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EFFECT OF 6-HYDROXYDOPAMINE ON POLYMERIZATION OF TUBULIN

PROTECTION BY SUPEROXIDE DISMUTASE, CATALASE, OR ANAEROBIC CONDITIONS*

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Abstract—Microtubular protein (tubulin) isolated from porcine brain was subjected to selected oxidative stresses, including incubation with the neurotoxin 6-hydroxydopamine (6-OHDA) under aerobic and anaerobic conditions. The functional capacity of the tubulin was determined on the basis of its ability to form microtubules as measured by alterations in the viscosity of the test mixtures, and confirmed by electron microscopy. 6-OHDA completely inhibited formation of microtubules at concentrations as low as 10 mM. Assembled microtubules were half as susceptible to destruction by 6-OHDA as unaggregated tubulin. Anaerobic conditions or the presence of catalase, superoxide dismutase, or a mixture of superoxide dismutase and catalase provided partial protection against 6-OHDA-induced destruction. In control reactions, tubulin-containing solutions incubated for up to 8 hr at ambient oxygen tensions, also showed significant decreases in ability to polymerize. Anaerobic conditions provided partial protection against this loss of function. In contrast, ascorbate accelerated the loss of activity upon standing, while glutathione or dithiothreitol offered no protection.

Microtubules

Microtubules have been important components of eukaryotic cells since they evolved about one billion years ago [1]. They occur in the mitotic spindle, centrioles, cilia and flagellae, axonemes, and neurotubules, the manchettes associated with spermatid nuclei during spermiogenesis and (through their association with trabecular structures) in the cytoskeleton [1, 2]. Microtubules have been implicated in cellular division, intracellular and axoplasmic transport [1], cellular motility and the maintenance of cellular shape and volume [3, 4]. The intimate relationship between form and function has directed much attention towards the regulation of their assembly and disassembly. Yet the basic mechanisms by which these processes are controlled remain among the outstanding problems of biology [5].

Disruption of the dynamic equilibria between tubulin subunits and fully-formed microtubules (whether by chemical agents, or by exposure to either low temperatures or high hydrostatic pressures) results in partial or complete loss of microtubular function [1, 6–9]. Abnormalities in microtubular structure and function are associated with a variety of human neuromuscular and central nervous disorders including presenile and senile dementia, Down's syndrome, analgesic abuse [10], and metal toxicity [11–13].

Redox stability of microtubular protein

Kuriyama and Sakai [14] studied the time-dependent changes in the polymerizing ability of tubulin in crude (50,000 *g*) supernatant fractions of porcine brain exposed to room air. The observed loss of activity was linear with respect to time, and inversely dependent on protein concentration. The estimated half-life for solutions containing tubulin concentrations of between 6.6 and 23.7 mg/ml ranged from 4 to 11 hr. Tubulin has a half-life of 3.5 hr in crude (105,000 *g*) supernatant fraction from rabbit brain [15]. Whether this instability reflects physical or chemical changes has not yet been determined. Since the sulfhydryl groups of tubulin are unstable to redox reagents, and crucial to its ability to polymerize, we wished to determine whether the stability of tubulin is affected by the presence or absence of oxygen or pro-oxidants.

There are between 8 and 13 cysteine residues per tubulin subunit [1, 16–23], and alteration of one or more of these may affect polymerization [24, 25]. Blockade of at least two of the sulfhydryl groups prevented polymerization and caused disintegration of preformed microtubules. These effects were reversible upon addition of dithiothreitol (DTT), although blockade of greater amounts of sulfhydryl groups resulted in irreversible changes. Up to 4 moles of sulfhydryl per mole of tubulin could be blocked before rapid decreases in colchicine binding were observed. On the basis of these results, sulfhydryl groups in tubulin reportedly serve different functions [24], 2 moles being functional in polymerization but not in colchicine binding. Only 2 or 3 sulfhydryl groups are immediately accessible to dithionitrobenzoic acid, the remaining groups becoming available only after certain conformational changes [18].

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6-Hydroxydopamine

We selected the neurotoxin 6-hydroxydopamine (6-OHDA) as the primary pro-oxidant probe. This choice was dictated in part by interest in its neurotoxic mechanisms, and in part by its ability to generate a mixture of free radical species, complex enough to yield interesting conclusions. Selected species of active oxygen, generated by its reactions with oxygen, were scavenged by addition of a variety of anti-oxidant enzymes, nutrients, or metabolites.

