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The Action of Cytochalasin A on the In Vitro Polymerization of Brain Tubulin and Muscle G-Actin

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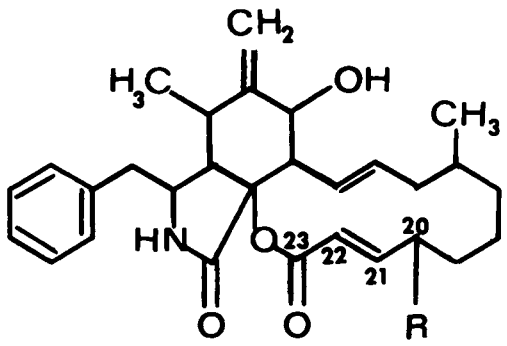
The presence of cytochalasin A inhibits the self-assembly of beef brain tubulin and rabbit muscle G-actin in vitro and also decreases the colchicine binding of tubulin. Prior reaction of cytochalasin A with 2-mercaptoethanol destroys its inhibitory effects. It is shown that cytochalasin A exerts its actions by reacting with sulfhydryl groups, possibly causing irreversible structural changes in the proteins. Cytochalasin B does not affect the tubulin assembly reaction.

Key words: cytochalasin A and B, G-actin, tubulin, microtubules, sulfhydryl modification, covalent modification, colchicine binding

INTRODUCTION

The cytochalasins are fungal metabolic products which have a wide range of effects on cellular activities (1). At least seven cytochalasins are known, but the actions of cytochalasin B (CB), because of its greater availability, are better known than the actions of the other cytochalasins. CB has been shown to stop cytokinesis with the production of multinucleated cells (1), inhibit hexose transport (2), interfere with cellular secretion (3), induce nuclear extrusion (1), inhibit cell movement (4), stop phagocytosis (5), and cause the "arborization" of some cells (6). A large number of other effects have also been noted. Extensive bibliographies of the effects of cytochalasins are contained in Refs. 1 and 7 and in publication A74 of the Aldrich Chemical Co. CB apparently acts by affecting microfilament structures (8–10). However, there is still some question as to the actual target(s) of CB (11,12).

Cytochalasin A (CA) differs from CB in chemical structure at only one position (Fig. 1). The hydroxy function at C-20 in CB is oxidized to a carbonyl in CA. Reports on the biological effects of CA are sparse. In one publication (13) it was noted that the inhibitory effect of CA on the uptake of 2-deoxy-D-glucose by chick embryo fibroblasts was irreversible while that of CB was reversible. CA also has been shown to have multiple effects on cellular processes in yeast (14). We provide data here to show that CA is a very effective sulfhydryl reagent and prevents the self-assembly of tubulin and G-actin in vitro by reacting with sulfhydryl groups of the proteins. Some of the results described in this report have been published previously (15).



Cytochalasin A - R = O

Cytochalasin B - R = OH,H

Fig. 1. Structures of CA and CB. Numbering system is that given in Ref. 7.

MATERIALS AND METHODS

Cytochalasin A and B were purchased from the Aldrich Chemical Co. Solutions of these compounds were made in dimethyl sulfoxide (DMSO). In all experiments in which CA or CB was used the final DMSO concentration was 1%. This concentration of DMSO was included in all controls and did not affect any of the reactions studied. GTP and colchicine were products of Sigma Chemical Co. ^{3}H-Colchicine was obtained from New England Nuclear Co. Beef brain tubulin was purified by the polymerization method of Shelanski et al. (16) as described previously (17). The extraction and reassembly buffer was 20 mM (2[N-morpholino] ethane sulfonic acid) (MES), 70 mM NaCl, 1 mM ethylene-glycol-bis-(β-amino-ethyl ether) N,N′ tetraacetic acid (EGTA), 0.5 mM $MgCl_2$, pH 6.4. The protein was purified through two polymerization cycles and was stored at $-80°$ in the buffer containing 2 M glycerol. G-actin was extracted from an acetone powder of rabbit muscle by the procedure of Reese and Young (18) and was dialyzed at 4° overnight against 0.5 mM ATP, 0.5 mM 2-mercaptoethanol, 0.2 mM $CaCl_2$, pH 7.7, before use. Colchicine binding was performed as described by Borisy (19). The sulfhydryl content of tubulin was determined by the reaction with 5,5′-dithiobis (2-nitrobenzoic acid) (DTNB) (20).

