Properties of highly viscous gels formed by neurofilaments *in vitro*

A possible consequence of a specific inter-filament cross-bridging

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Neurofilaments freshly isolated from bovine spinal cord form a reversible gel *in vitro*, consisting of nearly parallel and interlinked filaments organized in bundles. This phenomenon is obtained above a critical neurofilament concentration and is highly sensitive to denaturation. No gelation occurs with neurofilaments reconstituted from urea-solubilized subunits. The velocity of the gelation kinetics, optimum at a slightly acidic pH, is inhibited by low and high ionic strength and activated by millimolar concentrations of Mg^{2+} and other bivalent cations. No protein other than the purified neurofilament preparation itself (80–95% neurofilament triplet) is necessary for the formation of a gel. However, purified cytoskeletal proteins from microtubules and neurofilaments influence the viscosity of the native preparation. These observations suggest a reticulation *in vitro* between neurofilaments, dependent upon a fragile conformation of the polymers and possibly mediated through the high- M_r neurofilament subunits (200 kDa and 150 kDa). The significance of these results is discussed with regard to the inter-neurofilament cross-bridging *in situ* involving the 200 kDa subunit described by Hirokawa, Glicksman & Willard [(1984) *J. Cell Biol.* **98**, 1523–1536].

INTRODUCTION

Neurofilaments constitute a major component of the specialized cytoskeleton in mature neurons. They are cross-linked together, with microtubules and the membranes of organelles, through a dense microtrabecular network [1–4]. Although the precise physiological function of neurofilaments remains unclear, several reports demonstrate a close relationship between the axonal diameter and the neurofilament content [5,6]. Neurofilaments are slowly transported as a coherent network of fully polymerized structures [7]. Two separated pools of relatively stationary and moving filaments coexist in the axon [8] and the velocity of the neurofilament flow is modified during nerve growth and regeneration [9,10]. These observations suggest a regulatory mechanism of the neurofilament motion along the axon involving their lateral interactions and possibly modulated by phosphorylation [11]. Investigations on the connections between isolated axoskeleton structures were essentially focused for the last decade on microtubule-neurofilament interactions [12–16]. If the possibility of a direct linkage between neurofilaments *in situ* was repeatedly suggested, little information exists in the literature on the neurofilament cross-bridging potentiality *in vitro*. The present work describes the reversible formation *in vitro* of a highly viscous gel in freshly isolated bovine spinal-cord neurofilament preparations. The physicochemical studies and the examination of the spatial organization of the gels indicate that a reticulation *in vitro* between individual neurofilaments occurs and may be mediated via their high- M_r subunits.

MATERIALS AND METHODS

Preparation of neurofilaments, gliofilaments, reconstituted neurofilaments and purified high- M_r neurofilament subunits

Neurofilaments from bovine spinal cord were purified by a modification of the procedure described by Delacourte *et al.* [17]. The fresh tissue was homogenized in 1:1 (w/v) ratio with ice-cold buffer A (1 mM- $MgCl_2$ /1 mM-EGTA/0.1 M-Mes adjusted to pH 6.8 with 5 M-NaOH) in a Waring blender (3 × 10 s at maximal speed). The homogenate was centrifuged at 78000 *g* for 1 h (Kontron rotor 50, r_{av} 5.4 cm) at 4 °C. The supernatant was made 4 M with respect to glycerol and kept for 2 h at 4 °C before centrifugation at 78000 *g* for 1 h (Kontron rotor 50) at 4 °C. The first pellet was gently resuspended in buffer A (one-tenth of the homogenization volume) and was further centrifuged at 230000 *g* for 3 h on a discontinuous sucrose layer (5 ml of 1.5 M-sucrose and 10 ml of 0.9 M-sucrose, both in buffer A). The final pellet of purified neurofilaments was resuspended in buffer A containing 1.2 M-sucrose in a Teflon/glass homogenizer. Samples (6 mg/ml) were stored at –80 °C and used within a week.

