

# Preferential Phosphorylation of the 150,000 Molecular Weight Component of Neurofilaments by a Cyclic AMP-dependent, Microtubule-associated Protein Kinase

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**ABSTRACT** Highly purified preparations of bovine brain and rabbit nerve root neurofilaments were found to be lacking in protein kinase activity when either histone F<sub>IIA</sub> or the neurofilaments themselves were used as acceptors. There was no augmentation of activity in the presence of cyclic AMP. Addition of microtubule proteins prepared by cycles of assembly and disassembly resulted in phosphorylation of histone, phosphorylation of tubulin and the microtubule-associated proteins, and phosphorylation of neurofilament subunits. The phosphorylation of neurofilaments was predominantly in the 150,000-dalton species and was completely cyclic AMP dependent.

Intermediate filaments in most cell types are composed of protein subunits in the 50,000- to 60,000-dalton range (11). These include the filaments from smooth muscle cells (3) and glial cells (1) as well as the filaments of the cytokeratin (27, 33) and vimentin types (5). Mammalian neurofilaments, which represent a fifth class of antigenically and biochemically distinct intermediate filaments, are present in mature neurons. They are composed of three polypeptides with molecular weights of ~70,000 (NF1), 150,000 (NF2), and 200,000 (NF3) (1, 12, 19). The precise molecular weight of each of the components varies slightly from species to species (23).

Morphological studies of axoplasmic architecture have shown the presence of "arms" on neurofilaments (32) that, in many instances, appear to form bridges or links between adjacent neurofilaments and between neurofilaments and microtubules. In addition, radioactively labeled subunits of microtubules and neurofilaments move at the same rate in the slow component of axonal transport (7), suggesting the association of microtubules and neurofilaments, either directly or through a common third element.

A possible mechanism for the regulation of the interactions between these two cytoskeletal elements is phosphorylation of one or more of the protein elements present. In the case of tubulin, two phosphoprotein kinase activities have been reported, one cyclic AMP dependent (2, 6, 26) and the other cyclic AMP independent (16). These kinases are capable of phosphorylating both the MAP<sub>2</sub> (16, 26) and tau (2) microtubule accessory proteins. Recent work by Vallee (29) suggests

that the cyclic AMP-dependent protein kinase is located on the microtubule arm.

In the studies reported here we have examined different preparations of neurofilaments for the presence of phosphoprotein kinases and for phosphorylation in the presence and absence of microtubule proteins.

## MATERIALS AND METHODS

### *Preparation of Microtubules*

Microtubules were purified from bovine brain by the assembly-disassembly method as modified by Karr et al. (9). Microtubules obtained after two cycles of assembly were dissociated at 4°C and stored at -80°C in reassembly buffer (RB), (0.1 M 2-[N-morpholino]ethane sulfonic acid [MES]; 1 mM EGTA; 1 mM MgCl<sub>2</sub> and 1 mM GTP at pH 6.5) containing 8 M glycerol. Protein concentrations of these stock solutions were ~15 mg/ml.

### *Preparation of Neurofilaments*

Intradural rabbit spinal nerve root neurofilaments were prepared by the method of Liem et al. (12). Roots were carefully dissected, homogenized in 0.85 M sucrose in a 10 mM phosphate buffer containing 0.1 M NaCl and 1 mM EGTA (pH 6.8), and the myelinated axons were floated to the top of the sucrose by centrifugation at 10,000 rpm for 10 min in a SW 27 rotor (Beckman Instruments, Inc., Fullerton, Calif.). The myelin was stripped by a 1% Triton solution and the neurofilaments were collected by sucrose density gradient centrifugation as described (12).

Bovine brains were homogenized (three 30-s pulses at setting 1.5) in 0.32 M sucrose containing 1 mM EGTA (pH 7.0) with a tissue-to-buffer ratio (wt/vol) of 1:1 in a Sorvall omnimixer (Sorvall, DuPont Co., Newtown, Conn.). After the homogenate was centrifuged for 1 h at 105,000g, the supernatant fluid was separated and used to purify microtubules as described above. The pellet was

