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The proteolytic digestion of ox neurofilaments with trypsin and α-chymotrypsin

Thien K. CHIN, Peter A. M. EAGLES* and Alison MAGGS
Department of Biophysics, King's College, London, and M.R.C. Cell Biophysics Unit, 26–29 Drury Lane, London WC2B 5RL, U.K.

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Brief digestion of ox neurofilaments with trypsin liberates fragments that are soluble and have molecular weights ranging from 164000 to 97000. Peptide fingerprinting indicates that these regions, termed the tryptic head-regions, arise from the 205000- and 158000-mol.wt. components of the triplet. The remains of the parent polypeptides sediment with normal filaments and have been termed tail-regions. Digestion of neurofilaments with chymotrypsin also liberates soluble fragments (chymotryptic head-regions) but these have mol.wts. 171000 and 119000, though they too originate from the higher-molecular-weight triplet polypeptides. Tryptic and chymotryptic head-regions have extensive homology, and a low (⩽20%) helix content. Electron microscopy shows that chymotryptic digestion rapidly reduces the length of filaments, probably because this enzyme preferentially attacks the 72000-mol.wt polypeptide. In contrast, brief digestion with trypsin does not reduce filament length even though more than 90% of the two higher-molecular-weight components have been cleaved. These results indicate that the backbone of native filaments is formed from the 72000-mol.wt. polypeptide together with the tail-regions from the 205000- and 158000-mol.wt. polypeptides. The corresponding head-regions of these components, which can represent nearly 75% of each molecule, are not necessary for preserving the backbone of native neurofilaments and are therefore good candidates for being the side arms that connect these filaments in nerve cells.

Neurofilaments are major cytoskeletal components of nerve fibres. Electron microscopy of intact fibres shows that neurofilaments have diameters of around 11 nm and that arms, or microtrabeculae, of variable length link them to form an intricate network (Wuerker, 1970; Ellisman & Porter, 1980; Metuzals *et al.*, 1981). Connections also bridge neurofilaments to microtubules and possibly to organelles as well (Hirokawa, 1982; Schnapp & Reese, 1982). Clearly a most important question regarding the structure of the neurofilament and its relationship to processes like axonal transport is whether these connections are integral parts of the neurofilament or separate components.

It is now well established that mammalian neurofilaments are composed of three major polypeptides (the triplet) that have molecular weights of approx. 200000, 155000 and 70000 (e.g. Lasek & Hoffman, 1976; Willard & Simon, 1981). If a connection is part of the neurofilament then any of these polypeptides could be involved in its structure. Furthermore, the region of the polypeptide forming the link by its very nature would not be needed to stabilize the backbone of the filament and it might therefore be possible to remove it without destroying the filament's integrity.

Support for this idea that some of the polypeptides are arranged primarily in the backbone, whereas others are more peripheral to it, and may therefore represent cross-links, has so far come from two approaches: decorating neurofilaments with antibodies directed against the triplet components and reconstituting neurofilaments from purified polypeptides (Willard & Simon, 1981; Geisler & Weber, 1981; Sharp *et al.*, 1982; Liem & Hutchison, 1982; Zackroff *et al.*, 1982).

These studies support a model where the 70000-mol.wt. polypeptide is in the backbone and where the 200000- and probably the 155000-mol.wt. poly-

Abbreviations used: PMSF, phenylmethanesulphonyl fluoride; SDS, sodium dodecyl sulphate.

* To whom correspondence and reprint requests should be sent.

peptides are on the outside of the filament. If extensive regions of the 200000- and 155000-mol.wt. polypeptides are not needed for the backbone, then their removal by proteolysis, for example, would not be expected to disrupt the filament.

We report in the present paper our studies on the proteolytic digestion of native ox neurofilaments and show that it is indeed possible to cleave nearly all of the two higher-molecular-weight triplet polypeptides while maintaining a filament. During the process, about 75% of each of these components, the head-regions of the polypeptides, are liberated as soluble products, whereas the remaining 25%, the tail-regions, bind to the backbone composed predominantly of the 70000-mol.wt. polypeptide.

Preliminary results of this work have been published in abstract form [Brown *et al.* (1982) *Biol. Cell.* **45**, 207].

Materials and methods

The proteins that were used as molecular-weight markers were those previously described (Eagles *et al.*, 1981). Trypsin (diphenylcarbamoyl chloride-treated), α-chymotrypsin, papain and PMSF were purchased from Sigma. Lima-bean trypsin inhibitor was purchased from the Worthington Co. All other chemicals were of a high grade of purity.

Preparation of ox neurofilaments

Ox spinal cords were collected in ice from a local abattoir and processed immediately. The isolation procedure that was used is described in detail elsewhere (Carden & Eagles, 1983).

Digestion of neurofilaments with trypsin and chymotrypsin

Ox neurofilaments, normally at a concentration of 0.5–2 mg/ml in 10 mM-Tris/0.1 M-NaCl, pH 7.5, were digested for various times (0–40 min) with trypsin or chymotrypsin at final concentrations between 0.5 and 10 μg/ml. Incubations were carried out at room temperature (23–24°C) and the reactions were stopped with either 10-fold (w/w) excess trypsin inhibitor or 20-fold (w/w) excess PMSF; control samples, where inhibitor was added before the enzyme, showed no digestion under these conditions for up to 24 h. The fragments liberated by proteolysis were separated from filaments by centrifugation either in an MSE ultracentrifuge operated for 1–4 h at 100000 **g** ($r_{av.}$ 10 cm) or in a Beckman Airfuge operated for 1–2 h at 148000 **g** ($r_{av.}$ 1.32 mm). Samples of the supernatant and precipitate were then dissolved in SDS and subjected to electrophoresis.

A number of experiments were performed to monitor the activity of endogenous proteinases that are co-purified with neurofilaments. These enzymes are active soon after filament preparation but quickly die (Carden & Eagles, 1983). In the experiments described here, the activities of these proteinases were far less than those of the trypsin and chymotrypsin used, by a factor of 10^5 or more. Controls for the endogenous proteinases were carried out where samples were incubated without trypsin inhibitor or PMSF and were identical with the zero-time controls shown in the Figures. Experiments were also performed where neurofilaments were spun immediately before digestion with trypsin or chymotrypsin in order to remove any soluble fragments contaminating the neurofilament preparation, though results from these experiments were indistinguishable from those where filaments were not pre-spun in this way.

Peptide fingerprinting

This was carried out essentially as described previously (Eagles *et al.*, 1981) by the technique originally devised by Cleveland *et al.* (1977). Peptide fingerprints were generally developed first with Coomassie Blue and then, to reveal more detail, with the reduced silver-stain method of Morrissey (1981).

Polyacrylamide-gel electrophoresis

This was performed on 5–15% gradient gels under denaturing conditions in the presence of SDS using the discontinuous buffer system of Laemmli (1970). Non-denaturing gels at a single acrylamide concentration were also used to estimate native molecular weights (Hedrick & Smith, 1968); in this case the buffer system was essentially the same but with the omission of SDS. In both types of gel the proteins were stained with Coomassie Blue. Routine, rapid analyses were carried out on a high-voltage analytical gel system ('minigel'). Here, 2–10 μl samples containing 5 μg of protein were subjected to high-voltage electrophoresis (400–500 V) on thin polyacrylamide gels (0.9 mm) sandwiched between microscope slides. With this analytical system samples were well resolved after electrophoresis for 20 min and staining and destaining of gels could be performed in less than 1 h. Densitometry of gels was performed as described by Carden & Eagles (1983).

Chemical cross-linking of thiol groups

Cross-linking experiments with copper *o*-phenanthroline were performed essentially as described by Carden & Eagles (1983).

Protein determination

Protein was generally determined spectrophotometrically at 210 nm (Tombs *et al.*, 1959). In the range 1–10 μg of protein a modification of the assay devised by Bramhall *et al.* (1969) was used with a standard of bovine albumin.