Gliofilaments were extracted from the membrane pellet obtained by centrifugation of the spinal-cord homogenate described above. The upper white layer of the pellet was resuspended (1:5, w/v) in buffer A containing 0.1 M-NaCl and 1% (v/v) Triton X-100. After a vigorous homogenization (Ultra-Turrax, maximum speed 4 × 30 s), the suspension was layered on a 1 M-sucrose cushion (10 ml/tube) and centrifuged at

Abbreviation used: MAPs, microtubule-associated proteins.

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100000 *g* for 2 h (Kontron rotor 50). The pellets of purified gliofilaments sedimented through the sucrose layer were resuspended in buffer A containing 1 M-sucrose and stored at -80°C .

Neurofilaments were solubilized in urea buffer [10 mM-potassium phosphate buffer, pH 7.0, containing 1% (v/v) 2-mercaptoethanol, 8 M-urea (from a freshly deionized solution) and 1 mM-phenylmethanesulphonyl fluoride], in accordance with Liem & Hutchison [18], and cleared from insoluble aggregates by centrifugation at 300000 *g* for 1 h (Kontron rotor 65 Ti). Intermediate-filament-like polymers were reconstituted by dialysis of urea-solubilized neurofilaments against buffer A containing 1 M-sucrose and 1 mM-phenylmethanesulphonyl fluoride.

The 200 kDa and 150 kDa subunits were purified by the procedure of Liem & Hutchison [18] after the two subunits had been separated from other components by chromatography of the urea-solubilized filaments on Ultrogel AcA 22 in accordance with Chin & Norton [19]. The pure proteins were further simultaneously renatured and concentrated by dialysis under vacuum against buffer A containing 1 M-sucrose and 1 mM-phenyl-

methanesulphonyl fluoride. Concentrated samples (1 mg/ml) were stored at -80°C .

Preparation of microtubule proteins

Microtubules from rat brain were obtained by the two-cycle polymerization method of Shelanski *et al.* [20]. Purified tubulin and MAPs (0.35 M-NaCl elution fraction) were obtained by chromatography of the freshly depolymerized microtubules on phosphocellulose in accordance with Weingarten *et al.* [21].

Measurement of the viscosity

The viscosity kinetics were performed at 22°C in the falling-ball apparatus described by MacLean-Fletcher & Pollard [22]. Routinely, 10 cm-long glass tubes (internal diam. 2 mm) sealed with Parafilm (American Can Co.) were filled with 0.5 ml of the neurofilament samples, and the sedimentation velocity of a 1.5 mm-diameter stainless-steel ball (SKF) was measured over a 5 cm distance with angles of 20° , 45° or 80° over the horizontal line. The relative viscosity was obtained by comparison with the sedimentation velocity of the ball under the same conditions through glycerol at various concentrations.

Fig. 1. Purification pattern of neurofilaments

SDS/7.5%-polyacrylamide-gel electrophoresis of the successive steps of a standard neurofilament preparation from spinal cord (lanes 1–6), and of the purified gliofilaments (lane 7, 10 μg of proteins). Lane 1, cytosol obtained by centrifugation of the homogenate, containing the neurofilament triplet. Lane 2, second supernatant obtained after the removal of neurofilaments by centrifugation from the cytosol incubation at 4°C with 4 M-glycerol. Lane 3, crude neurofilament pellet. Lanes 4–6, fractions obtained by the purification of the resuspended crude neurofilaments by high-speed centrifugation on a discontinuous sucrose gradient: lane 4, upper layer containing soluble proteins and membranes (at the interface with the sucrose cushion) contaminating the crude filaments; lane 5, sucrose cushion; lane 6, purified neurofilament pellet. A 20 μl portion from each fraction was mixed with the same volume of solubilization buffer, and 20 μl of each mixture was analysed. Arrows indicate the positions in the gel of the three neurofilament subunits (apparent molecular masses 200 kDa, 150 kDa and 68 kDa), the α - and β -tubulins (T) and the gliofilament subunit (GF).

