

## Phenylmethanesulfonyl fluoride elicits and intensifies the clinical expression of neuropathic insults\*

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**Abstract.** It has been recently reported that phenylmethanesulfonyl fluoride (PMSF) when given to hens after a neuropathic organophosphate (OP) promotes organophosphate-induced delayed polyneuropathy (OPIDP). Chicks are resistant to OPIDP despite high inhibition/aging of neuropathy target esterase (NTE), the putative target of OPIDP initiation. However, when PMSF (300 mg/kg s.c.) is given to chicks after di-butyl 2,2-dichlorovinyl phosphate (DBDCVP, 1 or 5 mg/kg s.c.), OPIDP is promoted. Inhibition/aging of at least 30% of NTE was thought to be an essential prerequisite for promotion to be elicited in adult hens. However, we observed in hens that when NTE is maximally affected (>90%) by phenyl N-methyl N-benzyl carbamate (40 mg/kg i.v.), a non-ageable inhibitor of NTE, and then PMSF is given (120 mg/kg/day s.c. × 3 days) clinical signs of neuropathy become evident. Methamidophos (50 mg/kg p.o. to hens), which produces in vivo a reactivatable form of inhibited NTE, was shown either to protect from or promote OPIDP caused by DBDCVP (0.45 mg/kg s.c.), depending on the sequence of dosing. Because very high doses of methamidophos cause OPIDP, we considered this effect to be a "self-promoted" OPIDP. We concluded that NTE inhibitors might have different intrinsic activities for producing OPIDP once NTE is affected. Aging might differentiate highly neuropathic OPs, like DBDCVP, from less neuropathic OPs, like methamidophos, or from the least neuropathic carbamates, which require promotion in order for neuropathy to be expressed. Retrograde axonal transport in motor fibers was measured as the accumulation of <sup>125</sup>I-tetanus toxin in spinal cord after injection in the gastrocnemius muscle of

chicks treated either with DBDCVP (5 mg/kg s.c.) or with DBDCVP followed by PMSF (300 mg/kg s.c.). Retrograde axonal transport was reduced in both groups (to about 50%, 10 days after dosing) and returned to normal 27 days after dosing. However, DBDCVP-treated chicks had a mild neuropathy which recovered relatively quickly, whereas chicks to which PMSF was also given had more severe signs which did not recover by day 27. We concluded that promotion affects a site other than NTE and that it acts at a point downstream from initiation. PMSF was also shown to promote 2,5-hexanedione (2,5-HD) neuropathy. 2,5-HD was given to hens at doses (200 mg/kg/day i.p. × 8 days) which caused mild and reversible neuropathy. When PMSF (120 mg/kg/day × 2 days at the end of 2,5-HD treatment) was given, more severe and irreversible signs of neuropathy were observed. We conclude that promotion might be a common feature in neuropathies of different origin.

**Key words:** Organophosphate neuropathy – Neuropathy target esterase – Promotion – Phenylmethanesulfonyl fluoride – 2,5-Hexanedione – Carbamates – Methamidophos – Age sensitivity – Retrograde axonal transport

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### Introduction

Phenylmethanesulfonyl fluoride (PMSF) is an inhibitor of proteases and esterases (Prouty and Goldberg 1972) with low cholinergic toxicity. PMSF has been used in mechanistic studies of organophosphate-induced delayed polyneuropathy (OPIDP) because it protects hens from this toxicity (Johnson 1982). When neuropathy target esterase (NTE), the putative target for the initiation of OPIDP, is sulfonated in vivo by PMSF prior to neuropathic doses of organophosphates (OPs), no OPIDP develops (Johnson 1974; Lotti et al. 1987). PMSF shares this effect with other NTE inhibitors, e. g., some phosphinates and carbamates. It

is thought that OPIDP develops when enough NTE (>70%) is modified by a two-step mechanism, involving inhibition of the enzyme and a subsequent intramolecular rearrangement called "aging" (Johnson 1982). Protection occurs because sulfonylated, phosphinylated and carbamylated NTEs are not able to undergo the "aging" reaction (Johnson 1982). Consequently, by occupying NTE in a non-ageable form they prevent the phosphorylation/aging of NTE by neuropathic OPs.

