SULFHYDRALS AND THE IN VITRO POLYMERIZATION OF TUBULIN

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ABSTRACT

The free sulfhydryls of brain tubulin prepared by cyclic polymerization procedures both with and without glycerol have been examined. The average free sulfhydryl titer of tubulin prepared with glycerol (7.0 sulfhydryls/55,000 mol wt) is greater than that of tubulin prepared without glycerol (4.0 sulfhydryls/55,000 mol wt). Diamide, a sulfhydryl-oxidizing agent, inhibits the polymerization of tubulin. Diamide also disperses the 20S and 30S oligomers of tubulin seen in analytical ultracentrifuge patterns of tubulin solutions and, depending on the temperature at which diamide is added, converts all or part of the oligomeric material to 6S dimers. Electron microscopy demonstrates that diamide also destroys the 450-Å ring structures characteristic of tubulin solutions. All diamide effects are reversible by the addition of 10 mM dithioerythritol, a sulfhydryl-reducing agent. That diamide interacts with sulfhydryls on tubulin is directly demonstrated by a 50% decrease in the free sulfhydryl titer of tubulin measured after diamide treatment. Concentrations of CaCl₂ which inhibit polymerization also decrease the free sulfhydryl titer of tubulin.

Since the in vivo assembly and disassembly of microtubules is a basic cellular process about which little is known, potential regulatory mechanisms involving modifications of tubulin such as phosphorylation (4), tyrosylation (1), and the presence or absence of carbohydrate (7) are of considerable interest. Modifications of the reactivity of sulfhydryl groups on tubulin similarly suggest themselves as interesting possibilities for control. Previous work on tubulin isolated from mammalian brain by ion exchange procedures has demonstrated that there are 8-11 half-cystines/55,000 monomer (4, 16) and that most of the half-cystine residues exist as easily titratable sulfhydryls in the native tubulin molecule. Eipper (4) has reported that in rat brain tubulin there are 11 half-cystine residues all accounted for by easily titratable sulfhydryls. However, Lee et al. (16) have shown in calf brain that while 8 of the 10 sulfhydryls detected by amino acid analysis exist as available sulfhydryls, the remaining two sulfurs are involved in an interchain disulfide bond. The differences in these two reports may result from differences in the techniques used to isolate tubulin.

With the introduction of the in vitro polymerization conditions developed by Weisenberg (25), we can now investigate the sulfhydryls of polymerizable tubulin. We have determined average sulfhydryl titers of tubulin prepared with and without glycerol and we have also investigated the effects of diamide, a sulfhydryl-oxidizing agent, on tubulin polymerization. Diamide is a substituted di-azene introduced by Kosower (10, 11) which stoichiometrically oxidizes low molecular weight thiols to disulfides according to reactions 1 and 2 (10) and, in particular, rapidly converts reduced glutathione to oxidized glutathione. Diamide has recently been shown to inhibit cell division in sea...
urchin eggs and to cause the rapid disappearance of the in vivo mitotic apparatus (23). We report below that diamide inhibits the in vitro polymerization of brain tubulin and causes the dispersal of the 30S and 20S oligomers of tubulin, an effect which is reversible by disulfide-reducing agents. Our results are complementary to those of Kuriyama and Sakai (15) who have reported that the chemical blockage of two of the seven sulfhydryls of tubulin will inhibit polymerization.

\[
RS^- + (CH_3)NCON--NCON(CH_3)_2 \xrightarrow{H^+} \text{ "diamide"} \\
RS \xrightarrow{H^+} \text{RSSR} \\
(1)
\]

\[
RS \xrightarrow{H^+} \text{RSSR} \\
(2)
\]

**Materials and Methods**

**Materials**

Diamide, DTE (dithioerythreitol), EGTA [ethylene glycol bis(aminooxyethyl ether) tetraacetic acid], PIPES, [piperazine-N,N'-bis(2-ethane sulfonic acid)], DTNB, [5,5'-dithiobis(2-nitrobenzoic acid)], and GuHCl (guanidine HCl) were obtained from Sigma Chemical Co., St. Louis, Mo. 4,4-Dithiodipyridine was obtained from Aldrich Chemical Co., Milwaukee, Wis. All other chemicals were reagent grade.

**Purification of Tubulin**

Tubulin was prepared from brain tissue by repeated cycles of polymerization and depolymerization. Fresh rabbit or porcine brains were prepared within 2 h of sacrifice of the animal. Rapidly frozen rabbit or porcine brain was obtained from Pel-Freez Bio-Animals, Inc., Rogers, Ark., and maintained at -80°C until used. Fresh or frozen brain tissue regardless of source behaved identically in these experiments. After removal of the meninges, fresh or frozen brains were homogenized in 0.1 M PIPES, 1 mM EGTA, and 50 μM MgCl₂, pH 6.4 (PEM buffer), at a ratio of 1 g of tissue per milliliter of buffer. The homogenate was centrifuged for 30 min at 50,000 g at 4°C. The supernate was removed and polymerization was initiated by addition of GTP to a final concentration of 1 mM. After polymerization for 30 min at 37°C, microtubules were pelleted at 35,000 g for 20 min at 25°C and resuspended in PEM buffer. Solutions were then chilled for 30 min and clarified by centrifugation at 100,000 g at 4°C for 20 or 30 min. Further purification was accomplished by repeated cycles of polymerization—with each complete cycle consisting of a centrifugation at room temperature to pellet microtubules and a centrifugation at 2-4°C to remove material which does not depolymerize in the cold. For preparations at pH 6.8, tubulin was prepared through two cycles of polymerization at pH 6.4 and resuspended in PEM buffer at pH 6.8 during the last cycle of polymerization.