6-OHDA, a synthetic analogue of the endogenous neurotransmitter dopamine, causes rapid and selective destruction of catecholaminergic neurons [26–29]. It is extremely unstable, being rapidly oxidized by molecular oxygen to form a number of highly reactive intermediates and products including quinones, H_2O_2, superoxide and hydroxyl radicals [30–35]. Accumulation of norepinephrine (NE) in pre-terminal axons following 6-OHDA administration has been taken as an indication of disruption of axoplasmic transport processes resulting from a direct action of 6-OHDA on the neuronal microtubular system, on the grounds that similar effects have been demonstrated using both mechanical means and various other chemical agents [1, 6–9].

At low concentrations, 6-OHDA is taken up into synaptic vesicles via normal catecholamine reuptake systems. Destruction of cellular structures is observed when the neurotoxin surpasses a threshold concentration of approximately 5000 μg/g [28]. The precise mechanism of action of 6-OHDA and the site(s) at which it acts initially are not yet known. Not only are the time-dependent interrelationships between different phases of the contributing free radical reactions exceedingly complex [32–35], but the relative susceptibility of cellular components to the different radical species is difficult to assess.

Various neuronal structures have been examined as potential targets in the cytotoxicity of 6-OHDA [29]. Haeusler [36] demonstrated rapid impairment of membrane functions, the ability to conduct and generate action potentials being completely lost within 1 hr after administration. Similarly, application of 20 mM 6-OHDA to isolated axons led to rapid cessation of membrane electrical activity. Uptake of noradrenaline is also impaired rapidly [37, 38]. The effects of 6-OHDA on other potential targets, including microtubular function, invited investigation.

The current study emphasizes a neglected aspect of the biochemistry of the microtubular protein molecule, namely modification of its *in vitro* stability by the presence of oxygen, ascorbate, or the pro-oxidant 6-hydroxydopamine (6-OHDA). We also used catalase and superoxide dismutase to assess the roles of superoxide and hydrogen peroxide in the inhibitory effects of 6-OHDA.

MATERIALS AND METHODS

Reagents

L-Ascorbic acid, Analar grade, was from BDH Chemicals. Catalase (65,000 units/mg) was obtained from Boehringer because this commercial source has been shown to be free of superoxide dismutase. Crystalline bovine blood superoxide dismutase (2800 units/mg) was obtained from Sigma. All other reagents were of analytical grade.

Incubation media

To provide for effective polymerization of tubulin *in vitro*, neutral pH, moderate ionic strength and low concentrations of both magnesium ions [39–41] and calcium ions [42, 43] were provided. We confirmed that the use of a PIPES [piperazine-*N*, *n*-bis(2-ethanesulfonic acid] buffer (50 mM) obviated the need for addition of magnesium ions; EGTA [ethylene glycol-bis-(tetraacetic acid)], 1 mM, was used when it was desirable to chelate calcium ions. The pH of buffer solutions was adjusted to 6.94 at 23° by the addition of 5.0 N NaOH. Polymerization was initiated by the addition of GTP to a final concentration of 2.5 mM. Details concerning the concentrations of other reagents used in specific reaction mixtures are provided in the legends of the figures.

Isolation of tubulin

Pig brains obtained within 20 min of slaughter were placed on ice. All subsequent operations were performed at or near 0°. Blood vessels and the superficial meninges were removed before the tissue was minced with scissors and washed twice in ice-cold PIPES buffer without GTP. Buffer containing GTP was then added to the tissue to a volume of 1.5 ml/mg, and the mixture was homogenized in a Virtis homogenizer at a moderate speed. The homogenate samples were immediately centrifuged at 25,000 *g* for 35 min to remove cellular debris. The tubulin-containing S1 fraction was made 1 M in sucrose and 2.5 mM in GTP and then incubated in a waterbath maintained at 37° for between 30 and 60 min, while microtubule formation was followed viscometrically in separate 2-ml samples to determine the optimal incubation time. The incubated material was then centrifuged at 100,000 *g* in a Beckman L5 ultracentrifuge for 60 min at 37°. The supernatant fraction was discarded, and the pellet containing the microtubules was resuspended in PIPES buffer at 4°, and then centrifuged again at 100,000 *g* for 35 min to remove any debris and residual polymerized material. This pellet was discarded, and the supernatant fraction was made 1 M in sucrose and 2.5 mM in GTP, and incubated once more at 37°. After a further centrifugation at 100,000 *g* and 37°, the pellet was resuspended in ice-cold PIPES, made 1 M in sucrose and frozen using liquid nitrogen.

