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POSSIBLE ROLE OF MICROTUBULES AND ASSOCIATED PROTEASES IN ORGANOPHOSPHORUS ESTER-INDUCED DELAYED NEUROTOXICITY

JOSEF SEIFERT* and JOHN E. CASIDA
Pesticide Chemistry and Toxicology Laboratory, Department of Entomological Sciences, University of California, Berkeley, CA 94720, U.S.A.

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Abstract—Organophosphorus delayed neurotoxicants (phenyl saligenin cyclic phosphate and diisopropyl phosphorofluoridate) altered cyclic AMP (cAMP)-dependent phosphorylation and several other processes in brain homogenates and cytoplasmic microtubules. Phenyl saligenin cyclic phosphate slightly stimulated *in vitro* cAMP-dependent phosphorylation in brain homogenates of three species (rat, mouse and rabbit) that have been reported to be insensitive to delayed neurotoxicity, whereas it slightly decreased this phosphorylation in brain homogenates of three sensitive species (chicken, cow and pig) and in brain microtubules of chicken and pig. The microtubule-associated processes that were moderately inhibited by phenyl saligenin cyclic phosphate in sensitive species were: *in vitro* [^{3}H]cAMP binding to protein kinase, *in vitro* assembly when tubulin rings were absent, and cAMP-dependent phosphorylation of microtubule-associated proteins (MAPs) both *in vitro* and on intracerebral administration of $^{32}P_i$. The endogenous proteases that degrade the high molecular weight MAPs were strongly inhibited *in vitro* by phenyl saligenin cyclic phosphate and diisopropyl phosphorofluoridate. In contrast, treatment of chickens with diisopropyl phosphorofluoridate remarkably decreased the *in vitro* stability of their brain cytoplasmic high molecular weight MAPs, perhaps by enhancing the MAPs-degrading protease activity. These findings indicate that the MAPs-protease system is a possible target for organophosphorus delayed neurotoxicants.

Many organophosphorus esters induce axonal degeneration with secondary demyelination, evident in hens as ataxia appearing about 10–14 days after poisoning [1–4]. This syndrome is not related to acetylcholinesterase (AChE)† inhibition but correlates with selective inhibition of a hen brain neurotoxic esterase (NTE) with subsequent aging of the phosphorylated NTE [1–6]. The ratio of I_{50} values for NTE and AChE inhibition *in vitro* provides a useful indicator of the tendency for an organophosphorus (OP) compound to produce this type of neuropathy [5]. No studies to date have defined the specific biochemical lesion leading to OP delayed neurotoxicity.

There is some basis for focusing attention on microtubules (MT) and MT-associated proteins (MAPs) as possible target sites for OP neurotoxicants. Ethyl 2-nitro-4-methylphenyl *N*-isopropylphosphoramidothionate (amiprophos), a delayed neurotoxicant in hens [7], or related herbicides inhibit tubulin synthesis [8] and exert high antimitotic activity in algae [9, 10]. Other OP delayed neurotoxicants are reported to inhibit fast axoplasmic transport in rats [11], which could conceivably involve alterations in MT functions [12]. OP neurotoxicants are both phosphorylating agents and inhibitors of many proteases [7]. It is therefore of interest that MT undergo cyclic nucleotide-dependent phosphorylation [13] and also contain protease(s) that limit their stability and/or interfere with their assembly [14–18].

The present study evaluates the possible role of MT-associated processes in OP delayed neurotoxicity. It examines brain cytoplasmic MT as a model system. Emphasis was given to adult chickens as the classically sensitive organism; chicks are relatively insensitive [1–4]. Phenyl saligenin cyclic phosphate (PSCP) and diisopropyl phosphorofluoridate (DFP) were used as potent delayed neurotoxicants because they act rapidly in inhibiting NTE *in vitro* [1–4] and probably also in phosphorylating the primary neurotoxic target site both *in vitro* and *in vivo*. PSCP and DFP, being quite reactive phosphorylating agents [7], are therefore unlikely to persist for more than a few hours in birds or mammals.