Fig. 2. Simultaneous viscosity and turbidity measurements during the gelation *in vitro* of purified neurofilaments

(a) Gelation kinetics of the purified filament pellet resuspended in buffer A (which contains 1 mM-MgCl₂) containing 1.2 M-sucrose, and immediately assayed for their viscosity at 22 °C. Neurofilaments (5.3 mg/ml) were tested alone (○) and in the presence of either 20 mM-potassium phosphate (▲) or 300 mM-KCl (●). The viscosity of gliofilaments (3 mg/ml) resuspended in buffer A containing 1 M-sucrose was also recorded (■). After 6 h all samples were removed, vigorously agitated and re-introduced into the capillary tube before a second incubation (arrow). Insets: a sample of neurofilaments (30 μg) was solubilized in denaturing buffer and analysed by SDS/10% -polyacrylamide-gel electrophoresis before the experiment (left inset) and after 8 h at 22 °C and two gelation kinetics (right inset). (b) Turbidity of the neurofilament suspension (5 mg/ml in buffer A containing 1 M-sucrose) during a gelation kinetic. The absorbance at 350 nm decreased slightly during the first hour of incubation at 22 °C ($\Delta A_{350} = 0.05$), and remained unchanged for the next 3 h while the gel was obtained.

The gel state was defined as being that in which the ball does not fall with an angle of 80°.

Polarized-light microscopy

The formation of gels in the neurofilament preparation was observed by polarized-light microscopy (Zeiss Universal microscope) with Normarski interferential contrast optics (Zeiss), with 40× NA 0.65 and 63× Planachromat NA 1.4 objectives immersed in oil. A drop of the neurofilament suspension (200 μl) was introduced by capillarity between the microscope object slides 1 mm apart and sealed with paraffin.

Electron microscopy

The neurofilament samples (2 mg/ml) after gelation *in vitro* were fixed with 1% (v/v) glutaraldehyde/0.2% tannic acid. To avoid any disorganization of the fragile gel, an equal volume of 2% glutaraldehyde and 0.4% tannic acid in buffer A (containing 1 M-sucrose) was layered on the gelled neurofilaments and kept overnight at 4 °C. Control neurofilaments were mixed immediately after thawing with an equal volume of the fixation buffer and kept under the same conditions. These preparations were further post-fixed with 1% OsO₄ in buffer A, dehydrated in ethanol after staining *en bloc* with aq. 1% uranyl acetate, and embedded in Spurr resin. Ultrathin sections were post-stained with uranyl acetate and lead citrate and examined in a Philips EM 300 electron microscope.

Miscellaneous

Protein measurements were made by the method of Lowry *et al.* [23], with bovine serum albumin as standard.

SDS/polyacrylamide-gel electrophoresis was performed by the method of Laemmli [24] with a 10 cm × 10 cm slab-gel apparatus (1.5 mm width). Protein samples were denatured by boiling in the presence of 1% SDS in 0.12 M-Tris/HCl buffer, pH 6.8, containing 1% 2-mercaptoethanol before being loaded on the gel. Polypeptides were detected by Coomassie Brilliant Blue R250 staining [0.1% in 10% (v/v) acetic acid/50% (v/v) methanol] and destained in 10% acetic acid/10% methanol.

Chemicals

Mes and phenylmethanesulphonyl fluoride were from Sigma Chemical Co. Urea (99.5% pure) was from LKB. Usual chemical reactants were from Merck. Ultrogel AcA 22 was from LKB. Phosphocellulose P11 was from Whatman. The 8 M-urea stock solutions were deionized on AG 50/1-X8 (D) mixed-bed resin (Bio-Rad Laboratories).

RESULTS

Formation of gels in purified neurofilament preparations

The crude neurofilament pellet obtained after the sedimentation of the soluble extract incubated at 4 °C

Fig. 3. Birefringent structures in gelled neurofilaments observed by polarized-light microscopy

Neurofilaments (5 mg/ml) in buffer A containing 1 M-sucrose were examined during the course of a gelation kinetic. Birefringent domains appeared within 10–15 min after the initiation of the reaction by temperature (22 °C). Very long bundles (over 100 μm) were frequently found in semi-parallel arrays overlapping each other. Unoriented aggregates were also present (top and bottom left of the Figure). The size and length of the birefringent formations slowly increased with time and remained stable for several hours. The bar represents 30 μm .

with 4 M-glycerol is constituted at 70% neurofilament triplet and several contaminating proteins as assessed by electrophoretic analysis on SDS/polyacrylamide gel (Fig. 1, lane 3).

