Proc. Natl. Acad. Sci. USA
Vol. 83, pp. 6174–6178, August 1986
Neurobiology

Calcium and calmodulin-enhanced *in vitro* phosphorylation of hen brain cold-stable microtubules and spinal cord neurofilament triplet proteins after a single oral dose of tri-*o*-cresyl phosphate

(delayed neurotoxicity)

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Communicated by Edward M. Arnett, April 7, 1986

ABSTRACT **The effect of a single 750-mg/kg oral dose of tri-*o*-cresyl phosphate (TOCP) on the endogenous phosphorylation of brain microtubule preparations and spinal cord neurofilaments was assessed in hens after the development of delayed neurotoxicity. Protein phosphorylation with [γ-^{32}P]ATP was analyzed by one-dimensional and two-dimensional gel electrophoresis, autoradiography, and microdensitometry. TOCP treatment enhanced the Ca^{2+}- and calmodulin-dependent phosphorylation of tubulin in crude chicken brain cytosol (160% for α-tubulin and 140% for β-tubulin) and cold-stable microtubules (165% and 155% for α- and β-tubulin, respectively). Microtubule-associated protein 2 (MAP-2) phosphorylation was also increased in brain fractions studied—i.e., brain cytosol (145%), cold-stable microtubules (133%), and cold-labile microtubules (328%). There was significant increase in phosphorylation of a 70-kDa protein in the brain cytosol and in the cold-stable microtubule fractions. TOCP also stimulated the phosphorylation of spinal cord proteins of 70 kDa (119%) and 160 kDa (129%) in a Mg^{2+}-dependent manner. Addition of Ca^{2+} and calmodulin further enhanced the phosphorylation of these 70-kDa (563%) and 160-kDa (221%) proteins as well as of 52-, 59-, and 210-kDa proteins by as much as 126%, 160%, and 196%, respectively. Two-dimensional electrophoresis was carried out to identify these proteins. They were confirmed as α- and β-tubulin (52 and 59 kDa) in brain and spinal cord preparations and the neurofilament triplet proteins (70, 160, and 210 kDa) in the spinal cord preparation. The 70-kDa protein in brain was not neurofilament in origin. Peptide mapping using *Staphylococcus aureus* V8 protease showed the brain and spinal cord cytoskeletal proteins have identical phosphopeptide patterns in control and TOCP-treated hens, indicating that it was unlikely that the phosphorylation sites were altered by TOCP treatment.**

Studies from this laboratory have suggested that Ca^{2+}/calmodulin-regulated protein phosphorylation may play a role in the pathogenesis of organophosphorus compound-induced delayed neurotoxicity (OPIDN) (1). This condition is characterized by an ataxia that progresses to paralysis concurrent with a central-peripheral distal axonopathy after a delay period of 6–14 days in sensitive species (e.g., humans, cows, cats, dogs, chickens) after a single exposure to OPIDN-causing compounds—e.g., tri-*o*-cresyl phosphate (TOCP) (2). The increased brain protein phosphorylation in TOCP-treated chickens correlated with the time of the development of ataxia and paralysis in chickens. Further, we have recently found that a 750-mg/kg oral dose of TOCP increases *in vitro* Ca^{2+}/calmodulin-dependent phosphorylation of chicken brain tubulin and microtubule-associated protein 2 (MAP-2) (3).

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A number of protein kinase activities have been identified in the preparation of microtubules (MTs) and neurofilaments (NFs) (4–6). Among the Ca^{2+}/calmodulin-dependent protein kinases, a type II calmodulin kinase has been purified in association with a number of cytoskeletal elements, including tubulin (7), MT preparations (8, 9), and NFs (10). This suggests that phosphorylation by this kinase may play a dynamic role in the function of cytoskeletal proteins.

In the present report, we investigated the effects of a single 750-mg/kg dose of TOCP on MT preparations and spinal cord NFs in hens. *In vitro* Ca^{2+}/calmodulin-regulated phosphorylation of tubulin in crude chicken brain cytosol and cold-stable and cold-labile MT fractions after TOCP treatment was investigated. Alteration in MAP-2 phosphorylation was also examined in all brain fractions. Ca^{2+}/calmodulin-dependent phosphorylation of NF proteins as well as the tubulin that copurifies with the NFs was also studied in chickens after TOCP treatment.