Three complete cycles of the procedure described above yielded highly purified tubulin as judged by its ability to form cold-labile, colchicine-labile microtubules. At this point in the procedure, Shelanski *et al.* [44] determined that their preparation was 95% pure tubulin. Although further repetitions of this procedure allows the production of increasingly pure samples, there is a significant loss of protein with each cycle. On occasion poor yields were obtained using these methods. Higher *g* forces (50,000 *g*: 20,000 rpm) in the first centrifugation gave more consistent results and were used in the later preparations.

Protein determination

The protein content of tubulin samples was determined using the method of Lowry *et al.* [45] as described by Umbreit *et al.* [46]. A correction factor of 1.27 was applied to the observed value in order to account for the higher tyrosine content of tubulin (19 moles vs 15 moles for bovine serum albumin).

Viscometry

Samples for viscometry consisted of 1.8 ml of purified tubulin in 1 M sucrose/PIPES buffer at 0°. GTP (0.1 ml of 50 mM) was added and the viscometer was warmed to 37° to initiate polymerization.

Viscosity was measured using Oswald viscometers (1 m of 1.0 mM capillary) immersed in a water bath maintained at 37°. Outflow times for sample (OTs) and for buffer alone (OTb) (stopwatches calibrated to 0.1 sec) were converted to specific viscosities (Nsp) by application of the following formula [40]: Nsp = (OTs-OTb)/OTb.

Fifteen control samples were tested, to characterize the polymerization process and the reliability of the system, after which all reactions were run in triplicate.

Initial specific viscosity was determined from the OTs following thermal equilibration of the cold, purified tubulin. The final specific viscosity was determined by fitting a rectangular hyperbola to specific viscosity as a function of time, and extrapolating to infinite time. The ability to polymerize was estimated from the difference between the viscosity at time zero (unpolymerized) and at infinite time (maximally polymerized). Rates of depolymerization were estimated by differentiating the rectangular hyperbola, using the coefficients yielded by regression of the hyperbola as fitted to the depolymerization curves.

Electron microscopy

Electron microscopy was performed on selected samples following viscometric analysis, to provide confirmatory morphological data. Single drops of the sample were placed on Formvar coated electron microscopy grids. After 3 sec, four drops of a solution containing distilled water, cytochrome *c* (0.1%) and uranyl acetate (1.0%) were added. The grid was then air-dried for 20 min prior to examination using a Phillips 300 electron microscope set at 80 kV.

RESULTS AND DISCUSSION

Time-dependent loss of activity of tubulin in air-saturated buffer

Incubation of tubulin in PIPES–EGTA buffer at 4° for various lengths of time resulted in decreased ability to polymerize upon addition of GTP (Fig. 1). The rate of loss of activity was higher at lower concentrations of protein (data not plotted), confirming results of Kuriyama and Sakai [24]. The addition of DTT to a final concentration of 0.5 mM failed to restore activity at either low or high concentrations of tubulin, indicating that the loss of activity with time is not due to reversible oxidation of sulfhydryl groups.

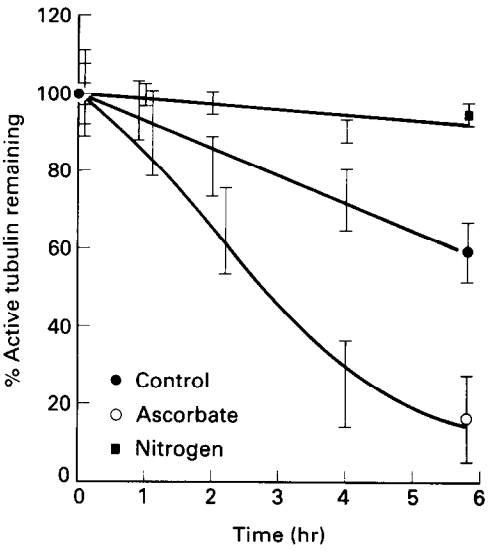

Fig. 1. Loss of activity of tubulin on standing: Effects of ascorbate or anaerobic conditions. Ascorbate (10 mM) was incubated with tubulin (2.0 mg/ml) in air-equilibrated PIPES–EGTA buffer. A second sample was equilibrated with high purity nitrogen prior to and after addition of tubulin. Both samples were maintained at 0° and sampled at the stated periods of time. Reactions were initiated by addition of GTP after quickly warming the samples to 37° in the viscometers. The maximum viscosity finally attained was determined as described in Materials and Methods.

