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THE FORMATION OF FILAMENTOUS STRUCTURES FROM IODINATED NEUROTUBULES

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The porcine neurotubule and its basic subunit were found to be modified in vitro by iodination of amino acids (principally tyrosine) using lactoperoxidase. Iodide ion, H_2O_2, or lactoperoxidase singly or in any pairwise combination had virtually no effect on neurotubules. However, when all three reagents were present, permitting covalent iodination, it was found that at 0.1 iodotyrosines per tubulin dimer the microtubules unravel to form structures which morphologically resemble strands of protofilaments twisted or wound around each other. These abnormal tubules are stable at room temperature and 4°C. Both monomers of tubulin are labeled to approximately the same extent. Iodinated tubulin (0.1 iodotyrosines/dimer) is unable to assemble in vitro under normal assembly conditions. Heavily iodinated microtubules (8 iodines per tubulin dimer) are similar in morphology to the slightly iodinated structures.

INTRODUCTION

In the assembled cytoplasmic microtubules there must be specific lateral and vertical interactions between molecules of the protein dimer tubulin. A wide variety of reagents are known to disrupt microtubules in vivo and in vitro. For example, microtubules disassemble in the presence of mitotic spindle inhibitors such as colchicine, the vinca alkaloids, and podophyllotoxin. Several other agents, such as sulfhydryl reagents, chloral hydrate, heavy cations such as Cu^{2+}, and general anesthetic gases, also disrupt microtubule function (1). In addition, assembly is known to depend on bound nucleotides, is promoted by Mg^{2+} and temperatures above 20°C, and is inhibited by Ca^{2+} and temperatures below 15°C (2, 3, 4). In most cases there is little hint as to whether lateral or vertical protein interactions are preferentially affected by use of these ligands or environmental

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factors. Little specific chemical modification of microtubules or tubulin has been reported to date. We chose to examine the effect of the lactoperoxidase (LP)-H_2O_2-NaI system of Phillips and Morrison (5). This system appears to preferentially iodinate surface exposed tyrosines. The major question of interest was whether such a relatively gentle form of covalent modification would perturb microtubule function and the ability to assemble and disassemble.

MATERIALS AND METHODS

Preparation of Tubulin and Microtubules

Porcine tubulin was purified from fresh brains by the assembly-disassembly procedure described by Shelanski et al. (6) and stored at −20°C in 0.1 M 2-(N-morpholino) ethane sulfonic acid (Mes), pH 6.6, containing 4 M glycerol, 10^{-3} M EGTA, and 5×10^{-4} M $MgCl_2$. Tubulin stored in this way has a half life of decay (based on ability to reassemble) that is greater than six weeks. All experiments were performed on protein stored less than two weeks. On the day of the experiment the stored tubulin was dialyzed for 3 hr at 4°C against 100 volumes of 0.1 M Mes, pH 6.6, containing 10^{-3} M EGTA and 5×10^{-4} M $MgCl_2$. After centrifugation at 4°C at 120,000 g for 30 min the tubulin was stored in 10^{-3} M GTP in an ice bath until ready for use. For microtubule preparations, the solution was warmed to 37°C for 30 min. Protein concentrations were determined by the method of Lowry et al. (7).

Iodination of Microtubules and Tubulin

LP was obtained from Sigma Chemical Co. and purified as described by Morrison and Hultquist (8). The concentration of LP was determined from absorbance at 412 using $\epsilon = 114\ mM^{-1}$ (5). Na ^{125}I, obtained carrier free from New England Nuclear, was usually used at a specific activity of 20 Ci/M.

Microtubules were iodinated at room temperature for several different periods of time. A typical reaction mixture contained 4 mg microtubule protein, 2 ml Mes buffer, 25 μl of 1×10^{-5} M LP, 20 μl of 2×10^{-3} M NaI, and 20–200 μl 8.75 mM H_2O_2. Components were added slowly with constant stirring to the microtubule protein solution. Each reaction mixture was brought to a final volume of 2.5 ml by adding Mes buffer. There was no detectable pH change during iodination. Controls were done by adding the appropriate amount of LP + H_2O_2, LP + Na ^{125}I, or H_2O_2 + Na ^{125}I to the protein solution. To separate microtubules from tubulin, samples were centrifuged at 120,000 g for 30 min at room temperature. The pellet was resuspended in a small volume of Mes buffer and homogenized. In a typical experiment 70% of the protein was pelletable both after assembly only and after iodination.

