Biochem. J. (1979) **181**, 275–284
Printed in Great Britain

The Polypeptides of Isolated Brain 10nm Filaments and their Association with Polymerized Tubulin

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(*Received* 4 *December* 1978)

Brain 10nm filaments were isolated from bovine, rabbit and rat brains by a modification of an existing procedure. The overall polypeptide composition of these preparations was similar to that previously reported for brain neurofilaments. In addition to the major polypeptide component, which has mol.wt. approx. 50000, three other polypeptides with chain mol.wts. approx. 210000, 155000 and 70000, which correspond to peripheral-nerve neurofilament polypeptides, were consistently found to be present. The mol.wt.-50000 species was found to be heterogeneous and may contain a component derived from the mol.wt.-70000 polypeptide. The three higher-molecular-weight polypeptides did not appear to be obviously homologous or to be homologous with myosin or *Myxicola* neurofilament polypeptides. These same three higher-molecular-weight components were shown to be identical with the polypeptides probably responsible for the 10nm filaments formed during the early cycles of the tubulin-purification protocol.

In addition to microtubules and microfilaments, a third fibrous cytoplasmic organelle with a diameter of about 10nm is now recognized. These latter filaments are referred to as 10nm filaments or intermediate filaments (Gaskin & Shelanski, 1976). Typical examples from various cell types are neurofilaments, glial filaments (Wuerker, 1970), tonofilaments (Gray *et al.*, 1977), smooth-muscle 10nm filaments (Small & Sobieszek, 1977), and fibroblast intermediate filaments (Starger *et al.*, 1978). Besides the morphological similarities between the different 10nm filaments, several also appear to contain one or more prominent polypeptides with mol.wts. in the range 50000–58000 (Benitz *et al.*, 1976; Steinert *et al.*, 1976; Lazarides & Hubbard, 1976; Davison & Hong, 1977; Small & Sobieszek, 1977; Starger & Goldman, 1977).

There are, however, a number of inconsistencies that have yet to be resolved before it can be accepted that 10nm filaments are a general class of fibrous organelles built from a common subunit(s) and, not least, within the neurofilament subclass there are serious discrepancies between results obtained on different preparations. Polypeptides with mol.wts. between 50000 and 212000 have been implicated as components of neurofilaments from various sources (Huneeus & Davison, 1970; Shelanski *et al.*, 1971; Davison & Winslow, 1974; Gilbert *et al.*, 1975; Hoffman & Lasek, 1975; Lasek & Hoffman, 1976; Yen *et al.*, 1976; Anderton *et al.*, 1976; Benitz *et al.*, 1976; DeVries *et al.*, 1976; Iqbal *et al.*, 1977; Micko & Schlaepfer, 1978; Schlaepfer & Freeman, 1978). In addition, preparations from brain are possibly contaminated with glial fibrillary acidic protein (Benitz *et al.*, 1976; DeVries *et al.*, 1976), which is responsible for 10nm glial filaments (Schachner *et al.*, 1977).

We now report a modified method for isolating brain 10nm filaments simply and in relatively large quantities that is based on the procedure of Yen *et al.* (1976); the method employs a final single flotation step in place of several discontinuous sucrose density gradients. We choose to refer to the resultant material as isolated brain 10nm filaments, since there are two morphologically distinct types of filament aggregates present and we cannot positively ascribe either or both to neural origin. We have previously compared the polypeptide complement of brain 10nm filaments with that of sciatic-nerve neurofilaments and have found common components (Anderton *et al.*, 1978). We have now attempted to evaluate the contribution to the overall protein composition of the brain 10nm filaments of the various polypeptides present and to establish possible homologies between them and with proteins of other fibrous organelles. We have also found evidence that the 10nm filaments that are formed in the early cycles of the tubulin-purification protocol (Delacourte *et al.*, 1977) are probably composed of neurofilament polypeptides.

Materials

All reagents were of AnalaR grade where available. Ampholines were obtained from LKB-Producter

A.B., Bromma, Sweden, and myoglobin was from Koch–Light Laboratories, Colnbrook, Bucks, U.K. Ammonium persulphate, acrylamide, *NN'*-methylenebisacrylamide, *NNN'N'*-tetramethyl ethylenediamine, Bromophenol Blue, glycerol, Fast Green, EDTA and Triton X-100 were from BDH Chemicals, Poole, Dorset, U.K.

Sodium dodecyl sulphate, Coomassie Brilliant Blue R, sodium deoxycholate, Tris (Trizma base, Sigma 7–9), bovine serum albumin, alcohol dehydrogenase, 2-mercaptoethanol, papain, Pronase (Protease VI), subtilisin BPN' (Protease VII), agarose (type 1), phenylmethanesulphonyl fluoride, *p*-chloromercuribenzoic acid and *N*-ethylmaleimide were all obtained from Sigma (London) Chemical Co., Poole, Dorset, U.K.

Methods

Preparation of brain 10nm filaments

Brain 10nm filaments were prepared from bovine, rat and rabbit brain white matter. Brain stem was the exclusive source of white matter. Fresh cattle brains were transported from the slaughterhouse packed in ice and either processed immediately or, alternatively, after dissection, the brain stems were frozen and stored in the deep freeze until required. Rabbit and rat brains were either processed within 30min *post mortem* or again, after dissection, the brain stems were frozen and stored until needed. When brain 10nm filaments were prepared from the frozen tissue, the white matter was thawed directly in the first buffer.

Brain 10nm filaments were prepared by two different methods, one was that of Yen *et al.* (1976) and the other was a modification of this procedure developed by us. The protocol described by Yen *et al.* (1976) was followed as closely as possible, the only changes being those of apparatus and certain assumptions made by us regarding volumes of solutions not specified by these authors. Routinely, however, preparations of brain 10nm filaments were made by the modified method irrespective of the tissue source and quantity; a constant ratio of wet weight of white matter to solution volume (350g of white matter/2 litres of solution A) was maintained and all operations were carried out at 4°C. EDTA (0.001M) was included in all solutions, whereas 2-mercaptoethanol was omitted. Otherwise the solution components were the same as those of Yen *et al.* (1976). The centrifugation conditions were similar to those described by Yen *et al.* (1976), except that to process greater quantities of material we used two 6×250ml angle rotors up to and including the sedimentation of released axoplasm and the discontinuous-sucrose-density-gradient centrifugations were substituted with a single flotation step. For this final flotation, the pellets of released axoplasm were combined and homogenized in 1.7M-sucrose, 0.001M-EDTA, 0.01M-Tris, pH8.6, to give a final volume of 65ml (for 350g of original white matter) and centrifuged at $80000g_{av.}$ for 30min with an MSE 3×23ml swing-out rotor and Superspeed 50 ultracentrifuge. The fluid including the floating material was carefully poured off and the inside walls of the centrifuge tubes wiped with tissue; the translucent pellets served as the preparation of brain 10nm filaments for subsequent experiments.

