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A THEORETICAL KINETIC ANALYSIS OF THE PROTECTIVE ACTION EXERTED BY ESERINE AND OTHER CARBAMATE ANTICHOLINESTERASES AGAINST POISONING BY ORGANOPHOSPHORUS COMPOUNDS

ALBERT L. GREEN

Department of Bioscience and Biotechnology, University of Strathclyde, The Todd Centre, 31 Taylor Street, Glasgow G4 0NR, U.K.

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Abstract—The protection exerted by carbamate anticholinesterases against the lethal effects of organophosphate anticholinesterases is interpreted kinetically. The protective action is shown to depend on the provision (by carbamylation) of a pool of sequestered cholinesterase resistant to organophosphates but which furnishes (by decarbamylation) sufficient active enzyme to essential cholinergic synapses to ensure survival until all free organophosphate is cleared from the tissues. The two main factors governing the extent of protection are the minimum cholinesterase activity compatible with survival and the "enzyme conservation index" defined essentially as the ratio of the rate of enzyme decarbamylation to the rate of enzyme phosphorylation; the lower the "minimum essential cholinesterase" and the higher the "enzyme conservation index", the greater the protection. The theory is shown to be in excellent qualitative agreement with experiment.

It has been known for over 30 years that cats could be protected against the lethal effects of several times the LD_{50} of di-isopropylphosphorofluoridate (DFP) by pretreatment with the carbamate anticholinesterase eserine [1]. Since carbamate and organophosphate anticholinesterases inhibit cholinesterase by essentially the same mechanism (carbamylation or phosphorylation of the enzyme active centre [2]), it might be expected that administration of a carbamate and an organophosphate together would lead to an enhancement of the toxic action. Such an additive toxic effect is observed if the carbamate is administered after the organophosphate [1]. No detailed explanation of this apparent paradox has been given, although the effect has been attributed qualitatively to a complex re-adjustment of reaction rates which allows an adequate level of active cholinesterase to be maintained until all free organophosphate has been cleared from the tissues [2, 3].

This paper presents a theoretical kinetic analysis of the principal reactions which are likely to occur when cholinesterase in a living animal is exposed to a carbamate and an organophosphate either simultaneously or sequentially. It is shown from this model that purely kinetic factors can account semiquantitatively for the main features of the protective action exerted by carbamates against poisoning by organophosphorus anticholinesterases. Some suggestions are given as to how this protective action might be increased.

THEORY

Carbamates and organophosphates both combine covalently with cholinesterase via an initial transient reversible complex, but at the inhibitor concentrations normally met in practice this transient reversible complex does not contribute significantly to the total level of inhibition. The inhibitory process can consequently be treated in both cases as though it were a simple bimolecular reaction between the inhibitor and the enzyme, with the inhibitor being present in large excess (e.g. see [2, 4]). Carbamylated cholinesterases hydrolyze at moderate speed to regenerate free active enzyme [5] and the extent of cholinesterase inhibition by carbamates can be best interpreted by assuming that the concentration of the carbamylated enzyme reaches a steady state where its rate of formation and rate of decomposition are equal [6]. Phosphorylated cholinesterases are more stable, and spontaneous hydrolysis is normally too slow to be relevant to the inhibitory potency or toxicity of organophosphates, but surplus free organophosphate disappears rapidly from the tissues of living animals by excretion or metabolism so that *in vivo* a steady level of cholinesterase inhibition is also attained within a short time after administration (e.g. see [7, 8]). Thus, the simplest kinetic description of the effect on cholinesterase of the administration of both a carbamate and an organophosphate *in vivo* can be made by assuming that there are only three relevant reactions, namely:

(i) $E + I \xrightarrow{k_1} A \xrightarrow{k_2} E$ + hydrolyzed carbamate
(ii) $E + P \xrightarrow{k_3} B$
(iii) $P \xrightarrow{k_4}$ waste products.

In these equations, E, I and P are free cholinesterase, carbamate and organophosphate, A and B are the inactive carbamylated or phosphorylated forms of the enzyme, and k_1, k_2, k_3 and k_4 are the rate constants for the reactions indicated. Since inhibition of butyrylcholinesterase appears to play no significant part in the lethal action of anticholin-

esterases, the name cholinesterase is used throughout this paper to refer exclusively to acetylcholinesterase.