resuspended in the 0.32 M sucrose solution and subjected to vigorous homogenization (three 30-s pulses at setting 9) in a Sorvall omimixer. The homogenate was centrifuged at 48,000g for 20 min. The neurofilament-containing supernatant fluid was carefully removed and reserved, and the pellet was reextracted by homogenization and centrifugation. The combined supernates were brought to a final concentration of 0.8 M KCl by the addition of solid KCl. Final purification of the filaments was achieved by layering this extract on a discontinuous sucrose gradient (0.85 M; 1 M; 2 M) in which the sucrose solutions are prepared in microtubule RB with the addition of glycerol and KCl to final concentrations of 4 and 0.8 M, respectively. Neurofilaments were harvested from the 0.85 M/1 M and 1 M/2 M interfaces, diluted 10-fold with RB containing 4 M glycerol and 0.8 M KCl, and harvested by centrifugation at 60,000 rpm for 1 h. The pellet was resuspended by gentle sonication in RB containing 2 M glycerol. All operations were done at 4°C.

### Phosphorylation Measurements

Phosphorylations were carried out in RB with 2 M glycerol.  $\text{ATP}\gamma^{32}$  ( $2 \times 10^{-5}$  M;  $0.5 \mu\text{Ci}/\text{assay}$ ) and cyclic AMP ( $5 \times 10^{-6}$  M) were added to protein samples in RB (0.1 or 0.2 ml final vol). Measurements were made by removing 25- $\mu\text{l}$  aliquots and precipitating them on 4  $\text{cm}^2$  squares of Whatman 3 MM filter paper (Whatman, Inc., Clifton, N.J.) that had been impregnated previously with 10% TCA containing 10 mM ATP. The reactions were started by incubation at 37°C, and 25- $\mu\text{l}$  samples were withdrawn for assay at 5 and 45 min. The TCA-impregnated filters were then washed in 150 ml of 10% TCA, 10 mM ATP for 1 h and then washed twice in 10% TCA, twice in 95% ethanol, once in 100% ethanol, once in a 1:1 (vol/vol) mixture of ethanol and ethyl ether, and once with ethyl ether. The first three sets of washes were for 30 min each, the remainder for 15 min. After drying, the papers were counted in 5 ml of  $\text{H}_2\text{O}$  by Cerenkov counting (40% efficiency).

### Preparation of the Protein Kinase Inhibitor

The specific, thermostable inhibition of cAMP-dependent protein kinases was isolated from pig brain by the method of Walsh (30). The purified inhibitor fraction was then lyophilized and redissolved for use in RB.

### Gel Electrophoresis

6–15% gradient polyacrylamide gels containing 0.1% SDS were used for the electrophoresis experiments. The gels were fixed in methanol, water, acetic acid (5:4:1), and stained with 1% Coomassie Blue. After destaining, the gels were dried on Whatman 3 MM paper. Autoradiograms were exposed for 24 h on Kodak XR-O-Mat film XR3. For quantitative measurements, the gels were scanned

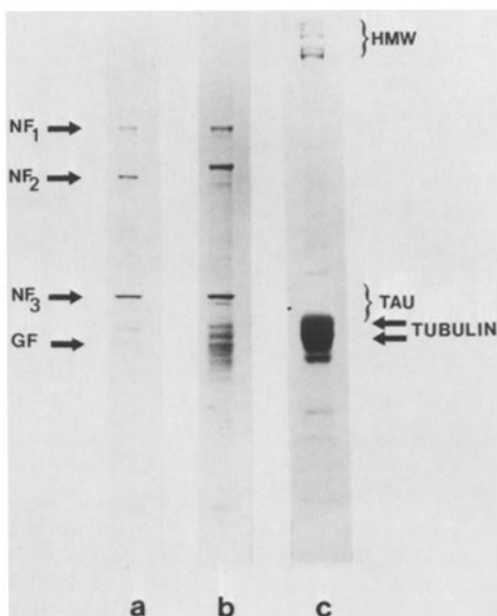


FIGURE 1 Polyacrylamide gel electrophoresis of preparations of (a) neurofilaments (NF) from rabbit intradural roots (8.1  $\mu\text{g}$ ); (b) NF from bovine brain (14  $\mu\text{g}$ ); and (c) microtubule proteins purified by two cycles of assembly-disassembly (20  $\mu\text{g}$ ).

before drying in a Zeiss PM 6 spectrophotometer, then sliced into 2-mm sections, and counted by the Cerenkov method. Histone type IIA, ATP (Na salt), MES, EGTA, and GTP (type IIs) were obtained from Sigma (Sigma Chemical Corp., St. Louis, Mo.).  $\text{ATP}\gamma^{32}$  (7.8 Ci/mmol) was obtained from New England Nuclear (Boston, Mass.). Radioactivity measurements were performed in a Beckman 7500 liquid scintillation counter (Beckman Instruments, Inc.).