RESULTS

When the in vitro self-assembly of brain tubulin is carried out in the presence of CA a pronounced inhibition occurs. The data in Fig. 2 show that at an approximate ten-fold excess of CA over tubulin, the inhibition is essentially complete. This light scattering data was confirmed with the electron microscope (Fig. 3). CB at the same concentration as CA had no effect. In other experiments it was shown that 200 μM CB also failed to inhibit the polymerization reaction. In the experiments described in Fig. 2 CA was incubated with tubulin for 2 min prior to the addition of GTP. Longer incubations of CA with

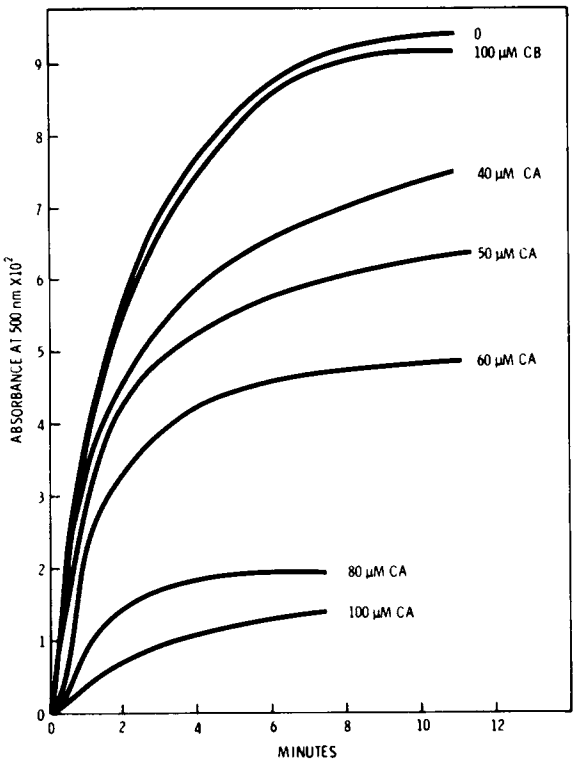


Fig. 2. Inhibition of self-assembly of tubulin by CA. Tubulin (10 μM) was preincubated for 2 min at 37° in 0.5 ml of reassembly buffer containing 0.32 M glycerol and the concentrations of CA and CB shown. GTP (final concentration, 0.5 mM) was then added to initiate the reaction.

the protein were found to increase the effectiveness of CA (Fig. 4). The observed inhibition by CA is irreversible. This was shown by the failure to achieve reversal after dialyzing the CA-reacted tubulin against reassembly buffer containing 1 M glycerol. This procedure removes free CA and does not inactivate unreacted tubulin.

To determine whether CA causes structural alterations in the protein the effect of the compound on the ultracentrifuge pattern and on the colchicine binding properties of the protein was investigated. As shown in Fig. 5 colchicine binding by tubulin is definitely inhibited by CA. Preparations of tubulin isolated by the polymerization-depolymerization method contain both the 6S tubulin dimer and a higher molecular weight aggregate usually having an S-rate between 20 and 30 and a ring-like structure (21–27). The aggregate presumably acts as an initiation site in the self-assembly process. As the data in Fig. 6 show CA causes a decrease in the amount of the aggregate (26S) present together with a corresponding increase in the 6S dimer. In the absence of CA the aggregate accounts for 51% of the protein; in its presence, 31%. In this experiment the molar ratio of CA to tubulin was approximately 2. It was not possible to achieve a higher ratio without increasing the DMSO concentration above 1%. The results suggest then that at the higher molar ratios used in the experiments reported in Figs. 2 and 4 even more conversion of aggregates to dimer would occur.