Purification according to the glycerol method of Shekstein et al. (24) was used in some preparations. In these cases the brain homogenate was diluted 1:1 with PEM buffer containing 8 M glycerol and polymerized at 37°C with 1 mM GTP. Microtubules were pelleted at 100,000 g at 25°C for 30 min and resuspended in PEM buffer without glycerol and thus contained the residual glycerol included in the pellet. We estimated the final concentration of glycerol in most preparations to be 0.5 M. The microtubules were depolymerized and the solution was clarified by centrifugation in the cold as described above. In all successive cycles of purification, the tubulin solution was made 4 M in glycerol before polymerization.

**Turbidity Measurements**

The polymerization reaction was followed by an increase in turbidity at 500 nm in a Gilford Recording Spectrophotometer (Gilford Instrument Laboratories, Inc., Oberlin, Ohio). The absorbance was measured against a blank containing depolymerized tubulin without GTP. For measurements of polymerization in the presence of diamide and DTE, the blank also contained equivalent concentrations of these reagents.

**Sulfhydryl Titrations**

The reaction of DTNB with -SH groups was carried out according to the method of Ellman (5). The assay mixture contained 1-5 μM tubulin, 0.1-0.2 mM DTNB, 0.1 M Tris HCl, pH 7.5, and, when required, 1 mM CaCl₂, 6 M GuHCl, or 8 M urea in a final volume of 1 ml. The reaction was followed at 25°C in a Gilford Recording Spectrophotometer by measuring increase in absorbance at 412 nm against a blank without tubulin. A separate blank with an appropriate concentration of tubulin in PEM buffer was run and the values obtained were subtracted from each experimental value.

For -SH determinations of diamide-treated tubulin, 0.1-0.2 mM tubulin in PEM was preincubated with 1 mM diamide for 15-30 min at 25°C and diluted to 2-5 μM in the final assay mixture. A blank value for diamide alone in the assay mixture was subtracted from the experimental values.

For -SH determinations of CaCl₂-treated protein, 0.1-0.2 mM tubulin in PEM buffer was preincubated for 15-30 min at 25°C with 3 mM CaCl₂ to overcome the 1 mM EGTA in the PEM buffer and then diluted to 2-5...
μM in the final assay mixture which was supplemented with 1 mM CaCl₂.

The reaction of 4,4-dithiodipyridine (4-TP) with −SH groups was carried out according to the method of Grassetti and Murray (8). The assay mixture contained 2–5 μM tubulin, 1 mM 4-TP, 0.1 M phosphate buffer, pH 6.5, and, where present, 6 M GuHCl or 1 mM CaCl₂ in a final volume of 1 ml. The reaction was followed by measuring the increase in absorbance at 324 nm against a blank without tubulin. The protocol for CaCl₂-treated protein was the same as described for the DTNB reaction. Since diamide absorbs strongly at 324 nm, the measurement of the −SH groups for diamide-treated protein was done only after a 2-h dialysis to remove diamide.

**Ultracentrifugation**

Sedimentation velocity experiments were done with a Beckman Model E analytical ultracentrifuge (Beckman Instruments, Inc., Fullerton, Calif.) and recorded with a Schlieren optical system. All samples were run at a rotor speed of 48,000 rpm with the schlieren phase angle set at 65°.

**Electron Microscopy**

For electron microscopy, samples of tubulin at 3–4 mg/ml were negatively stained with 1% uranyl acetate and examined with a Hitachi HU11E1 electron microscope.

**Protein Concentrations**

Protein concentrations were determined by the method of Lowry et al. (17) using bovine serum albumin as a standard. Since PIPES and glycerol interfere with the Lowry reaction, standard curves were run with appropriate concentrations of these reagents and proper corrections applied.

**RESULTS**

**Sulfhydryl Titers of Tubulin Prepared with and without Glycerol**

In Table I, sulfhydryl values are shown for tubulin prepared by cyclic polymerization with and without glycerol. Tubulin was analyzed after three complete cycles of polymerization in the presence or absence of glycerol and was at least 90% pure on SDS acrylamide gels. The approximately 10% impurity was primarily due to the presence of high molecular weight proteins which appear to copurify with tubulin (2). The number of free sulfhydryls was determined with DTNB and 4-TP and was expressed per 55,000 mol wt monomer of tubulin.