MATERIALS AND METHODS

Brain homogenates and MT. Brains of chicks, chickens (over 90 days old), rats, mice and rabbits

* Address all correspondence to: Dr. Josef Seifert, Pesticide Chemistry and Toxicology Laboratory, Department of Entomological Sciences, 201 Wellman Hall, University of California, Berkeley, CA 94720, U.S.A.

† Abbreviations and names used: AChE, acetylcholinesterase; amiprophos, ethyl 2-nitro-4-methylphenyl *N*-isopropylphosphoramidothionate; BP, basic protein; cAMP, cyclic AMP; DFP, diisopropyl phosphorofluoridate; EGTA, ethyleneglycol-bis-(β-aminoethyl ether) *N,N'*-tetraacetic acid; Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; HMW, high molecular weight; i.p., intraperitoneal; LMW, low molecular weight; MAPs, microtubule-associated proteins; MT, microtubules; NTE, neurotoxic esterase; OP, organophosphorus; P_i, inorganic phosphate; PSCP, phenyl saligenin cyclic phosphate or 2-phenoxy-4H-1,3,2-benzodioxaphosphorin-2-oxide; and SDS, sodium dodecyl sulfate.

were used immediately following sacrifice while those of pigs and cows were held 1–4 hr at 0° before use. Brain homogenates were prepared in 0.32 M sucrose (1:4, w/v) with a Potter–Elvehjem homogenizer followed by a 1:10 dilution with 0.32 M sucrose [19]. MT from pig and chick brain were isolated and purified by temperature- and glycerol-dependent reversible assembly and disassembly [13] using two cycles unless specified otherwise. The resulting MT preparations behaved in accordance with expectations relative to assembly and disassembly assayed both turbidimetrically [20] and viscometrically [21] and to protein profile examined electrophoretically (see Results).

Cyclic nucleotide-dependent phosphorylation. Typical reaction mixtures [19] consisted of 40 μl of 250 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (Hepes) buffer (pH 7.0), 20 μl of 100 mM $MgSO_4$ in water, 20 μl of 100 μM cyclic AMP (cAMP) in water (or 20 μl water to determine non-cAMP phosphorylation), 100 μl of brain homogenate (120–220 μg protein) or MT preparation (300–1000 μg protein), and 20 μl of 10 μM [γ-^{32}P]ATP (~100 Ci/mmole, 97% radiochemical purity, Amersham, England). Enzymatic phosphorylation was initiated by addition of [^{32}P]ATP, and after 15 sec at 25° it was terminated by adding 2 ml of 10% trichloroacetic acid followed by 0.2 ml of 0.6% (w/v) bovine serum albumin in water. The precipitated protein was recovered and dissolved by adding 0.2 ml of 0.5 M NaOH; the acid precipitation and alkaline dissolution cycle was repeated three times before liquid scintillation counting in toluene–methyl cellosolve (2:1) containing 2.5% diphenyloxazole.

The activities of chicken spinal cord and sciatic nerve homogenates and MT fractions were low and lacked sufficient consistency for further studies. Cyclic GMP was not a suitable replacement for cAMP because with pig MT the former compound induced a phosphorylation rate only 2–8% of that induced by cAMP in the range of 3.2 to 26 μM.

Phosphoproteins of MT preparations were separated after cAMP-dependent phosphorylation [19] by sodium dodecyl sulfate (SDS) electrophoresis on polyacrylamide slabs (7.5% linear or 5–20% gradient) (Isolab, Akron, OH) using glycine–tris-(hydroxymethyl)aminomethane buffer, pH 8.3. The proteins were stained with Coomassie blue [19], and the ^{32}P-phosphoproteins were determined, after slicing the gel, by counting directly in scintillation mixture. Resolution of the ^{32}P-phosphoproteins was confirmed by subjecting the stained gels to autoradiography after wrapping in Saran plastic film.

To examine its effect on cyclic nucleotide-dependent phosphorylation, PSCP or DFP was added in 5 μl acetone to 0.5 ml of brain or MT preparation, yielding a final concentration of 10^{-5} M. After 15 min at 25°, an aliquot was added to the cAMP phosphorylation assay system for determination of total ^{32}P-phosphoproteins and/or the distribution of ^{32}P-phosphoproteins separated by polyacrylamide gel electrophoresis.