Tubulin was iodinated at 4°C for 60 min. The appropriate controls were done as described above.

Identification of the Iodinated Species

To determine the amount of covalent ^{125}I incorporation, proteins were precipitated

by the addition of 12.5% trichloroacetic acid (TCA) to a final concentration of 10% TCA. The solutions were chilled on ice, and the precipitate was isolated on a millipore filter after washes with cold 5% TCA. The filters were dried and counted in 10 ml of PPO-POPOP scintillation fluid containing 4 gm 2.5 diphenyloxazol (PPO) and 0.1 gm 1,4-bis-2-(5-phenyloxazolyl)-benzene (POPOP) per liter of toluene. Both PPO and POPOP were obtained from Packard Instrument Co., Inc. A Packard Tri-Carb 3375 scintillation counter was used at a gain setting of 17%.

Samples were reduced and alkylated and analyzed for incorporation of ^{125}I into specific proteins by electrophoresis on sodium dodecyl sulfate–8 M urea polyacrylamide gels (9). Gels were stained with fast green (10) and scanned with a Gilford spectrophotometer for quantitation of protein. Areas under the peaks were usually determined by tracing the curves on high-quality graph paper, cutting them, and weighing them. When there was overlap of the peaks, a Du Pont 310 curve resolver was used to estimate areas.

For radioactive determinations gels were cut into 2 mm slices. The slices were put into scintillation vials, dried for 25 hr at 75°C, and dissolved in 0.4 ml of 30% H_2O_2 by incubation at 70°C for 16 hr. Ten ml of scintillation fluid [1,152 ml xylene, 1,152 ml distilled dioxane, 696 ml ethanol, 240 gm naphthalene, 12 gm of 2,5-bis-2-(tert-butylbenzoxazolyl)-thiophene] was added to each vial. Samples were counted as described above. The efficiency for ^{125}I was approximately 60%.

To determine which amino acids were iodinated, samples were analyzed as described by Hubbard and Cohn (11). After enzymatic hydrolysis with crude trypsin (Grand Island Biological Co.), TCA precipitation, and rinsing, samples were applied to a Sephadex G-25 Fine column 1 × 50 cm. The radioactivity was eluted with 1 M acetic acid and counted in PPO-POPOP scintillation fluid as previously described.

Electron Microscopy

All samples were examined in an electron microscope (Hitachi HU-12, Perkin Elmer Corp., Palo Alto, Calif.) For routine analysis, 1 drop of sample was applied to a formvar grid for 15 sec and stained by rinsing with 6 drops of 1% uranyl acetate and blotting dry with filter paper. Thin-layer sections of microtubules and iodinated microtubules were prepared as follows. The solutions were centrifuged for 30 min at 120,000 g at room temperature. The supernatant was discarded and the pellet was fixed in 5% glutaraldehyde in phosphate buffer (0.1 M, pH 7.4). The pellets were thin sectioned and negatively stained for electronmicroscopy.

RESULTS

Iodination of Microtubules

Microtubules were iodinated and analyzed for structural changes with the electron microscope. Since the basic microtubule subunit is tubulin, MW 110,000, we will use "I/D" to indicate iodines per tubulin dimer. Very low degrees of iodination cause appreciable disruption of microtubule structure. To learn the minimum number of iodinated amino acids required to disrupt the normal mircotubule, the initial concentration of H_2O_2 was

varied to control the extent of reaction. After 30 min grids were made and the samples examined. At approximately 0.02 I/D in the pellet a mixture of abnormal and normal microtubules was found. No structure was ever seen in which a single tubule was partially normal and partially abnormal (Fig. 1 and Table I). Abnormal forms predominated at higher iodination levels (0.05 to 8 I/D). The supernatant fraction contained 2–3 times as many iodinated amino acids as the pellet fraction. This is not surprising since a good fraction of the amino acid side chains must be involved in subunit interactions in the microtubule. These must be protected from reaction at least to some extent. In addition, residues exposed on the inside of the tubule would probably be labeled only slowly because LP would first have to diffuse into the tubule.