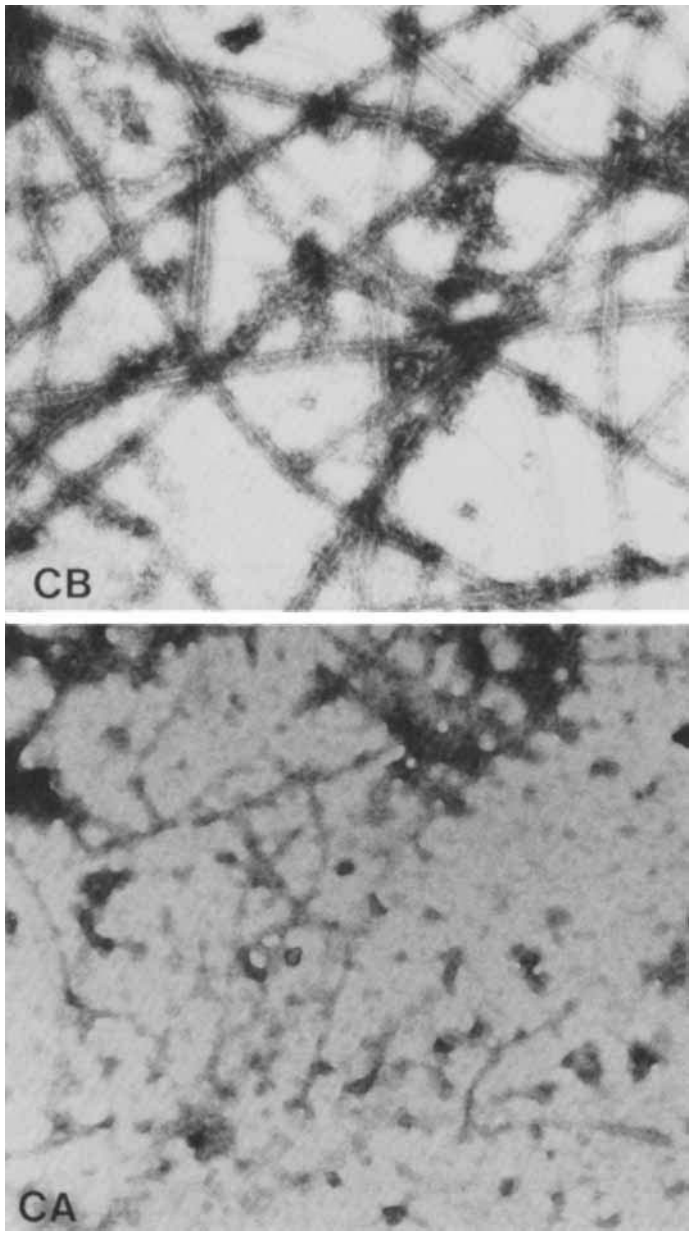


Fig. 3. Electron micrographs of tubulin polymerized after incubation with CA and CB. Tubulin (10 μM) was incubated at 37° in reassembly buffer with 100 μM CA or CB for 15 min before addition of GTP. Samples were negatively stained with uranyl acetate after an additional 10 min. Magnification is 57,600.

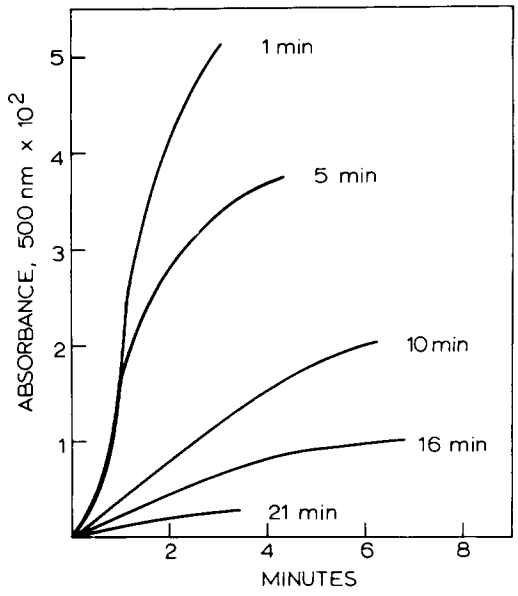


Fig. 4. Effect of time of incubation of tubulin with CA on the inhibition of polymerization. Tubulin (10 μM) was incubated with 50 μM CA at 37° in 0.5 ml of reassembly buffer containing 0.32 M glycerol for the times shown before the addition of GTP. Taken from Ref. 15 with permission.