[^{3}H]*cAMP binding to MT.* The reaction mixture consisted of 50–100 μl of MT preparation (75–200 μg protein), 10 μl of 27 μM [^{3}H]cAMP (37 Ci/mmole, Amersham) and (in inhibition studies) 10 μl of PSCP (final concentration 5×10^{-6} M) in acetone, with a 20-min preincubation before starting the reaction by addition of [^{3}H]cAMP. Two conditions were examined: 50 mM pH 4.0 acetate buffer with 120 min incubation at 0° [22]; and the pH 6.9 disassembly MT buffer (without GTP) [14] with 15 min incubation at 25°. At the end of the incubation, samples were diluted with 2 ml of a wash buffer (20 mM acetate, pH 6.0) and filtered through prewashed Millipore-type HA filters (0.45 μm pores). The filter discs were rinsed subsequently four times with the wash buffer, then dissolved in scintillation mixture, and counted.

In vitro *assembly of MT.* The 25,000 *g* supernatant fraction of cow brain homogenate in disassembly buffer [14] (1:1, w/v) at 0° was quickly brought to 35° for incubation to observe viscosity changes with the Ostwald viscometer. For comparison, assembly at 35° was measured following treatment with PSCP (10^{-4} M, 20-min preincubation at 0°) or colchicine (10^{-5} M, added immediately before temperature increase) or with PSCP followed by colchicine using the aforementioned schedule.

The effect of PSCP was also examined under conditions in which most of the tubulin rings are dissociated [23]. Pig MT were separated from low molecular weight (LMW) components and transferred into low ionic strength buffer by passage through a Sephadex G-25 column and elution with 10 mM Hepes buffer (pH 6.8) with 0.1 mM ethyleneglycol-*bis*-(β-aminoethyl ether) *N,N'*-tetraacetic acid (EGTA) and 0.5 mM $MgCl_2$. One milliliter of MT (100–150 μg protein) in the low ionic strength solution was preincubated with 5 μl of an acetone solution of PSCP (final concentration 10^{-5} M) before addition of concentrated buffer (80 μl of 1.6 M Hepes buffer, pH 6.8, with 8 mM $MgCl_2$, 16 mM EGTA and 16 mM GTP). The assembly of MT was monitored turbidimetrically at 350 nm and 35° and/or after overnight incubation at 25°. Samples phosphorylated with cAMP and ATP were used as negative controls.

In vivo *incorporation of* $^{32}P_i$ *into chick brain MT.* Chicks (white leghorn, 2 weeks old) were treated i.p. with PSCP (4.6 mg/kg; administered in 10 μl of methoxytriglycol) as a single dose or as two identical doses 5 days apart. One day after completing the PSCP treatment schedule, chicks were dosed by injecting directly into the cerebrum $^{32}P_i$ (0.1 to 0.5 mCi per chick; carrier free; Amersham) in 10 μl of saline. After 45 min the chicks were decapitated, the brains were removed, and MT were isolated and purified by a single cycle of assembly–disassembly. ^{32}P-phosphoproteins were analyzed after electrophoretic separation by slicing the gels and direct liquid scintillation counting.

Stability of high molecular weight (HMW) microtubule-associated proteins (MAPs). Hens were treated with a physostigmine–atropine mixture (0.1 and 20 mg/kg respectively) or with the physostigmine–atropine mixture followed after 10 min by DFP (i.p., 1.7 mg/kg) (physostigmine and atropine as prophylactic agents ensure survival of the DFP cholinergic effect, see Ref. 24). MT from both groups (stored up to 18 hr at −15°) were incubated alone or with 10^{-5} M PSCP or 10^{-4} M DFP for

up to 6 hr at 37° and then for up to 42 additional hr at 25°. They were subjected to electrophoresis, as previously described, and the gels were scanned at 620 nm in the transmittance/absorbance mode for Coomassie blue-stained proteins using an RFT Scanning Densitometer (Transidyne General Corp., Ann Arbor, MI) connected to a computer system for quantitation of the HMW-MAPs fraction.