In order to better understand the structural effect of iodination, various tubule samples were pelleted, fixed with glutaraldehyde, thin sectioned, stained with 1% uranyl acetate, and examined in the electron microscope. Figure 2 demonstrates the differences between thin sections of normal microtubules and tubules after iodination to a level of 0.6 I/D. After iodination to this extent, no normal microtubules remained in solution. Instead, only filamentous structures which have no apparent rigidity are found. It is striking that more than 99% of the control microtubules (H_2O_2 + LP, H_2O_2 + NaI, NaI + LP) are normal, whereas all of the iodinated microtubules are abnormal.

In another series of experiments aliquots of H_2O_2 were added every 20 min over a period of 2 hr. Again there were mixtures of normal and abnormal microtubules at very low I/D ratios and all abnormal microtubules above 0.1 I/D.

TABLE I. Morphology of Microtubules After Iodination

Morphology*		
Normal	Abnormal	I/D†
+	0	0.006
+	0	0.014
+	very few	0.015
0	+	0.010
+	some	0.018
+	+	0.02
+	+	0.05
0	+	0.05
0	+	0.05
0	+	0.08
0	+	0.09
+	some	0.10
0	+	0.57
0	+	3.1
0	+	7.7
0	+	8.0

*Based on electron microscopy.
†Microtubules were pelleted, and I/D is the number of iodines per 110,000 daltons.

