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INTERACTION OF THE FOUR STEREOISOMERS OF SOMAN (PINACOLYL METHYLPHOSPHONOFLUORIDATE) WITH ACETYLCHOLINESTERASE AND NEUROPATHY TARGET ESTERASE OF HEN BRAIN

MARTIN K. JOHNSON,* DAVID J. READ* and HENDRIK P. BENSCHOP†
*Molecular Toxicology Section, Medical Research Council Laboratories, Woodmansterne Road, Carshalton, Surrey SM5 4EF, U.K. and †Prins Maurits Laboratory, TNO, 2280AA Rijswijk, The Netherlands

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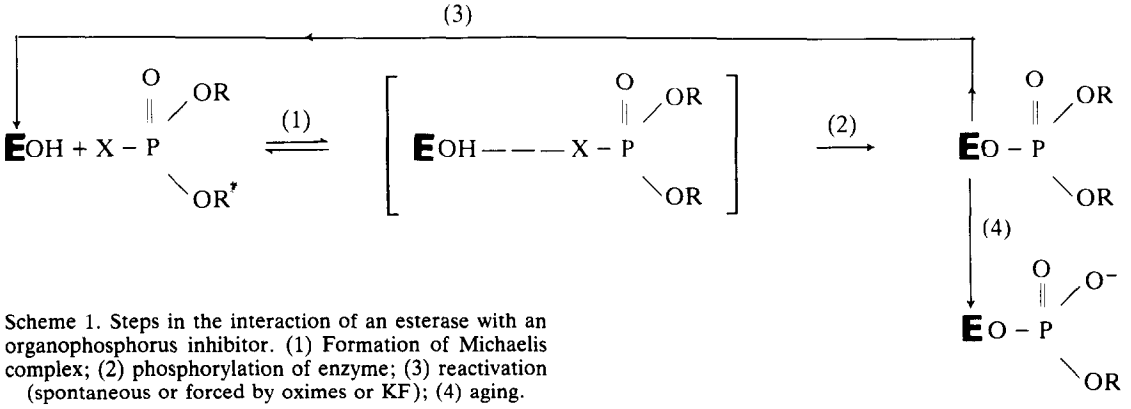
Abstract—Dilute solutions in cold dry ethyl acetate of 98–100% pure specimens of each of the four stereoisomers of soman were tested against enzymes in hen brain homogenate at 37° and pH 8.0. Rate constants for progressive inhibition of acetylcholinesterase were 10^7–10^8/mole/min for both P(−) isomers and less than 10^5 for both P(+) isomers. All isomers inhibited neuropathy target esterase non-progressively to some degree. Rate constants for progressive inhibition of neuropathy target esterase were 2.7–3.8 × 10^5/mole/min for C(−) P(+) and 2–6 × 10^4 for the others. Forced reactivation by KF was 90% initially and aging was slow in each case. Spontaneous reactivation of inhibited neuropathy target esterase was substantial during 18 hr for both P(−) isomers but not for P(+). By comparison of rate constants for the two enzymes we predict that pure P(+) isomers may cause delayed neuropathy in hens dosed at about unprotected LD_{50}: prophylaxis and therapy against acute cholinergic effects would have to raise LD_{50} 1000-fold before birds could tolerate potentially neuropathic doses of P(−) isomers.

Two major toxic effects of OP‡ esters are known. These are the acute toxicity initiated by covalent organophosphylation of the active centre of AChE and delayed neuropathy initiated by a similar reaction on the active site of NTE [1–4]. For delayed neuropathy a second essential step in the initiation process is aging of the inhibited enzyme [Reaction (4) in Scheme 1] [5, 6]. The structural features in OP esters associated with inhibitory power against AChE and NTE are different [7–9] so that some anticholinesterase compounds are known which are neuropathic below LD_{50}, while others are not neuropathic in animals even at doses far above the unprotected LD_{50}: such doses can only be tolerated when given in conjunction with prophylaxis and therapy against acute toxicity. Among the nerve agents, sarin (isopropyl methylphosphonofluoridate) has been shown to cause delayed neuropathy associated with high inhibition of NTE at about 30 times the unprotected LD_{50} [10, 11], while soman (pinacolyl methylphosphonofluoridate) was not neuropathic at 38 times the LD_{50}, although brain NTE in dosed hens was 55% inhibited: it was predicted that a further threefold increase in the dose of soman would be required to increase NTE inhibition above the threshold level which initiates neuropathy. Recently Willems *et al.* [12] managed to improve therapy such that one out of six dosed birds survived 100 times the LD_{50} of soman and did indeed develop severe delayed neuropathy. Soman is a mixture of four optical isomers due to the presence of centres of asymmetry at phosphorus (P) and in the pinacolyl moiety (C). These isomers, designated as C(+)P(+),

‡ Abbreviations: OP, organophosphorus; AChE, acetylcholinesterase; NTE, neuropathy target esterase (formerly called neurotoxic esterase); ChE, cholinesterase.



