Partial Purification of Neurofilament Subunits from Bovine Brains and Studies on Neurofilament Assembly

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ABSTRACT The 200,000-dalton neurofilament subunit (P200) and the 160,000-dalton (P160) and 78,000-dalton (P78) neurofilament subunits were partially purified from bovine brain. Intact neurofilaments were prepared by high-speed and sucrose-zone centrifugation. The crude neurofilament was solubilized in 8 M urea solution containing pyridine, formic acid, and 2-mercaptoethanol. The solubilized neurofilament was purified by carboxymethyl (CM) cellulose column and hydroxylapatite column chromatography. The P200 was purified as separate from P160 and P78, but the P160 and P78 subunits were copurified on CM cellulose, hydroxylapatite, Bio-Gel A150m, and Sephadex G-150 column chromatography. Electron microscopy of these purified neurofilament subunits revealed the P200 subunit as a globular structure, and the P160 and P78 subunits as a rod-shaped structure extending up to 120 nm with a 8- to 12-nm width. In the presence of 200 mM KCl, 15 mM $MgCl_2$, and 1 mM ATP, the purified subunits assembled into long filaments. Under the assembly condition, P160 and P78 subunits elongated up to 500 nm, but the longer filament formation required the presence of P200 subunits. The filaments formed in vitro were of two types: long straight filaments and intertwined knobby-type filaments. From these results, we have suggested that P160 and P78 form the neurofilament backbone structure and P200 facilitates the assembly of the backbone units into longer filaments.

The neurofilament is a 10-nm filament specific to neurons of the central and peripheral nervous system (5). The neurofilament from vertebrates seems to be composed of three unidentical polypeptide subunits of ~200,000, ~160,000 and ~68,000 daltons (2, 4, 7, 11, 13, 16). However, other 10-nm filaments present in nonneuronal cells and invertebrate neurons seem to be composed of only one or two subunits (5). Studies on the neurofilament have been mainly directed toward the identification and characterization of these subunits. Studies on the neurofilament-assembly mechanism require purified individual neurofilament subunits. Recently, Willard et al. (20) purified 200,000-dalton neurofilament subunit from rabbit and others have used individual neurofilament subunit polypeptides eluted from polyacrylamide gel slabs for immunological studies. Neurofilament subunits obtained in these ways may not be suitable for studies of neurofilament assembly in terms of both quality and/or quantity.

We have made efforts to purify neurofilament subunits from calf brain and report in this communication the methods for solubilization of neurofilament, the partial purification of subunits, and the results of studies of their assembly.

MATERIALS AND METHODS

Preparation of Neurofilaments

Crude neurofilament was prepared by use of a modified procedure of Runge et al. (15) and Mori and Kurokawa (13). Fresh bovine brains were obtained from a local beef purveyor and homogenized for 2 min in a Waring blender in 1 vol (wt/vol) of 0.1 M MES (2[*N*-morpholino]ethane sulfonic acid) buffer, pH 6.6, containing 0.5 mM $MgCl_2$ and 5 mM EGTA (buffer A). For routine preparation, we used three brains with a total weight of about 1 kg. The homogenate was centrifuged twice, first at 5,000 rpm for 5 min and then for 10 min at 10,000 rpm in a Sorvall GSA rotor (DuPont Company, Biomedical Products Div., Wilmington, Del.). The supernate was centrifuged at 35,000 rpm for 1 h in a Beckman 45 Ti rotor (Beckman Instruments, Inc., Spinco Div., Palo Alto, Calif.). The resultant supernate was centrifuged at 40,000 rpm in the same rotor for 1 h. The resulting pellets were suspended in buffer B, which contained 0.01 M MES pH 6.4, 0.1 M NaCl, 0.5 mM EDTA, and 5 mM EGTA to make up a total volume of 20–30 ml. This suspension was divided into aliquots of ~7 ml. Each aliquot was layered on a sucrose gradient made from three 5-ml solutions of 2.2 M, 1 M, and 0.5 M

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sucrose in buffer B. The sucrose gradient tubes were centifuged for 90 min at 40,000 rpm in a Beckman 60 Ti rotor. The interface formed between 2.2 M and 1 M sucrose was used for further purification.

Solubilization of Neurofilament

The interface formed between 2.2 M and 1 M sucrose was dissolved in an 8 M urea solution (3) containing 0.8 ml of pyridine, 4.8 ml of formic acid, and 1 ml of mercaptoethanol per liter of solution (buffer C) to make up a final volume of 20 ml. The sample was dialyzed against buffer C with three buffer changes.

Purification of Neurofilament Subunits

CARBOXYMETHYL (CM) CELLULOSE COLUMN CHROMATOGRAPHY: CM cellulose column chromatography was performed according to the published method of Hindennach et al. (3). The column (2.5 × 40 cm) was equilibrated with buffer C. Sample protein (0.4–1.0 g) was dialyzed against the same buffer and then loaded onto the column at a flow rate of 10 ml/h. Proteins were eluted by use of a linear gradient consisting of 400 ml of buffer C and 400 ml of 8 M urea containing 48 ml of pyridine, 19.2 ml of formic acid, and 1 ml of mercaptoethanol per liter. 3-ml fractions were collected. Every other fraction was analyzed by SDS polyacrylamide gel electrophoresis (SDS PAGE).