## RESULTS

### Protein Preparations

Neurofilament proteins purified from the intradural nerve roots of the rabbit were composed of the neurofilament triplet proteins (NF1, NF2, and NF3) plus a trace component with a molecular weight of 60,000 (Fig. 1a). The major contaminants in the bovine neurofilament preparations were tubulin and glial filament proteins (Fig. 1b). The tubulin gel was deliberately overloaded to demonstrate the presence of both high molecular weight and tau accessory proteins. Actin was a variable contaminant in these preparations (Fig. 1c). The neurofilament preparations from brain are greatly reduced in tubulin and glial filament contamination compared with those made by methods previously reported by our group (12) and by others (1, 17, 19, 21).

### Phosphorylation of Histone $F_{IIA}$ and Autophosphorylation of Neurofilaments

To determine whether phosphoprotein kinases were present in our preparations, histone  $F_{IIA}$  was used as an acceptor molecule. Incubations were carried out over a period of 15 min in the presence of  $5 \times 10^{-5}$  M cAMP. There was no detectable phosphorylation of the histone by the rabbit intradural root neurofilaments, and the bovine brain filaments showed only

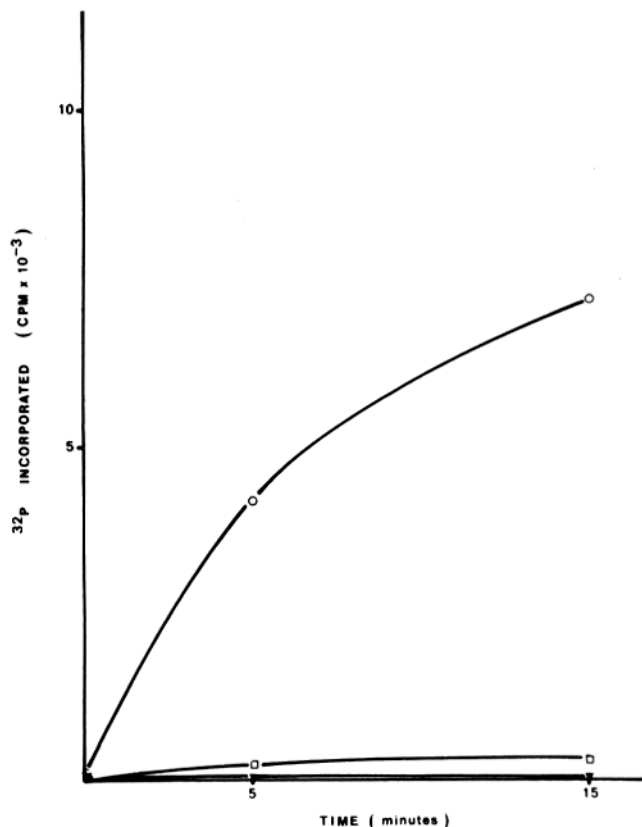


FIGURE 2 Phosphorylation of histone  $F_{IIA}$  by root neurofilaments (0.16  $\mu\text{g}/\text{ml}$ , ▼); bovine brain neurofilaments (0.28  $\mu\text{g}/\text{ml}$ , □); and two cycle microtubule proteins (0.4  $\mu\text{g}/\text{ml}$ , ○). cAMP,  $5 \times 10^{-6}$  M.