Scheme 1. Steps in the interaction of an esterase with an organophosphorus inhibitor. (1) Formation of Michaelis complex; (2) phosphorylation of enzyme; (3) reactivation (spontaneous or forced by oximes or KF); (4) aging.

C(+)P(−), C(−)P(+) and C(−)P(−), have been resolved and studied separately: it appeared that only two of them account for most of the anticholinesterase activity and acute toxicity of the mixture [13]. Previous studies on stereospecificity of OP compounds with respect to NTE have been limited to fixed-time determinations of I_{50} for the isomers of EPN oxon (ethyl 4-nitrophenyl phenylphosphonate) (ref. 14 and Lotti and Johnson, unpublished). The stereoisomers of several phenylphosphonothioates have been shown to have different neuropathic potential *in vivo* [15–17]. Therefore it seemed of interest to examine the four stereoisomers of soman with respect to each aspect of their interaction with NTE and their delayed neuropathic potential. This paper reports *in vitro* studies and a preliminary report has been given [18].

MATERIALS AND METHODS

Reagents

Isomers of soman were resolved as described previously [13]. Two separate preparations [called (A) and (B)] were made in The Netherlands with an interval of 5 months: the isomers were dissolved in dry ethyl acetate at concentrations of 1–10 mM. To discourage epimerization before use the following procedure was used. Sealed ampoules of the fresh solutions were cooled to −20° and packed in polystyrene boxes which were shipped immediately to arrive at Carshalton 24 hr later. The solutions were then stored at −5° for up to 24 hr prior to dilution. Concentrated samples were diluted to about 10–100 μM in dry ethyl acetate and were then kept double-packed at −5° in closed containers with an open dish of ethyl acetate in the outer one to discourage evaporative loss of solvent from the samples: the whole bulk was warmed to ambient temperature before withdrawing a sample for study. Screening studies using AChE as a monitor showed that the samples of P(+) isomers underwent about 1% epimerization within 2 days of receipt and dilution at Carshalton but no more than 2% after several weeks: the P(−) isomers could not be checked in this way. Samples of the dilute solutions were returned to the lab of origin about 8 weeks after receipt for gas liquid chromatography analysis on an optically active column [13]. Details are not given here but less than 3% epimerization had occurred for most samples. Bovine erythrocyte AChE was from Sigma Chemical Co. (Poole, U.K.). Other inhibitors and reagents were as described previously [8, 19, 20].

Buffers

For NTE studies Tris (50 mM or 10 mM)/0.2 mM EDTA was adjusted to pH 8.0 with HCl (about 12 M) or to 5.2 by admixture with necessary amounts of 50 mM-citric acid/0.2 mM-EDTA: these buffers are subsequently only identified by pH and molarity. For assay of AChE, solutions of NaH_2PO_4 and Na_2HPO_4 (each 100 mM) were mixed to give the desired pH.

Inhibition, aging, reactivation and assay of NTE

The activity of NTE is normally measured as the difference in phenyl valerate esterase activity between two samples which have been preincubated with a non-neuropathic progressive inhibitor (paraoxon or benzenesulphonyl fluoride) ± the neuropathic inhibitor, mipafox. The reasons for the choice of inhibitors used in aging studies along with a standard protocol have been described [6, 20]. However, modifications have been made for the present case where aging is slow and other improvements have been introduced so that a full description is given here. Glass and plastic-ware were specially cleaned as described previously [20].

