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Monoclonal antibodies distinguish phosphorylated and nonphosphorylated forms of neurofilaments *in situ*

(chemoarchitectonics/electroblot/immunocytochemistry/monoclonal peroxidase-antiperoxidase/neuronal individuality)

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ABSTRACT **The immunocytochemical staining patterns of 37 neuron-specific monoclonal antibodies previously described fell into four groups: (*i*) anti-synapse-associated, (*ii*) anti-neurofibrillar, (*iii*) anti-perikaryonal-neurofibrillar, and (*iv*) a single antibody reactive with a widely distributed epitope that covered the patterns of groups *ii* and *iii*. Antibodies of groups *ii*, *iii*, and *iv* were shown to be specific to neurofilament triplet subunits, even though there was little overlap in staining patterns between groups *ii* and *iii*. We examined nine of these antibodies as to their ability to distinguish functional states of neurofilaments dependent upon phosphorylation. Upon digestion with phosphatase, electroblot staining of neurofilament components was abolished with the five antibodies from group *ii*, enhanced with the three antibodies from group *iii*, and unaffected with antibody *iv*. Immunocytochemical staining of Bouin-fixed paraffin sections of rat brain was unaffected by phosphatase pretreatment. With antibodies of group *ii*, digestion with trypsin also left staining unaffected, but when followed by digestion with phosphatase, staining was diminished with three out of five antibodies. In contrast, digestion with trypsin abolished all staining with each antibody from group *iii*. If followed by digestion with phosphatase, staining reappeared, but the group *iii* pattern was replaced by a group *ii* pattern. Staining of this pattern was again abolished upon a second treatment with trypsin. The antibody from group *iv* lost most of its groups *ii* and *iii* staining patterns when sections were digested with trypsin. The group *ii* pattern reappeared and, indeed, was enhanced upon a subsequent phosphatase treatment and was reduced again upon a second trypsin treatment. Staining by four out of five antibodies from group *ii* was inhibited by inorganic phosphate. The data indicate that certain nerve cell bodies, their dendrites, and at least proximal axons possess nonphosphorylated neurofilaments and that long fibers, including terminal axons, possess phosphorylated neurofilaments. We propose that phosphorylation may be a factor in stabilizing compacted forms of neurofilaments and that heterogeneity of the compacted structures may play a role in a possible multiplicity of function within individual nerve cells.**

Out of 135 monoclonal antibodies previously obtained upon immunization with hypothalamus, 37 were neuron-specific and delineated heterogeneous antigens (1, 2). Their general staining distribution permitted classification into four groups: (*i*) an anti-synapse-associated group that stained gray matter to the exclusion of the interior of cell bodies or discernible nerve fibers and reacted with isolated synaptosomes; (*ii*) an anti-neurofibrillar group that generally stained white and gray matter fibers, basket cell fibers in the cerebellum, and transverse fibers in the cerebral cortex, but never any cell bodies or their proximal axons or dendrites; (*iii*) an anti-perikaryonal-neurofibrillar group that stained selected perikarya, their dendrites and proximal axons, including Purkinje axons and dendrites in the cerebellum and pyramidal cells as well as longitudinal fibers in the cortex, but never any basket cell fibers, transverse cortical fibers, or white matter fibers; (*iv*) a single, broadly reacting antibody (02-40) that exhibited the staining patterns of both groups *ii* and *iii*.

There was considerable heterogeneity of staining patterns for each antibody in the first three groups, but with the exception of antibody 02-40, there was little overlap of staining of antibodies from one group to another. Thus, all structures stained by antibodies of the anti-neurofibrillar group were not stained by antibodies from the anti-perikaryonal-neurofibrillar group, or vice versa. Nevertheless, analysis of electroblots of brain homogenates, cytoskeletal preparations (3, 4), and isolated neurofilaments (5) showed that antibodies of both groups reacted exclusively with doublet M_r 200,000 bands and one M_r 150,000 band of the M_r neurofilament triplet (three major polypeptides of M_rs 200,000, 150,000, and 68,000) (6). These data and differences in immunocytochemical reaction of each antibody in each group suggested two levels of heterogeneity of neurofilaments, one detectable by difference of reaction between the anti-neurofibrillar and anti-perikaryonal-neurofibrillar group and the other by differences with antibodies within each of these groups. However, it was proposed to us by Maurice Rapport (personal communication) that some of this heterogeneity may not be pretranslational but may be due to functional states of neurofilament activity expressed by posttranslational processes. The present paper examines this proposal.

