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ORGANOPHOSPHATE INHIBITORS

THE STEREOSPECIFICITY OF HYDROLYSIS OF METHYL *n*-BUTYL *p*-NITROPHENYL PHOSPHATE BY SERUM PHOSPHOTRIESTERASES (EC 3.1.1.2) AND BY ACETYLCHOLINESTERASES (EC 3.1.1.7)

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Summary

A chiral phosphotriester, methyl *n*-butyl *p*-nitrophenyl phosphate was used to determine the stereospecificity of hydrolysis catalysed by serum phosphotriesterases (aryl-ester hydrolases, EC 3.1.1.2) from horse, ox and rabbit. Each enzyme hydrolysed the (—)-enantiomer more quickly. The same phosphate was used to inhibit acetylcholinesterase (acetylcholine hydrolase, EC 3.1.1.7) from ox, rabbit and electric eel, and in each case, the (+)-enantiomer caused more rapid inhibition. Serum phosphotriesterase did not catalyse the dephosphorylation of dialkylphosphoryl-acetylcholinesterase or dialkylphosphoryl-carboxylesterase. Levels of serum phosphotriesterase in rabbits which received sub-lethal injections of the phosphotriester remained unchanged after one or several injections. In the same rabbits, the levels of blood acetylcholinesterase fell sharply following injections, but normal values were regained in 2—8 days. Serum phosphotriesterases seem incapable either of preventing acute phosphotriester poisoning or of regenerating active enzyme from phosphorylated acetylcholinesterase. However, phosphotriesterases would act in cases of chronic exposure by catalysing the hydrolysis of such organophosphate poisons as remain in the blood.

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Abbreviations: MBNP, methyl *n*-butyl *p*-nitrophenyl phosphate; BNMP, bis(*p*-nitrophenyl) methyl phosphate; sarin, isopropyl methylphosphonofluoridate; tabun, ethyl *N,N*-dimethylphosphoramidocyanidate.

Introduction

Organophosphates cause rapid poisoning by covalently inhibiting synaptic acetylcholinesterase (acetylcholine hydrolase, EC 3.1.1.7) [1], yielding an inactive enzyme derivative in which a particular serine residue is phosphorylated [2]. In this paper, we report on the stereospecificity with which acetylcholinesterase reacts with an optically active phosphotriester, and on the stereospecificity with which serum phosphotriesterase (aryl-ester hydrolase, EC 3.1.1.2, also variously known as A-esterase [3], arylesterase [4], paraoxonase [5], and phosphorylphosphatase [6]) catalyses the hydrolysis of the optically active phosphotriester. These observations lead us to consider whether, in higher animals, phosphotriesterase is capable of protecting the animal from poisoning by organophosphates.

Phosphorylations of acetylcholinesterase by phosphonates which are asymmetric at the phosphorus atom are stereoselective. For example, the (−)-enantiomer of sarin reacts 4200 times faster with ox erythrocyte acetylcholinesterase than does the (+)-enantiomer [7—9]. Conversely, in the inhibition of rat brain acetylcholinesterase by ethyl *S*-2-choroethyl ethylphosphonothiolate, the (+)-enantiomer reacts twice as fast as the (−)-enantiomer [10].

The hydrolysis of asymmetric organophosphates by serum phosphotriesterase can also be stereoselective. For example, sheep serum phosphotriesterase hydrolyses (+)-*S*-*n*-pentyl *p*-nitrophenyl methylphosphonothiolate 16 times faster than the (−)-enantiomer [11]. However, no stereoselectivity was observed in the hydrolysis of tabun by the human enzyme [12], or of sarin by the enzyme from rat serum [13]. Concerning the hydrolysis of asymmetric phosphotriesters, only the hydrolysis of (±)-methyl *n*-butyl *p*-nitrophenyl phosphate ((±)-MBNP) by horse and ox serum has been reported [14]; in this instance the (−)-enantiomer was preferentially hydrolysed.

In principle, serum phosphotriesterase could protect animals against organophosphate poisoning by two mechanisms: (i) the phosphotriesterase could hydrolyse organophosphates in serum so rapidly that they would be unable to inhibit acetylcholinesterase; (ii) the phosphotriesterase could catalyse rapid dephosphorylation of phosphoryl-acetylcholinesterase.

Serum phosphotriesterase would undoubtedly hydrolyse activated organophosphates in serum, and it is present in serum in relatively high concentrations. For example, the phosphotriesterase present in the blood of a single large rabbit would be capable of catalysing the hydrolysis of approx. 100 mg of paraoxon per min under conditions described by Aldridge [3]. Phosphotriesterase levels in the sera of other species are lower but still substantial: horse serum is fairly typical with approx. 5% of the activity of rabbit serum [3]. However, the rate constants for the inhibition of acetylcholinesterase by organophosphates are so high (second-order rate constants of 10^5—10^7 $M^{-1} \cdot min^{-1}$ [15]) that some organophosphates would probably react with acetylcholinesterase at significant rates, even at concentrations much lower than the expected K_m for the inhibitor as a substrate for phosphotriesterase.

