Study of the 10-nm-filament fraction isolated during the standard microtubule preparation

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The cold non-depolymerizable fractions obtained during the standard procedure for the isolation of microtubules from ox brain stem-cerebral hemispheres and spinal cord have been studied. The cerebral-hemisphere preparation was composed of 10-nm filaments but also contained large amounts of membranes. The polypeptide content included tubulin, microtubule-associated proteins and minor proteins corresponding to the neurofilament triplet of proteins of mol.wt. 210 000, 160 000 and 70 000 respectively. The brain-stem preparation contained more 10-nm filaments than membranes. The polypeptide content consisted of the neurofilament triplet (35%), tubulin (30%) and minor proteins. In contrast, the spinal-cord preparation was mainly composed of 10-nm filaments, free of membranes and containing essentially the neurofilament protein triplet (64%). These filaments appeared very similar to the peripheral-nervous-system neurofilaments described by several authors. Since the best neurofilament preparations from the central nervous system often contained less than 15% of the neurofilament protein triplet, our spinal-cord preparation is an improvement on the usual neurofilament preparation. This simple and rapid method gave large amounts of 10-nm filaments (100 mg per 100 g of spinal cord) characterized by the absence of membranous material, a low content of tubulin and the 50 000-mol.wt.-protein component, and a high content of neurofilament polypeptides. Thus, the presence of tubulin in 10-nm filament preparations seems to be related to the contaminant membranous material and not to be linked to the interaction *in vitro* of tubulin or microtubules with neurofilaments, as has been suggested previously.

Microtubules are found in all eukaryotic cells. They are part of a large number of structures such as mitotic spindles, eukaryotic flagella, cilia and cytoplasmic cytoskeleton (Dustin, 1978).

Neurofilaments are intracellular-protein filaments approx. 10 nm in diameter and of indefinite length. They are prominent structures of the neurons of most animal species, vertebrate and invertebrate (for general review see Shelanski & Liem, 1979). Tubulin, the microtubule subunit and neurofilament proteins co-migrate with the slow wave of axonal transport (Lasek & Hoffman, 1976).

Microtubules are usually prepared from beef or pig brain, as described by Shelanski *et al.* (1973). This method consists of microtubule polymerization

cycles *in vitro* at 37°C and microtubule depolymerization cycles at 4°C. The cold non-depolymerizable fraction obtained during this standard procedure is widely composed of 10-nm filaments (Delacourte *et al.*, 1977; Berkowitz *et al.*, 1977). The polypeptide content of the fraction consists of tubulin (60%) and a protein triplet with mol.wts. 210 000, 160 000 and 70 000 (20%) (Thorpe *et al.*, 1979).

Evidence has been accumulated that this triplet is associated with the structure of neurofilaments (Anderton *et al.*, 1978; Liem *et al.*, 1978; Schlaepfer & Freeman, 1978), and it has been suggested that 10-nm filaments isolated during microtubule preparation are neurofilaments (Thorpe *et al.*, 1979; Runge *et al.*, 1979a,b).

The significance of the large amount of tubulin

Abbreviation used: SDS, sodium dodecyl sulphate.

present in the filament fraction is not clear. It has been suggested that 10-nm filaments (devoid of tubulin) may interact with tubulin and microtubules *in vitro* and that such interactions may also occur *in vivo* as well (Runge *et al.*, 1979a,b).

We now report that 10-nm-filament fractions isolated during microtubule preparation cycles also contain large quantities of membranes. Moreover, it has been reported that microtubule proteins bind strongly to membranous material (Caron & Berlin, 1979).

In addition, the 10-nm-filament preparation protocol applied to spinal cord instead of cerebral hemispheres gives 10-nm-filament preparations devoid of membranes, and with a lower content of tubulin (10% instead of 60%).

Thus, we suggest that tubulin in 10-nm-filament preparation is essentially a membrane-bound material. These results lead to an improvement of central-nervous-system 10-nm-filament preparation. Furthermore, the biochemistry of 10-nm filaments commonly isolated from microtubule preparations should be re-examined, in view of the large amount of membranes present in the preparation.

Experimental

Chemicals were all obtained from Sigma (St. Louis, MO, U.S.A.).

Methods

10-nm filaments were prepared from cerebral hemispheres, the brain stem and the spinal cord of ox. This material was transported packed in ice and either processed immediately or, alternatively, the brain stems and the spinal cords were frozen and stored in the deep freeze until required. Cerebral hemispheres were always processed within 30 min *post mortem*. The method of 10-nm-filament preparation relies on the principle that the cold non-depolymerizable material pelleted during the standard microtubule purification protocol described by Shelanski *et al.* (1973) is largely composed of 10-nm filaments. The isolation of 10-nm filaments is as described by Delacourte *et al.* (1977).