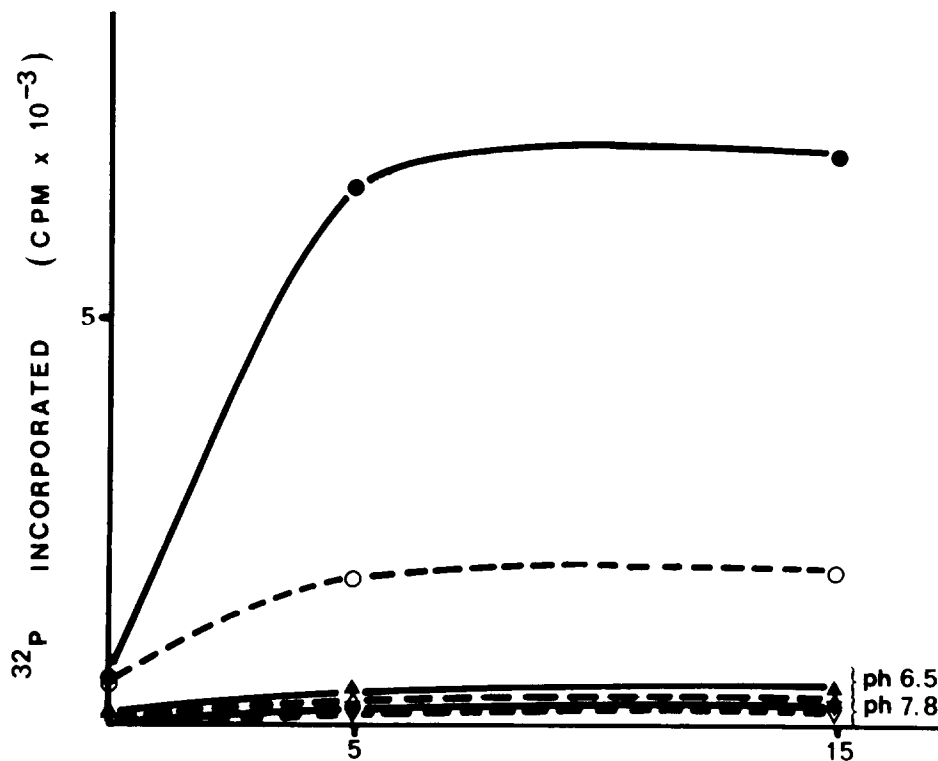


FIGURE 3 Kinetics of autophosphorylation of bovine brain neurofilaments in the presence and absence of microtubule proteins. (NF, 0.35 mg/ml; MT, 0.8 mg/ml; cAMP,  $5 \times 10^{-6}$  M). Neurofilaments with cAMP, pH 6.5 ( $\blacktriangle$ ); neurofilaments without cAMP, pH 6.5 ( $\triangle$ ); neurofilaments with cAMP, pH 7.8 ( $\blacktriangledown$ ); neurofilaments without cAMP, pH 7.8 ( $\triangledown$ ); neurofilaments with microtubule proteins and cAMP, pH 6.5 ( $\bullet$ ); neurofilaments and microtubule proteins without cAMP, pH 6.5 ( $\circ$ ).

weak activity. In contrast, the microtubule preparation showed a high activity (Fig. 2) measured at 44 pmol/min per mg histone per 0.1 mg microtubule protein. Analysis of the incubation mixtures by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) showed the major portion of the incorporated counts to be in histones.

The autophosphorylation of cow brain neurofilament proteins in the presence and absence of cAMP as well as in the presence and absence of microtubule protein was measured. The cow neurofilaments showed only a trace of phosphorylation at either pH 6.5 or pH 7.8 whether or not cAMP was present (Fig. 3). The addition of microtubule proteins at a concentration approximately twice that of the neurofilaments resulted in a marked increase in  $^{32}\text{P}$  incorporation, which was augmented over twofold by cAMP (Fig. 3). Analysis of these experiments by SDS-PAGE and autoradiography showed that, in the absence of cAMP (Fig. 4A, column 12), labeling is found only in the MAP<sub>2</sub> and tau regions. The same pattern is seen with microtubule protein alone (Fig. 4B, column 3).

When cAMP is added to an incubation mixture containing only microtubule proteins (Fig. 4B, column 4), there is an increase in labeling of the tau proteins, MAP<sub>2</sub> and a protein with a molecular weight of  $\sim 240,000$ . Addition of cAMP to the mixture of tubulin and neurofilaments results in the strong labeling of NF2 in the cow filaments. Weaker labeling in the region of NF3 is also seen in these preparations (Fig. 4a, column 11). Intracellular root filaments showed similar cAMP-dependent phosphorylation in the presence of microtubule protein.