EXPERIMENTAL PROCEDURES

Animals. Adult Leghorn laying hens (*Gallus gallus domesticus*), 18 months old and weighing approximately 1.6 kg, were obtained from Featherdown Farms (Raleigh, NC). Each of six hens was given a single oral dose of 750 mg/kg of TOCP (tri-*o*-tolyl phosphate, 99% pure, Eastman Kodak, Rochester, NY) in gelatin capsules. Another group of six served as control animals. Body weights were monitored weekly and animals were observed for neurological dysfunction. Twenty-one days after treatment, hens were anesthetized with CO_2 and killed by decapitation.

Preparation of MT and NF Proteins. Cold-stable and cold-labile MT proteins were obtained from *in vitro* polymerization of chicken brain tubulin by the method of Larson *et al.* (8), while spinal cord NFs were prepared as described (6) with only one axonal flotation step.

Phosphorylation Assays. The standard phosphorylation reaction for the brain proteins (8) was initiated by the addition of a 25-μl sample containing 100 μg of brain cytosol protein or 10 μg of either cold-labile or cold-stable MT to the reaction mixture. The standard reaction mixture contained, in a final volume of 200 μl, 10 mM Pipes (1,4-piperazinediethanesulfonic acid) at pH 7.4, 1 mM EDTA (ethylenediaminetetraacetic acid), 2 mM EGTA [ethylene glycol bis(β-aminoethylether)-*N,N,N',N'*-tetraacetic acid], 5 μM [γ-^{32}P]ATP (2900 Ci/mmol, New England Nuclear; 1 Ci = 37 GBq), and, where indicated, 10 mM $MgCl_2$, 5 mM $CaCl_2$, and 5 μg of calmodulin (Sciogen, Detroit, MI). Incubations were at 37°C and reactions were terminated after 1 min by the

Abbreviations: OPIDN, organophosphorus compound-induced delayed neurotoxicity; NF, neurofilament; MT, microtubule; TOCP, tri-*o*-cresyl phosphate; MAP, microtubule-associated protein.
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addition of 100 μl of "stop solution" [0.125 M Tris·HCl, pH 6.8/4.5% $NaDodSO_4$/20% (vol/vol) glycerol/10% (vol/vol) 2-mercaptoethanol]. The NF phosphorylation assay (6) was initiated by adding 25 μl containing 10 μg of NF proteins to a reaction mixture (total volume of 200 μl) containing 50 mM Pipes at pH 6.5, 5 μM [γ-^{32}P]ATP, and the following components as indicated in figures and tables: 10 mM $MgCl_2$ and 500 μM $CaCl_2$/5 μg of calmodulin. Incubations were at 37°C and the reactions terminated after 4 min by the addition of 100 μl of stop solution.

$NaDodSO_4$/Polyacrylamide Gel Electrophoresis ($NaDodSO_4$/PAGE). One-dimensional gel electrophoresis was performed by the method of Laemmli (11) on 8% resolving gel for brain proteins, 7% for NF proteins, and 4% stacking gels. Some samples from brain and spinal cord NF proteins received additions of tubulin purified by phosphocellulose P-11 (Whatman) chromatography (12) or NF proteins purified by Sepharose CL-6B (Pharmacia) chromatography (13), respectively, before they were subjected to two-dimensional electrophoresis (14). Gels from one- and two-dimensional electrophoresis were stained with Coomassie blue, dried under reduced pressure, and subjected to autoradiography.

Peptide Mapping. Individual ^{32}P-labeled polypeptides obtained from two-dimensional gel electrophoresis were digested with *Staphylococcus aureus* V8 protease (Miles Laboratories, Naperville, IL) as described (15), using 0.5 μg of protease. The products were subjected to $NaDodSO_4$/PAGE, and the gels were stained with silver stain (16), dried under reduced pressure, and subjected to autoradiography.

RESULTS

Clinical Assessment. During the 21-day experiment, TOCP-treated hens lost 33% of their body weight, while the control birds had no significant change in their weights. In TOCP-treated hens, neurologic dysfunction, expressed as mild ataxia characterized by diminished leg movement and reluctance to walk, was observed at day 10. Clinical signs progressed to disturbances in gait, unsteadiness, and frequency of falling on the floor. By day 15, treated animals were completely paralyzed.