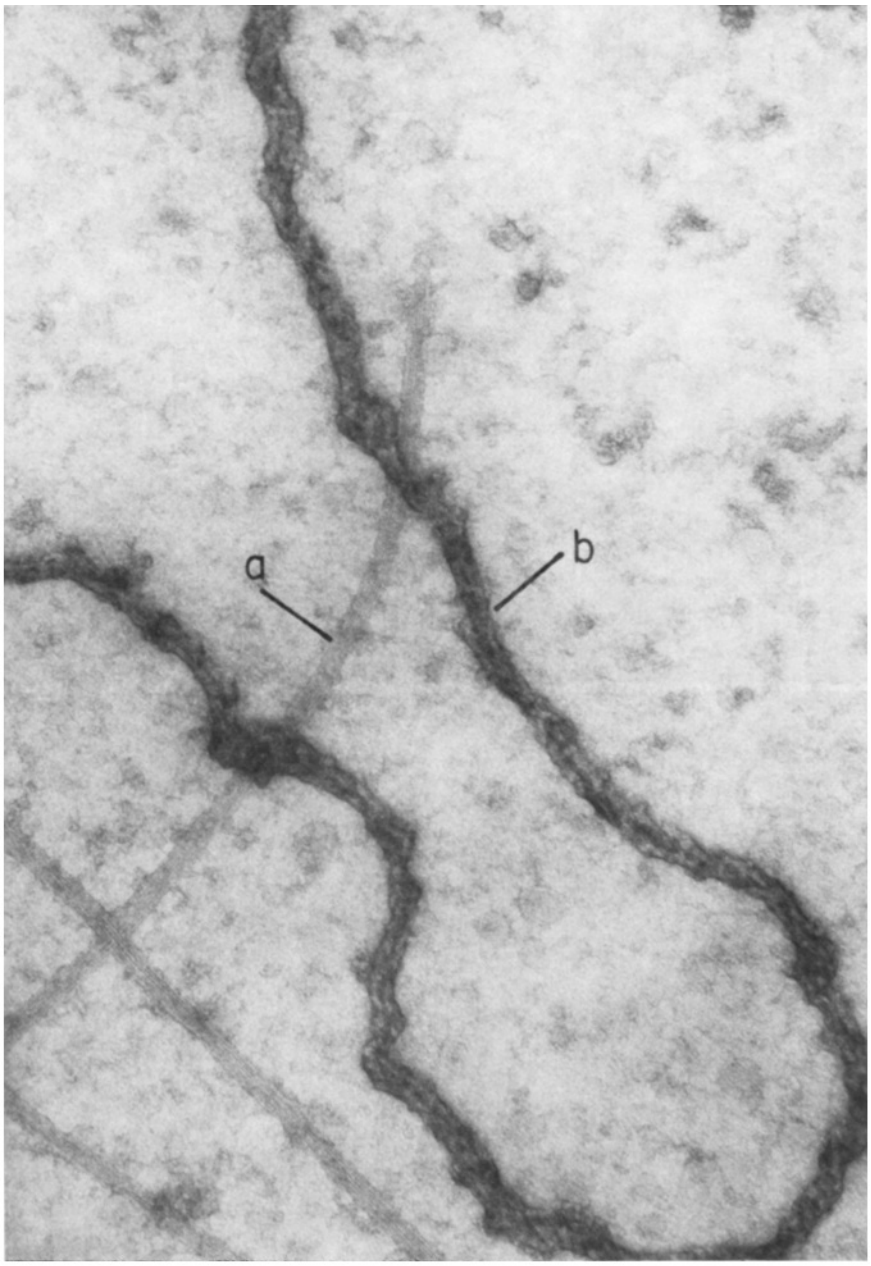

Fig. 1. Mircotubules after iodination (I/D = 0.02 in solution. Structure a is a normal microtubule. Structure b is an abnormal microtubule. (× 110,000)

