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Neurofilament degradation in the nervous system of rats intoxicated with acrylamide, related compounds or 2,5-hexanedione

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Abstract. Degradation of neurofilament (NF) proteins by Ca^{2+}-activated neutral protease (CANP) was studied in the nervous system of rats treated with neurotoxic or non-neurotoxic compounds. In the tibial nerve, the degradation of NF 68K was depressed by five neurotoxic compounds: acrylamide, N-hydroxymetylacrylamide, N-isopropylacrylamide, methacrylamide and 2,5-hexanedione. A non-neurotoxic compound, diacetone acrylamide, did not show any effect on the degradation. An immunoblot analysis confirmed the reduction in the degradation and revealed a difference in the degradation pattern between the control and acrylamide-treated rats. In the spinal cord, the degradation of the three subunits of NF was depressed in animals treated with acrylamide. Although the exact mechanism of the reduction in the degradation of NF is not yet known, the present results suggest that an inhibitory effect on CANP activity might be relevant to the mechanism of neurotoxic action of acrylamide derivatives.

Key words: Acrylamide – N-Hydroxymethylacrylamide – N-Isopropylacrylamide – Methacrylamide – Diacetone acrylamide – 2,5-Hexanedione – Axonopathy – Calcium activated neutral protease – Neurofilament

Introduction

Repeated exposure to monomeric acrylamide produces peripheral neuropathy in man and in experimental animals (Spencer and Schaumburg 1974, 1977). The neurotoxicity has also been reported for some analogues of acrylamide in experimental animals (Barnes 1970; Edwards 1975; Hashimoto et al. 1981; Tanii and Hashimoto 1983). The neuropathy due to acrylamide is characterized by multifocal axonal swellings which occur preferentially at distal nodes of Ranvier (Spencer and Schaumburg 1974) and in the gracile nucleus (Prineas 1969). In addition, the swelled area of myelinated axons contains a massive accumulation of 10 nm neurofilaments (NFs) (Suzuki and Pfaff 1973). Such changes are also described in some other toxic neuropathies; *n*-hexane (Spencer and Schaumburg 1977; Couri and Milks 1982), carbon disulfide (Seppalainen and Haltia 1980), aluminum salts (Wisniewski et al. 1984) and β,β′-iminodipropionitrile (Clark et al. 1980; Griffin et al. 1982). As for the mechanism of neuofilamentous accumulation, Schoental and Cavanagh (1977) suggested that toxic substances such as acrylamide may enter the nerve fibers at the terminals or nodes of Ranvier, and can interact with neurofilaments and other molecules within an axon.

NFs are part of the structural network of neuronal cells and the major component of the axoplasm. NFs in rat peripheral nerve consist of 200K, 160K and 68K triplet proteins (Mori and Kurokawa 1980). After being synthesized in the cell body, NFs are subject to post-translational modifications by a protein kinase (Julien and Mushynski 1982; Nixon and Lewis 1986) and by a calcium-activated neutral protease (CANP) (Schlaepfer and Micko 1978; Kamakura et al. 1983). There have been several reports on CANP in invertebrate (Gilbert and Newby 1975) and vertebrate (Schlaepfer and Micko 1979) nerves. This enzymic activity has been shown to be involved in turnover of NF (Gallant et al. 1986) and in Wallerian degeneration of peripheral nerves (Schlaepfer 1977).

CANP is a thiol protease (Malik et al. 1981), and since acrylamide reacts with non-protein and protein sulfhydryl (Hashimoto and Aldridge 1970), it is expected that acrylamide inhibits CANP by interacting with the sulfhydryl residue on CANP. The present study was undertaken to examine the degradation of NF by CANP in the nervous system of rats intoxicated with acrylamide, related compounds or 2,5-hexanedione to elucidate the mechanism of neurofilamentous accumulation. The effects of neurotoxic compounds on neurofilamentous degradation are compared with those of non-neurotoxic ones, and the results are discussed with regard to neurotoxicity of the compounds.