Circular dichroism

This was performed on a Cary 61 spectropolarimeter. All measurements were carried out at

room temperature in either 50mM-Tris/0.1M-NaCl/1mM-EGTA, pH 7.5, or in 50mM-sodium phosphate/0.1M-NaCl, pH 7.5. The content of α-helix and random coil in the samples was estimated by a modified procedure of that originally devised by Saxena & Wetlaufer (1971) and Chen *et al.* (1972). They showed that the mean residue ellipticity, $[\theta]$, at any fixed wavelength can be related to the fraction of α-helix (f_H), β-structure (f_β) or random coil (f_R), by the equation:

$$[\theta] = f_H[\theta]_H + f_\beta[\theta]_\beta + f_R[\theta]_R$$

where $[\theta]_H$, $[\theta]_\beta$ and $[\theta]_R$ are reference values for pure α-helix, β-structure and random coil respectively and where $f_H + f_\beta + f_R = 1$. The reference values were taken from Chen *et al.* (1974).

Electron microscopy

Electron microscopy was carried out on a Philips 200 electron microscope working at 80kV. A solution of 1% uranyl acetate was used as the negative stain.

Results

Molecular weights of ox neurofilament polypeptides

Estimates for the molecular weights of the ox neurofilament polypeptides were obtained by calibrating gels with about 20 different proteins. From 14 separate determinations, the average values (±S.D.) for the molecular weights of the major neurofilament polypeptides in the gel system were 205000±4000, 158000±5000 and 72000±3000; these estimates will therefore be used throughout the present paper.

Digestion of ox neurofilaments with trypsin

A typical digestion experiment is shown in Fig. 1. The procedure used was similar to that used in a

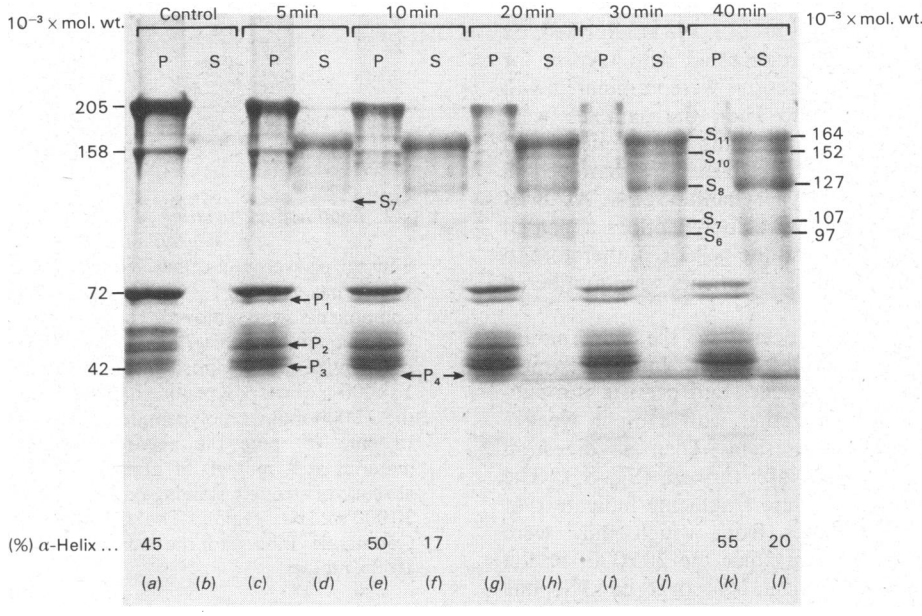

Fig. 1. *Digestion profile of ox neurofilaments with trypsin*

Purified neurofilaments (1–2mg/ml) were spun from solution by centrifugation for 1½h at 120000*g* ($r_{av.}$ 6.5cm) and the pellet was suspended by gentle homogenization in 80mM-sodium phosphate/0.1M-NaCl/14mM-β-mercaptoethanol/0.01% azide/1mM-EGTA, pH 7.2. For this gel, 50μl samples of the resuspended filaments were incubated with 5μl of trypsin (6μg/ml) at room temperature for the times indicated. The reactions were stopped with inhibitor and the samples were then spun at room temperature in a Beckman Airfuge operated at 148000*g* ($r_{av.}$ 1.32mm) for 1½h. Pellets (P) and supernatants (S) were dissolved in SDS and run on the gel. The digestion times (0–40min) are indicated above the lanes labelled(*a*)–(*l*). Larger samples of neurofilaments without azide or β-mercaptoethanol were digested for 10min and 40min and used for the measurement of α-helix content. The bands in the supernatant are labelled S_{11}, S_{10}, S_8, S_7', S_7 and S_6 and in the pellet P_1, P_2, P_3 and P_4. This experiment was carried out about 14 days after the filaments were purified. During this time, endogenous proteinases that contaminated the preparation have degraded the polypeptides to a very small extent. This is seen in lane (*b*), the supernatant of the control, which shows some minor proteolytic fragments, one of which has a similar mobility to the trypsin-derived S_{11} band. The filaments in the pellet (lane *a*) are also slightly altered from those freshly prepared, there being less of the 158000-mol.wt. component than normal and an increase in intensity of the bands in the 40000–60000-mol.wt. region. The numbers at the side of the photograph represent 10^{-3} × mol.wt.

Table 1. *The molecular weight and origins of the major soluble proteolytic fragments produced after digestion of ox neurofilaments with trypsin and chymotrypsin*

The numbers of determinations are given in parentheses. Values are means ± S.D.

	10^{-3} × Mol.wt.	Origin of fragment
Tryptic fragments		
S_{11}	164 ± 4 (12)	Head-end of 205 000-mol.wt. polypeptide
S_{10}	152 ± 4 (8)	
S_8	127 ± 7 (13)	
$S_{7'}$	119 ± 2 (5)	Head-end of 158 000-mol.wt. polypeptide
S_7	107 ± 5 (11)	
S_6	97 ± 6 (12)	
Chymotryptic fragments		
M_1	171 ± 6 (10)	Head-end of 205 000-mol.wt. polypeptide
M_2	121 ± 4 (10)	Head-end of 158 000-mol.wt. polypeptide
M_3	119 ± 4 (7)	

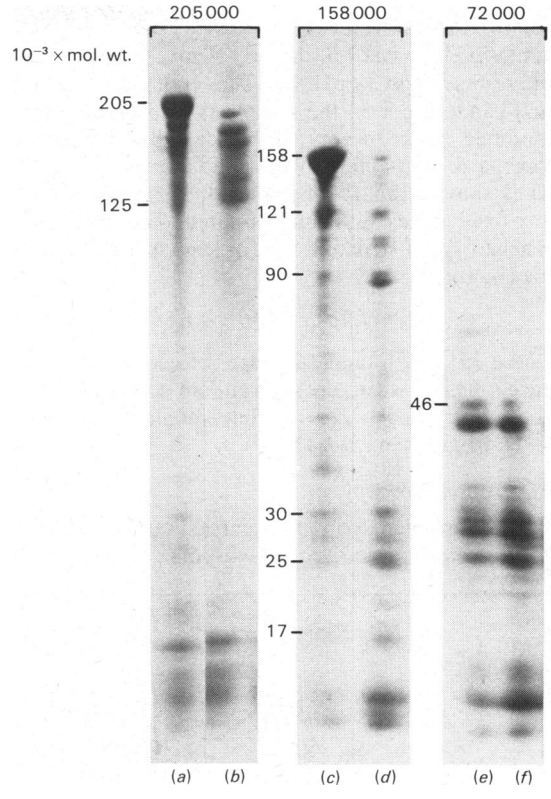

Fig. 2. *Peptide fingerprinting of the major polypeptides of ox neurofilaments*

Source gels were run of purified neurofilaments from which the 205 000-, 158 000- and 72 000-mol.wt. components were removed. These were then mapped with papain (0.5–5 ng/lane). The 205 000-mol.wt. polypeptide was mapped in lanes (*a*) and (*b*), the 158 000-mol.wt. polypeptide in lanes (*c*) and (*d*), and the 72 000-mol.wt. polypeptide in lanes (*e*) and (*f*). In lane (*b*) note the apparent accumulation of material at a mol.wt. of approx. 125 000 and the absence of strongly staining peptides in the 125 000–30 000-mol.wt. region. The gel was stained with Coomassie Blue and the numbers correspond to 10^{-3} × mol.wt.

previous study (Eagles *et al.*, 1981). Purified ox neurofilaments were first digested with trypsin for various times and the reactions were terminated with excess trypsin inhibitor. Then the samples were centrifuged to sediment filaments and the pellet and supernatant from this procedure were dissolved in SDS and run on polyacrylamide gels. At brief digestion times, a number of polypeptides appeared in the supernatant (S) and the pellet (P); therefore to help in the analysis these fragments were numbered (see Fig. 1).