Julien and Mushynski (7) have shown that neurofilaments are largely phosphorylated and that digestion with phosphatase reduces the size of the subsequently electrophoresed M_r 200,000 component of the neurofilament triplet. We examined whether neurofilaments can be similarly affected by phosphatase after separation into their triplet components and whether some of our monoclonal antibodies are specific to phosphorylated and others to nonphosphorylated forms. We compared the observations on separated neurofilament components with effects of phosphatase on insoluble neurofilaments in fixed tissue. The data showed that neurofilaments of certain perikarya, their dendrites, and at least their proximal axons are nonphosphorylated and that the neurofilaments of many long projection fibers and terminal axons are largely phosphorylated. We propose that phosphorylated neurofilaments are a more compact and organized form of this heterogeneous protein than nonphosphorylated neurofilaments and that compacted forms of neurofilaments, alone or in association with other neural constituents, have a function in gating multiple neurotransmitters within single neurons towards selected synaptic sites.

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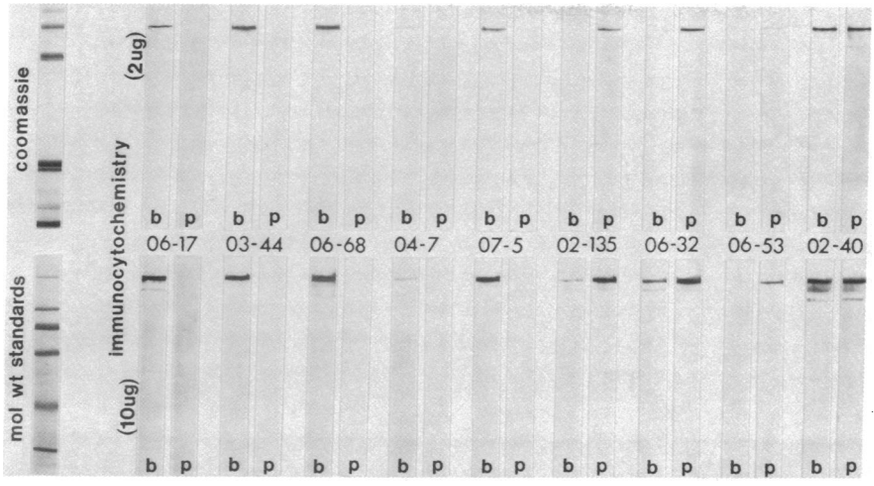

FIG. 1. Electroblot of cytoskeletal preparations. The far-left lane is stained with Coomassie blue: cytoskeletal preparation (10 μg) (*Upper*); molecular weight standards (top to bottom M_rs, 200, 116, 92, 66, 45, and 31) (*Lower*). Remaining lanes are stained immunocytochemically with the antibodies listed. Stacks were loaded with 2 μg (*Upper*) or with 10 μg (*Lower*) of cytoskeletal preparation. Lanes: b, preparations incubated in buffer; p, preparations incubated in phosphatase-containing buffer after electroblotting and prior to immunocytochemical staining.

MATERIALS AND METHODS

Cytoskeletal preparations from rat brain stems obtained by the method of Chiu *et al.* (3) and Eng *et al.* (4) were applied in amounts ranging from 2 to 10 μg on gels containing 8% acrylamide, 0.375 M Tris·HCl (pH 8.8), and 0.1% $NaDodSO_4$ and were electrophoresed and electroblotted along with molecular weight standards by the method of Towbin *et al.* (8) as detailed (6). One strip containing M_r standards and one containing cytoskeletal preparations in each blot were stained by Coomassie blue, and the rest of the strips containing cytoskeletal preparations were incubated at 32°C for 2.5 hr either in 0.1 M Tris·HCl, pH 8.0/0.01 M phenylmethylsulfonyl fluoride containing 43 μg of calf intestinal alkaline phosphatase (type VII, Sigma) per ml or in the same solution devoid of phosphatase.