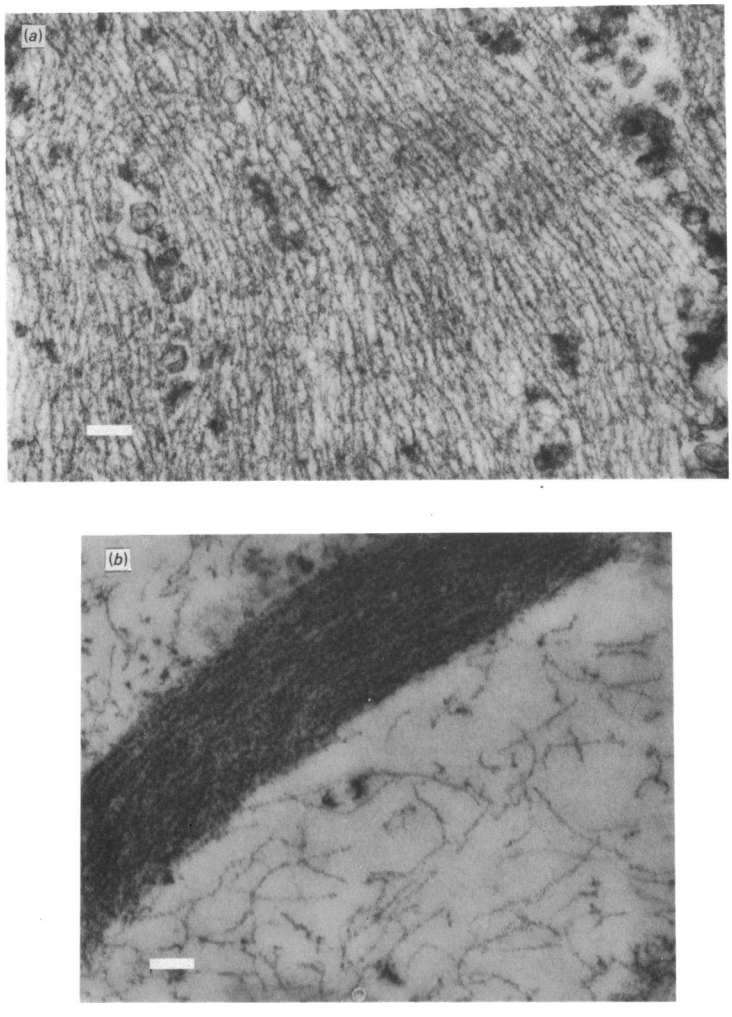
The cold non-depolymerizable material (bovine) from the first cycle of the tubulin polymerization–depolymerization purification protocol was used as the source of tubulin-associated 10nm filaments (Delacourte *et al.*, 1977).

Protein estimation

For the purpose of comparing preparation procedures, approximate estimates of total protein were made by measuring solution absorbance at 280nm. Samples of the initial homogenate and final brain-10nm-filament preparation were diluted into 5% (w/v) sodium dodecyl sulphate and heated in a boiling-water bath for 10min. The A_{280} was then measured with a Unicam SP.1800 u.v. spectrophotometer with 5% (w/v) sodium dodecyl sulphate as the reference solution. Protein concentrations were always adjusted with 5% (w/v) sodium dodecyl sulphate such that the measured absorbance was in the range 0.3–0.7; the A_{280} of the 5% (w/v) sodium dodecyl sulphate solution was also checked (usually 0.2–0.3) to ensure that the protein absorbance was not measured against a high background of u.v.-absorbing material that is present in some batches of sodium dodecyl sulphate. An absorbance of 1.0 at 280nm was assumed to represent a protein concentration of 1mg/ml.

Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis

Analytical polyacrylamide-gel electrophoresis was performed by using the apparatus and buffers described by Laemmli (Laemmli, 1970; Laemmli & Favre, 1973). All gels were made from a stock solution of acrylamide that contained 2.5% (w/v) of the total acrylamide as *NN'*-methylenebisacrylamide. The separating gel consisting of a gradient of polyacrylamide was generated between glass plates by using a three-channel peristaltic pump; normally, gradients of 5–15% (w/v) total acrylamide were used, and sucrose to a final concentration of 15% (w/v) was included in the 15% (w/v) acrylamide solution to aid stabilization of the gradient during formation. A stacking gel of 3.0% (w/v) total acrylamide was



EXPLANATION OF PLATE 1

Electron micrographs of bovine brain 10 *nm filaments prepared by the flotation method illustrating* (*a*) *loosely packed filaments and* (*b*) *a tight bundle of filaments*
Bar = 0.1 μm.

cast above the separating gel with a plastic comb inserted at the top to form loading slots. Samples were prepared for electrophoresis by incubation for 5min at 100°C in 2% (w/v) sodium dodecyl sulphate, 2% (v/v) 2-mercaptoethanol, 0.1M-Tris, pH 6.8. Just before loading, a solution of sucrose and Bromophenol Blue was added to each sample to give final concentrations of 5% (w/v) and 0.0005% (w/v) respectively. Gels were run at a constant current of 40mA per gel with air cooling from a fan and finally were stained with 0.025% (w/v) Coomassie Brilliant Blue R in 45.5% (v/v) methanol, 9% (v/v) acetic acid, 45.5% (v/v) water and destained by soaking in several changes of 5% (v/v) methanol, 7.5% (v/v) acetic acid, 87.5% (v/v) water; destained gels were stored in 5% (v/v) acetic acid.

One-dimensional peptide 'mapping' by partial proteolysis in sodium dodecyl sulphate/polyacrylamide gels was performed essentially by the method of Cleveland *et al.* (1977*a*).