Materials and methods

Anti-NF (68K) mouse monoclonal antibody was obtained from Boeringer Mannheim, FRG, Vectastain ABC (peroxidase mouse IgG) Kit from Vector Laboratories, Inc. (Burlingame, USA), bovine albumin (globulin free) from Sigma Chemical Co. (St Louis, USA). Chemicals used for polyacrylamide gel electrophoresis were purchased from Wako Pure Chemical Industries (Osaka, Japan). All other chemicals used were of reagent grade. Acrylamide and analogues had a purity of greater than 95% in gas chromatog-

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Abbreviations: CANP, calcium-activated neutral protease; CBB, Coomassie Brilliant Blue R-250; NF, neurofilament; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis

raphy (Hashimoto et al. 1981). The purity of 2,5-hexanedione from Tokyo Kasei Co. (Tokyo, Jaoan) was more than 95%.

Animals. Male rats of Wistar strain were randomly placed in plastic cages containing wooden flakes. They were fed laboratory chow (MF, Oriental Yeast Co., Tokyo, Japan) and water containing the test compounds ad lib. Control animals were fed the chow and water only.

Treatment of animals. Rats were dosed for 120 days with each of the test compounds dissolved in drinking water at concentrations used in our previous report (Tanii and Hashimoto 1983). The concentration of 2,5-hexanedione was chosen to induce neuropathy in 120 days of treatment.

In vitro incubation of the rat tibial nerve. The test chemical-treated or untreated rats were killed by decapitation and tibial nerve segments 20 mm in length proximal to the ankle were removed. They were washed with saline and cut into four segments of 5 mm each. The four segments were incubated according to Kamakura et al. (1983) in 1 ml of the following solution; 5 mM Tris-HCl (pH 7.4), 130 mM NaCl, 4 mM KCl, 1 g/l glucose, 100 U/ml penicillin G, 10 mM $CaCl_2$, 10% (vol/vol) triton X-100 and with or without 2 mM leupeptin (acetyl-L-leucyl-L-leucyl-L-argininal). Triton X-100 10% (vol/vol) was used to stimulate the penetration of Ca^{2+} into the tibial nerve. The nerves were incubated at 37°C with shaking in 1.5 ml test tubes.

SDS-PAGE. The four segments were homogenized in 100 µl of a solution consisting of 65 mM Tris-HCl (pH 6.8), 2% (wt/vol) SDS, 5% (vol/vol) 2-mercaptoethanol, 10% (vol/vol) glycerol and 0.001% (wt/vol) bromophenol blue (sample buffer) in a Teflon-glass homogenizer, followed by boiling at 100°C for 5 min. An aliquot of the solubilized homogenate was resolved on a 7.5% polyacrylamide slab gel with a 3% polyacrylamide stacking gel according to Laemmli's method (1970). Bands were visualized by staining with Coomassie Brilliant Blue R-250 (CBB) and the destained gel was scanned with a chromatoscanner (Shimazu CS-930, Kyoto, Japan). The protein content of the solubilized sample was determined by the method of Bradford (1976) using bovine serum albumin as the standard.

Degradation of NFs from spinal cords. Rats untreated or treated with acrylamide for 90 days were killed by decapitation and their spinal cords were removed. Pellets (P_2) enriched in NFs were prepared according to Chiu and Norton (1982), and suspended in the buffer; 50 mM Tris-HCl (pH 7.6), 25 mM KCl and 10 mM $MgCl_2$. Incubation mixtures contained a 0.2 ml portion of the NF suspension (0.28 mg protein), 5 mM $CaCl_2$ and 10 mM Tris-HCl (pH 7.6) in a final volume of 1.0 ml. The mixtures were incubated with shaking in 1.5-ml test tubes at 37°C for 0 or 60 min. After incubation, the reaction was stopped by the addition of 0.2 ml ice-cold 70% trichloroacetic acid. The trichloroacetic acid-insoluble protein was pelletted by centrifugation, washed successively with ethanol, ethanol-ether (1:1) and ether, dried and dissolved in sample buffer. Aliquots of 20 µg protein were subjected to SDS-PAGE as mentioned before.

Immunoblotting. Proteins of the tibial nerve homogenates were separated on SDS-PAGE as described above. Electrophoretic transfer of proteins from gels to nitrocellulose paper was performed according to Towbin et al. (1979) with a slight modification, i.e., for 4 h at 4°C, 80 V, and 200–300 mA. The paper was then removed, and the reaction was blocked with bovine serum albumin for 30 min. After four washes in a solution consisting of 10 mM phosphate buffer (pH 7.5), 0.9% (wt/vol) NaCl and 0.05% (vol/vol) Tween 20, (TPBS), the paper was incubated with a 1:500 dilution of anti-NF (68K) mouse monoclonal antibody in 0.1% bovine serum albumin in TPBS for 1 h at room temperature. After four washes in TPBS, the second antibody (biotynylated horse anti-mouse antibody) was applied for 30 min. The paper was washed four times with TPBS and then incubated with an avidin-biotin-peroxidase complex (ABC) for 30 min at room temperature. After four washes in phosphate-buffered saline, the bound peroxidase on the paper was developed in 100 ml Tris-buffered saline, 20 mg diaminobenzidine and 20 µl fresh H_2O_2.