In spite of the fact that neurofilament proteins are phosphorylated when mixed with microtubule proteins under appro-

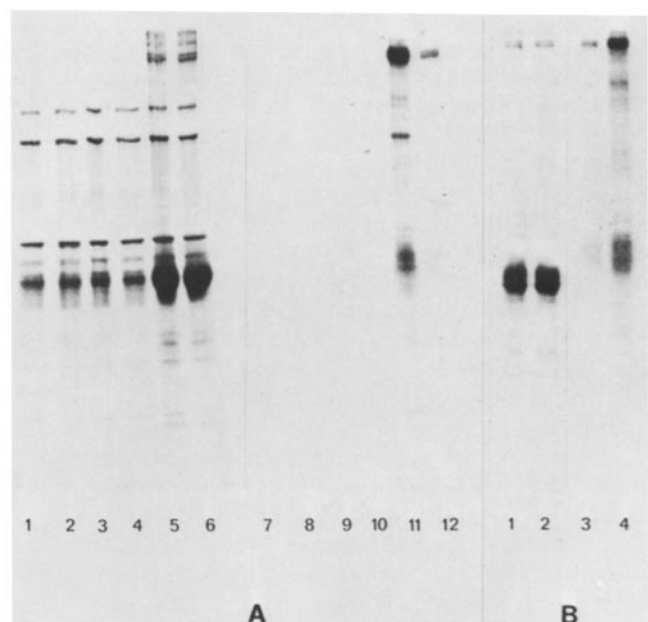


FIGURE 4 (A) Polyacrylamide gel electrophoresis of the neurofilament (NF) preparations, with and without microtubule proteins, from the experiment in Fig. 3. Lanes 1-6 are stained with Coomassie Blue and contain the following: (1) NF and cAMP, pH 6.5; (2) NF minus cAMP, pH 6.5; (3) NF and cAMP, pH 7.8; (4) NF minus cAMP, pH 7.8; (5) NF with MT and cAMP, pH 6.5; and (6) NF with MT minus cAMP, pH 6.5. Lanes 7-12 are autoradiographs of lanes 1-6. (B) Polyacrylamide gels of microtubule proteins alone incubated in the presence and absence of cAMP. (1) MT and cAMP; (2) MT without cAMP; Lanes 3 and 4 are autoradiographs of 1 and 2.