During the further purification step, the neurofilaments were found both in the 0.9 M- and 1.5 M-sucrose layers and as a pellet (Fig. 1, lanes 4, 5 and 6). About 40% of the total purified filaments was systematically recovered in the sucrose fraction, suggesting the existence of two sedimentation states of the polymers. The final preparation was constituted of 80–90% neurofilament subunits, a few contaminating MAPs (0.5%), variable amounts of gliofilaments (5–10%) and several polypeptides in the 50–70 kDa molecular-mass range. No or very little tubulin was found associated with the purified filaments. When kept at 22 °C, both the crude preparation and the purified neurofilaments became highly gelatinous with time (Fig. 2). The viscosity of the purified neurofilaments increased rapidly during the first hour of incubation, after which a gel was obtained (Fig. 2a). No solation of the gel occurred when the sample was further maintained at 4 °C. Nevertheless, the velocity of the viscosity change was partially temperature-dependent: a much slower but significant kinetic occurs at 4 °C, and a gel was formed only after an overnight incubation (results not shown). The gel obtained *in vitro* is labile: when the gelled sample was sheared by vortex agitation, the viscosity returned to its basal level (Fig. 2a, arrow) and a second gelation kinetic was recorded. The alternation of gel formation and disruption by shearing could be repeated several times, with a significant slowing down of the velocity after three cycles (results not shown).

In contrast, purified gliofilaments incubated under the

same conditions never undergo any viscosity change (Fig. 2a, $\blacktriangle$ symbols). No modification of the composition of the sample was observed during the gelation of neurofilaments (Fig. 2a insets).

The formation of a gel suggests a physicochemical modification of the filamentous suspension. This cannot be attributed to an equilibrium between the free subunits and the polymers: the turbidity of the sample (measured by the absorbance at 350 nm) was recorded during a gelation kinetic in which the relative viscosity changed from 0.01 Pa·s (10 centipoise) to over 5 Pa·s (gel) (Fig. 2b). No simultaneous change of the turbidity occurred, reflecting the constant mass of the total polymers of the preparation. Instead, a small decrease (10%) of the absorbance was constantly found within the first half-hour of the kinetic. Moreover, the neurofilaments are never in a depolymerized form (free subunits) in the buffer used, and can be completely sedimented by centrifugation at any moment of the gelation process (results not shown).

Morphology of the gelled neurofilaments

Given the structural stability of neurofilaments *in vitro*, the formation of a gel in freshly purified preparations could result from a rearrangement of individual filaments into a reticulated network. This assumption was supported by the morphological examination of the gelled sample. In Fig. 3 is shown the polarized-light-microscopy observation of a neurofilament preparation under the optimal gelation conditions. Large birefringent bundles appeared rapidly (within 15 min) after the deposit of the drop between the slides, and slowly increased in size and length with time.

Electron-microscopy observation of the gelled neuro-

Fig. 4. Electron-microscopic observation of the control (a) and the gelled [(b) and (c)] neurofilaments

The initial suspension (2 mg/ml) consisted of a homogeneous filament population randomly distributed

Fig. 5. Protein-concentration-dependent viscosity of native and reconstituted neurofilaments

The viscosity of freshly purified filaments at increasing concentrations (in buffer A containing 1 M-sucrose) was measured after 6 h at 22 °C (●). An identical preparation was solubilized in 6 M-urea buffer and reassembled by dialysis against buffer A containing 1 M-sucrose before the assay (▲).

filament sample showed that the bundles were constituted of many densely packed and nearly parallel filaments (Fig. 4b). Such a spatial organization was never found in the control preparation (non-gelled neurofilaments), where individual filaments have a random orientation (Fig. 4a). Under higher magnification, tiny fuzzy cross-bridges were found between neurofilaments of the gelled sample, originating from periodic discontinuities along the filament axis (Fig. 4c).