The relative stereospecificities of acetylcholinesterase and phosphotriesterase would also be important. Hoskin and Trick [16] have reported that rat serum stereospecifically hydrolyses the (+)-enantiomer of tabun. Racemic tabun is

highly toxic to mice, but after hydrolysis with rat serum the residuum is very much less toxic. Since the toxicity is presumably due to inhibition of the mouse acetylcholinesterase, it is reasonable to conclude that the mouse enzyme is preferentially inhibited by (+)-tabun. Ooms and Boter [11] studied the hydrolysis of several *S*-alkyl *p*-nitrophenyl methylphosphonothiolates by sheep serum phosphotriesterase. In each case, the enantiomer of the phosphonothiolate which was hydrolysed least rapidly was the more powerful inhibitor of ox erythrocyte acetylcholinesterase. If the latter result rather than the former represents the general case (i.e. if the enantiomer which best inhibits acetylcholinesterase is hydrolysed very slowly by phosphotriesterase), protection by phosphotriesterase against such inhibitors could only occur by mechanism (ii) above.

Augustinsson [17,18] noted that "acetylcholinesterase completely or almost completely inhibited by tabun is reactivated by incubation with (phosphoryl-)-phosphatase." After further work, however, Augustinsson and Heimbürger [19] strongly modified this view, reporting that the phosphotriesterase was not capable of reactivating the inactivated acetylcholinesterase, although some reactivation of paraoxon-inhibited cholinesterase by the phosphotriesterase was observed.

In this paper, we have examined the stereoselective phosphorylation of acetylcholinesterase from ox, electric eel and rabbit by MBNP. The stereoselective hydrolysis of this phosphotriester by serum phosphotriesterase from ox, horse, and rabbit is also reported. These results allow the relative stereoselectivities of acetylcholinesterase and phosphotriesterase from the same species (in this case both ox and rabbit) to be compared for the first time. A reexamination of the possibility that serum phosphotriesterase is capable of catalysing dephosphorylation of phosphoryl-acetylcholinesterase led ut to an investigation of the catalysis of reactivation of methyl *n*-butyl phosphoryl-acetylcholinesterase, and also of diethylphosphoryl-carboxylesterase, by ox serum phosphotriesterase.

In addition, we have monitored the levels of plasma phosphotriesterase and blood acetylcholinesterase of rabbits before and after injecting the rabbits on several occasions with phosphotriesters. We wished to see whether the rabbits would become less sensitive to organophosphate poisons after repeated exposures, as gauged by: (1) their behaviour after poisoning; (2) the extent and duration of loss of their blood acetylcholinesterase after poisoning; and (3) the rate of recovery of their acetylcholinesterase.

Materials and Methods

Ox and horse sera were prepared from blood collected from freshly slaughtered animals at local abattoirs. The blood was allowed to stand for several hours at room temperature and serum which separated was centrifuged to remove cell debris. The sera were stored at 4°C and used within a few days, or stored frozen at −20°C and used later. Rabbit serum was prepared likewise from blood taken by venipuncture from the ear veins of adult female New Zealand White rabbits obtained from Tillside, New South Wales, and maintained at about 25°C with ample standard lab chow. Ox erythrocyte acetylcholin-