HYDROXYLAPATITE COLUMN CHROMATOGRAPHY: Hydroxylapatite (Bio-Rad Laboratories, Richmond, Calif.) was suspended in buffer C containing 0.01 M potassium phosphate, pH 7.0 (buffer D). After removal of fine particles by settling and decanting the buffer, the suspension was packed into a 1.2 × 17 cm column and equilibrated with buffer D. After sample protein (1–10 mg) was dialyzed against buffer D, it was loaded onto the column at a flow rate of 5 ml/h. Proteins were eluted with a linear gradient consisting of 50 ml of buffer D and 50 ml of buffer D containing 0.6 M potassium phosphate, pH 7.0. 2-ml fractions were collected and every other one was analyzed by SDS PAGE.

PAGE and Molecular Weight Determination

For analysis of polypeptides, SDS slab gels with a straight 7% acrylamide separation gel and 3% acrylamide stacking gel were used. Details have been described elsewhere (12). The molecular weights of the neurofilament subunits were estimated by comparing their electrophoretic mobilities with known molecular weight standards as described by Weber and Osborn (19) except that a 5–20% polyacrylamide gradient SDS gel slab electrophoresis was used (12). We found that in the gradient gel the electrophoretic mobilities were directly proportional to the molecular weights in the range of 200,000–43,000, as shown in Fig. 1. Myosin (200,000), β-galactosidase (130,000), phosphorylase *b* (98,000), bovine serum albumin (BSA, 68,000), and ovalbumin (43,000), obtained from Bio-Rad Laboratories, were used as protein standards.

Neurofilament Assembly

Incubation mixtures to study neurofilament assembly contained, in a total volume of 200 μl, 11–16 μg of P200, 16–39 μg of P160 and P78, 10 mM MES (pH 6.4), 200 mM KCl, 15 mM $MgCl_2$, and 1 mM ATP. The reaction mixture was incubated for 30 min at 37°C and then observed by EM.

The stoichiometry of three neurofilament subunits in the filaments assembled in vitro was determined as follows: purified P160 and P78 (260 μg/ml) and P200 (110 μg/ml) were centrifuged at 30,000 rpm for 1 h in a Beckman 75 Ti rotor to remove aggregates. The resulting supernates (with P160 and P78 at a new concentration of 160 μg/ml and P200 at 110 μg/ml) were incubated in 1-ml volume as described above for assembly. The incubations were centrifuged for 90 min at 40,000 rpm in a 75 Ti rotor on two layers of preformed sucrose zones consisting of 2 ml of 1 M sucrose and 2 ml of 0.5 M sucrose in buffer B. The pellets were dissolved in 50 μl of sample buffer for gel electrophoresis and subjected to SDS gel electrophoresis on a 7% polyacrylamide gel. Protein bands were stained with Coomassie (R-250) Brilliant Blue and densitometer tracings were made using Zeineh soft laser scanning densitometer. From the area of each protein peak, relative amount of protein was determined.

Electron Microscopy

NEGATIVE STAINING: Carbon films for negative staining were prepared on freshly cleaved mica and transferred to copper grids (300–400 mesh). Before the application of the sample, the carbon-coated copper grids were glow-discharged. The sample was applied onto the film by use of a 15-μl Eppendorf pipette (Brinkmann Instruments, Inc., Westbury, N. Y.). After 60 s, the grid was drained with filter paper and rinsed with three drops of glass-distilled water. Staining was accomplished by the sequential addition of five drops of 2% uranyl acetate, with the last drop remaining on the grid for 40 s. The stain was removed with filter paper and the grid was air-dried before examination under either a Philips 300 electron microscope at 80 kV or a Hitachi HU-11E at 75 kV. Micrographs were taken at magnifications of × 28,000–70,000 on the Philips 300 or × 22,000–43,000 on the Hitachi HU-11E.

PROTEIN DETERMINATION: Protein determination was carried out by the method of Lowry et al. (8) or Bradford (1). Unless stated otherwise, all materials were obtained from commercial sources.

RESULTS

Preparation of Neurofilament-enriched Fraction

Our strategy for the purification of neurofilament subunits from calf brain was to first purify intact neurofilaments; second,

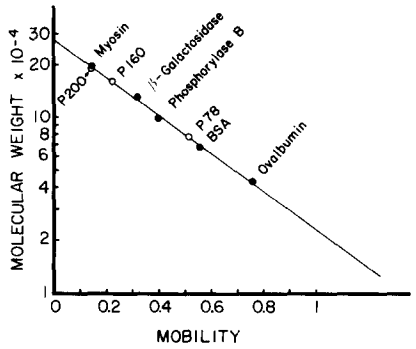


FIGURE 1 Estimation of molecular weights by SDS PAGE. Details of experimental conditions are described in Materials and Methods. 1 μg each of myosin (200,000), β-galactosidase (130,000), phosphorylase *b* (98,000), bovine serum albumin (BSA, 68,000), and ovalbumin (43,000) were used as protein standards. P200: 200,000 mol wt neurofilament subunit; P160: 160,000 mol wt neurofilament subunit; P78: 78,000 mol wt neurofilament subunit.

