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2,5-Hexanedione Alters Microtubule Assembly

II. Enhanced Polymerization of Crosslinked Tubulin[1]

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2,5-Hexanedione Alters Microtubule Assembly. II. Enhanced Polymerization of Crosslinked Tubulin. BOEKELHEIDE, K. (1987). *Toxicol. Appl. Pharmacol.* **88,** 383–396. The toxic syndrome resulting from *in vivo* exposure to *n*-hexane or *n*-hexane derivatives may, in part, be a manifestation of altered tubulin and microtubule properties. The effect of *in vitro* γ-diketone derivatization was first studied using purified bovine brain tubulin and the results were then verified in tubulins purified from target organs of an experimental species. Microtubule assembly and structure were modified after *in vitro* incubation with 2,5-hexanedione (2,5-HD) as follows: (1) 2,5-HD derivatization of purified tubulin resulted in an alteration in microtubule assembly kinetics, most prominantly a decrease in the length of the nucleation phase, (2) the alteration in assembly kinetics was accompanied by the formation of a covalently crosslinked tubulin dimer, (3) mixing experiments which combined different proportions of control and treated tubulin showed that only a small amount of derivatized tubulin need be present to induce altered assembly properties, and (4) as a result of the more rapid nucleation phase, a greater number of nucleating seeds produced more numerous and shorter assembled polymers. *In vitro* incubation with the 2,5-HD congener 3,4-dimethyl-2,5-hexanedione produced similar alterations in microtubule assembly. Thus, both the kinetics of tubulin polymerization and the morphology of the final assembly product were modified by *in vitro* γ-diketone incubation.

Chronic human exposure to the solvent *n*-hexane, or its derivative, methyl *n*-butyl ketone, results in a severe peripheral neuropathy (Spencer *et al.*, 1980). In animal studies, the peripheral nervous system disease is accompanied by testicular atrophy (Chapin *et al.*, 1982). 2,5-Hexanedione (2,5-HD)[2] has been identified as the ultimate *in vivo* toxic metabolite of *n*-hexane (Krasavage *et al.*, 1980). 2,5-HD reacts with primary amines to form 2,5-dimethyl-substituted pyrroles, the Knorr-Paal condensation (Broadbent *et al.*, 1968). Pyrroles are reactive five-membered heterocyclic aromatic compounds which, in an autocatalytic oxygen-dependent free-radical process, form an assortment of polymeric species covalently attached through ring carbon-to-carbon bonds. Pyrrylpyrrolidinones have been identified as specific dimeric polymerization products (Hoft *et al.*, 1967; Smith and Jensen, 1967). Both conversion of protein lysines to pyrryl derivatives and intermolecular protein crosslinks have been identified following *in vivo* intoxication with 2,5-HD (Anthony *et al.*, 1983; DeCaprio *et al.*, 1983) and *in vitro* incubation of proteins with 2,5-HD under physiologic and near physio-

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[2] Abbreviations used: SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; tV_{max}, the time required to achieve maximal velocity of assembly; 2,5-HD, 2,5-hexanedione; DMHD, 3,4-dimethyl-2,5-hexanedione; GTP, guanosine 5′-triphosphate.

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logic conditions (DeCaprio *et al.*, 1982; Graham *et al.*, 1982, 1984).

Microtubules are ubiquitous cytoskeletal elements which influence cell shape, movement, secretion, and nuclear division (Dustin, 1984). These polymers are composed of tubulin which has a monomer molecular mass of 50 kDa and exists free in solution as noncovalently bound ($\alpha + \beta$) tubulin dimer. Porcine brain tubulin dimer contains a total of 34 lysines in the α- and β-tubulin primary sequences (Ponstingl *et al.*, 1981; Krauhs *et al.*, 1981). Studies with mono- and bifunctional imidoesters have documented the presence of spatially adjacent lysyl residues on native tubulin which readily participate in the formation of intermolecular crosslinks. The covalently bound tubulin dimers produced in crosslinking reactions tended to be α-β heterodimers, though α-α and β-β homodimers also were formed (Luduena *et al.*, 1977). Solutions of extensively crosslinked tubulin (greater than 50% multimeric) were still capable of polymerization in the presence of microtubule-associated proteins (Galella and Smith, 1982).

Microtubule assembly is a multiphase process. An initial nucleation step is followed by the addition of tubulin subunits onto nucleating seeds during an elongation phase. This culminates in a steady-state rate of exchange between tubulin subunit and polymer (Purich and Kristofferson, 1984). A lag during the initial portion of assembly represents the time during which nucleating seeds are formed and is a function of tubulin concentration (Johnson and Borisy, 1977). The end result of the nucleation phase is the generation of a relatively fixed number of seeds which provide the nidus for rapid tubulin addition during elongation (Voter and Erickson, 1984). Elongation occurs as an apparent first-order process of addition of tubulin subunits to the ends of protofilaments and continues until the rate of subunit addition equals the rate of subunit loss (Carlier and Pantolini, 1978).

In the preceding article, *in vivo* evidence of a γ-diketone-induced alteration in microtubule assembly is associated with the occurrence of testicular atrophy, not nervous system toxicity, in treated rats (Boekelheide, 1987). This study examines tubulin structure and biochemical properties following *in vitro* reaction with 2,5-HD and 3,4-dimethyl-2,5-hexanedione (DMHD).

MATERIALS AND METHODS

All ultracentrifugations were performed using a Type 269 rotor in a B-60 ultracentrifuge (International Equipment Co., Needham Heights, MA) at 100,000*g* at 4°C ("cold") or 37°C ("warm"). All chemicals were obtained from Sigma Chemical Co. (St. Louis, MO) unless otherwise indicated.

Tubulin purification. Tubulin was purified according to Hamel and Lin (1981) with several modifications. Bovine brains were homogenized for 1 min in a Waring blender 100 g at a time in 75 ml of 0.1 M 2-(N-morpholino)ethane sulfonic acid, 1 mM ethylene glycol bis(β-aminoethyl ether)*N,N,N',N'*-tetraacetic acid, 0.5 mM $MgCl_2$, 4 M glycerol, pH 6.75, at 4°C. The supernatant from a cold ultracentrifugation was frozen in liquid nitrogen and stored at −70°C until needed. The buffer for the remaining purificaton steps was 1 M sodium glutamate, 0.1 M guanosine 5'-triphosphate (GTP), pH 6.6. DEAE-Sephacel (Pharmacia Inc., Pascataway, NJ) was repeatedly separated for washing and elution steps by gentle sedimentation in a clinical centrifuge rather than by filtration. Tubulin, eluted with 1 M NaCl in glutamate buffer, was further purified by two cycles of temperature-dependent assembly and disassembly to give the starting material for incubation experiments. The average yield was 150 μg twice cycled tubulin per gram bovine brain. Modifications to the above procedure for the purification of rat brain and testis tubulin are described in the preceding article (Boekelheide, 1987).

