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Competitive inhibition of colchicine binding to tubulin by microtubule-associated proteins

(microtubules/tau protein/high molecular weight proteins)

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ABSTRACT **Microtubule-associated proteins (MAPs) promote tubulin polymerization, whereas colchicine inhibits this process. In this paper, MAPs have been shown to inhibit colchicine binding to tubulin in a competitive manner. Attempts were made to identify which of the MAPs fraction(s) was responsible: both tau protein (a thermostable molecule with a molecular weight of ≈70,000) and a high molecular weight fraction (HMW) were able to compete with colchicine. In contrast, Mg^{2+}, which also induces microtubule assembly *in vitro*, had no effect on colchicine binding to tubulin.**

Microtubules are organelles found in all eukaryotic cells and are implicated in several cellular processes. They are formed by polymerization of 6S tubulin subunits. The assembly process is inhibited by colchicine both *in vivo* and *in vitro* when this drug binds to a specific site on tubulin (1–3). We report herein that endogenous brain factors inhibit the binding of colchicine to tubulin, competitively. To identify these factors we assumed that they should have, like colchicine, a high affinity for tubulin. Thus, if such factors exist, one might predict that they would copurify with tubulin in the microtubule assembly process *in vitro*. Microtubules assembled *in vitro* contain several minor protein components that have been referred to as microtubule-associated proteins (MAPs). Several of these proteins are believed to play a role in the microtubule assembly process (4–8). We have therefore investigated whether MAPs inhibit colchicine binding to tubulin.

EXPERIMENTAL PROCEDURE

Rat brain microtubules were purified by two cycles of polymerization, using the assembly–disassembly procedure described by Shelanski *et al.* (9). Pure tubulin was prepared by the method of Weingarten *et al.* (4) as follows: Microtubule protein was resuspended to a final concentration of 10 mg/ml in 25 mM 2-(*N*-morpholino)ethanesulfonate (Mes) buffer/0.5 mM $MgCl_2$/1 mM 2-mercaptoethanol/0.1 mM EDTA/0.1 mM GTP, pH 6.4, kept 30 min at 0°C, and spun at 105,000 × *g* for 30 min. The supernatant was applied to a phosphocellulose (Whatman P11) column (2.5 mg of protein per ml of phosphocellulose) equilibrated with the same buffer. Tubulin was eluted in the void volume and immediately made up to 100 mM Mes/1.0 mM ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA), 1.0 mM GTP/4 M glycerol when necessary.

MAPs were obtained either by elution from a phosphocellulose column with 25 mM Mes buffer, pH 6.4, containing 0.75 M NaCl, or by boiling the purified microtubules resuspended in 25 mM Mes buffer containing 2 mM dithiothreitol and 0.75 M NaCl, as described previously (10). The MAPs fraction thus obtained was either dialyzed against 100 mM Mes buffer (100 mM Mes/0.5 mM $MgCl_2$/1.0 mM EGTA/0.1 mM EDTA/1.0 mM 2-mercaptoethanol/1 mM GTP, pH 6.4) or filtered on Sephadex G-25 equilibrated with the same buffer to remove the NaCl. Two fractions present in the MAPs were purified by column chromatography. These fractions were the tau protein described by Weingarten *et al.* (4) and high molecular weight (HMW) components isolated by Murphy and Borisy (5). Tau and HMW proteins were purified by chromatography on an LKB Ultrogel AcA 34 polyacrylamide/agarose column. The total MAPs fraction eluted from the phosphocellulose column was applied (5 mg protein in 1 ml) to an AcA 34 column (40 × 1.5 cm) equilibrated with the same buffer. Fractions were collected and dialyzed against 100 mM Mes buffer, pH 6.4, to remove NaCl and then tested for their activity (in promoting the assembly of pure tubulin). Their protein contents were determined by the Lowry method (11). The purity of tubulin and MAPs fractions was established by sodium dodecyl sulfate gel electrophoresis as described by Weber *et al.* (12). The molecular weight of tau protein was estimated in polyacrylamide gel electrophoresis with the tubulin subunit (molecular weight 55,000) as a reference marker.

Tubulin assembly *in vitro* at 37°C was followed by turbidimetric measurements at 345 nm with a Zeiss PM 6 KS spectrophotometer with an automatic thermostated four-sample changer.