The sulfhydryl values obtained with DTNB and 4-TP for tubulin prepared through three complete cycles of polymerization with glycerol were 7.3 and 7.2, respectively, and agree with the value of 7.2 reported by Kuriyama and Sakai (15) for tubulin prepared with glycerol. In contrast, the values determined for tubulin prepared without glycerol were 4.0 and 4.1. In tubulin prepared both with and without glycerol, no additional sulfhydryls became available when assays were performed in the presence of the denaturing agents 8 M urea or 6 M GuHCl; apparently no sulfhydryls had been masked in the native molecule. Assuming that the tubulin prepared under both conditions had an equal number of half-cystines per molecule, it is likely that the lower sulfhydryl titer in tubulin prepared without glycerol represents sulfhydryls unavailable to DTNB or 4-TP because they have been covalently modified—probably oxidized to disulfides.

**Oxidizing Agents Inhibit Polymerization**

We also examined the effect of oxidizing agents on tubulin polymerization. Diamide inhibits the polymerization of tubulin in a dose-dependent manner. Fig. 1 shows the effect of diamide incubated with tubulin for 10 min at 37°C before initiation of polymerization with 1 mM GTP. Both the rate and the extent of polymerization are de-
FIGURE 1 Effect of diamide on tubulin assembly. Tubulin prepared through one cycle of polymerization without glycerol, pH 6.4, was preincubated with diamide for 10 min before initiation of polymerization with 1 mM GTP. The turbidity in each experiment was followed at 37°C against a blank containing an equivalent concentration of diamide. (a) - - - - No additions; (b) - - - - 7.5 x 10^-5 M diamide; (c) - - - - 10^-4 M diamide; (d) - - - - 3 x 10^-4 M diamide. Protein concentration was 5.0 mg/ml.

Increased with increasing concentrations of diamide. Complete inhibition occurs at 3 x 10^-4 M diamide. When 3-5 x 10^-4 M diamide is added to tubulin at the same time as GTP, some polymerization occurs initially but the microtubules formed disappear within 30 min. 1 mM sodium tetraphthionate and 10 mM oxidized glutathione also inhibit tubulin polymerization.

Diamide will also cause the disassembly of preformed microtubules although we have observed variable rates of disassembly. Upon addition of 1 mM diamide, some preparations of microtubules as monitored by turbidity were completely depolymerized in 5 min, while in others the process took as long as 2 h. The differences in rates of disassembly are not yet understood.

Reversal of Inhibition with DTE

The inhibition of polymerization by diamide can be reversed by the subsequent addition, in excess, of the reducing agents DTE or mercaptoethanol. Fig. 2 shows the effect of the addition of 10 mM DTE after a 5- or 30-min incubation with 1 mM diamide and 1 mM GTP. Upon addition of DTE, the ability to polymerize is recovered rapidly, although the extent of the recovery depends on the length of the incubation with diamide. After 10 min in diamide, the extent of the polymerization on addition of DTE was 87% of maximum control value; after 30 min in diamide and GTP, the level of polymerization was only 62% of control. No polymerization can be detected upon addition of DTE to tubulin after a 1-h treatment with diamide. Excess reducing agents, e.g., reduced glutathione or DTE, added concomitantly with diamide will prevent the inhibition of polymerization. The ability of sulfhydryl-reducing agents to both prevent and reverse the inhibition of polymerization by diamide strongly suggests that diamide is interacting with sulfhydryls on tubulin.

Diamide Lowers Free Sulfhydryl Titer

Direct evidence that diamide is acting on free sulfhydryls has been obtained by measuring the number of free sulfhydryls after incubation of tubulin with diamide (Table II). The number of free sulfhydryls in tubulin prepared with and without glycerol decreases by approximately 50% after diamide treatment at 25°C for 15 min. Incubations for 1 h or more at higher temperatures result in a 70% decrease in the sulfhydryl titer (Table II). There is no increase in the number of sulfhydryls when titrations are performed in the presence of 6 M GuHCl, indicating that diamide does not mask sulfhydryls but probably covalently modifies them. Even after removal of diamide by dialysis, the number of sulfhydryls in tubulin remains low.

FIGURE 2 Reversal of diamide inhibition of polymerization with DTE. (A) 1 mM diamide was added to tubulin for 5 min at 37°C before addition of 1 mM GTP to control and experimental samples. 5 min after the addition of GTP 10 mM DTE was added to the diamide-inhibited sample. (B) Diamide and 1 mM GTP were added at time zero to the experimental sample, while GTP alone was added to the control. After 30 min, 10 mM DTE was added to the diamide-inhibited sample. Protein concentration for both A and B was 6.0 mg/ml. Different batches of tubulin prepared through one cycle of polymerization, pH 6.4, without glycerol were used in the two experiments.

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### Table II

**Effect of Diamide on Sulphydryl Titers of Tubulin**

| Additions                  | Conditions | Sulphydryl assay (Sulhydryls/55,000 mol wt) | DTNB | 4-TP |
|----------------------------|------------|------------------------------------------|------|------|
|                           |            |                                          |      |      |
| Prepared with glycerol     |            |                                          |      |      |
| None                      | 15-30' 25°C| 7.4 ± 0.5                                 | (3)  |      |
| Diamide 1 mM              | 15-30' 25°C| 3.8 ± 0.7                                 | *    |      |
| Diamide 1 mM + 6 M GuHCl  | 15-30' 25°C| 3.6                                      | (3)  |      |
| Diamide 1 mM              | 1 h 37°C   | 2.0                                      | (1)  |      |
| Diamide 1 mM              | 15' 25°C   |                                          |      |      |
|                        | dialyzed 2 h|                                      |      |      |
| Diamide 1 mM              | 15' 25°C   | 3.5                                      | (1)  |      |
|                        | dialyzed 20 h|                                    |      |      |
| Prepared without glycerol |            |                                          |      |      |
| None                      | 15-30' 25°C| 3.4 ± 0.3                                 | (2)  |      |
| Diamide 1 mM              | 15-30' 25°C| 2.0 ± 0.1                                 | (2)  |      |

* Number of different preparations on which determinations were made.