Statistics. Differences in values between control and treated groups were evaluated by one-way analysis of variance, followed by Dunnett's multiple comparisons with the control value (Dunnett 1964).

Results

Body weight and neurotoxicity

Rats were treated with acrylamide, related compounds or water only, and their body weights and clinical signs of ataxia were assessed 120 days after the first treatment (Table 1). Significant suppressions of body weight gain were seen during the 120-day treatment of animals with five compounds: acrylamide, N-hydroxymethylacrylamide, N-isopropylacrylamide, diacetone acrylamide, and 2,5-hexanedione, whereas no significant change was seen in rats treated with methacrylamide. Neurological signs were detected in rats after treatment with five compounds:

Table 1. Body weight and neuropathy of rats treated with acrylamide, related compounds or 2,5-hexanedione

Compound	Dose level (mM)	Body weight (% of control)	Neurotoxicity
Control	0	100 ± 11	
Acrylamide	2.81	76 ± 11*	+
N-Hydroxymethylacrylamide	8.65	64 ± 2*	+
N-Isopropylacrylamide	2.65	80 ± 4*	+
Methacrylamide	23.5	90 ± 12	+
Diacetone acrylamide	171	44 ± 11*	−
2,5-Hexanedione	42.0	52 ± 5*	+

Rats were treated with each compound in drinking water for 120 days, and their body weights and clinical signs of ataxia were assessed. A solution for each group (four rats/test compound) was prepared freshly at least twice per week. Body weights of animals are shown as the mean ± SD (controls, 576 ± 64 g). Neurotoxicity of the test compound was expressed as: +: rats showed signs of ataxia, i.e., weakness and a tendency toward spreading and dragging of hindlimbs, −: animals did not show any signs of neuropathy. Significant differences between mean values of the control and experimental groups are indicated by asterisks: * $p < 0.05$

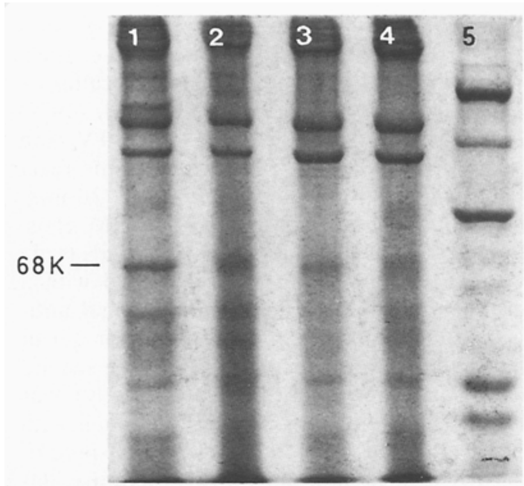

Fig. 1. Time course of in vitro neurofilament degradation. The tibial nerves from untreated rats were incubated with Ca^{2+} at 37°C in the presence or absence of leupeptin. *Lane 1* respresents the homogenate from the nerve incubated in the presence of leupeptin for 10 h. *Lanes 2–4* show the homogenates from the nerves incubated in the absence of leupeptin for 2,6 and 10 h, respectively. *Lane 5* shows the molecular weight standards (180, 140, 100, 42 and 39K daltons from top to bottom). *68K* denotes neurofilament with molecular weight of 68K