esterase (Sigma Chemical Co., type I) was dissolved in, and dialysed against 0.1 M sodium phosphate buffer (0.1 M in NaCl, pH 7.61) before use. Electric eel acetylcholinesterase (Sigma, type III) was dissolved in, and dialysed against 0.1 M phosphate buffer, pH 6.95. Ox serum phosphotriesterase as used in experiments measuring the dephosphorylation rates of dialkylphosphoryl-acetylcholinesterase or dialkylphosphoryl-carboxylesterase, was partially purified as follows. To 100 ml of serum was added 1 ml of 1.0 M Tris · HCl buffer (500 mM in $CaCl_2$ and 5 mM in EDTA, pH 8.0), and the resulting solution was subjected to ammonium sulphate fractionation. The 37—70% precipitate was redissolved in the same buffer diluted 100-fold, and rapidly desalted by passing through a Sephadex G-25 column (20 × 9 cm) in diluted buffer at 4°C. The peak protein fractions were combined and chromatographed through a Sephadex G-200 column (30 × 9 cm) in the same buffer. The yellow fractions containing phosphotriesterase were concentrated by ultrafiltration and clarified by centrifugation. The specific activity of the preparation towards 328 μM (±)-MBNP at 25°C in 0.1 M Tris · HCl buffer, pH 8.0, was 29 (nkat/l)/$A_{280\ nm}$. Chicken liver carboxylesterase, purified in this laboratory [20], was a gift from Dr. P.A. Inkerman. The specific activity of this enzyme was 1.05 (mkat/l)/$A_{280\ nm}$ towards *p*-nitrophenyl acetate at pH 7.61, 25°C. Racemic and (+)-MBNP, and bis(*p*-nitrophenyl) methyl phosphate (BNMP) were prepared as described previously [14]. BNMP was recrystallised from chloroform/carbon tetrachloride; m.p. 143°C, ref. 21: 142—143°C. Paraoxon, obtained from Albright and Wilson, was purified by passing a chloroform solution through a column of neutral alumina, activity grade I (Woelm). After removal of chloroform a stock solution (10.2 mM) in acetonitrile was prepared. The material was 99.1% pure by release of *p*-nitrophenol upon alkaline hydrolysis. Phenyl acetate, purified by molecular distillation, was a gift from Dr. J.K. Stoops; *p*-nitrophenyl acetate (Aldrich Chemical Co.) was recrystallised from chloroform/hexane. Acetylcholine perchlorate, a useful derivative of acetylcholine because it is not significantly hygroscopic [22], was prepared from acetylcholine chloride and recrystallised twice from ethanol; m.p. 115—116°C, ref. 22: 116—117°C. Sephadex G-25 and Dextran Blue were obtained from Pharmacia.

Acetonitrile was Spectro grade from Eastman Kodak. Buffers were prepared using analytical grade reagents. pH measurements were made on a Radiometer pH meter 4c at 25°C, standardised according to Bates [23], and are accurate to ±0.01 pH unit.

All spectrophotometric measurements were performed on a Cary 14 recording spectrophotometer equipped with 0—0.1 absorbance slide wires, and a cell compartment maintained at 25.0 ± 0.1°C. The activity of chicken liver carboxylesterase was measured by adding an aliquot (10—50 μl) of enzyme to a cell containing 3.0 ml of 0.05 M Tris · HCl buffer (5 mM in EDTA, pH 7.61), and then adding 50 μl of *p*-nitrophenyl acetate solution (65.63 mM in acetonitrile) and observing the increase in absorbance at 400 nm. The same assay was used for measuring the activity of electric eel acetylcholinesterase in the experiment in which the enzyme was inhibited with (+)-MBNP and its rate of reactivation in the presence or absence of ox phosphotriesterase was observed.

The stereoselectivity of horse and rabbit serum phosphotriesterase was observed by adding 0.2 ml of serum to 2.55 ml of 0.115 M Tris · HCl buffer

(0.15 M in NaCl and $CaCl_2$, 25 μM in EDTA, pH 8.1), and then adding 0.25 ml of an acetonitrile solution of (±)-MBNP (final concentration 245.9 μM) or (+)-MBNP (final concentration 121.7 μM) and observing the change in absorbance at 400 nm. The stereoselectivity of ox serum phosphotriesterase was calculated from data reported previously [14].

Phosphorylated enzymes for the reactivation experiment were prepared as follows. Electric eel acetylcholinesterase in 0.1 M phosphate buffer (0.1 M in NaCl, pH 6.95; 0.3 ml; $A_{280\ nm} = 10.57$) was inhibited with (+)-MBNP (25 μl of a 9.63 mM solution in acetonitrile) for 18.5 min at 25°C, after which the residual activity was $\leqslant$0.1%. The mixture was passed through a Sephadex G-25 column (7 × 0.5 cm) in the same buffer, and the inhibited enzyme ($A_{280\ nm} = 0.426$) collected free of MBNP. Chicken liver carboxylesterase in 50 mM Tris · HCl buffer (5 mM in EDTA, pH 7.61; 250 μl; 213 μM) was inhibited with paraoxon (25 μl of a 10.21 mM solution in acetonitrile) for 20 min at 25°C, after which the residual activity was $\leqslant$0.15%. The inhibited carboxylesterase was freed of paraoxon by passage at 25°C through the same Sephadex G-25 column in 0.1 M Tris · HCl buffer (5 mM in $CaCl_2$, 50 μM in EDTA, pH 8.0).

Rabbit ear veins were used for removal of blood by venipuncture, and for injection of organophosphate solutions or solvents. Blood was collected in syringes containing heparin (Boots Co., Nottingham, U.K.; approx. 10 μl of heparin solution containing 50 units of heparin per ml of blood), immediately cooled to 4°C, and stored at that temperature. Just before samples were taken for acetylcholinesterase assays, the blood was thoroughly mixed by gentle inversion (12 times). After these assays, the blood was centrifuged to separate the plasma to be used for assays of phosphotriesterase activity and protein concentration.