Two-dimensional polyacrylamide-gel electrophoresis was performed by the method of O'Farrell (1975). Ampholines were a mixture comprised of 0.8% (w/v) pH range 4–6 solution, 0.8% (w/v) pH range 5–7 solution and 0.4% (w/v) pH range 3.5–10 solution. For the second dimension in sodium dodecyl sulphate a 5–15% (w/v) gradient polyacrylamide gel was used. It was not found necessary to pre-equilibrate the focused rods with sodium dodecyl sulphate.

Electron microscopy

Brain 10nm filaments for electron microscopy were fixed as a pellet from the final centrifugation of the isolation protocols. The pellets were first washed in 0.1M-sodium cacodylate buffer, pH 7.2, and fixed in 2.5% (v/v) glutaraldehyde in cacodylate buffer for 2h. After overnight rinsing in buffer the pellets were post-fixed in 1% (w/v) OsO_4 for 2h, rinsed in water, dehydrated and embedded in Spurs resin. Sections were cut on an LKB III microtome, stained with uranyl acetate and lead citrate and examined in a Phillips 301 microscope.

Results

Quantity and yield of brain 10nm filaments

The described modification to the method of Yen *et al.* (1976) has resulted in several improvements in the isolation of brain 10nm filaments. We found that the discontinuous-sucrose-density-gradient steps (Yen *et al.*, 1976) severely limited the quantity of material that could be processed, since a large impenetrable pad formed at the interfaces when concentrated suspensions of axoplasm were centrifuged and hardly any material was sedimented further down the tube. The single flotation step overcame these difficulties since the major contaminant of the released axoplasm appears to be membranous material that floats in 1.7M-sucrose. This step has enabled us to commence with more than 100 times the amount of white matter and handle up to 50 times more concentrated suspensions of released axoplasm. On the basis of the method of Yen *et al.* (1976), typically, 430mg of protein in the initial homogenate yielded 0.7mg of 10nm-filament protein (0.16% recovery), whereas in our modified procedure we were able to process, for example, 52600mg of homogenate protein to give 70mg of 10nm-filament protein with a comparable recovery of 0.13%.

The modifications have also enabled us to isolate routinely rabbit and rat brain 10nm filaments, whereas we were repeatedly unsuccessful (a total of four experiments) in attempts to prepare 10nm filaments from rabbit and rat brain by the method of Yen *et al.* (1976).

Ultrastructure of isolated brain 10nm filaments

A representative sample of pelleted bovine brain 10nm filaments is shown in the electron micrograph in Plate 1. The material was isolated by the flotation modification and is in the form of 10nm filaments packed as both tight and loose bundles. Membranous structures were also present but not in any great quantity and always apparently entrapped in the filament bundles. The overall appearance is very much like that described by others for brain neurofilaments (Shelanski *et al.*, 1971; DeVries *et al.*, 1972).

Polypeptide-chain composition of brain 10nm filaments

The polypeptide-chain composition of bovine brain 10nm filaments isolated by the method of Yen *et al.* (1976) is like that of the 10nm filaments prepared by our flotation modified method (Fig. 1, gels *a–e*); rabbit brain 10nm filaments are included for comparison. The differences between the preparations are quantitative and were assessed by densitometry of gels that had been stained with Coomassie Brilliant Blue R. To ensure that the results approximated to the Beer–Lambert law, electrophoresis was carried out with three different known loadings of each sample, which overall covered up to a 10-fold range in quantity, and the gels were scanned. The peaks were cut from the densitometric traces and weighed and the weights were plotted against the volume of sample loaded. In all cases these plots were linear and the slopes were calculated. For each preparation the proportionate contribution to the total protein of the individual polypeptides was calculated from

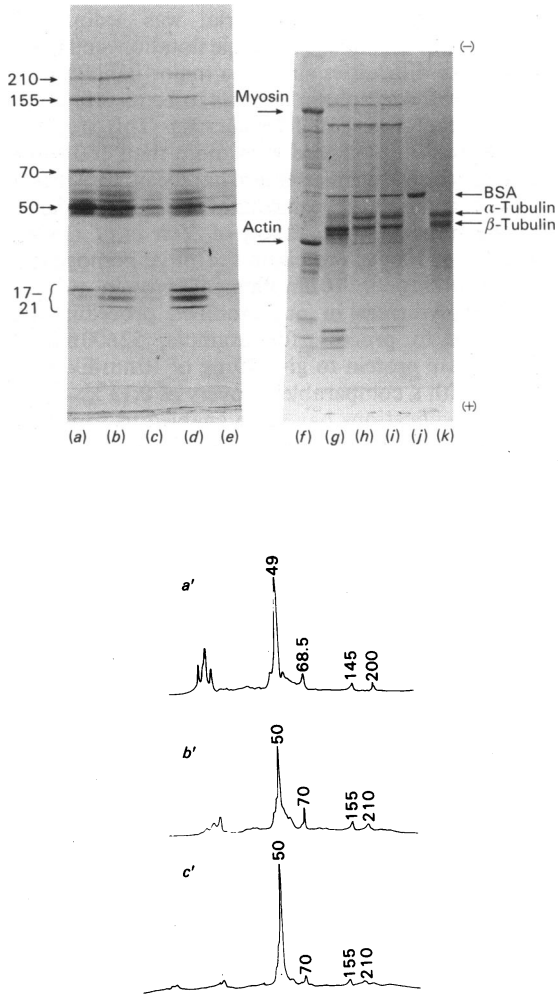


Fig. 1. *Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis of bovine and rabbit brain 10nm filaments and the cold non-depolymerizable tubulin-associated material*

(*a*) Bovine brain 10nm filaments prepared by the method of Yen *et al.* (1976); (*b*, *c*, *d*, and *g*) bovine brain 10nm filaments prepared by the flotation modified method; (*e*) rabbit brain 10nm filaments prepared by the flotation method; (*h* and *i*) bovine cold non-depolymerizable tubulin-associated polypeptides; (*f*) rabbit myofibrils; (*j*) bovine serum albumin; (*k*) purified tubulin. The two gels were made with 5–15% (w/v) gradients of acrylamide. Densitometric traces of SDS/polyacrylamide gels of brain 10nm filament preparations are also shown from (*a'*) rabbit (flotation method), (*b'*) cattle (flotation method), (*c'*) cattle [Yen *et al.* (1976) method]. Gels were stained with Coomassie Brilliant Blue R. Gel bands and densitometric peaks are identified by their molecular weights ($\times 10^{-3}$). Abbreviation used: BSA, bovine serum albumin.

the ratios of these slopes. Approximate molecular weights of the polypeptides from bovine and rabbit brain 10nm filaments are also given in Table 1.