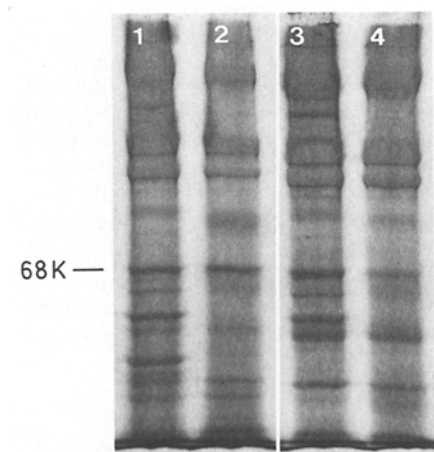

Fig. 2. Degradation of tibial nerve neurofilaments from acrylamide-treated or untreated rats. The tibial nerves from acrylamide-treated or untreated rats for 120 days were incubated with Ca^{2+} in the presence *(lanes 1, 3)* or absence (*lanes 2, 4)* of leupeptin at 37°C for 6 h. *Lanes 1, 2* show the homogenates from acrylamide-treated rats. *Lanes 3, 4* represent the homogenates from untreated rats. *68K* denotes neurofilament with molecular weight of 68K

acrylamide, N-hydroxymethylacrylamide, N-isopropylacrylamide, methacrylamide, and 2,5-hexanedione. Animals treated with diacetone acrylamide showed no neurological signs in spite of the suppression of body weight gain.

Degradation of NF from tibial nerve

To determine the condition of the degradation of NF by CANP (Fig. 1), tibial nerves from the untreated rats were incubated with Ca^{2+} in the presence (lane 1) or absence (lanes 2–4) of leupeptin. The amount of protein applied to gel lanes 1, 2, 3 and 4 was 6.1, 5.9, 7.0 and 7.3 μg, respectively. NF 68K was retained in the presence of leupeptin after 10 h incubation, while the NF in the absence of leupeptin was degraded with time, although a diffuse band remained after 6–10 h incubation. Based on this experiment, 6 h was selected for the time of incubation.

A comparison was made between the acrylamide-treated and the control rats for degradation of NF 68K determined on SDS-PAGE (Fig. 2). The tibial nerves from intoxicated (lanes 1 and 2) and control (lanes 3 and 4) rats were incubated with Ca^{2+} in the presence (lanes 1 and 3) or absence (lanes 2 and 4) of leupeptin at 37°C for 6 h. The amount of protein applied to the gel lanes 1, 2, 3 and 4 was 7.1, 6.8, 8.5 and 7.2 μg, respectively. After incubation, NF 68K was retained in lanes 1, 2 and 3, while a diffuse band was visible in lane 4, indicating that the degradation of NF 68K from intoxicated rats was reduced compared with the control. Analysis of NFs 200K and 160K could not be performed because of their paucity in the tibial nerve.

The degradation of NF 68K in the tibial nerve by CANP was also studied in rats treated with each of the test chemicals other than acrylamide for 120 days. There was a reduction in the degradation in rats treated with four neurotoxic compounds as well, compared to the controls. They were: N-hydroxymethylacrylamide, N-isopropylacrylamide, methacrylamide and 2,5-hexanedione. No reduction in the degradation, however, was seen in rats treated with diacetone acrylamide, a non-neurotoxic compound (data not shown).

Immunoblot analysis

The degradation of NF 68K by CANP was further confirmed by immunoblot analysis (Fig. 3). The tibial nerves from rats untreated (lanes 1 and 2) or treated (lanes 3 and 4) with acrylamide for 60 days were incubated with Ca^{2+} in the presence (lanes 1 and 3) or absence (lanes 2 and 4) of leupeptin at 37°C for 6 h. Anti-NF (68K) antibody bound to NF 68K (a) and to an unknown polypeptide (c) in the presence of leupeptin (lanes 1 and 3). In the absence of leupeptin, NF 68K from the control was completely degraded into a polypeptide (d) (lane 2), while NF 68K from intoxicated rats (lane 4) was partly degraded into two polypeptides (b and d), the former of which could not be detected in the control nerve (lane 2).

Degradation of NFs from spinal cord

Figure 4 shows chromatoscan of the time-dependent degradation of NFs from spinal cord incubated with Ca^{2+}. Three subunits of NFs from control rats were markedly degraded in 60 min (Fig. 4A), while those from animals treated with acrylamide for 90 days were tolerable to the proteolysis (Fig. 4B). Per cent degradation of 70, 160 and 210K NF subunits was 65, 85 and 63 for the control, and 22, 35 and 13 for treated rats (Table 2).