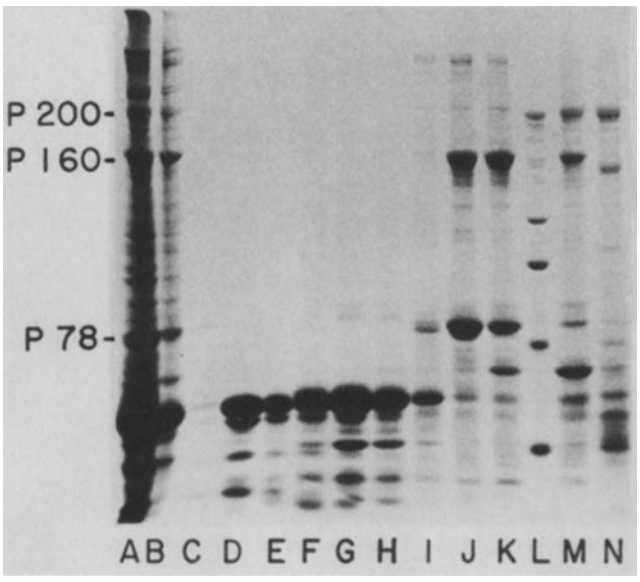


FIGURE 2 Electrophoretic pattern of protein fractions from the purification steps. SDS PAGE performed as described in Materials and Methods. Columns *A* (60 μg of protein) and *B* (20 μg of protein): electrophoretic pattern of neurofilament-enriched fractions from the 1–2.2 M sucrose interface. Columns *C–I*: proteins eluted before the neurofilament subunits. Columns *J* and *K*: some of the P160- and P78-rich fractions from CM cellulose column. Column *L*: myosin (200,000), β-galactosidase (130,000), phosphorylase *b* (98,000), BSA (68,000), and ovalbumin (43,000) (expressed in daltons). Columns *M* and *N*: some of the P200-rich fractions from CM cellulose column. 30-μl aliquots taken from each fraction were analyzed by SDS electrophoresis.