The changes that occur in the supernatant fraction will be considered first. Initially a predominant band, S_{11}, appears and persists throughout the time course together with a much weaker band S_7', which is transient. Then as digestion proceeds many bands are formed (S_{10}–S_6). The molecular weights of these fragments indicate that the parent polypeptides from which they were derived must have been either the 205 000- or the 158 000-mol.wt. component. Moreover, band S_{11} has a molecular weight greater than 158 000 (Table 1), which suggests that it must contain a substantial contribution by fragments derived from the polypeptide with mol.wt. 205 000.

In an attempt to unravel the origin of the major components of these bands we used the technique of peptide fingerprinting. Fingerprinting with papain clearly demonstrates that the two higher-molecular-weight components of the triplet have substantially different peptide maps (see Fig. 2). Fingerprinting can therefore be used to help identify the origin of the fragments derived after trypsin digestion.

Generally maps of the 205 000-mol.wt. component show few peptides, the major ones being confined to regions corresponding to molecular weights between 205 000 and 125 000 and below about 30 000; the region between molecular-weight values of 125 000 and 30 000 was consistently devoid of strongly-staining peptides. Another characteristic feature of these maps, despite the variation caused by the extent of digestion, was the build-up of a peptide at a molecular weight corresponding to 125 000, suggesting the presence here of a relatively stable species (see Fig. 3). In contrast peptide maps of the 158 000-mol.wt. component showed a wealth of structural detail that was critically dependent on the extent of digestion. Two

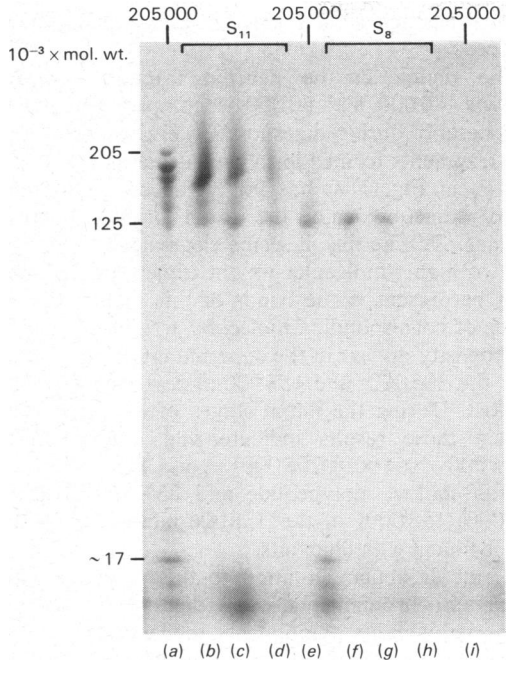

Fig. 3. *Peptide fingerprinting of bands S_{11} and S_8*
Bands S_{11} and S_8 were excised from gels similar to that shown in Fig. 1 and were fingerprinted with papain. Lanes (*a*), (*e*) and (*i*) correspond to maps of the 205000-mol.wt. components with increasing amounts of papain from (*a*)–(*i*). Lanes (*b*), (*c*) and (*d*) correspond to maps of S_{11} with increasing papain from (*b*)–(*d*) and lanes (*f*), (*g*) and (*h*) correspond to maps of S_8 with increasing papain loads. In all fingerprints note the build-up of the papain-resistant fragment at a mol.wt. of approx. 125000. The gel was stained with Coomassie Blue and the numbers correspond to 10^{-3} x mol.wt.

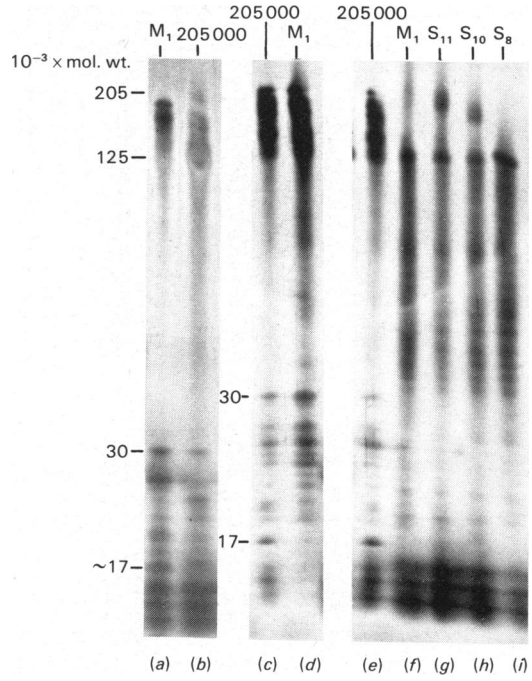

Fig. 4. *Peptide fingerprinting of bands M_1, S_{11}, S_{10} and S_8*
The chymotryptic-derived band M_1 and the tryptic-derived bands S_{11}, S_{10} and S_8 were excised from gels and fingerprinted with papain (0.5–5 ng/lane). Lanes (*b*), (*c*) and (*e*) correspond to maps of the 205000-mol.wt. component. Lanes (*a*), (*d*) and (*f*) are from band M_1 and (*g*), (*h*) and (*i*) are from bands S_{11}, S_{10} and S_8 respectively. Note the relatively stable peptide at mol.wt. approx. 125000. The gel was stained with silver (see the Materials and methods section) and the numbers given at the side of the photograph are 10^{-3} x mol.wt.

features of note in maps of this component were a strongly staining doublet of mol.wt. approx. 90000 and a peptide with mol.wt. 46000 (see, for example, Fig. 6, lanes *k* and *p*), both of which appear only after prolonged digestion.

Fig. 3 shows the results of fingerprinting some of the major bands liberated into the supernatant fraction after brief (5–10 min) digestion of neurofilaments with trypsin. Fingerprints of S_{11} are similar to peptide maps of the 205000-mol.wt. component. For example, they contain the papain-resistant fragment with mol.wt. 125000 and show no features characteristic of maps from the 158000-mol.wt. polypeptide, Therefore band S_{11} appears to be composed solely of a fragment from the 205000-mol.wt. component. By similar reasoning (see Figs. 3 and 4) bands S_{10} and S_8 also appear to be derived from fragments of this triplet polypeptide.

Bands S_7', S_7 and S_6, however, are derived from a different component. Figs. 5 and 6 show that these bands contain a substantial contribution by fragments derived from the 158000-mol.wt. component.

The data therefore indicate that under the conditions of digestion used here trypsin liberates large fractions of the 205000- and of the 158000-mol.wt. components and that the polypeptides are lost from the filament as soluble products. One obvious question to be answered is what happens to the remains of the parent polypeptides after these soluble fractions are released. Band S_{11} has a mol.wt. of approx. 164000 (Table 1) and as it is derived solely from the triplet component with mol.wt. 205000, a fragment of mol.wt. approx. 41000 should also be formed during digestion. In Fig. 1 (lanes *c*, *e* and *g*) it is seen that a band at near this molecular weight (band P_3 of mol.wt. approx.

