# Sulfhydryls of Platelet Tubulin: Their Role in Polymerization and Colchicine Binding†

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ABSTRACT: Sulfhydryls and disulfides of platelet tubulin have been quantified, their accessibility and reactivity measured, and their role in polymerization and colchicine binding evaluated. Platelet tubulin isolated by two cycles of temperature-dependent polymerization-depolymerization was found to contain 12 free sulfhydryl groups per tubulin monomer all of which reacted rapidly with *p*-chloromercuribenzoate. One sulfhydryl was inaccessible to dithiobis(nitrobenzoic acid). Under anaerobic conditions of tubulin extraction, one intrachain disulfide bridge was found per tubulin monomer. Polymerization of tubulin reduced the number of sulfhydryls by one which were able to react with *p*-chloromercuribenzoate or dithiobis(nicotinic acid) but did not affect the disulfide bridge. Polymerizability of platelet tubulin was very sensitive to blocking of free sulfhydryl groups. Complete inhibition of microtubule assembly was obtained when the number of free sulfhydryls per tubulin was reduced by 3 but could be reversed by the addition of dithiothreitol. Colchicine binding, on the other hand, was only minimally influenced by blocking of sulfhydryls.

Mechanisms and regulation of tubulin assembly into microtubules are topics of continuing interest. Protein kinase mediated phosphorylation, nucleotide binding, and tyrosylation of tubulin have been studied as potential regulatory mechanisms of the reversible assembly-disassembly of microtubules. Previous studies by a number of authors have indicated that tubulin contained a considerable number of sulfhydryl groups (Renaud et al., 1968; Stephens, 1970; Lee et al., 1973; Eipper, 1974; Mellon & Rebhuhn, 1976). Surprisingly few reports, however, have dealt with the physiological role of sulfhydryl groups in microtubules. Kuriyama & Sakai (1974) in a brief communication pointed out the importance of free thiols for the assembly of microtubules. A similar conclusion was reached by Mellon & Rebhuhn (1976) who showed that diamide, a sulfhydryl-oxidizing agent, inhibits in vitro polymerization of tubulin and disperses 20S and 30S oligomers of tubulin. Sulfhydryl groups were also found to be important for in vivo assembly of microtubules which was shown to be influenced by intracellular levels of reduced glutathione (Oliver et al., 1976).

We believe that a precise quantification of sulfhydryl and disulfide groups and determination of their reactivity may prove to be of great importance for an understanding of the molecular structure of tubulin and possibly its mechanism of assembly into microtubules. In this communication we report our studies on the quantification and characterization of sulfhydryl and disulfide groups of platelet tubulin and their role in polymerization and colchicine binding activity.

## Experimental Section

### *Methods*

*Isolation of Platelet Tubulin.* Tubulin was prepared from human platelets by the method of Shelanski et al. (1973) with minor modification described previously (Ikeda & Steiner, 1976). It was dissolved in 0.1 M Pipes[1] buffer, pH 6.94, containing 4 mM EGTA, 2 mM $MgSO_4$, and 2 mM GTP (PM buffer). Platelet tubulin recovered after two cycles of temperature-dependent polymerization-depolymerization was 96% pure as judged by poly(acrylamide) gel electrophoresis (Ikeda & Steiner, 1976). It was used immediately for in vitro polymerization studies and quantification of sulfhydryl groups. In order to prevent inadvertent oxidation of sulfhydryl groups during isolation of platelet tubulin, the extraction procedures were performed with deaerated solutions. All extraction steps beginning with the sonication of platelets were carried out under $N_2$. During centrifugation a layer of mineral oil placed on top of the tubulin solution sealed the contents of the centrifugation tube toward the atmosphere. When disulfides were to be measured, a tenfold excess of NEM over the expected number of free sulfhydryl groups was added to the tubulin preparation after the last centrifugation.

*Quantification of Free Sulfhydryl Groups in Platelet Tubulin.* (a) Filtration Method Utilizing *p*-Chloro[$^{203}$Hg]mercuribenzoate. Nitrocellulose filters of 0.45-$\mu$m pore size were presoaked in 0.02 M Tris-HCl buffer, pH 7.8, containing 0.05 M NaCl for 30 min at room temperature. A solution containing [$^{203}$Hg]PCMB (10 $\mu$Ci) was prepared fresh daily by dissolving 5 mg of PCMB in 1 mL of 0.04 N NaOH and diluting with 0.01 M Tris-HCl buffer, pH 7.8, to the desired volume. To determine the final concentration of PCMB the optical absorbance was read at 233 nm using a molar absorbance coefficient of $1.69 \times 10^4$.