2,5-HD incubation. Twice-cycled rat or bovine brain tubulin or thrice-cycled rat testis tubulin was adjusted to 1–2 mg/ml and incubated for 16 hr at 37°C in 1 M sodium glutamate, 1 mM GTP, pH 6.6, alone (control) or with added 2,5-HD (treated) (Eastman Kodak Co., Rochester, NY). DMHD derivatization of rat brain tubulin was identical except 5 mM DMHD replaced 2,5-HD in the 16-hr incubation. Following a 30-min warm ultracentrifugation, pellets were resuspended in cold glutamate buffer without GTP and incubated at 0°C for 20 min. The solution was then sheared by passage through a 26-gauge needle three times and incubated at 0°C for an additional 10 min. Shearing increased the yield of tubulin, but did not alter assembly. Cold ultracentrifugation for 30 min gave the working tubulin preparation. An overall 50% recovery was attained for both control and treated tubulin for the incubation and cycle procedure.

For the time-course experiment, aliquots were withdrawn at 1, 4, 8, and 16 hr of incubation, glycerol was added to a final concentration of 3–4 M, and the samples were then frozen in liquid nitrogen for storage at −70°C. When needed, the samples were incubated at 37°C for 45 min and then cycled as described above. In some cases, the tubulin was passed through a 1.5 × 15-cm Sephadex G-25 (Pharmacia Inc.) column equilibrated with glutamate buffer to remove residual 2,5-HD before performing the Ehrlich's reaction for detection of pyrroles (see below). Assembly parameters were not altered by this chromatographic procedure.

Anaerobic samples included 200 μg/ml catalase, 1 mM deferoxamine (CIBA Pharmaceutical Co., Summit, NJ), and 1 mM ascorbic acid in the incubation solution. The samples were bubbled with nitrogen for 5 min prior to incubating for 16 hr in sealed containers and processing through the postincubation cycle procedure described above.

Assembly. The extent of assembly was measured by monitoring absorbance at 350 nm in a Gilford 252 spectrophotometer equipped with a Gilford 6051 recorder. Assembly temperatures were recorded from the bath that circulated water through the jacketed cuvette holder housing. After preequilibration of semimicro cuvettes, the standard assembly reaction was initiated by addition of ice-cold tubulin in glutamate buffer, GTP, to a final concentration of 0.25 mM (unless otherwise indicated) and coreactants in a final volume of 0.5 ml. Zero time was taken as the time of initiation of recording and excludes a 5–10 sec delay involved in the addition and mixing of reactants.

For experiments examining the effect of GTP concentration, the tubulin was first passed through a 1.5 × 15-cm Sephadex G-25 (Pharmacia, Pascataway, NJ) column. The peak and prepeak protein fractions were combined to provide GTP-depleted tubulin for subsequent assembly (Maccioni and Seeds, 1982).

Electrophoretic procedures. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE) was performed according to Laemmli (1970) with staining by Coomassie brilliant blue R or the silver technique as described by Merril *et al.* (1981). Prior to electrophoresis, samples were boiled for 5 min in buffer containing a final concentration of 3 M urea and 0.5% β-mercaptoethanol. Molecular mass standards included rabbit muscle myosin (205 kDa), *Escherichia coli* β-galactosidase (116 kDa), bovine serum albumin (66 kDa), and ovalbumin (45 kDa). Densitometry was performed using an EC910 transmission densitometer and Hewlett–Packard 3390A integrator (E-C Apparatus Corp., St. Petersburg, FL) on silver-stained polyacrylamide gels using appropriate dilutions to construct standard curves of density *versus* concentration. Decreased protein staining due to crosslinking was an unlikely problem given the small percentage of amine derivatization by 2,5-HD (Leffak, 1983). Protease-facilitated electrophoretic transfer to nitrocellulose was performed as described by Gibson (1981) except chymotrypsin (50 μg/ml) was used in place of pronase. The primary anti-α-tubulin mouse monoclonal antibody recognizing epitopes of chick brain tubulin was obtained commercially (Amersham Corp., Arlington Heights, IL) and the anti-β-tubulin mouse monoclonal antibody recognizing epitopes of sea urchin tubulin was the kind gift of Dr. Richard McIntosh, University of Colorado (Boulder, CO) (Scholey *et al.*, 1984). The secondary antibody was affinity-purified biotinylated anti-mouse IgG (H + L) immunoglobulin prepared in horse (Vector Laboratories, Burlingame, CA) and visualized using the Vectastain ABC Kit (Vector Laboratories) and 3,3′-diaminobenzidine.

Electron microscopy. Negative stains of pure tubulin were prepared by leaving a drop of assembled microtubules on a carbon and Formvar-coated grid for 1 min. The grid was then dipped rapidly three times in 2% uranyl acetate and dried with filter paper. The specimens were viewed in a Phillips 410 electron microscope at 100 kV. Polymer length was determined directly from electron microscopic negatives at 10,000× (treated) and 10,000× and 2400× (control) using a Bausch and Lomb 7× measuring magnifier equipped with a 20-mm scale. Measurements were standardized to a 21,600 lines/cm grating.

Pyrrole determination. Ehrlich's reagent was used for the detection of pyrroles. Treated and control tubulin were first put through a 1.5 × 15-cm Sephadex G-25 (Pharmacia) column equilibrated with distilled water or 1 M sodium glutamate, pH 6.6, to remove residual 2,5-HD. Aliquots (100 μl) of control and treated tubulin containing approximately 100 μg of protein were proteolyzed by incubation at 37°C for 10 min with 20 μg of chymotrypsin. Dimethyl sulfoxide (400 μl) was added and the samples were boiled for 5 min followed by addition of Ehrlich's reagent (12 mg/ml *p*-dimethylaminobenzaldehyde in 50% aqueous methanol with 1% HCl), boiling for 5 min, incubation at 37°C for 15 min, cooling and measurement of absorbance at 530 nm. A standard curve was constructed by adding appropriate concentrations of 2,5-dimethylpyrrole (Aldrich Chemical Co., Milwaukee, WI) to control samples and, therefore, the pyrrole content of treated tubulin is reported as equivalents 2,5-dimethylpyrrole.