Enhanced Phosphorylation of Brain Proteins in TOCP-Treated Hens. An increased phosphorylation in the presence of Mg^{2+}, Ca^{2+}, and calmodulin of proteins of 52, 59, 70, and 300 kDa was observed in brain cytosol and the cold-stable MT fraction of TOCP-treated hens, while in the cold-labile MT fraction only the phosphorylation of the 300-kDa protein seemed to be enhanced (Fig. 1). There were no significant changes in protein staining. The percentage increase in phosphorylation of the proteins was quantified as shown in Table 1: 160–165% for 52-kDa, 140–155% for 59-kDa, 139–166% for 70 kDa and 133–328% for 300-kDa, depending on the brain protein fractions, with the cold-stable MT fraction being, in general, the most heavily phosphorylated. The stimulation of phosphorylation in the cold-stable fraction was about 10 times greater than in brain cytosol, indicating that Ca^{2+}/calmodulin kinase activity was enriched in this fraction. Two-dimensional gel electrophoresis (Fig. 2) and one-dimensional peptide mapping (data not shown) utilizing phosphocellulose-purified α- and β-tubulins were run to identify the 52- and 59-kDa proteins. They were confirmed to be α- and β-tubulin. The 300-kDa protein was identified as MAP-2, while the identity of the 70-kDa protein is not known.

Ca^{2+}/Calmodulin-Stimulated Phosphorylation of Hen Spinal Cord NF Triplet Proteins After TOCP Treatment. Fig. 3 shows the effect of TOCP treatment on spinal cord NF proteins. There was a slightly enhanced Mg^{2+}-dependent phosphorylation of the 70-kDa (119%) and 160-kDa (129%) proteins (Table 2). Addition of Ca^{2+} and calmodulin further enhanced the incorporation of ^{32}P into these proteins (Table 3). The Ca^{2+}/calmodulin-stimulated phosphorylation was observed alone, after the Mg^{2+}-only baseline had been subtracted. The increase in protein phosphorylation in treated animals due to the Ca^{2+}/calmodulin-dependent kinase was determined in this manner. There was a 563% increase in protein phosphorylation of the 70-kDa protein, while the phosphorylations of the 160- and 210-kDa proteins were increased 221% and 196%, respectively (Table 3). Again, two-dimensional gel electrophoresis was carried out, with phosphocellulose-purified tubulin and Sepharose CL-6B-purified NF triplet proteins added to the NF samples (Fig. 4). These proteins were identified as α-tubulin (52 kDa), β-tubulin (59 kDa), and the three NF subunits of 70, 160, and 210 kDa. One-dimensional peptide mapping using *S. aureus* V8 protease was also carried out to further confirm that the spinal cord 52- and 59-kDa proteins were α- and β-tubulin. Identical polypeptide patterns were seen in these and the phosphocellulose-purified tubulin (data not shown).

Phosphopeptide Mapping. Limited proteolysis by *S. aureus* V8 protease of phosphorylated peptides that had been obtained from two-dimensional gel electrophoresis was used to examine the phosphopeptide patterns. There were identical

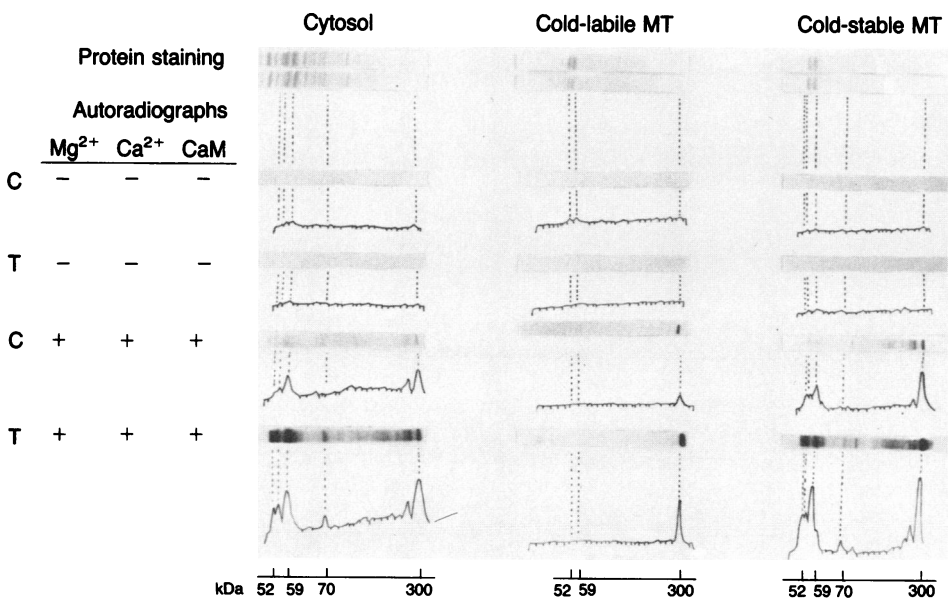

FIG. 1. Protein staining, autoradiography, and microdensitometry of proteins from hen brain. Proteins from control (C) and TOCP-treated (T) hens were incubated under the standard phosphorylation assay conditions in the presence or absence of the indicated cofactors (CaM, calmodulin). Brain cytosol (25 μg) and either cold-labile or cold-stable MTs (2.5 μg) were then subjected to gel electrophoresis and autoradiography. The amount of protein and the amount of phosphate incorporated into the specific proteins were quantified by densitometry (tracing below each autoradiograph).