In general, the reaction mixtures contained 50–60 $\mu$g of platelet tubulin (0.1 mL) and 200 nmol of [$^{203}$Hg]PCMB (0.1 mL). Their volume was made up to 2 mL with 0.02 M Tris-HCl, pH 7.8. Unless otherwise indicated these mixtures were incubated for 60 min at 37 °C. Aliquots (usually 0.5 mL) were passed through nitrocellulose filters under weak negative

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[1] Abbreviations used: Pipes, piperazine-*N*,*N*′-bis(2-ethanesulfonic acid); PM buffer, 0.1 M Pipes, pH 6.94, containing 4 mM EGTA, 2 mM $Mg^{2+}$, and 2 mM GTP; EGTA, ethylene glycol bis($\beta$-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; PCMB, *p*-chloromercuribenzoic acid; DTNB, 5,5′-dithiobis(nitrobenzoic acid); CPDS, 6,6′-dithiodinicotinic acid; $NaDodSO_4$, sodium dodecyl sulfate, DCC, diphenylcarbamoyl chloride.

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pressure. The filters were then washed with 15 mL of 0.02 M Tris-HCl, pH 7.8, containing 0.05 M NaCl. The radioactivity of [$^{203}$Hg]PCMB platelet tubulin adducts adsorbed onto the filters was measured in a liquid scintillation spectrometer using Bio-Solv BBS (Beckman Inst.) as solubilizer and 0.4% (w/v) 2,5-diphenyloxazole plus 0.05% (w/v) 1,4-bis[2-(4-methyl-5-phenyloxazolyl)]benzene in toluene as scintillation fluid. Filtration assays were always performed in triplicate. Protein was measured by the method of Lowry et al. (1951) using bovine serum albumin as standard.

(b) Spectrophotometric Method Using 5,5′-Dithiobis(2-nitrobenzoic acid) or 6,6′-Dithiodinicotinic Acid. The reaction mixtures contained 1 mL of 1 M phosphate buffer, pH 8.1, and 0.1 mL of 3 mM DTNB or CPDS in 10 mM phosphate buffer, pH 8.1. These mixtures were incubated for 30 min at 22 °C. When DTNB was used, the optical absorbance was read at 412 nm against blanks consisting of reagent mixture without DTNB. The optical readings were also corrected for the absorbance of the reagent blank. The concentration of released thiol anion, 3-carboxy-4-nitrothiophenolate, was calculated using a molar absorbance coefficient of $1.36 \times 10^4$. With CPDS as the reagent, the optical absorbance was measured at 344 nm and a molar absorbance coefficient of $1 \times 10^4$ was applied.

*Determination of Disulfide Bonds.* (a) Filtration Method Utilizing *p*-Chloro[$^{203}$Hg]mercuribenzoate and Sodium Sulfite. Disulfide bonds of platelet tubulin were measured according to Ando & Steiner (1973). Tubulin solution (0.1 mL) containing 50–60 μg of protein was added to 1.9 mL of 0.02 M Tris-HCl buffer, pH 9.0, 8 M urea, 0.2 M sodium sulfite, and 200 nmol of [$^{203}$Hg]PCMB. The radioactivity of the [$^{203}$Hg]PCMB adducts adherent to the nitrocellulose filters was measured as described above. Filtrations were performed in triplicate.

(b) Spectrophotometric Determination of Disulfide Bonds. The method of Zahler & Cleland (1968) was used with minor modifications (Ando & Steiner, 1973).

*In Vitro Polymerization of Platelet Tubulin.* Platelet tubulin in PM buffer (≥1 mg/mL) was polymerized by raising the temperature to 37 °C. The reaction was followed using the increase in optical absorbance at 500 nm as an indicator of microtubule formation (Houston et al., 1974). As previously demonstrated (Ikeda & Steiner, 1976), electron microscopy verified the tubular nature of the polymerized tubulin.

*Colchicine Binding Assay.* Colchicine binding activity was measured by filter assay according to Wilson (1970) using Whatman DE 81 filter paper discs. Aliquots of purified tubulin were incubated for 60 min at 37 °C with [$^3$H]colchicine at a final concentration of 10 μM. Incubation mixture (0.1 mL) was passed through a moistened filter paper disc which was then washed with 15 mL of 10 mM phosphate buffer containing 0.5 mM $MgCl_2$, pH 6.95. The radioactivity which remained on the paper disc was measured by liquid scintillation counting (see above). All filtration assays were performed in triplicate.