Protein determination. Protein concentrations were determined in triplicate according to Lowry *et al.* (1951) using bovine serum albumin as a daily standard with concentrations normalized to the extinction coefficient of tubulin in 6 M guanidine HCl at 275 nm (Na and Timasheff, 1981).

A Coomassie blue protein dye binding assay concentrate (Bio-Rad, Rockville Centre, NY) was used at a 1:4 dilution, adding 2.5 ml to control and treated aliquots with measurement of absorbance at 595 nm.

RESULTS

2,5-HD Induces Tubulin Crosslinking

A range of 2,5-HD concentrations was examined for effect upon microtubule assembly

TABLE 1

EFFECT OF 2,5-HD CONCENTRATION DURING INCUBATION UPON MICROTUBULE ASSEMBLY

2,5-Hexanedione concentration (mM)	$t_{V_{max}}$ (min)	V_{max} (ΔOD_{350}/min)	Final OD_{350}
1	7.5	0.015	0.249
10	3.5	0.030	0.201
50	1.5	0.039	0.171
100	1.0	0.027	0.165

Note. Twice-cycled DEAE-Sephacel purified bovine brain tubulin was incubated with 2,5-HD at 37°C in 1 M sodium glutamate, 1 mM GTP, pH 6.6. After 16 hr the tubulin was purified by a cycle of warm and cold ultracentrifugations and analyzed for assembly characteristics at a concentration of 0.39 mg/ml by measuring the increased turbidity at 350 nm associated with polymer formation.

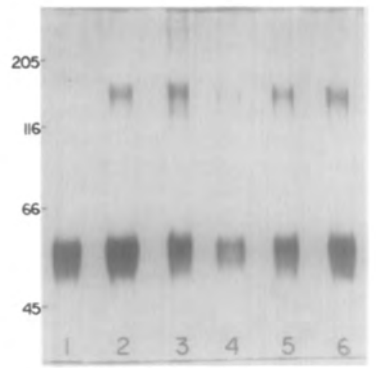

FIG. 2. SDS–PAGE analysis of control and treated tubulin. Control tubulin (lane 1) is compared with the various stages of tubulin incubated with 100 mM 2,5-HD for 16 hr in glutamate buffer with 1 mM GTP at 37°C and then processed in the usual manner with warm and cold ultracentrifugations: cold ultracentrifugation pellet (lane 3), warm ultracentrifugation supernatant (lane 4), after 16 hr incubation prior to cycle (lane 5), final treated tubulin preparation (cold ultracentrifugation supernatant, lane 6). Lane 2 contains control plus final treated tubulin. Samples of 1.4–1.8 μg were applied to a 7.5% polyacrylamide minigel and stained by the silver technique. Molecular mass standards are indicated in kDa.

(Table 1). Overnight incubation with 1 mM 2,5-HD produced microtubule assembly behavior similar to that of control tubulin, while 10, 50, and 100 mM 2,5-HD produced earlier and more rapid assembly. Tubulin incubation with 100 mM 2,5-HD was chosen for subsequent *in vitro* studies, since this 2,5-HD concentration produced the most extreme assembly alterations.

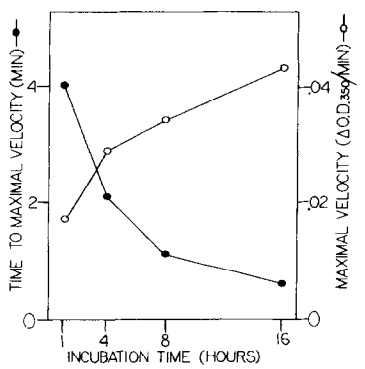

FIG. 1. Progressive alteration of assembly parameters with 2,5-HD incubation time. Purified bovine brain tubulin at 2 mg/ml in 1 M sodium glutamate, 1 mM GTP, pH 6.6, at 37°C was incubated with 100 mM 2,5-HD. Aliquots were withdrawn at 1, 4, 8, and 16 hr and processed as described under Materials and Methods. The time required to achieve maximal velocity of assembly and the maximal velocity of assembly were determined for the time-course samples at a concentration of 0.32 ± 0.03 mg/ml (mean ± SD) in glutamate buffer with 0.25 mM GTP at 37°C. An increased time of incubation resulted in an increased maximal velocity of assembly and a decreased time required to reach maximal velocity of assembly.

The time course of the tubulin assembly alteration produced by 100 mM 2,5-HD was examined by removing aliquots after 1, 4, 8, and 16 hr of incubation. Following a purification cycle of warm and cold ultracentrifugations, the 2,5-HD-treated tubulin was analyzed for assembly characteristics. The maximal velocity of assembly (V_{max}) increased progressively with incubation time while the time required to reach maximal velocity ($t_{V_{max}}$) decreased progressively with incubation time (Fig. 1).

SDS–PAGE analysis of the tubulin preparation following incubation with 2,5-HD showed the development of covalently cross-linked tubulin multimers (Fig. 2). The postincubation warm and cold ultracentrifugation cycle was used to insure that the final tubulin preparation was largely free of 2,5-HD and