In another type of experiment, MT from control and DFP-treated hens were phosphorylated with [^{32}P]ATP and cAMP after 6 hr incubation at 25° with 10^{-5} M PSCP or without an OP inhibitor. ^{32}P-Phosphoproteins of MAPs and tubulins were determined after electrophoretic separation, cutting the corresponding gel bands and counting directly.

RESULTS

Effects of PSCP in vitro *on cAMP-dependent phosphorylation in various species.* The rates of cAMP-dependent phosphorylation in brain homogenates (Table 1) did not correlate with species sensitivity to delayed neurotoxicants [1, 3], rates in rabbit and rat homogenates being high compared with rates in cow, mouse, pig and chicken. However, there were apparent species-dependent differences in the effect of PSCP on cAMP-dependent phosphorylation since it increased the activity in the OP-insensitive species (rat, mouse and rabbit) whereas it had little effect or decreased the activity in sensitive species (~40% in chicken and less so in cow and pig). Overall cAMP-dependent phosphorylation was also decreased by PSCP in fresh MT preparations from pig and chicken brain. An unusual time-dependent phenomenon was associated with PSCP acting on both chicken MT (Table 1) and brain homogenates, inhibiting cAMP phosphorylation in fresh samples but stimulating phosphorylation in stored preparations (3 days at −5°).

SDS electrophoresis revealed that a short (10 min) preincubation of fresh chicken MT with PSCP before the addition of cAMP and [^{32}P]ATP resulted in a decreased incorporation of ^{32}P in HMW-MAPs (23%) but not in other fractions.

Effect of PSCP on [^{3}H]*cAMP binding to MT.* The binding of [^{3}H]cAMP to chicken MT was decreased by PSCP (5×10^{-6} M) to the extent of 7, 11 and 14% in three preparations when preincubated at pH 4.0 (0°, 120 min; optimal pH and temperature for cAMP binding [22]) and 6% when preincubated at pH 6.9 (25°, 15 min; pH used for MT disassembly and assembly [13]).

Effect of PSCP on in vitro *assembly of MT.* PSCP (10^{-4} M) did not alter the rate of *in vitro* assembly of cow MT and did not affect the efficiency of colchicine (10^{-5} M) in completely blocking MT assembly. However, the assembly of MT was diminished by PSCP (10^{-5} M) under conditions in which the number of tubulin rings was reduced, i.e. turbidimetric measurements revealed a decrease in MT formation by 20% with PSCP (compared to 35% after cAMP-dependent phosphorylation).

Effect of PSCP in vivo *on incorporation of* $^{32}P_i$ *in chick MT.* Two treatments with PSCP at 6 and 1 days before intracerebral administration of $^{32}P_i$ reduced ^{32}P-incorporation into HMW-MAPs by 25% and into the tubulin fraction by 13% based on peak areas (Fig. 1). The LMW-MAPs were strongly affected but quantitation was difficult due to the small amounts of ^{32}P-phosphoproteins formed. A single dose of PSCP 1 day before $^{32}P_i$ treatment did not interfere with ^{32}P-incorporation into MT.

Table 1. Effect of 10^{-5} M phenyl saligenin cyclic phosphate (PSCP) on cyclic AMP-dependent phosphorylation of brain homogenates and microtubules prepared from various species

Species and preparation	Cyclic AMP-dependent phosphorylation*	
	Control [pmoles ^{32}P·(mg protein)$^{-1}$·(15 sec)$^{-1}$]	With PSCP relative to control (%)
Animals insensitive to delayed neurotoxicity		
Rat homogenate	8.0, 7.6	116, 114
Mouse homogenate	4.6, 4.7	113, 116
Rabbit homogenate	11.0, 9.1	127, 100
Animals sensitive to delayed neurotoxicity		
Cow homogenate	5.3, 6.5	81, 88
Pig homogenate†	3.5	96
Pig microtubules		
Fresh	61, 51	99, 81
Stored‡	56, 49	95, 103
Chicken homogenate§	1.9, 2.1, 2.3	57, 51, 71
Chicken microtubules		
Fresh	9.8, 8.1	96, 82 61, 38‖
Stored‡	7.2, 6.1	125, 97

* Each value is the average of duplicate determinations for an individual animal given in the same sequence for the phosphorylation and PSCP effect.