*Tryptic Digestion and Preparation of Peptide Maps.* For these experiments tubulin was reduced and alkylated with [$^{14}$C]iodoacetamide. Platelet tubulin was dialyzed against 8 M urea, 0.35 M Tris-HCl, 0.12 M 2-mercaptoethanol, 0.1% EDTA, pH 8.5 for 24 h under $N_2$. Then [$^{14}$C]iodoacetamide was added at a final concentration of 0.12 M. The reaction was allowed to proceed in the dark for 60 min at room temperature and was stopped by dialysis against 8 M urea, 0.02 M Tris-HCl, and 1% thiodiglycol, pH 7.3. The tubulin was then placed on pH 8.7 urea-$NaDodSO_4$/poly(acrylamide) gels (5%) prepared according to Eipper (1974). The concentration of $NaDodSO_4$ was 0.1% in all gel solutions and in the electrode buffer which contained 0.01 M Tris and 0.077 M glycine. In general, gels were loaded with 20–40 μg of protein. Electrophoresis was carried out at room temperature at 1 mA per gel. Fixation and staining with Coomassie blue were performed by the method of Fairbanks et al. (1971). The bands representing α and β subunits were excised and the protein eluted by incubating the finely sliced gel pieces in 8–10 volumes of 5 mM phosphate buffer containing 0.05% $NaDodSO_4$, pH 7.5, with agitation at 37 °C. After 12–18 h the gel pieces were spun down and the supernatant passed through Sephadex G-25. The eluted protein was lyophilized and reelectrophoresed on $NaDodSO_4$–poly(acrylamide) (7.5%) gels to assure purity of the tubulin subunits. The process of excision, elution, passage through Sephadex G-25 and lyophilization was then repeated. The freeze-dried protein was suspended in 0.2 M ammonium bicarbonate and digested for 6 h at 37 °C with HCl-dissolved (1 mM), $CaCl_2$-containing (1 mM) DCC-trypsin at an enzyme to protein ratio of 1:20. After a second addition of trypsin at a ratio of 1:40, digestion was continued for another 6–8 h. The reaction mixture was then freeze-dried, dissolved in 20% acetic acid, and applied to a 0.9 × 19 cm cation exchange column (Aminex A-5, Bio-Rad) which had been equilibrated with 0.2 M pyridine–acetic acid buffer, pH 3.1. The column was developed at 50 °C with 450 mL of a linear gradient of pyridine–acetic acid beginning at pH 3.1, 0.2 M pyridine, and ending at pH 5.0, 2.0 M pyridine. At the completion of the gradient, an additional 50–75 mL of the final buffer was passed through the column. Developing buffer was passed through the column at a rate of 30 mL/h and effluent fractions, 2.1 mL in volume, were collected. One aliquot of each fraction was used for measurement of ninhydrin-reactive material (Moore & Stein, 1954) while another was used to determine the radioactivity. Completeness of trypsin digestion of tubulin was measured essentially as described by Eipper (1974). The amounts of arginine and lysine released by carboxypeptidase B ranged from 78 to 84% of the total amount of these amino acids present in platelet tubulin.

## *Materials*

*p*-Chloro[$^{203}$Hg]mercuribenzoate (specific activity, 99.8 mCi/g) was obtained from Amersham/Searle Corp., Arlington Heights, Ill. [$^3$H]Colchicine (specific activity, 9.35 Ci/mmol) was purchased from New England Nuclear, Boston, Mass. *N*-Ethylmaleimide and *p*-chloromercuribenzoate were obtained from Mann Research Lab, New York, N.Y. Dithiothreitol and 5,5′-dithiobis(2-nitrobenzoic acid) were products of Calbiochem, Los Angeles, Calif.; 6,6′-dithiodinicotinic acid was purchased from Aldrich Chemical Co., Milwaukee, Wis.; pipes and colchicine were obtained from Sigma Chemical, St. Louis, Mo. GTP was a product of P-L Biochemicals, Milwaukee, Wis., and nitrocellulose membrane filters were obtained from Schleicher & Schuell Inc., Keene, N.H.

## Results

*Quantification of Sulfhydryl Groups in Depolymerized Platelet Tubulin.* (a) PCMB-Reactive Sulfhydryl Groups. Under standard conditions of assay the [$^{203}$Hg]PCMB retained on nitrocellulose filters was linearly related to the amount of platelet tubulin in the range from 0 to 100 μg. The number of sulfhydryl groups reacting with the mercurial agent could be titrated as seen in Figure 1. The end point at the intersection of the two straight lines occurs at 6 nmol of sulfhydryl groups per 0.5 nmol of platelet tubulin. Further experiments established that under standard conditions platelet tu-

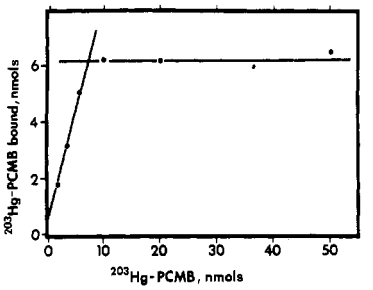


FIGURE 1: Titration of free sulfhydryl groups of platelet tubulin with $^{203}$Hg-labeled PCMB. Tubulin (0.5 nmol) was incubated at 37 °C for 60 min under standard conditions with increasing amounts of mercurial reagent. The mercurial adducts formed were measured by passing the reaction mixtures through nitrocellulose filters.