† After diamide addition, tubulin solutions were dialyzed against PEM buffer at 0°C. Conditions for DTNB and 4-TP assays are as described in Fig. 1. Diamide was added to 0.1–0.2 mM solutions of tubulin. Aliquots of each sample were taken to give 2–5 μM protein in the final assay mixture. All tubulin was prepared through three cycles of polymerization either with or without glycerol.

Thus, the reaction of diamide with tubulin apparently results in a covalent modification of the sulphydryls on tubulin, not spontaneously reversible in the absence of soluble diamide. These observations are consistent with the suggestion that diamide oxidizes free sulphydryls on tubulin to disulfides in the same manner as it oxidizes thiols of low molecular weight to their corresponding disulfides (Eq. 1 and 2 in the introductory paragraphs).

### Ultracentrifugation Patterns

Depolymerized microtubules exhibit characteristic patterns when examined in the ultracentrifuge, depending on the presence or absence of glycerol and the final pH of the solution. At pH 6.4, the ultracentrifuge pattern of tubulin prepared without glycerol has two peaks sedimenting at about 6S and 30S. At pH 6.8 there are three peaks, the 30S and an additional peak sedimenting at 20S (2, 22). The 6S peak is the 110,000-mol wt dimer of tubulin, and the 30S peak in our preparations consists predominantly of 450-Å double ring forms. The 20S peak may represent a single ring structure but has not yet been conclusively characterized. Ring structures reported to run at either 30S (2) or 36S (9) have been proposed as intermediates in in vitro polymerization.

When 1 mM diamide is added at 0°C to tubulin prepared without glycerol at either pH 6.4 or pH 6.8, the faster sedimenting species (30S and 20S) disappear while the 6S dimer peak increases in size (Fig. 3). Analysis of the areas under the peak of the plates in Fig. 3 shows that the area of the 6S peak after diamide treatment is equal to the area under all the peaks in the respective controls (Table III). Thus, the material in the faster sedimenting peaks has been completely converted to 6S dimers by diamide treatment at 0°C.

Fig. 4 shows the effect of diamide added at 37°C to tubulin prepared at pH 6.4. As when added at 0°C 1 mM diamide abolished the 30S peak and enhanced the size of the 6S peak. However, in this case the area under the 6S peak after diamide treatment constitutes only 57% of the total area under the two peaks in the control (Table III). Examination of the same preparation treated with a lower concentration of diamide (0.5 mM) reveals the presence of a broad peak sedimenting ahead of the 30S peak which apparently contains a heterogeneous collection of fragments with S values greater than 30S, probably aggregate forms of the 30S component. In the case of the 1 mM diamide treatment, similar large heterodisperse fragments unable to sediment as a single peak may account for that fraction of the control tubulin undetectable as either 6S or 30S components after diamide treatment. Thus, at 37°C the 30S component may be either dispersed into 6S dimers as occurs at 0°C or converted into large heterodisperse fragments.

As expected, both effects of diamide on the 30S
FIGURE 3  Effect of diamide added at 0°C. 1 mM diamide was added at 0°C to tubulin at either pH 6.4 or pH 6.8 which had been prepared through one cycle of polymerization without glycerol. The samples were analyzed in the ultracentrifuge at 6°C. (a) pH 6.4 no additions; (b) pH 6.4 + 1 mM diamide; (c) pH 6.8 no additions; (d) pH 6.8 + 1 mM diamide. Protein concentrations were 9 mg/ml at pH 6.4 and 6 mg/ml at pH 6.8.

Peak areas are reversed with excess DTE (Fig. 4), although the extent of the recovery depends on the length of the period of incubation with diamide before DTE addition. 10 mM DTE added to tubulin subsequent to incubation with 1 mM diamide for 5 or 15 min at 37°C causes the restoration of the 30S peak with a concomitant decrease in the size of the 6S peak (Fig. 4). The size of the reformed 30S peak is larger if DTE is added 5 min after diamide than if added 15 min after diamide addition. The total area under the 6S and 30S peaks in the sample which had been incubated with diamide for 5 min was 94% of the total area under the corresponding peaks in the control (Fig. 4). The material not sedimenting as either 6S or 30S components may be present as large fragments. Thus, the DTE-induced reformation of the 30S peak is correlated with the reversal of the diamide-induced inhibition of polymerization, the extent of which is also dependent on the length of the incubation in diamide. The subsequent addition of 10 mM DTE to tubulin prepared at pH 6.4 and treated with diamide at 0°C also causes the reappearance of the 30S peak. In tubulin prepared at pH 6.8 both the 20S and 30S reappear on addition of DTE.