The most abundant single polypeptide species is the approximately mol.wt.-50000 chain in both bovine and rabbit brain 10nm filaments. Significant contributions to the overall composition are also made by the polypeptides with molecular weights indicated in Table 1. Molecular-weight estimations were made by comparison with those of bovine serum albumin, alcohol dehydrogenase, myoglobin, haemoglobin α-chains and rabbit muscle actin and myosin.

The major differences between filaments prepared by the two methods is that the mol.wt.-50000 polypeptide makes a larger contribution to the total protein in preparations by the method of Yen *et al.* (1976) and there is also less material in the mol.wt. 17000–21000 range (Fig. 1 and Table 1). We have not observed any significant differences in the polypeptide-chain patterns of preparations made by the flotation method with fresh brain compared with frozen white matter; we have, however, found it impossible to prepare 10nm filaments from frozen brain by the method of Yen *et al.* (1976). Rabbit brain 10nm filaments appear to contain relatively less material that is not associated with the polypeptides identified in Table 1 than do bovine brain 10nm filaments and in this sense might be considered a 'cleaner' preparation (see also the densitometric traces in Fig. 1). Rabbit (and rat) brain 10nm filaments have a similar complement of polypeptides with slight differences in individual chain molecular weights (Fig. 1).

The polypeptide composition of the cold non-depolymerizable material from the first polymerization–depolymerization cycle of the tubulin-purification protocol (Delacourte *et al.*, 1977) was compared with the complement of isolated brain-10nm-filament polypeptides (bovine). It is clear that the three largest (mol.wts. 210000, 155000 and 70000) prominent bands in both preparations have identical electrophoretic mobilities (Fig. 1, gels *g* and *h*). However, the major components in the two preparations differ, since in the cold non-depolymerizable tubulin-associated material the principal protein species migrate with α- and β-tubulin.

Two-dimensional electrophoretic analysis of bovine brain 10nm filaments was performed by the method of O'Farrell (1975) (Fig. 2). With four different preparations (flotation method) we have found that the mol.wt.-50000 polypeptide is heterogeneous and gives rise to the triplet of spots clearly shown in Fig. 2 (arrowed on gel), additional components with the same molecular weight also being present. On the other hand, the mol.wt.-155000 polypeptide appears to be homogeneous as assessed by this criterion whereas the mol.wt.-70000 band

Table 1. *Relative distribution of protein in various polypeptides present in isolated brain 10nm filaments*

Bovine brain-10nm-filament preparation			Rabbit brain 10nm-filament preparation	
	Protein (% of total protein)			
Polypeptide molecular weight	Flotation modified method	Method of Yen *et al.* (1976)	Polypeptide molecular weight	Protein (% of total protein by the flotation method)
210000	2	2	200000	2
155000	3	3	145000	2
70000	4	5	68500	6
50000	24	37	49000	34
Low-molecular-weight complex (17000–21000)	16	8	Low-molecular-weight complex (17000–21000)	25
Remainder	51	45	Remainder	31

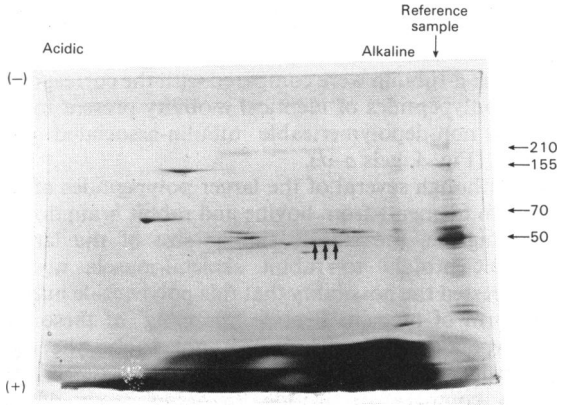


Fig. 2. *Two-dimensional electrophoresis of bovine brain 10nm filaments (O'Farrell, 1975)*
Isolectric focusing in the first dimension was over the approximate pH range 4.5–6 (left to right in the Figure). A reference sample of 10nm-filament proteins were loaded at the right hand side of the gel to act as markers for the spots in the second dimension. Bands are identified by molecular weight ($\times 10^{-3}$). The three intense spots in the mol.wt. 50000 range are indicated by arrows.

may consist of two components. The mol.wt.-210000 polypeptide 'streaks' and does not focus readily.

Attempts to decrease or eliminate possible proteolytic effects were made by inclusion of 10^{-4}M-phenylmethanesulphonyl fluoride and 10^{-4}M-*p*-hydroxymercuribenzoate in all solutions for several preparations (flotation method) of bovine brain 10nm filaments. No significant differences in the polypeptide-chain patterns of preparations made with or without these potential proteinase inhibitors were observed. An additional preparation (flotation method) of bovine brain 10nm filaments was performed in which 10^{-4}M-*N*-ethylmaleimide was included in all solutions and 1mM-*N*-ethylmaleimide was substituted for 2-mercaptoethanol when the 10nm filament preparation was finally treated with 2% (w/v) sodium dodecyl sulphate before electrophoresis. No change to the usual polypeptide distribution in the gels was observed, which indicated that the various polypeptides present in these 10nm-filament preparations are unlikely to be disulphide linked *in vivo*.