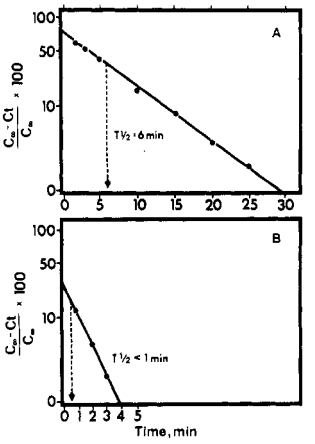


FIGURE 2: Reaction of depolymerized tubulin with DTNB. The number of nonreacted free sulfhydryl groups ($C_\infty - C_t$) expressed as a percentage of the total available half-cysteines ($C_\infty$) were plotted against time of reaction. The upper panel illustrates the result obtained with nondenatured tubulin, the lower panel those obtained with tubulin which had been denatured with 1% $NaDodSO_4$. Incubations were carried out under standard conditions for the time intervals indicated in the figure.

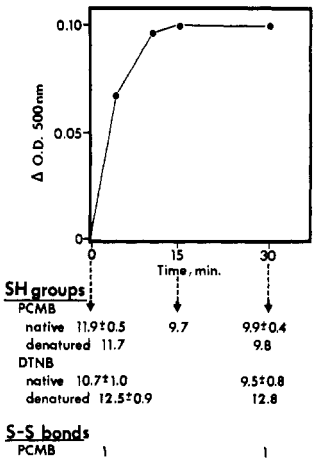


FIGURE 3: Quantification of sulfhydryls and disulfides in platelet tubulin undergoing assembly into microtubules. Tubulin suspended in PM buffer at a concentration of 1 mg/mL was polymerized by raising the temperature to 37 °C. The increase in optical absorbance at 500 nm, indicative of polymerization, was recorded at the indicated time intervals. At 0, 15, and 30 min after raising the temperature to 37 °C aliquots of the tubulin suspension were withdrawn for assay of free sulfhydryl groups and disulfide bonds. The conditions of assay were standard and described under Methods. Tubulin was denatured by addition of $NaDodSO_4$ to a final concentration of 1%. The number of sulfhydryl groups given in the figure represent the means of three experiments. Those values for which ±1 SD are shown are the means of five experiments.

TABLE I: Sulfhydryl Groups of Platelet Tubulin.

| reagent | mol per 55 000 g of tubulin[a] undenatured | denatured |
|---|---|---|
| PCMB | 11.9 ± 0.5 | 11.7 ± 0.6 |
| DTNB | 10.7 ± 1.0 | 12.5 ± 0.9 |
| CPDS | 11.6[b] | |

[a] Mean ± 1 SD (six experiments). [b] Mean of three experiments.

bulin has 11.9 ± 0.5 mol of sulfhydryl groups per mol of tubulin monomer (mean ± 1 SD; six experiments). Denaturation with 8 M urea did not change the total number of mercaptides formed. The rate of reaction of sulfhydryl groups with excess PCMB was very fast. Within 1 min mercaptide formation had already reached a plateau. This suggests easy accessibility of all reactive sulfhydryl groups to this reagent. Preincubation of tubulin with colchicine under saturating conditions did not change the number of PCMB-reactive thiols.

Evaluation of the number of reactive sulfhydryl groups in denatured, sulfite-treated tubulin revealed 1 more thiol than in native, nonreduced microtubule protein which indicates the presence of 1 disulfide bond per monomer in depolymerized tubulin. For this experiment free sulfhydryl groups were first alkylated with NEM. In preliminary studies we were able to show that this alkylating agent could block all PCMB-reactive thiols of platelet tubulin. After cleavage by sodium sulfite, 0.93 nmol of mercurial adducts formed in 1 nmol of alkylated platelet tubulin. A similar result was obtained when [$^{14}$C]-iodoacetamide was used instead of PCMB. Finally, spectrophotometric determination of disulfide bonds with dithiothreitol and DTNB (Zahler & Cleland, 1968) also gave evidence of 1 disulfide bond per tubulin monomer.