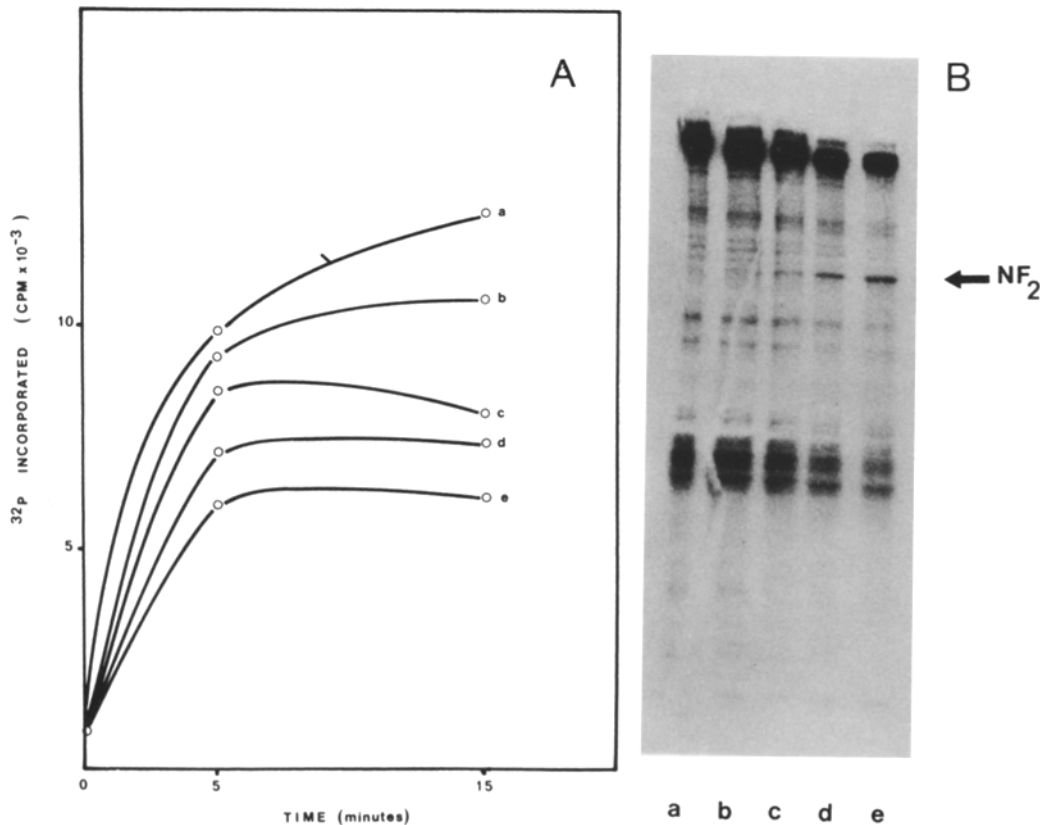


FIGURE 5 (A) Kinetics of phosphorylation of microtubule proteins in the presence of increasing concentrations of bovine brain neurofilaments. (MT, 1 mg/ml) Neurofilament protein concentrations as follows: (a)  $\text{O}$ ; (b) 35  $\mu\text{g/ml}$ ; (c) 87.5  $\mu\text{g/ml}$ ; (d) 175  $\mu\text{g/ml}$ ; and (e) 350  $\mu\text{g/ml}$ . (B) Autoradiographs of polyacrylamide gel electropherograms of samples a-e in Fig. 5A. The position of  $\text{NF}_2$  is indicated by the arrow.

appropriate incubation conditions, the addition of increasing amounts of neurofilaments to the incubation mixtures results in a decrease of total phosphorylation in the mixture. This can be seen clearly in the kinetic results in Fig. 5A. When the respective mixtures are analyzed by PAGE and autoradiography, one finds a decrease in  $\text{MAP}_2$  labeling and an increase in the labeling of  $\text{NF}_2$ . The labeling of minor proteins in the microtubule preparation is also decreased (Fig. 5B).

When the autophosphorylation of microtubule protein is measured in the presence of increasing concentrations of  $\text{CaCl}_2$  (Fig. 6a), one sees a total abolition of both cAMP-dependent and -independent activities at 5 mM  $\text{CaCl}_2$ . Addition of the thermostable inhibitor of cAMP-dependent phosphoprotein kinases (30) abolishes the cAMP-dependent activity at 0.12 mg/ml whereas the cAMP-independent kinase is only slightly inhibited at fivefold greater concentrations (Fig. 6).

When similar experiments are carried out to determine the effect of the thermostable inhibitor on mixtures of root filaments and microtubules and cow brain filaments and microtubules with and without cAMP, one also finds that neurofilament phosphorylation is almost totally cAMP dependent and that it is abolished by the inhibitor at a concentration of 0.6 mg/ml. As was seen in the bovine filaments in Fig. 4, the greatest labeling occurred in  $\text{NF}_2$  though there was some incorporation into bands comigrating with  $\text{NF}_1$  and  $\text{NF}_3$ . In the root neurofilaments, only  $\text{NF}_2$  is labeled among the triplet proteins (Fig. 7).

To determine the stoichiometry of  $^{32}\text{P}$  labeling, standardized quantities of purified NF proteins were analyzed by spectro-

photometric gel scanning after protein determination by the method of Lowry et al. (13). These data were used to standardize gel scanning data. Actual experimental mixtures were then scanned and the concentration of  $\text{NF}_2$  was determined. Due to the proximity of  $\text{NF}_3$  and  $\text{NF}_1$  to other proteins in the mixture, accurate measurement was difficult and the values obtained are subject to error. Quantitative data were obtained on NF phosphorylation by counting gel slices after scanning (Fig. 8). Over 85% of the incorporated counts were in  $\text{NF}_2$  for a total incorporation of 0.33 mol  $\text{PO}_4/\text{mol}$   $\text{NF}_2$ . Because  $\text{NF}_1$  and  $\text{NF}_3$  are seen on the shoulders of larger peaks in these data, direct quantification is not possible. As in the previous experiments, the phosphorylation of  $\text{NF}_2$  is extremely weak in the absence of cAMP in the bovine (0.02 M/M) preparations.

## DISCUSSION

Preparations of neurofilament proteins obtained by the methods described here are of various degrees of purity. The filaments isolated from the intradural roots of rabbits are composed almost solely of the triplet proteins and are free of contamination by tubulin and glial filament (GF) protein. Unfortunately, quantities in excess of 1–2 mg require extensive effort to obtain, and necessitate the sacrifice of large numbers of animals. The bovine brain filament preparation described here avoids the extensive GF contamination of earlier preparations although traces of both tubulin and GF are present (Fig. 1). Although these preparations are more highly defined than previous preparations, the possibility remains that pro-