Colchicine binding to tubulin was assayed in 100 mM Mes buffer with, or without, 4 M glycerol. Pure tubulin (concentrations ranging from 0.2 mg/ml to 0.25 mg/ml depending on the experiment) was incubated at 37°C for 2 hr in a final volume of 0.5 ml in the presence of different colchicine concentrations, labeled with 0.1 μCi (1 Ci = 3.7×10^{10} becquerels) of [^{3}H]colchicine per assay. Bound colchicine was measured according to a modification (13) of the procedure described by Wiesenberg *et al.* (1). Corrections for the first-order time-dependent decay were made, when necessary. The results are the average of four values. In double reciprocal plot representation, free colchicine $(C)_F$ was determined by subtracting the concentration of the bound species from the initial colchicine concentration.

RESULTS AND DISCUSSION

Fig. 1 shows the effect of MAPs, obtained by phosphocellulose chromatography, on the binding of colchicine to tubulin in the absence of glycerol. It is clear that total MAPs inhibit colchicine binding. Similar results have been obtained with MAPs purified by thermal treatment of microtubules (Fig. 2 *upper*). Control experiments (results not shown) showed that total MAPs do not bind colchicine.

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Abbreviations: MAPs, microtubule-associated proteins; HMW proteins, high molecular weight proteins; Mes, 2-(*N*-morpholino)ethanesulfonate.

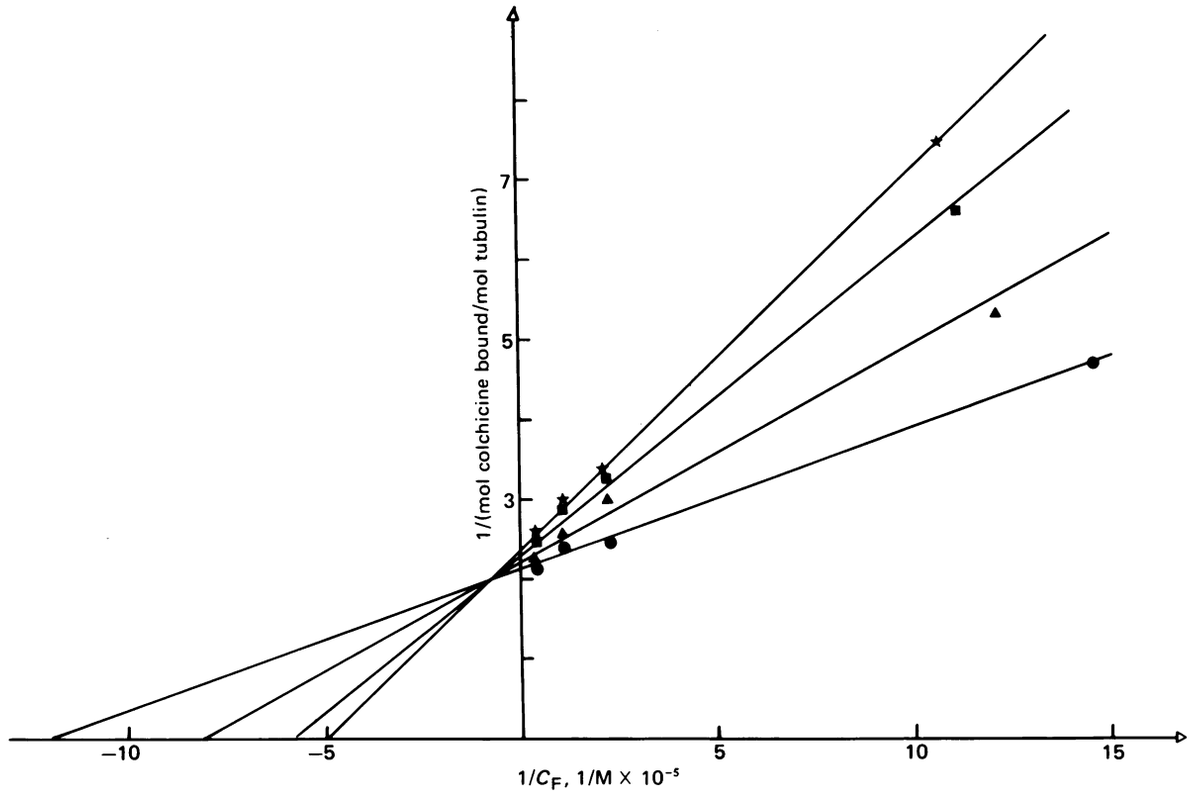


FIG. 1. Effect of total MAPs on colchicine binding to tubulin (double reciprocal plot). Pure tubulin (200 μg/ml) was incubated at 37°C in 100 mM Mes buffer without glycerol for 2 hr (final volume 0.5 ml) with various concentrations of colchicine (1–25 μM) labeled with 0.1 μCi of [^{3}H]colchicine per assay, in the absence (●) and in the presence of increasing amounts of MAPs obtained by phosphocellulose chromatography: 100 μg/ml (▲), 150 μg/ml (■), and 200 μg/ml (★).