One-dimensional peptide 'mapping' by partial proteolysis in sodium dodecyl sulphate/polyacrylamide gels

Peptide 'mapping' of several of the brain-10nm-filament polypeptides was carried out (Cleveland *et al.*, 1977*a*). Notwithstanding the difficulties inherent in comparing polypeptides of grossly different molecular weights, we have not found any evidence that suggests that the mol.wt.-70000, -155000 and -210000 polypeptides of bovine brain 10nm filaments are homologous. The proteinases papain, Pronase and subtilisin BPN' over a range of concentrations all gave rise to a unique set of digestion products with each of the above three polypeptides. For example the subtilisin BPN'-digestion products from the mol.wt.-155000 and -210000 polypeptides (bovine) are shown in Fig. 3 (gels *b* and *c*) from which it is clear that they do not give rise to common fragments. When the mol.wt.-49000 polypeptide of rabbit brain 10nm filaments was compared with the mol.wt.-68500 polypeptide, it was apparent that they appear to share some digestion products and that the mol.wt.-68500 polypeptide gives rise to a product that co-migrates with the authentic mol.wt.-49000 polypeptide (Fig. 3, gels *d* and *c*). For this experiment a rabbit preparation was used because the mol.wt.-49000 region of the gel is less complex, therefore facilitating interpretation of the peptide 'map'.

Iqbal *et al.* (1977) have reported that the mol.wt.-

50000 neurofilament polypeptide isolated from human autopsy material gives a tryptic 'fingerprint' that is very similar to β-tubulin. We have reinvestigated this intriguing possibility by the peptide-'mapping' method, but with rabbit brain 10nm filaments as the source of this polypeptide and compared this with rabbit brain tubulin (prepared by the method of Sheterline, 1977). Although α- and β-tubulin gave similar peptide 'maps' [Fig. 4 and Cleveland *et al.* (1977*b*)], we failed to demonstrate any homology between the brain-10nm-filament mol.wt.-50000 polypeptide with either α- or β-tubulin (Fig. 3, gels *f* and *g*).

The three higher-molecular-weight polypeptides (mol.wts. 210000, 155000 and 70000) found in the cold non-depolymerizable tubulin-associated protein fraction were then compared by peptide 'mapping' with the corresponding polypeptides from bovine brain 10nm filaments. The same three proteinases were again used and with each enzyme identical patterns of digestion products were obtained when the corresponding polypeptides from the isolated brain 10nm filaments and the cold non-depolymerizable tubulin-associated proteins were compared e.g. mol.wt.-70000 polypeptides (Fig. 3, gels *h* and *i*).

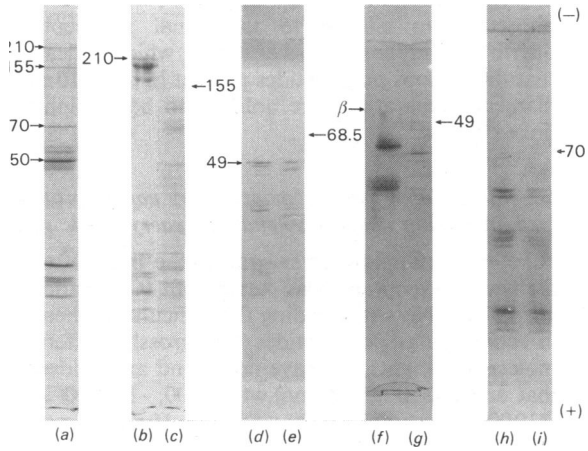


Fig. 3. *Comparison of various brain-10nm-filament polypeptides by peptide 'mapping'*

The 'maps' shown are taken from representative gels. (*a*) Bovine brain 10nm filaments (as a reference); (*b* and *c*) comparison of the bovine mol.wt.-210000 polypeptide (*b*) with bovine mol.wt.-155000 polypeptide (*c*) by using subtilisin BPN′; (*d* and *e*) comparison of rabbit mol.wt.-49000 polypeptide (*d*) with rabbit mol.wt.-68500 polypeptide (*e*) by using papain; (*f* and *g*) comparison of rabbit β-tubulin (*f*) with rabbit brain-10nm-filament mol.wt.-49000 polypeptide (*g*) by using papain; (*h* and *i*) comparison of the mol.wt.-70000 polypeptides from bovine brain 10nm filaments (*h*) and from bovine cold non-depolymerizable tubulin-associated material (*i*) by using papain. The positions of the undigested polypeptides are identified by their molecular weights ($\times 10^{-3}$). Gels (*a*), (*b*), (*c*), (*h*) and (*i*) were 7.5–15% (w/v) acrylamide, gels (*d*) and (*e*) were 10–17.5% (w/v) acrylamide and gels (*f*) and (*g*) were 12.5–20% (w/v) acrylamide. Abbreviation used: β, β-tubulin.

Although the cold non-depolymerizable proteins appear to contain tubulin as the major component, microtubules seem to be absent and the only morphologically discernible regular structures present are 10nm filaments (Delacourte *et al.*, 1977). It was of interest therefore to determine whether the major polypeptide components of this protein mixture were identical with highly purified α- and β-tubulin (purified by six cycles of polymerization–depolymerization), at least by the criterion of identical partial-digest peptide 'maps'. Again, papain, Pronase and subtilisin BPN′ were used and in all cases identical partial-digest patterns were obtained when α- and β-tubulin were compared with the corresponding polypeptides of identical mobility present in the cold non-depolymerizable tubulin-associated proteins (Fig. 4, gels *a–d*).

Although several of the larger polypeptides of the 10nm filaments from bovine and rabbit brain do not co-migrate, the closely similar size of the largest rabbit protein to rabbit skeletal-muscle myosin suggested the possibility that this polypeptide may be a form of myosin. Peptide 'mapping' of these two polypeptides has shown this not to be the case (results not shown).

A comparative peptide 'mapping' study of the mol.wt.-70000, -155000 and -210000 polypeptides from bovine and the corresponding polypeptides from rabbit brain 10nm filaments was performed to determine if these apparently similar polypeptides are homologous and therefore likely to have equivalent roles in the two species, even though they do not possess identical chain molecular weights. With all the three proteinases that were used, similar proteolysis patterns were observed when the corresponding pairs of 10nm filament polypeptides were compared (results not shown).

The mol.wt.-155000 polypeptide from bovine brain 10nm filaments was found to co-migrate with the smaller of the two major neurofilament polypeptides from *Myxicola* (Gilbert *et al.*, 1975). Comparison of peptide 'maps' of the brain protein with the peptide 'map' of the smaller *Myxicola* polypeptide revealed no obvious homologies (Fig. 4, gels *e* and *f*), although the two *Myxicola* neurofilament polypeptides are clearly related (Fig. 4, gels *g* and *h*).