Effects of decreased oxygen pressure, DTT, GSH or ascorbate on time-dependent inactivation

In a second set of experiments designed to assess the effects of decreased oxygen concentration, loss of the ability to polymerize was followed in either air or high purity nitrogen (Fig. 1). After incubation for 8 hr, less than half the original activity was present in the control mixture while over 80% remained in the sample incubated anaerobically. This protection was highly significant statistically ($P < 0.001$), supporting the hypothesis that the loss of activity is due to oxidation by atmospheric oxygen. Although no protection was provided by the addition of glutathione or DTT to the incubation medium, this does not rule out the possibility that sulfhydryl groups are the primary target, since in an analogous system [18], reducing agents were ineffective in protecting against the loss of sulfhydryl groups. Figure 1 also shows that addition of ascorbate, far from protecting, approximately doubled the rate of loss of activity on standing.

Effects of 6-hydroxydopamine on ability of tubulin to polymerize

Preliminary experiments in this series were performed using 6-OHDA concentrations of up to 20 mM, on the basis that we should attempt to attain the presumed threshold intraneuronal concentrations required for ultrastructural damage [28, 29, 37]. However, concentrations between 10 and 20 mM were sufficient to block polymerization completely within 5 min. A much lower range of concentrations

was used, therefore, for the dose-dependency relationships shown (for both polymerization and depolymerization) in Fig. 2. The curve for unpolymerized tubulin subunits displays an approximately first-order dependence of destruction on 6-OHDA concentration, and an approximately 0.5 order dependence when the subunits were initially polymerized. In these data, the inactivations induced by all 6-OHDA concentrations over 0.006 mM were statistically significant ($P < 0.01$) in the case of tubulin subunits, but in the case of microtubules significance at this level was not attained until 6-OHDA concentrations of 0.01 mM were used.

The foregoing findings were confirmed in electron micrographs prepared from the 6-OHDA-treated samples which instead of formed microtubules showed abundant amorphous aggregates, visible as clumps of stained protein. These formations were seen upon ("observer-blind") examination of all of the grids prepared from samples with 6-OHDA concentrations as low as 0.006 mM. Some apparently normal microtubules were also found in almost all of the above samples. However, the samples containing 0.250 mM 6-OHDA were almost devoid of microtubules, only a few isolated filaments being observed.

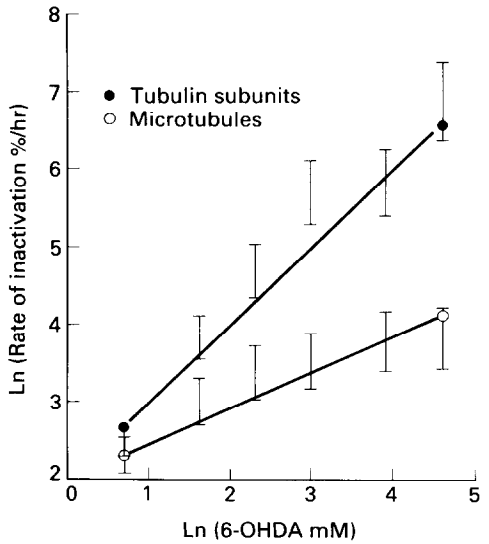

Fig. 2. Loss of activity of tubulin induced by 6-OHDA: Dose/response curve comparison of tubulin subunits with polymerized microtubules. Polymerization: Samples containing tubulin (1.5 mg/ml) in PIPES–EGTA buffer were thermally equilibrated in prewarmed viscometers. In each reaction, 6-OHDA was added to the concentration shown and polymerization was initiated with GTP. Ability to polymerize was then measured repeatedly over the succeeding 8 hr by cooling to 0° after polymerization, and then reinitiating polymerization by rewarming the viscometer to 37°. Results are expressed as the estimated maximum specific viscosity attained, taken as a percentage of initial control values. Other procedures and conditions were as described in the legend of Fig. 1. Depolymerization: Samples were allowed to polymerize until a plateau was observed in recorded specific viscosity values, whereupon 6-OHDA was added in 0.1-ml aliquots. Data are expressed as the estimated minimum specific viscosity, taken as a percentage of control values.