Physicochemical properties of the neurofilament gelation

A non-linear relationship between the viscosity and the neurofilament concentration was obtained with a critical concentration between 1.5 and 2.0 mg of protein/ml. Below this value the gel was never obtained, and above it the velocity of the gelation kinetics was directly proportional to the amount of neurofilaments (Fig. 5). In contrast, neurofilaments solubilized in 6 M-urea and reconstituted by dialysis against buffer A have totally lost the gelation capacity when tested under the same conditions (Fig. 5, ▲ symbols). A similar result was obtained when the native preparation was submitted to

throughout the sample [(a); the bar represents 0.25 μ m]. In the gelled preparation, dense aggregates of approximately parallel neurofilaments were present in addition to the dispersed pattern shown in (a) [(b); the bar represents 0.25 μ m]. Empty spaces were frequently observed surrounding these structures, resembling the birefringent bundles in Fig. 3. Higher magnification of the gelled sample showed occasional fuzzy cross-bridges between adjacent neurofilaments (arrows) [(c); the bar represents 0.1 μ m].

Table 1. Physicochemical conditions for the formation of a gel in neurofilament preparations

Assay conditions . . .	Temperature	pH	Low-ionic-strength buffers	NaCl, KCl	Sodium phosphate, potassium phosphate	Bivalent cations
Influence on neurofilament gelation	Slow gelation at 4 °C; maximum velocity between 20 and 35 °C; no dissociation at 4 °C of the preformed gel	Maximum velocity of the viscosity kinetics between pH 6.5 and pH 6.8	No gelation; disruption of the 10 nm-filament (pellets in 10 mM-Tris, pH 7.0)	No effect at 10–20 mM; inhibition above 100 mM	No effect under 10 mM; inhibition at 20 mM and above	Strong activation with $Mg^{2+} > Mn^{2+} > Ca^{2+}$; precipitation with Zn^{2+}
Assay conditions . . .	Mercaptoethanol	Glycerol, sucrose	Antimitotic drugs	Acrylamide	Proteolytic enzymes	Protein denaturation
Influence on neurofilament gelation	No effect at 10 mM (induction of linear kinetics)	Protection of the gelation capacity upon storage (–80 °C) above 0.2 M	No effect of 10 μM -colchicine or 10 μM -podophyllotoxin	Inhibition of the gelation: 50% inhibition between 50 and 100 mM	No gelation with α -chymotrypsin or trypsin (1:10, w/w) with 5 min boiling, sonication, or several cycles of freezing–thawing	Progressive and rapid loss of the gelation capacity with 5 min boiling, sonication, or several cycles of freezing–thawing

various denaturing conditions and to low-ionic-strength buffers, where they were disrupted into ball-like aggregates [25] (Table 1). These observations show that the formation of a gel depends upon a fragile conformation of the native neurofilament structure. The addition of either 1 M-sucrose or 2 M-glycerol allowed the long-term conservation of the preparations, which were still active after several weeks at –80 °C.

Investigation of the effect of ions revealed an inhibition of the gelation in the presence of 20 mM-potassium phosphate or -sodium phosphate and 300 mM-NaCl or -KCl, and was more pronounced in the course of the second kinetic recorded after shearing (Fig. 2a). In contrast, a strong activation was found with Mg^{2+} and other bivalent cations. The dependence of the viscosity kinetics upon the presence of Mg^{2+} ions was higher with the higher purity of neurofilaments (Fig. 6). Moreover, homogenization of the tissue in buffer A with $MgCl_2$ omitted did not affect the yield of neurofilaments recovered in the crude fraction (15 mg of protein in the pellet per 100 mg of total soluble proteins were routinely obtained). The critical neurofilament concentration necessary for the formation of a gel was not modified between 0 and 10 mM- $MgCl_2$ or - $CaCl_2$ (results not shown). The cations influenced only the velocity of the phenomenon (Fig. 6b).

Influence of cytoskeletal proteins on the viscosity of neurofilaments

To explore the molecular basis of the reticulation *in vitro* of the neurofilament suspension, the effect of several proteins on the viscosity of the purified preparations was investigated. Gliofilaments did not influence the phenomenon in the concentration range tested (Fig. 7). In contrast, MAPs, known to bind on a specific site on the neurofilament core [26], strongly inhibited the viscosity of the preparation. Tubulin, which cannot polymerize into microtubules under the experimental conditions used (absence of GTP), evoked an increase in the viscosity of the neurofilaments, with a maximum effect at 0.15 mg of tubulin/ml. With higher concentrations, the viscosity returned progressively to the control value, and an inhibition was obtained above 1–1.5 mg of tubulin/ml (results not shown).