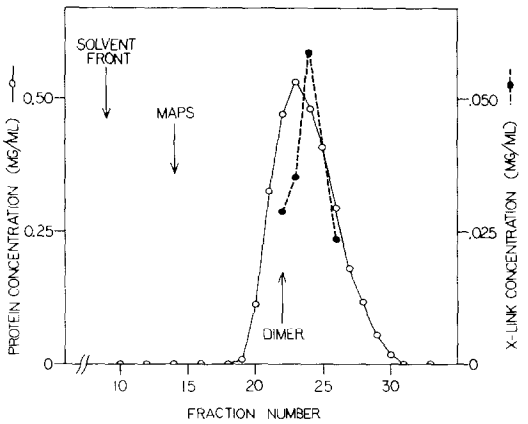

FIG. 3. Crosslinked tubulin coeluted with tubulin dimer. Treated tubulin (2 mg in 0.75 ml) was added to a Sepharose 6B (Pharmacia) 1 × 30-cm column equilibrated at 4°C with 1 M sodium glutamate, pH 6.6. Fractions of 0.6 ml were analyzed for the content of covalently crosslinked tubulin (X-link concentration) by densitometric scanning of silver-stained polyacrylamide gels and for total protein (protein concentration). The peak of covalently crosslinked tubulin coeluted with tubulin dimer. The column was calibrated by applying cycle tubulin (Shelanski *et al.*, 1973) for determination of the elution location of the solvent front of the applied sample (solvent front), microtubule associated proteins (MAPS, molecular masses 275–350 kDa), and native tubulin dimer (dimer, molecular mass 100 kDa).

contained only assembly-competent tubulin. SDS–PAGE analysis (7.5% gel) of the final tubulin preparation showed only tubulin monomer and a single species of crosslinked tubulin which migrated at a relative molecular mass of 145 kDa (Fig. 2, lane 6). The crosslinked species migrated at a relative molecular mass of 120 kDa following SDS–PAGE analysis on a 5% gel. Of the final tubulin product, approximately 8% was covalently crosslinked tubulin as determined by densitometric scanning.

The postincubation-cycled 2,5-HD-derivatized tubulin was subjected to additional cycles of temperature-dependent assembly/disassembly. Through two additional cycles, the proportion of covalently crosslinked tubulin remained approximately constant and assembly alterations persisted. Thus, the covalently crosslinked tubulin product was assembly competent and the 2,5-HD-induced covalent tubulin modification was a stable structural alteration.

Biochemical Properties of Crosslinked Tubulin

Gel filtration of 2,5-HD-treated tubulin was performed on Sepharose 6B to determine if the treated preparation contained a multimeric aggregate in cold solution. The covalently crosslinked tubulin coeluted with tubulin dimer (Fig. 3). The protein containing fractions demonstrated the assembly characteristics of treated tubulin preparations.

SDS–PAGE of control and 2,5-HD-treated tubulin was followed by electrophoretic transfer to nitrocellulose and reaction with specific anti-α- and anti-β-tubulin mouse monoclonal antibodies for determination of the subunit composition of the covalently crosslinked tubulin. The covalently crosslinked tubulin was composed of similar amounts of α- and β-tubulin, supporting a heterodimeric tubulin as the crosslinked entity (Fig. 4).

Basic Features of Altered Assembly

A 16-hr incubation period was adopted for additional studies of derivatized tubulin. Postincubation-cycled control and treated tubulin preparations were compared for assembly behavior (Fig. 5). The tV_{max} was used as a measure of the length of the nucleation phase. The nucleation phase of assembly was markedly different between samples: at a tubulin concentration of 0.4 mg/ml, the tV_{max} for treated tubulin was less than 1 min while the control tV_{max} was 7–8 min. The maximal velocity of assembly also differed with the treated sample elongating more rapidly than control. The control polymerization reached a higher maximal absorbance than the treated polymerization. Both preparations disassembled when cooled, as indicated by a decrease in absorbance, though the rate of

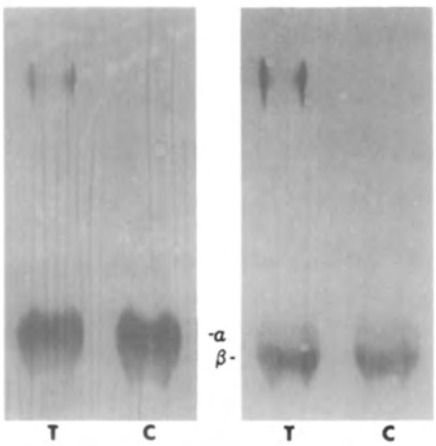

FIG. 4. Crosslinked tubulin was composed of both α- and β-tubulin. Control (C) and treated (T) tubulin (6 μg per lane) were electrophoresed on a 7.5% polyacrylamide minigel followed by protease-facilitated electrophoretic transfer to nitrocellulose. Monoclonal antibodies specific for α-tubulin (left-hand panel) and β-tubulin (right-hand panel) reacted with the covalently crosslinked tubulin and the appropriate monomer.

disassembly was slower in the treated sample. Following cold disassembly, the treated sample did not return to baseline but retained a small amount of absorbance. Negative stains of cold disassembled 2,5-HD-treated tubulin prepared with precooled pipets and grids obtained after 60 min of cold disassembly (the 110-min point in Fig. 5) revealed rare intact closed microtubules; no cold stable microtubules were observed in control material.

Concentraton Dependence of Assembly Parameters

The concentration dependence of assembly parameters was examined by absorbance spectroscopy. Control tubulin had a higher apparent critical concentration of assembly (0.07 mg/ml) than treated tubulin (0.03 mg/ml) and also demonstrated a higher absorbance at 350 nm for a given amount of polymer formation (Fig. 6A). The ratio of slopes of the best fit lines produced by least squares linear regression analysis indicated a 32% greater absorbance at 350 nm for a given amount of control assembly product relative to treated assembly product.

The tV_{max} showed a strong concentraton dependence in control assemblies varying from 7 to 8 min at 0.4 mg/ml to greater than 40 min at 0.2 mg/ml (Fig. 6B). The treated preparations showed little concentration dependence for this parameter between 0.2 and 0.4 mg/ml (usually less than one min). At a concentration of 0.1 mg/ml, the tV_{max} increased to 3 min (Fig. 6B). In general, for a given amount of polymer formed, the nucleation phase of assembly lasted at least 10 times longer in control than in treated assemblies.

The maximal velocity of assembly was consistently different between control and treated samples (Fig. 6C). For a given amount of polymer formed, this parameter of elongation phase kinetics was approximately three times higher in treated than in control assemblies.