On injection, the organophosphate may be assumed to be distributed instantaneously throughout the tissues to reach an initial uniform maximum concentration directly related to the dose. This concentration is then assumed to decline at a first order rate as a result of metabolic and excretory processes unrelated to cholinesterase [equation (iii) above]. Strictly, a similar equation should be included to allow for the time-dependent metabolic inactivation or excretion of the carbamate, but this would result in excessive mathematical complication, and this problem has been surmounted indirectly as explained later. The other essential assumption required to allow for manageable calculations is that the animal body is homogeneous. This obviously is not wholly true, but it has been shown in a previous kinetic analysis [9] that semiquantitative deductions can be made about the treatment of organophosphate poisoning by oximes and atropine which are reasonably consistent with experiment despite the neglect of any non-uniformity in drug and enzyme distribution. The initial organosphate and carbamate concentrations (P_o and I) are likely to be much larger than the cholinesterase concentration and no correction has been made for the small amounts lost as A, B, or hydrolyzed carbamate.

If concentrations are represented by italicized capital letters, the rate equations arising from (i) to (iii) are:

$$dE/dt = k_2A - (k_1I + k_3P)E \quad (1)$$
$$dB/dt = k_3P \cdot E \quad (2)$$
$$dP/dt = -k_4P \quad (3)$$

It follows directly from (3) that $P = P_o \exp(-k_4t)$.

Equations (1) and (2) may be simplified by putting $E/E_o = y$ and $B/E_o = z$, where E_o is the initial concentration of enzyme, and E and B are the concentrations of free and phosphorylated enzyme after time t. It then follows that $A/E_o = (1 - y - z)$. A further simplication can be achieved by replacing $\exp(-k_4t)$ with a new variable (x). Equations (1) and (2) now become:

$$dy/dt = k_2(1 - y - z) - y(k_1I + k_3P_ox) \quad (4)$$
$$dz/dt = k_3P_ox \cdot y \quad (5)$$

Since $dy/dt = (dy/dx) \times (dx/dt)$, $dz/dt = (dz/dx) \times (dx/dt)$ and $dx/dt = -k_4x$, (4) and (5) can be arranged to give:

$$dy/dx = (k_3P_o/k_4)\,y + (k_2/k_4) \times [(1 + k_1I/k_2)(y/x) - (1 - z)/x] \quad (6)$$
$$dz/dx = -(k_3P_o/k_4)y \quad (7)$$

Approximate numerical values for all the constants appearing in (6) and (7) can be given from published experiments with the exception of k_4, the average rate constant for the disappearance of free organophosphate from the tissues. This constant is not amenable to direct measurement, but (6) and (7) can be modified as follows so as to avoid the need to allocate a specific value to k_4. In the absence of carbamate, $z = 1 - y$, hence (7) becomes $dy/dx = (k_3P_o/k_4)y$, which can be readily integrated to give $\ln(1/y) = (k_3P_o/k_4)(1 - x)$. Thus, in animals given an organophosphate alone, the cholinesterase activity will approach a minimum value (E_∞) as x becomes small (i.e. as t approaches infinity). At this time, $\ln(1/y)$ (which equals $\ln E_o/E_\infty$) $= k_3P_o/k_4$. In an earlier kinetic model for the action of organophosphates *in vivo* [9], it was proposed that if P_o were the mean tissue concentration of organophosphate calculated from the uniform distribution throughout the body of one LD_{50} dose, then the corresponding value of E_∞/E_o could be regarded as the minimum fraction of the available cholinesterase which was required to maintain life in 50% of the animals concerned. This minimum cholinesterase level probably lies between 10 and 0.1% of normal (for a more detailed discussion of this point see [9]). Thus k_3P_o/k_4 can be replaced by a single constant J ($= \ln E_o/E_\infty$) having value between 2.3 and 6.9, and k_2/k_4 can be replaced by $J(k_2/k_3P_o)$.

The term k_2/k_3P_o represents the ratio of the rate at which free active cholinesterase is regenerated from the carbamylated enzyme to the rate at which it disappears again to form the inactive phosphorylated enzyme. As this is likely to be an important factor in maintaining a sufficient level of free cholinesterase for life to continue it is referred to below as the "enzyme conservation index".