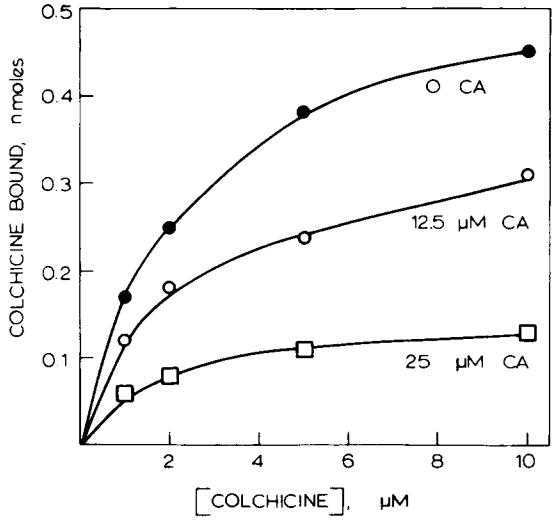


Fig. 5. Inhibition of colchicine binding by CA. Tubulin (1.5 nmoles) was incubated at 37° in the absence and presence of CA for 20 min in reassembly buffer. At this time different aliquots of a colchicine solution (1 mM, 5 mc/ml) were added and incubation was continued for 60 min. The reactions were stopped by placing the samples on ice and the amout of colchicine bound was determined by the filter assay method (19). Taken from Ref. 15 with permission.

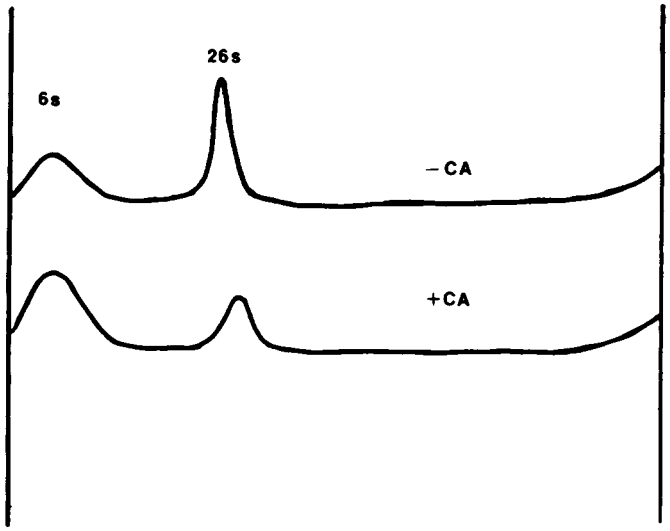


Fig. 6. Effect of CA on the ultracentrifuge pattern. Tubulin was polymerized, microtubules were collected by centrifugation and suspended in 2 ml of cold reassembly buffer. The protein concentration was 5 mg/ml. To 1 ml, 10 μl of a 10 mM CA solution was added. The samples were then centrifuged at 42,040 RPM in a Spinco Model E Ultracentrifuge. The patterns shown are after 16 min.

The irreversibility of the inhibition as well as the effects on the structure and colchicine binding activity of the protein suggested that covalent modification of tubulin by CA might explain its action. The only difference in the structures of CA and CB is at one position (Fig. 1). This difference, replacement of a keto function for a hydroxyl at position 20, however, causes a significant change in the reactivity of the double bond between carbons 21 and 22. α,β-Unsaturated esters and amides such as methyl acrylate and acrylamide are known to act as sulfhydryl reagents toward proteins (28). CA, which is an α,β-unsaturated, γ-keto lactone would be even more reactive toward nucleophiles. With this in mind we determined whether CA does react with sulfhydryl groups of tubulin and 2-mercaptoethanol.

Incubation of tubulin with a ten-fold molar excess of CA does indeed cause a rapid decrease in the free sulfhydryl content (Fig. 7). The reaction appears to occur in two phases, a rapid phase in which there is a decrease of about 10% of sulfhydryl content followed by a slower reaction. Within 25 min 40% of the sulfhydryl groups disappear. Since tubulin contains 16–18 sulfhydryl groups per 110,000, this is approximately the theoretical value expected under the conditions of the experiment. These results suggest that prior incubation of CA with a thiol compound would reduce its effectiveness as an inhibitor. This is indeed the case. 2-Mercaptoethanol almost completely abolishes the inhibitory activity of CA (Fig. 8). As a further confirmation of the interaction between CA and sulfhydryl groups, the reaction between CA and 2-mercaptoethanol was measured directly. CA has a higher absorbance in the 240 nm region than does CB because of the α,β-unsaturated keto-structure. Addition of a nucleophile across the double bond should cause this absorbance to disappear. The data in Fig. 9 shows that this does happen upon the addition of 2-mercaptoethanol. The latter compound has no effect on the absorbance of CB.