However, it has been shown recently that when PMSF or other non-ageable inhibitors are given after a neuropathic OP they promote OPIDP (Pope and Padilla 1990; Lotti et al. 1991). Thus far promotion was caused only by non-ageable NTE inhibitors at doses which were inhibitory to NTE (when given to naive birds), and when about 30% of NTE was affected by the initiating OP (Lotti et al. 1991).

We report in this paper further studies aimed at understanding the significance of promotion. In particular, we addressed some known exceptions in OPIDP to determine whether they remain as such when promotion is given. Thus, we observed that PMSF promotes OPIDP in chicks, which are known to be resistant to this toxicity (Johnson and Barnes 1970). Age-related resistance occurs despite the high inhibition/aging of NTE in the nervous tissues of young animals (Moretto et al. 1991), even though it can be partially overcome by increasing the single dose of the neuropathic OP (Peraica et al. 1991).

A notable exception as far as aging is concerned has been recently reported. Methamidophos (O,S-dimethyl phosphorothioamidate) causes OPIDP in the hen at exceedingly high doses but inhibited brain NTE can be fully reactivated by KF, suggesting that aging had not occurred (Johnson et al. 1991). We report in this paper another exception of this type. High doses of phenyl N-methyl N-benzyl carbamate (PMBC), which caused high levels of non-ageable carbamylated NTE, produced neuropathy in hens only when a subsequent promoting dose of PMSF was given. Just as PMBC can both protect and promote neuropathy (Lotti et al. 1991) methamidophos was also found to cause both protection and promotion in hens, depending on the sequence of dosing with a neuropathic OP.

The results of these experiments on exceptions in OPIDP indicate that the mechanism of promotion might be different from that of initiation. This contention was supported by the following findings.

First, retrograde axonal transport, which is selectively reduced in OPIDP (Moretto et al. 1987), was not affected by promotion when assessed in young chicks.

Second, the peripheral neuropathy caused by 2,5-hexanedione (2,5-HD), which has a different mechanism of initiation from that of OPIDP (DeCaprio 1987), but also proceeds with a reduction of retrograde axonal transport (Braendgaard and Sidenius 1986), was also promoted by PMSF in hens.

## Materials and methods

**Chemicals.** Acetylacetone (2,5-hexanedione; 2,5-HD), 97% purity and atropine sulfate were purchased from Aldrich-Chemie, Steinheim, FRG. O,S-dimethyl phosphorothioamidate (methamidophos) was purchased

from Societa' Italiana Chimici, Rome, Italy. Phenylmethanesulfonyl fluoride (PMSF) and diisopropylphosphorofluoridate (DFP) were purchased from Fluka AG Chem. Fabrik, Buchs, Switzerland. Phenyl N-methyl N-benzyl carbamate (PMBC) was purchased from the Medic Research Council, Carshalton, UK. Di-butyl 2,2-dichlorovinyl phosphatate (DBDCVP) was purchased from Ash Stevens, Detroit, MI, USA. Diethyl p-nitrophenyl phosphate (paraoxon) was purchased from Sigma Chemical Co., St Louis, MO, USA. Phenyl valerate and N,N'-diisopropylphosphorodiamidofluoridate were purchased from Lark Enterprise Webster, MA, USA. Tetanus toxin was kindly supplied by Dr. Montecucco, Dept of General Pathology, University of Padua and N-succinylmidyl 3-(4-hydroxy-5-[<sup>125</sup>I]-iodophenyl) propionate was purchased from Amersham, UK.

**Animals.** Randomly bred adult hens (1.7–2.7 kg body wt) and Warren strain female chicks (36–40 days of age) were purchased from a local breeder, caged in groups of five to ten, and allowed food and water *ad libitum*.