Table 1. Stimulation of phosphorylation of brain proteins in hens treated with TOCP

Fraction	Protein mass, kDa	Phosphorylation (Densitometer units) Control	TOCP-treated	Treated/control, %
Brain cytosol	52	6.30 ± 0.96	10.1 ± 0.57	160
	59	7.96 ± 1.58	11.1 ± 0.81	140
	70	1.88 ± 0.18	2.61 ± 0.11	139
	300	3.83 ± 0.57	5.55 ± 0.52	145
Cold-stable MT	52	5.04 ± 1.5	8.30 ± 0.89	165
	59	6.14 ± 1.22	9.49 ± 0.73	155
	70	1.98 ± 0.20	3.28 ± 0.56	166
	300	9.22 ± 1.16	12.3 ± 0.73	133
Cold-labile MT	52	0.56 ± 0.24	0.75 ± 0.15	NS
	59	0.37 ± 0.16	0.54 ± 0.28	NS
	70	ND	ND	—
	300	1.68 ± 0.59	5.51 ± 0.48	328

Incorporation of phosphate into specific brain cytosolic proteins in the presence of 10 mM $MgCl_2$, 5 mM $CaCl_2$, and 5 μg of calmodulin. Proteins (25 μg of brain cytosol or 2.5 μg of either cold-labile or cold-stable MT per well) from six individual TOCP-treated or control animals were subjected to electrophoresis. Gels were then exposed on x-ray film for the same period of time. All lanes were scanned twice with an LKB densitometer. Values are in arbitrary units, mean ± SEM of at least 12 independent observations. Values from control and treated hens were compared by Student's *t* test; the difference was significant ($P < 0.05$) unless indicated by NS. ND, bands were not detectable by densitometer. There was no significant difference in Coomassie blue protein staining between control and treated.

phosphopeptide patterns of brain α- and β-tubulin, as well as of the spinal cord NF triplet proteins, in control and treated animals, indicating that the phosphorylation sites were most likely unaltered (Fig. 5). Furthermore, there was an enhanced ^{32}P incorporation in treated compared to control animals.

DISCUSSION

Cold-stable MTs have been reported to be insensitive to free Ca^{2+} at millimolar concentrations, a condition that causes rapid disassembly of cold-labile MTs. However, addition of Ca^{2+}/calmodulin at a substoichiometric concentration in the presence of ATP causes rapid disassembly of these same MTs (17). Since TOCP also enhanced Ca^{2+}/calmodulin-dependent phosphorylation of tubulin and MAP-2 (3), we investigated if the increased MT protein phosphorylation was increased in one of the two physical states in which MTs are found.

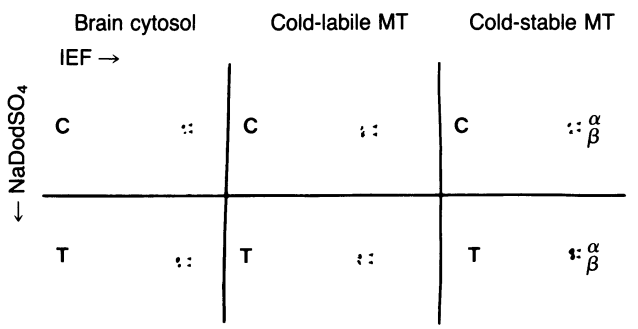

FIG. 2. Autoradiograph of two-dimensional gel electrophoresis of phosphorylated brain proteins from control (C) and TOCP-treated (T) hens. Proteins were phosphorylated in the presence of 10 mM $MgCl_2$, 5 mM $CaCl_2$, and 5 μg of calmodulin. To 50 μg of brain cytosol and 5 μg of either cold-labile or cold-stable MT, 5 μg of hen brain tubulin purified by chromatography on a phosphocellulose P-11 column was added and the mixture was subjected to two-dimensional gel electrophoresis and autoradiography. IEF, isoelectric focusing. The gel of the cold-labile MT was exposed 5 times longer than the gels of the cytosol and cold-stable MT. The 52- and 59-kDa proteins comigrated with the phosphocellulose-purified α- and β-tubulins (arrowheads).