At yet higher concentrations (within the range 0.5 to 20 mM) there was no viscometrically detectable polymerization, and grids were completely devoid of microtubules. Although the effects of 6-OHDA on polymerization were apparent within approximately 5 min, the effects of depolymerization occurred much more slowly, over a period of several hours. Fully-formed microtubules are presumably much less sensitive to oxidative stresses because of the relatively smaller area of exposed protein. Another difference was that, in the depolymerization studies, even concentrations as high as 0.250 mM 6-OHDA never produced more than a 50% decrease in specific viscosity. Presumably this limit reflects oxidant-induced cross-linking of damaged or native tubulin subunits, and "tanning" reactions in the mixture of quinonoid products.

Requirement for oxygen in actions of 6-OHDA

A further series of experiments was performed at diminished oxygen concentrations. Bubbling the incubation medium with high purity nitrogen provided 42% protection ($P < 0.001$) against destruction by 6-OHDA, and electron micrographs of the anaerobic samples showed an abundance of apparently normal microtubules in addition to many amorphous aggregates.

Role of quinonoid products in actions of 6-OHDA

In an attempt to determine the relative contributions of free radical species and quinonoid autoxidation products, ascorbate was added to tubulin-containing samples. Ascorbate can maintain 6-OHDA in a reduced state, by rereducing the quinonoid products of autoxidation [30]. Protection by ascorbate would argue in favour of a role for the quinonoid products. However, addition of ascorbate to those incubation mixtures which contained 6-OHDA failed to protect against the destructive actions of 6-OHDA significantly (Fig. 3). Since inactivation by 6-OHDA was essentially complete, enhancement of destruction by ascorbate would not have been detected.

Having failed to show an effect from removal of quinonoid products, we then investigated the effect of increasing the concentration of quinones. This next experiment then involved incubation of tubulin with fully-oxidized 6-OHDA (6-OHDA that had been allowed to stand exposed to room air for 6 hr). The addition of this quinone-containing mixture to the tubulin sample inhibited polymerization by 47% ($P < 0.001$) (cf. 92% inhibition for fresh 6-OHDA). Thus, some (about half) of the destructive actions of 6-OHDA in this system can be attributed to the quinonoid products. On the other hand, allowing accumulation of quinonoid products certainly did not enhance damage to the tubulin subunits over that induced by fresh 6-OHDA, and prevention of formation of quinonoid products by addition of ascorbate (Fig. 3) did not decrease damage.

Electron micrography after incubation to allow polymerization revealed morphologically normal microtubules as well as the amorphous aggregates described previously in the samples treated with either ascorbate/6-OHDA, or with oxidized 6-OHDA. The microtubules in the latter sample were

shorter and fewer in number than those in either anaerobic or control samples. This series of experiments then, as summarized in Fig. 3, suggests that the damaging effects of 6-OHDA are for the most part mediated by free radicals generated by autoxidative processes rather than by the quinonoid products of oxidation. This conclusion is consistent with the hypothesis of Heikkila and Cohen [30, 47, 48] and Cohen and Heikkila [49].

Effects of catalase and superoxide dismutase on actions of 6-OHDA

In the final experiments of this series, an attempt was made to determine, by using appropriate scavenging agents, to what extent superoxide or hydrogen peroxide was participating in the mechanisms of inactivation (Fig. 4). Catalase (at a final concentration of 50 units/ml) provided 44% ($P < 0.001$) protection, although the damage which did occur remained significantly different ($P < 0.001$) from control. SOD alone (50 units/ml) produced 32% protection, but the simultaneous presence of both superoxide dismutase and catalase offered no increase in protection over catalase alone.