In addition, the gel formed by neurofilaments *in vitro* was profoundly affected by isolated neurofilament subunits (Fig. 8). The reconstituted neurofilaments, which have lost their gelation ability (Fig. 5), lowered the viscosity of the native preparation in a linear concentration-dependent relationship. A strong inhibition of the reaction was obtained by the addition of the purified high- M_r neurofilament subunits, isolated from the urea-solubilized filaments. The 200 kDa subunit lowered the viscosity of the preparation considerably at concentrations as low as 0.1 mg/ml. The purified 150 kDa molecule influenced the process above a concentration of 0.1–0.2 mg/ml and lowered the viscosity significantly only at 0.3 mg/ml. This suggests that the effects of the two proteins examined are distinct from each other.

DISCUSSION

This work was initiated from the observation that the neurofilament sedimentation behaviour changed dramatically during the purification procedure between the centrifugation of the homogenate (where the neuro-

Fig. 6. Activation of the gelation kinetics by Mg^{2+} ions

(a) Viscosity kinetics of the crude neurofilament fraction (10 mg/ml) in buffer A devoid of $MgCl_2$ (prepared in the absence of bivalent cation). A linear kinetic was recorded in the absence of added $MgCl_2$ (●). $MgCl_2$ (5 mM) strongly activated the velocity (▲). The gel was obtained in both samples after several hours of incubation at 22 °C. (b) Relationship between the viscosity of purified neurofilaments (2.6 mg/ml) and the concentration of $MgCl_2$. Measurements were made after 6 h at 22 °C. A very small but significant viscosity change occurred in the sample devoid of $MgCl_2$.

filaments are recovered in the soluble fraction) and the second centrifugation of the soluble extract incubated at 4 °C with glycerol (yielding neurofilaments in the pellet). Under the experimental conditions adopted, no microtubule polymerization occurs, and the selective sedimentation of neurofilaments could result from their association in large aggregates. The gelation process described here could explain this phenomenon. However, the fact that the purification of neurofilaments is independent of added Mg^{2+} suggests that other factors from the tissue extract might affect their interactions. Such a possibility is further supported by the finding that associated proteins in the crude filament pellet influence the gelation kinetics of the pure neurofilaments in a concentration-dependent manner (J.-F. Leterrier & J. Eyer, unpublished work).

The gel obtained with pure neurofilaments exhibits several characteristics in sharp contrast with the properties of the neurofilament-induced microtubule gels described elsewhere that consist of an antimetabolic-drug- and cold-sensitive network strictly dependent upon the polymerization state of microtubules [12–16,27,28]. Thus the bivalent-cation-activated neurofilament reticulation process represents a novel intrinsic property *in vitro* of these specialized intermediate filaments. The gelation seems to be specific for neurofilaments (no viscosity change in gliofilaments purified from the same tissue) and does not require any essential molecule other than the purified preparation itself. An explanation of the activation of the neurofilament viscosity by low tubulin concentrations in the absence of GTP (Fig. 7) may be found in the demonstration that tubulin strongly binds to neurofilaments *in vitro* in a cold- and colchicine-insensitive manner [27]. Such a stable binding, independent of the polymerization mechanism, could mediate the cross-linkage between adjacent filaments at low tubulin concentration, thus increasing the extent of

reticulation. In contrast, the inhibitory effect of MAPs raises the possibility that their binding site on neurofilaments [13], shown recently to be restricted to the core of the polymer [26], could be involved in an inter-filament association.