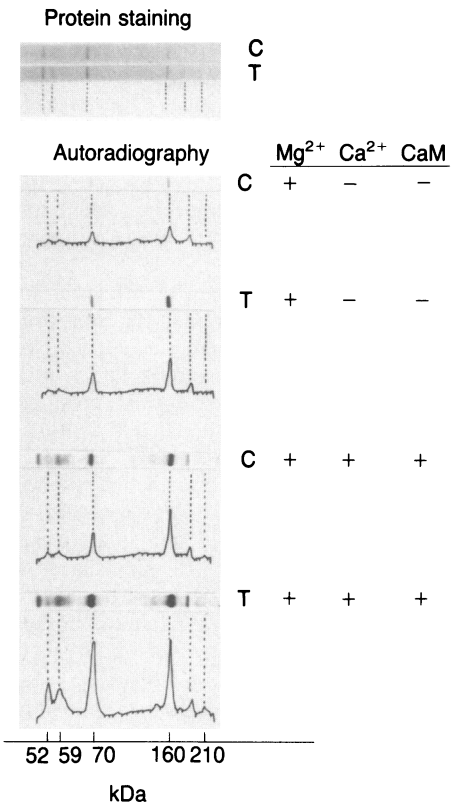

FIG. 3. Protein staining, autoradiography, and microdensitometry of hen spinal cord crude NF proteins. The load was 2.5 μg of protein per well. See legend of Fig. 1.

In this investigation, we have found that tubulin phosphorylation was enhanced in the cold-stable MT fraction as well as in crude brain cytosol from TOCP-treated animals in comparison to control. In comparison to the starting cytosolic preparation, the kinase activity in the cold-stable MT preparation was enriched by at least 10-fold. Furthermore, the cold-stable MT fraction also showed a 20-fold increase in phosphorylation of MAP-2. The relative specific activity of Ca^{2+}/calmodulin-dependent phosphorylation of α- and β-tubulin, as well as MAP-2, is in agreement with the report by Larson *et al.* (8); this activity has subsequently been shown to be calmodulin kinase II (9). This leads to the speculation that the same enzyme is involved in the enhanced phosphorylation of tubulin in the TOCP-treated hens. There was also an enhanced phosphorylation of a 70-kDa protein in the TOCP-treated animals in both the crude cytosol and the cold-stable MT. The identity and the functional significance of the 70-kDa protein is unknown. A previous study indicated that this 70-kDa protein was not the smaller subunit of the NFs (3). It is of interest to examine if this 70-kDa protein is one of the stable tubule-only polypeptides [STOPs (18)], which can confer cold stability to the cold-labile MTs. The Ca^{2+}/calmodulin-dependent phosphorylation of a 72-kDa STOP has been reported to abolish the cold-stabilizing activity of this protein on MTs (18).

In the cold-labile MT fraction, tubulin was very lightly phosphorylated, while MAP-2 phosphorylation was greatly enhanced in TOCP-treated animals. MAP-2 has been reported to be a better substrate than tubulin for calmodulin kinase II, which copurifies with cold-stable MTs (5). The evidence that MAP-2 was more phosphorylated than tubulin, in addition to the diminished activity of the kinase in the cold-labile

Table 2. TOCP-enhanced *in vitro* phosphorylation of hen spinal cord NF proteins

Protein mass, kDa	Phosphorylation: 10 mM $MgCl_2$ — Densitometer units: Control	10 mM $MgCl_2$ — Densitometer units: TOCP-treated	10 mM $MgCl_2$ — Treated/control, %	10 mM $MgCl_2$/500 μM $CaCl_2$/5 μg CaM — Densitometer units: Control	10 mM $MgCl_2$/500 μM $CaCl_2$/5 μg CaM — Densitometer units: TOCP-treated	10 mM $MgCl_2$/500 μM $CaCl_2$/5 μg CaM — Treated/control, %
52	ND	ND	—	1.93 ± 0.15	2.43 ± 0.19	126
59	ND	ND	—	5.82 ± 0.58	9.29 ± 0.63	160
70	6.07 ± 0.58	7.22 ± 0.49	119	6.74 ± 0.56	9.37 ± 0.82	139
160	6.67 ± 0.72	8.62 ± 0.62	129	7.46 ± 0.90	10.3 ± 0.51	138
210	2.21 ± 0.19	2.54 ± 0.18	NS	2.47 ± 0.17	3.05 ± 0.20	123

The incorporation of ^{32}P into spinal cord crude NF proteins was measured under the standard phosphorylation assay conditions with the indicated cofactors. CaM, calmodulin. Values are in arbitrary units, mean ± SEM of at least 12 independent observations. Differences were evaluated as described for Table 1. There were no significant differences in protein staining.

fraction compared to the cold-stable fraction, leads to the speculation of calmodulin kinase II being involved here, as well.