Rabbit blood acetylcholinesterase was assayed as follows. Rabbit blood (200 μl) was added to 10 ml of a solution of acetylcholine perchlorate (4.85 mM, 100 mM in NaCl and 40 mM in $MgCl_2$) in a mechanically stirred tube at 38°C. Hydrolysis of acetylcholine was followed by titrating the acetic acid released with 10 mM NaOH from an Agla microsyringe at pH 7.34, using a recording pH stat (Radiometer TTTlc, and SBR2c).

Rabbit plasma phosphotriesterase was assayed as follows. Rabbit plasma (200 μl) was equilibrated at 25°C with 2.60 ml of 0.115 M Tris · HCl buffer (0.15 M in NaCl and in $CaCl_2$, 25 μM in EDTA, pH 8.1) in a spectrophotometer cell for 15 min, and the reaction was started by the addition of 200 μl of a (±)-MBNP solution (3.92 mM in 32% (v/v) acetonitrile). The Cary recorder was started 15 s after addition of MBNP, the hydrolysis of which was followed by measuring the release of *p*-nitrophenolate ion at 400 nm.

Rabbit plasma protein concentration was measured by diluting plasma with 0.125 M NaCl and measuring the absorbance at 280 nm ($A_{280\ nm}$). A protein concentration of 1 mg/ml was taken to have an absorbance of 1. Rabbit blood acetylcholinesterase and plasma phosphotriesterase activities have been divided by the plasma $A_{280\ nm}$ and expressed as specific activities [(μkat/l)/$A_{280\ nm}$]. Initially, all assays of these two enzyme activities were done in duplicate for each sample of blood. However, as the average error for 30 pairs of duplicate assays of acetylcholinesterase was ±1.26% and of phosphotriesterase was ±1.29%, assays after day 115 were done singly.

Results

The hydrolysis of (±)-MBNP catalysed by ox, rabbit and horse serum phosphotriesterases

Reaction of ox serum with (±)-MBNP resulted in an initial fast release of one half of the total *p*-nitrophenol, followed by a much slower, apparently zero-order increase in absorbance. After the zero-order part of the reaction had been reached, residual unhydrolysed MBNP was extracted from the reaction mixture with cyclohexane, and proved to be virtually pure (+)-enantiomer [14]. Thus the initial fast reaction was hydrolysis of the (—)-enantiomer. That the rate of hydrolysis of (—)-MBNP was at least 50.4-fold greater than that of (+)-MBNP under the conditions used was established by a comparison of the rates of the initial fast hydrolysis of (±)-MBNP (373 nM) and the subsequent slower hydrolysis of (+)-MBNP. The horse serum phosphotriesterase-catalysed hydrolysis of (±)-MBNP (245.9 μM) and of (+)-MBNP (121.6 μM) in separate experiments showed that this enzyme also favoured the (—)-MBNP by a factor of 88.3. Rabbit serum phosphotriesterase likewise favoured the (—)-enantiomer by a factor of 76.3.

Stereospecific inhibition of acetylcholinesterase from ox erythrocytes and from electric eel by MBNP

A preliminary study showed that acetylcholinesterase from electric eel had no detectable hydrolytic activity towards (±)-MBNP at pH 7.6. In a second experiment, 0.5 mg of ox erythrocyte acetylcholinesterase in 2.3 ml of 0.1 M sodium phosphate buffer (0.1 M in NaCl, pH 7.61) was equilibrated at 25 ±

TABLE I

INHIBITION OF OX ERYTHROCYTE ACETYLCHOLINESTERASE BY (±)-MBNP AND BY (+)-MBNP at 25°C

[MBNP] at which enzyme incubated (nM)	Enzyme activity after inhibition for 350 s * (μM/min)	Predicted enzyme activity after inhibition for 350 s ** (μM/min)
(±)-MBNP		
451	15.3	(15.3)
45.1	89.6	90.3
4.51	112	110
(+)-MBNP		
458	2.24	1.99
45.8	74.7	73.6
4.58	105	105
Control (enzyme not reacted with MBNP)	110	

* Initial rate of hydrolysis of phenyl acetate, observed after adding phenyl acetate to the solution of acetylcholinesterase and MBNP at 350 s and starting the recorder at 375 s.

** These activities were calculated using $k'_1 = 0.33_9\ min^{-1}$ for the inactivation of acetylcholinesterase by 451 nM (±)-MBNP, assuming that only (+)-MBNP inhibits (see text).

1°C with 0.1 ml of an acetonitrile solution of (±)-MBNP at the concentrations shown in Table I. After 350 s, phenyl acetate solution (1 ml) was added to the enzyme and MBNP reaction mixture, bringing the phenyl acetate concentration to 6.43 mM; at 375 s, the rate of release of phenol was observed. Similarly the enzyme was equilibrated with (+)-MBNP at the concentrations noted in Table I, and then assayed for residual activity using phenyl acetate as before.