Discussion

Recently, many studies have been done on the inhibition of axoplasmic flow (Jakobsen and Sidenius 1983; Miller and Spencer 1984; Brændgaard and Sidenius 1986) and NF accumulations in the nerve (Prineas 1969; Spencer and Schaumburg 1974; Veronesi et al. 1983) in acrylamide or 2,5-hexanedione neuropathy. It is possible that these ef-

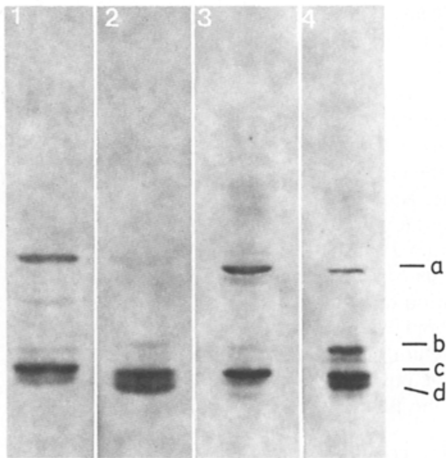

Fig. 3. Immunoblots of homogenates of tibial nerves from control *(lanes 1, 2)* and acrylamide-treated *(lanes 3, 4)* rats. Rats were given water only or water containing acrylamide for 60 days. After treatment, they were killed by decapitation, and the tibial nerves were removed and incubated with Ca^{2+} in the presence *(lanes 1, 3)* or absence (*lanes 2, 4)* of leupeptin at 37°C for 6 h. The nerves were homogenized in sample buffer. The amount of protein applied to gel lanes 1, 2, 3 and 4 was 5.8, 6.5, 5.9 and 5.4 µg, respectively. They were resolved on 7.5% polyacrylamide gels followed by electronic transfer of proteins from gels to nitrocellulose paper. The paper was cut into strips and processed with anti-neurofilament (68K) antibody. 68K neurofilament *a* was degraded into polypeptides *b* and *d*. An unknown polypeptide *c* reacted with anti-neurofilament (68K) even after being blocked with albumin

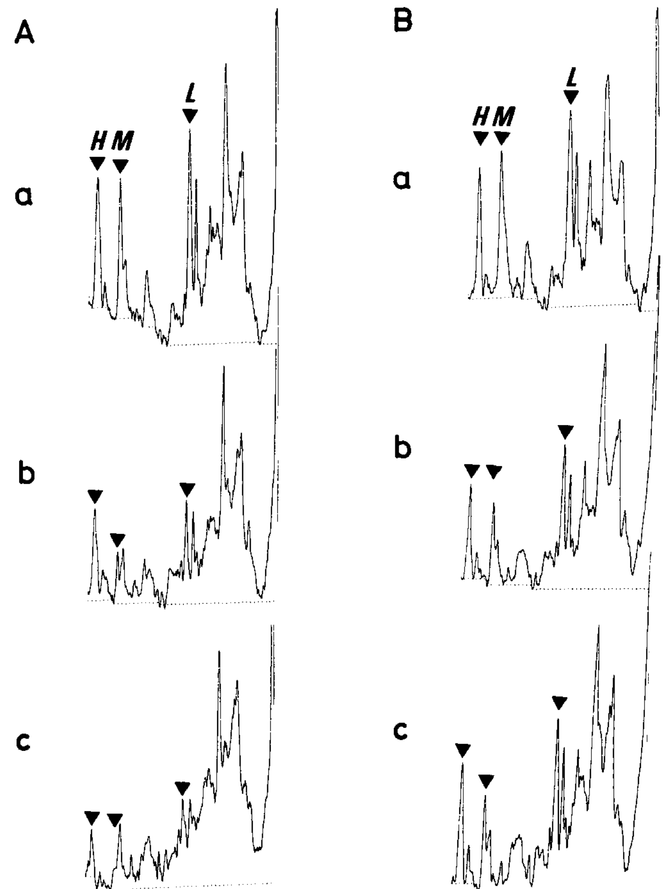

Fig. 4. Time course of the degradation of spinal cord neurofilaments from control rats **A** and from rats treated with acrylamide for 90 days **B.** The neurofilament-enriched fractions were incubated with Ca^{2+} at 37°C for 0 *a*, 30 *b* and 60 *c* min. After incubation, the samples were analysed by SDS-PAGE followed by staining with CBB. The proteins on gels were quantified with a chromatoscanner. *H, M* and *L* denote neurofilaments with molecular weight of 210K, 160K and 70K, respectively

Table 2. Degradation of NFs of spinal cord from rats treated with or without acrylamide