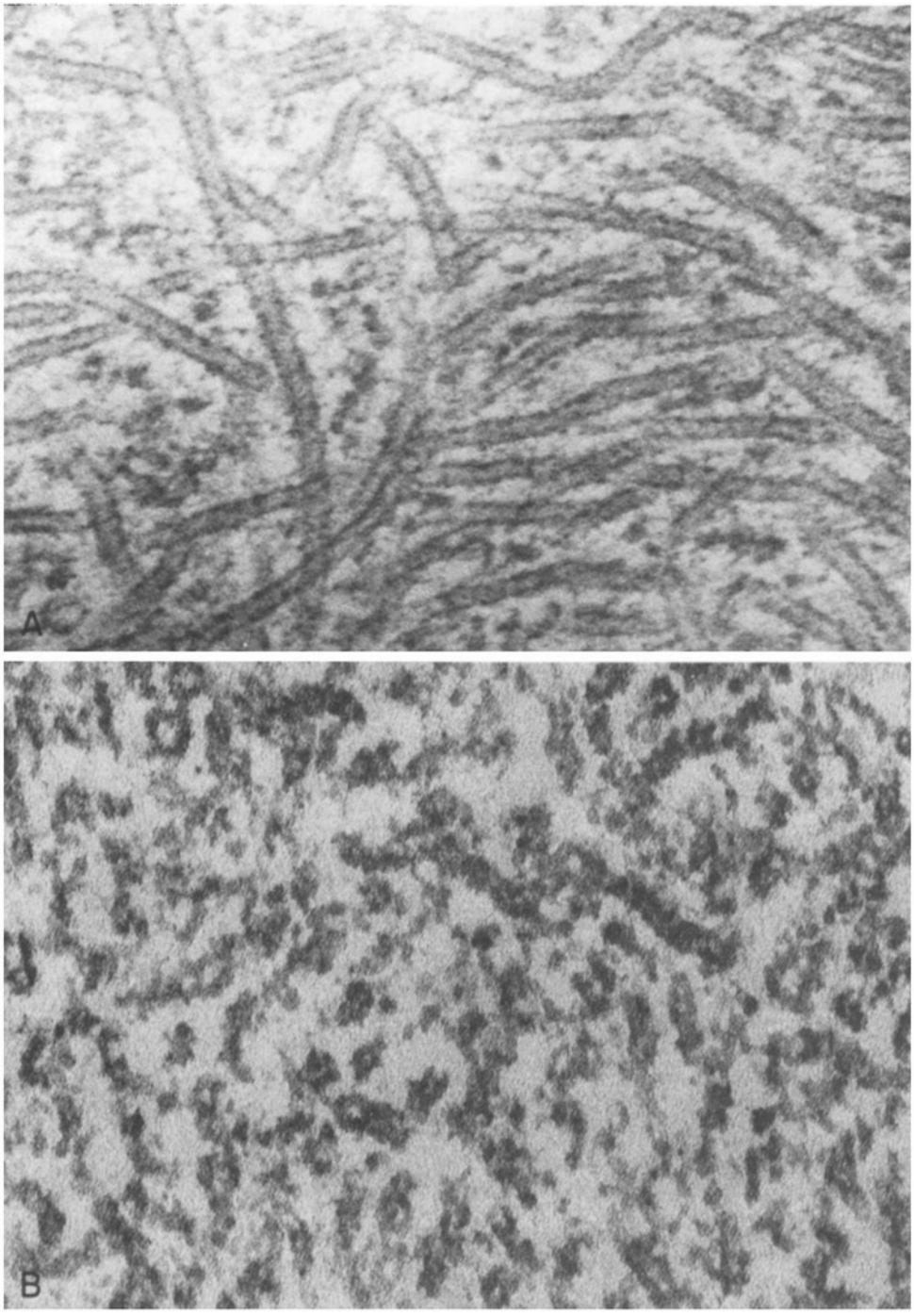

Fig. 2. (A) Thin section of pelleted microtubules (control containing H_2O_2 and LP). (B) Thin section of pelleted iodinated microtubules (I/D = 0.6). (× 135,000).

Timed experiments were also done to learn how fast iodination occurs and when the abnormal forms appear. Table II gives results from a typical experiment. In the range of about 0.08–0.15 I/D, iodination was over within 1 min. However, the percentage of filamentous structures increased over a period of several minutes. At higher levels of iodination, both maximum iodination and all abnormal forms appeared within 1 min. These results suggest that at low levels of iodination there is a slow unraveling of the microtubules to form stable filamentous structures.

TABLE II. Time Dependence of the Iodination of Microtubules

Time after Adding H_2O_2 (min)†	I/D (total protein)	Fraction of normal tubules remaining*
0	–	1.00
0.5	0.12	1.00
1.5	0.12	0.98
2.5	0.10	0.90
4	0.13	0.85
6	n.d.**	0.60
8	0.14	0.20
10	n.d.	0.10
15	0.11	0
30	n.d.	0

†1×10^{-5} M MTP, 9.0×10^{-5} M NaI, 6×10^{-8} M LP, 1.1×10^{-4} M H_2O_2.

**n.d. = not determined.

*It should be noted that there was a decrease in the total number of filamentous structures with time.

Iodination of Tubulin

The next experiments studied the effect of iodination of both tubules and tubulin on subsequent assembly or disassembly. Normal microtubules rapidly disassemble at 4°C and can be reassembled by raising the temperature to 37°C. However, it was found that iodinated microtubules are stable at 4°C and, based on electron microscopy, there was no disassembly. In a separate experiment, iodinated structures (I/D = 8) and microtubules were pelleted at 120,000 g for 30 min at 25°C, resuspended and homogenized in Mes buffer, and kept at 4°C. This resulted in less than 10% of the iodinated protein in the supernatant (representing unassembled protein), whereas 80% of an uniodinated control was found in the supernatant after centrifugation at 4°C.

Tubulin preparations were iodinated for 1 hr at 4°C at 0.07–4.4 I/D. No microtubules were found using electron microscopy upon raising the temperature to 37°C for 30 min (see Table III). Mixtures of control tubulin and iodinated structures were then combined and analyzed for assembly by electron microscopy. When assembly did occur it resulted primarily in normal microtubules. In the cases where a few abnormal microtubules were found, one can postulate that normal microtubules were formed first and then iodinated, since there should still be LP present, as well as excess NaI in the iodinated sample and unused H_2O_2 in the control. Finally, when iodinated samples were dialyzed for several hours at 4°C and warmed to 37°C for either 30 min or 2 hr, no microtubules or other assembled forms were found in the electron microscope, whereas the uniodinated control sample contained many normal microtubules.