The protective effects of catalase suggest that H_2O_2 plays a major role in the destruction of tubulin by 6-OHDA. Nevertheless, added H_2O_2 was much less destructive than 6-OHDA (Fig. 5), although the level of damage by 0.25 mM H_2O_2 (42%) was significant ($P < 0.001$), with a substantial decrease in the number of formed microtubules being visible in the electron micrographs. Another depolymerization study was carried out using a final concentration of 0.20 mM H_2O_2, again yielding values significantly different from controls ($P < 0.001$). Evi-

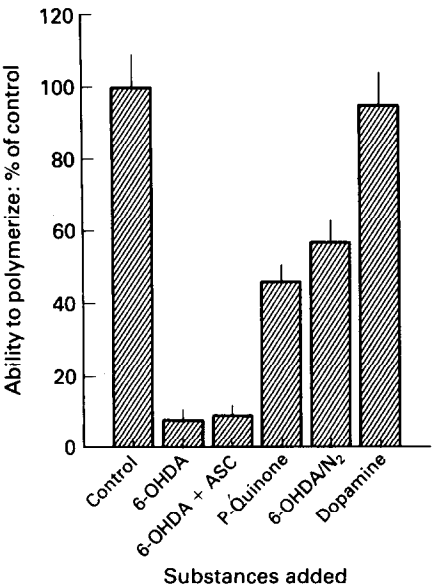

Fig. 3. Loss of activity of tubulin induced by 6-OHDA: Effects of paraquinone, ascorbate or nitrogen. Tubulin (1.65 mg/ml) in PIPES–EGTA buffer was incubated with 6-OHDA (0.1 mM), 6-OHDA (0.1 mM) and ascorbate (10 mM), or fully-oxidized 6-OHDA (0.1 mM) either air equilibrated or anaerobically. Other procedures and conditions were as described in the legend of Fig. 1.

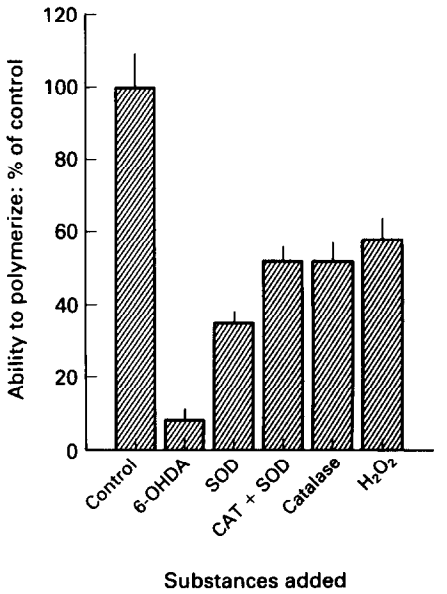

Fig. 4. Loss of activity of tubulin induced by 6-OHDA: Effects of catalase, SOD or H_2O_2. Tubulin samples (1.65 mg/ml) in PIPES–EGTA buffer were incubated with catalase (50 units/ml) or SOD (50 units/ml) alone or in combination. Samples were thermally equilibrated in prewarmed viscometers prior to the addition of 6-OHDA (0.25 mM) or H_2O_2 (0.25 mM) and subsequently GTP (2.5 mM) to start the reactions. Other procedures and conditions were as described in the legend of Fig. 1.

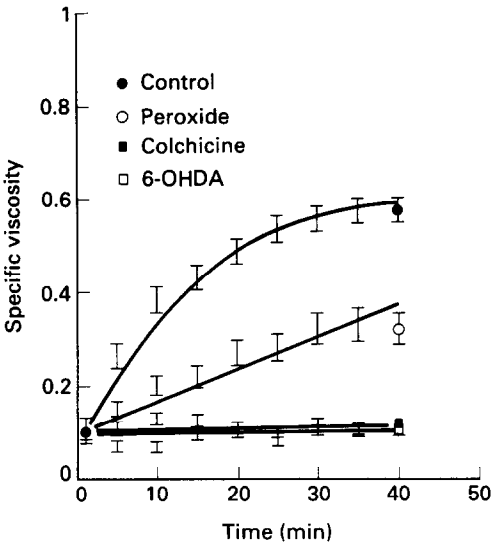

Fig. 5. Progress of polymerization: Effects of H_2O_2 6-OHDA, or colchicine. The relative potencies of 6-OHDA (0.25 mM), H_2O_2 (0.25 mM) and colchicine (100 μM) in inhibiting polymerization of tubulin samples were assessed by the addition of these reagents to tubulin samples of various concentrations (1.65 mg/ml for samples containing 6-OHDA and H_2O_2 and 1.58 mg/ml for samples with colchicine) in PIPES–EGTA buffer. All reactions, with the exception of those involving colchicine, were initiated by the addition of GTP. Other procedures and conditions were as the legend of Fig. 1.