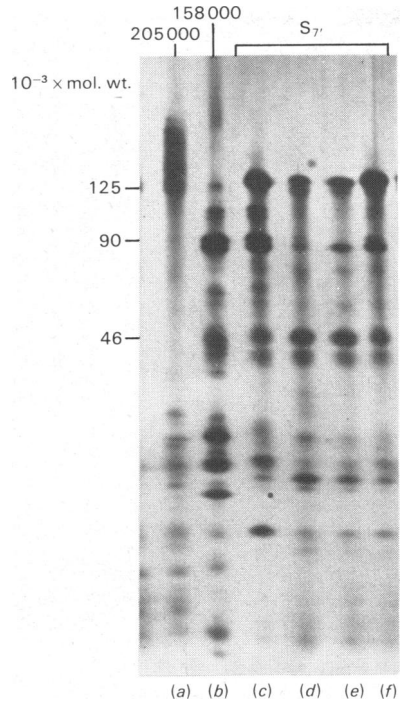

Fig. 5. *Peptide fingerprinting of band S_7'*
The tryptic-derived band S_7' was fingerprinted with papain together with controls. Lanes (*a*) and (*b*) are controls of the 205000- and 158000-mol.wt. components and lanes (*c*)–(*f*) are from band S_7' digested at different papain concentrations. Note the presence of the doublet with mol.wt. approx 90000 and 46000-mol.wt. peptide in the maps of S_7'. The gel was stained with silver.

44000) appears only in the pelleted fraction where undigested filaments are also found. Bands S_{10} and S_8 appear to be derived for the most part from S_{11} and therefore would not generate pelletable fragments. This can be seen in Fig. 1, where after digestion for about 20 min, when most of the parent polypeptide with mol.wt. 205000 has disappeared, band S_{11} decreases with a corresponding increase in S_8. The $S_{11} \rightarrow S_8$ transition can also be seen if the supernatant fraction (e.g., lane *d* in Fig. 1) is re-digested with trypsin.

A similar situation where part of the parent polypeptide is rendered soluble also explains the appearance of bands S_7', S_7 and S_6. These bands are derived mainly from the parent polypeptide with mol.wt. 158000 and they have mol.wts. of approx. 119000, 107000 and 97000 respectively (Table 1). The corresponding fragments from these cleavages would therefore have mol.wts. of approx. 39000, 51000 and 61000. Bands in this region (e.g. band P_2, mol.wt. 52000) are indeed generated in the pelleted fraction (Fig. 1, lanes *c* and *e*) as digestion proceeds.

The region in the pelleted fraction between mol.wts. 40000 and 60000 is complex and alters considerably during digestion. By fingerprinting the new fragments formed in this area (bands P_1, P_2, P_3 and P_4 in Fig. 1) it has not yet been possible to identify unequivocally the exact origin of these remains. Despite this, it seems clear that the ends of the two higher-molecular-weight triplet polypeptides must be present in the bands of this region, for no bands of corresponding molecular weight or matching intensity appear in the supernatant fraction until after the 205000- and 158000-mol.wt. polypeptides are lost. During the initial stages of digestion with trypsin these results indicate that approx. 20% [(205000 − 164000)/205000] of the original 205000-mol.wt. polypeptide and 25% [(158000 − 119000)/158000] of the 158000-mol.wt. polypeptide sediment with filaments.

A not dissimilar situation to this is found with *Myxicola* neurofilaments, where digestion of the two major polypeptide components renders about 30% of each pelletable in a filamentous form (Eagles *et al.*, 1981). By analogy with this previous study, we have termed the end of the triplet polypeptide that is liberated into the supernatant fraction the head and the opposite end, i.e. that which sediments together with normal filaments, the tail. To characterize further the head and tail regions we investigated the digestion of ox neurofilaments with another proteinase with different specificity.

The digestion of neurofilaments with α-chymotrypsin

A comparison of a digestion profile of ox neurofilaments with α-chymotrypsin and trypsin is represented in Fig. 7. There are some qualitative similarities between the two profiles. For example, digestion with either α-chymotrypsin or trypsin liberates high-molecular-weight polypeptides into the supernatant and, together with this process, lower-molecular-weight fragments (in the 40000–60000-mol.wt. region) are generated that remain with the pelleted filaments. The sizes of the fragments liberated into the supernatant by α-chymotrypsin digestion, however, differ from those produced by trypsin (Table 1) and there are also fewer of them. With α-chymotrypsin two major products (M_1 and M_2) are produced in the supernatant at brief digestion times (2–5 min) that have mol.wts. of 171000 (M_1) and 121000 (M_2) and they are apparently quite stable to further digestion. To determine the origin of the fragments that constitute M_1 and M_2, the bands were excised and subjected to peptide fingerprinting. The results of this study are shown in Figs. 4 and 6.

Fingerprints of M_1 (Fig. 4) show many features in common with maps of the 205000-mol.wt. compo-

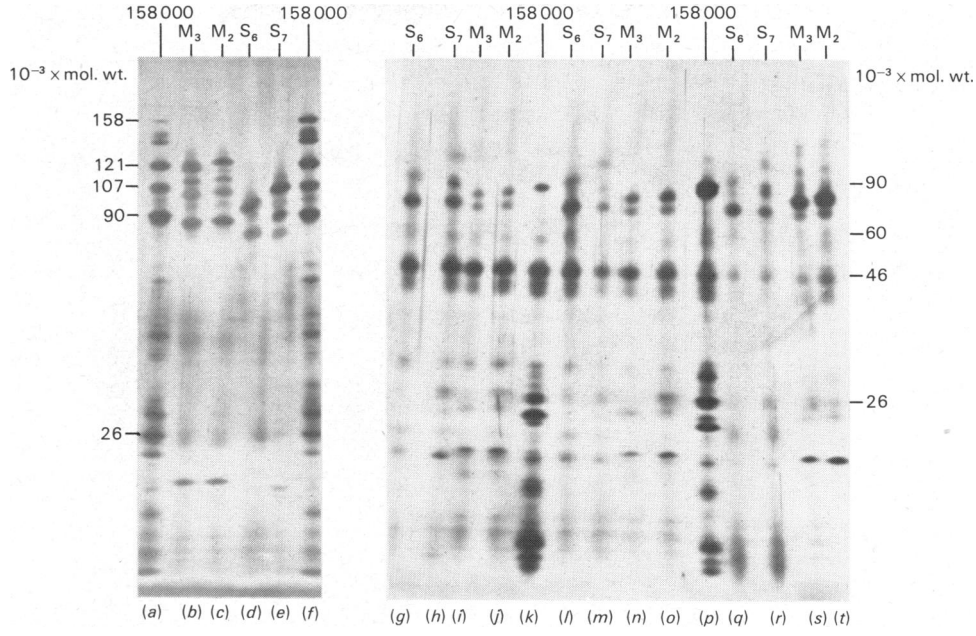

Fig. 6. *Peptide fingerprinting of bands M_2, M_3, S_6 and S_7*

The chymotryptic-derived bands M_2 and M_3 and the tryptic-derived bands S_6 and S_7 were excised from gels and fingerprinted with papain. Lanes (*a*), (*f*), (*k*) and (*p*) are controls of the 158000-mol.wt. component. Lanes (*c*), (*j*), (*o*) and (*t*) are from band M_2; lanes (*b*), (*i*), (*n*) and (*s*) are from band M_3; lanes (*d*), (*g*), (*l*) and (*q*) are from band S_6 and lanes (*e*), (*h*), (*m*) and (*r*) are from band S_7. All these samples were digested with different concentrations of papain (0.5–5 ng/lane). Note the presence of the 46000-mol.wt. species in lanes (*g*)–(*t*), and the similarity of fingerprints of S_7 and the 158000-mol.wt. component in the high-molecular-weight region (lanes *e* and *f*). The gel was stained with silver.

nent in both the high- and the low-molecular-weight regions (e.g. lane *d*). This, in addition to the fact that the molecular weight of the component is around 171000, greater than the 158000-mol.wt. component, leads us to conclude that M_1 must have originated from the largest of the triplet polypeptides. Fig. 4 also shows, as might be expected, that fingerprints of M_1 are similar to fingerprints of the tryptic fragments S_{11}, S_{10} and S_8. All these fingerprints have, for example, the papain-resistant polypeptide with mol.wt. 125000 and a number of smaller fragments in common.

The chymotryptic fragment M_2 has a mol.wt. of 121000 and Fig. 6 shows that it has a fingerprint in common with the 158000-mol.wt. polypeptide. Fragment M_2 also has a fingerprint similar to peptide maps of the tryptic fragments S_7', S_7 and S_6, suggesting that all these fragments may come from the same part of the parent polypeptide.