† Stored for 10 hr at 0° before assay.

‡ Stored for 3 or 4 days at −5° before assay.

§ DFP (10^{-5} M), instead of PSCP, gave a value of 70%.

‖ These two values, each from one animal, are the cpm with PSCP relative to control cpm; corresponding values for the specific activities were not determined.

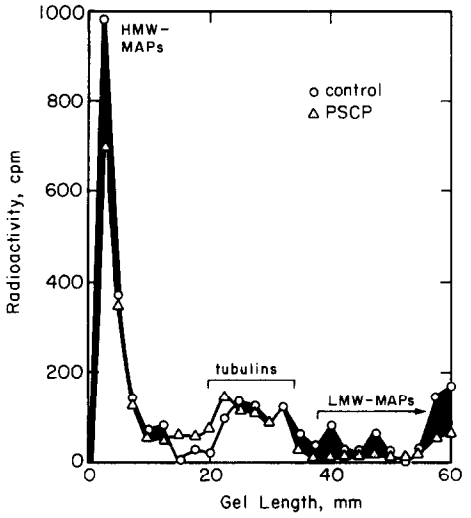

Fig. 1. Effect *in vivo* of phenyl saligenin cyclic phosphate (PSCP) pretreatment of chicks on intracerebral incorporation of $^{32}P_i$ into microtubule phosphoproteins. Chicks were administered 4.6 mg/kg of PSCP intraperitoneally on days 6 and 1 before intracerebral administration of $^{32}P_i$, with sacrifice 45 min after $^{32}P_i$ treatment. Duplicate determinations were made on each chick; each point is the mean of two chicks.

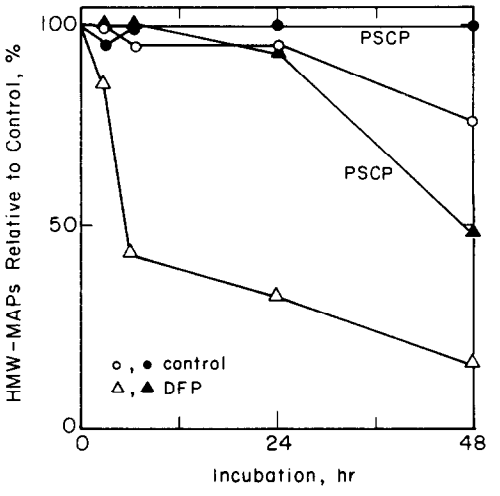

Fig. 3. Effect *in vivo* of diisopropyl phosphorofluoridate (DFP) treatment on spontaneous *in vitro* degradation of high molecular weight MAPs. MT isolated from control chickens or those treated 14 days earlier with DFP were incubated alone or with 10^{-5} M PSCP.

Effects of PSCP and DFP in vitro *and DFP* in vivo *on the stability of HMW-MAPs.* Trypsin digests MAPS *in vitro* with a high preference for the HMW components [14, 25]. We confirmed this observation with pig HMW-MAPs (700 μg protein incubated with 220 μg trypsin for 30 min at 37°) and found that PSCP inhibited MAPs digestion in parallel with its inhibition of the amidase activity of trypsin (data not shown). Chicken MT were less stable than pig MT and underwent slow breakdown even without trypsin, converting HMW-MAPs to fragments of lower molecular weight (Fig. 2) [14]. The rate of disappearance of HMW-MAPs was significantly greater for MT from DFP-treated than from control chickens with similar findings at 14 days (Fig. 3) and 10 days (data not shown) after DFP administration. In both cases, PSCP (10^{-5} M) (Figs. 2 and 3) and DFP (10^{-4} M) (results not shown) acted *in vitro* to retard degradation of HMW-MAPs.

Effects of PSCP in vitro *and DFP* in vivo *on cAMP-dependent phosphorylation in MT.* Twenty-four hour preincubation of pig MT with PSCP (10^{-5} M) markedly decreased the cAMP-dependent phosphorylation of LMW fragments (Fig. 4.). In contrast, 6-hr preincubation of chicken MT with PSCP (10^{-5} M) enhanced cAMP-dependent phosphorylation to a small degree for tubulins and to a greater extent for HMW-MAPs (Table 2). This effect was more prominent for HMW-MAPs isolated from DFP-treated than from control chickens.