TABLE III. Effect of Iodination* of Tubulin at 4°C on Subsequent Assembly at 37°C

Solution	H_2O_2 M × 10^5	I/D	Assembly at 37°C*
1	1.25	0.07	0
2	6	0.09	0
3	10	0.12	0
4	40	4.4	0
5	no H_2O_2	–	+, normal
6	8×10^{-4} M H_2O_2, no NaI	–	+, normal
7	4×10^{-4} M H_2O_2, no LP	–	+, normal

*30 min, 10^{-5} M tubulin, 6×10^{-8} M LP, 1.8×10^{-4} M NaI.

Identification of the Iodinated Species

Since only 0.1 I/D were required to cause disruption of the microtubules into the abnormal form, polyacrylamide gel analysis was done to see if any specific polypeptide was preferentially labeled. Preparations of porcine neurotubules purified by the assembly-disassembly procedure contain at least 80% tubulin (α and β monomers) and high-molecular-weight polypeptides (370,000 and 355,000) (12). Figure 3 illustrates representative scans of gels in two polyacrylamide systems after staining of iodinated samples with fast green. Also shown is the radioactivity of slices of the same gel. Table IV compares the protein concentration and cpm ^{125}I of each peak. All the proteins found in the gels are labeled to essentially the same extent. This argues against the notion that a minor protein component of the neurotubule is a specific target for iodination-induced conversion to the abnormal form.

Samples after iodination were digested and applied to a Sephadex G-25 column (Fig. 4). Eighty percent of the material eluted with monoiodotyrosine, and no detectable material eluted with diiodotyrosine. The rest of the radioactivity elutes just after NaI and may represent undigested peptides or another amino acid.

DISCUSSION

The abnormal structures formed after iodination of microtubules are interesting for many reasons:

(1) Less than 10% of the tubulin molecules need to be modified to convert normal tubules into these abnormal structures. This suggests that what has occurred is more complex than simple denaturation of the protein.

(2) The abnormal structures can be formed directly from microtubules but probably not directly from tubulin dimers. Free tubulin is labeled 2–3 times as much as assembled tubulin. Thus it can be postulated that tyrosines are involved in the subunit interactions required for assembly and that some but not all of these are protected in the microtubule. Once formed, the iodinated tubules are very stable and are not disrupted at 4°C, a condition known to disrupt normal microtubules.

(3) Iodination of microtubules at low levels proceeds faster than does the appearance of the abnormal structures. Thus the protofilaments of the microtubules appear to partially unravel and then rewind or twist to form the abnormal filamentous structures.