At digestion times of 10 min or longer, chymotrypsin often liberates a band just below M_2 (Fig. 7, lane *h*). This band, M_3, has a mol.wt. of approx. 119000. Fingerprints of band M_3 differ slightly from M_2 in the high-molecular-weight region (see Fig. 6). In this region the patterns from M_3 (Fig. 6, lane *b*) have similar intensities to those from M_2 (Fig. 6, lane *c*) but each peptide is displaced by the same amount, suggesting that papain attacks the opposite end of the molecule to that which was cleaved by chymotrypsin. In the low-molecular-weight region, peptide maps of M_3 and M_2 are indistinguishable (e.g. Fig. 6, lanes *i* and *j*).

Our results therefore indicate that the chymotryptic fragment M_1 is derived from the parent polypeptide with mol.wt. 205000 and that M_2 and M_3 are derived from the 158000-mol.wt. polypeptide. From the origin of these fragments one would expect that as they are formed, other peptides, representing the remaining parts of the parent molecules, would be produced that would have mol.wts. of 34000, 37000 and 39000 respectively. Bands of mol.wts. of approx. 38000–40000 are produced in the pelleted fraction during digestion (Fig. 7, lane *k*) and thus they are good candidates for these peptides.

In parallel with the nomenclature that we have used for the trypsin-derived fragments we have termed the soluble polypeptides M_1, M_2 and M_3 the chymotryptic head-regions of the parent molecules.

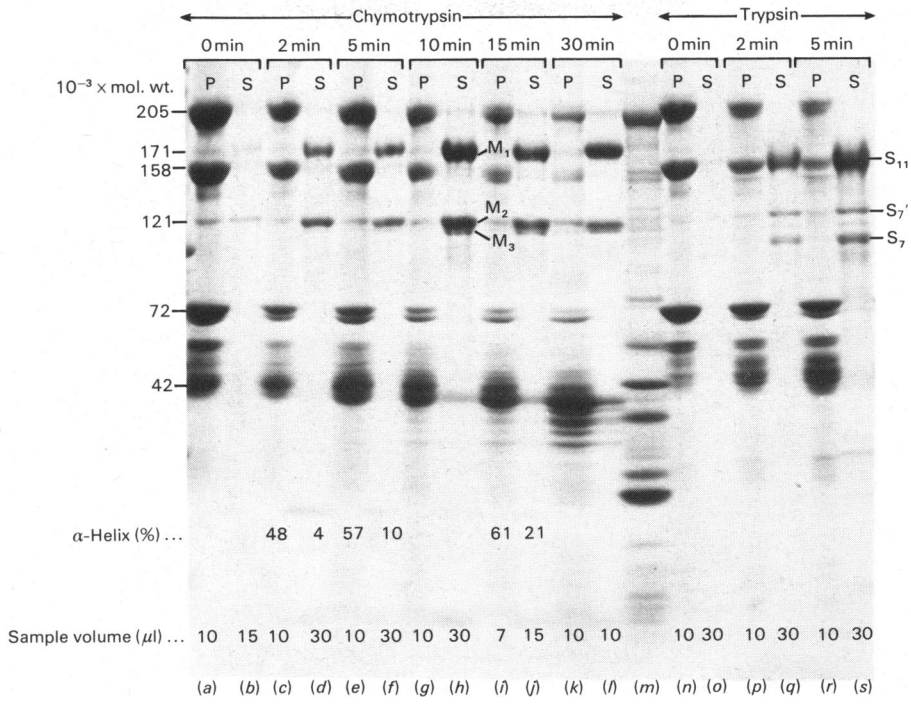

Fig. 7. *Comparison of the digestion profiles of ox neurofilaments with chymotrypsin and trypsin*
Filaments were prepared in a similar manner to that described in the legend to Fig. 1. For this gel, filaments were incubated with chymotrypsin (final concentration 3.1 μg/ml) or trypsin (final concentration 1.2 μg/ml) for the times indicated. The reactions were stopped with either PMSF or trypsin inhibitor. The samples were then spun and the precipitates (P) and supernatants (S) dissolved in SDS and run on the gel. Larger samples were also prepared by chymotryptic digestion that would yield enough material for α-helix measurements. Lanes (*c*)–(*l*) contain the samples digested with chymotrypsin and lanes (*p*)–(*s*) contain samples digested with trypsin. Lanes (*a*)–(*b*) and (*n*)–(*o*) were zero-time controls. Lane (*m*) contains various molecular-weight markers. The major soluble digestion products M_1 and M_2, derived by chymotryptic digestion, and the minor product M_3 are marked in lane (*h*). For the gel, all samples were prepared in a similar manner and the amount of each applied is indicated in μl.

Having defined the regions in this way, it is important to establish the extent of overlap between the chymotryptic head-regions and the tryptic head-regions that are obtained from the same parent polypeptide.

It is evident from the fingerprints of M_1 and S_{11} (Fig. 4) that they both contain the papain-resistant peptide with mol.wt. 125000 and they have extensive similarity below a mol.wt. of about 30000. These facts suggest that M_1 and S_{11} have at least 73% of their sequence in common and that they probably come from the same end of the 205000-mol.wt. parent molecule. Fingerprints of M_2, M_3, S_6, S_7 and S_7' are less easy to interpret in the high-molecular-weight region (Fig. 6, lanes *a–f*). However, prolonged papain digestion (Fig. 6, lanes *g–t*, and Fig. 5) shows that they all share a peptide with mol.wt. approx. 46000 and that the similarity extends to peptides having mol.wts. of 80000–90000 (see Fig. 5 and lanes *g*, *h*, *i* and *j* in Fig. 6).

In conclusion, the digestion pattern of ox neurofilaments with chymotrypsin shows qualitative similarities with the pattern produced by trypsin digestion. These are that, under the conditions used, the parent polypeptides with mol.wts. 205000 and 158000 are digested to yield soluble products that represent about three-quarters of the original molecules and the remaining insoluble fragments sediment with filaments. In addition, peptide fingerprinting of the soluble fragments shows that the chymotryptic- and tryptic-derived head-regions of the 205000-mol.wt. polypeptides have at least 73% homology and that the chymotryptic- and tryptic-derived head-regions of the 158000-mol.wt. polypeptide have more than 66% of their length in common. Together these facts suggest that each of the two higher-molecular-weight triplet components is attacked by trypsin and chymotrypsin to liberate soluble fragments that most probably come from the same end of the parent polypeptide.

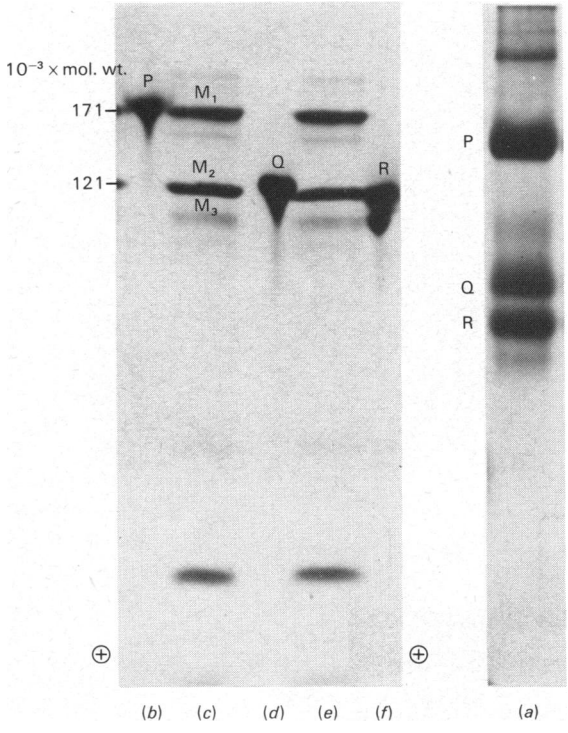

Fig. 8. *Analysis of the soluble chymotryptic fragments M_1, M_2 and M_3*

Filaments at a concentration of 10mg/ml were digested with chymotrypsin (3 μg/ml) for 10min and the reaction stopped with 24-fold excess (w/w) PMSF. Samples were then spun for 4h at 120000 *g* ($r_{av.}$ 6.5cm) and the supernatant removed. Of the supernatant 48 μg was run on a 9% acrylamide gel and stained with Coomassie Blue (lane *a*). The major bands in lane (*a*) are labelled P, Q and R. These bands were removed and analysed on a 5–15% polyacrylamide gel in the presence of SDS. Lane (*b*) corresponds to band P, lane (*d*) to band Q and lane (*f*) to band R. Lanes (*c*) and (*e*) are chymotryptic controls containing bands M_1, M_2 and M_3. It is clear that band P is composed solely of polypeptide M_1 and thus is derived from the 205000-mol.wt. component and bands Q and R are composed of polypeptide M_2, and M_2 and M_3 respectively and are therefore derived from the 158000-mol.wt. component.