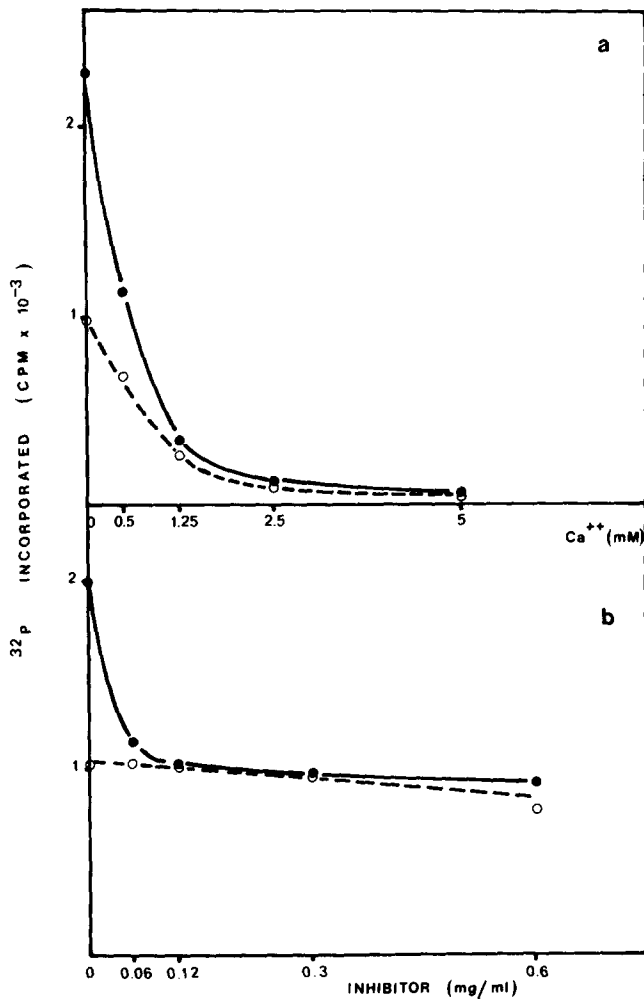


FIGURE 6 (a) The effect of increasing concentrations of  $\text{CaCl}_2$  on the autophosphorylation of microtubule proteins. Conditions: Microtubule proteins 0.2 mg/ml; cAMP  $5 \times 10^{-6}$  M;  $\text{ATP}^{32}$   $2 \times 10^{-5}$  M.; 50- $\mu\text{l}$  aliquots from 1-ml reaction mixture. (b) Effect of increasing concentrations of the thermostable inhibitor of cAMP-dependent protein kinases on autophosphorylation of microtubule proteins. (●, with cAMP); (○, without cAMP).

teins that might normally be associated with the neurofilament *in vivo* are removed in the process.

The intradural root neurofilament preparations were totally lacking in phosphoprotein kinase activity when measured with histone  $F_{11A}$  as an acceptor protein. Low levels of activity were found in cow neurofilaments. This may represent a residual amount of the kinase activity reported in less highly purified neurofilament preparations (18, 20). On the other hand, microtubule preparations prepared by modifications of the reassembly method (22) show an active phosphoprotein kinase activity that is stimulated by cAMP (Fig. 2). This activity has been reported to label both tau (2) and  $\text{MAP}_2$  (16, 26) proteins, and the cAMP-dependent portion of the activity has been localized to the microtubule "arms" (29).

With microtubule proteins containing the cAMP-dependent kinase we see the expected phosphorylation of  $\text{MAP}_2$  and tau as well as the incorporation of label into the components of the neurofilament triplet. Under the conditions used, this incorporation was greatest in the NF2 component, with 0.33 mol  $\text{PO}_4$  being incorporated per mol of NF2. The labeling of NF1 and NF3 was considerably lower on a molar basis and appar-

ently absent in the root filament preparations.

Phosphorylation of the entire triplet complex by a kinase present in less highly purified filament preparations has been reported by two groups (18, 21). In neither case is the phosphorylation cAMP dependent nor does it show preferential labeling of one of the components of the triplet. Therefore, the kinase activity and phosphorylation described here appear to differ from those described previously. The activity present in less well defined preparations might be a contaminant or an activity that was associated with the filaments *in vivo* and eliminated in the course of purification.