The covalent inhibition of acetylcholinesterase by phosphorylating agents is described as a two-step process consisting of the formation of an enzyme-inhibitor complex, followed by phosphorylation [24]. At concentrations of inhibitor well below the apparent K_m of the inhibitor, the reaction follows second-order kinetics:

$$\frac{-d[\text{enzyme}]}{dt} = k_2'[\text{enzyme}][\text{organophosphate}] \quad (1)$$

and when [organophosphate] $\gg$ [enzyme], Eq. 1 may be written as

$$\frac{-d[\text{enzyme}]}{dt} = k_1'[\text{enzyme}].$$

An approximate value of the apparent first-order rate constant k_i' may be calculated, knowing the activity of acetylcholinesterase before inhibition and the activity after inhibition for a specified time (say 350 s). For example, when [(±)-MBNP] = $4.51 \cdot 10^{-7}$ M, $k_i = 0.33_9$ min^{-1}, and this value, together with the known concentrations of MBNP to which the enzyme was exposed, may be used to calculate the expected residual enzymatic activity after 350 s in contact with various concentrations of MBNP. The predicted residual rates in Table I were obtained in this way, but k_i for the reaction of (±)-MBNP with acetylcholinesterase was taken to be one half of the value of k_i for the reaction of the enzyme with (+)-MBNP, at a given inhibitor concentration. The value of k_2' for the inhibition of ox erythrocyte acetylcholinesterase, averaged from four measurements taken at higher concentrations of (+)-MBNP, was $1.50 \pm 0.05 \cdot 10^6$ M$^{-1} \cdot$ min^{-1}, and for (±)-MBNP, $7.99 \pm 0.05 \cdot 10^5$ M$^{-1} \cdot$ min^{-1}.

The assumption that inhibition follows first-order kinetics with respect to enzyme was tested separately by following the course of inactivation of the enzyme in 45.8 nM (+)-MBNP. Several spectrophotometer cells containing the enzyme in 2.3 ml buffer were equilibrated at 25.0°C. Triester solution in acetonitrile (0.1 ml) was added to a cell, and after a suitable time phenyl acetate (1.0 ml, 21.3 mM) was added, and the rate of release of phenol was measured, starting 40 s after addition of phenyl acetate. A plot of enzymatic activity against time, to 1000 s, showed that the inactivation did indeed follow first-order kinetics, with $k_1' = 7.02 \cdot 10^{-2}$ min^{-1}, corresponding to $k_2' = 1.53 \cdot 10^6$ M$^{-1} \cdot$ min^{-1}, in agreement with the value already observed.

The inhibition of electric eel acetylcholinesterase by both (±)- and (+)-MBNP was investigated in the same way as the inhibition of ox erythrocyte enzyme reported in Table I. For the inhibition of the eel enzyme by (+)-MBNP, $k_2' = 1.50 \pm 0.07 \cdot 10^6$ M$^{-1} \cdot$ min^{-1}, and for (±)-MBNP, $k_2' = 7.9 \pm 1.2 \cdot 10^5$ M$^{-1} \cdot$ min^{-1}.

Effect of ox serum phosphotriesterase on the rate of reactivation of phosphorylated enzymes

Equal volumes of methyl *n*-butyl phosphoryl-acetylcholinesterase (eel) and either 10 mM Tris · HCl buffer (0.5 mM in $CaCl_2$, 5 μM in EDTA, pH 8.0) or ox serum phosphotriesterase (802 nkat/l) in the same buffer were kept at 25°C, and assayed periodically for activity. Activity in each sample increased progressively, with no significant difference in the rate of reactivation occurring between the two samples. The recovery of activity in the samples was 21.6 ± 0.4% of the expected specific activity after 8 h and 27.4 ± 0.5% after 83 h. Similarly, aliquots (200 μl) of diethylphosphoryl-carboxylesterase (50.5 μM) were treated with equal aliquots of phosphotriesterase solution or buffer at 25°C and the return of activity monitored. The contribution of phosphotriesterase to the measured rate of hydrolysis of *p*-nitrophenyl acetate was determined in a control experiment. Again there was no significant difference between the rates of reactivation in the presence and absence of phosphotriesterase. The carboxylesterase samples had regained 23.5 ± 1.4% of the expected specific activity after 2 h, 64.1 ± 3.2% after 11.5 h, and 100% after 83.5 h.