Sagittal paraffin sections of brains (7 μm thick) from Sprague–Dawley rats perfused with Bouin's fixative under Nembutal anesthesia were incubated (*i*) with trypsin (GIBCO) at 37°C for 10 min (400 μg/ml) in 0.05 M Tris·HCl, pH 7.6/0.3 M sodium chloride/0.02 M calcium chloride; or (*ii*) with calf intestinal phosphatase at 32°C for 2.5 hr (130 μg/ml) or for 18 hr (400 μg/ml) in 0.1 M Tris·HCl, pH 8.0/0.01 M phenylmethylsulfonyl fluoride; or (*iii*) with both trypsin- and phosphatase-containing buffers sequentially; or (*iv*) in these buffers without enzymes.

The incubated electroblots and the paraffin sections were stained immunocytochemically (9, 10) with mouse peroxidase-anti-peroxidase prepared from monoclonal anti-peroxidase (Clono PAP) (2, 6, 11) diluted 1:100 to contain 4.3 μg of peroxidase and 11.6 μg of anti-peroxidase per ml. Ascites fluids containing the following nine monoclonal antibodies were used as primary antibodies: five anti-neurofibrillar antibodies (06-17, 03-44, 06-68, 04-7, and 07-5), three anti-perikaryonal-neurofibrillar antibodies (02-135, 06-32, and 06-53), and the broadly reacting antibody 02-40. Antibodies were diluted in 0.05 M Tris·HCl, pH 7.6/0.3 M sodium chloride/1% normal goat or rabbit serum [depending on the species of secondary antibodies (9, 10)], except in special cases where 0.15 M sodium potassium phosphate (pH 7.6) was substituted for 0.5 M Tris·HCl. For electroblots, antibodies were applied in dilutions of 1:2,000 for 1 hr. For the paraffin sections, in each experiment, a series of antibody dilutions were applied for 24 hr, ranging from 1:1,000 to 1:320,000.

RESULTS

Treatment of electroblots with phosphatase abolished the immunocytochemical staining with the five anti-neurofibrillar antibodies (06-17, 03-44, 06-68, 04-7, and 07-5) (Fig. 1). It enhanced the staining with the three anti-perikaryonal-neurofibrillar antibodies (02-135, 06-32, and 06-53). Staining with the broadly reacting antibody 02-40 did not appear to be significantly affected.

No effect was noted when paraffin sections were treated with phosphatase at 130 μg/ml for 2.5 hr. However, if digested by trypsin prior to phosphatase, there was a slight decrease of staining with the anti-neurofibrillar antibody 04-7. Tryptic digestion alone had no effect on the staining with any of the anti-neurofibrillar antibodies. Yet, when tryptic digestion was followed by phosphatase at 400 μg/ml for 18 hr, staining was inhibited to about equal extents with the anti-neurofibrillar an-

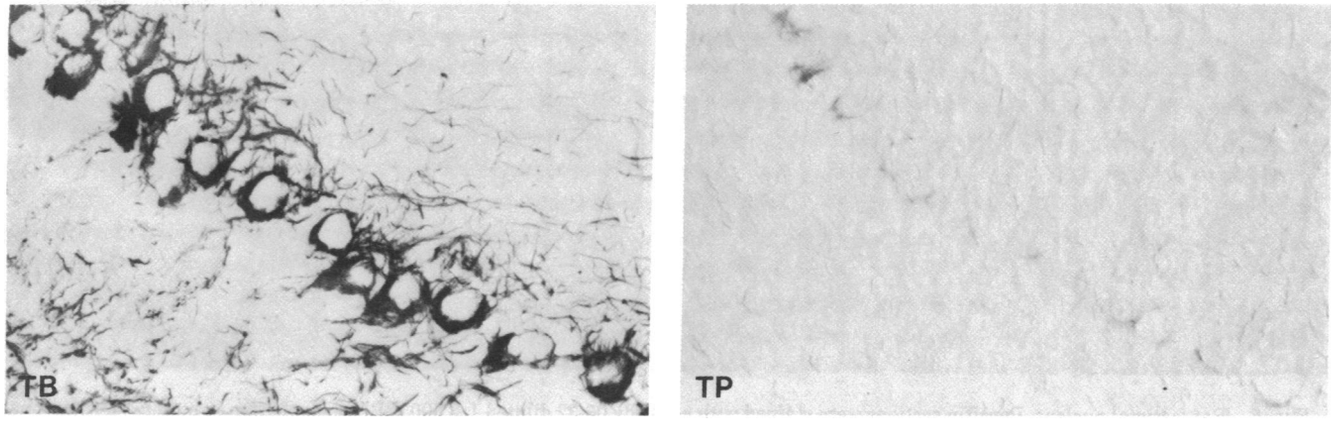

FIG. 2. Rat cerebellar cortex. Paraffin sections were stained with anti-neurofibrillar antibody 06-17 diluted 1:20,000. TB, preincubation in trypsin followed by incubation in buffer. TP, preincubation in trypsin followed by incubation with phosphatase. (×200.)