Cerebral hemispheres, brain stems and spinal cords were homogenized in 'Mes buffer' at 4°C (250 g/400 ml). Mes buffer is 0.1 M-Mes (4-morpholine-ethanesulphonic acid) buffer, containing 1 mM-EGTA and 0.5 mM-MgCl₂, adjusted to pH 6.5 with 1.0 M-NaOH, at room temperature. The different materials were homogenized in a 1 litre-capacity Waring Blendor apparatus (ref. 8011 S; Waring Products Corp., New Hartford, CT, U.S.A.) at low speed for 3 × 10 s and at high speed for 3 × 10 s. Each 10 s period was followed by a 15 s delay to

avoid heating. The homogenate was centrifuged for 15 min at 4°C and 29 000 g (r_{av} , 10 cm) and the resulting supernatant at high speed, for 30 min at 4°C and 78 000 g (r_{av} , 7.8 cm). To the high-speed supernatant was added 20% (v/v) glycerol and it was warmed at 37°C for 20 min. The solution was then centrifuged for 45 min at 30°C and 78 000 g (r_{av} , 7.8 cm). The different pellets were combined and homogenized in 60 ml of Mes buffer with a Teflon Potter homogenizer (2.5 cm in diameter, 1500 rev./min) at 4°C for 1 min. The homogenate was centrifuged for 30 min at 4°C and 105 000 g (r_{av} , 5.9 cm). The resulting pellets correspond to the first-cycle 10-nm-filament fraction. The supernatants were pooled and used for a second-cycle 10-nm-filament preparation. The solution was made 20% (v/v) in glycerol, warmed at 37°C for 30 min and centrifuged in six tubes of 10 ml volume for 30 min at 30°C and 105 000 g (r_{av} , 5.9 cm). The sedimented material is the second-cycle 10-nm-filament fraction. It is the neurofilament preparation when dealing with spinal cord as starting material.

Protein estimation. Pellets of the filament preparation were homogenized in a known volume of Mes buffer/0.1% SDS and the protein concentration was determined by the method of Lowry *et al.* (1951), with bovine serum albumin in 0.1% SDS as standard.

SDS/polyacrylamide-gel electrophoresis. Analytical polyacrylamide-gel electrophoresis was performed by the method of Laemmli (1970) and Laemmli & Favre (1973), with acrylamide reticulation gradient from 3 to 15% (Thorpe *et al.*, 1979).

Electron microscopy. The final pellets of the isolation protocols were washed in 0.1 M-sodium cacodylate buffer, pH 7.2, and fixed in 2% (v/v) glutaraldehyde in 0.1 M-cacodylate buffer for 2 h at 4°C. After overnight rinsing in buffer, the pellets were post-fixed with 1% (w/v) OsO₄ in 0.1 M-cacodylate buffer for 1 h at room temperature, rinsed with buffer, dehydrated and embedded in Araldite. Ultrathin sections were treated for 1 min with 1% uranyl acetate in 50% ethanol and for 10 min with lead citrate, by the method of Reynolds (1961), to increase contrast. The sections were examined with a 100 C Jeol electron microscope.

Samples were also examined in negative staining before the centrifugation steps. The sample was applied on 200 mesh grids covered with a formvar film; after rinsing with 10 drops of Mes buffer, one drop of 0.5% uranyl acetate solution in water was applied during 30 s.

Gel-filtration chromatography. This was carried out with Bio-Gel A 50m (100–200 mesh) obtained from Bio-Rad Laboratories. All columns were equilibrated with Mes buffer/0.5 M-NaCl and the samples applied to gel-filtration columns had protein concentrations between 10 and 15 mg/ml.

EXPLANATION OF PLATE 1

Representative electron micrographs of negative stainings and ultrathin sections of 10-nm-filament fractions obtained from three different sources, and analysis of their protein content by SDS/polyacrylamide-gel electrophoresis

The upper photographs show negative stainings and the lower photographs show ultrathin sections. (a) Cerebral-hemisphere preparation. We observe essentially membranes and vesicles on the electron micrographs of ultrathin sections. The inset shows the protein composition of the preparation (mainly α - and β -tubulin and microtubule-associated proteins). (b) Brain-stem preparation. The proportion of 10-nm filaments is increased and we observe few membranes. On SDS/polyacrylamide-gel electrophoresis, the neurofilament triplet represents 40% and the microtubule proteins 30% of the overall composition. (c) Spinal-cord preparation, showing 10-nm filaments free of membranes and containing essentially the neurofilament triplet proteins. The negative stainings (upper parts of a, b and c) of the three preparations present the same aspects, i.e. long flexible unbranched 10-nm filaments, and no membranous material was detected. Gel bands are identified by their molecular weights ($\times 10^{-3}$). Bars represent 100 nm. Abbreviations used: Tu, tubulin; MAPs, microtubule-associated proteins.