(1) *Preparation of pairs of tissue samples (P and M) and study of the time course of inhibition.* Whole brain from freshly killed hens was homogenized (10% w/v) in pH 8 buffer (50 mM) as usual [2, 6] and large cellular debris was removed by sedimentation at 100 g_{av} for 5 min only. Procedure (1b) of ref. [6] was then followed to give a pair of sedimented pellets which were identical except that one (routinely referred to as P) contained active NTE while the other (M) did not. The pellets were either used the same day or stored at −20° for up to 4 days with no more than 10% loss of NTE. Prior to assay, each pellet (derived from 300 mg brain) was dispersed in pH 8 buffer (50 mM) containing Triton X-100 (0.01%) using a power-driven close-fitting Perspex/glass homogenizer with cooling and diluted to 45 ml. Inclusion of Triton was found to improve the reproducibility of subsequent assays. Just before the assay commenced, solutions of soman (101 × final test concentration) in ethyl acetate (20 μl) were added to ice-cold buffer (1 ml) in chilled tubes. At half-minute intervals tubes were transferred to a 37° bath and, after 3 min, preinhibited P or M suspensions (each 1 ml and containing particles from 6.67 mg brain) were added. Inhibition by soman at 37° was allowed to progress for periods up to 40 min before it was halted by addition of the substrate (2 ml): 30 min later the hydrolysis reaction was halted and colour developed as usual [19]. The differential extinction from control samples with added solvent was about 0.5, representing a phenyl valerate hydrolysis rate due to NTE of 1131 ± 74 (S.D., N = 5) nmole/min in freshly prepared P preparations from 1 g brain. The ethyl acetate (1%) had reduced control NTE activity to 82% of that measured in the absence of solvent: this effect was greater than the 5–10% inhibition by 1% acetone which has commonly been used as solvent for inhibitors [19].

(2) *Preparation of tissue for aging studies.* Homogenization of brain and removal of cell debris was performed as in (1) above. A sample of the supernatant (5 ml containing particles from about 500 mg of brain) was incubated at 37° in 50 mM pH 8 buffer with inhibitor (50 μl in ethyl acetate to give a chosen final concentration): controls were treated with solvent only. At the end of the inhibition period ice-cold pH 8 buffer (25 ml) was added and the mixture was cooled to 4° and centrifuged at 27,000 g_{av} for 30 min. The pellet was resuspended in 32 ml of ice-cold pH 8 buffer (10 mM only).

(3) *Aging and induced reactivation.* Suspensions of inhibited and control tissue prepared as in (2) above were incubated at 37°. At zero-time two samples (each 7 ml containing particles from 109 mg

brain) were removed from each and mixed with prewarmed solutions (25 ml) of either KCl or KF (each 250 mM) in Tris-Citrate-EDTA buffer pH 5.2. These samples were used as points of reference for a further set which was processed identically after a measured period of incubation at 37° in the aging medium. After 10 min at 37° in the KF (reactivation) or KCl (control) medium, the mixtures were cooled to 4° and centrifuged at 27,000 g_{av} for 60 min. The sedimented particles were resuspended in pH 8 buffer/Triton (10 ml) as in (1) above. Neuropathy target esterase activity was then determined by the standard method with phenyl valerate as the substrate [19] but with benzenesulphonyl fluoride (250 μM final) as the inhibitor in place of paraoxon, since the latter would react with residual KF [20]. Particles from approx. 11 mg of the original brain which were put through the whole inhibition/aging and reactivation process as controls (ethyl acetate as "inhibitor" and KCl as "reactivator") gave a differential extinction of about 0.5 due to NTE after 20 min incubation with substrate: this represented a mean value of 1126 ± 80 (S.D., N = 11) nmoles of phenyl valerate hydrolysed/min for particles derived from 1 g brain.

Inhibition and assay of acetylcholinesterase

Whole homogenates (10% w/v) of hen brain in pH 8 buffer (50 mM) were freed from the debris as above and portions (0.33 ml) were incubated at 37° in Tris buffer (50 mM, pH 8) (4.66 ml) with added inhibitor (20 μl in ethyl acetate) for chosen times before an aliquot (0.2 ml containing tissue from about 1.3 mg original brain) was transferred to a cuvette containing reagents (3.05 ml) for assay at 37°, as described by Ellman *et al.* [21]. Mean control rate for such debris-free homogenates, preincubated with solvent only, was 23.5 ± 1.46 (S.D., N = 5) μmoles/min/g of brain.

RESULTS

Determinations were made of the various interactions using two separate preparations, (A) and (B), of the four isomers prepared and delivered about 5 months apart. Assays were performed as soon as possible after delivery and dilution of the isomers. However, preparation (A) of the C(−) P(−) isomer was found to have epimerized substantially soon after delivery (see Methods) and no reliable data were obtained for inhibition of NTE by that sample.