Nucleotide Dependence of Assembly

Control and treated samples were compared for assembly behavior at different con-

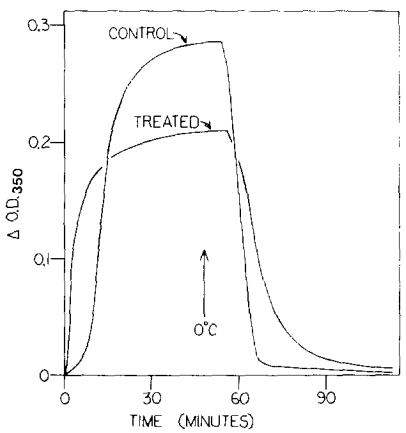

FIG. 5. Comparison of assembly with control and 2,5-HD-treated tubulin. Assembly of control tubulin (0.39 mg/ml) and treated tubulin (0.41 mg/ml) in 1 M sodium glutamate, 0.25 mM GTP, pH 6.6, at 37°C was monitored by measuring increased absorbance at 350 nm. Disassembly was induced by circulating ice-cold water through the cuvette holder housing. Treated tubulin nucleated and elongated more rapidly, reached a lower maximal absorbance, disassembled more slowly with cold, and contained relatively cold stable microtubules.

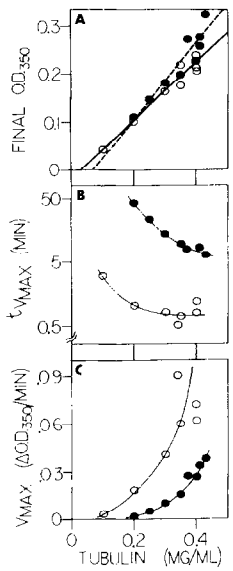

FIG. 6. Variation of assembly parameters with tubulin concentration. The final absorbance at 350 nm (Final OD_{350}), the time required to achieve maximal velocity of assembly (tV_{max}), and the maximal velocity of assembly (V_{max}) were determined for treated and control tubulin concentrations of 0.1–0.4 mg/ml. (A) The best fit lines were determined by least squares linear regression analysis for the concentration dependence of the final maximal absorbance of the assembly product at 350 nm. Control tubulin (closed circles, dashed line) had a higher critical concentration for assembly than treated tubulin (open circles, solid line) and greater absorbance for a given amount of polymer formation. (B) The time required to achieve maximal velocity of assembly in minutes (log scale) was determined for control and treated assemblies. Treated tubulin nucleated more rapidly and showed less concentration dependence of the length of the nucleation phase. (C) The maximal velocity of assembly was about three times higher with treated tubulin.

centrations of GTP. The samples were first freed of unbound GTP by Sephadex G-25 chromatography, a procedure reported to yield tubulin with slightly in excess of two GTPs per dimer (Maccioni and Seeds, 1982). The presence of 1000 μM, 500 μM, or 200 μM GTP produced an identical maximal assembly response. At concentrations below 200 μM GTP, the alterations produced in control and treated assemblies were strikingly different (Fig. 7). With decreasing GTP concentration, control preparations exhibited a progressive prolongation of the nucleation phase and a slowing of the maximal velocity of assembly. Without added GTP, only a minimal increase in absorbance was observed.

For treated assemblies, there was no apparent dependence of the length of the nucleation phase upon GTP concentration. Assembly, verified morphologically by negative staining and electron microscopy, occurred without added GTP with an unusual monophasic increase in absorbance. With no added GTP, the assembly product was also more cold stable than treated assemblies which contained added GTP. The treated assembly without added GTP retained 70% of maximal absorbance after 45 min at 0°C, whereas the typical treated assembly with 250 μM GTP retained only 7% of maximal absorbance. Electron microscopy showed the presence of numerous cold stable open and closed protofilamentous polymers in assemblies without added GTP.

Temperature Dependence of Assembly

Control preparations at 0.4 mg/ml assembled readily at 37°C. However, decreasing the

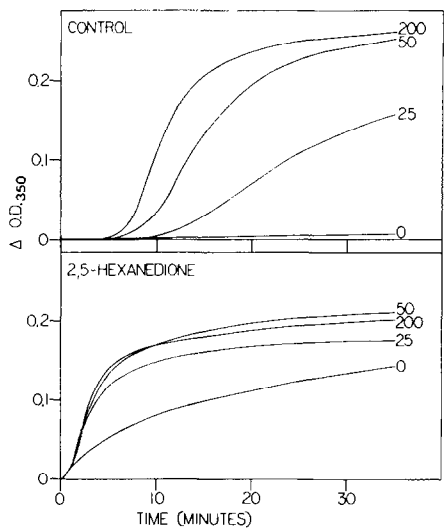

FIG. 7. Dependence of assembly upon GTP concentration. Control tubulin (0.41 mg/ml) showed an increase in lag time and a decrease in maximal velocity of assembly at GTP concentrations below 200 μM. No control assembly occurred at 0 μM added GTP. 2,5-HD-derivatized tubulin (0.40 mg/ml) showed little alteration in assembly behavior at low GTP concentrations. At 0 μM added GTP, treated tubulin assembled with a monophasic increase in absorbance.

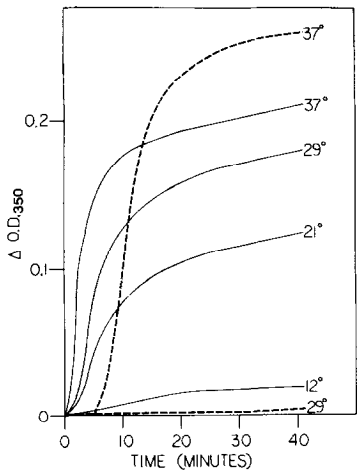

FIG. 8. Temperature dependence of tubulin assembly. Derivatized tubulin (solid lines, 0.36–0.41 mg/ml) assembled readily at temperatures as low as 12°C while control tubulin (dashed lines, 0.41 mg/ml) demonstrated a marked prolongation of the nucleation phase for assembly when the temperature was reduced from 37 to 29°C.

temperature to 29°C resulted in a markedly prolonged nucleation phase of greater than 30 min. The temperature dependence of the 2,5-HD-treated polymerization was much different (Fig. 8). Assembly occurred readily at temperatures as low as 12°C. The tV_{max} increased to about 3 min upon lowering the temperature to 12°C. Both the maximal velocity of assembly and the final extent of assembly decreased at lower temperatures.