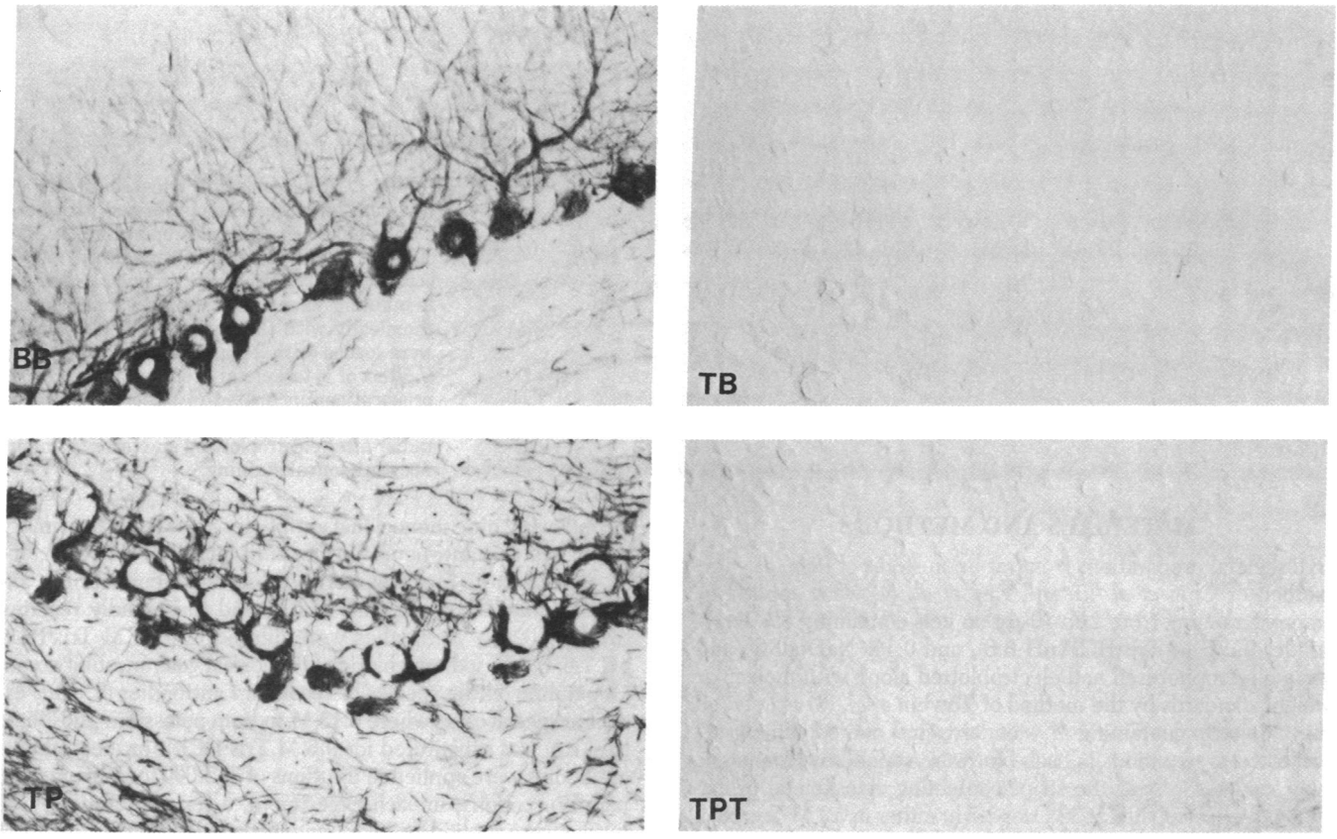

FIG. 3. Rat cerebellar cortex. Paraffin sections are stained with anti-perikaryonal-neurofibrillar antibody 02-135 diluted 1:2,000. BB, preincubation in buffers. TB, preincubation in trypsin-containing buffer followed by incubation in buffer. TP, preincubation with trypsin followed by incubation with phosphatase. TPT, preincubation with trypsin followed by incubation with phosphatase and again by incubation with trypsin. (×200.)

tibodies 06-17, 04-7, and 07-5 (Fig. 2). No effect was noted with antibodies 03-44 and 06-68.