One explanation for these results might be the following: The assembly of pure tubulin is markedly enhanced by MAPs (4, 8, 10); self-assembly is achieved in the absence of MAPs, but only at protein concentrations higher than 2.5 mg/ml (14). In the presence of MAPs, the minimal concentration [critical concentration, C_c, (15, 16)] that is required is ≈10 times lower. Because colchicine binds only to free tubulin (1–3) or rings (17) and not to microtubules (1–3), the inhibition by MAPs of colchicine binding (Figs. 1 and 2) could therefore be due to the masking of the colchicine binding sites in the intact microtubule. This explanation is, however, not valid, because the data recorded in Figs. 1 and 2 were obtained with tubulin concentrations lower than C_c. Furthermore, double reciprocal plot representations of the effect of MAPs on colchicine binding to tubulin in the absence (Fig. 1) and in the presence (Fig. 2) of glycerol show that MAPs did not modify the C_{max}, the number of sites available to colchicine. A decrease in the number of sites would be expected if the MAPs reduced the number of free tubulin molecules—i.e., molecules having an unmasked colchicine binding site. In Fig. 1 the affinity constant K_A was changed from 1.2×10^6 to 1.06×10^5 M^{-1} and in Fig. 2, in the presence of glycerol, from 2.75×10^5 to 1.06×10^5 M^{-1}. A Scatchard plot of these data (Fig. 2 *lower*) clearly shows that, in the presence of MAPs, there is no change in the number of colchicine binding sites. These data therefore show: (*i*) that the affinity of colchicine for tubulin is decreased by glycerol; (*ii*) that MAPs produce a decrease in the value of K_A in both the presence and absence of glycerol. A modification in the number of colchicine binding sites by tubulin antibodies has been observed by Aubin *et al.* (18), but no attempt was made by these authors to measure the affinity constant.

It is possible that the decrease in the colchicine binding affinity described in Figs. 1 and 2 could be due to the formation of rings, which would have a lower affinity for colchicine than free tubulin.

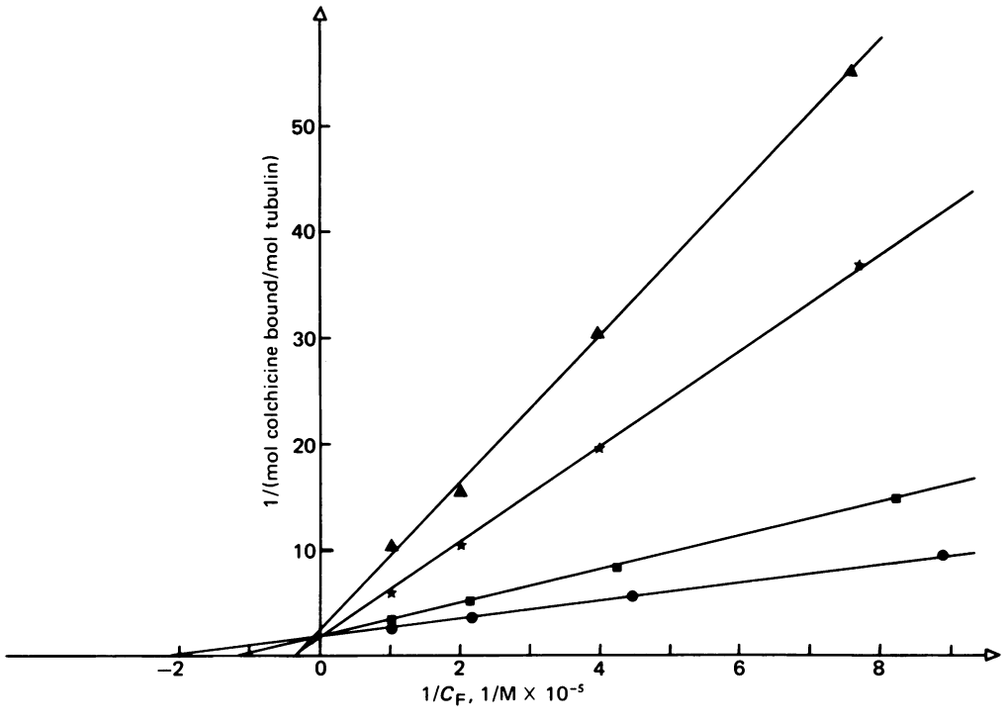
Because MAPs are made up of several entities (4–8), an attempt was made to identify the factor(s) responsible for this inhibition more precisely. It has been reported that two protein fractions from MAPs promote *in vitro* tubulin polymerization: tau factor, described by Weingarten *et al.* (4), and HMW proteins ($MAPs_1$ and $MAPs_2$) described by several authors (5–8). These two groups of proteins therefore could be candidates as inhibitors of colchicine binding to tubulin.