Crosslinking Correlates with Assembly Enhancement

Incubation of tubulin under anaerobic conditions in the presence of free-radical scavengers was used to inhibit protein crosslinking by 2,5-HD. The anaerobic incubation mixture contained 200 μg/ml catalase, 1 mM deferoxamine, 1 mM ascorbic acid, and 100 mM 2,5-HD in glutamate buffer. The mixture was bubbled for 5 min with nitrogen prior to incubation for 16 hr at 37°C. The amount of crosslinked tubulin formed under these anaerobic conditions was 18% of that formed in the presence of oxygen.

Mixtures of control and treated tubulin were used to construct a curve correlating the assembly parameter tV_{max} with the proportion of treated tubulin (Fig. 9). The presence of only a small amount of treated tubulin caused a large decrease in tV_{max}. In addition, the tV_{max} of the anaerobically incubated sample was appropriate for the amount of crosslinked tubulin (Fig. 9), supporting crosslinking per se as the cause of treatment-induced assembly alterations.

Physical and Chemical Characteristics of Derivatized Tubulin

Electron microscopy of negatively stained assembled control and treated tubulin showed both closed and open protofilamentous tu-

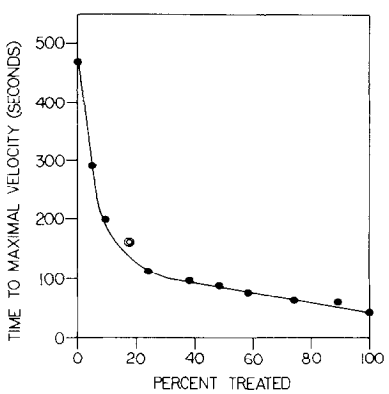

FIG. 9. Anaerobic incubation of tubulin with 2,5-HD alters crosslinking and assembly. Treated (0.35 mg/ml) and control (0.37 mg/ml) tubulin were mixed in various proportions and the time required to reach maximal velocity of assembly was determined (closed circles). A marked alteration in assembly occurred in the presence of only a small percentage of treated tubulin. Tubulin incubated anaerobically with 2,5-HD (double open circle) had an increased time required to reach maximal velocity of assembly appropriate for its small amount of crosslinked tubulin (equivalent to 18% of treated tubulin) and the slightly lower assembly concentration (0.31 mg/ml). The amount of crosslinked tubulin in the anaerobic sample was determined by densitometric scanning of silver-stained polyacrylamide gels of the anaerobic sample and the control plus treated mixtures.

bular profiles (Fig. 10). Treated tubulin tended to form a higher proportion of simple, narrow sheets or closed tubules than control tubulin; however, these morphologic features were inconstant and varied from preparation to preparation. A striking qualitative difference in length was verified by measuring polymer profiles. The 2,5-HD-treated polymers averaged 1.3 μm (n = 229) compared with 3.8 μm (n = 331) for controls (Fig. 11).

2,5-HD-treated and control tubulin preparations were passed through a 1.5 × 15-cm Sephadex G-25 column equilibrated with distilled water for spectrophotometric studies and chemical analyses. The uv and visible spectra (230–400 nm) of the two samples in 6 M guanidine HCl were indistinguishable with a peak at 275 nm. A Coomassie blue protein dye binding assay, the Lowry protein assay, and measurement of absorbance at 275 nm all gave similar and reproducible values for protein content of control and 2,5-HD-treated tubulin.

The Ehrlich's reaction was used to provide a measure of the pyrrole content of treated tubulin. 2,5-Dimethylpyrrole was added to control tubulin as a standard. The pyrrole content of 2,5-HD-derivatized tubulin was 1.53 ± 0.08 2,5-dimethylpyrrole equivalents per tubulin dimer (mean ± SE, four different preparations). Anaerobic incubation produced derivatized tubulin with similar pyrrole reactivity, 1.25 ± 0.16 2,5-dimethylpyrrole equivalents per tubulin dimer (mean ± SE, four different preparations). Assuming a similar Ehrlich's reactivity for proteolyzed-treated tubulin and proteolyzed control tubulin with added 2,5-dimethylpyrrole, approximately 5% of available lysines of tubulin dimer have been derivatized by 2,5-HD.

In Vitro Modification of Rat Brain Tubulin by 2,5-HD

Purified rat brain tubulin was derivatized by overnight incubation with 100 mM 2,5-HD followed by a cycle of warm and cold ultracentrifugations to give 2,5-HD-treated rat

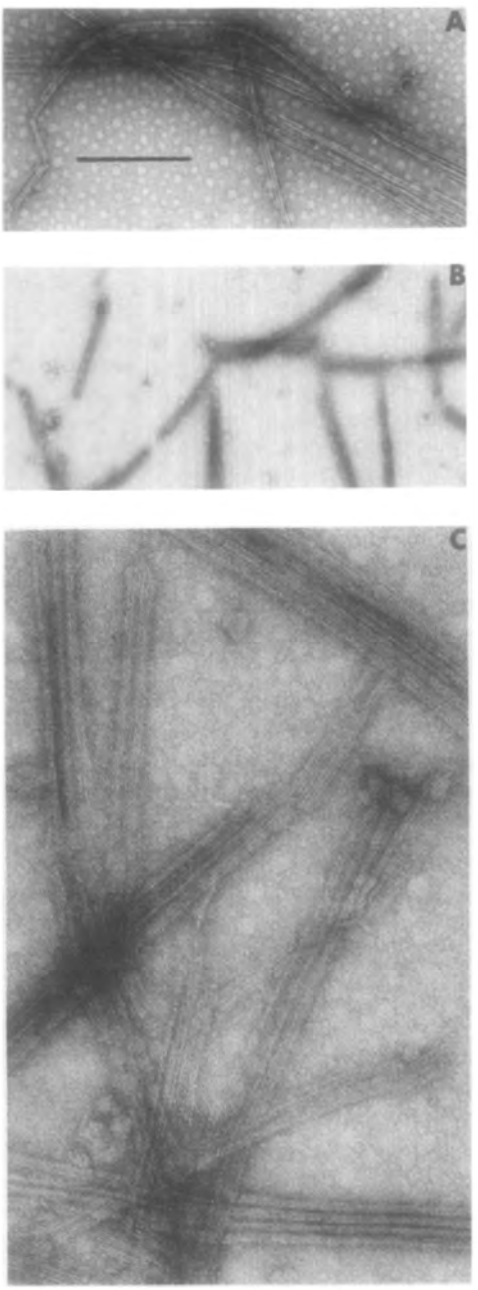

FIG. 10. Ultrastructure of the tubulin assembly product. Control and treated tubulin preparations at 0.4 mg/ml were polymerized for 45 min at 37°C in glutamate buffer prior to negative staining with uranyl acetate. The contrasting length of control tubulin polymers ((A) magnification, ×30,000; bar = 0.5 μm) and treated tubulin polymers ((B) same final magnification as (A), ×30,000) was apparent at low magnification. At high magnification, the treated tubulin polymers ((C) magnification, ×110,000) were composed of both closed tubules and open protofilamentous sheets.