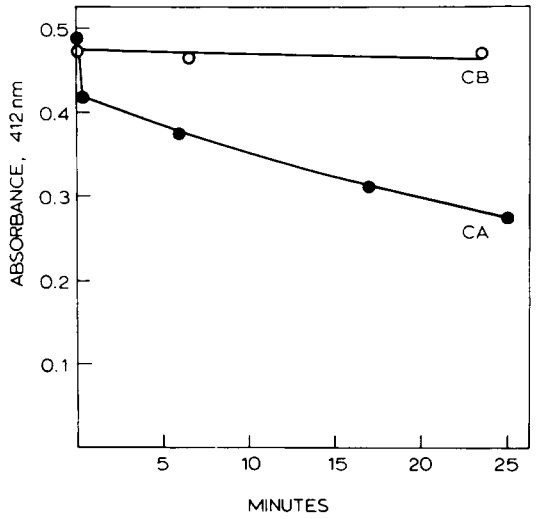


Fig. 7. The effect of CA on the sulfhydryl content of tubulin. Tubulin (0.7 mg) was incubated in 0.6 ml of reassembly buffer containing 0.3 M glycerol at 37°. A sample (0.1 ml) was removed and added to 0.4 ml of triethanolamine. HCl, pH 8.0, containing SDS and DTNB. The final concentrations of the latter two were 0.1% and 1 mM, respectively. The absorbance at 412 nm was read immediately. CA (5 μl of a 10 mM solution in DMSO) was added to the above tubulin solution and samples were removed at different times for sulfhydryl determinations as described. Taken from Ref. 15 with permission.

It is known that the increase in viscosity which occurs during the conversion of G-actin to F-actin is lower in the presence of CB (9). CA has an even greater effect on this transformation as is shown in Table I. The actin preparation contains 0.5 mM 2-mercaptoethanol. Thus in the experiment shown in Table I the low molecular weight thiol and actin compete for the CA. The sulfhydryl content of the G-actin present is 0.17 mM. Prior incubation of CA with 2-mercaptoethanol clearly reduces the inhibitory effect of CA.

DISCUSSION

The results clearly show that CA inhibits tubulin self-assembly by reacting with protein sulfhydryl groups, most likely via an addition reaction to the double bond between the carbon atoms at position 21 and 22. This double bond would be highly reactive because it is α,β to a lactone and it is situated next to a carbonyl function. Evidence to support this mechanism of action include: 1) the same molar ratio of CA to tubulin which inhibits polymerization causes a rapid decrease in the free sulfhydryl content of tubulin; 2) prior reaction of CA with 2-mercaptoethanol destroys its inhibitory effect; 3) the reaction of 2-mercaptoethanol with CA can be demonstrated by measuring the decrease in absorbance at 240 nm; 4) CB, which lacks the carbonyl group at position 20, shows none of the effects exhibited by CA. A suggested reaction between thiols and CA is presented in Fig. 10. The reaction with CA causes large structural changes as evidenced by a destruction of colchicine binding activity and a conversion of aggregated material to 6S dimer. Previous studies have shown that sulfhydryl reagents inhibit the in vitro self-assembly and colchicine binding activities (29). CA must be a very reactive sulfhydryl reagent since in

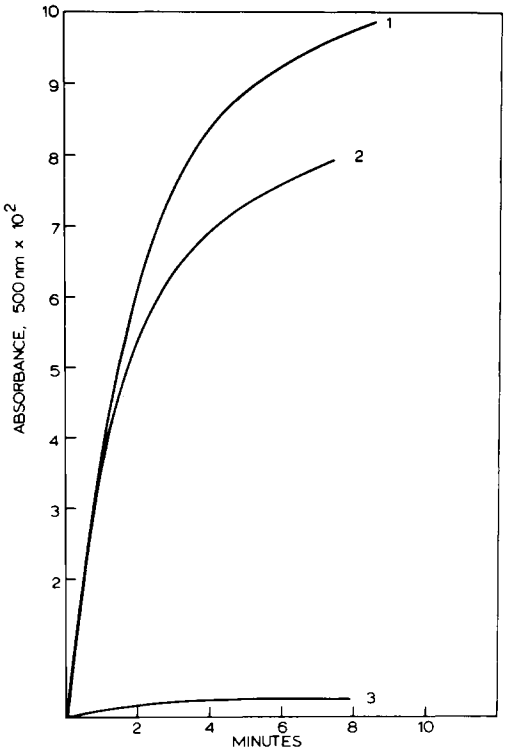


Fig. 8. The effect of 2-mercaptoethanol on inhibition by CA. 1) Polymerization of tubulin in reassembly buffer (0.84 mg/ml) without CA. 2) CA (100 μM) and 2-mercaptoethanol (1 mM) were incubated together at 37° in 0.42 ml of reassembly buffer for 1 min. Tubulin (0.42 mg in 0.08 ml) was added and after 11 min the polymerization was initiated by the addition of GTP. 3) CA (100 μM) and tubulin (0.84 mg/ml) were incubated at 37° for 11 min before the addition of GTP. The glycerol concentration in all experiments was 0.32 M. Taken from Ref. 15 with permission.