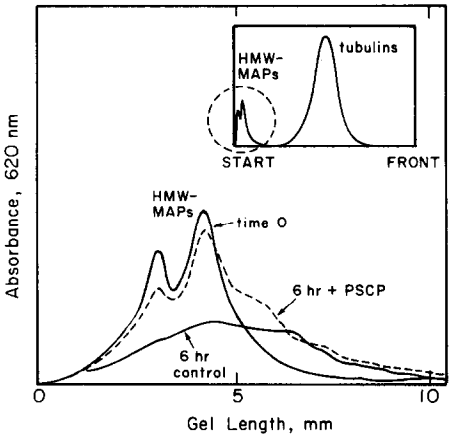

Fig. 2. Effect *in vitro* of phenyl saligenin cyclic phosphate (PSCP) on spontaneous degradation of high molecular weight MAPs. MT isolated from DFP-treated (14 days *in vivo*) chickens were incubated 0 or 6 hr at 37° alone or with 10^{-5} M PSCP. The insert figure gives the electrophoretic pattern for all MT proteins of which the encircled MAPs portion is expanded for the main figure.

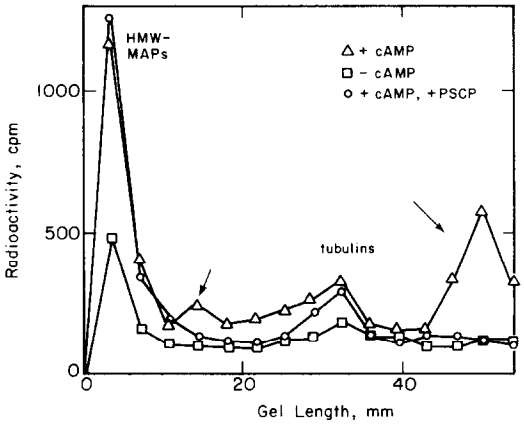

Fig. 4. Effect *in vitro* of phenyl saligenin cyclic phosphate (PSCP) on cAMP-dependent phosphorylation of pig microtubules. MT incubated for 24 hr at 25° alone or with 10^{-5} M PSCP were phosphorylated with [γ-^{32}P]ATP (± cAMP). Arrows designate LMW fragments in stored preparation undergoing cAMP-dependent phosphorylation.

Table 2. Effect of 10^{-5} M phenyl saligenin cyclic phosphate (PSCP) on cyclic AMP-dependent phosphorylation of HMW-MAPs and tubulins separated electrophoretically from brain microtubules of normal or DFP-treated chickens

Pretreatment of chickens	cAMP phosphorylation* (with PSCP relative to control, %)	
	Tubulins	HMW-MAPs
None	110 ± 9	133 ± 16
DFP	120 ± 10	163 ± 24

* Average and standard deviations based on four normal chickens and six DFP chickens (7–14 days after treatment).

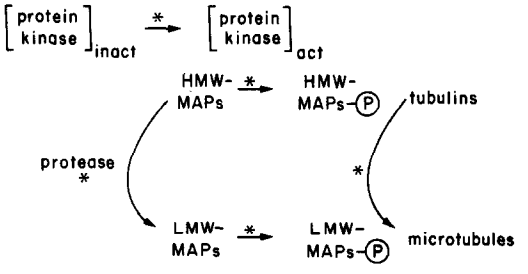

Fig. 5. Proteins involved in microtubule system. Asterisks designate hypothetical sites for disruption by organophosphorus delayed neurotoxicants.

DISCUSSION

The species specificity in delayed neurotoxicity may result from differences in the nature and/or significance of the target site. Comparison of cAMP-dependent phosphorylation in brain homogenates of sensitive and insensitive species revealed such a difference, with inhibition by PSCP in chickens and possibly other sensitive species and stimulation in refractory species. However, it would be premature to conclude any cause and effect relationship from these observations because of the small numbers of species and animals examined.