The tau protein and HMW fractions were isolated from the MAPs as previously described (10) and their purities and compositions were determined by sodium dodecyl sulfate gel electrophoresis (10). Purified tau factor migrated as a broad peak composed of several closely spaced bands with molecular weights ranging from 57,000 to 63,000; i.e., the factor was very similar to the thermostable factor described by Witman *et al.* (19). HMW proteins were mainly composed of several high molecular weight species; this is in agreement with data obtained by other authors (8).

As seen in Fig. 3, the tau factor inhibits colchicine binding to tubulin very efficiently; tau factor did not modify the C_{max}, but markedly decreased the K_A. The *inset* of figure 3 shows that this fraction is very active in inducing 6S tubulin polymerization.

The second main component of MAPs, HMW proteins, likewise inhibits colchicine binding to tubulin (Fig. 4) and also has a moderate effect on tubulin assembly (Fig. 4, *inset*).

These findings are consistent with the idea that both tau and HMW proteins inhibit colchicine binding either to the free



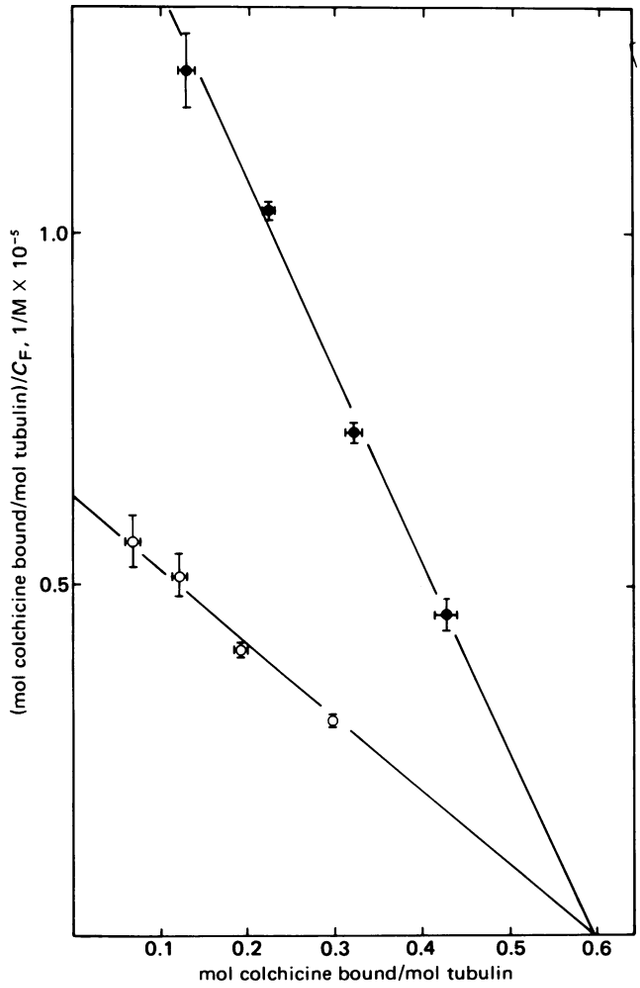


FIG. 2. Effect of thermostable MAPs on colchicine binding to tubulin. Pure tubulin (200 μg/ml) was incubated at 37°C in 100 mM Mes buffer containing 4 M glycerol for 2 hr (final volume 0.5 ml) with various concentrations of colchicine (1.35–10 μM) labeled with 0.1 μCi of [^{3}H]colchicine per assay, in the absence and the presence of different amounts of MAPs obtained by thermal denaturation of microtubules assembled *in vitro*. (*Upper*) Double reciprocal plot: control (●), 80 μg/ml (■), 160 μg/ml (★), and 240 μg/ml (▲) of MAPs. (*Lower*) Scatchard plot: control (●), and 80 μg/ml (○) of MAPs. Bars indicate standard error.