The value of k_2 will depend on the nature of the carbamylating residue, being about $0.03\,min^{-1}$ for monomethylcarbamates such as eserine [5]. Values for k_3 and P_o will vary with the organophosphate and animal species, but generally P_o decreases as k_3 increases (i.e. the LD_{50} is inversely related to the rate constant for cholinesterase inactivation). Thus the product of the two constants is likely to lie within a fairly restricted range of values. For DFP, paraoxon and sarin, k_3P_o varies from about 1 to $10\,min^{-1}$ [9] and this range of values is likely to be typical. Thus, the "enzyme conservation index" (k_2/k_3P_o) is unlikely to have a value exceeding 0.05, and would normally be appreciably less than this. Equations (6) and (7) can now be rewritten as:

$$dy/dx = J[y + (k_2/k_3P_o) \times \{(1 + k_1I/k_2)(y/x) - (1 - z)/x\}] \quad (8)$$
$$dz/dx = -Jy \quad (9)$$

Appropriate values for the other coefficient appearing in (8) namely $(1 + k_1I/k_2)$, may be deduced as follows. Winteringham and Fowler [6] showed that for inhibition of cholinesterase by eserine *in vitro*, the enzyme activity reached a steady state level where $E/E_o(y)$ equalled $1/(1 + k_1I/k_2)$. Thus the term $(1 + k_1I/k_2)$ can be simply equated to the reciprocal of the residual level of cholinesterase produced by administration of the carbamate alone once this has reached the steady state. Since the dose of carbamate must be non-lethal, $(1 + k_1I/k_2)$ will normally lie between 1 (0% inhibition) and 10 (90% inhibition) and it is unnecessary to allocate individual values to k_1 and I.

The final requirement before calculation can begin is to specify the initial value conditions. Three sets of these have been considered. In the first, the carbamate and organophosphate were assumed to be given together, hence $y_o = 1$ (i.e. $E = E_o$) and $z_o = 0$ (i.e. $B = 0$), and the carbamate was assumed to

Table 1. Simulation study on the minimum level of cholinesterase reached in animals given both a carbamate and an organophosphate

"Minimum essential cholinesterase" (% activity remaining after one LD_{50} dose of organophosphate)	"Enzyme conservation index" (k_2/k_3P_o)	Minimum cholinesterase level (% of normal) produced by the carbamate and organophosphate in combination for the following steady-state cholinesterase levels (% of normal) due to the carbamate alone						
		67	50	33	20	10	5	1
10	0.05	<10	<10	<10	<10	<10	—	—
1	0.05	1.53	1.76	1.98	2.17	2.09	—	—
	0.03	1.23	1.34	1.44	1.51	1.49	—	—
	0.02	1.10	1.14	1.18	1.19	1.15	—	—
	0.01	<1	<1	<1	<1	<1	—	—
0.1	0.03	0.35	0.48	0.65	0.85	1.03	1.07	0.66
	0.01	0.16	0.19	0.24	0.29	0.36	0.42	0.35
	0.003	0.11	0.11	0.12	0.13	0.14	0.15	0.14
	0.002	0.103	0.105	0.107	0.111	0.115	0.118	0.110
	0.001	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1

The carbamate and organophosphate were assumed to have been administered together. The carbamate was also assumed to remain at its initial concentration until all free organophosphate had disappeared from the tissues.

remain at its initial concentration (I) till all the organophosphate had disappeared. In both the second and third, the carbamate was assumed to be given a sufficient time before the organophosphate so that the steady state inhibition level by the carbmate was reached before any reaction commenced between the cholinesterase and organophosphate, hence in both cases $y_o = 1/(1 + k_1I/k_2)$ and $z_o = 0$. However, whereas in the second set of conditions the carbamate was again assumed to remain at a constant concentration, in the third it was assumed to disappear instantaneously from the tissues immediately before administration of the organophosphate, so that the $(1 + k_1I/k_2)$ term in (8) was put equal to 1. The true situation for carbamate pretreatment would lie somewhere between those predicted by the second and third set of conditions. As will be seen from the results (Tables 2 and 3), there is very little difference between the two. Thus, inclusion of an additional rate equation to allow for time-dependent spontaneous disappearance of the carbamate would be unlikely to produce more than a minor effect on the conclusions reached from the simpler model which has been used here.

