (52, 53). If [<sup>3</sup>H]taxol is allowed to bind directly to such cytoskeletons, a major fraction of the binding seen with unextracted cells is retained (Table III). Since these cytoskeletons have been washed free of their dimer pool, unassembled tubulin does not appear to contribute to the binding of [<sup>3</sup>H]taxol, and thus it is unlikely that the addition of 0.3  $\mu\text{M}$  [<sup>3</sup>H]taxol to intact cells is affecting the extent of microtubule polymerization. This also supports our *in vitro* demonstration that taxol binding is not dependent on microtubule treadmilling of a free tubulin dimer pool (41); there is a taxol-binding site on the microtubule.

If the cytoskeleton is extracted in the presence of 5 mM  $\text{CaCl}_2$ , ~70% of the specific binding is lost without an appreciable change in nonspecific binding (Table III). Calcium extraction disrupts the microtubule cytoskeleton in these preparations (Fig. 7c; 51). SDS polyacrylamide gel analysis of these extracts reveals a band comigrating with bovine brain tubulin in the calcium extract but not in the control (data not shown); the calcium treatment facilitates the release of tubulin (57). Thus, when the calcium concentration is increased, the resulting release of tubulin causes a disruption of the microtubule

TABLE III  
Effect of 0.1% NP-40 Extraction on Binding of [<sup>3</sup>H]Taxol to J774.2

|  | Nonspecific     | Specific        |
|--|-----------------|-----------------|
|  | pmol/mg protein | pmol/mg protein |
| Unextracted cell                           | 5.1 ± 1.1       | 18.7 ± 2.0      |
| Ca <sup>++</sup> -treated unextracted cell | 7.5 ± 0.4       | 18.2 ± 2.6      |
| Cytoskeleton                               | 4.6 ± 1.1       | 17.0 ± 1.3      |
| Ca <sup>++</sup> -treated cytoskeleton     | 4.6 ± 0.4       | 6.2 ± 1.2       |

Subconfluent 35-mm dishes of J774.2 were washed twice at room temperature with 2 ml of stabilization buffer (SB) and then incubated for 5 min at 37°C with 1 ml of SB alone (unextracted cells), SB containing 5 mM  $\text{CaCl}_2$  (Ca<sup>++</sup>-treated unextracted cells), SB containing 0.1% NP-40 (cytoskeletons), or SB containing 0.1% NP-40 and 5 mM  $\text{CaCl}_2$  (Ca<sup>++</sup>-treated cytoskeletons). Cells were subsequently washed twice at room temperature with 2 ml of SB and the extraction procedure was repeated. After two additional washes with SB at room temperature, the dishes were further incubated with 2 ml of SB containing 0.3  $\mu\text{M}$  [<sup>3</sup>H]taxol ± 30  $\mu\text{M}$  unlabeled taxol for 45 min at 37°C. Cells were washed four times with 2 ml of SB at room temperature, and lysed in 1 ml of 0.1 N NaOH. Radioactivity and protein concentration were determined as described in Materials and Methods. Since NP-40 extraction releases two-thirds of the cellular protein (28), the bound radioactivity in the extracted samples was related to the amount of protein in the unextracted cell samples. Values are the average of six determinations ± one standard deviation.

peated. Cells were processed for immunofluorescence as described in Materials and Methods. The arrows in c point to the limits of the cell. Bar, 20  $\mu\text{m}$ .

cytoskeleton, and a concomitant decrease in the binding of [<sup>3</sup>H]taxol. The specific binding of [<sup>3</sup>H]taxol remaining after calcium extraction (Table III) is probably due either to cells which are not completely lysed or to extracted cells in which the microtubules are not totally depolymerized.

In contrast to intact cells, treatment of cytoskeletons with 0.3 μM [<sup>3</sup>H]taxol in the presence of 30 μM unlabeled taxol for 60 min does not alter the tubulin cytoskeletal organization as seen by tubulin immunofluorescence. The organizing center remains visible and no bundle formation occurs. An intact cell appears to be necessary for this unusual cytoskeletal rearrangement to occur.

## DISCUSSION

All of the effects of taxol in cells appear to be related specifically to the tubulin-microtubule system. Taxol added to PtK<sub>1</sub> cells at anaphase retards chromosome movement and blocks spindle elongation (8). Taxol-resistant Chinese hamster ovary (CHO) cells have an altered α-tubulin (9), while taxol requiring CHO cells have a normal cytoplasmic microtubule complex but have an impaired ability to form a mitotic spindle (10). The migration of 3T3 cells through gold particles (48) as well as chemotaxis in rabbit leukocytes (38) is inhibited by taxol. Taxol injected into unfertilized *Xenopus* eggs induces asterlike tubulin structures (25). In unfertilized sea urchin eggs, taxol likewise induces the formation of punctate microtubule-containing asters. In fertilized eggs, the sperm aster is incompetent to complete migration of the egg nucleus (46). Taxol inhibits the growth factor-induced deciliation of primary cilia in 3T3 cells (61). It induces postmitotic myoblasts to assemble interdigitating microtubule-myosin arrays that exclude actin filaments (3). In the trypanosome, *Trypanosoma cruzi*, taxol inhibits cytokinesis, yet duplication of cellular organelles continues (5). The drug induces abnormal microtubule arrays in primary mouse dorsal root ganglion-spinal cord cultures (33).