All chemicals given to animals were dissolved immediately before administration in glycerol formal (Fluka) except methamidophos and 2,5-HD, which were dissolved in water. Pretreatment with atropine sulfate (20 mg/kg *i.p.*) to counteract cholinergic toxicity was given in all experiments, except in those with 2,5-HD, DBDCVP (1 mg/kg) and PMSF. Maximal volumes used were the following: 0.5 ml/kg *p.c.*, 1.5 ml/kg *s.c.*, 1.0 ml/kg *i.p.*, and 0.13 ml/kg *i.v.*

Clinical signs of ataxia were evaluated on a 0–8 point scale according to Lotti et al. (1991).

Animals were killed by decapitation and brain, lumbosacral spinal cord and sciatic nerve were excised immediately and either placed in ice-cold 50 mM TRIS-HCl buffer (pH 8.0 at 23°C) containing 0.2 mM EDTA and assayed for NTE or stored at –80°C prior to assay.

**NTE assays.** NTE was measured in the brain according to Johnson (1977) and in the peripheral nerve according to Caroli and Lotti (1982), modified by Moretto et al. (1989). NTE activities of control hens were always within the range of historical values from our laboratory: 2.0–2.5 in brain, 0.5–0.7 in the spinal cord and 0.1–0.15 in the peripheral nerve ( $\mu\text{mol}/\text{min}/\text{g}$  wet wt of tissue).

**Retrograde axonal transport.** Retrograde axonal transport was measured in motor fibers of sciatic nerve of chicks as the accumulation of [<sup>125</sup>I]-tetanus toxin in the spinal cord 28 h after injection of labelled tetanus toxin into the gastrocnemius muscle according to Moretto et al. (1987). [<sup>125</sup>I]-tetanus toxin was prepared according to Rogers and Snyder (1981). The specificity of iodination was checked by autoradiography of a gel electrophoresis togram of the eluate containing high radioactivity.

Animals were injected with [<sup>125</sup>I]-tetanus toxin 5, 10, and 27 days after the treatments.

## Results

The promoting effect of PMSF in chicks is shown in Table 1. DBDCVP (1 mg/kg *s.c.*) and DFP (1.5 mg/kg *s.c.*) failed to cause OPIDP when given alone, but promotion occurred when PMSF was given afterwards. However, mild OPIDP was observed with a higher dose of DBDCVP (5 mg/kg *s.c.*), which was also promoted by subsequently administered PMSF. The promoting effect of PMSF was stronger in DFP-treated chicks (clinical score increased from 0 to 5.1) than that in DBDCVP-treated chicks (clinical score increased from 1.8 to 5.1).

Table 2 shows that when PMBC was used instead of an OP to "initiate OPIDP", then clinical neuropathy occurred after PMSF treatment. However, when the sequence of dosing of the same compounds was reversed, a marginal response was observed in two animals only.

**Table 1.** OPIDP promotion by PMSF in chicks<sup>a</sup>

Initial dosing (mg/kg s. c.)	PMSF <sup>b</sup> (300 mg/kg s. c.)	Clinical score <sup>c</sup> (mean ± SD)
DBDCVP (1) <sup>d</sup>	Solvent	0 (5)
DBDCVP (1)	+	2.0 ± 1.4 (4)
DBDCVP (5)	Solvent	1.8 ± 1.3 (12)
DBDCVP (5)	+	5.1 ± 1.5 (10)
DFP (1.5) <sup>e</sup>	Solvent	0 (8)
DFP (1.5)	+	5.1 ± 1.3 (8)

<sup>a</sup> 39 days of age at the beginning of the experiment

<sup>b</sup> Given 24 h later. This dose does not cause OPIDP and inhibits more than 90% of brain NTE (Lotti et al. 1991)

<sup>c</sup> Maximal scores are reported and were reached 12–15 days from the beginning of the experiments. Number of animals in brackets

<sup>d</sup> This dose given to 20-day-old chicks causes about 80% NTE inhibition in peripheral nerve (Moretto et al. 1991)