Inhibition of acetylcholinesterase

For inhibition by the two P(−) isomers semilog progress lines were linear with time and the slopes were proportional to inhibitor concentration and intercepted the ordinate at log 100% activity. Derived rate constants are in Table 1. For both preparations of the C(+) P(+) isomer the fresh samples caused negligible inhibition in 20 min at the highest concentration tested (0.20 μM and 0.13 μM, respectively). A fresh sample of preparation (A) of C(−) P(+) caused negligible inhibition at 0.030 μM. Over the next 2 days the inhibitory power of this diluted sample, which had been kept sealed at −5°, increased

Table 1. Second order rate constants (k_a)(/mole/min) for inhibition of AChE and NTE by stereoisomers of soman at 37°, pH 8.0

Isomer: Designation / Preparation	C(−) P(+) (A)	C(−) P(+) (B)	C(−) P(−) (A)	C(−) P(−) (B)	C(+) P(+) (A)	C(+) P(+) (B)	C(+) P(−) (A)	C(+) P(−) (B)
AChE								
Bovine erythrocyte	≪10^6*	—	3.1×10^7	—	≪2×10^5*	—	9.1×10^7	—
Hen brain	≪1.2×10^6*	8.3×10^4	3.8×10^7	5.4×10^7	≪1.7×10^5*	≪2.6×10^5*	6.3×10^7	7.6×10^7
NTE								
Hen brain	3.8×10^5	2.7×10^5	—	4.6×10^4	4.7×10^4	4.4×10^4	1.8×10^4	1.7–2.9×10^4
Approximate ratio of rate constants: k_a(NTE)/k_a(AChE)	>1		0.001		>1		0.001	

For AChE, residual activity was determined after preincubation of enzyme with test inhibitor for periods up to 20 min. Rate constants were derived from the slope of semilog plots (not shown). Error of the slopes was trivial compared with inter-experiment variation. For P(−) isomers values are mean of three determinations with *range* ±15% for preparation (A) and single determinations for (B); for P(+) only the highest available concentration was tested in each case. For NTE, paired samples of P and M particles prepared as described in Methods were incubated with test inhibitor for periods up to 40 min: NTE activity was calculated from the difference in their residual phenyl valerate esterase activity. Semilog plots are shown in Fig. 1 and the derived rate constants are given as *mean within a range* less than ±10% except for preparation (B) of the C(+) P(−) isomer.

* Numbers associated with "≪" sign are the rate constants which would have been obtained if 50% progressive inhibition had occurred with the highest concentration tested; negligible inhibition was seen in each case so that the actual constant would be expected to be at least 10-fold less than the written number.