RESULTS AND DISCUSSION

The simultaneous differential equations (8) and (9) cannot be solved analytically, but numerical solutions may be obtained using the Merson–Runge–Kutta method. The calculations were carried out using a Data General NOVA 2 minicomputer, and

Table 2. Simulation study on the minimum level of cholinesterase reached in animals given both a carbamate and an organophosphate

"Minimum essential cholinesterase" (% activity remaining after one LD_{50} dose of organophosphate)	"Enzyme conservation index" (k_2/k_3P_o)	Minimum cholinesterase level (% of normal) produced by the carbamate and organophosphate in combination for the following steady-state cholinesterase levels (% of normal) due to the carbamate alone				
		67	50	33	20	10
10	0.05	11.8	11.2	<10	<10	<10
1	0.05	5.1	5.9	6.0	5.4	4.1
	0.03	3.8	4.5	4.6	4.1	3.2
	0.01	2.4	2.5	2.5	2.2	1.8
	0.003	1.4	1.4	1.3	1.1	<1
	0.002	1.2	1.2	1.1	<1	<1
0.1	0.01	1.25	1.49	1.66	1.64	1.45
	0.003	0.58	0.67	0.72	0.70	0.63
	0.002	0.47	0.52	0.55	0.53	0.48
	0.001	0.31	0.34	0.35	0.33	0.29
	0.0002	0.14	0.14	0.13	0.12	>0.1

The administration of the organophosphate was assumed to have been deferred until after the steady-state cholinesterase level due to the carbamate alone had been attained. The carbamate was also assumed to remain at its initial concentration until all free organophosphate had disappeared from the tissues.

Table 3. Simulation study on the minimum level of cholinesterase reached in animals given both a carbamate and an organophosphate

"Minimum essential cholinesterase" (% activity remaining after one LD_{50} dose of organophosphate)	"Enzyme conservation index" (k_2/k_3P_o)	Minimum cholinesterase level (% of normal) produced by the carbamate and organophosphate in combination for the following steady-state cholinesterase levels (% of normal) due to the carbamate alone				
		67	50	33	20	10
10	0.05	12.8	12.6	11.5	<10	<10
	0.05	5.4	6.4	7.1	7.1	6.4
	0.03	4.1	4.9	5.2	5.0	4.6
1	0.01	2.4	2.6	2.7	2.5	2.1
	0.003	1.5	1.4	1.4	1.2	<1
	0.002	1.2	1.2	1.1	<1	<1
	0.01	1.23	1.53	1.75	1.78	1.70
	0.003	0.57	0.67	0.74	0.73	0.67
0.1	0.002	0.45	0.52	0.56	0.54	0.50
	0.001	0.30	0.34	0.35	0.34	0.29
	0.0002	0.14	0.15	0.13	0.12	<0.1

The administration of the organophosphate was assumed to have been deferred until after the steady-state cholinesterase level due to the carbamate alone had been attained. The carbamate was also assumed to remain at its initial concentration until the steady-state cholinesterase level had been attained, but then to disappear completely from the tissues before administration of the organophosphate.

a BASIC programme based on those described by Bull [10] and by Wilkins *et al.* [11]. The procedure adopted was to specify values for J (i.e. $\ln E_o/E_\infty$), for the "enzyme conservation index" (k_2/k_3P_o), and for the residual enzyme activity which would be produced by carbamate treatment alone (i.e. $1/(1 + k_1I/k_2)$) and then to calculate the value of the residual cholinesterase activity y (i.e. E/E_o) as x (i.e. $\exp(-k_4t)$) decreased (in steps of $\geqslant 0.1$ units) from 1 ($t = 0$) to 0 ($t = \infty$). The minimum value of y was noted from each calculation. These minimum values are summarized in Tables 1–3 for the three sets of initial value conditions described in the Theory section.

Interpretation of the results summarized in Tables 1–3 is based on the premise that survival of an animal poisoned with an anticholinesterase depends on the cholinesterase activity not being depressed below some minimum essential level. If, in animals treated with a carbamate and organophosphate in combination, the cholinesterase activity is always higher than that which would be produced by the organophosphate on its own, then the carbamate can be regarded as exerting a protective action against the organophosphate. If the level falls below that which would be produced by the organophosphate alone, the carbamate would be considered to enhance the toxic action.