One additional peptide-'mapping' study was carried out that demonstrated the presence of actin in these 10nm-filament preparations. The polypeptide in bovine brain 10nm filaments that co-migrates

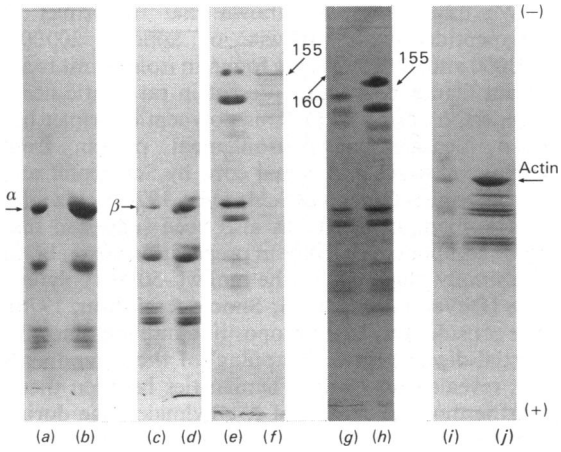


Fig. 4. *Comparison of various polypeptides by peptide 'mapping'*

The 'maps' are taken from representative gels. (*a–d*) Comparison of authentic α- and β-tubulin (bovine) with suspected α- and β-tubulin from bovine cold non-depolymerizable tubulin-associated material by using papain; (*a*) α-tubulin, (*b*) suspected α-tubulin, (*c*) β-tubulin, (*d*) suspected β-tubulin; (*e* and *f*) comparison of the mol.wt.-155000 polypeptide from bovine brain 10nm filaments (*f*) with the smaller *Myxicola* neurofilament polypeptide (*e*) by using papain; (*g* and *h*) comparison of the two major neurofilament polypeptides from *Myxicola* by peptide 'mapping' by using papain; (*i* and *j*) comparison of rabbit skeletal-muscle actin (*j*) with co-migrating material from rabbit brain 10nm filaments (*i*) by using papain. The positions of the undigested polypeptides are identified by name or by their molecular weights ($\times 10^{-3}$). Gels (*a*)–(*h*) were 7.5–15% (w/v) acrylamide and gels (*i*) and (*j*) were 10–17.5% (w/v) acrylamide. Abbreviations used: α,α-tubulin; β,β-tubulin.

with actin was compared by peptide 'mapping' with rabbit skeletal-muscle actin and several components were seen to be identical (Fig. 4, gels *i* and *j*). The differences in digestion patterns were probably due to contamination of the actin from the 10nm-filament preparation with other protein(s) present in this material.

Discussion

Brain 10nm filaments isolated by our flotation modified method based on that of Yen *et al.* (1976) are both morphologically and biochemically similar to preparations that other authors have assumed to be isolated neurofilaments (Shelanski *et al.*, 1971; Davison & Winslow, 1974; Shook & Norton, 1975; Yen *et al.*, 1976). We have chosen to refer to them as brain 10nm filaments for several reasons. There are morphologically two clearly distinct types of aggregates of 10nm filaments present and it has been suggested previously that the tightly packed filaments might represent glial filaments, and the loose bundles, neurofilaments (Shelanski *et al.*, 1971). It is of course possible that the two different aggregation states are only artefactually induced during isolation, but the fact that sciatic-nerve axonal material does not contain tight bundles of 10nm filaments of the type seen in brain preparations may favour the attribution of the tightly packed bundles of filaments to glial origin (Anderton *et al.*, 1978). The proportion of filaments in each type of aggregate is impossible to assess accurately from electron-microscopic examination, even ignoring possible differential distribution of the two types of aggregate in the pelleted material. Immunochemical evidence also suggests that preparations of brain 10nm filaments of 'neurofilaments' do contain material originating from glial cells. Independently, DeVries *et al.* (1976) and Benitz *et al.* (1976) have demonstrated with an antiserum specific to glial fibrillary acidic protein, a protein present in glial filaments, that brain 'neurofilament' preparations are contaminated with glial filaments. We, like Lee *et al.* (1976), have found by immunofluorescence that an antiserum to bovine brain 10nm filaments labels both glia and neurons in rat cerebellum (B. H. Anderton, R. Thorpe, J. Cohen, S. Selvendran & P. Woodhams, unpublished work). However, since antisera to glial fibrillary acidic protein stain specifically glial cells (Schachner *et al.*, 1977) we suggest caution be exercised in interpreting results that appear to demonstrate cross-reactivity between neurofilaments, glial filaments and other intermediate filaments when antisera to brain 'neurofilaments' are employed.

The polypeptide composition of preparations of brain 10nm filaments is complex, with the major component found by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis possessing a mol.wt. of approx. 50000. Our results qualitatively resemble those of Davison & Winslow (1974), Shook & Norton (1975, 1976), DeVries *et al.* (1976), Benitz *et al.* (1976) and Yen *et al.* (1976). However, the relative proportions of the different polypeptides in reports from different laboratories seems variable. Although the mol.wt.-50000 polypeptide (which most authors assume to be the neurofilament subunit) is quantitatively the major species present in our preparations, other polypeptides were consistently found by us in 10nm filaments isolated from bovine (25 preparations), rabbit (three preparations) and rat (two preparations) brain white matter. In devising the present protocol for the isolation of brain 10nm filaments, we deliberately avoided the use of protein denaturants and reasoned that all soluble and membranous material that was not trapped in

filamentous aggregates should be removed from the final pellet by repeated centrifugation of the filaments from solutions of appropriate density. The final pellet of brain 10nm filaments is much more translucent than the membrane-containing pellets obtained at earlier stages in the procedure, and electron-microscopical examination of the final preparation has shown it to contain very little membranous material. We have found that repeated sedimentations of the filaments from 1.7M- or even 1.85M-sucrose did not alter the polypeptide composition. This is not unlike the observation made by Shook & Norton (1975) who reported that 'purified neurofilaments' have a similar polypeptide composition to the 'crude neurofilaments' initially released by osmotic shock of axonal segments. Inclusion of either 2.5% (w/v) sodium deoxycholate or 5% (w/v) Triton X-100 in the final centrifugation step also did not significantly change the sodium dodecyl sulphate/polyacrylamide-gel-electrophoresis pattern, further indicating that the prominent polypeptides found in the final preparation are unlikely to originate from membranous contamination. The consistent differences between the protein composition of brain 10nm filaments isolated by the flotation procedure and that of the original method of Yen *et al.* (1976) are probably due to the different centrifugation protocols employed.