Irrespective of whether platelet tubulin was completely reduced and carboxamidomethylated or not its molecular weight as judged by $NaDodSO_4$-polyacrylamide gel electrophoresis remained unchanged at 55 000.

(b) DTNB- and CPDS-Reactive Sulfhydryl Groups. The relation between formation of 3-carboxy-4-nitrothiophenolate and the amount of platelet tubulin reacted with DTNB was linear. The number of sulfhydryl groups determined by this method was 10.7 ± 1.0 mol per mol of tubulin monomer (mean ± 1 SD; six experiments). Denaturation of platelet tubulin with 1% $NaDodSO_4$ increased the number of DTNB-accessible thiols by approximately 2 (Table I). The rate of 3-carboxy-4-nitrothiophenolate formation when excess DTNB was reacted with free sulfhydryl groups of platelet tubulin was also evaluated. A semilogarithmic plot of the number of nonreacted free tubulin thiols expressed as percentage of the total available sulfhydryl groups vs. time showed a straight line for all the measured rate values which intersected the ordinate approximately at the 65% point (Figure 2). Thus 35% of the sulfhydryl groups had reacted by the time the first rate measurement at 2 min after DTNB addition was taken. Because of technical difficulties no attempt was made to obtain reaction rates at less than 1 min. For the observed values, the $T_{1/2}$ was found to be 6 min. Upon denaturation with 1% $NaDodSO_4$ again 2 groups of thiols could be distinguished, one in which the reaction with the sulfhydryl agent had gone to completion by the time the first reading could be taken and the other of a $T_{1/2}$ of <1 min, markedly faster than in the nondenatured molecule.

Reaction of tubulin with CPDS yielded 11.6 mol of free sulfhydryl groups per mol of tubulin monomer.

*Quantification of Sulfhydryl Groups in Polymerized Tubulin.* The number of reactive sulfhydryl groups in polymerized

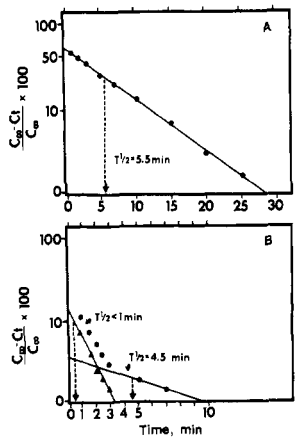


FIGURE 4: Reaction of polymerized tubulin with DTNB. The number of nonreacted free sulfhydryl groups ($C_\infty - C_t$) expressed as a percentage of the total available half-cysteines ($C_\infty$) was plotted against time of reaction. The results obtained with nondenatured microtubules are shown in the upper panel, those of $NaDodSO_4$-treated (1%) polymerized tubulin in the lower panel. Incubations were carried out under standard conditions described under Methods.

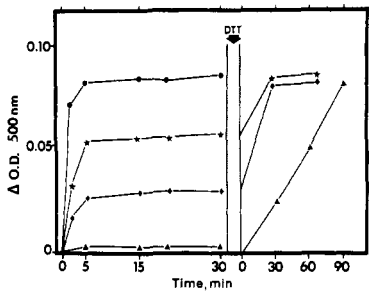


FIGURE 5: Effect of PCMB on polymerization of platelet tubulin. Tubulin suspended in PM buffer (1 mg/mL) was incubated at 37 °C with varying concentrations of PCMB. The extent of polymerization was measured by the increase in optical absorbance at 500 nm as described under Methods. (Circles) No PCMB; (stars) $8.4 \times 10^{-9}$ M PCMB; (diamonds) $17 \times 10^{-9}$ M PCMB; (triangles) $25 \times 10^{-9}$ M PCMB. After 30-min incubation an excess of DTT (400 nmol/mL) was added to the tubulin suspensions and the optical absorbance at 500 nm monitored.

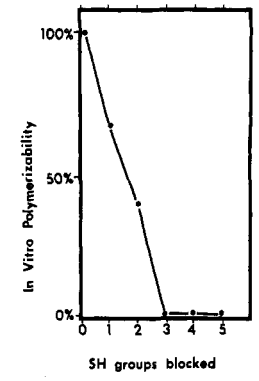


FIGURE 6: Relation between number of sulfhydryl groups blocked and inhibition of polymerization of tubulin. Platelet tubulin in PM buffer (1 mg/mL) was incubated at 37 °C with increasing amounts of PCMB. The maximal optical absorbance at 500 nm was recorded and plotted as a percentage of the absorbance value obtained with non-PCMB-treated tubulin. The number of free sulfhydryls blocked was measured and related to the decrease in polymerizability.