<sup>e</sup> This dose given to 36-day-old chicks causes 70 ± 11% NTE inhibition in peripheral nerve ( $n = 3$ ; mean ± SD; activity of control was 1.71 ± 0.06 nmol/min/mg of protein)

**Table 2.** Promotion of neuropathy in hens treated with a combination of PMSF and PMBC

Dosing	Clinical score
PMBC (40 mg/kg i. v. × 3) <sup>a</sup>	0 ( $n = 9$ )
PMSF (120 mg/kg s. c. × 3) <sup>b</sup>	0 ( $n = 2$ )
PMBC (40 mg/kg i. v.) <sup>c</sup> + PMSF (120 mg/kg s. c. × 3) <sup>d</sup>	2, 2, 2, 2
PMSF (120 mg/kg s. c. × 3) + PMBC (40 mg/kg i. v.) <sup>e</sup>	0, 0, 0, 1, 1

<sup>a</sup> Treatments were given 5 h apart. In one animal killed 7 h after the last dose, brain NTE activity was 93% inhibited

<sup>b</sup> Treatment was given 12 h apart. In one animal killed 24 h after the last dose, brain and peripheral nerve NTE activities were 98% and 88% inhibited, respectively. PMSF (120 mg/kg s. c.) caused about 93% inhibition of brain NTE when measured 24 h after treatment (Lotti et al. 1991). No ataxia was observed when either a single dose of 480 mg/kg s. c. or 9 daily doses of 30 mg/kg s. c. of PMSF were given

<sup>c</sup> PMBC (40 mg/kg i. v.) causes 80–90% inhibition of brain NTE (Lotti et al. 1991) when measured 5 h after treatment

<sup>d</sup> Given 5, 17, and 29 h after PMBC

<sup>e</sup> Given 24 h after the last dose of PMSF

**Table 3.** Promotion of and protection from DBDCVP neuropathy by methamidophos in hens

Dosing (mg/kg) <sup>a</sup>	Clinical score mean ± SD (n)	% NTE activity <sup>b</sup>		
		Brain	Spinal cord	Peripheral nerve
DBDCVP (0.45 s. c.)	3.1 ± 1.4 (9)	10 ± 1 <sup>c</sup>	17 ± 3 <sup>c</sup>	46 ± 9 <sup>c</sup>
DBDCVP (0.45 s. c.) + methamidophos (50 p. o.)	6.2 ± 0.4 (5)	8	12	12
Methamidophos (50 p. o.)	0 (3)	50	52	45
Methamidophos (50 p. o.) + DBDCVP (0.45 s. c.)	0 (4)	12	12	24
Methamidophos (25 p. o.)	–	76	73	73
Methamidophos (25 p. o.) + DBDCVP (0.45 s. c.)	4.3 ± 0.6 (3)	–	–	–
Methamidophos (5 p. o.)	–	83	67	100
Methamidophos (5 p. o.) + DBDCVP (0.45 s. c.)	4.6 ± 1.1 (3)	–	–	–

<sup>a</sup> Treatments were given 24 h apart

<sup>b</sup> Measured 24 h after last dosing. Expressed as percentage of NTE activity of a concurrent control. Control activity was within historical values from our laboratory (see Materials and methods)

<sup>c</sup> Data from Moretto et al. (1989)

Table 3 shows that methamidophos (50 mg/kg p. o.) both protected from and promoted DBDCVP neuropathy, depending on the sequence of dosing. However, lower doses of methamidophos given prior to DBDCVP failed to protect birds, because most of the NTE activity was left for phosphorylation by DBDCVP. The severity of OPIDP in such birds was not different from that seen in birds treated with DBDCVP only.