TABLE I. The Effects of Cytochalasin A and B on the Polymerization of G-Actin.

Additions	KCl	η_{sp}
DMSO	–	0.08
DMSO	+	1.55
CB, 100 μM	+	1.30
CA, 20 μM	+	**1.06**
CA, 50 μM	+	0.67
CA, 100 μM	+	0.35
CA, 100 μM + 2–MET	+	1.19

Five μl of DMSO, 10 mM CA or 10 mM CB were added to 0.5 ml of G-actin (1.5 mg/ml) in 0.5 mM ATP, 0.5 mM 2-mercaptoethanol, 0.2 mM $CaCl_2$ at 22°. After 2 min 10 μl of 4M KCl were added. Thirty minutes later the viscosity of the solutions was determined using a Cannon semi-microviscometer. In the experiment in which CA was first reacted with 2-mercaptoethanol, 167 μM CA was incubated with 2.17 mM 2-mercaptoethanol in 0.3 ml for 1 min prior to the addition of the actin in a 0.2 ml vol. The experiment was then continued as described above. Taken from Ref. 15 with permission.

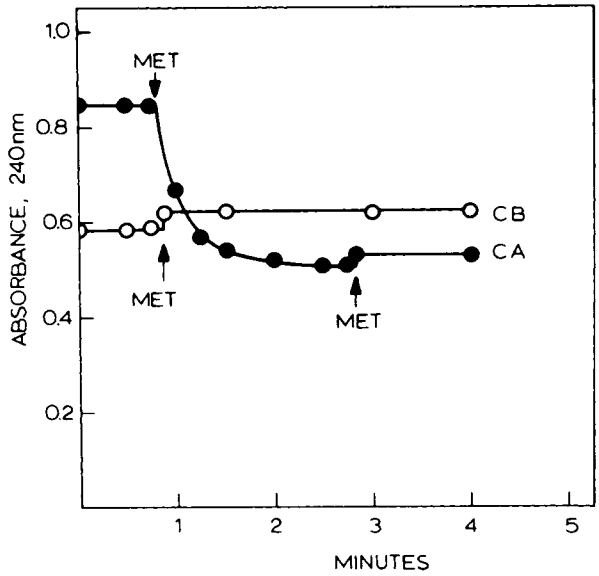


Fig. 9. The effect of 2-mercaptoethanol on the A_{240} of CA. CA or CB (100 μM) was incubated at 37° in 0.5 ml of reassembly buffer. Where shown 5 μl of a 100 mM solution of 2-mercaptoethanol was added.

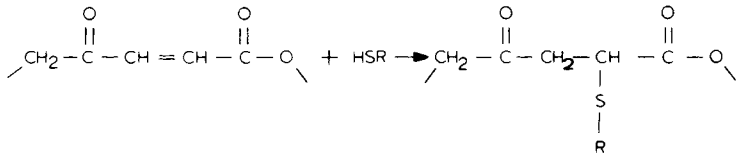


Fig. 10. Proposed reaction between sulfhydryl groups and CA.

most of these studies the molar ratio of CA to protein sulfhydryl groups was only about 0.6. CA at the concentrations used exhibited no effect on the structure of microtubules formed in vitro suggesting that either the sulfhydryl groups become inaccessible in the polymerized state or that their reaction with CA does not disrupt the tubule. The latter suggestion is unlikely in light of the fact that other thiol reagents depolymerize microtubules (29). Conceivably CA could react with other nucleophiles in addition to thiol groups. However, since methyl acrylate and acrylamide are selective for thiol groups at the pH used in the studies reported here (28) it is probable that only sulfhydryl modification accounts for inhibition of tubulin and G-actin polymerization.

The reactivity of CA toward sulfhydryl residues observed in these in vitro experiments probably explains the in vivo actions of CA, i.e., CA will react with proteins in a non-specific manner. The results do not explain the actions of CB and other cytochalasins. However, if results of experiments in which CB is used are to be attributed solely to the action of CB, the preparations must be free of contamination by CA. Such a contamination is not unlikely, especially when CA is used to prepare labelled CB.

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