dently the protective actions of catalase lie not so much in preventing the small toxic effects of H_2O_2 itself, as in preempting formation of other toxic species in Fenton-type reductions of H_2O_2 (by 6-OHDA, its semiquinone, or O_2^-). These protective actions of catalase are consistent with those reported by Oliver *et al.* [50], although their explanation (that maintenance of microtubules was prevented by decreases in levels of GSH as a result of the oxidizing action of H_2O_2) cannot be applied to the present (*in vitro*) studies. Clearly, H_2O_2 has an effect independent of a possible action mediated by GSH oxidation. However, the effect in the current system is also likely to be indirect. Since the actions of SOD and catalase are not additive, they are likely to involve an action on the same mechanism. The metal catalyzed Haber–Weiss reaction (or a superoxide regenerated Fenton reaction) is the most likely candidate.

In conclusion, then, in the current studies the instability of tubulin resulted, in part, from the presence of oxygen and was aggravated by the presence of pro-oxidants. Selected scavengers of active oxygen had major protective effects on the inactivation induced by 6-OHDA. The exact mechanisms by which intermediates in the reaction of oxygen with the pro-oxidant may exert effects on tubulin can only be described in general terms at this time, but in view of the magnitude of the effects further research to delineate their scope and mechanisms is warranted. In particular, the relative roles, on the one hand, of electrophilic attack on the -SH groups and, on the other, less specific free radical attack need to be determined. Moreover, the roles of hydroxyl radicals, transition metal ions, quinones and semiquinones need to be investigated using appropriate scavengers and traps in both 6-OHDA itself, and in the products of its reaction with oxygen.