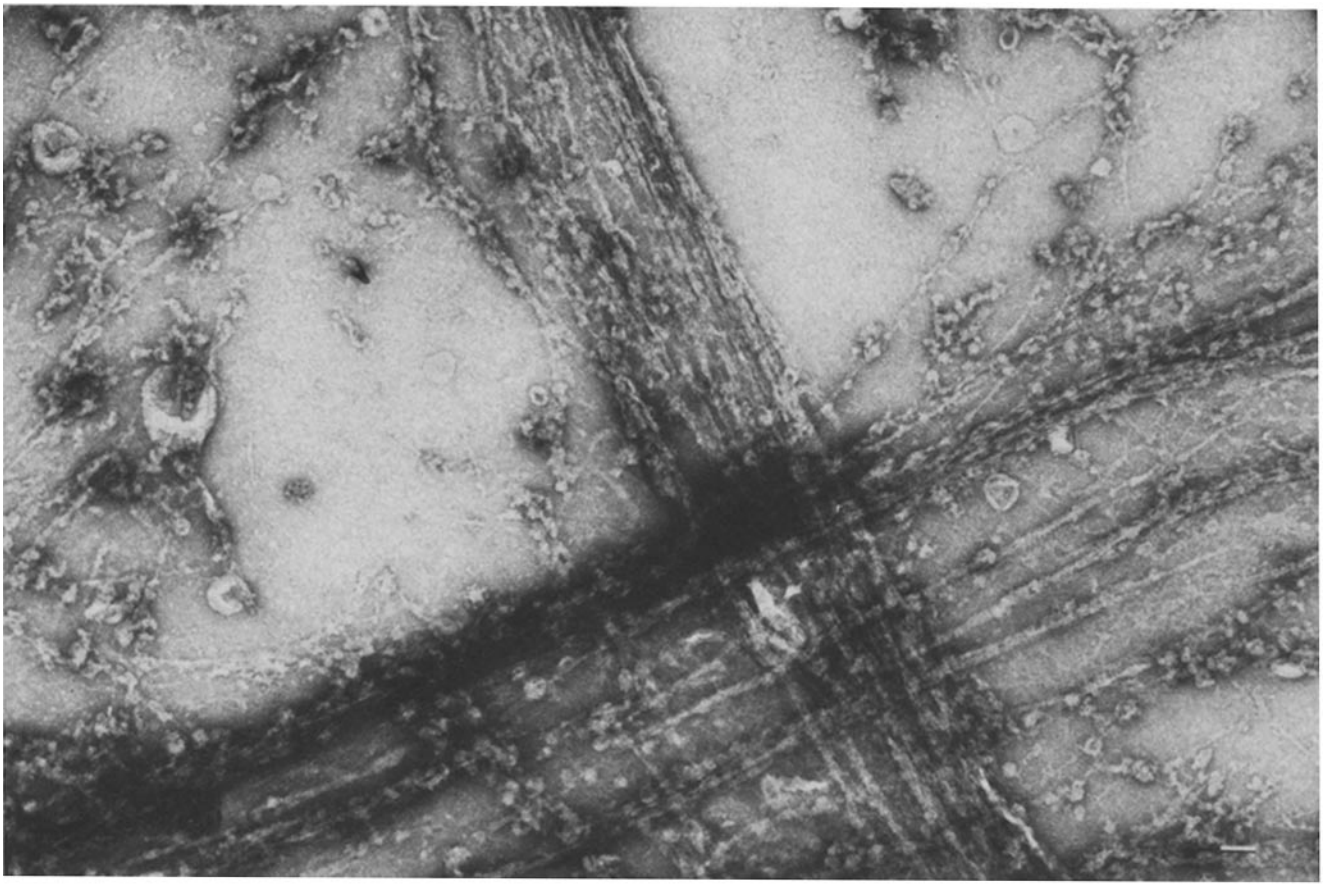


FIGURE 3 Electron micrograph of crude neurofilament preparation. Fraction from the 1–2.2 M sucrose interface suspended in buffer A. A drop of sample was used for negative staining as described in Materials and Methods. Bar, 0.1 μm. × 45,000.

to solubilize the relatively clean neurofilament into subunits; and, finally, to purify the subunits. To isolate the crude neurofilament, we used a combination of high-speed centrifugation (15) and sucrose-zone centrifugation (13). The methods allowed for a large-scale preparation to be completed in a relatively short period of time. Molecular weights of neurofilament subunits determined by SDS gel electrophoresis (12, 19) are 200,000 (P200), 160,000 (P160), and 78,000 (P78) (Fig. 1). The molecular weight of the smallest subunit seems to be somewhat larger than the molecular weight reported in the literature. The reason for this is not clear at this moment, but it could be a result of different gel electrophoretic conditions. Lanes *A* and *B* of Fig. 2 show the polyacrylamide gel electrophoresis pattern of the crude neurofilament preparation obtained by high-speed centrifugation and sucrose-zone centrifugation. In addition to neurofilament triplet bands (P200, P160, and P78), there are numerous contaminating protein bands. Among the contaminating protein bands are a 41,000 mol wt protein (presumably actin), a 55,000 mol wt protein (presumably tubulin), a 63,000 mol wt protein, and polypeptides of ~300,000 mol wt. These proteins were cochromatographed with the neurofilament subunits, even in the Bio-Gel A-150m column. Therefore, further purification of the crude neurofilament fraction on the basis of the size difference of proteins did not seem possible. Furthermore, the P200 band preferentially disappeared as it passed through the Bio-Gel A-150m column, presumably because of solubilization (6, 20) and/or proteolysis (17).

Numerous filamentous structures were observed singly and in bundles by electron microscopy of the negatively stained preparation (Fig. 3). Long filaments 8–22 nm in diameter were seen on numerous squares of the grid. Contaminating cellular

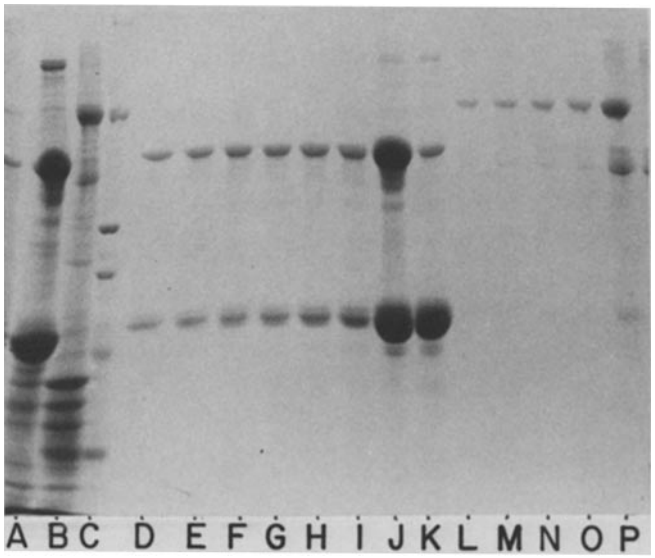


FIGURE 4 Electrophoretic pattern of neurofilament protein from hydroxylapatite chromatography. SDS PAGE was performed as described in Materials and Methods. *A*: P160- and P78-rich fractions (30 μg of protein) pooled from CM cellulose column chromatography. *B*: P200-rich fractions (25 μg of protein) pooled from CM cellulose column chromatography. *C*: protein standards, myosin (200,000), β-galactosidase (130,000), phosphorylase *b* (98,000), BSA (68,000), and ovalbumin (43,000) (expressed in daltons). Columns *D–K*: P160- and P78-rich fractions from hydroxylapatite column chromatography. Columns *L–P*: P200-rich fractions from hydroxylapatite column chromatography. 30-μl aliquots taken from each fraction were analyzed by SDS electrophoresis.