The estimate by densitometry of the distribution of protein in the different brain-10nm-filament polypeptides is not absolute since Coomassie Brilliant Blue R may bind differentially to the various polypeptides. Furthermore, components in the mol.wt.-50000 region are not sufficiently resolved to enable their accurate quantification from the densitometric profile; the values for the major mol.wt.-50000 polypeptide included in Table 1 were obtained by interpolation. Possible deviation by Coomassie Brilliant Blue R from the Beer–Lambert law was controlled by taking quantitative estimates of polypeptides from gels that had been run with three different protein loadings varying over a 10-fold range. Similar results were obtained when Fast Green was used as a protein stain, the absorbance of which has been reported to obey more closely the Beer–Lambert law than Coomassie Brilliant Blue R (Gorovsky *et al.*, 1970). Because of the problems associated with making quantitative estimates of the proportional contribution of different polypeptides to the total protein composition of brain 10nm filaments, it is difficult to compare quantitatively our results with those of Yen *et al.* (1976). These authors have claimed that the mol.wt.-50000 polypeptide represents at least 90% of the total protein present. However, in our hands and by our estimates their method of isolating brain 10nm filaments has never resulted in the mol.wt.-50000 component representing greater than 35–40% of the total protein.

We have previously shown that the triplet of polypeptides with mol.wts. of approx. 200000, 150000 and 70000 that are found in isolated rat brain 10nm filaments are also present in rat sciatic nerve (Anderton *et al.*, 1978). This polypeptide triplet has been identified as neurofilament protein from peripheral nerve and spinal cord by Schlaepfer and his colleagues (Micko & Schlaepfer, 1978; Schlaepfer & Freeman, 1978). It has also been suggested that these components present in preparations from brain are simply oligomers of the mol.wt.-50000 polypeptide (DeVries *et al.*, 1976; Shook & Norton, 1976). We consider this latter proposition unlikely since the partial-digest peptide 'mapping' of the components has revealed no obvious similarities between them. Furthermore, inclusion of *N*-ethylmaleimide during isolation and in the sample during preparation for sodium dodecyl sulphate/polyacrylamide-gel electrophoresis did not alter the electrophoretic-separation pattern of the brain-10nm-filament preparation.

There are small species differences in the individual molecular weights of the triplet polypeptides. However, we have found that the corresponding polypeptides from different species are homologous by the partial-digest peptide-'mapping' criterion. This result suggests that the presence of these larger polypeptides in preparations of brain 10nm filaments is not simply fortuitous contamination, but that they possess an equivalent functional role associated with brain 10nm filaments in the different species. Further evidence that implicates a functional role for this triplet of polypeptides in 10nm filaments is the finding that these same polypeptides are associated with the 10nm filaments that form *in vitro* during the early polymerization cycles of tubulin purification. In the cold non-depolymerizable tubulin-associated material, this triplet originates from the soluble-protein fraction of a brain homogenate, whereas in isolated brain 10nm filaments, the proteins are readily sedimented in an aggregated form. The cold non-depolymerizable tubulin-associated material has been previously shown to contain many 10nm filaments concomitant with a sparsity of microtubules (Berkowitz *et al.*, 1977; Delacourte *et al.*, 1977). As subsequent polymerization–depolymerization cycles are completed, a progressive and parallel decrease in the quantity of 10nm filaments and the above triplet of polypeptides is found in the tubulin fraction and highly purified tubulin does not form 10nm filaments when induced to polymerize under the conditions employed for its purification. Furthermore, this triplet of polypeptides is not found in association with highly purified tubulin, unlike the so-called high-molecular-weight-microtubule-associated proteins and tau (Kirkpatrick *et al.*, 1970; Keates & Hall, 1975; Murphy & Borisy, 1975; Weingarten *et al.*, 1975; Sloboda *et al.*, 1976; Cleveland *et al.*, 1977*b*). Thus this triplet of polypeptides with mol.wts. of

approx. 210000, 155000 and 70000 may be principally responsible for the formation *in vitro* of the tubulin-associated 10nm filaments.

We suggest therefore that the high-molecular-weight triplet of polypeptides are brain neurofilament components and that brain spinal-cord and peripheral-nerve neurofilaments do share certain common polypeptides (Anderton *et al.*, 1978). The status of the mol.wt.-50000 component remains to be determined, particularly since it has been previously shown that such a component is absent from peripheral nerve (Anderton *et al.*, 1978; Micko & Schlaepfer, 1978). We have now demonstrated that this material is heterogeneous, and partial-digest peptide-'mapping' experiments have shown that it may be partially composed of a proteolytic-digestion product of the mol.wt.-70000 species. We have also found evidence to suggest that during the first few minutes after killing the animal, rat brain-10nm-filament polypeptides undergo proteolysis that results in a relatively greater contribution by the mol.wt.-50000 species to the overall polypeptide composition (A. I. Matus, D. H. Jones & B. H. Anderton, unpublished work). Subsequent precautions to minimize proteolysis taken some hours *post mortem* apparently have no effect, since we have found that addition of potential proteinase inhibitors did not change the polypeptide distribution in preparations of bovine brain 10nm filaments. These findings are reminiscent of the rapid but short-lived Ca^{2+}-activated proteolytic activity found in *Myxicola* axoplasm that leads to degradation of *Myxicola* neurofilaments to products in the mol.wt.-50000–80000 range (Gilbert *et al.*, 1975). Iqbal *et al.* (1977, 1978) have suggested that the mol.wt.-50000 species is similar to β-tubulin. Our results do not support this view since the partial-digest peptide 'maps' obtained from α- and β-tubulin do not show obvious similarities with that obtained from the mol.wt.-50000 component. It is also pertinent to point out that the 10nm filaments that appear to polymerize *in vitro* from the soluble-protein fraction of a brain homogenate do not contain a prominent polypeptide constituent with a mobility identical with the major mol.wt.-50000 polypeptide of isolated brain 10nm filaments, suggesting that this latter polypeptide may not play an essential structural role.