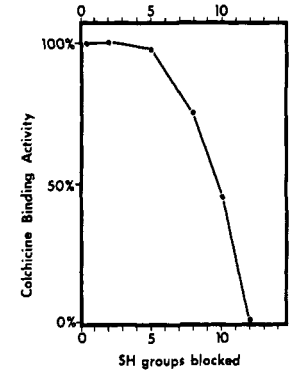


FIGURE 7: Relation between number of sulfhydryl groups blocked and inhibition of colchicine binding activity of platelet tubulin. Solutions of tubulin in PM buffer (0.5–1 mg/mL) were incubated with varying concentrations of PCMB for 15 min at 37 °C following which their colchicine binding activity was measured as described under Methods. The number of sulfhydryl groups which had formed mercurial adducts was related to the ligand activity expressed as a percentage of that obtained in non-PCMB-treated tubulin. The latter was subjected to the same preincubation period (15 min) at 37 °C as the PCMB-treated experimental samples.

tubulin was found to be less than in the depolymerized form of the protein (Figure 3). The mercurial adducts decreased by 2, while DTNB-reactive thiols decreased by 1. The number of disulfide bonds did not change when tubulin polymerized. Denaturation of polymerized tubulin with 8 M urea did not make the 2 thiols which had become unreactive following polymerization available to reaction with PCMB or DTNB but solubilization of microtubules with 1% $NaDodSO_4$ did.

The rate of reaction of DTNB with native polymerized tubulin was virtually identical with that of depolymerized tubulin (Figure 4). However, treatment of polymerized tubulin with $NaDodSO_4$ produced a marked difference in the rate of reaction. A composite semilogarithmic plot was constructed by drawing a straight line corresponding to the slowest reaction and subtracting for each observed time from the measured value of the remaining free sulfhydryl groups their extrapolated value predicted by the straight line. In this manner, the reaction of DTNB with $NaDodSO_4$-treated polymerized tubulin could be resolved into at least three simultaneous rates. As no measurements were taken at time intervals less than 1 min, this estimate of three types of reactive sulfhydryl groups presents a minimal value. The rate of 3-carboxy-4-nitrothiophenolate formation by one group of thiols, constituting 2.3 mol per mol of tubulin monomer, was virtually identical with that observed in depolymerized tubulin. Compared with the latter, however, only approximately half as many thiols had this rate in polymerized tubulin. A small fraction of the sulfhydryl groups in polymerized tubulin, 0.7 mol per mol of tubulin monomer, reacted with a $T_{1/2}$ of 4.5 min.

*Effect of PCMB on in Vitro Polymerization of Tubulin.* In vitro polymerization of platelet tubulin was markedly inhibited by PCMB (Figure 5). At concentrations >0.025 $\mu$M, PCMB completely abolished microtubule formation. The specificity of the inhibition was demonstrated by the fact that DTT restored the ability of tubulin to polymerize. To determine the number of free sulfhydryl groups necessary for polymerization, platelet tubulin was exposed to titrated amounts of PCMB (Figure 5). When ≥3 sulfhydryl groups had formed mercaptides, polymerizability was completely lost (Figure 6). The effect of PCMB on polymerized tubulin was also studied. Upon addition of an excess of mercurial reagent (20 nmol/nmol of tubulin), polymerized tubulin became aggregated displaying some decrease of optical absorbance at 500 nm but more importantly showing complete loss of microtubules as demonstrated by electron microscopy.

*Effect of Sulfhydryl Agents on Colchicine Binding Activity.* The ability to bind colchicine is one of the most specific functions of tubulin. As colchicine is known to inhibit polymer-