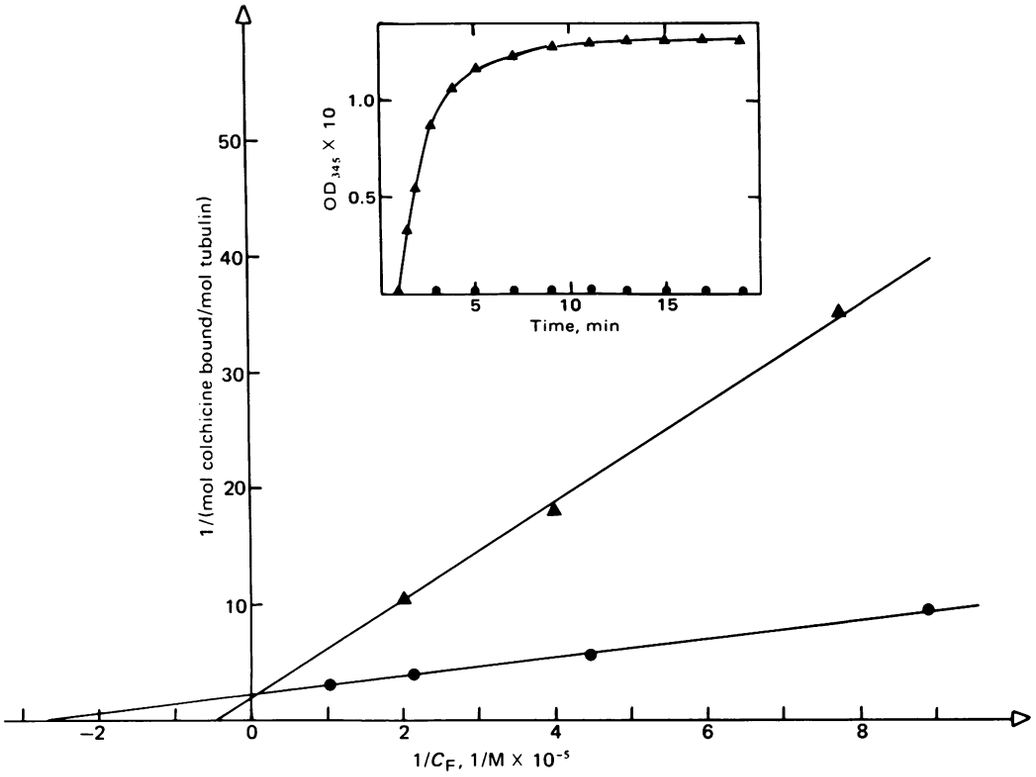


FIG. 3. Effect of purified tau factor on colchicine binding to tubulin (double reciprocal plot) and on purified tubulin assembly (*Inset*). Pure tubulin (254 μg/ml) was incubated at 37°C for 2 hr in 100 mM Mes containing 4 M glycerol (final volume 0.5 ml) with various concentrations of colchicine (1.35–5.1 μM) labeled with 0.1 μCi of [^{3}H]-colchicine per assay in the absence (●) and in the presence (▲) of tau factor (70 μg/ml) purified by Ultrogel AcA 34 chromatography. (*Inset*) Time course of assembly of pure tubulin (1.16 mg/ml) in the absence (●) and in the presence (▲) of tau factor (130 μg/ml) used in the main figure.

tubulin or to the rings and other protofilaments that might be formed below the critical concentration for tubulin assembly (19, 20). In either case, however, the colchicine binding site is not completely masked by the two MAPs, tau and HMW. Our data show that it is not the number of colchicine binding sites that is reduced in the presence of MAPs, but rather a modification in the affinity of tubulin for this drug, which accounts for these results. Frigon and Timasheff (21) have, however, reported the formation of rings (and microtubule-like structures) in the absence of MAPs, and in the presence of high concentrations of Mg^{2+}. The incubation of pure tubulin in the presence of 16 mM Mg^{2+} does not modify either the number of colchicine binding sites or the affinity of colchicine for tubulin (Fig. 5). Thus colchicine binding to low concentrations of tubulin is modified by tau and HMW factors but not by Mg^{2+}.