**Electron Microscopy**

Further evidence that diamide causes a disappearance of rings which can be reversed with DTE has been obtained with the electron microscope. In Fig. 5, negatively stained preparations of tubulin treated with 1 mM diamide at 37°C exhibit no rings, while in the control sample 450-A rings are abundant. When this same sample of diamide-treated tubulin was further incubated with DTE and then examined in the electron microscope, numerous rings were again present. It is clear that the DTE can cause the reformation of rings dispersed by diamide. In preparations treated with diamide at 0°C, some rings are still present after 20 min but have disappeared after 2 h.

**Effect of DTE on Polymerization and Ultracentrifugation Patterns**

Tubulin prepared without glycerol has a relatively low sulfhydryl titer, and reducing agents

**Table III**

*Comparison of the Total Areas under the Peaks on Ultracentrifuge Patterns of Diamide- and DTE-Treated Tubulin*

| Conditions                  | Total area under all peaks | Total area on control pattern | g   | %  |
|-----------------------------|----------------------------|-------------------------------|-----|----|
| Fig. 3                      |                            |                               |     |    |
| 0°C, pH 6.4                 |                            |                               | 0.047 | 100 |
| Control                     | 0.050                      |                               |     |    |
| Diamide 1 mM                | 0.051                      | 100                           |     |    |
| 0°C, pH 6.8                 |                            |                               |     |    |
| Control                     | 0.032                      |                               |     |    |
| Diamide                     | 0.032                      | 100                           |     |    |
| Fig. 4                      |                            |                               |     |    |
| 37°C control                |                            |                               | 0.047 | 100 |
| 37°C 5 M diamide            | 0.048                      | 102                           |     |    |
| 37°C 1 mM diamide           | 0.027                      | 57                            |     |    |
| 37°C 1 mM diamide 5' min + 10 mM DTE | 0.044 | 94 |     |    |
| 37°C 1 mM diamide 15 min + 10 mM DTE | 0.038 | 81 |     |    |

Peak areas were compared by transferring outlines of peaks to tracing paper by using a Nikon comparator, cutting out the peaks, and then weighing the papers.

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Effect of diamide or diamide and DTE added at 37°C Tubulin prepared through one cycle of polymerization without glycerol at pH 6.4 was incubated with diamide for 15 min at 37°C. (a) No additions; (b) 0.5 mM diamide; (c) 1 mM diamide. Tubulin from the same preparation which had been incubated with 1 mM diamide for 5 min (d) or 15 min (e) was further incubated for 10 min at 37°C with 10 mM DTE. All samples were analyzed in the ultracentrifuge at 20°C. Protein concentration was 10 mg/ml.

Calcium Lowers Sulphydryl Titer

In the presence of concentrations of CaCl₂ which inhibit polymerization, the average number of free sulphydryls on tubulin is decreased (Table IV). Tubulin prepared both with and without glycerol and assayed in the presence of 1 mM Ca²⁺ consistently showed a 10–20% reduction in the free sulphydryl titer. The calcium effect on sulphydryls requires the presence of 1 mM Ca²⁺ in the assay mixture. If tubulin is pretreated with 3 mM Ca²⁺ and diluted 1:50 into unsupplemented sulphydryl assay mixture, the initially depressed sulphydryl titer quickly reattains the control number of sulphydryls. However, if 1 mM Ca²⁺ is present in the assay mixture, the level of sulphydryls remains 10–20% less than that of the control. Thus, the reduction of the sulphydryl titer is apparently reversible in the absence of high Ca²⁺ concentrations.

Two further observations suggest that Ca²⁺ is not specifically interfering with the sulphydryl assay by DTNB and 4-TP: (a) 1 mM Ca²⁺ does not affect the sulphydryl titer of small molecule thiols determined with DTNB or 4-TP; and (b) the sulphydryl titer of tubulin preincubated with 3 mM Ca²⁺ and 1 mM diamide is the same as the sulphydryl titer of tubulin treated in diamide alone (Table III). If Ca²⁺ were nonspecifically interfering with the sulphydryl assay, a further decrease over that occurring in diamide alone would have been expected. This result also indicates that the sulphydryls available to diamide include those available to calcium.

DISCUSSION

Effect of Diamide

Since diamide is known to oxidize thiols of low molecular weight to disulfides and would be expected to oxidize unhindered acidic protein thiols (10), it is reasonable to suggest that the mechanism of the interaction of diamide with tubulin is through the oxidation of its free sulphydryls. Most of the sulphydryls of tubulin are accessible to titrating agents and are therefore exposed to the solvent. The observation that diamide-induced inhibition of polymerization can be reversed with excess reducing agents is strong support for this suggestion. The fact that diamide modifies the sulphydryls on tubulin is directly demonstrated by the 50% reduction in the number of free sulphydryls available to two sulphydryl-titrating agents after diamide treatment. Since the number of
sulfhydryls does not increase when titrations are done in the presence of protein-denaturing agents, the reaction of diamide with tubulin induces a modification which is probably covalent in nature. This observation is consistent with the idea that the sulfhydryls have not been masked by some indirect effect of diamide but in fact have been oxidized to disulfides.