An entirely different pattern was observed with the three antibodies of the anti-perikaryonal-neurofibrillar group. Here digestion with trypsin alone abolished all immunocytochemical staining (Fig. 3). If tryptic digestion was followed by phosphatase at 130 μg/ml for 2.5 hr, staining reappeared, but the perikaryonal pattern (including attached axons and dendrites) remained invisible and was replaced by staining indistinguishable from the neurofibrillar pattern (basket cell fibers, transverse fibers in the cerebral cortex, and long fiber tracts) (Figs. 3 and 4). These fibers were never stained by the anti-perikaryonal-neurofibrillar antibodies in the absence of trypsin and phosphatase pretreatments. If the trypsin and phosphatase-treated sections were treated with trypsin a second time, the newly emerged neurofibrillar type of staining again disappeared (Fig. 3). Buffer post-treatment had no effect.

When the broadly reacting antibody 02-40, which stained both structures represented by the anti-neurofibrillar and the anti-perikaryonal-neurofibrillar antibodies, was applied to trypsin-treated sections, all staining became diminished (Fig. 5). If trypsin incubation was followed by phosphatase, the perikaryonal-neurofibrillar pattern of staining remained weak, but the neurofibrillar pattern became intensified beyond that of sec-

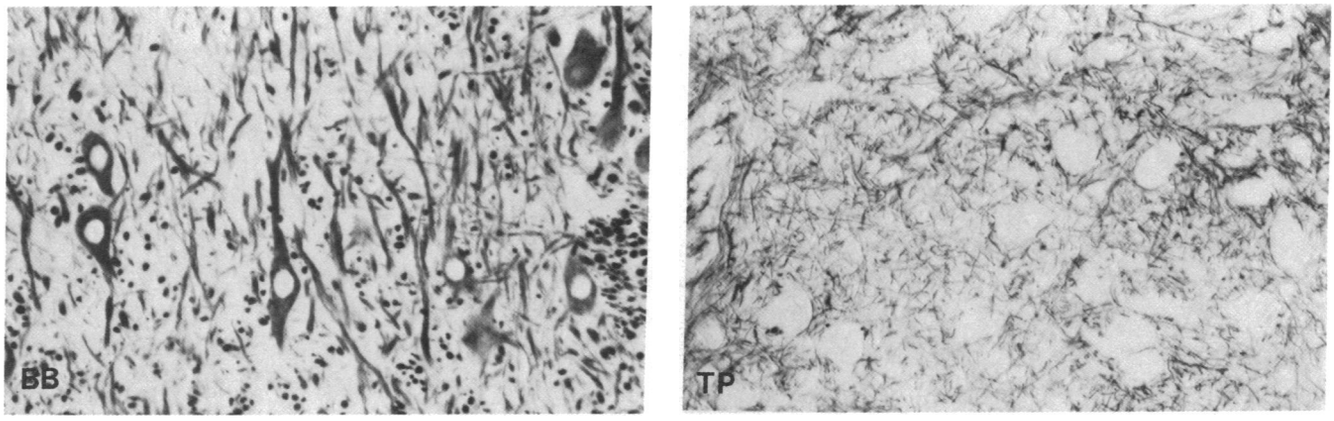

FIG. 4. Rat fastigial nucleus. Paraffin sections were stained with antibody 06-32 diluted 1:2,000. BB, preincubation in buffers (perikaryonal-neurofibrillar pattern); TP, preincubations with trypsin followed by incubation with phosphatase (neurofibrillar pattern). Sections were entirely blank (not shown) when preincubated with trypsin only or with trypsin followed by incubation with phosphatase and again by incubation with trypsin. (×200.)