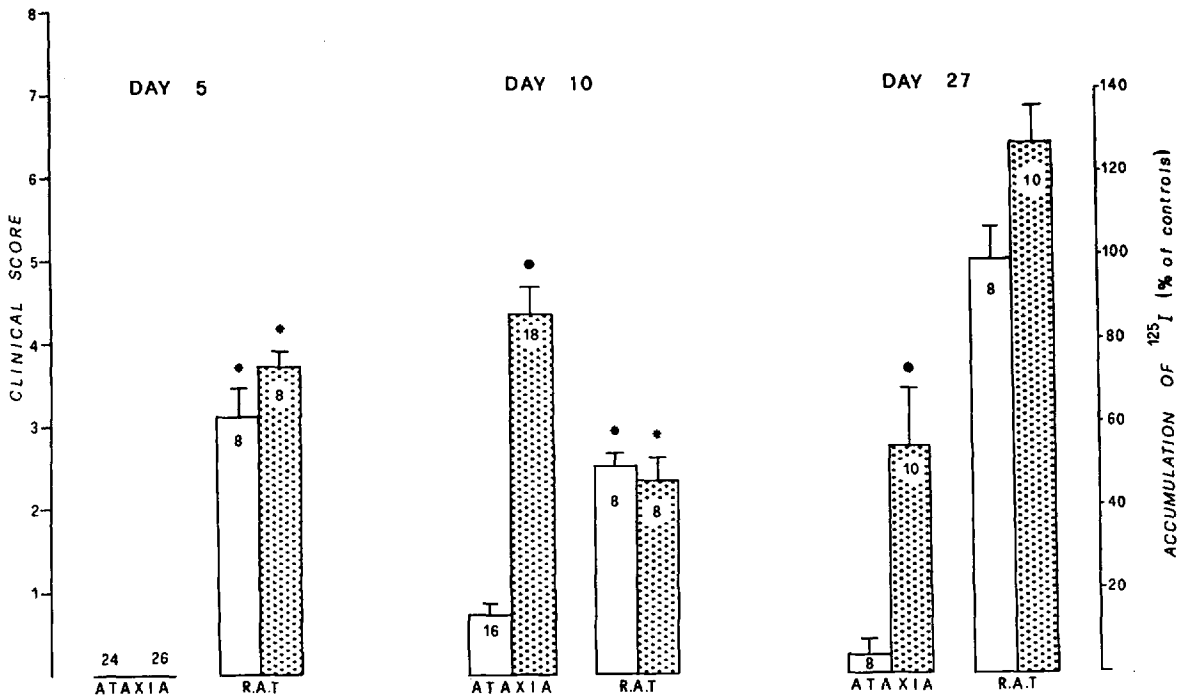
Figure 1 shows the clinical effects of DBDCVP and of DBDCVP followed by PMSF in chicks, together with the reduction of retrograde axonal transport. DBDCVP caused a mild and reversible OPIDP, whereas when PMSF was also given, birds were more severely affected and recovery was much delayed. In contrast with these time-courses of clinical effects, the accumulation of <sup>125</sup>I-tetanus toxin showed a progressive reduction and then a full recovery in both groups.

Figure 2 shows the promoting effect of PMSF when given after repeated doses of 2,5-HD which caused on its own a mild neuropathy in some hens only. PMSF exacerbated the severity of signs, caused all animals to be affected, and delayed the recovery.