The cAMP-dependent phosphorylation system consists of many components, perhaps only a few of which are affected by DFP and PSCP. The basic protein (BP) of myelin is of interest because of its encephalitogenic properties [26] which might take on pathological significance when modified, e.g. by phosphorylation. We found that PSCP and [^{3}H]DFP do not react with BP but instead phosphorylate only LMW proteins present in BP preparations as impurities and/or formed by spontaneous degradation (unpublished observations). MT, therefore, appear to be of greater interest for reasons given at the beginning of the paper and because their response to PSCP and loss of sensitivity on storage are similar to that of brain homogenates.

Several sites in the *in vitro* MT system were considered as possible targets for OP attack (Fig. 5). PSCP decreased [^{3}H]cAMP binding to protein kinase but the magnitude of the decrease was small, and this type of interaction may involve competition between cyclic phosphates (PSCP and cAMP) not applicable to the acyclic neurotoxicants such as DFP. MT assembly was diminished by PSCP but only under conditions in which the tubulin rings were dissociated. Since cAMP-dependent phosphorylation was also inhibitory under these conditions (see also Ref. 23) and others [27], PSCP may interfere with MT assembly by phosphorylating some of the same sites involved with cAMP-dependent phosphorylation. The decrease in cAMP-dependent phosphorylation of HMW- and/or LMW-MAPs by PSCP both *in vitro* and *in vivo* suggests that changes in the MAPs fractions may be relevant to the OP-induced delayed neurotoxicity. Changes in PSCP sensitivity during storage of MT and/or brain homogenates and the known instability of chicken MT [14, 15] served to focus attention on the stability of MAPs fractions.

The OP neurotoxicants had a dramatic effect on the stability of HMW-MAPs which, in turn, was governed at least in part by endogenous MAPs-degrading proteases (Figs. 5 and 6). The OP neurotoxicants acted *in vitro* to stabilize HMW-MAPs. PSCP decreased the cAMP-dependent phosphorylation of LMW fragments, possibly having prevented their formation rather than their subsequent phosphorylation. These observations are consistent with

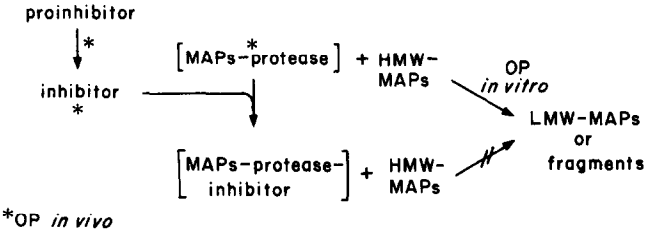

Fig. 6. Possible action of organophosphorus neurotoxicants on MAPs protease(s) and its hypothetical endogenous inhibitor.

OP inhibition of the endogenous MAPs-degrading protease(s). However, DFP acted *in vivo* in a most surprising manner since, in contrast to stabilizing HMW-MAPs *in vitro*, it apparently enhanced their degradation in treated birds. In a possibly related phenomenon, DFP treatment of hens enhanced the *in vitro* PSCP effect on cAMP-dependent phosphorylation of HMW-MAPs. An attempt to rationalize these apparently contradictory observations is given in Fig. 6. Direct OP attack on the proteolytic site of the MAPs-protease (or on its zymogen) would stabilize the HMW-MAPs, as occurs *in vitro*. The basis for *in vivo* destabilization is therefore more indirect, such as by altering a critical regulatory mechanism [28, 29], e.g. interaction of the OP compound with an endogenous inhibitor or with its release from an inactive form and/or modification of the MAPs protease by reaction at a site other than its proteolytic site (perhaps with subsequent dealkylation [1, 2, 7, 30]). An elevation in MAPs protease activity might affect normal nerve functioning by altering the MAPs-related filaments of MT [25, 31, 32] or by changing the dynamic equilibrium of HMW- and LMW-MAPs and physiological processes dependent on this equilibrium.

The present investigation suggests that the MAPs protease system is a possible target for OP delayed neurotoxicants. HMW-MAPs were destabilized *in vivo* by DFP, but it remains to be seen if this destabilization is specific for neurotoxic compounds and is a cause rather than an effect of the neurotoxicity.

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