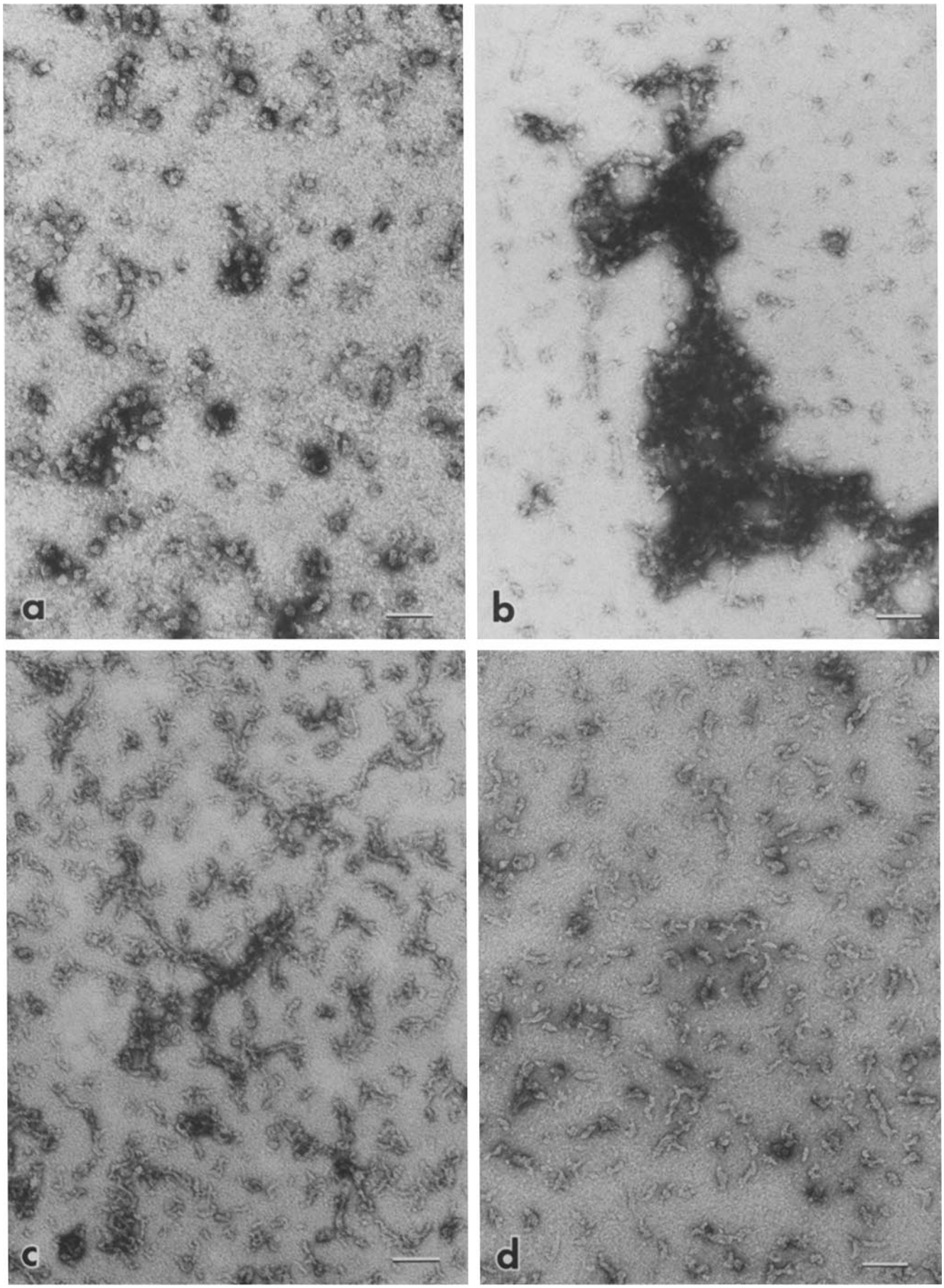


FIGURE 5 Electron micrographs of purified neurofilament peptides. Samples were negatively stained for EM as described in Materials and Methods. *a*: P200 subunits in urea buffer from hydroxylapatite column chromatography. *b*: same samples dialyzed against buffer E. *c*: P160 and P78 subunits in urea buffer from hydroxylapatite column. *d*: same samples dialyzed against buffer E. Bars, 0.1 μm. × 86,000.

debris adhered to the filaments and was also distributed across the background.

Solubilization of Neurofilament

It has been reported that neurofilaments are partially soluble in low-salt buffer (6). Using this property, Willard et al. (20) purified P200 subunit from rabbit brain. However, the other two subunits have not been purified this way, probably because of their low level of solubility. Therefore, we selected 8 M urea solution containing pyridine, formic acid, and β-mercaptoethanol. This solution yielded a clear solution of neurofilament up to 20 mg of protein/ml.