The gelation is a labile phenomenon (Fig. 5 and Table 1). In our hands, the most critical condition for obtaining a reversible gel was the preservation of the native state of the polymers. The neurofilament interaction is sensitive to low and high ionic strength, which profoundly influence their conformation as shown by Carden & Eagles [25], who also described the formation of a gel in nearly native neurofilament preparations. That the establishment of a network between these stable structures (no equilibrium between subunits and polymers *in vitro*) is very sensitive to the various denaturing conditions assayed recalls the similar lability of the neurofilament-induced tubulin polymerization [27], which activity is essentially borne by the 200 kDa subunit [15]. This instability could be related to a fragile conformation of the lateral domains of the high- M_r subunits [29–31], whose arm-like projections could possibly extend freely in the surrounding space of the neurofilament axis (allowing interactions with other organelles) or stick to the same axis, depending on the physicochemical environment. An analogy with the microtubule packing volume, which depends partially on the high- M_r MAPs exclusion zone [32], is likely, since large parts of MAP2 and of the 200 kDa and 150 kDa neurofilament molecules are similarly accessible to mild proteolysis, releasing large soluble polypeptides from the polymers [33,34]. Moreover, the hypothesis that the high- M_r neurofilament subunits might share some common ubiquitous determinants with high- M_r MAPs has already received support from published data [15,35,36].

To the gelation process there corresponds a reorganiz-

Fig. 7. Influence of cytoskeletal proteins on the viscosity of neurofilaments

Purified neurofilaments (2.9 mg/ml) were incubated with increasing concentrations of gliofilaments (■), MAPs (●) and tubulin (▲). No GTP or other nucleotide was present. All samples were in buffer A but with 5 mM-MgCl₂ and containing 1 M-sucrose. The viscosity, measured after 6 h at 22 °C, was expressed as a percentage of the control value (neurofilaments alone). Inset: SDS/7.5%-polyacrylamide-gel electrophoresis of 30 µg of tubulin (lane 1), 5 µg of MAPs (0.35 M-NaCl fraction from the phosphocellulose chromatography of microtubules) (lane 2) and 10 µg of gliofilaments (lane 3).

ation of the neurofilaments in semi-parallel arrays where they are connected through wispy structures (Figs. 3 and 4). This pattern is consistent with the hypothesis of an inter-filament cross-bridging mediated by the high- M_r subunits proposed by Hirokawa *et al.* [2]. Further support is provided in the present work since the purified 200 kDa and 150 kDa molecules inhibit the viscosity kinetics of neurofilaments differentially (Fig. 8), suggesting a more direct participation of the 200 kDa subunit in the cross-bridging mechanism as demonstrated morphologically by the previous authors [2]. Moreover, the possibility of a binding site on the 68 kDa neurofilament core for the high- M_r neurofilament subunits (inter-filament interaction) is raised by the chemical cross-linking studies performed by Carden & Eagles [25].

The physiological significance of the gelation process in purified neurofilaments remains to be found, since they are not involved in the fast axonal transport [37,38]. A

Fig. 8. Inhibition of the viscosity of native neurofilaments by reconstituted neurofilaments and purified neurofilament high- M_r subunits

To the native filaments (2.9 mg/ml in the same buffer as in Fig. 7) were added increasing concentrations of neurofilaments reassembled by dialysis after urea solubilization (○) and of the two high- M_r subunits [200 kDa (■) and 150 kDa (●)] purified from the urea-solubilized neurofilaments. The viscosity kinetics were measured during 6 h at 22 °C and the last value was expressed as a percentage of the control (neurofilaments alone). Inset: SDS/7.5%-polyacrylamide-gel electrophoresis of the 200 kDa (lane 1) and 150 kDa (lane 2) molecules (15 µg of protein).

possible direct relationship between the inter-neurofilament reticulation and the modulation of their delivery *in vivo* might be deduced from developmental studies of the neurofilament axonal flow [39]. According to several authors [8–11,39], such a mechanism could underlie the regulation of the local neurofilament density, which affects the axonal diameter and secondarily the conductance and excitability of nerve [40,41]. More information concerning the physiological role of neurofilaments in neurons could be provided by studies performed *in vitro*, like the present work, focusing on the regulatory events such as the role of the high phosphate content of the high- M_r subunits. In our hands, the gelation phenomenon was separately influenced by a filament-associated hydrolysis of ATP and the extent of phosphorylation of the neurofilament proteins (J. Eyer & J.-F. Leterrier, unpublished work), suggesting some unexpected dynamic properties of their bundles *in situ*, whose role in the cellular function remains to be defined.

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