In tubulin prepared with glycerol, the difference in the sulfhydryl titer between the control and diamide-treated samples indicates that 3.6 sulfhydryls are lost upon treatment with diamide. Although the impurity of the preparation (i.e. the presence of high molecular weight proteins of unknown sulfhydryl content) and the possible heterogeneity of the tubulin preparations precluded exact integral figures for the number of sulfhydryls per tubulins, the figures suggest that, on the aver-

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**Figure 5** Effect of diamide and DTE on the 30S circles of tubulin. Tubulin was prepared through three cycles of polymerization at pH 6.4. Samples were examined in the electron microscope after the following additions. (a) None; (b) 1 mM diamide for 15 min at 37°C; (c) 1 mM diamide for 15 min at 37°C + 10 mM DTE for 15 min at 37°C. × 130,000. Bar = 0.1 μM.

**Figure 6** Effect of DTE added alone on tubulin polymerization. 10 mM DTE was added for 10 min at 37°C before initiation of polymerization with 1 mM GTP. Tubulin was prepared through three cycles of polymerization at pH 6.4. Protein concentration was 3 mg/ml.
FIGURE 7 Effect of DTE on the sedimentation patterns of tubulin. 10 mM DTE was added at 0°C to tubulin at pH 6.4 or 6.8. The tubulin at pH 6.4 had been prepared through three cycles of polymerization and at pH 6.8 through two cycles. Samples were analyzed at 4°C. (a) pH 6.4, no additions; (b) pH 6.4 + 10 mM DTE; (c) pH 6.8, no additions; (d) pH 6.8 + 10 mM DTE. Protein concentrations were 8 mg/ml at pH 6.4 and 9 mg/ml at pH 6.8.

In the absence of glycerol, diamide has caused the formation of two disulfide bonds in tubulin prepared with glycerol. The loss of sulfhydryl titer in tubulin prepared without glycerol is 1.4, and suggests that in this type of preparation one disulfide bond is formed.

Another possibility is that diamide inhibits polymerization by binding to the tubulin sulfhydryls, as occurs in the inhibition of polymerization by N-ethyl maleimide, DTNB, or p-chloromercuribenzenesulfonate, as reported by Kuriyama and Sakai (15). In the latter case, the bound reagents probably cause steric hindrance of tubulin-tubulin interactions. However, in the case of diamide, the thiol-diazene adduct formed with small molecule thiols is unstable (12), and therefore, by analogy, we consider the possibility of a stable protein-diamide compound unlikely. In addition, DTNB or 4TP analysis of tubulin after diamide treatment indicates that not all of the free sulfhydryls are modified by diamide treatment. If a stable diamide-tubulin complex could be formed, it seems reasonable that all of the sulfhydryls which are in fact easily accessible to DTNB or 4-TP would have reacted with diamide. However, this did not occur. A likely explanation for the fact that diamide modifies only some of the free sulfhydryls on tubulin is available if it is assumed that diamide acts to induce formation of protein disulfides. In this case, diamide action would require spatially adjacent sulfhydryls, and not all of the sulfhydryls on

| Preincubations    | Sulphydryl assay (Sulfhydryls/55,000 mol wt) |
|-------------------|---------------------------------------------|
|                   | DTNB | 4-TP |
| Prepared with glycerol |      |      |
| None              | 7.4 ± 0.5 (3)* | 7.1 ± 0.1 (3) |
| CaCl₂ 3 mM†       | 6.5 ± 0.5 (3) | 5.8 ± 0.6 (3) |
| Diamide 1 mM      | 4.2 ± 0.6 (2) | – |
| Diamide 1 mM + CaCl₂ 3 mM† | 4.3 ± 0.3 (2) | – |
| Prepared without glycerol |      |      |
| None              | 3.4 ± 0.3 (2) | – |
| CaCl₂ 3 mM†       | 2.9 ± 0.2 (2) | – |

* Number of different preparations on which determinations were done.
† Reaction mixtures contained 1 mM CaCl₂.

Conditions for DTNB and 4-TP reactions are as described in Table I. Tubulin 0.1–0.2 mM was preincubated with each reagent for 15°–30° at 25°C. Aliquots of each sample were taken to give 2–5 μM protein in the final assay mixture. Tubulin was prepared through three cycles of polymerization either with or without glycerol.
tubulin would be correctly oriented for the formation of a disulfide bond. Also, the reversibility of the inhibition of polymerization with DTE can be readily explained as the reduction of the protein disulfide bonds, while it is not known if the addition of a reducing agent would cause the disassociation of a diamide tubulin complex. However, until direct measurements of the binding of diamide to tubulin can be obtained, the question of a diamide-tubulin complex remains open.