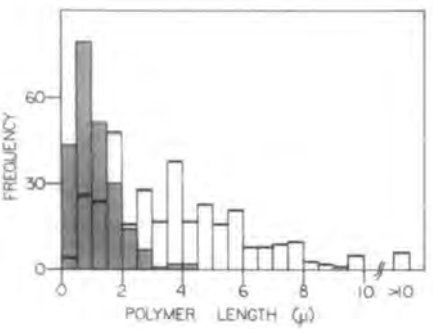

FIG. 11. Control polymers were three times as long as treated polymers. The frequency distribution of polymer length was determined for control (heavy bar, average polymer length 3.8 μm, n = 331) and treated (shaded area, average polymer length 1.3 μm, n = 229) assemblies (0.4 mg/ml) after a 45-min incubation at 37°C.

brain tubulin adequate for assembly and biochemical analyses. The Ehrlich's reaction for pyrroles indicated the formation of 1 DMP equivalent per tubulin dimer. Measurements of tubulin concentrations by the method of Lowry *et al.* (1951) or of absorbance at 275 nm in 6 M guanidine HCl gave similar and reproducible results. [^{3}H]Colchicine binding was unaltered by 2,5-HD derivatization. Silver staining of the 2,5-HD-derivatized rat brain tubulin sample followng SDS–PAGE revealed a high-molecular-weight component representing 5–10% of the total protein.

In vitro 2,5-HD-treated rat brain tubulin assembled after a minimal nucleation phase with a greater V_{max} and to a lower final optical density at 350 nm when compared with untreated *in vitro* control. Mixtures of various proportions of *in vitro* control and 2,5-HD-derivatized tubulin were analyzed for assembly kinetics; the tV_{max} decreased dramatically with only a small percentage of 2,5-HD-treated tubulin. *In vitro* derivatization of rat brain tubulin by 2,5-HD induced a stable modified tubulin which demonstrated reproducible assembly alterations during many hours of experimentation. In sum, *in vitro* 2,5-HD-treated rat brain tubulin demonstrated altered assembly similar to that described for *in vitro* 2,5-HD-derivatized bovine brain tubulin.

A mixture of 90% *in vitro* control rat brain tubulin plus 10% *in vitro* 2,5-HD-derivatized rat brain tubulin was chosen for further study in comparison with purified *in vitro* control and *in vitro* 2,5-HD-treated rat brain tubulin. Assembly in the presence of 2.5 mM calcium or at reduced temperature markedly altered control assembly behavior (Fig. 12). The assembly of the 90% *in vitro* control plus 10% *in vitro* treated sample was moderately inhibited under these stringent conditions while the pure 2,5-HD *in vitro*-treated rat brain assembly was minimally altered. The 90% *in vitro* control plus 10% *in vitro* treated sample accurately mimicked the assembly behavior of the

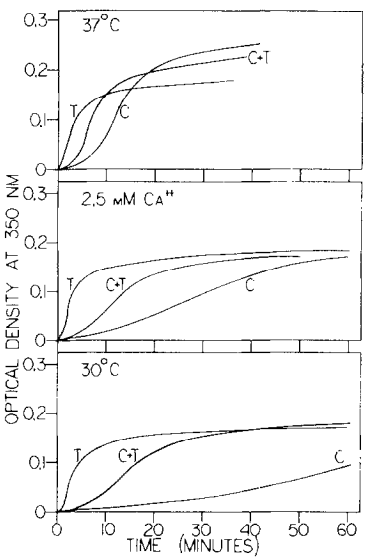

FIG. 12. Assembly of *in vitro* control and *in vitro* 2,5-HD-derivatized rat brain tubulin. *In vitro* control (C) and *in vitro* 2,5-HD-treated (T) rat brain tubulin and a mixture of 90% *in vitro* control plus 10% *in vitro* treated (C + T) tubulin were assembled at a tubulin concentration of 0.40 mg/ml in 1 M sodium glutamate, pH 6.6, with 0.25 mM GTP. As shown in the top panel, treated tubulin demonstrated markedly altered assembly with a minimal nucleation phase under standard conditions at 37°C. The 90% control plus 10% treated tubulin mixture assembled with an intermediate tV_{max} and V_{max} when compared with control and treated tubulin. As shown in the middle panel, addition of 2.5 mM calcium markedly altered control assembly behavior. As shown in the bottom panel, assembly in the standard buffer at 30°C delayed control tubulin assembly.

in vivo purified brain tubulin assemblies from 2,5-HD-intoxicated rats (Boekelheide, 1987).

In Vitro Modification of Rat Brain Tubulin by DMHD

Incubation of purified rat brain tubulin with 5 mM DMHD at 37°C for 16 hr in 1 M sodium glutamate, pH 6.6, followed by a postincubation cycle of assembly and disassembly, gave modified tubulin with altered assembly. SDS–PAGE of DMHD-derivatized tubulin identified a high-molecular-weight component representing approximately 1% of the total protein. When compared to control assembly at 0.36 mg/ml, *in vitro* DMHD derivatization of rat brain tubulin decreased tV_{max} from 7 to 3 min, decreased the total change in optical density from 0.223 to 0.150, and minimally altered V_{max} (control, 0.021; DMHD, 0.016 absorbance units per minute).

In Vitro Modification of Rat Testis Tubulin by 2.5-HD

In vitro incubation of purified rat testis tubulin with 2,5-HD altered assembly in a manner analogous to that found with rat and bovine brain tubulin and similarly produced a high-molecular-weight contaminant by SDS–PAGE. When compared to *in vitro* control testis tubulin assembled at a similar concentration (0.35 mg/ml), the 2,5-HD-treated testis tubulin tV_{max} decreased from 10 to 0.5 min while the V_{max} increased from 0.039 to 0.066 absorbance units per minute, and the total change in optical density decreased from 0.417 to 0.207.