## Discussion

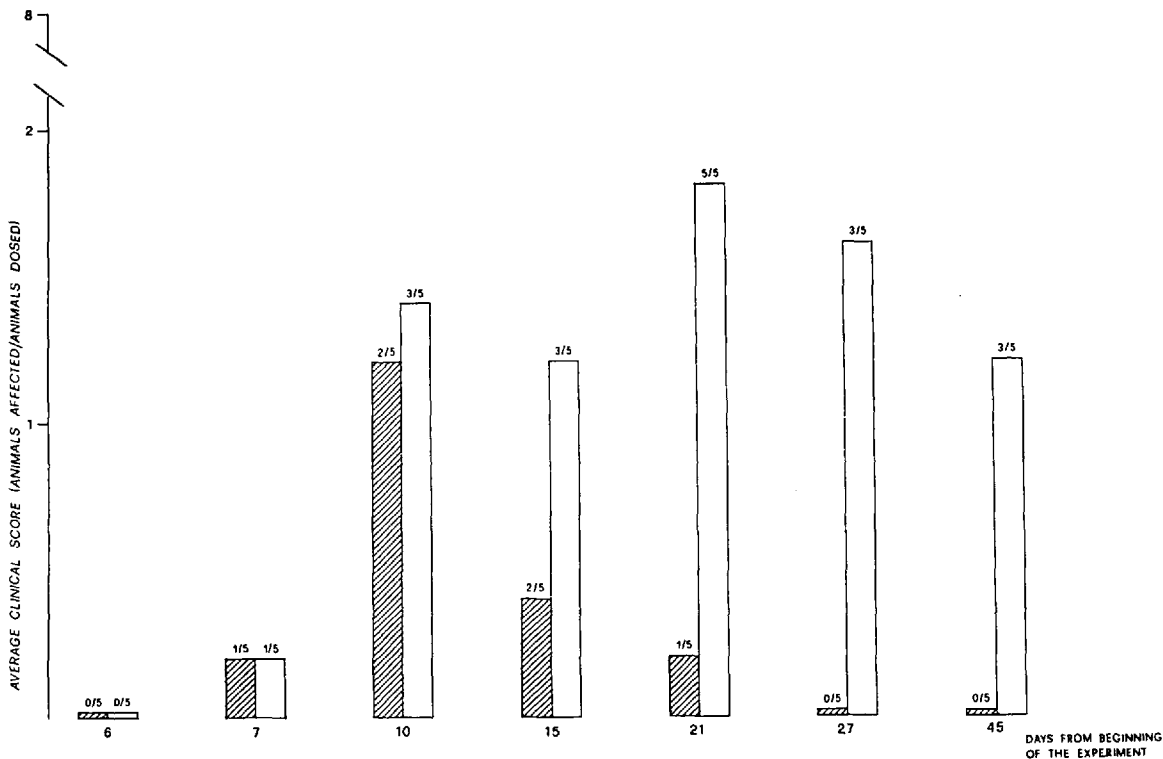
OPIDP promotion by PMSF was shown in the hen after OP doses which do not cause overt clinical signs (Pope and Padilla 1990; Lotti et al. 1991). This effect was confirmed in chicks but it is different in as much as promotion overcomes their resistance to OPIDP whereas in hens causes a "subthreshold" inhibition/aging of NTE to be expressed clinically (Lotti et al. 1991). Because maximal inhibition/aging of NTE in chicks is not enough to trigger clinical OPIDP (Moretto et al. 1991), they might differ from the hen either because of lack of progression of the lesion or of a more efficient repair. Consequently, promotion by PMSF in chicks should affect one or both of these functions, which seem unrelated to NTE.

Promotion caused clinical responses of similar size independently from the initial effect of different compounds. In fact, the clinical score was higher in chicks treated with DBDCVP (5 mg/kg) than that in birds treated with DFP, whereas after PMSF it was the same in both groups. A similar phenomenon was observed when promotion was given to chicks of different age (Peraica et al. 1991; Pope



**Fig. 1.** Effects on clinical behavior and retrograde axonal transport (R. A. T.) in motor fibers of sciatic nerve, in chicks treated with DBDCVP alone ( $\square$ ) and in combination with PMSF ( $\boxtimes$ ). Fifty chicks of 40 days of age were injected with DBDCVP (5 mg/kg s. c.) after pretreatment with atropine and 24 h later either with solvent or with PMSF (300 mg/kg s. c.).  $^{125}$ I-tetanus toxin was injected on days 5, 10 and 27. Data are expressed as mean percentage ( $\pm$  SE) of the mean of controls. The same preparation of  $^{125}$ I-tetanus toxin was used on days 5 and 10 but

differently diluted. Control values for  $^{125}$ I-accumulation were: day 5 =  $5698 \pm 738$  CPM (mean  $\pm$  SD, 8 animals), on day 10  $3834 \pm 1277$  CPM (8 animals) and on day 27 =  $1712 \pm 496$  CPM (7 animals). \* Significantly different from controls and day 27, by one-way analysis of variance (Kruskal-Wallis test,  $p < 0.05$ ). ● Significant different from animals treated with DBDCVP only, Wilcoxon test ( $p < 0.001$ )