In control animals, Ca^{2+} and calmodulin stimulated the phosphorylation of the three NF subunits (70-, 160-, and 210-kDa), in comparison to Mg^{2+} only, while tubulin phosphorylation was apparent only in a Ca^{2+}- and calmodulin-dependent reaction. This is in agreement with reports (10, 19) of at least two kinases, both of which are Mg^{2+} dependent, being associated with the NF proteins. Mg^{2+} was found to significantly increase the phosphorylation of the 70- and 160-kDa NF proteins from TOCP-treated animals, while Mg^{2+}, Ca^{2+}, and calmodulin significantly increased the phosphorylation of all three NF proteins. When the relative amount of Ca^{2+}/calmodulin-stimulated phosphorylation was examined, a marked increase was found in the phosphorylation of all three NF subunits from TOCP-treated animals. Several investigators have determined that the interaction of the three NF subunits is delicately balanced by the extent of phosphorylation (6, 20). TOCP treatment may interfere with this balance and promote the association of NFs into uncharacteristic arrangements.

The effect of TOCP treatment on the tubulin that copurified with NF was striking. There was a significant increase in phosphorylation of spinal cord α- and β-tubulin in a Ca^{2+}- and calmodulin-dependent manner. Since calmodulin kinase II also copurifies with NF proteins, it is very likely that this same kinase is responsible for the enhancement in phosphorylation of these proteins as well (7). NFs have also been reported to possess a firmly associated protein kinase activity that is stimulated by the presence of MAP-2 *in vitro* in a Mg^{2+}- and Ca^{2+}-dependent manner (19). However, since the baseline (Mg^{2+} only) NF phosphorylation is much greater than the tubulin phosphorylation, there is most likely more than one kinase acting on them.

Morphological and biochemical studies have suggested that neuronal cytoskeletal proteins (α- and β-tubulin, NF triplet proteins, and MAPs) interact with each other. MAPs have been proposed to mediate the interactions between MT and NF. Heimann *et al.* (20) have demonstrated that MAPs bind specifically to the 70-kDa NF subunit. Runge *et al.* (21) have also reported an ATP-induced formation of an associated complex between MTs and NFs. An increased Ca^{2+}/calmodulin-dependent phosphorylation of MAP-2 was observed to inhibit MT assembly (22). Calmodulin kinase II is known to phosphorylate tubulin, MAP-2, and NF proteins. Calmodulin kinase II has also been suggested to play an important role in regulating MT and cytoskeletal dynamics (8,

Table 3. Relative amounts of calcium/calmodulin-stimulated phosphorylation of spinal cord NF proteins in hens after TOCP treatment

Protein mass, kDa	Phosphorylation: Densitometer units: Control	Phosphorylation: Densitometer units: TOCP-treated	Treated/control, %
70	0.35 ± 0.21	1.97 ± 0.74	563
160	0.79 ± 0.30	1.74 ± 0.44	221
210	0.26 ± 0.17	0.51 ± 0.09	196

The stimulation of ^{32}P incorporation into spinal cord crude NF proteins was measured in the presence of Mg^{2+}, Ca^{2+}, and calmodulin. Values express the difference between proteins phosphorylated in a Mg^{2+}-, Ca^{2+}-, and calmodulin-dependent manner relative to that of the solely Mg^{2+}-dependent. All numbers are mean ± SEM of at least 12 independent observations. Control and treated phosphorylations differed significantly ($P < 0.05$ by Student's t test). The Coomassie blue staining patterns of control and TOCP-treated NF proteins did not significantly differ, either qualitatively or quantitatively.