Three values were used for J, namely 2.3, 4.6 and 6.9, corresponding to "minimum essential cholinesterase" levels of 10%, 1% and 0.1% of normal. This "minimum essential cholinesterase" level may be species dependent, so may the LD_{50} of the organophosphate and the numerical values of the rate constants for the various reactions involved. However, for any given carbamate and organophosphate in a particular species, these values will be fixed. Suppose for one LD_{50} of organophosphate in this particular species the "enzyme conservation index" (k_2/k_3P_o) had a value of 0.03. As can be seen from Tables 1–3 the minimum cholinesterase activity produced for this value of k_2/k_3P_o by an organophosphate–carbamate combination under any of the specified treatment regimes is above the "minimum essential cholinesterase" provided this is not greater than 1% of normal. Hence, under these conditions more than 50% of the animals would survive, and the carbamate would have exerted a protective action. The extent of the protection that can be achieved may be assessed crudely as follows. Suppose the dose of organophosphate administered was raised to ten times the LD_{50}, then the numerical value of k_2/k_3P_o would fall from 0.03 to 0.003. If, for this lower value of k_2/k_3P_o the minimum cholinesterase activity reverts to the "minimum essential cholinesterase" level, as indicated in Tables 2 and 3, then treatment with the carbamate may be considered to have raised the LD_{50} of the organophosphate about ten times.

The first clear conclusion that can be reached from the above reasoning is that the protective action is essentially prophylactic. For any specified "minimum essential cholinesterase" level and "enzyme conservation index" the potential protection given when the two drugs are administered together (Table 1) is far inferior to that which may be achieved if the carbamate is given some time before the organophosphate (Tables 2 and 3). This conclusion is in full agreement with the original experiments of Koster [1] on the protection given by eserine against DFP poisoning in cats. Subsequent studies on the protection given by eserine and other carbamates against soman poisoning in guinea pigs [3, 12] showed that optimum protection was achieved if the interval between giving the two drugs was about 30–60 min. This is also the time required for carbamates to produce their steady state level of inhibition of blood cholinesterase when injected subcutaneously into guinea pigs [13]. Comparison of Tables 2 and 3 shows

that once this steady state level of inhibition has been reached, the continued presence of free carbamate becomes irrelevant, the degree of protection being slightly greater in fact if the carbamate disappears than if it stays. This clearly shows that the mechanism of the protective action does not depend on competition between the two drugs for the active site of cholinesterase but, as suggested by Berry and Davies [3], on the provision (by carbamylation) of a pool of sequestered inactive cholinesterase which is resistant to attack by the organophosphate, but which furnishes (by decarbamylation) a slow but steady supply of fresh enzyme to the essential cholinergic synapses until such time as all the free organophosphate in the tissues has been disposed of by metabolic detoxification or by excretion. Some further experimental support for this view is provided by the work of Berry *et al.* [14], who showed that a similar protective action against soman poisoning could be achieved by prior administration of an oxime (such as P2S) and an organophosphate which, unlike soman, produced a form of phosphorylated cholinesterase which was readily amenable to reactivation by the oxime.

Another important conclusion from the calculations is that the extent of the protective action of the carbamate is very dependent on the minimum level of cholinesterase consistent with survival. As can be seen from Tables 2 and 3, the minimum value of the "enzyme conservation index" needed to maintain the cholinesterase activity above that produced by one LD_{50} of organophosphate alone decreases by a factor of about 10 for each tenfold decrease in the "minimum essential cholinesterase". Since the effect of concurrent administration of anticholinergic drugs would be to decrease the amount of cholinesterase necessary for survival, one might expect a strong synergism in protective action if atropine were given as well as carbamate. In soman poisoned guinea pigs, eserine alone (0.16 mg kg^{-1}) raised the LD_{50} of soman only 1.5 times, atropine alone (17.4 mg kg^{-1}) raised it 1.8 times, but the two together, at these same doses, increased the LD_{50} of soman 8.2 times [3]. Koster [1] originally proposed using atropine along with eserine to suppress the autonomic effects of the eserine itself, and thus permit larger doses to be given. However, Berry and Davies [3] showed that the atropine–eserine combination was equally effective even if administration of the atropine was deferred until immediately after injection of the soman, and that the optimum dose of eserine was the same in atropinised and non-atropinised animals.