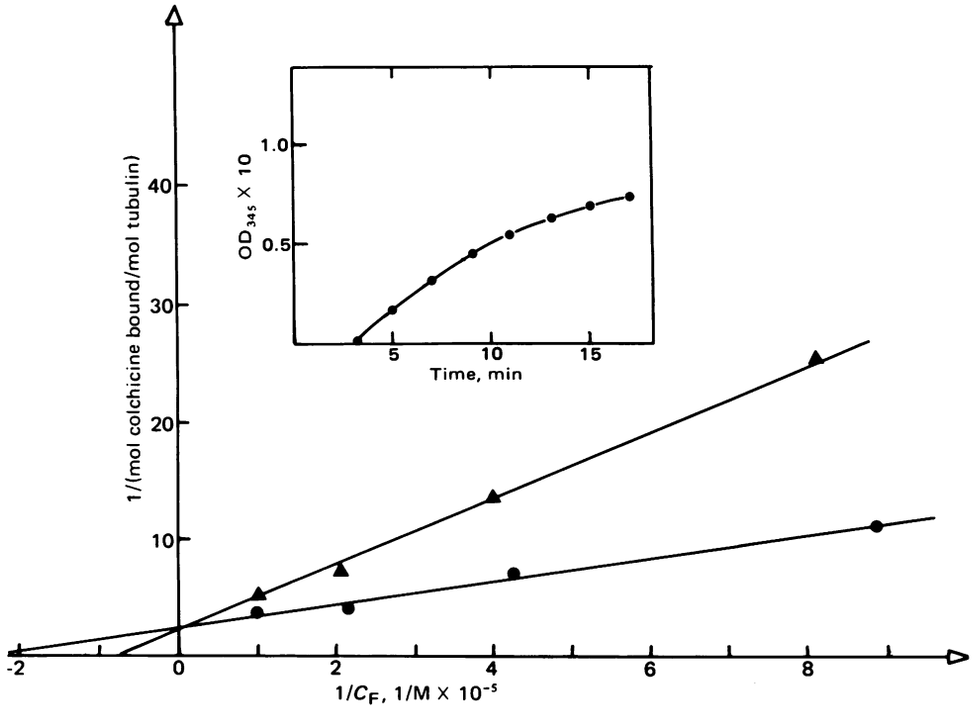


FIG. 4. Effect of HMW proteins on colchicine binding to tubulin (double reciprocal plot) and on purified tubulin assembly (*Inset*). Pure tubulin (200 μg/ml) was incubated as for Fig. 3 with various colchicine concentrations (1.35–25 μM) in the absence (●) and the presence (▲) of HMW proteins (130 μg/ml) purified by Ultrogel AcA 34 chromatography. (*Inset*) Polymerization of pure tubulin (1.16 mg/ml) in the presence of HMW proteins (150 μg/ml). Time course of tubulin assembly alone was the same as in *inset* of Fig. 3.

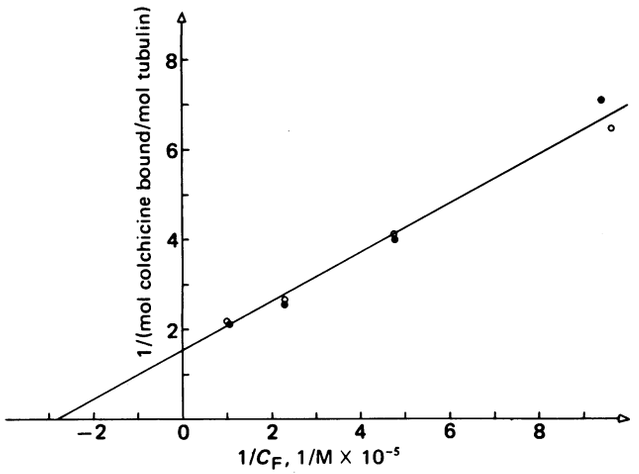


FIG. 5. Effect of Mg^{2+} (16 mM) on colchicine binding to tubulin (double reciprocal plot). Pure tubulin (200 μg/ml) was incubated in 100 mM Mes buffer containing 4 M glycerol and 0.5 mM Mg^{2+} (●) or 16 mM Mg^{2+} (○) (final volume 0.5 ml) with various concentrations of colchicine (1.35–10 μM) labeled with 0.1 μCi of [^{3}H]colchicine per assay.

In conclusion, the data reported above are consistent with the idea that endogenous factors competitively inhibit colchicine binding by the free tubulin molecule or by the rings that may be formed below the critical concentration required for microtubule assembly. Either the factors and colchicine bind to the same site or the factors modify the affinity of the colchicine binding site in the process of ring formation. The most reasonable mechanism is a simple reversible binding of MAPs to tubulin in a region that overlaps with (or fully contains) the colchicine binding site.

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