There is currently no evidence concerning the *in vivo* phosphorylation of the neurofilament triplet in mammals, though neurofilament phosphorylation has been reported in the squid (14). The physiological implications of our findings are not clear. However, in view of the close association between neurofilaments and microtubules that is suggested by morphological and axonal transport studies, it is conceivable that NF2 and  $\text{MAP}_2$  are involved in filament-to-tubule bridging. Because the association between filaments and tubules is regulated in development (15), in disease (24), and in situations in which the microtubules are depolymerized by spindle inhibitors (31) or by cold (28), a possible role of a cAMP-dependent phosphorylation-regulated mechanism controlling either cross-link formation or cross-link stability can be hypothesized. Other data obtained in our laboratory (25) have shown saturable high-affinity binding of phosphocellulose-purified MAPs to a neurofilament triplet, further supporting a role of these elements in cross-linking. Whereas the major protein, on a molar basis, in the neurofilament triplet is NF1 (68,000 mol. wt.) and

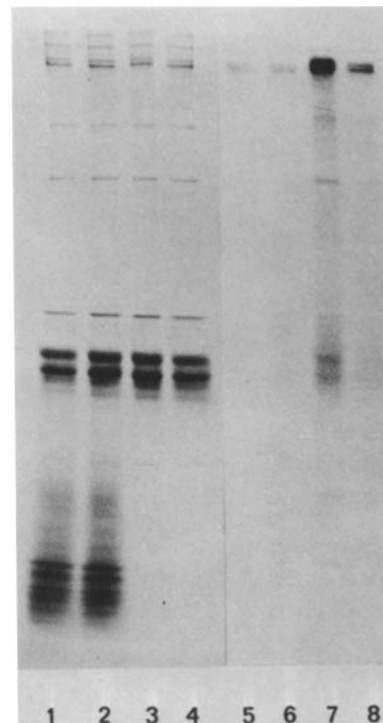


FIGURE 7 Polyacrylamide gel electrophoresis of rabbit root neurofilaments after phosphorylation for 15 min with microtubule proteins in the presence and absence of cAMP and protein kinase inhibitor. Lanes are labeled as follows: (1) with inhibitor and cAMP; (2) with inhibitor and without cAMP; (3) with cAMP and without inhibitor; (4) without cAMP and without inhibitor. Lanes 5-8 are autoradiographs of lanes 1-4.

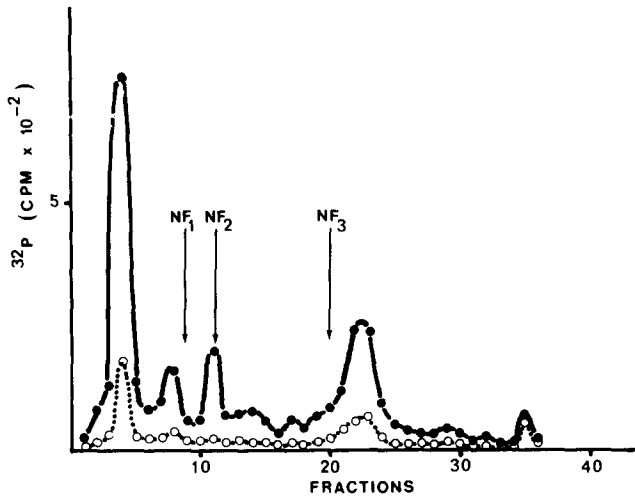


FIGURE 8 Incorporation of  $^{32}\text{P}$  into microtubule proteins and neurofilament proteins from calf brain in the presence (●) and absence of cAMP (○). Arrows indicate the positions of neurofilament proteins on the stained gel. After staining and destaining, 2-mm slices were cut and counted. The large peak to the left is composed of high molecular weight MAPs.

because it is likely that this forms the filament "core," it is possible that NF2 might form part of the arm seen on the filaments and that the actual link is NF2-MAP<sub>2</sub> in a phosphorylation-regulated arm-to-arm cross-bridge.

Axoplasmic transport studies have failed to show the presence of MAP<sub>2</sub> in transported, radioactively labeled material (10), but studies using monoclonal antibodies against MAP<sub>2</sub> in neuroblastoma cells have shown selective localization of MAP<sub>2</sub> to neurites in these cells (8). These studies raise the possibility that different types of filament-tubule links may exist in the cell body and axon or in different cell types. Such variations might permit functional specialization of the cytoskeleton in the axon as compared to the dendrite, or differences between cells of different architecture or functions. Derangements of the cross-link and phosphorylation mechanism might be the key to at least a portion of the neurological diseases marked by neurofilamentous accumulations.

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