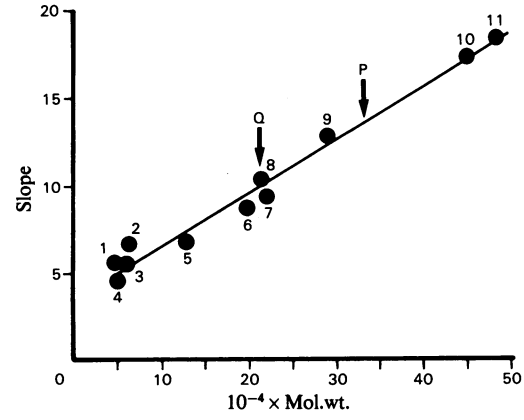

Fig. 9. *Graph of slope against molecular weight used to estimate native molecular weight of chymotryptic fragments P and Q*

Gels at polyacrylamide concentrations between 4 and 12% were run overnight with standard proteins and P and Q. After staining, the mobility of the proteins was measured and the log (relative mobility) for each protein was plotted against the acrylamide concentration. The slope obtained from this graph has been plotted here against the molecular weight of the protein (Hedrick & Smith, 1968). The standard proteins were: 1, triose phosphate isomerase; 2, albumin; 3, pepsin; 4, ovalbumin; 5, β-galactosidase; 6, phosphorylase *b*; 7, catalase; 8, β-amylase; 9, xanthine oxidase; 10, ferritin; 11, urease. The positions of the chymotryptic fragments P and Q are marked with arrows and in this particular experiment they had mobilities corresponding to mol.wts. of 330000 and 216000 respectively.

Analysis of the chymotryptic head-regions M_1, M_2 and M_3 on non-denaturing gels

In an attempt to determine more about the structure of the head-regions of the 205000- and 158000-mol.wt. polypeptides we subjected these soluble fractions to electrophoresis under non-denaturing conditions. Chymotryptic- rather than tryptic-derived fragments were used because of the smaller number of polypeptides formed. Lane (*a*) of Fig. 8 shows the results of such an experiment. Three major components labelled P, Q and R are visible under non-denaturing conditions and to determine their composition they were removed and run in a denaturing buffer system (Fig. 8, lanes *b*–*f*). It is seen that component P is composed of polypeptide M_1, Q is composed of polypeptide M_2 and that R is composed of M_2 and probably M_3. These results therefore suggest that the isolated head-regions derived from the parent polypeptides with mol.wts. 205000 and 158000 do not directly interact with each other, at least under the conditions used here.

Further evidence for the absence of a direct association between the head-regions of these two parent polypeptides comes from experiments where we have tried to cross-link the soluble chymotryptic fragments with copper phenanthroline. Under conditions where the 205000- and 158000-mol.wt. polypeptides were readily cross-linked in normal

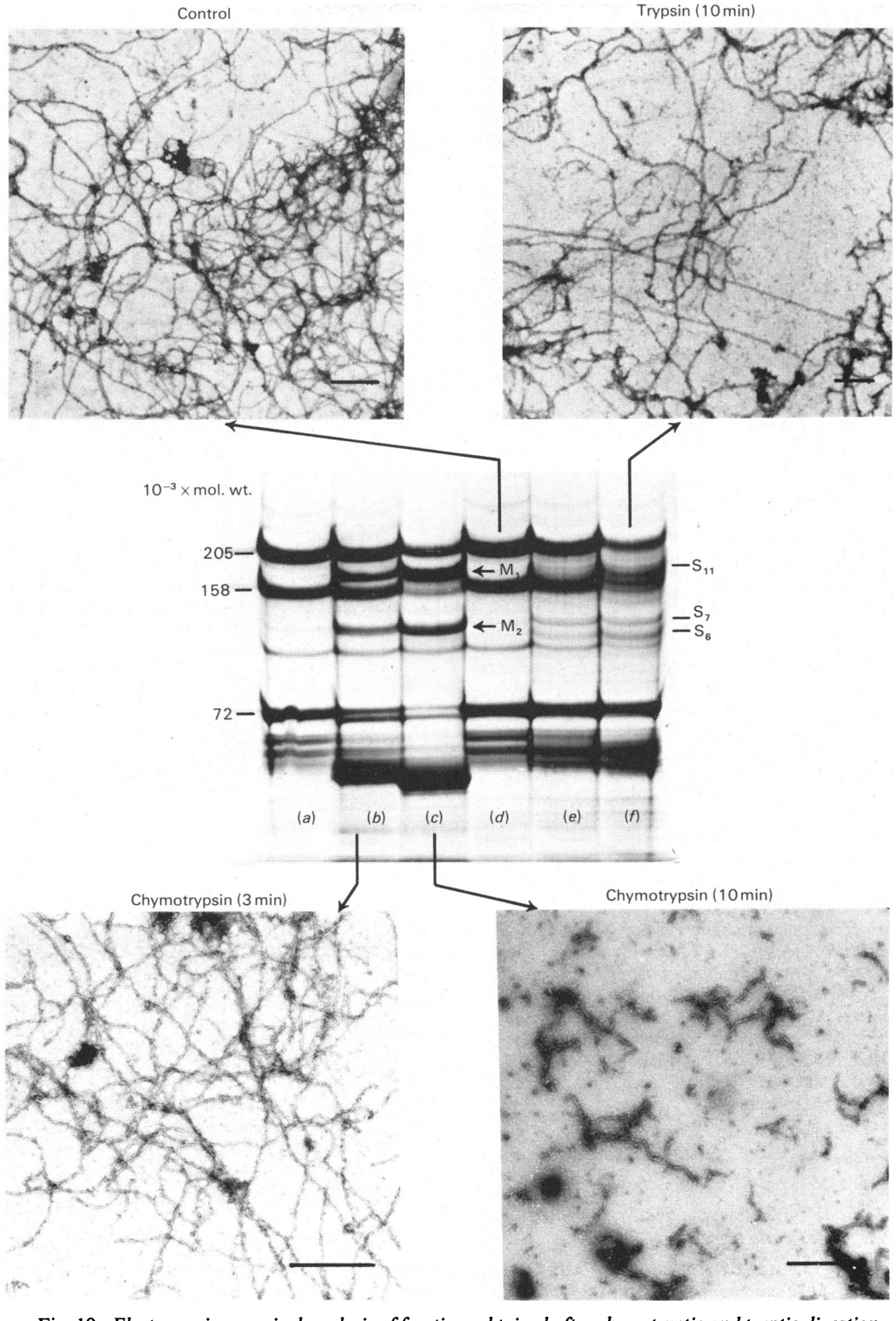

Fig. 10. *Electron-microscopical analysis of fractions obtained after chymotryptic and tryptic digestion*
Purified neurofilaments (0.5 mg/ml) were digested with chymotrypsin (10 μg/ml) and trypsin (10 μg/ml). The reactions were stopped after 3 min and 10 min with either PMSF or lima-bean trypsin inhibitor. Samples were then removed from the reaction mixture; for gel analysis they were dissolved in SDS and for electron microscopy they

filaments, no cross-linked material was obtained with the soluble chymotryptic fragments. Various attempts to obtain cross-linked complexes after treatment of the soluble fragments with glutaraldehyde have also been unsuccessful so far.