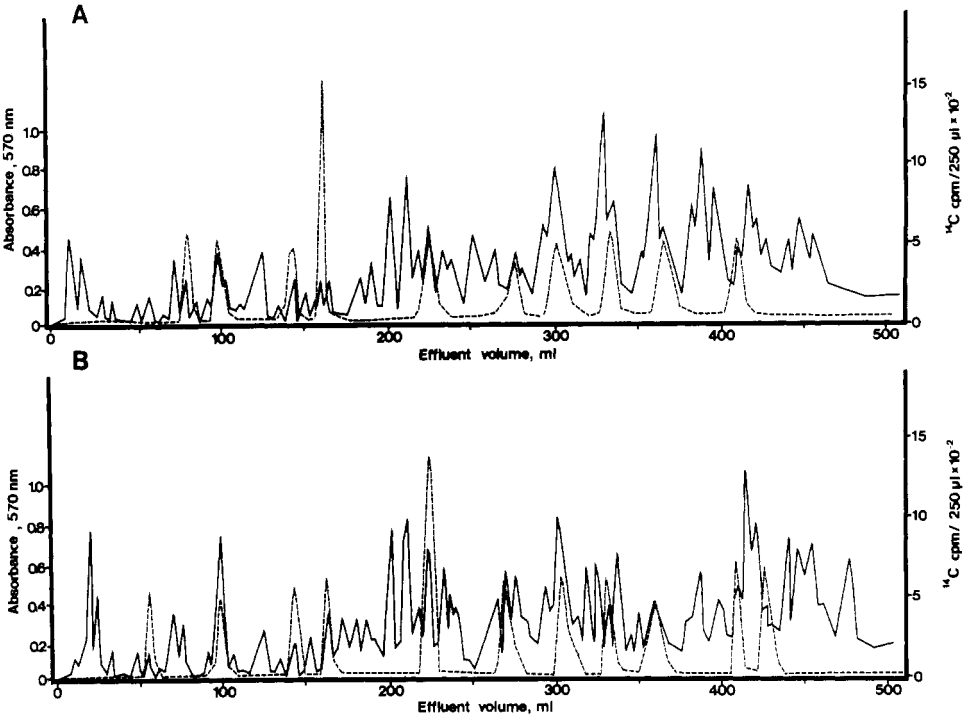


FIGURE 8: Pattern of soluble tryptic peptides of [$^{14}$C]carboxamidomethylated $\beta$ (A) and $\alpha$ subunits (B) of platelet tubulin. Details of the isolation and preparation of tubulin subunits and their digestion with trypsin are described under Methods. Soluble tryptic peptides from 2 mg of tubulin subunits were applied to a cation exchange column which was developed with a pyridine–acetic acid gradient starting at 0.2 M pyridine, pH 3.1, and rising to 2 M pyridine, pH 5.0. The exact conditions of chromatographic separation are described under Methods. Aliquots of 2.1-mL fractions were analyzed for ninhydrin-reactive material (solid line) and for radioactivity (interrupted line).

ization of tubulin, we investigated the effect of PCMB and NEM on the colchicine ligand activity of tubulin. The latter was completely inhibited in the presence of ≥16 nmol of PCMB or 20 nmol of NEM/nmol of tubulin monomer. DTT was able to abolish the inhibition due to mercaptide formation. Titration of the thiols necessary for colchicine binding revealed that, even when 5 sulfhydryl groups were blocked, the ligand activity was virtually unchanged (Figure 7). Complete loss of colchicine binding occurred only when all free sulfhydryl groups of tubulin were blocked.

*Analysis of Tryptic Peptides of* [$^{14}$C]*Iodoacetamide-Treated Tubulin.* The tryptic peptides of $\alpha$ and $\beta$ subunits of carboxamidomethylated tubulin were prepared as described under Methods and were separated by ion-exchange chromatography. The pattern of soluble peptides obtained is shown in Figure 8. Measurement of radioactivity demonstrated 10 $^{14}$C-labeled peptides in the $\alpha$ subunit and 11 $^{14}$C-labeled peptides in the $\beta$ subunit. Both subunits had one peptide which contained higher radioactive counts than the others. This probably indicates the presence of more than one sulfhydryl group in these peptides. There was considerable congruence in the tryptic peptides of the 2 tubulin subunits and also of the peptides containing sulfhydryl groups. However, the high activity peptide and at least two others seem to differ in the 2 subunits.

## Discussion

There is general consensus that tubulin isolated from brain contains 8–11 easily titratable sulfhydryl groups. Lee et al. (1973) were the only investigators who described one disulfide bond per brain tubulin subunit. Their studies provided persuasive evidence for an intrachain location of the disulfide bridge. Stephens (1970) found one intrachain disulfide bond in B tubulin of the flagellar outer doublet of *Strongylocentrotus droebachiennsis*. However, their experiments do not rule out the possibility that the disulfide bridge formed during the extraction of tubulin. None of the other investigators were able to demonstrate the presence of disulfide bonds in tubulin. It was specifically for this reason that we conducted our extraction procedure under a nitrogen atmosphere to prevent formation of disulfides during the isolation of the protein from platelets. Our results clearly established that one disulfide bridge existed per tubulin subunit of 55 000 molecular weight. Evidence obtained from gel electrophoretic analysis of reduced and alkylated denatured platelet tubulin indicates that the disulfide bridge is intrachain.