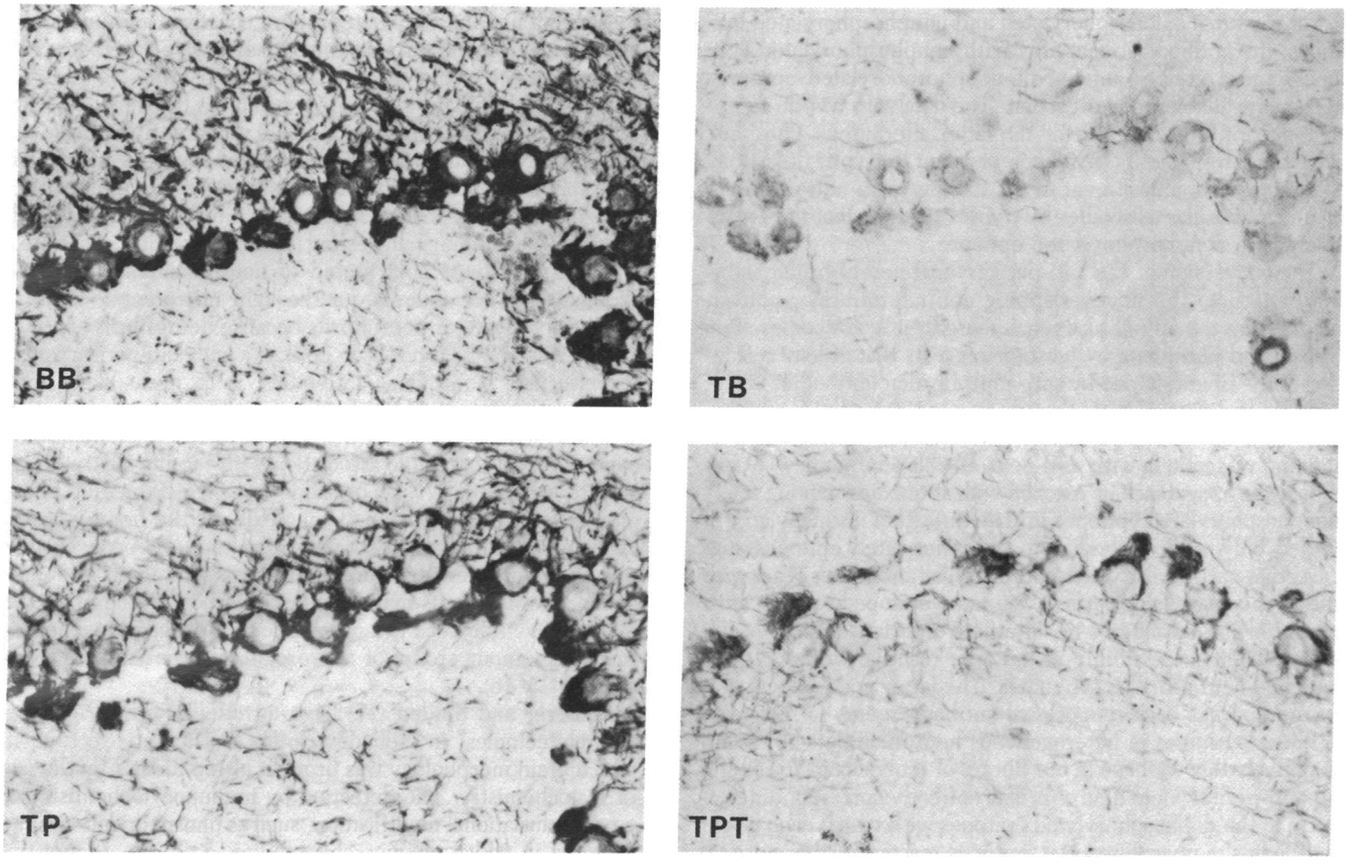

FIG. 5. Rat cerebellar cortex. Paraffin sections were stained with the broadly reactive antibody 02-40 diluted 1:2,000. BB, preincubation in buffer; TB, preincubation in trypsin-containing buffer followed by incubation in buffer; TP, preincubation with trypsin followed by incubation with phosphatase; TPT, preincubation with trypsin followed by incubation with phosphatase and again by incubation with trypsin. (×200.)

tions only incubated in buffers. If followed by a second trypsin incubation, most staining again disappeared.