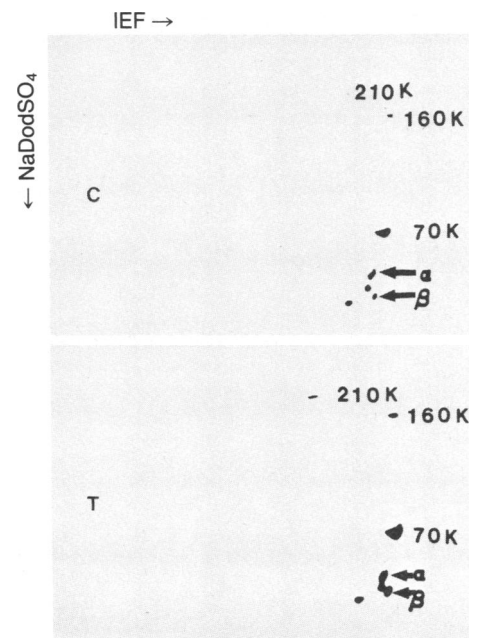

FIG. 4. Autoradiography of two-dimensional gel electrophoresis of hen spinal cord crude NF proteins phosphorylated in the presence of 10 mM $MgCl_2$, 500 μM $CaCl_2$, and 5 μg of calmodulin. Five-microgram protein samples were mixed with 5 μg of Sepharose-CL-6B-purified NF proteins and 5 μg of phosphocellulose-purified tubulin. The 70-, 160-, and 210-kDa proteins (70 K, 160 K, and 210 K, respectively) comigrated with the Sepharose CL-6B-purified NF triplet proteins and the 52- and 59-kDa proteins comigrated with α- and β-tubulins.

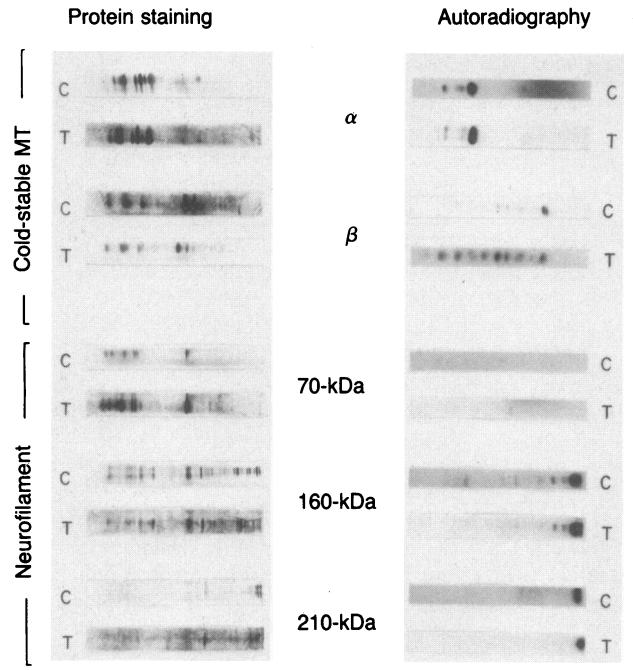

FIG. 5. Peptide mapping and autoradiography of hen brain cold-stable MTs and spinal cord NFs. Corresponding phosphorylated peptide spots from two-dimensional electrophoresis were excised and subjected to limited proteolysis and then electrophoresis. Gels were then subjected to autoradiography. There were identical phosphopeptide and protein staining patterns of corresponding proteins in control (C) and treated (T) animals. Enhanced incorporation of phosphate was seen in treated compared to control animals.

10). Furthermore, DeLorenzo (23) has reported that endogenous Ca^{2+}/calmodulin-dependent phosphorylation of rat brain synaptoplasmic tubulin transformed it into twisted filamentous polymers distinct from MTs. From these observations several possibilities exist on the role of the increased phosphorylation and the pathogenesis of OPIDN. The increased phosphorylation may interfere with normal association of NFs, tubulin, and MAPs, leading to the formation of "tangled filaments" observed in OPIDN (24). Hence, the evidence that TOCP perturbs the endogenous phosphorylation of tubulin, MAP-2, and NFs suggests that phosphorylation may play a role in the normal interactions between these neuronal cytoskeletal proteins. Alterations of these interactions by TOCP may contribute to the resulting axonopathy.

The increase in endogenous phosphorylation of these cytoskeletal proteins after TOCP treatment can be explained by (*i*) phosphatase inhibition, (*ii*) an increased number of phosphorylatable sites, or (*iii*) an increased protein kinase activity/availability. The first possibility was ruled out, because a study of *in vitro* rates of phosphorylation and dephosphorylation by means of the pulse–chase technique showed that phosphatase activity was not altered by TOCP treatment (1). In the present study, peptide mapping of the phosphorylated cytoskeletal proteins showed identical phosphopeptide patterns in both TOCP-treated and control animals, indicating that the phosphorylation sites were not altered by TOCP treatment. Phosphopeptide mapping also showed that the incorporation of ^{32}P into the cytoskeletal proteins was enhanced in the TOCP-treated compared to control animals. Since there was no alteration in the amount of substrate protein, the data suggest that TOCP treatment altered the kinase activity. It remains to be determined whether the activity or the amount of kinase was increased after TOCP treatment.