Condition	% Degradation of neurofilament		
	70K	160K	210K
Control	65±8	85±3	63±3
Acrylamide	22±4	35±3	13±5

Rats were treated with acrylamide or water only for 90 days, and their spinal cords were removed. Partially purified NF fractions from the spinal cords were incubated with Ca^{2+} at 37°C for 0 or 60 min. After solubilization by SDS, aliquots of the samples were analysed by SDS-PAGE followed by staining with CBB. NF bands on gels were quantified with a chromatoscanner. The degree of the degradation was determined by comparison between the protein peaks from the NF fractions incubated with Ca^{2+} for 0 and 60 min. Each value represents the mean±SD of four rats

fects are associated with each other and are involved in the mechanism of neurotoxicity, although our knowledge about the interactions is insufficient at present. As for the disturbance of axoplasmic transport, much attention has been focused on the inhibition of glycolytic enzymes in nerves by these neurotoxicants (Howland et al. 1980; Sabri 1984; Tanii and Hashimoto 1984, 1985; Sakamoto and Hashimoto 1985 a, b), since the axonal transport system has been known to be energy dependent (Adams 1982).

With regard to NF accumulation in these neuropathies, several mechanisms could be postulated, e.g., an inhibition of NF degradation at the distal end of the nerve, being due either to NFs becoming more resistant than controls to the action of proteolytic enzymes such as CANP, after a modification in their physico-chemical properties, and/or inhibition of the activity of the enzymes by neurotoxicants, and/or others. Concerning the NF modification, Sayre et al. (1985) have proposed a hypothesis based on the charge-neutralization theory, i.e., γ-diketones such as 2,5-hexanedione would result in the formation of pyrrole groups on lysyl residues, leading to covalent crosslinking between proteins (Graham et al. 1982, 1984), and acrylamide would add to lysyl ε-amino group, leading to neutralization of lysyl side chains.

The results obtained in the present study have shown that degradation of NF 68K by CANP in the tibial nerves was inhibited in rats treated with neurotoxic compounds, but was not inhibited in rats treated with non-neurotoxic compounds, nor was it inhibited in the control. We could not demonstrate the same effect on 200 and 160K subunits, because of the paucity of these units in the tibial nerve, but we could show the inhibitory effect of acrylamide on degradation of the triplet NFs in the spinal cord. Roots (1983) has found an accumulation of NF in synaptic terminals of the optic nerve by injecting leupeptin, an inhibitor for CANP, into the optic tectum of goldfish, which is also

observed in acrylamide (Prineas 1969) and 2,5-hexanedione (Veronesi et al. 1983) neuropathies. Biochemical studies have revealed that degradation of NF was induced by Ca^{2+} and inhibited by the sulfhydryl inhibitors, indicating that CANP is a thiol protease (Malik et al. 1981). It is therefore possible that acrylamide inhibits CANP as do sulfhydryl inhibitors, because acrylamide and its derivatives react with non-protein and protein sulfhydryl (Hashimoto and Aldridge 1970) and inhibit glycolytic enzymes by reacting with the sulfhydryl on the protein (Howland et al. 1980; Tanii and Hashimoto 1985).

Immunoblot analysis confirmed the reduction in degradation of NF 68K and showed the difference in the degradation pattern between the control and acrylamide-treated rats. This result suggests another possibility, namely that NF 68K from the treated animals might have been modified by acrylamide to become resistant to the complete degradation seen in the control, although no direct interaction has been found between NF and acrylamide (to be published).

Concerning 2,5-hexanedione, the reduction in NF degradation could be accounted for by the chemical modification of NF as pointed out by Sayre et al. (1985). 2,5-Hexanedione appears not to share with acrylamide the mechanism responsible for NF accumulation, since there has been no report on the reactivity of 2,5-hexanedione with the sulfhydryl.

Another type of modification of NFs is a phosphorylation by protein kinase. A current study on phosphate content of NF found that 200, 155 and 70K subunits contain approximately 100, 25 and 1 μmol phosphate per mole of subunit, respectively (Jones and Williams 1982). Howland and Alli (1986) showed altered phosphorylation of NF from rat spinal cords in acrylamide-induced neuropathy, but it is not known at the present whether the alteration in phosphorylation affects the degradation of NF. However, a possibility that the altered phosphorylation might change the interaction between NF and CANP cannot be ruled out at the present.

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