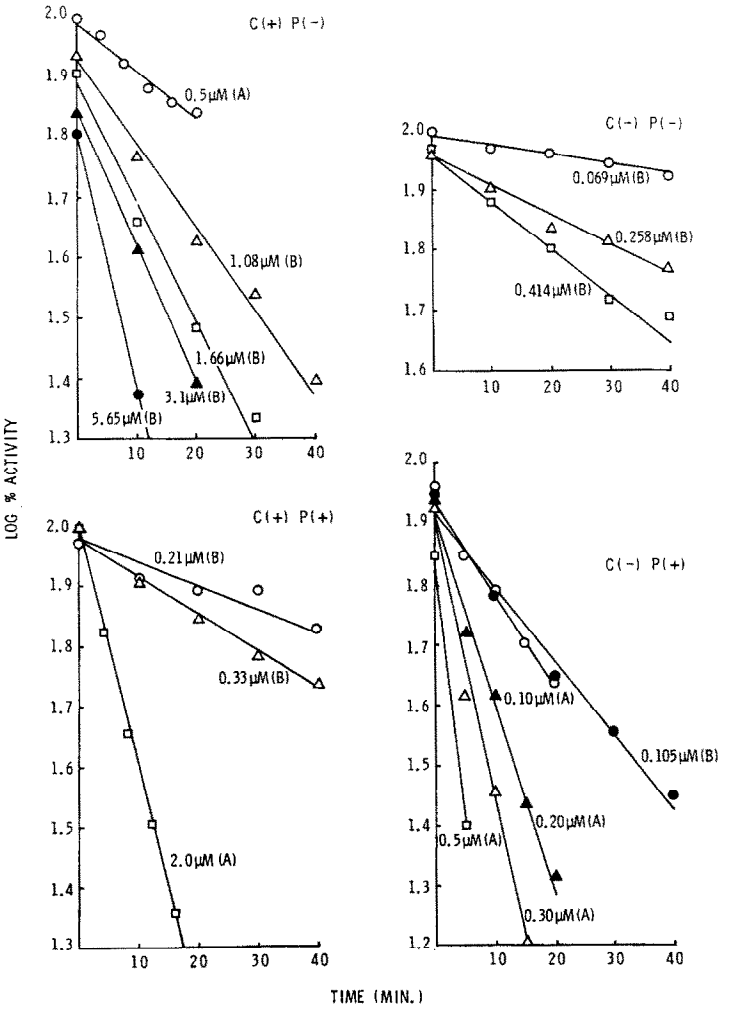


Fig. 1. Semilog plots of progressive inhibition of NTE by isomers at concentration shown and at pH 8.0 and 37°.

to a level equivalent to that expected if about 1% of C(−) P(−) isomer were present and this increased to about 2% in a further 12 days. A more concentrated fresh sample of preparation (B) had sufficient inhibitory power for a single progress line to be determined and the rate constant to be derived.

The rate constants for inhibition of hen brain AChE were similar to those determined for bovine erythrocyte AChE and these were in reasonable agreement with those obtained by Benschop *et al.* in their own laboratory but at 25° and pH 7.4 [13].

Inhibition of neuropathy target esterase

During the course of the study some erratic values were obtained: this variability was traced to incomplete dispersion of the P and M pellets which had been stored at −20° for 1–4 days before homogenizing them in buffer without detergent (see Methods). Addition of Triton X-100 at a final concentration of 0.01% to the dispersing buffer effected a marked improvement in the linearity of subsequent assays. Since NTE assays are differential and each value requires two measurements, only five time-points were used for each concentration of inhibitor.

Semilog plots of activity vs time (Fig. 1) were linear. For each isomer, except C(+) P(−), the slopes were proportional to inhibitor concentration and the derived second-order rate constants are given in Table 1. For the C(+) P(−) isomer, slopes were not proportional to concentration and substantial intercepts were obtained on the ordinate at higher concentrations. These digressions from first-order kinetics may signify formation of a reversible inhibitor-enzyme complex [22, 23]. However, when the slopes of the four lines obtained using preparation (B) were replotted in the format of slope/inhibitor concentration vs inhibitor concentration, according to Wilkinson [24] (graph not shown), no linear relationship was seen and we are unable to interpret the data: in Table 1 we have set out the range of rate constants derived from Fig. 1 for this isomer.

Because of the technical limitations and the small quantities of isomers available, few progress lines were obtained and the rate-constants for progressive

inhibition of NTE given in Table 1 must be regarded as only semiquantitative: no further statistical evaluation is appropriate. The C(−) P(+) isomer stood out as 6–10 times more inhibitory than the other isomers.

Reactivation and aging of inhibited neuropathy target esterase

Because of the limited quantities and concentrations of isomers it was necessary to incubate samples for up to 2 hr in order to obtain conveniently high inhibition. Since aging was found to be slow this delay was acceptable. The inhibitions caused by incubating concentrated brain particle suspensions under the conditions given in Table 2 were noticeably less than the degree expected by extrapolation of the data shown in Fig. 1, which was obtained with very dilute tissue suspensions; this discrepancy is considered in the Discussion.

Comparison of values in columns (e) and (a) of Table 2 show that no spontaneous reactivation was detected in 18 hr incubation at pH 8.0 (37°) for the two P(+) isomers whereas reactivation was seen with both the P(−) isomers.

In aging studies KF-induced reactivation measured immediately after inhibition was always better than 90%. [Table 2, column (d)]; the reference values were activities of KCl-treated samples which had been "inhibited" only with solvent but processed identically in all other respects. The degree of aging was assessed first by comparing the extent of reactivation at this early time with that seen after the specified time [column (h)]. This procedure is correct theoretically under ideal conditions and proved satisfactory for the short aging periods studied by Clothier and Johnson [6, 20]. However, column (g) shows a progressive decline in activity of control samples treated with KF after a long period at 37°. Moreover, spontaneous reactivation occurred in some samples which means that the numerical change in measured activity associated with a certain percentage of aging was not the same in all circumstances. Also in these complex experiments, we do not consider measured activity changes of 5% or less of control uninhibited activity to be meaningful. Therefore we have preferred to be cautious and to use both the theoretically correct, [(h) with (d)] and *ad hoc* [(g–f) with (c–b)] comparisons to score aging and we report only the lesser score where the two do not agree.