A number of studies were performed to investigate whether the chymotryptic head-regions from the same parent polypeptide represented monomeric species or polymeric complexes. Estimates for the native molecular weights of P, Q and R were obtained in non-denaturing gels (see Fig. 9). For P the molecular weight was calculated to be between 330000 and 336000, for Q between 216000 and 252000 and for R between 210000 and 246000. As the molecular weights of P, Q and R in SDS are about half these values, the results imply that the head-regions exist as homodimers under native conditions. The absence of any cross-linked complexes after treatment of the head-regions with glutaraldehyde, however, would argue against this proposal. Although these results, overall, indicate that the head-regions from the 205000- and 158000-mol.wt. polypeptides under native conditions are composed of either monomeric or dimeric species, more work is clearly needed to distinguish between these two possibilities.

The content of α-helix in the head- and tail-regions of the 205000- and 158000-mol.wt. polypeptides

In a number of digestion experiments the α-helix content of fractions was measured by using circular dichroism (see Figs. 1 and 7). In general, pelleted fractions after proteolytic digestion became very sticky and were difficult to disperse evenly. Even so, in all the experiments that were performed there was a consistent trend in that the α-helix content of the pellet increased as digestion proceeded. In these experiments values for the α-helix content of the pellet varied from 50 to 70%. At short digestion times when only the head-regions of the 205000- and 158000-mol.wt. polypeptides were present, measurement of the α-helix content of the supernatant fractions showed that they contained less than 20% α-helix.

These data indicate that the regions that are pelleted with filaments and that at least in part represent the tail-ends of the 205000- and 158000-mol.wt. polypeptides are areas relatively rich in α-helix.

Electron microscopy of trypsin- and chymotrypsin-digested filaments

The studies so far show that digestion of ox neurofilaments with either trypsin or chymotrypsin cleaves off soluble fragments of the 205000- and 158000-mol.wt. polypeptides, whereas the remaining parts sediment with filamentous material. The implication of this is that the soluble polypeptides that are removed are not needed to maintain the integrity of a filament. To explore this possibility further we examined samples by electron microscopy after digestion. Fig. 10 shows results from one of these experiments, where samples were digested for 3 min and 10 min with trypsin and chymotrypsin and subsequently analysed by electron microscopy and by SDS/polyacrylamide-gel electrophoresis.

From experiments like this it is seen that digestion with trypsin still results in the presence of filaments. Measurements of the lengths ($\geqslant 3\ \mu m$) and diameters ($10.4\ nm \pm 1\ nm$) of these digested filaments show that they differ little from undigested filaments. In this experiment (Fig. 10, lane *f*) analysis of the gel pattern shows that at least 70% of the original 205000-mol.wt. polypeptide has been lost after 10 min with trypsin. It therefore appears that the loss of the fragments S_{11}–S_8 does not alter considerably the appearance of the filaments under negative staining conditions.

A different picture is seen after digestion with chymotrypsin. In this case disruption of the normal filament structure occurs after short digestion times. One reason for this may be that chymotrypsin liberates larger fractions of the 205000- and 158000-mol.wt. polypeptides than does trypsin and that the extra regions are important in some way for the filament's integrity. Another reason may be that as digestion with chymotrypsin causes a more rapid removal of the 72000-mol.wt. component than digestion with trypsin (5% remaining after 10 min compared with 93%) the stability of filaments may be determined by the intactness of this polypeptide.

To help distinguish between these possibilities we

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were diluted (four to ten times) in 0.1 M-NaCl/10 mM-Tris/HCl, pH 7.0. The gel used was a minigel and is represented here at its actual size. It was 0.9 mm thick. Samples of 7 μl were applied to each lane and were as follows: lane (*a*), control; lanes (*b*) and (*c*), chymotryptic digestion for 3 min and 10 min respectively; lane (*d*), control; lanes (*e*) and (*f*), tryptic digestion for 3 min and 10 min respectively. The gel was run for 15 min at 450 V. Densitometry showed that after 10 min digestion with chymotrypsin 29% of the 205000- and 5% of the 72000-mol.wt. polypeptides remained and 27% of the 205000- and 93% of the 72000-mol.wt. polypeptide remained after using trypsin for 10 min. Some of the major proteolytic fragments are marked and the numbers at the side of the gel represent $10^{-3} \times$ mol.wt. For electron microscopy, the diluted samples were stained with uranyl acetate. Representative micrographs of the control, the tryptic digestion for 10 min and the chymotryptic digestion for 3 min and 10 min are shown. The bars on the micrographs correspond to 0.2 μm.

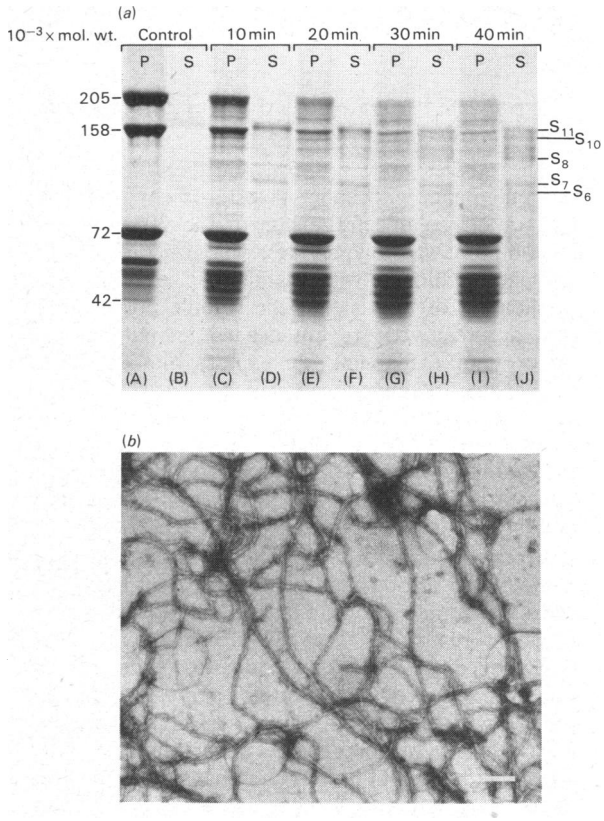

Fig. 11. *Digestion profile of ox neurofilaments with trypsin*

Purified neurofilaments (1–2 mg/ml) at 1 day after preparation were homogenized and digested at room temperature with trypsin (300 ng/300 μl of neurofilament suspension) for 10–40 min. The reactions were stopped with trypsin inhibitor at which time samples were taken for electron microscopy. The rest of the samples were spun at room temperature in an Airfuge at 140000 ***g*** for 1½h ($r_{av.}$ 1.32 mm). The pellets (P) and supernatant (S) were removed and run on the gel (*a*). Lanes (A) and (B) correspond to the control pellet and supernatant where inhibitor was added before trypsin. Lanes (C) and (D) correspond to the pellet and supernatant after 10 min digestion, lanes (E) and (F) after 20 min, lanes (G) and (H) after 30 min and lanes (I) and (J) after 40 min digestion. Comparison by densitometry of lanes (A) and (I) showed that after 40 min digestion >97% of the 205000-mol.wt. component and >93% of the 158000-mol.wt. component had been lost, whereas more than 70% of the 72000-mol.wt. component was still present. Electron microscopy of the original sample corresponding to lane (I) showed well-preserved filaments similar to those represented in (*b*). The filaments in (*b*), however, were obtained from a different experiment where SDS/polyacrylamide gels of the sample showed no detectable 205000- or 158000-mol.wt. polypeptides. The bar in (*b*) corresponds to 0.1 μm.

investigated how much of each of the 205000- and 158000-mol.wt. polypeptides could be removed while still maintaining an intact filament. Attempts to achieve this have centred around digesting neurofilaments with trypsin in the presence of sucrose, which helps to stabilize the filaments. Fig. 11(*a*) shows that digestion under these conditions results in preparations having more than 70% of the original 72000-mol.wt. component and having lost more than 93% of the two higher-mol.wt. polypeptides. Electron microscopy of these samples shows that they contain intact filaments. Similar experiments indicate that it is possible to retain filaments even though more than 99% of the original amount of the 205000- and 158000-mol.wt. polypeptides have been lost from them (Fig. 11*b*).