The inhibition of polymerization by diamide correlates with the disappearance of the 20S and 30S components characteristic of the ultracentrifuge patterns of depolymerized tubulin. The disappearance of the oligomer peaks is accompanied by an increase in the size of the 6S dimer peak which accounts for all or a part of the material in the original oligomer peaks, depending on the temperature of the diamide addition. Electron microscopy confirms the dispersal of the 30S rings with diamide. The 20S and 30S peaks represent oligomers of tubulin and therefore depend on tubulin-tubulin interactions. The dispersal of the oligomers into 6S dimers suggests that diamide can prevent subunit interactions between tubulin dimers but does not prevent monomer-monomer interactions (i.e. ultracentrifuge patterns show no decrease in the S value of the 6S dimer peak on addition of diamide). Tubulin subunits unable to form rings are probably also unable to form microtubules. Since the probable effect of diamide is to oxidize certain of the tubulin sulfhydryls, we suggest that the presence of free sulfhydryls is necessary for the dimers to be capable of subunit-subunit interaction and that the oxidation of these sulfhydryls to disulfides destroys this capacity. As expected, the addition of excess reducing agents allows the partial restoration of rings as demonstrated by both the reappearance of the 30S peak in the ultracentrifuge patterns and the reformation of circles observable with the electron microscope. An additional possibility is that diamide modification affects the interaction of tubulin and the high molecular weight proteins and thus interferes with the ability of tubulin to polymerize or form rings.

Analysis of ultracentrifuge patterns of tubulin treated with diamide at 0°C shows that all of the material in the 30S peak is converted to 6S dimers. However, analysis of the ultracentrifuge patterns of tubulin treated with diamide at 37°C indicates that, in addition to a conversion of some of the 30S components to 6S, as occurs at 0°C, the 30S component also aggregates into heterogeneous fragments of high molecular weight. Both of these effects of diamide are reversed with DTE. The aggregation of 30S material may be due to the direct formation of intermolecular disulfides by diamide or to disulfide exchange reactions occurring subsequent to intramolecular disulfide formation.

**Tubulin Prepared with and without Glycerol**

Tubulin preparations purified through three cycles of polymerization with and without glycerol do not have the same sulfhydryl titer; the average number of sulfhydryls in tubulin prepared without glycerol is four while the sulfhydryl titer of tubulin prepared with glycerol is seven. Since the number of free sulfhydryls in tubulin prepared without glycerol is not increased on denaturation with guHCl or urea, the unavailability of sulfhydryls to titrating reagents in such preparations indicates that they have been covalently modified, probably oxidized to disulfide bonds. The persistence of the differences in sulfhydryl titer between tubulin prepared with and without glycerol in the presence of denaturing agents argues against the possibility that the sulfhydryls have been noncovalently "masked" (as, for example, by the high molecular weight proteins) in tubulin prepared without glycerol.

It should be emphasized that these experiments compare purified tubulin samples whose preparation through a multistep process has included or not included glycerol. At the polymerization and depolymerization step of each cycle of purification, tubulin of unknown composition has been discarded and the discarded tubulin may or may not have the same composition in tubulin prepared with and without glycerol. Thus, after three cycles of polymerization, the resultant purified tubulin is only a sample of tubulin originally present in the homogenate and may represent quite different populations of tubulin for the two types of preparation. The effect of glycerol is probably best considered as a cumulative, selective effect occurring during the total process of purification, rather than a direct effect on tubulin sulfhydryls. Preliminary evidence indicates that the addition of glycerol to tubulin purified without glycerol will not raise the sulfhydryl titer of the solution, i.e. glycerol does not directly reduce disulfide bonds. Possible more indirect effects of glycerol occurring...
during purification are that (a) glycerol protects tubulin sulfhydryl from auto-oxidation, and that (b) in the presence of glycerol, highly reduced tubulin is selected for at either the polymerization or depolymerization step.

Correlation of Free Sulfhydryls and the Polymerizability of Tubulin

Certain observations suggest that the polymerizability and free sulfhydryl content of tubulin may be related. (a) Glycerol is known to stabilize the tubulin on storage (14, 24) and to enhance the yield of microtubules on polymerization (24), and these phenomena may be related to a protection of free sulfhydryls by glycerol. (b) Diamide and calcium both of which inhibit the polymerization of tubulin also lower the free sulfhydryl titer of tubulin.

However, other results indicate that the correlation between polymerizability and the number of free sulfhydryls may not be simple. For example, the number of free sulfhydryls in tubulin prepared without glycerol and the diamide-inhibited tubulin prepared with glycerol have roughly equivalent sulfhydryl titers (3-4), yet tubulin prepared without glycerol will polymerize and the diamide-treated tubulin will not. The differences in the polymerizability of tubulin with similar sulfhydryl contents may reflect the complex nature of a solution of tubulin. It is likely that the sulfhydryls on tubulin are not all equivalent. There may be some sulfhydryls whose presence is critical to polymerizability, for which the presence of others may be extraneous. Thus, although polymerizable tubulin prepared without glycerol and diamide-treated tubulin may have similar sulfhydryl titers, the diamide-treated tubulin may have lost the critical sulfhydryls while these may be retained in tubulin prepared without glycerol. Average sulfhydryl titers do not provide information on which sulfhydryls have oxidized under given conditions and are therefore of limited value. It is also possible that tubulin populations may be heterogeneous with respect to sulfhydryl content.