Finally, we have found no evidence for homology between brain-10nm-filament polypeptides and skeletal-muscle myosin or *Myxicola* neurofilaments, although the two major *Myxicola* neurofilament polypeptides are obviously similar to each other. Actin, on the other hand, does appear to be present in preparations of brain 10nm filaments and any possible functional role for this protein associated with 10nm filaments remains to be determined. For the present, we cannot ascribe a structural role to the polypeptides in the mol.wt.-20000 region.

This work was supported by grants from The Royal Society and the Medical Research Council to B.H.A. and by an EMBO Short-Term Fellowship to A.D., and by grant no. LA 04-0268 of Centre National de la Recherche Scientifique.

References

Anderton, B. H., Bell, C. W., Newby, B. J. & Gilbert, D. S. (1976) *Biochem. Soc. Trans.* **4**, 544–548

Anderton, B. H., Ayers, M. & Thorpe, R. (1978) *FEBS Lett.* **96**, 159–163

Benitz, W. E., Dahl, D., Williams, K. W. & Bignami, A. (1976) *FEBS Lett.* **66**, 285–289

Berkowitz, S. A., Katagiri, J., Binder, H. & Williams, R. C., Jr. (1977) *Biochemistry* **16**, 5610–5617

Cleveland, D. W., Fischer, S. G., Kirschner, M. W. & Laemmli, U. K. (1977*a*) *J. Biol. Chem.* **252**, 1102–1106

Cleveland, D. W., Hwo, S.-Y. & Kirschner, M. W. (1977*b*) *J. Mol. Biol.* **116**, 207–225

Davison, P. F. & Hong, B.-S. (1977) *Brain Res.* **134**, 287–295

Davison, P. F. & Winslow, B. (1974) *J. Neurobiol.* **5**, 119–133

Delacourte, A., Plancot, M.-T., Han, K. K., Hildebrand, H. & Biserte, G. (1977) *FEBS Lett.* **77**, 41–46

De Vries, G. H., Norton, W. T. & Raine, C. S. (1972) *Science* **175**, 1370–1372

De Vries, G. H., Eng, L. F., Lewis, D. L. & Hadfield, M. G. (1976) *Biochim. Biophys. Acta* **439**, 133–145

Gaskin, F. & Shelanski, M. L. (1976) *Essays Biochem.* **12**, 115–146

Gilbert, D. S., Newby, B. J. & Anderton, B. H. (1975) *Nature (London)* **256**, 586–589

Gorovsky, M. A., Carlson, K. & Rosenbaum, J. L. (1970) *Anal. Biochem.* **35**, 359–370

Gray, R. H., Brabec, R. K., Byrsk, M. M. & Bernstein, I. A. (1977) *J. Histochem. Cytochem.* **25**, 1127–1139

Hoffman, P. N. & Lasek, R. J. (1975) *J. Cell Biol.* **66**, 351–366

Huneeus, F. C. & Davison, P. F. (1970) *J. Mol. Biol.* **52**, 415–428

Iqbal, K., Grundke-Iqbal, I., Wisniewski, H. M. & Terry, R. D. (1977) *J. Neurochem.* **29**, 417–424

Iqbal, K., Grundke-Iqbal, I., Wisniewski, H. M. & Terry, R. D. (1978) *Brain Res.* **142**, 321–332

Keates, R. A. B. & Hall, R. H. (1975) *Nature (London)* **257**, 418–421

Kirkpatrick, J. B., Hymas, L., Thomas, V. L. & Howley, P. M. (1970) *J. Cell Biol.* **47**, 384–394

Laemmli, U. K. (1970) *Nature (London)* **227**, 680–685

Laemmli, U. K. & Favre, M. (1973) *J. Mol. Biol.* **80**, 575–599

Lasek, R. J. & Hoffman, P. M. (1976) in *Cell Motility* (Goldman, R., Pollard, T. & Rosenbaum, J., eds.), vol. 3, pp. 1021–1049, Cold Spring Harbor Laboratories, Cold Spring Harbor

Lazarides, E. & Hubbard, B. D. (1976) *Proc. Natl. Acad. Sci. U.S.A.* **73**, 4344–4348

Lee, V., Yen, S.-H. & Shelanski, M. L. (1976) *J. Cell. Biol.* **70**, 331a

Micko, S. & Schlaepfer, W. W. (1978) *J. Neurochem.* **30**, 1041–1049

Murphy, D. B. & Borisy, G. G. (1975) *Proc. Natl. Acad. Sci. U.S.A.* **72**, 2696–2700

O'Farrell, P. H. (1975) *J. Biol. Chem.* **250**, 4007–4021
Schachner, M., Hedley-Whyte, E. T., Hsu, D. U., Schoonmaker, G. & Bignam, A. (1977) *J. Cell Biol.* **75**, 67–73
Schlaepfer, W. W. & Freeman, L. A. (1978) *J. Cell Biol.* **78**, 653–662
Shelanski, M. L., Albert, S., De Vries, G. H. & Norton, W. T. (1971) *Science* **174**, 1242–1245
Sheterline, P. (1977) *Biochem. J.* **168**, 533–539
Shook, W. J. & Norton, W. T. (1975) *Trans. Am. Soc. Neurochem.* **6**, 214
Shook, W. J. & Norton, W. T. (1976) *Brain Res.* **118**, 517–522
Sloboda, R. D., Dentler, W. L. & Rosenbaum, J. L. (1976) *Biochemistry* **15**, 4497–4506
Small, J. V. & Sobieszek, A. (1977) *J. Cell Sci.* **23**, 243–268
Starger, J. M. & Goldman, R. D. (1977) *Proc. Natl. Acad. Sci. U.S.A.* **74**, 2422–2426
Starger, J. M., Brown, W. E., Goldman, A. E. & Goldman, R. D. (1978) *J. Cell Biol.* **78**, 93–109
Steinert, P. J., Idler, W. W. & Zimmerman, S. B. (1976) *J. Mol. Biol.* **108**, 547–567
Weingarten, M. D., Lockwood, A. H., Hwo, S.-Y. & Kirschner, M. W. (1975) *Proc. Natl. Acad. Sci. U.S.A.* **72**, 1859–1862
Wuerker, R. B. (1970) *Tissue Cell* **2**, 1–9
Yen, S.-H., Dahl, D., Schachner, M. & Shelanski, M. L. (1976) *Proc. Natl. Acad. Sci. U.S.A.* **73**, 529–533