REFERENCES

1. P. Dustin, *Microtubules*, Springer, New York, (1978).
2. J. J. Wolosewick, R. P. Becker and J. S. Candeelis, in *Microtubules and Microtubule Inhibitors 1980* (Eds. M. De Brabander and J. De Mey), p. 3. Elsevier/North Holland Press, New York (1980).
3. R. N. Melmed, P. Karanian and R. D. Berlin, in *Microtubules and Microtubule Inhibitors 1980* (Eds. M. De Brabander and J. De Mey), p. 85. Elsevier/North Holland Press, New York (1980).
4. F. Solomon, *Trends Neurosci.* 7, 17 (1984).
5. R. D. Berlin, C. S. Regula and J. R. Pfeiffer, in *Microtubules and Microtubule Inhibitors 1980* (Eds. M. De Brabander and J. De Mey), p. 145. Elsevier/North Holland Press, New York (1980).
6. A. Dahlstrom, in *New Aspects of Storage and Release Mechanisms of Catecholamines; Bayer-Symposium II* (Eds. H. J. Schuman and G. Kroneberg), p. 20. Springer, New York (1970).
7. J. S. Dixon, *Acta Anat.* **76**, 493 (1970).
8. T. Hockfelt and A. Dahlstrom, *Z. Zellforsch. mikrosk. Anat.* **119**, 460 (1971).
9. L. Karlstrom and A. Dahlstrom, *J. Neurobiol.* **4**, 191 (1973).
10. R. M. Murray, J. M. Greene and J. H. Adams, *Lancet* **11**, 242 (1971).
11. M. Wallin, H. Larsson and A. Edstrom, *Expl. Cell Res.* **107**, 219 (1977).
12. R. A. Goyer, in *Disorders of Mineral Metabolism* (Eds. F. Bronner and J. W. Coburn), Vol. 1, p. 160. Academic Press, New York (1981).
13. K. Miura, M. Inokawa and N. Imura, *Toxic. appl. Pharmac.* **73**, 218 (1984).
14. R. Kuriyama and H. Sakai, *J. Biochem., Tokyo* **76** 651 (1974).
15. R. H. Haschke, M. R. Byers and B. R. Fink, *J. Neurochem.* **22**, 837 (1974).
16. H. Sakai, *Biochim. biophys. Acta* **112**, 132 (1966).
17. J. C. Lee, R. P. Frignon and S. N. Timasheff, *J. biol. Chem.* **248**, 723 (1973).
18. F. L. Renaud, A. J. Rowe and I. R. Gibbons, *J. Cell Biol.* **36**, 79 (1973).
19. J. Bryan, *Fedn. Proc.* **33**, 152 (1974).
20. B. A. Eipper, *J. Cell Biol.* **249**, 1407 (1974).
21. M. G. Mellon and L. I. Rebhun, *J. Cell Biol.* **70**, 226 (1976).
22. M. G. Mellon and L. I. Rebhun, in *Cell Motility* (Eds. R. Goldman, T. Pollard and J. Roenbaum), p. 1149. Cold Spring Harbor Lab. Press, Cold Spring Harbor, N.Y. (1976).
23. M. Little, E. Krauhs, H. Postingl, R. Luduena and N. Rice, in *Microtubules in Microorganisms* (Eds. P. Capuccinelli and N. R. Morris), p. 1. Marcel Dekker, New York (1982).
24. R. Kuriyama and H. Sakai, *J. Biochem. Tokyo* **75**, 463 (1974).
25. E. Nishida and T. Kobayashi, *J. Biochem. Tokyo* **81**, 343 (1977).
26. H. Thoenen, in *Perspectives in Neurophamacology* (Ed. S. H. Snyder), p. 302. Oxford University Press, New York (1972).
27. H. Thoenen and J. P. Tranzer, *A. Rev. Pharmac.* **13**, 169 (1973).
28. M. W. Kostrezewa and D. M. Jacobowitz, *Pharmac. Rev.* **26**, 199 (1974).
29. C. H. Sachs, *Biochem. Pharmac.* **24**, 1 (1975).
30. R. E. Heikkila and R. G. Cohen, *Molec. Pharmac.* **8**, 241 (1972).
31. G. Cohen, in *Pathology of Oxygen* (Ed. A. P. Autor), p. 115. Academic Press, New York (1982).
32. P. Gee, *Ph.D. Thesis*. Simon Fraser University, Burnaby, B. C., Canada (1984).
33. A. J. Davison and P. Gee, *Archs. Biochem. Biophys.* **233**, 761 (1984).
34. P. Gee and A. J. Davison, *Archs. Biochem. Biophys.* **231**, 164 (1984).
35. P. Gee and A. J. Davison, *Biochim. biophys. Acta* **838**, 183 (1984).
36. H. Haeusler, in *6-Hydroxydopamine and Catecholamine Neurons* (Eds. T. Malmfors and H. Thoenen), p. 193. Elsevier/North Holland Press, New York (1971).
37. G. Jonsson, in *6-Hydroxydopamine and Catecholamine Neurons* (Eds. T. Malmfors and H. Thoenen), p. 243. Elsevier/North Holland Press, New York (1971).
38. G. Jonsson and C. H. Sachs. *J. Neurochem.* **19**, 2577 (1972).
39. J. B. Olmsted and G. G. Borisy, *Biochemistry* **12**, 4282 (1973).
40. J. B. Olmsted and G. G. Borisy, *A. Rev. Med.* **24**, 831 (1973).
41. J. B. Olmsted, J. M. Marcum, K. A. Johnson, D. B. Murphy and G. G. Borisy, *Ann. N.Y. Acad. Sci.* **36**, 598 (1974).
42. R. C. Weisenberg, *Science* **177**, 1104 (1972).
43. L. I. Rebhun, D. Jemiolo, T. Keller, W. Burgess and R. Kretsinger, in *Microtubules and Microtubule Inhibitors 1980* (Eds. M. De Brabander and J. De Mey), p. 243. Elsevier/North Holland Press, New York (1980).
44. M. L. Shelanski, F. Gaskin and C. R. Cantor, *Proc. natn. Acad. Sci. U.S.A.* **70**, 465 (1973).

45. O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. biol. Chem.* **193**, 265 (1951).
46. W. W. Umbreit, R. H. Burris and J. F. Stauffer, *Manometric and Biochemical Techniques*, (5th Edn.). Burges, Minneapolis (1972).
47. R. E. Heikkila and R. G. Cohen, *Science* **181**, 456 (1974).
48. R. E. Heikkila and R. G. Cohen, *Science* **172**, 1257 (1971).
49. G. Cohen and R. E. Heikkila, *J. biol. Chem.* **249**, 2447 (1974).
50. J. M. Oliver, D. F. Albertini and R. D. Berlin, *J. Cell Biol.* **71**, 921 (1976).