**Fig. 2.** Development of 2,5-HD neuropathy ( $\boxtimes$ ) in hens (200 mg/kg i. p.  $\times$  8 daily) and the influence of two subsequent doses of PMSF (120 mg/kg s. c. daily) ( $\square$ ). Two birds dosed with PMSF prior to 2,5-HD had mild neuropathy with a time course similar to that in birds treated with 2,5-HD only

and Padilla 1991). Indeed, regardless of age susceptibility to OPs, PMSF promoted OPIDP to a similar degree of severity. These observations imply either a maximal promoting effect, or that phosphorylated NTE at different ages or with different OPs varies in its capability to initiate OPIDP, or both. Besides their inhibitory power, NTE inhibitors might therefore display different intrinsic activities with respect to initiation of OPIDP, once attached to NTE. Thus, PMSF promotion may represent a combination of different degrees of OPIDP initiation plus the same maximal promoting effect.

The hypothesis that NTE inhibitors might have different intrinsic activities (Lotti et al. 1991) is supported by results on PMBC and methamidophos. N-Methyl N-benzyl carbamylated NTE does not age (Johnson 1982) and consequently no neuropathy develops when this compound is given on its own. However, PMBC becomes neuropathic when PMSF treatment follows. Methamidophos causes OPIDP at very high doses when almost all NTE is affected in a non-ageable form (Johnson et al. 1991). When given at lower doses it protects from and promotes DBDCVP neuropathy, depending on the order of dosing. Looking at NTE as a receptor, inhibitors will behave like partial agonists (such as PMBC and methamidophos, causing protection or weak initiation) or like full agonists (such as DBDCVP, causing neuropathy), where the capability to age would confer the property of a full agonist. Therefore, PMBC neuropathy is expressed only by PMSF promotion, whereas methamidophos neuropathy is "self-promoted" when exceedingly high doses are given. OPIDP in resistant chicks caused by very high doses of OPs (Peraica et al. 1991) may also be interpreted as "self-promotion".

Promotion has no effect on retrograde axonal transport, because the clinical scores of DBDCVP-treated chicks are less severe than those of chicks treated also with PMSF, whereas the reductions of retrograde axonal transport are similar in the two groups. Because reduction of retrograde axonal transport is involved in the pathogenesis of OPIDP (Moretto et al. 1987) measurement of retrograde axonal transport might discriminate between progression of the lesion and other mechanisms affecting the clinical outcome.

Given the results of NTE inhibition in the hen after much smaller doses of DBDCVP (Moretto et al. 1987) we might assume that almost all NTE was inhibited in chicks by DBDCVP before PMSF was given. Taking into account also methamidophos (Johnson et al. 1991) and PMBC data where promotion occurred after maximal NTE inhibition, we conclude that NTE is probably not the target of promotion.

The mechanism of 2,5-HD neuropathy is thought to involve the formation of adducts to  $\epsilon$ -amino groups of lysine residues in subunit proteins of neurofilaments and subsequent crosslinking (DeCaprio 1987). Because this target is different from that of OPIDP and yet PMSF promotes both neuropathies, promotion must act at a point in common downstream from initiation. It is possible that neuropathies produced by different means will also be promoted by PMSF.

In conclusion, these experiments suggest that the aging of inhibited NTE is not necessary to initiate neuropathy.

However, the initiation of neuropathy correlates with NTE inhibition. Inhibitors might have a very low intrinsic activity (PMSF < PMBC < methamidophos) or high intrinsic activity (DFP < DBDCVP), once they are attached to NTE. The aging reaction might differentiate full agonists from others. Promotion seems to be separate from progression of the initial lesion and to reach a maximum effect. Perhaps promotion involves a mechanism of either degeneration in the cascade of events which follow initiation and/or repair common to all degenerating axons. It also appears that promotion affects a site other than NTE, although it has to be explained why, so far, only NTE inhibitors act as promoters (Lotti et al. 1991).

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