DISCUSSION

2,5-HD reacts with protein lysyl ϵ-amines to give at least two distinct products: (1) the aromatic pyrryl derivative of lysine, ϵ-(2,5-dimethylpyrrolyl)-norleucine (DeCaprio *et al.*, 1982) and (2) intramolecular and intermolecular covalent crosslinks bridging lysyl residues (Graham *et al.*, 1982; Anthony *et al.*, 1983). The crosslinking reaction is a free-radical-dependent process enhanced by oxygen (Smith and Jensen, 1967). When compared to the usual aerobic incubation, anaerobic incubation of tubulin with 2,5-HD produced a less extensively crosslinked product while pyrryl derivatization remained unaltered. The less extensively crosslinked anaerobic incubation product showed concomitantly less assembly enhancement. This identified an oxygen-dependent reaction, presumably tubulin crosslinking, as the underlying chemical basis for the promotion of assembly caused by 2,5-HD.

The Ehrlich's test for pyrroles indicated only a low level of tubulin derivatization. On average, only one or two lysines per tubulin dimer reacted with 2,5-HD. Three different analytic methods for protein determination, a protein dye binding assay, the Lowry protein assay and absorbance at 275 nm, revealed no differences in reactivity between 2,5-HD-derivatized and control tubulin. Since the Coomassie blue protein dye binding assay is based upon attachment to protein basic amines (Sedmak and Grossberg, 1977), the similarity of control and treated tubulin emphasizes the small degree of 2,5-HD-induced lysine modification. Alkylpyrroles characteristically absorb at 210–215 nm (Hinman and Theodoropulos, 1963). The pyrrole content of derivatized tubulin did not noticeably alter the absorption spectrum from 230 to 400 nm. The low level of covalent modification is apparently insufficient to affect these assays for protein determination.

The 2,5-HD crosslinked tubulin behaved anomalously by SDS–PAGE analysis, migrating at an apparent molecular mass of 145 kDa on 7.5% gels and 120 kDa on 5% gels, inconsistent with any multiple of tubulin monomer (55 kDa). Additional evidence was most consistent with an α-β heterodimer as the crosslinked tubulin. First, the crosslinked tubulin coeluted with native tubulin dimer by gel filtration chromatography. A crosslinked trimer in solution, as either trimer or

tetramer, would elute from a gel-filtration column prior to native tubulin dimer. Second, both anti-α- and anti-β-tubulin monoclonal antibodies reacted with the crosslinked tubulin, indicating an α-β heterodimeric structure for the intermolecular covalently bound species.

Control and 2,5-HD-treated tubulin were compared for basic features of the assembly reaction. For the purposes of this study, the use of absorbance at 350 nm allowed a qualitative comparison of assembly behavior in control and 2,5-HD-treated tubulin preparations (Gaskin *et al.*, 1974; Johnson and Borisy, 1977). The assembly-altering effects of 2,5-HD derivatization were most prominent in the nucleation phase. The lag time for assembly was markedly shorter with treated tubulin and there was a decrease in the concentration dependence of the length of the lag time. The treated tubulin critical concentration of assembly was 2–3 times lower than that of control as determined by the tubulin concentration dependence of the final optical density. An increase in the number of nucleating sites can be inferred from the shorter length of derivatized polymers and the increased rate of elongation. Assembly at low temperature, decreased dependence of assembly upon GTP concentraton, slower rate of cold-induced depolymerization, and the presence of relatively cold stable polymers were all features which supported assembly promotion of treated tubulin and stabilization of the polymeric state.

These studies of *in vitro* 2,5-HD modification of purified bovine brain tubulin were extended to include examination of the γ-diketone DMHD and purified rat brain and testis tubulins. 2,5-HD and DMHD produced qualitatively similar alterations in microtubule assembly. Purified brain tubulin showed similar assembly behavior whether derived from rat or cow. Testis tubulin differed from brain tubulin by manifesting more rapid assembly with a greater final optical density. Nonetheless, the different tubulins responded to *in vitro* γ-diketone modification in a similar manner with earlier and more rapid assembly to a lower final optical density.

Limited proteolysis by subtilisin produces a modified tubulin with enhanced assembly properties (Bhattacharyya *et al.*, 1985; Sackett *et al.*, 1985). Taxol, a low-molecular-weight plant taxane with antimitotic activity (Wani *et al.*, 1971), promotes microtubule assembly both *in vitro* and *in vivo* (Manfredi and Horwitz, 1984). The underlying mechanism of assembly enhancement produced by 2,5-HD derivatization of tubulin must differ from that produced by taxol but may share some similarities with that produced by proteolytic cleavage. With both 2,5-HD-treated tubulin and proteolytically cleaved tubulin, a stable pool of permanently modified subunits is present at initiation of assembly. Taxol, on the other hand, exerts its effect by preferentially and reversibly binding and stabilizing polymeric tubulin (Carlier and Pantaloni, 1983; Horwitz *et al.*, 1982). 2,5-HD-derivatized tubulin is covalently crosslinked while in the polymeric state, suggesting a fixation of the dimer conformation into that of the stable polymeric form. The most prominent alteration in assembly of 2,5-HD-derivatized tubulin is enhanced nucleation. One possible explanation for this altered assembly behavior lies in a stabilization of the oligomeric and/or polymeric state by a decreased rate of dissociation of derivatized tubulin from the polymer.

The microtubule assembly alterations produced by *in vivo* 2,5-HD intoxication were reproduced by *in vitro* 2,5-HD derivatization of purified brain and testis tubulin. A mixture of 90% *in vitro* control plus 10% *in vitro* 2,5-HD-treated rat brain tubulin mimicked the nucleation and elongation alterations as well as assembly behavior under stringent conditions characteristic of the *in vivo* 2,5-HD preparations (Boekelheide, 1987). One striking result of the analysis of assembly behavior of mixtures of *in vitro* control and *in vitro* treated tubulin was the small amount of 2,5-HD-derivatized tubulin needed to make a large change in the kinetics of microtubule assembly: the tV_{max} of a 90% *in vitro* control

plus 10% *in vitro* treated sample was half that of pure *in vitro* control tubulin. From the biologic perspective, this suggests that a small degree of 2,5-HD derivatization can effect a large alteration in microtubule assembly behavior.

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