Taxol has no effect on the uptake of nucleotides, glucose, or amino acids in primary mouse embryo cells (13), nor on the synthesis of DNA, RNA, or protein in exponentially growing HeLa cells (47) or J774.16 macrophagelike cells (26). The drug has no effect on <sup>125</sup>I-thrombin and <sup>125</sup>I-EGF (epidermal growth factor) binding to specific surface receptors and their subsequent internalization in primary mouse embryo cells (13). No alteration of Fc-mediated phagocytosis in J774.16 cells occurs (26).

In vitro, taxol does not influence actin polymerization nor does it bind to intermediate filaments or DNA (41). The drug also does not affect the interaction of microtubules with their associated proteins in vitro (27, 50, 62) or in cells (14). Taxol assembles highly purified tubulin, and these microtubules bind [<sup>3</sup>H]taxol and are relatively stable to podophyllotoxin-induced disassembly (41, 50).

Our experiments demonstrate a single set of saturable, specific binding sites for taxol in cells. Since conditions which depolymerize microtubules in cells inhibit binding of [<sup>3</sup>H]taxol, it is suggested that this single set of sites represents cellular tubulin in its polymerized form. Binding to the J774.2 cell line saturates at 21 pmol taxol/mg total cellular protein. Assuming 1 mol of taxol bound/mol of polymerized tubulin (41), simple calculations reveal that 0.3% of the total cellular protein is tubulin in the polymer form. Such a value is well within the range of total tubulin content reported for human peripheral unseparated monocytes (0.6%) and purified lymphocytes (0.4%; 54), as well as guinea pig peritoneal macrophages (0.3%, 43).

Its limited solubility in aqueous solution and its molecular structure suggest that taxol is hydrophobic and hence is probably transported across the plasma membrane by passive diffusion. It is reasonable that the specific binding of taxol to the microtubule, a cytoplasmic receptor, results in a saturating binding curve for the drug (Fig. 2 a). Since the binding of [<sup>3</sup>H]taxol saturates, is diluted by an excess of unlabeled taxol, and is inhibited by conditions which depolymerize microtubules, it is unlikely that the specific binding of the drug reflects its partitioning into the cell membrane or cellular interior. Thus, taxol binds directly to the preexisting cytoplasmic microtubule complex (CMTC; Table III). It does not bind vinblastine-induced paracrystals (Fig. 4), but, rather, specifically recognizes the microtubule.

In the original studies with taxol, Schiff and Horwitz (48) used primary BALB/c fibroblasts in which it is difficult to identify a microtubule organizing center. Bundle formation was demonstrated but it was difficult to discern whether these bundles were MTOC-associated. DeBrabander et al. (18), using a cell line with a more prominent MTOC (PtK<sub>2</sub>), were able to definitively observe bundles independent of the organizing center. Using 3T3 cells and colcemid reversal, Simone et al. (55) corroborated this finding. Our studies here with the J774.2 cell line concur. The clearly visualized organization of the macrophage cytoskeleton makes it an ideal tool for such study.

The mechanism of microtubule bundle formation is not clear. This taxol-induced process requires ATP (Table II) as well as an intact, unextracted cell (Table III). Since conditions for in vitro microtubule bundle formation have yet to be identified, it is likely that there are specific cellular conditions which are necessary for this unusual cytoskeletal reorganization. De Brabander et al. (18) have studied the time course of taxol-induced cytoskeletal rearrangements in control and nocadazole-reversed PtK<sub>2</sub> cells visualized by peroxidase-antiperoxidase immunostaining. They suggest that, in fact, the preexisting tubulin cytoskeleton depolymerizes as bundles form. This idea is supported by our observation that depolymerization of the CMTC appears to facilitate bundle formation. Treatment with 0.3 μM taxol for 60 min does not induce bundles in J774.2 (Fig. 5 a). However, if the CMTC is depolymerized by nocodazole, washed, and 0.3 μM taxol is then added, bundles do form within 60 min (Fig. 6 b). Simone et al. (55) using colcemid reversal have reported similar results.

Depletion of cellular ATP can inhibit depolymerization by colchicine or vinblastine, but does not affect repolymerization after nocodazole reversal (6, 17, 36). Since ATP is also required for taxol-induced bundle formation, it is interesting to speculate that microtubule depolymerization may be an important part of the cytoskeletal reorganization caused by taxol. In vitro data demonstrating that taxol suppresses microtubule treadmilling (27, 59) would tend to discredit depolymerization as an intermediate step in bundle formation. As yet, however, treadmilling of microtubules in cells has not been demonstrated, nor is it unreasonable to envisage unique cellular mechanisms that could overcome taxol-induced microtubule stability.

In conclusion, we have shown that taxol binds to cellular microtubules and that such binding of taxol and subsequent bundle formation are related but separable events. Extraction with NP-40 or treatment with azide inhibits the cytoskeletal reorganization without affecting the binding of [<sup>3</sup>H]taxol. Further studies of conditions such as these may prove helpful in elucidating how the binding of this low molecular weight plant alkaloid can mediate a major rearrangement of the cellular



tubulin cytoskeleton. Indeed, an understanding of this process will further our knowledge of the regulation of microtubule assembly and organization in cells. In addition, the ability of [<sup>3</sup>H]taxol to stoichiometrically bind to cellular microtubules may prove to be an ideal assay for quantifying tubulin in its polymer form in cells. Such an assay is sorely lacking in the current arsenal of microtubule technology and is necessary if we are to satisfactorily answer key questions concerning the role of the tubulin polymer-dimer equilibrium in a variety of cellular functions.

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