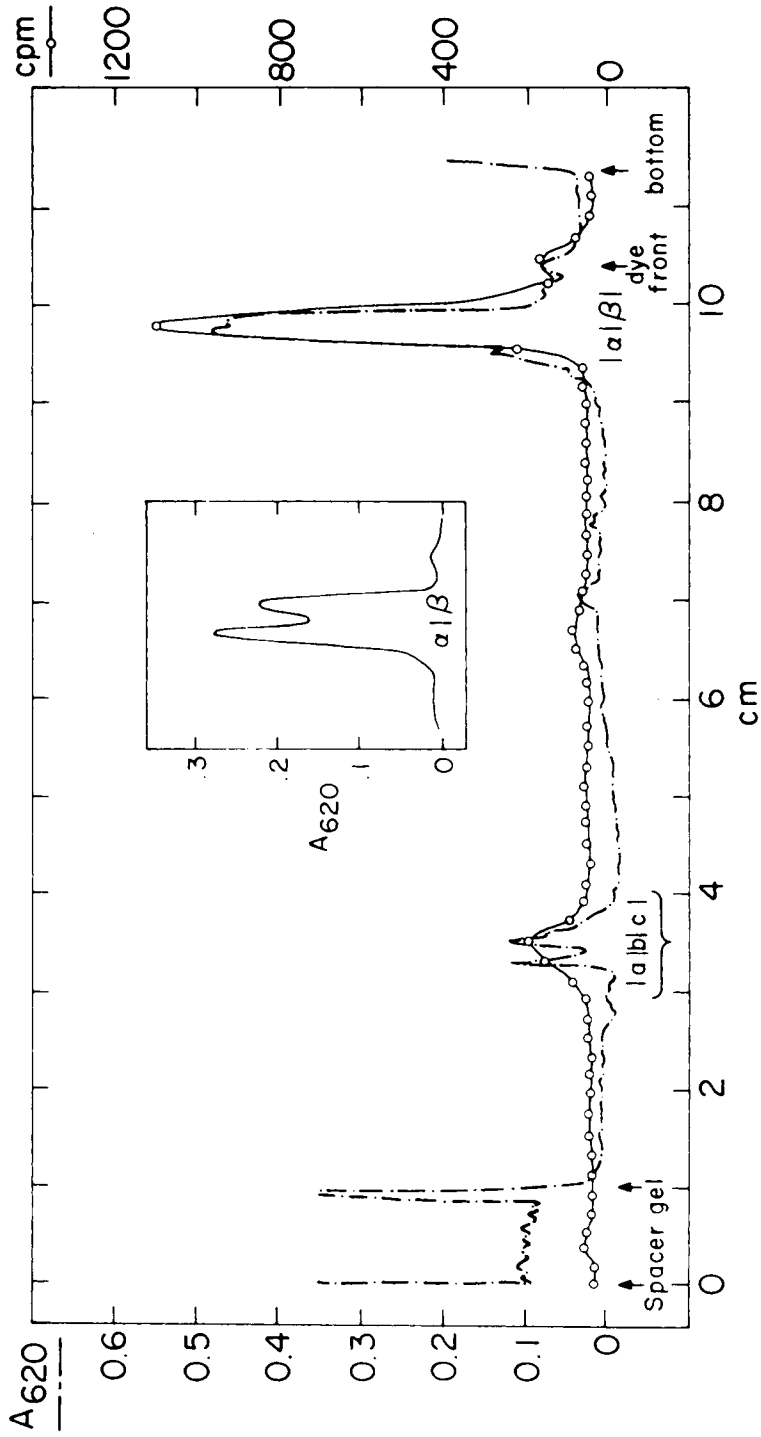

Fig. 3. Five percent polyacrylamide gel electrophoresis pattern of iodinated microtubules (I/D = 0.09). Designations a, b, and c are major contaminants, α and β are the tubulin monomers. Cpm were determined by cutting the gel into 2 mm fractions as described in Materials and Methods. Inset is the pattern of α, β subunits on 7% polyacrylamide gels.

TABLE IV. Separation of Iodinated Proteins by Polyacrylamide Gel Electrophoresis

A. Use of 7% polyacrylamide gels to separate iodinated α and β subunits of tubulin (see inset to Fig. 3). Areas were determined using the Du Pont 310 curve resolver.

Morphology	I/D	area α / area ($\alpha + \beta$)	cpm α / cpm ($\alpha + \beta$)
All abnormal	0.05	55 ± 3%	70 ± 5%
All abnormal	0.09	55 ± 3%	60 ± 2%

B. Use of 5% polyacrylamide gels to separate major contaminants (a, b, c) from tubulin (α, β). See Fig. 3.

Morphology	I/D	cpm/area tubulin / cpm/area total protein
Normal and abnormal	0.02	1.25
All abnormal	0.05	1.31
All abnormal	0.09	1.23
All abnormal	7.7	1.41

(4) Mixtures of all normal and all abnormal tubules are found at very low levels of iodination. This suggests that the iodination reaction may be cooperative. Once a defect has been created by the reaction, subsequent reactions may be facilitated. Alternatively, once a certain number of defects are made, they cause rapid disruption of the normal tubule.