We interpret these studies to mean that a major factor responsible for the integrity of native neurofilaments is the presence of the 72000-mol.wt. polypeptide and that the backbone formed by these polypeptides may be additionally stabilized by tail-regions from the 205000- and 158000-mol.wt. components.

Discussion

Our results show that digestion of ox neurofilaments with trypsin releases soluble fractions from populations of the 205000- and 158000-mol.wt. components and that this loss does not, at least at low resolution, affect the structure of the filament. The arrangement of this population of polypeptides is presumably such that at least 75% of their mass is not directly involved in the filament backbone and a model depicting this is shown in Fig. 12. The head-regions of these molecules do not appear to interact with each other, at least under the conditions that we have used. The tail-regions, which represent the major binding areas of these polypeptides to the filament, account for about 25% of the mass of the parent polypeptide; there is evidence from cross-linking studies (Carden & Eagles, 1983) that these regions from the 205000- and 158000-mol.wt. components do interact in the intact filament.

Measurement of the α-helix content indicates that the head-regions have a relatively low amount (⩽20%), though further work is clearly needed to clarify the content of α-helix in the head-regions of each of the parent polypeptides. From our studies it might be expected that extensive proteolysis would remove all the head-regions, leaving behind tail-regions that together with fractions of the 72000-mol.wt. polypeptide would represent structures relatively rich in α-helices. This may be the explanation for the findings of Day (1980), who obtained X-ray patterns from a digested neurofilament prep-

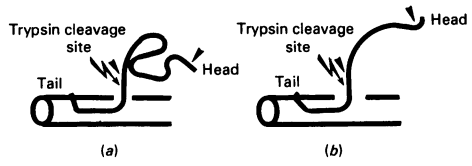

Fig. 12. *Diagrammatic representation of the positions of the proteinase-sensitive areas on the 205000- and 158000-mol.wt. polypeptides that are cleaved at brief digestion times*

The model shows a 205000-mol.wt. polypeptide (*a*) and a 158000-mol.wt. polypeptide (*b*) that are representative of the arrangements of a population of these polypeptides. Each polypeptide is depicted as a thick band with a tail embedded in the backbone of the filament and a head-end that is free. The actual amount of each polypeptide within the backbone is estimated at $\leqslant$25% of the molecule. An arrow indicates the approximate position of the major cleavage site with trypsin at brief digestion times which in (*a*) would liberate the S_{11} fragment and in (*b*) would liberate the S_7' fragment. Further cleavage sites (small arrowheads) near this point or at the extreme head-end of the polypeptides would generate S_{10} from S_{11} and probably S_7 and S_6 from S_7'. (The scheme shown here is not meant to imply that all the 205000- and 158000-mol.wt. polypeptides are initially cleaved by trypsin in this manner: their arrangement in the filament backbone may mean that some sites are less exposed and therefore less susceptible to attack than others so that a small population of 205000-mol.wt. polypeptides, for example, may be cleaved initially to produce fragment S_8 directly.) It is likely that the positions of the major cleavage sites depicted here for trypsin are not far removed from the positions of the chymotryptic sites that would produce the fragments M_1 and M_2. The head-regions of the 205000 and 158000-mol.wt. polypeptides do not appear to interact with each other but there is evidence that the tail-regions can be cross-linked (Carden & Eagles, 1983) and are therefore close together at the filament backbone. The shapes drawn for the head-regions should not be taken to imply the existence of any regular tertiary structure, though we have evidence from shadowing that under certain conditions they may assume a globular appearance.

aration that indicated reflections from coiled-coils of α-helices.

There is recent evidence suggesting that each neurofilament is composed of the three polypeptides and that each polypeptide is differently disposed in the filament (Willard & Simon, 1981; Sharp *et al.*, 1982). The 205000-mol.wt. component appears to have a peripheral location not dissimilar to that found for the 158000-mol.wt. component. The 72000-mol.wt. component is not peripheral, however, and probably constitutes a core or backbone. Moreover, reconstitution studies show that the 72000-mol.wt. polypeptide is competent to assemble into a filament that is capable of being decorated by the 205000-mol.wt. component (Geisler & Weber, 1981). The studies that we report here are basically in agreement with these ideas and in addition define the location of particular regions of the polypeptides in native neurofilaments.

It is noteworthy that compared with trypsin digestion, chymotrypsin causes rapid filament disruption. This may be because chymotrypsin liberates larger head-region polypeptides. However, as the 72000-mol.wt. polypeptide is extremely susceptible to digestion with chymotrypsin this could also contribute to the more rapid loss of filament structure during digestion with this proteinase.

Our studies indicate that the major parts of the 205000- and 158000-mol.wt. polypeptides, the head-regions, are peripheral to a central backbone. Such an arrangement may well cause these polypeptides to extend far beyond the normally accepted limits of the filament, and indications of this are seen after decorating neurofilaments with antibodies directed against these components (Willard & Simon, 1981; Sharp *et al.*, 1982). The peripheral nature of the major portion of these polypeptides might also explain the appearance of filaments after digestion that have similar diameters to normal filaments. Presumably the head-regions of the polypeptides in native filaments have low contrast under the staining conditions used here and are therefore not well visually detected. A similar situation to this is found with microtubule-associated proteins, which are thought to project like arms from the microtubule though they are only clearly seen in sectioned material (Amos, 1979) and by shadowing (Voter & Erickson, 1982). It is noteworthy in this respect that recent studies here show that head-regions, when shadowed, can assume a near-spherical structure approx. 25 nm in diameter, though more work is needed to elucidate the shape of these regions, which may well vary with conditions.

In our previous work it was apparent that contaminating proteinases were present in the ox neurofilament preparation that were responsible for digesting the 205000-mol.wt. component. Such digestion was only clearly detected after these preparations were centrifuged, when regions of this polypeptide were found to be soluble with mol.wts. of approx. 160000–170000. It is now apparent that digestion of the 205000-mol.wt. component with trypsin, chymotrypsin or papain also liberates soluble peptides in this molecular-weight range, suggesting that this molecule has a proteolytically susceptible area. Unless extreme care is taken to prevent proteolysis during the preparation and storage of ox filaments soluble fragments will thus be

produced from the 205 000-mol.wt. component that will run on SDS/polyacrylamide gels in the region of the 158 000-mol.wt. polypeptide. It is clearly important that the fragments that are derived from the 205 000-mol.wt. component are demonstrated to be absent from preparations against which antibodies are to be tested. Otherwise antibodies to this component may well show cross-reactivity to the 158 000-mol.wt. region. Moreover, as the tail-regions resulting from proteolysis stay attached to filaments it would not be surprising to find that antibodies to the two higher-molecular-weight triplet components would bind in the area of the gel where the tail-regions are found, and this has been observed by a number of workers (Shelanski & Liem, 1979; Anderton *et al.*, 1980; Autilio-Gambetti *et al.*, 1981; Dahl, 1981; Brown *et al.*, 1983).

It is interesting that the model that we have produced here, although preliminary, does share features with the model previously derived for another neurofilament, that from the marine fan worm *Myxicola* (Eagles *et al.*, 1980, 1981). In *Myxicola* the polypeptides are arranged such that if they are digested by the Ca^{2+}-activated proteinase then about 70% of their mass can be removed without destroying the filament, indicating that probably only about 30% of each polypeptide is needed to form the backbone.

It is possible that by studying two quite different types of neurofilaments, common features will emerge which will enable us to define the roles that particular areas might play. In particular we hope to elucidate the detailed structure of the head-regions of the polypeptides, which are clearly good candidates for being the cross-links that conjoin neurofilaments in the cytoskeleton (Wuerker, 1970; Wuerker & Kirkpatrick, 1972; Hirokawa, 1982).

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