CM Cellulose Column Chromatography

When an 800-ml gradient was used to elute protein from the CM cellulose column (2.5 × 40 cm), both P160 and P78 were eluted between 420 ml and 450 ml and P200 was eluted between 456 ml and 500 ml. SDS gel electrophoresis analysis of the column fractions is shown in Fig. 2. Lanes *C–I* in Fig. 2 represent early fractions containing contaminants, lanes *J* and *K* contain some of the P160- and P78-rich fractions, and lanes *M* and *N* show the P200-rich fractions. Lane *L* in Fig. 2 shows the electrophoretic pattern of protein standards. Using protein standards as references and comparing the protein bands seen in crude neurofilament sample with those seen in CM cellulose fractions, we were able to identify neurofilament subunits. This column separated out most of the 55,000 mol wt protein, 41,000 mol wt protein and many other proteins. P160- and P78-rich fractions were contaminated mainly with a 62,000 mol wt protein, and P200 was contaminated with a substantial amount of 62,000 mol wt protein and other smaller molecular weight proteins.

Hydroxylapatite Column Chromatography

Each of the two pooled fractions from the CM cellulose column was further purified on a hydroxylapatite column. The hydroxylapatite column (1.2 × 17 cm) was eluted with a 100-ml potassium phosphate buffer concentration gradient. Typically, both P200 and P160 and P78 started to elute from 50 ml and trailed up to the last fraction. Lanes *A* and *B* in Fig. 4 represent the pooled P160- and P78-rich CM cellulose column fractions and the pooled P200 CM cellulose column fractions, respectively. Comparing these with the fractions eluted from the hydroxylapatite columns, we found it clear that most of the contaminating protein was removed. Lanes *D–K* in Fig. 4 represent P160- and P78-rich fractions and lanes *L* through *P* represent P200-rich fractions. Some relatively minor impurities were still present. Purities estimated from the densitometer tracings of each band showed that P200 is at least 90% pure and the P160 and P78 fraction is >80% pure.

One precaution should be taken in the hydroxylapatite column step: the protein sample should not be kept too long in the pH 7 phosphate buffer. After several days, there was indication that P78 was being modified, probably by cyanate, leading to changes in electrophoretic mobility. One additional protein band moving slightly slower than P78 (Fig. 4, lanes *D–K*) had not been seen in the fraction from the CM cellulose column (Fig. 2, lanes *J* and *K*) but did appear as the sample eluted from the hydroxylapatite column was kept in the same buffer for several days.

Electron Microscope Observation of Subunits

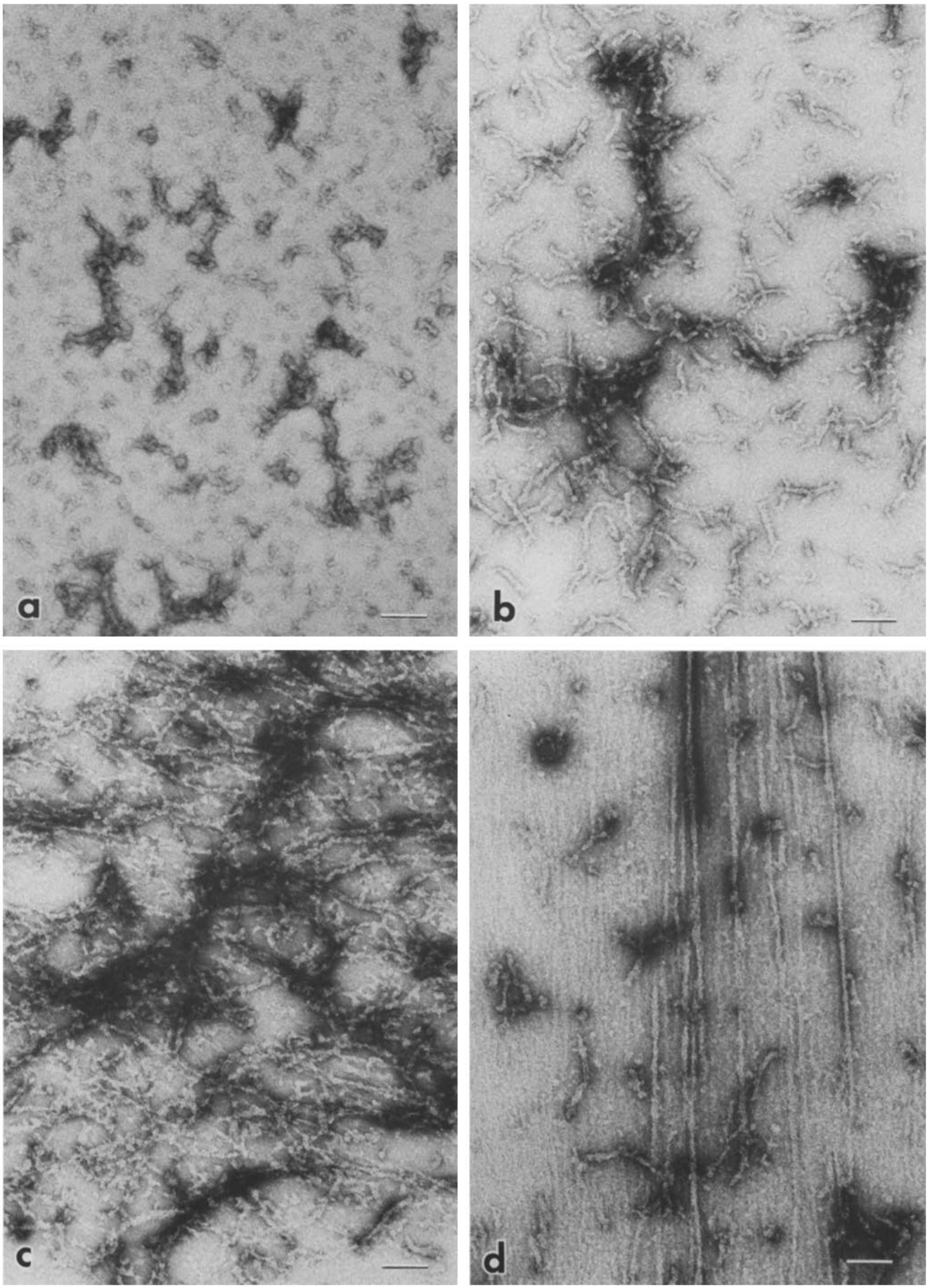
Electron microscopy of the pooled P200 polypeptide fractions eluted from the hydroxylapatite column in buffer C revealed mainly 15- to 30-nm smooth spheres, distributed across the grid singly or in small aggregates (Fig. 5 *a*). After removal of the urea by dialysis with 20 mM MES buffer, pH 6.6, with 10 mM mercaptoethanol (buffer E), these spheres had an irregular outline and formed large aggregates (Fig. 5 *b*). A few rods up to 75 nm in length and 10–20 nm in diameter were now revealed.