EXPLANATION OF PLATE 2

Purification of 10-nm filaments by gel filtration

An ox brain homogenate supernatant was fractionated on a column (2.5 cm × 85 cm) containing Bio-Gel A 50m and equilibrated in Mes buffer plus 0.5 M-NaCl to dissociate the associated forms of tubulin at 4°C (rings 36S and short protofilaments). Elution (12 ml/h) was followed by protein determination (Lowry *et al.*, 1951). Gel filtration was carried out at 4°C and the 10-nm filaments were located in the exclusion peak, as demonstrated by Berkowitz *et al.* (1977). This fraction has the following protein content: microtubule-associated proteins (mol.wt. 300 000), the neurofilament proteins (mol.wts. 210 000, 160 000 and 70 000), tubulin (mol.wts. 57 000 and 53 000) and actin (mol.wt. 43 000). 10-nm filaments of the exclusion peak were pelleted and fixed. Ultrathin sections were observed by electron microscopy, and showed the presence of large amounts of membranes. The bar represents 100 nm.

Results

Microtubules are usually prepared from the whole brain or from cerebral hemispheres that are rich in grey matter (Shelanski *et al.*, 1973). But neurofilaments are specially abundant in white matter, and central-nervous-system neurofilaments are generally prepared from the brain stem (Yen *et al.*, 1976; Thorpe *et al.*, 1979).

With that in mind, we have compared the 10-nm-filament fraction of the microtubule preparation protocol (Delacourte *et al.*, 1977; Berkowitz *et al.*, 1977) from three different sources more or less rich in neurofilaments: cerebral hemispheres, brain stem and spinal cord of ox.

Sections of these 10-nm-filament preparations were observed by electron microscopy. Representative electron micrographs of the different preparations are shown in Plate 1.

The material isolated from cerebral hemispheres is mainly composed of membranes and vesicles (Plate 1a). In SDS/polyacrylamide-gel electrophoresis, tubulin and microtubule-associated proteins are the prominent polypeptides (60%) and the neurofilament triplet is also present (10%) (Plate 1a, inset).

With the brain stem, a marked change was observed in the filament preparation. 10-nm filaments are easily discernible and membranes are only minor components (Plate 1b). Simultaneously, we observed on SDS/polyacrylamide-gel electrophoresis a decrease in the proportion of tubulin and microtubule-associated proteins (30%), and a large increase in the triplet protein content (approx. 40% of the overall composition) (Plate 1b, inset).

Working with the spinal cord, a filament preparation was obtained free of membranes (Plate 1c). The triplet proteins represent 64% of the overall composition; the other proteins are tubulin (10.5%) and a double band of a 50000-mol.wt. component. We obtained, at the first and second cycle of preparation, 100 mg of neurofilaments per 100 g of spinal cord.

With the cerebral hemispheres and the brain stem, the filament pellet was opalescent. With the spinal cord, it was translucent in the first cycle and crystalline in the second cycle.

10-nm-filament preparations were also observed by negative staining. In all cases, 10-nm filaments possess the same morphological aspects of flexible unbranched filaments with a 10-nm diameter and of indefinite length. Filaments from the spinal-cord preparation are presented in Plate 1.

Study of 10-nm filaments purified by a further gel-filtration step

It was shown that 10-nm-filament fractions isolated during microtubule depolymerization cycles and further purified by a gel-filtration step were

associated with a protein composition of tubulin, microtubule-associated proteins and the neurofilament triplet proteins (Berkowitz *et al.*, 1977). Thus, it could be that tubulin is strongly attached to 10-nm filaments, or is part of their structure. Another possibility is that 10-nm-filament fractions are still contaminated with membrane-bound tubulin.

We isolated 10-nm filaments from brain homogenate supernatant and from cold non-depolymerizable fraction of the first microtubule cycle in the exclusion volume of a Bio-Gel A 50M gel-filtration column; on electron microscopy, we detected, in the two samples, large quantities of membranous material (Plate 2).

Thus, separation of 10-nm filaments from membranous contaminants is not improved by the gel filtration, and it is possible that 10-nm-filament fractions obtained by this technique were contaminated by membrane-bound tubulin.