In summary, we have examined the biochemical events occurring in the nervous tissues as the result of delayed neurotoxicity induced by TOCP in chickens. Enhanced phosphorylation of cytoskeletal proteins has a role in the mechanism of OPIDN.

This study was supported in part by National Institute of Occupational Safety and Health Grant OHO 2003 and National Institute of Environmental Health Sciences Grant ESO 2717.

1. Abou-Donia, M. B., Patton, S. E. & Lapadula, D. M. (1984) in *Cellular and Molecular Neurotoxicology*, ed. Narahashi, T. (Raven, New York), pp. 265–283.
2. Abou-Donia, M. B. (1981) *Annu. Rev. Pharmacol. Toxicol.* **21,** 511–548.
3. Suwita, E., Lapadula, D. M. & Abou-Donia, M. B. (1986) *Brain Res.* **374,** 199–203.
4. Bennett, M. K., Erondy, N. E. & Kennedy, M. B. (1983) *J. Biol. Chem.* **258,** 12735–12744.
5. Goldenring, J. R., Gonzalez, B., McGuire, J. S., Jr., & DeLorenzo, R. J. (1983) *J. Biol. Chem.* **258,** 12632–12640.
6. Julien, J. P. & Mushynski, W. E. (1982) *J. Biol. Chem.* **257,** 10467–10470.
7. Goldenring, J. R., Casanova, J. E. & DeLorenzo, R. J. (1984) *J. Neurochem.* **43,** 1669–1679.
8. Larson, R. E., Goldenring, J. R., Vallano, M. L. & DeLorenzo, R. J. (1985) *J. Neurochem.* **44,** 1566–1574.
9. Vallano, M. L., Goldenring, J. R., Buckholz, T. M., Larson, R. E. & DeLorenzo, R. J. (1985) *Proc. Natl. Acad. Sci. USA* **82,** 3202–3206.
10. Vallano, M. L., Buckholz, T. M. & DeLorenzo, R. J. (1985) *Biochem. Biophys. Res. Commun.* **130,** 957–963.
11. Laemmli, U. K. (1970) *Nature (London)* **227,** 680–685.
12. Brenner, S. L., Pardue, R. L., Wible, L., Reading, C. L. & Brinkley, B. R. (1984) in *Laboratory Method Manual for Hormone Action and Molecular Endocrinology*, eds. Schrader, W. T. & O'Malley, B. W. (Houston Biological, Houston, TX), pp. 12-3–12-5.
13. Chiu, F.-C. & Norton, W. T. (1982) *J. Neurochem.* **39,** 1252–1260.
14. O'Farrell, P. H. (1975) *J. Biol. Chem.* **250,** 4007–4021.
15. Cleveland, D. W., Fisher, S. G., Kirschner, M. W. & Laemmli, U. K. (1977) *J. Biol. Chem.* **252,** 1102–1106.
16. Oakley, B. R., Kirch, D. R. & Morris, N. R. (1980) *Anal. Biochem.* **105,** 361–363.
17. Job, D., Fischer, E. H. & Margolis, R. L. (1981) *Proc. Natl. Acad. Sci. USA* **78,** 4679–4682.
18. Job, D., Rauch, C. T., Fischer, E. H. & Margolis, R. L. (1983) *Proc. Natl. Acad. Sci. USA* **80,** 3894–3898.
19. Runge, M. S., El-Maghrabi, M. R., Claus, T. H., Pilkis, S. J. & Williams, R. C., Jr. (1981) *Biochemistry* **20,** 175–180.
20. Heimann, R., Shelanski, M. L. & Liem, R. K. H. (1985) *J. Biol. Chem.* **260,** 12160–12166.
21. Runge, M. S., Laue, T. M., Yphantis, D. A., Lifsics, M. R., Saito, A., Altin, M., Reinke, K. & Williams, R. C., Jr. (1981) *Proc. Natl. Acad. Sci. USA* **78,** 1431–1435.
22. Yamamoto, H., Fukunaga, K., Tanaka, E. & Miyamoto, E. (1983) *J. Neurochem.* **41,** 1119–1125.
23. DeLorenzo, R. J. (1982) *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **41,** 2265–2272.
24. Bischoff, A. (1967) *Acta Neuropathol.* **9,** 158–174.