When the P160-P78 fractions were examined with urea present, three different structures were seen (Fig. 5 *c*). Rings measuring 22–30 nm and 45–50 nm were observed. The wall diameters were 9–12 nm in each case but the electron-dense centers were 4–6 or 12–15 nm. The second element apparently comprised broken rings 40–60 nm long and 8–12 nm wide. Finally, there were branched short filaments in which each branch was 35–60 nm long and 8–12 nm wide. After the removal of the urea, 20- to 120-nm-long and 8- to 12-nm-wide straight filaments (Fig. 5 *d*) were observed, some of which were tangled randomly. This suggests that the ring structure, seen in the presence of urea, was attributed to the denaturing conditions.

Neurofilament Assembly

Previously, 10-nm filament subunits prepared from BHK-21 (22), glial (9, 14), and squid (23) cells have been successfully assembled into intermediate filaments. When low-salt buffer was used, GTP was required (9). When assembly conditions included high-salt buffer, GTP was not needed (14, 22, 23). In accordance with these results, we made efforts to assemble partially purified neurofilament subunits. The assembly was monitored by EM, as the optical density increase at 300 nm could not be applied in our assembly condition. An assembly mixture containing 10 mM MES (pH 6.4), 200 mM KCl, 15 mM MgCl, and 1 mM ATP was best. As sources of monovalent cation, KCl, NaCl, and $(NH_4)_2SO_4$ were individually tested. Of these, KCl stimulated the largest amount of growth. NaCl worked to some extent; however, with NaCl, more nonspecific clumping of protein was observed. ATP was used throughout the experiment, although GTP worked as well. Without ATP or GTP and without $MgCl_2$, we observed only small filament fragments. To find out the optimum concentration of each component, we started from a low concentration and gradually increased each amount until the best assembly was seen. However, because electron microscopy is not a good quantitative measure of filament formation, these observations of optimum may be inaccurate.

FIGURE 6 In vitro assembly of purified neurofilament peptides. Conditions for assembly are described in Materials and Methods. Negative staining for EM was performed as described in Materials and Methods. *a*: P200 subunits after incubation in assembly buffer. *b*: P160 and P78 subunits after incubation in assembly buffer. *c* and *d*: P200, P160, and P78 after incubation in assembly buffer. Two structures are routinely seen. Bars, 0.1 μm. × 86,000.


a
b
c
d

P200 after incubation in assembly buffer revealed 15- to 30-nm spheres (Fig. 6 *a*) singly and as 40- to 400-nm-long aggregates. After incubation of the P160-P78 polypeptides in assembly buffer, a definite elongation of 8- to 12-nm filaments from 20–120 nm (Fig. 5 *d*) to 75–500 nm (Fig. 6 *b*) was observed. These filaments have smooth sides, tend to branch into arms, and form intertwining aggregates.

Structures similar to those described above were observed when the P200 and P160-P78 polypeptides were mixed and incubated in assembly buffer. Two other structures were routinely seen. The first, 8- to 12-nm intertwining filaments (Fig. 6 *c*), had apparent knobs adhering to them. The end of the individual filaments was difficult to discern. These filaments were very similar to some of the filaments observed in the crude preparation before solubilization (Fig. 4). The second structure, long, straight, 8- to 12-nm filaments, extended across the squares in an oriented direction (Fig. 6 *d*). They were seen singly and in parallel rows of two filaments lying next to each other measuring 22 nm in diameter. A comparison of these assembled filaments and those observed in the crude preparation reveals virtually no morphological difference in structure except for the contaminating cellular debris in the crude preparation.

The stoichiometry of neurofilament subunits in the assembled filament has been estimated from densitometer tracings of electrophoretic pattern of the filament assembled in vitro. As shown in the Table I, the ratio of P78:P160:P200 in the assembled filament is 2.4:2:1.