Table 2 shows that in three experiments aging scored + or ++ 18 hr after inhibition by C(−) P(+), but there was variable and only marginal (doubtful significance) aging after inhibition by the other three isomers. After inhibition by the P(−) isomers the numerical changes possible were limited by the concomitant spontaneous reactivation: in future studies this problem could be prevented by deferring the separation of enzyme and inhibitor until the end of the aging period.

DISCUSSION

Several technical improvements in the procedures compared with our earlier methods [6, 20] should be noted. Since aging of soman-inhibited NTE turned out to be slow we used extended inhibition times and separated inhibited enzyme from the unreacted inhibitor by sedimentation before commencing the timed aging period—this enabled us to achieve reasonable inhibition while economizing on scarce inhibitors and also lessened the extent of ongoing inhibition and/or reinhibition during aging. We eliminated some errors due to variable loss of enzyme during the processes of centrifugation and resuspension by using only a single sample of particles (instead of preincubated P and M preparations) for the steps of inhibition, aging and induced reactivation and then dissecting out the NTE in the final washed and resuspended particles just prior to measurement of phenyl valerate esterase activity. It was necessary to use benzenesulphonyl fluoride as a selective inhibitor rather than paraoxon in these circumstances because a potent NTE inhibitor is generated by reaction of paraoxon with (otherwise innocuous quantities of) KF carried over from the reactivation step: alternatively this KF could be removed by yet another washing step. Use of Triton X-100 (0.01%) in the final resuspension medium prior to assay also improved reproducibility. As noted in the Results, use of Triton seemed essential when dispersing stored frozen pellets.

The P(−) isomers of soman were 2–3 orders more inhibitory than the P(+) against hen brain AChE. We confirmed this differential for erthrocyte AChE as reported previously [13]. However, there was only a 6–10-fold range of potency among the four isomers against NTE. For horse serum ChE there is a range of 100-fold in the inhibitory power of the four isomers [25]. The occurrence of some non-progressive inhibition of NTE is discussed in the Results.

The degree of inhibition of NTE achieved using concentrated tissue suspensions in the aging studies was consistently 2–3-fold less than that anticipated from the progress lines in Fig. 1, where dilute tissue was used. It is possible that the inhibitor was being destroyed by non-specific binding or by enzyme-catalysed hydrolysis in the former case and we have evidence (unpublished) for such processes in analogous experiments with other OP esters.

Aging of inhibited NTE was slow for each isomer and the changes in reactivatibility were only marginal except for the C(−) P(+) isomer. We were unable to extend aging periods beyond 18 hr because NTE losses became significant and more so in samples which were treated with KF after aging. Initial experiments (unpublished) suggest that this instability may be less in samples incubated at pH 7 rather than at 8.0. In the light of the results presented here, the fact that NTE inhibited by unresolved soman aged measurably in 5 hr [20] suggests that the inhibition was due principally to the C(−) P(+) isomer: this deduction fits with the fact that this isomer is the more potent when tested in isolation against NTE.

In the only other report on aging of enzymes inhibited by specific isomers of soman, Keijer and Wolring [25] used the resolved epimeric pairs to inhibit serum ChE and obtained a mixture of C(−) P(−)- and C(−) P(+)-inhibited enzymes. One of these aged at a rate at least three orders faster than the other (negligible in 24 hr) and it was presumed

Table 2. Spontaneous reactivation and aging of hen brain NTE inhibited by individual stereoisomers of soman