(5) Finally, the iodinated neurotubules morphologically resemble twisted neurofilaments found in aged monkey brain (13). Whether this is a coincidence or represents something of actual biological relevance is not yet known. However, it suggests that iodination affects lateral interactions more than vertical interactions.

Further experiments will be needed if one is to understand both the reason for the extreme sensitivity of the neurotubule to iodination and the origin of the cooperative effects seen. Colchicine, calcium ion, and nucleotide binding studies are in progress to learn if these properties of tubulin and/or the tubule are affected by modification of a small number of tyrosines.

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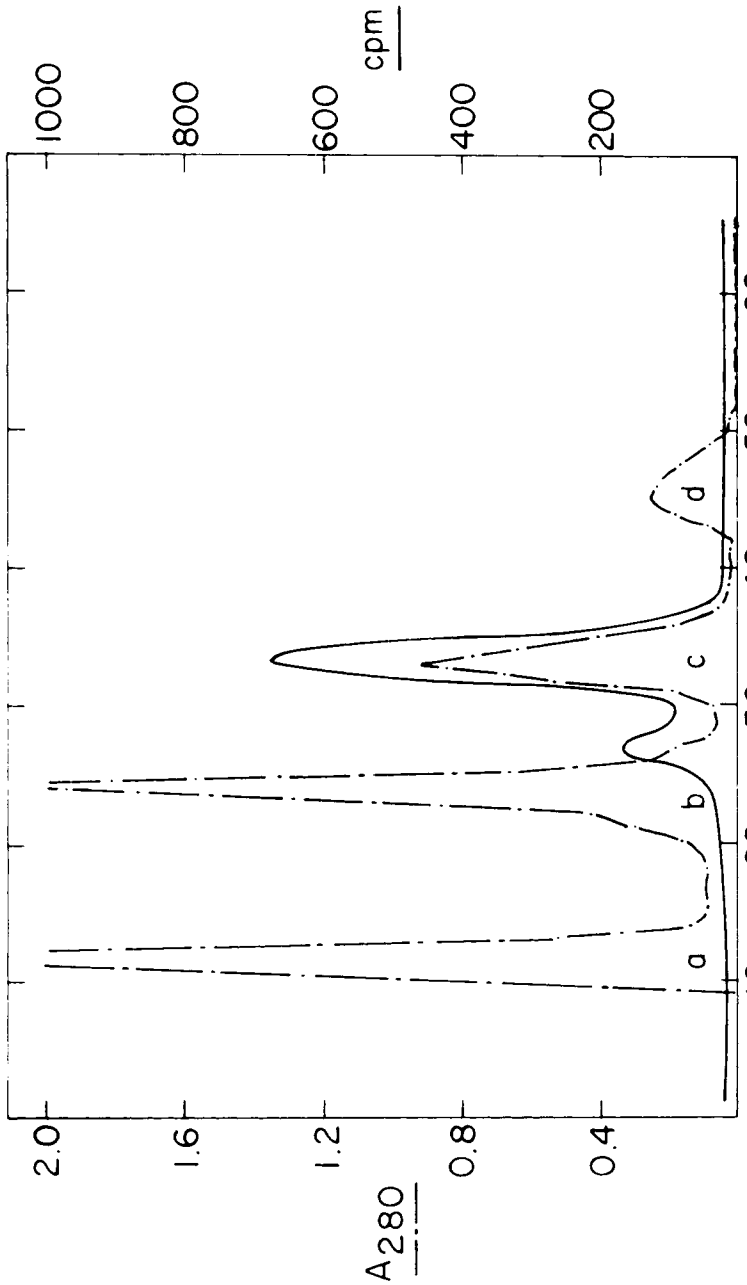

Fig. 4. Elution profile of digested iodinated microtubules (I/D = 0.04) on a Sephadex G-25 Fine column. See Materials and Methods for details. NaI is unstable in acid and forms I_2, which has an appreciable A_{280}. However, the conductivity due to NaI coincides with this A_{280} peak (b), so it is designated as NaI. Peak a is blue dextran; peak c, monoiodotyrosine; and peak d, diiodotyrosine.

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