The possible requirement for the nucleation of polymerization (2, 6, 9) may also complicate any direct proportionality between the number of free sulfhydryls and the extent of polymerization. In the presence of a limiting number of nucleating centers, 6S dimers could remain unincorporated into microtubules regardless of their fitness with respect to available sulfhydryls. For example, the fact that excess reducing agents do not consistently cause an increase in the extent of polymerization in tubulin prepared without glycerol (which has a relatively low sulfhydryl titer) may suggest that other factors, perhaps the number of available nucleating centers, are limiting the extent of polymerization.

It is interesting to note that the difference between tubulin in 6S dimers and 30S circles does not depend on the number of free sulfhydryls. The ultracentrifugation patterns of tubulin prepared without glycerol are similar in the presence and absence of reducing agent, i.e. the addition of reducing agent cannot produce a homogeneous population of either 6S or 30S molecules. However, reducing agents can apparently have some effect on the shape or size of the 30S molecules. However, quantitative comparison of the two preparations with respect to parameters such as rings vs. spirals, length of spirals, etc., are needed to answer this question.

The Effect of Calcium

CaCl₂ inhibits polymerization of tubulin at 1 mM free Ca⁺⁺ in a reversible manner, i.e. the removal of Ca⁺⁺ with EGTA will allow polymerization to occur. However, inhibitory concentrations of calcium do not destroy the rings as do inhibitory concentrations of diamide. Ca⁺⁺, like diamide, consistently causes a decrease in the free sulfhydryl titer of tubulin. The decrease in sulfhydryl titer which occurs in tubulin prepared both with and without glycerol is always smaller (10-20%) than the decrease seen with diamide (50%). The fact that calcium interacts with fewer sulfhydryls than diamide may be related to the fact that calcium does not affect the stability of circles while diamide does.

The nature of the calcium-sulfhydryl interaction is not known. The formation of disulfide bonds (21), noncovalent Ca⁺⁺-sulfur complexes, i.e. Ca⁺⁺ dithiolate bonds (13), or other conformational changes in the region of the protein close to the sulfhydryls could conceivably block access to the sulfhydryl-titrating reagents. The inhibition of tubulin polymerization by calcium cannot be prevented or reversed with 10 mM DTE.

It is interesting to note that Ca⁺⁺ has been
previously shown to cause the formation of disulfide bonds in a protein. As reported by Price et al. (21), the addition of 4 mM CaCl₂ to reduced pancreatic deoxyribonuclease causes the formation of two disulfide bonds and thus activates the enzyme. Calcium causes the formation of one of the two disulfides and activates the enzyme even in the presence of a 1,000-fold molar excess of mercaptoethanol, although the Ca⁺⁺ is only loosely bound to the enzyme. In view of these results, the idea that Ca⁺⁺ could induce the formation of disulfide bonds, even in the presence of excess reducing agent, becomes an interesting possibility. In the present case, however, it would be expected that removal of the calcium would not cause an increase in the number of free sulfhydryls as is found with tubulin. This argues for a more subtle and reversible effect of calcium on tubulin sulfhydryls, perhaps formation of a weak Ca⁺⁺ dithiolate bond (13) which might be expected to compete with the DTNB or 4-TP for access to sulfhydryls and so reduce the number measurable with those compounds.

Mechanism of Diamide In Vivo

1 mM diamide inhibits cell division in sea urchin eggs and causes the rapid disappearance (2–3 min) of the in vivo mitotic apparatus (19, 22). Although diamide can cause the disassembly of brain microtubules in vitro, it is unlikely that diamide acts directly on the microtubules in the spindles of marine eggs. Sea urchin eggs contain a high concentration of reduced glutathione (6–11 mM) (3, 20), which is known to react very rapidly with diamide (10). The diamide is probably consumed in the oxidation of reduced glutathione before a direct reaction with tubulin can occur. In support of this contention, we have observed in vitro that the presence of 10 mM reduced glutathione will protect tubulin polymerization from the inhibitory effects of 1 mM diamide (18). We have also found that the diamide-induced disassembly of microtubules in vitro occurs more slowly (5 min–2 h) than the observed in vivo rate of spindle disappearance. Thus, the disappearance of the mitotic apparatus in sea urchin eggs is probably not due to the direct oxidation of tubulin by diamide but rather to an indirect effect of diamide on glutathione, possibly through tubulin and the sulfhydryl enzyme Ca⁺⁺ ATPase, as discussed elsewhere (23).

CONCLUSION

In conclusion, we have shown that diamide inhibits tubulin polymerization and disperses the 20S and 30S oligomers characteristic of tubulin solutions. The addition of excess reducing agents reverses the effect of diamide on tubulin polymerization and allows for the reformation of the 20S aggregate and the 30S rings. Diamide treatment decreases the number of free sulfhydryls available to titrating agents and probably acts by oxidizing free sulfhydryls on tubulin to disulfide bonds. This suggests that tubulin capable of protomer-proto-mer interaction requires the presence of some free sulfhydryls. These findings may be important in developing optimum conditions for tubulin polymerization. Also, alterations in the sulfhydryls of tubulin represent possible control mechanisms for tubulin polymerization in vivo, either by direct oxidation and reduction of sulfhydryls or via calcium (19, 23).

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