Isomer and inhibition condition (μM/min)	Activity of unaged samples (%)					Activity of aged samples (%)				Scoring of:	
	Soman-inhibited and then treated with:		Solvent-"inhibited" and then treated with:	Induced reactivation before aging [(b − a) (c − a)] (%)	Time of Aging	Soman-inhibited and then treated with:		Solvent "inhibited" and then treated with:	Induced reactivation after aging + spontaneous reactivation [(f − e) (g − e)] (%)		
	KCl (a)	KF (b)	KF (c)	(d)	(hr)	KCl (e)	KF (f)	KF (g)	(h)	Spontaneous reactivation*	Aging
C(−) P(+)											
1.28/20	21	97	105	90	1.5	21	96	101	94	−	−
2.56/20	4	94–103	103	90–99	5	4	83–91	86	96–106	−	−
2.56/20	2	87	96	90	18	4	51	86	57	−	++
0.64/20	42	97	91	112	18	38	75	86	77	−	+
0.42/60	46	89	87	104	18	33	59	72	67	−	+
C(−) P(−)											
1.65/60	29	105	94	117!	5	44	87	92	90	+	?
1.65/60	12	80, 83	87	93	18	39	55	72	48	++	+
1.65/120	23	84	88	94	18	53	73	81	71	++	±
C(+) P(+)											
1/40	56	100	105	90	1.5	56	95	101	87	−	−
2/40	25	90	96	92	18	22	76–83	86	84–95	−	?
0.67/120	32	93	97	94	18	30	83	92	85	−	±
0.67/120	27	89	95	91	18	30	85	87	93	−	−
C(+) P(−)											
1/40	55	99	100	98	1.5	50	100	100	100	−	−
1/40	43	100	105	92	1.5	46	101	101	100	−	−
2.1/60	22	92	94	97	5	34	84	92	86	+	±
1.5/60	28	104	91	121	18	65	81	86	76	++	±
5.2/60	4	86	87	99	18	44	63	72	68	++	±
5.2/120	10	87	88	99	18	54	70	81	59	++	+

Activity values are % of activity of control particles processed in parallel with the inhibited samples but "inhibited" only with solvent (ethyl acetate, 1% v/v final), "aged" for zero time, "reactivated" with KCl and stored at 2° until all aged samples were ready for assay. Each value represents a single sample processed but is the mean of duplicate determinations of NTE: duplicates agreed within 4% except where shown. In six experiments recovery of NTE from control, uninhibited suspensions which were "aged" for 18 hr/37° and subsequently treated with KCl was 85–99% (mean 92%); recovery of NTE in control particles treated with KF was less (2–13: mean 7%) than that from KCl-treated particles "aged" for similar lengths of time.

* Spontaneous reactivation was assessed by comparing values in columns (e) and (a).

† Aging was assessed both by comparing values in columns (h) and (d) and also [(g) − (f)] with [(c) − (b)]. See text for a discussion of these two procedures.

Extent of reactivation and of aging have been scored as follows: ++, change >25%; +, >10%; ±, >5%; —, <5%. Changes scored ± are at the limit of technical reproducibility of the procedures.

that the rapid aging form had been inhibited by C(−) P(−).

We are unaware of any report of spontaneous reactivation of an esterase after inhibition by any isomer of soman. This is the first time that spontaneous reactivation of OP-inhibited NTE has been reported in detail and it is interesting that the reactivation appears specific to the P(−) isomers. The present experiments suggest that there must be marked differences in the regions surrounding the phosphorylation sites of AChE and NTE.

Extrapolation from potencies measured *in vitro* to predict effects *in vivo* is not straightforward. However, the ratio of inhibitory potencies measured *in vitro* against AChE and NTE, respectively, should indicate which enzyme is most liable to attack *in vivo*. For a range of compounds such ratios were shown to correlate with the ratio of doses which cause either acute or delayed effects [9]. Predictions on this basis for the dose of unresolved soman needed to cause neuropathy seem to have been justified [11, 12] although the method is strictly not valid when a mixture of isomers which will be metabolized differently is used. However, the ratio of rate constants for separate isomers given in Table 1 should be a valid predictor and suggests that the pure P(+) isomers individually should cause delayed neuropathy at doses around the LD_{50}. By comparison of rate constants for the two enzymes we predict that pure P(+) isomers may cause delayed neuropathy in hens dosed at about unprotected LD_{50}; prophylaxis and therapy against acute cholinergic effects would have to raise LD_{50} 1000-fold before birds could tolerate potentially neuropathic doses of P(−) isomers, and even then, initiation of neuropathy might be vitiated by spontaneous reactivation of the inhibited NTE.

The data in Table 2 suggest that aging of NTE inhibited *in vivo* by the C(−) P(+) isomer would be substantial within a few days and sufficient to initiate neuropathy but this might not be so for C(+) P(+). We hope to investigate these possibilities soon.

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