Injection of rabbits with MBNP or with BNMP: levels of acetylcholinesterase and phosphotriesterase

The levels of blood acetylcholinesterase, plasma phosphotriesterase, and plasma $A_{280\ nm}$ of five rabbits were monitored over a period of 41 weeks. In five experiments during this time, the rabbits were injected with MBNP or BNMP or solvent, as detailed in Table II. After being injected, each rabbit was observed for signs of organophosphate poisoning, such as muscular weakness, fasciculation, difficulty in breathing, and salivation for up to 90 min, by which time any signs had invariably become negligible. These observations

TABLE II

INJECTION OF RABBITS WITH ORGANOPHOSPHATES

Rabbits were intravenously injected with organophosphate solutions (2.1—4.9 mg/ml) at a dose of 50 μg/kg body weight, except that a dose of 150 μg/kg was received by rabbits A and C, injection 5.

Rabbit	Injection				
	1*	2**	3*	4***	5***
	Days after start of enzyme assays				
	81	121	145	221	253
A	(+)-MBNP	(+)-MBNP	(+)-MBNP	BNMP	BNMP
B	(+)-MBNP	(+)-MBNP	(+)-MBNP	BNMP	
C	(±)-NMBP	(±)—MBNP	(±)-MBNP	(±)-MBNP	BNMP
D	control	control	control	control	control
E	control	control	control	control	control

* Injections dissolved in 50% (v/v) ethanol/water. Controls were injected with this solvent.

** Injections were as in the above footnote, except that (±)-MBNP for rabbit C was dissolved in 50% (v/v) acetonitrile/water.

*** All injections were dissolved in, and controls injected with, neat ethanol.

TABLE III

RABBITS INJECTED WITH ORGANOPHOSPHATES: INTENSITY AND DURATION OF PHYSICAL SIGNS OF POISONING

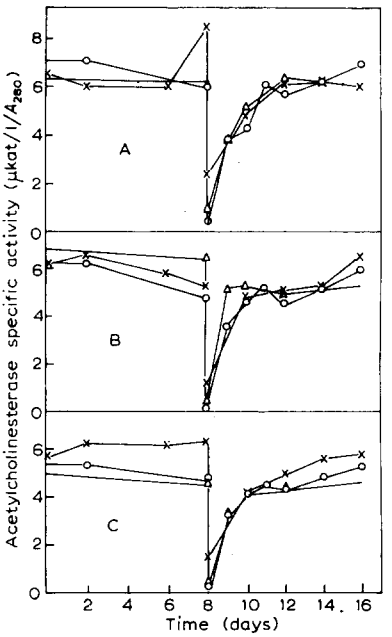
See Table II for the injection protocol. Intensity of poisoning: ++++, very intense, with much shaking, muscular weakness and difficulty in standing, convulsive movements of abdomen and difficulty in breathing; +++, intense, with shaking and muscular weakness, and difficulty in standing; +, mild, with some shaking and slight weakness; —, no signs.

Rabbit	Injection number				
	1	2	3	4	5
A	+++ (30 min)	+++ (30 min)	+++ (30 min)	—	—
B	++++ (30 min)	+++ (30 min)	++++ (>60 min)	—	
C	+	—	—	+	—
D	—	—	—	—	—
E	—	—	—	—	—

are summarised semi-quantitatively in Table III. They show that at the levels administered, (+)-MBNP is qualitatively a much more effective poison than (±)-MBNP; BNMP produced no immediate signs of poisoning at all.

The assays of $A_{280\ nm}$ and of the blood acetylcholinesterase and phosphotriesterase levels before injection 1 were carried out weekly and then at intervals of 2—4 days for a total of 12 weeks, in order to establish "normal" values. After injections 1 and 2, assays were performed generally at 1- or 2-day intervals for 2 weeks, and at longer intervals thereafter. These assays showed: (i) Specific activity levels of plasma phosphotriesterase were not at all affected by injection of the rabbits with either (+)-MBNP or (±)-MBNP, up to 41 days after injection. (ii) Specific activity levels of blood acetylcholinesterase fell immediately after each injection of MBNP, then quickly recovered about half of the lost activity in 1 or 2 days, and the remainder of the activity more slowly in up to 7 days (Fig. 1). As detailed in Table IV, rabbits injected with (+)-MBNP showed greater acetylcholinesterase inhibition than the rabbits injected with (±)-MBNP, in agreement with the observation that (+)-MBNP produced much more severe symptoms of poisoning than the same dose of (±)-MBNP.