When antibodies were diluted in sodium potassium phosphate instead of Tris·HCl, staining was inhibited with four of the five anti-neurofibrillar antibodies (06-17, 06-68, 04-7, and 07-5) (Fig. 6). Staining was unaffected with antibody 02-40 or any of the three antibodies from the anti-perikaryonal-neurofibrillar group.

DISCUSSION

The effect of phosphatase on the electroblots stained with the five anti-neurofibrillar antibodies and the inhibition by phosphate buffer of the staining of paraffin sections with four of the five anti-neurofibrillar antibodies suggest that these antibodies are specific for phosphorylated epitopes. Because neurofilaments are extensively phosphorylated (7) and because each anti-neurofibrillar antibody exhibits its own distinct immunocytochemical staining pattern on sections (1) and in electroblots (6), it is unlikely that each of the anti-neurofibrillar antibodies reacts with the same phosphorylated epitope.

The enhancement of staining of electroblots by the three anti-perikaryonal-neurofibrillar antibodies after incubation in phosphatase suggests two interpretations. Conceivably, the antibodies react with phosphorylated and nonphosphorylated epitopes, but better with the latter. Alternatively, the 200 M_r doublet and the 150 M_r band of cytoskeletal preparations con-

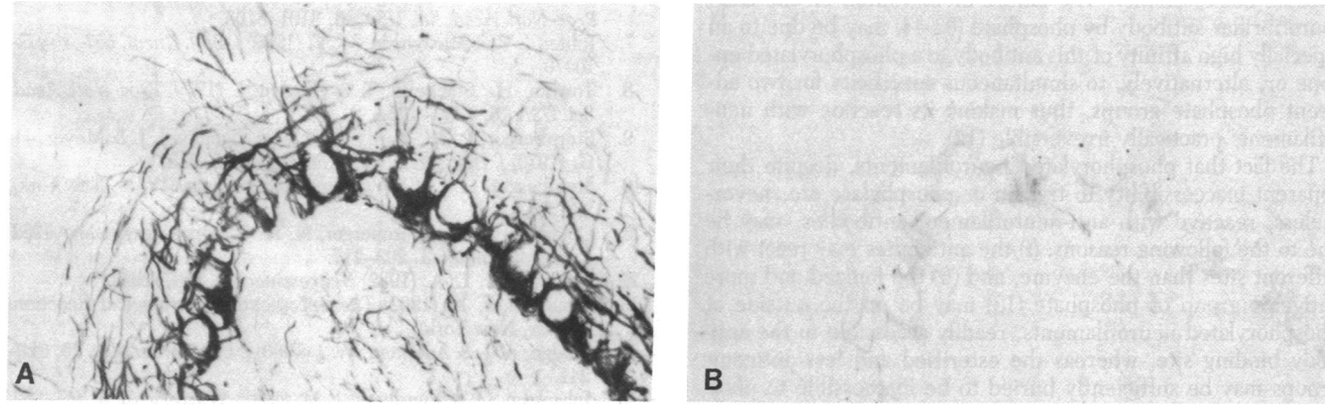

FIG. 6. Rat cerebellar cortex. Paraffin sections were stained with anti-neurofibrillar antibody 07-5 diluted 1:40,000. A, antibody diluted in Tris buffer (pH 7.6); B, antibody diluted in phosphate buffer (pH 7.6). (×200.)

sist of mixtures of phosphorylated and nonphosphorylated epitopes; the antibodies react only with nonphosphorylated epitopes, and a greater amount of nonphosphorylated epitopes becomes available upon digestion. The data with tryptic digestion of paraffin sections favor the latter alternative. Phosphorylated neurofilaments have been resistant to tryptic digestion. Therefore, the abolishment of all staining with anti-perikaryonal-neurofibrillar antibodies by trypsin suggests that they react solely with nonphosphorylated epitopes.