DISCUSSION

The three individual subunits of neurofilament protein have never been purified. The major reason for this seems to be that neurofilament from mammalian sources is relatively insoluble under mild conditions. Therefore, we had to choose 8 M urea solution containing pyridine and formic acid. One main objection, however, to the use of urea has been its cyanate impurity, causing carbamylation of the lysine groups of protein. At acidic pH, however, the modification of NH_2 groups should be minimal. The pH of our urea solution was <4. Further, it is well known that protein conformation is a function of its primary structure. Therefore, if the primary structure of any given protein molecule is preserved with no modification of amino acids, some of the polypeptide molecule should go back to its original conformation upon removal of denaturing agent.

TABLE I

Stoichiometry of Neurofilament Subunits in the Assembled Filament

Subunits	Area of densitometer tracing	Area/mol wt	Normalized ratio
	cm^3		
P78	169	2.17	2.4
P160	288	1.80	2
P200	179	0.90	1

P160 and P78 (160 μg) and P200 (110 μg) were incubated under the filament assembly condition for 30 min. The reaction mixture was centrifuged on a sucrose gradient as described in Materials and Methods. The resulting pellets were dissolved in 50 μl of sample buffer for gel electrophoresis. 35-μl aliquots were electrophoresed on a 7% gel. The relative amount of each neurofilament subunit was estimated from the density of the Coomassie Brilliant Blue-stained bands. Controls containing 110 μg of P200 or 160 μg of P160 and P78 were similarly analyzed. The relative amount of each control was subtracted from its corresponding complete incubation. These values were divided by the molecular weight factors of 200, 160, and 78 to obtain the relative ratio of each subunit in the assembled filament.

The 8 M urea with pyridine, formic acid, and β-mercaptoethanol did not seem to completely solubilize neurofilament into subunits. The largest subunit (P200) was completely separated from P160 and P78 by the CM cellulose column. However, P160 and P78 were not separated either by the ion-exchange column (CM cellulose) or the adsorption column (hydroxylapatite), or by gel filtration on the Sephadex G-150 column (data not presented). Furthermore, the two subunits (P160 and P78) were cochromatographed on a Bio-Gel A150m column and eluted in the void volume, which indicates a molecular weight $>150 \times 10^6$. Electron microscope observation of the two subunits in urea solution showed relatively large structures similar to short pieces of filament (35–60 nm long and 8–12 nm wide). However, P200 subunits were shown to be in globular form. Some rod-shaped structures seen in the P200 subunit fraction were believed to be contaminating P160 and P78 subunits, because the electrophoretic analysis of P200 from the hydroxylapatite column showed some contamination by P160 and P78 (Fig. 2, lane *P*). These results indicate that the 8 M urea solution containing pyridine, formic acid, and β-mercaptoethanol may have stripped off P200 subunits from the neurofilament. By doing so, the filament was broken down to small pieces containing P160 and P78 subunits. Removal of urea by dialysis against buffer E resulted in clear solutions of P200 and P160-P78. EM observation of these two fractions was similar to the EM picture of these two fractions in urea, except that some aggregates were formed and P160 and P78 pieces were straighter in the absence of urea. Whether these relatively large globular structures, which might consist of several P200 molecules and rod-shaped molecules presumably composed of P160 and P78 molecules, are artifacts or molecules present in vivo as precursors to neurofilament formation is not clear at this moment.

Incubations of these two separate fractions under neurofilament assembly condition did not change the morphology of P200 subunit. However, there was a definite indication that P160 and P78 were elongated. Long filaments were formed when P160 and P78 were incubated with P200 subunits, suggesting that P200 may have certain role(s) in the formation of long filaments.

On the basis of these observations, we propose two possible mechanisms for the assembly of neurofilament: the P200 subunit may act as a joint between short filaments formed of P160 and P78 subunits; or it may bind to short filaments and induce a conformational change in the P160 and P78 complex, which then can join together with other complexes, forming long filaments. Wuerker (21) proposed a molecular model for neurofilament structure based on his EM studies of rabbit neurofilament. According to the model, four globular molecules form a unit and these units stack up to form a long filament. Other studies also revealed that the neurofilament is formed from four protofilaments (10, 18). In view of these results and our finding that the molar ratio of the three subunits is 2.4:2:1 (P78:P160:P200) in the assembled filament and that the two subunits (P78 and P160) without P200 subunit can form short filaments, the second possibility seems to be a more plausible mechanism for neurofilament assembly. More explicitly, several units of four globular subunits form a certain short length of filament with each unit composed of two molecules of P78 and two molecules of P160. But, when one molecule of P200 binds to each unit, a proper conformation is induced in the complex, enabling many units to stack up and form a relatively long and

stable polymer. However, more studies must be conducted before any definite conclusions can be drawn, specifically with respect to the role of subunits and the partial reactions involved in the formation of neurofilament.

Why we see two different types of filaments—long, straight filaments (Fig. 6*d*) and tangled filaments (Fig. 6*c*)—is not clear at this moment. The optimal assembly condition still has to be found. This requires a good quantitative assay method for filament formation.

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