The recovery of acetylcholinesterase activity could be due to dephosphorylation of inhibited acetylcholinesterase or to synthesis of new enzyme. In an attempt to distinguish between these two possibilities, some rabbits were injected with bis(*p*-nitrophenyl) methyl phosphate, a reagent which covalently inhibits serine hydrolases, releasing at least 2 mol of *p*-nitrophenol per enzyme active site, and producing a phosphorylated enzyme very stable to dephosphorylation [25]. We considered that recovery of acetylcholinesterase activity in rabbits injected with BNMP would be caused only by synthesis of new enzyme. Rabbits injected with BNMP at 50 μg/kg showed a slight loss of acetylcholinesterase activity (rabbit A, 15.9%; rabbit B, 1.8%) and a greater loss after a dose of 150 μg/kg (rabbit A, 24.3%; rabbit B, 23.4%). In contrast to acetylcholinesterase levels in rabbits injected with MBNP, the levels in rabbits injected with BNMP showed no rapid increase in the first 2 days (see Fig. 2) but returned to normal levels in 3 or 4 days. This experiment was complicated by the low solubility of BNMP in the less toxic polar solvents which we tried. Thus rabbits



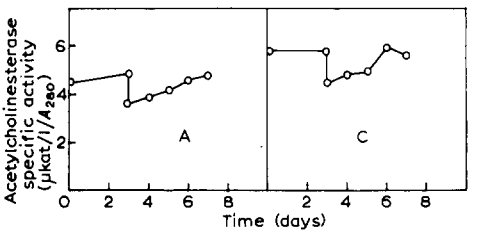


Fig. 1. Blood acetylcholinesterase levels of rabbits A, B, and C, before and after injection, respectively, with (+)-MBNP, (+)-MBNP, and (±)-MBNP. Injection 1, X———X; injection 2, ○———○; injection 3, △———△. Day 8 represents the time of injection.

Fig. 2. Blood acetylcholinesterase levels of rabbits A and C before and after injection 5, of BNMP. Day 3 represents the time of injection.

were injected with an ethanolic solution of BNMP almost saturated at 38°C. Injection of the rabbits with the larger volumes of BNMP solution led to some thrombosis in the ear veins, due to administration of relatively large volumes of anhydrous alcohol. Hence injection of the animals with sufficient ethanolic BNMP to inhibit the acetylcholinesterase to, say, less than 25% of its original level would have been impracticable.

Injection of control rabbits with solvent (50% or neat ethanol) produced no

TABLE IV

EXTENT OF ACETYLCHOLINESTERASE INHIBITION IN RABBITS INJECTED WITH (+)- OR (±)-MBNP

See Table II for the injection protocol.

Rabbit	Treatment	Residual acetylcholinesterase activity (%) after injection			
		1	2	3	4
A	(+)-MBNP	25.3	4.02	0	
B	(+)-MBNP	22.6	2.33	6.29	
C	(±)-MBNP	28.2	7.83	14.4	25.0
D	solvent *	—	99.4	96.8	—
E	solvent	99.8	107	94.8	94.3

* Clotting of blood from rabbit D precluded activity measurements after injections 1 and 4.

marked change in specific activities of phosphotriesterase, or of acetylcholinesterase where levels after injection were 98.6 ± 3.9% of pre-injection values.

Discussion

Measurement of the stereoselectivity of the serum phosphotriesterases of horse, ox and rabbit in hydrolysing MBNP showed that in each case the (−)-enantiomer was hydrolysed more rapidly than the (±)-MBNP by a factor of at least 50. The magnitude of this stereoselectivity, which varies from species to species, is not yet known accurately because the values reported here were obtained without using either pure phosphotriesterase or pure (−)-MBNP. As the magnitude will vary somewhat depending also on the K_m and K_i of each enantiomer of MBNP, and on the concentration of each used, the values should only be taken as showing that ox, horse and rabbit phosphotriesterase are all strongly stereoselective in favour of (−)-MBNP.

Strong stereoselectivity is also shown by acetylcholinesterase in its inhibition by MBNP. Thus (+)-MBNP is the more rapid inhibitor of the enzyme from electric eel, and from ox erythrocytes. The observations that rabbit blood acetylcholinesterase is inhibited more completely by (+)-MBNP than by (±)-MBNP (Table IV) and that rabbits are more effectively poisoned by (+)-MBNP (Table III) show that rabbit acetylcholinesterase is stereoselective in its inhibition by MBNP. As in ox, (+)-MBNP is the more effective inhibitor of rabbit acetylcholinesterase and the poorer substrate for serum phosphotriesterase. The possibility that acetylcholinesterases of higher animals are generally inhibited by the same enantiomer of a particular chiral organophosphate is raised by the parallel behaviour of the enzymes from such disparate sources as ox, rabbit, and electric eel towards MBNP.

Further, the k_2' values for inhibition of ox and electric eel acetylcholinesterase by either (+)-MBNP or (±)-MBNP are remarkably similar. Because of the strong stereoselectivity shown by both ox and eel enzymes, the magnitude of k_2' for (−)-MBNP cannot be estimated from the data obtained with (+)-MBNP and (±)-MBNP; hence, the stereoselectivity cannot be quantified. Our results demonstrating that the serum phosphotriesterase and acetylcholinesterase of particular species show opposite stereoselectivity towards enantiomers of MBNP are therefore consistent with and amplify the results of Ooms and Boter [11].