The conversion of the anti-perikaryonal-neurofibrillar staining pattern to the nonoverlapping anti-neurofibrillar pattern revealed by the anti-perikaryonal-neurofibrillar antibodies after trypsin and phosphatase digestion suggests that at least a fraction of the fibers stained by anti-neurofibrillar antibodies is susceptible to digestion with phosphatase at 130 μg/ml for 2.5 hr. However, this fraction is insufficient to greatly reduce the total staining of sections with anti-neurofibrillar antibodies. In addition, the appearance of reaction with antibodies staining solely nonphosphorylated epitopes in structures that normally are revealed only with antibodies to phosphorylated epitopes suggests that anti-perikaryonal-neurofibrillar antibodies react with the dephosphorylated form of the same epitope as do the anti-neurofibrillar antibodies or, alternatively, that dephosphorylation permits accessibility to epitopes reactive with anti-perikaryonal-neurofibrillar antibodies. The latter possibility is preferred because each monoclonal antibody seems to recognize different epitopes in heterogeneous neurofilaments (6), and it is unlikely that we have in our library of monoclonal antibodies an anti-perikaryonal-neurofibrillar antibody corresponding exactly to the dephosphorylated epitopes with which each of our antibodies to phosphorylated epitopes reacts. Furthermore, as we shall see below, the phosphorylated form of neurofilaments seems to be less accessible to enzymes than the nonphosphorylated form, again supporting the concept of masking of epitopes by phosphorylation.

The reabolishment of the neurofibrillar staining by anti-perikaryonal-neurofibrillar antibodies after a second treatment with trypsin suggests again that nonphosphorylated forms of neurofilaments, irrespective of their location, are more susceptible to tryptic digestion than the phosphorylated forms, which have been unaffected by trypsin. Apparently, phosphorylation increases compactness and order in neurofilament structure. Further support to this conclusion was obtained by the ineffectiveness of phosphatase on intact phosphorylated neurofilaments in fixed tissue, unless the tissue had been pretreated with trypsin and unless, in addition, large amounts of phosphatase (400 μg/ml for 24 hr) were used during prolonged incubation.

The resistance to phosphatase digestion of epitopes reactive with antibodies 03-44 and 06-68 is probably explained by their extreme inaccessibility. The failure of inhibition of one anti-neurofibrillar antibody by phosphate (03-44) may be due to an especially high affinity of this antibody to a phosphorylated epitope or, alternatively, to simultaneous specificity for two adjacent phosphate groups, thus making its reaction with neurofilaments practically irreversible (12).

The fact that phosphorylated neurofilaments, despite their apparent inaccessibility to trypsin or phosphatase are, nevertheless, reactive with anti-neurofilament antibodies, may be due to the following reasons: (*i*) the antibodies may react with different sites than the enzyme, and (*ii*) the ionized and more antigenic group of phosphate (13) may be on the outside of phosphorylated neurofilaments, readily accessible to the antibody binding site, whereas the esterified and less antigenic groups may be sufficiently buried to be inaccessible to phosphatase.

Antibody 02-40 does not seem to react with a phosphorylated epitope or an epitope unmasked by dephosphorylation. It may react with a nonphosphorylated, more constant, and universal region of neurofilaments not subject to extensive heterogeneity and readily accessible to destruction by trypsin. Sequential trypsin and phosphatase treatment enhances the neurofibrillar staining component of antibody 02-40, apparently because of the existence of additional 02-40-reactive epitopes in buried forms that are unmasked by the enzymes. After this unmasking, the second trypsin treatment again diminishes the enhanced neurofibrillar staining, suggesting further loss of the intact site.

Any compactness contributed to neurofilaments by phosphorylation is not essential for the light microscopic neurofibrillar appearance of neurofibrils because neurofibrils are easily discernible at high power in perikarya with anti-perikaryonal-neurofibrillar monoclonal antibodies. It is more likely that phosphorylation provides a further compacting or ordering of neurofilament structure, which may involve either neurofilaments alone or a possible association with tubulin (14) or other neurite constituents. The possibility of significance of phosphorylation in the functional complexity of the brain is made likely by the heterogeneity of neurofilaments (6). A conceivable role of the ordered structure, which compacting of heterogeneous neurofilaments may permit, could be a gating effect for the selective release at given synaptic sites of individual neuroregulators among groups of neuroregulators coexisting in a single cell (15–16).

Scharrer and Sinden (17) have introduced the term "chemoarchitectonics" in their description of the optic tectum, in which brain morphology was brought a step further by the use of histochemistry. The detection by immunocytochemistry of a post-translational modification, such as phosphorylation, is an example in which chemoarchitectonics—i.e., the demonstration of metabolic or allosteric differences—extends the definition of neural structures indistinguishable by morphological criteria alone.

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