Attempts were made to separate these components by centrifugation in discontinuous sucrose gradients and by a specific solubilization of the membranes with non-ionic detergents (Triton X-100 and Nonidet P40), but both attempts were unsuccessful.

Conclusion

The cold non-depolymerizable material pelleted during the depolymerization cycles of the microtubule preparation protocol contains large amounts of 10-nm filaments (Delacourte *et al.*, 1977; Berkowitz *et al.*, 1977; Runge *et al.*, 1979a,b). To obtain a better appreciation of the homogeneity and purity of 10-nm-filament preparations, we observed ultra-thin sections of the pelleted filaments. Furthermore, preparations were made from three different sources: the cerebral hemispheres, the brain stem and the spinal cord of ox.

The cerebral-hemisphere filament preparation contains a large quantity of membranous materials. Its polypeptide content is mainly tubulin and microtubule-associated proteins. Contribution to the overall composition is also made by a neurofilament protein triplet with mol.wts. 210000, 160000 and 70000 as demonstrated by Thorpe *et al.* (1979). The brain-stem filament preparation contained a greater amount of 10-nm filaments than membranes. The polypeptide content consisted mainly of the neurofilament triplet proteins (40%) and tubulin (30%). In contrast, the spinal-cord preparation was mainly composed of 10-nm filaments free of membranes and contained principally the neurofilament triplet (64%).

Thus, a progressive disappearance of the membranous material from the cerebral hemisphere to the spinal-cord-filament preparations was correlated with a decrease of tubulin and microtubule-asso-

ciated proteins in the preparations. Since it has been demonstrated that microtubule proteins bind strongly to membranes (Caron & Berlin, 1979), the tubulin present in the filament preparation appears to represent membrane-bound material.

It is noteworthy that the 50000-mol.wt. protein is the major component of the central-nervous-system 10-nm-filament preparation described previously (Yen *et al.*, 1976; Thorpe *et al.*, 1979), whereas it is a minor one in our preparation (<9%).

The origin of the 50000-mol.wt. component is not clear, since it may be of a glial contaminant (Bignami & Dahl, 1977) or a proteolytic product of the 70000-mol.wt. component (Thorpe *et al.*, 1979). In the latter case, the small amount of this protein could be explained by the fact that our preparation is processed in a very short time (less than 8 h), thus avoiding proteolytic degradation.

Since our spinal-cord 10-nm-filament preparation is essentially composed of the neurofilament triplet proteins, and the 10-nm filaments appear similar to neurofilaments described by several authors previously (Anderton *et al.*, 1978; Schlaepfer, 1977; Schlaepfer & Freeman, 1978; Liem *et al.*, 1978), it is very likely that our spinal-cord preparation is mainly composed of neurofilaments.

This simple and rapid method gives high and pure concentrations of 10-nm filaments (100 mg/100 g of spinal cord) characterized by the absence of membranous material, a low content of tubulin and 50000-mol.wt. protein component and a high content of neurofilament polypeptides. The procedure therefore appears to be an improvement on previous methods for the neurofilament preparation. In addition, ox spinal cord is readily available in slaughterhouses and can be stored at -20°C without any modification of the preparation viability.

One other aspect demonstrated is that 10-nm filaments isolated from the non-depolymerizable material of the microtubule preparation and purified by a further gel-filtration step still contain large amounts of membranes. This contamination was not detected by authors who monitored the preparations by negative staining (Delacourte *et al.*, 1977; Berkowitz *et al.*, 1977; Runge *et al.*, 1979a,b). In our experiments, the amounts of membranes in the preparations seemed to be lower when observed by negative staining than when studied by ultrathin section of fixed pellets, perhaps because membranous material does not bind efficiently to the grid. Thus it is likely that the contamination, when evaluated by negative staining, is markedly underestimated. In addition, membranes possess a low content of proteins and their presence is not revealed by SDS/polyacrylamide-gel electrophoresis after a colouration with Coomassie Brilliant Blue R.

We know from experience that 10-nm-filament pellets are opalescent when membranes are present

and crystalline in absence of membranous material. The contamination of 10-nm filaments may be explained by their similar behaviour during the centrifugation and gel-filtration steps. These two components have probably the same sedimentation coefficient distribution and a comparable apparent molecular weight.

In our experiments, the improvement of neurofilament preparation is affected probably by the choice of spinal cord as a source for the preparation; cerebral hemispheres contain mainly cell bodies, whereas the spinal cord contains a majority of axons and, in consequence, more neurofilaments. Accordingly, the presence of tubulin in the preparations seems to be related to the presence of membranous contaminants, and not due to the interaction *in vitro* between tubulin or microtubules and neurofilaments.

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