In a recent review of enzyme stereospecificity, Hanson and Rose [26] state that non-stereospecificity in the formation of products can be taken as evidence for a non-enzyme-directed step. This statement has some validity when the enzyme being considered has a narrow substrate specificity. However, it is meaningless when considering enzymes with a broad substrate specificity. In such enzymes, e.g. α-chymotrypsin, phosphotriesterase, carboxylesterases, the stereospecificity varies greatly with the substrate. Further, as discussed in this paper and elsewhere, synthetic substrates and inhibitors can be prepared which show the whole range of stereospecificity, including inversion of the normal specificity [27].

In the Introduction, it was suggested that phosphotriesterase could protect animals from organophosphates by catalysing the dephosphorylation of dialkylphosphoryl-acetylcholinesterase. The results reported here show clearly that ox

serum phosphotriesterase is incapable of catalysing the dephosphorylation of either diethylphosphoryl-carboxylesterase from chicken, or methyl *n*-butyl phosphoryl-acetylcholinesterase formed by the reaction of the eel enzyme with (+)-MBNP. It should be noted that reaction of enzyme with (+)-MBNP would be expected to result in inversion at the asymmetric phosphorus atom. Hence, the methyl *n*-butyl phosphoryl-acetylcholinesterase formed should have the configuration most susceptible to hydrolysis by phosphotriesterase.

Our results strongly indicate that serum phosphotriesterase will neither protect acetylcholinesterase against immediate inhibition by high doses of some organophosphate poisons, nor regenerate the resulting phosphorylated acetylcholinesterase. This conclusion is supported by the rabbit experiments, in which the level of plasma phosphotriesterase did not change significantly after one or several injections with MBNP or BNMP. However, the high levels of phosphotriesterase present in sera of many animals [3] presumably act in cases of chronic exposure by hydrolysing any organophosphates remaining in the blood.

The question of whether the rabbits were less (or more) sensitive towards organophosphate after their second, third, or fourth injection than after their first is not unequivocally answered by the work reported here. Subjectively, the same doses of (+)-MBNP seemed to affect a particular rabbit to different extents on different occasions (Table III). The data in Table IV, with the exception of rabbit A, injection 3, suggest that after injection 2, subsequent injections inhibit blood acetylcholinesterase to a decreasing extent. Any such trend might be caused by the increased level of one of the enzymes, other than plasma phosphotriesterase, which metabolise organophosphates. Other enzymes which could be involved are: a transferase which transfers specifically a methyl group from organophosphates containing a methyl ester group to the sulphydryl group of glutathione [28]; an hydroxylase [29] which results in dealkylation of phosphotriesters containing methyl or ethyl groups; and a reductase [30] which catalyses reduction of the *p*-nitro group of paraoxon to the *p*-amino group (diethyl *p*-aminophenyl phosphate is not strongly inhibitory [31]).

Measurement of the rate of dephosphorylation in vitro of methyl *n*-butyl phosphoryl-acetylcholinesterase, formed by the reaction of the electric eel enzyme with (+)-MBNP, gave a first-order rate constant of approx. $5 \cdot 10^{-6}$ s^{-1} ($t_{1/2} \approx 40$ h) at 25°C, pH 7.53. The time scale for the recovery of acetylcholinesterase in rabbit blood after inhibition with (+)-MBNP or (±)-MBNP (Fig. 1) is therefore consistent with either dephosphorylation or synthesis of new enzyme. The recovery of acetylcholinesterase activity after BMNP inhibition (Fig. 2) occurs on a similar time scale. However, the BNMP results should be interpreted cautiously in view of the low solubility of the material and the effects of injecting relatively large volumes of ethanol into rabbits.

The idea that rabbit acetylcholinesterase can be rapidly dephosphorylated in vivo was first proposed many years ago by Barnes [32]. His data indicated that sufficient of the diethylphosphoryl-acetylcholinesterase which formed when rabbits were injected with paraoxon could be dephosphorylated in a few minutes, so that a rabbit could survive a large dose of paraoxon if sustained through respiratory failure for about 5 min. Fairly complete reactivation of

respiratory and leg muscle enzyme seemed to have occurred by 100 min after injection.

Considering the half-life for the uncatalysed dephosphorylation of MBNP-inhibited acetylcholinesterase, these rates of recovery indicate that dephosphorylation in rabbits may be a catalysed process. The data of Fig. 1 are consistent with a rapid reactivation of part of the phosphorylated acetylcholinesterase in rabbits dosed with MBNP. Our experiments appear to rule out catalysis by serum phosphotriesterase, and suggest that the slow but significant reactivation of diethylphosphoryl-cholinesterase reported by Augustinsson and Heimbürger [19] and attributed to phosphotriesterase activity may in fact have been caused by some other agent.

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