The values used for the coefficient $1 + k_1I/k_2$ were 1.5, 2, 3, 5 and 10, which correspond to steady-state cholinesterase levels produced by the carbamate alone of 67, 50, 33, 20 and 10% of normal respectively. The steady-state level of activity indicating optimum protection by the carbamate against the organophosphate was almost always within this range. The only exception occurred when the two anticholinesterases were given simultaneously (Table 1) and the "minimum essential cholinesterase" was only 0.1% of normal; here, the calculation was extended to include steady-state cholinesterase levels of 5 and 1% of normal. Experimentally the degree of cholinesterase inhibition produced by the optimum dose of eserine is not known with certainty, but both Koster [1] and Berry and Davies [3] suggest that the optimum dose is about the maximum which can be given on its own without producing significant signs of autonomic dysfunction (about one fifth to one tenth of an LD_{50}). In similar studies on the protection of rhesus monkeys and marmosets against soman poisoning, Dirnhuber *et al.* [15] reported that at the maximum sign-free dose of the related carbamate pyridostigmine, the steady state level of blood cholinesterase was reduced to 40–50% of normal. Tables 2 and 3 show that this is about the range of inhibition which the kinetic model predicts would exert a maximum protective action irrespective of the "minimum essential cholinesterase" activity. Dirnhuber *et al.* [15] also showed that almost as much protection could be obtained against soman poisoning by using as little as one quarter of the maximum sign free dose of pyridostigmine, which produced only 30–33% inhibition of blood cholinesterase. Tables 2 and 3 confirm that this low level of cholinesterase inhibition by the carbamate is still sufficient to give substantial protection against organophosphate poisoning.

The results in Tables 2 and 3 can also account for the marked species variability found in the protective action of carbamates. As pointed out earlier, the greater the value of the "enzyme conservation index" (k_2/k_3P_o) for one LD_{50} of any particular organophosphate the greater the number of times the LD_{50} can be raised by carbamate pre-treatment. Thus for any given carbamate–organophosphate combination, the lower the LD_{50} of the organophosphate, the greater the "enzyme conservation index" and the greater the potential protection. This conclusion is also well supported experimentally. Eserine [3] and pyridostigmine [12] gave much more protection against soman poisoning in rabbits and guinea pigs (LD_{50} of soman 20–28 μg kg^{-1}) than in rats and mice (LD_{50} 160 μg kg^{-1}). Even greater protection has been obtained with pyridostigmine against soman poisoning in marmosets and rhesus monkeys (LD_{50} 8–13 μg kg^{-1}) [15].

The kinetic model thus agrees well with the direct experimental evidence on the protective action of carbamates against organophosphorus anticholinesterases. Although actions of the carbamate other than cholinesterase inhibition, as well as differential distribution effects of the two inhibitors at specific cholinergic sites, may well have a bearing on the extent of protection observed, it is unnecessary to invoke such effects as a basic explanation for it. Does it provide any indication as to whether any improvement might be possible? One conceivable way would be to raise the "enzyme conservation index" by using a carbamate which provided a carbamylating moiety with a higher value of k_2. The k_2 value for decarbamylation of cholinesterase inhibited by *N,N*-dimethylcarbamates is slightly greater than that for monomethylcarbamates such as eserine [5], and pyridostigmine (dimethyl-carbamoyloxy-1-methylpyridinium bromide) appeared marginally more effective against soman poisoning than any of the monomethylcarbamates tested [12]. However, the k_2 value for decarbamylation of cholinesterase inhibited by

carbamylcholine is much greater than that for cholinesterase inhibited by derivatives of *N*-methylated carbamic acids, such as eserine or pyridostigmine. Although carbamycholine itself was ineffective against soman poisoning in guinea pigs when given at the maximum sign-free dose (0.025 mg/kg) [3], this compound possesses such strong direct actions on cholinergic receptors that this dose may have caused only marginal inhibition of cholinesterase. A suitable non-alkylated carbamate which inhibited cholinesterase without exerting direct autonomic or neuromuscular effects might prove more successful. However, even a carbamate anticholinesterase possessing ideal kinetic properties would not overcome the weakness that carbamate treatment is only really effective when used prophylactically. Even so, there may be special circumstances where the potential protection against poisoning by a particular organophosphate would be sufficiently worth having to outweigh the risks of prophylactic administration of low doses of a compound as inherently toxic as eserine or one of its close relatives.

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