The total number of free sulfhydryl groups in platelet tubulin is somewhat greater than in tubulin extracted from brain. The difference ranges between 1 and 3 sulfhydryl groups depending on whose data one uses for comparison. Analysis of $^{14}$C-labeled carboxyamidomethylated tryptic peptides of individual tubulin subunits revealed 11 labeled peptides for $\alpha$ and 10 for $\beta$ subunits, representing 12 and 11 cysteine residues, respectively. This falls short of the number of sulfhydryls measured in the intact tubulin molecule but tryptic digestion was incomplete as approximately 20% of the lysines and arginines in platelet tubulin remained inaccessible to carboxypeptidase B. All of the sulfhydryls in depolymerized tubulin were readily accessible to mercurial reagent, both denatured and nondenatured tubulin forming the same number of mercaptides. Analysis of the reaction of sulfhydryls with DTNB showed very clearly that there are at least two types of sulfhydryls, one reacting almost instantaneously, the other with a $T_{1/2}$ of about 6 min. As expected denaturation increased the rate at which the sulfhydryl groups reacted. It is of interest that the rate of mixed disulfide formation between DTNB and nondenatured polymerized tubulin was almost the same as in the depolymerized native protein. However, the number of sulfhydryls accessible to PCMB and DTNB decreased slightly in polymerized tubulin. These results suggest that conformational changes occur

in tubulin as the protein undergoes polymerization. The reason why denaturation of microtubules did not make all the thiols available to the mercurial reagent is not known. The disulfide bridge of platelet tubulin was accessible to DTNB only in the uncoiled form of the protein. Our results show that polymerization did not result in formation of interchain disulfide bridges as polymerized tubulin had the same number of disulfide bonds as depolymerized tubulin.

Sulfhydryl groups are clearly involved in the assembly of microtubules. In platelet tubulin 3 sulfhydryl groups appear to be involved in the formation of microtubules. Kuriyama & Sakai (1974) reported a similar behavior for tubulin of porcine brain in which the blocking of 2 sulfhydryl groups abolished its polymerizability. The complete reversal by DTT of the inhibitory effect of PCMB on the ability to form microtubules confirms the importance of free sulfhydryls for this process. In sharp contrast is the effect of sulfhydryl blocking agents on the colchicine binding activity of tubulin. This phenomenon which occurs only when tubulin has a specific conformation (Ventilla et al., 1972) seems to be little affected by free sulfhydryl groups. As the heterodimer is essential for ligand activity, one can conclude that sulfhydryls are not critical for the interaction of the 2 tubulin subunits.

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# Aminoacyl-tRNA Synthetases from Yeast: Generality of Chemical Proofreading in the Prevention of Misaminoacylation of tRNA†

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ABSTRACT: The specificity of valyl-, phenylalanyl-, and tyrosyl-tRNA synthetases from yeast has been examined by a series of stringent tests designed to eliminate the possibility of artefactual interference. Valyl-tRNA synthetase, as well as activating a number of amino acid analogues, will accept alanine, cysteine, isoleucine, and serine in addition to threonine as substrates for both ATP-$PP_i$ exchange and transfer to some $tRNA^{Val}$ species. The transfer is not observed if attempts are made to isolate the appropriate aminoacyl-$tRNA^{Val}$-C-C-A but its role in the overall aminoacylation can be suspected from both the formation of a stable aminoacyl-$tRNA^{Val}$-C-C-A(3′$NH_2$) compound and from the stoichiometry of ATP hydrolysis during the aminoacylation of the native tRNA. Similar tests with phenylalanyl-tRNA synthetase indicate that this enzyme will also activate and transfer other naturally occurring amino acids, namely, leucine, methionine, and tyrosine. The tyrosine enzyme, which lacks the hydrolytic capacity of the other two enzymes (von der Haar, F., & Cramer, F. (1976) *Biochemistry 15,* 4131-4138) is probably absolutely specific for tyrosine. It is concluded that chemical proofreading, in terms of an enzymatic hydrolysis of a misacylated tRNA, plays an important part in maintaining the specificity in the overall reaction and that this activity may be more widespread than has so far been suspected.

The importance of fidelity in the aminoacylation of tRNAs has been appreciated for a long time (Pauling, 1958) but proposals have only recently been put forward for the way in which specificity is maintained (for a review, see Igloi & Cramer, 1978). Such mechanisms, in whichever detailed form one expresses them, are a necessary property of protein synthesizing systems if only to reduce the well-known isoleucyl-tRNA synthetase/valine misactivation (Baldwin & Berg, 1966) which, although efficient in the isolated systems, results in maximally 1 error in 3000 in the presence of the complex protein synthesizing machinery (Loftfield & Vanderjagt, 1972). Has the intricate mechanism of chemical proofreading (von der Haar & Cramer, 1976) which brings about the reduction in the error rate in the isoleucyl-tRNA synthetase/

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