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Inhibition of Brain Carboxylesterases by Neurotoxic and Non-Neurotoxic Organophosphorus Compounds

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SUMMARY

Carboxylesterases (EC 3.1.1.1) of chicken brain were investigated by applying kinetic analysis of organophosphorus inhibition. By iterative elimination of exponential inhibition curves and by sequential inhibition experiments using a combination of two organophosphorus inhibitors, 11 different carboxylesterases of chicken brain were characterized with respect to their phenyl valerate-hydrolyzing activity (milliunits per gram of brain) and their inhibition by *O,O*-diethyl *O*-4-nitrophenyl phosphate (Paraoxon), *O,O*-diisopropylphosphorofluoridate, and *N,N'*-diisopropylphosphorodiamidic fluoride (Mipafox). The bimolecular inhibition rate constants (liters · mole^{-1} · min^{-1}) were calculated for the 11 enzymes and 3 organophosphorus compounds. The corresponding data for acetylcholinesterase (EC 3.1.1.7) in chicken brain were determined. The importance of inhibition rate constants for the development of acute cholinergic symptoms, delayed neurotoxicity, and atypical organophosphate effects is shown.

INTRODUCTION

A group of esterases hydrolyzing carboxylesters of both aliphatic and aromatic alcohols is classified as carboxylesterases (EC 3.1.1.1). Although the physiological substrates and the functional significance of these enzymes are unknown (1), a distinct isoenzyme of carboxylesterases occurring in the vertebrate central nervous system is of special interest, since its inhibition by certain organophosphorus compounds has been shown to be one of the events in the development of irreversible nerve damage known as delayed neurotoxicity (2, 3).

The toxic effects of organophosphorus compounds can be classified into acute cholinergic and delayed neurotoxic signs (4). While acute cholinergic symptoms do occur immediately after ingestion of the toxic compound if neural acetylcholinesterase (EC 3.1.1.7) is inhibited to about 10–15% of normal (5), delayed neurotoxic signs such as ataxia, weakness of the limbs, and flaccid paralysis are to be expected after a characteristic 8- to 14-day delay period (6) if the carboxylesterase target is inhibited to about 15–30% of normal combined with a dealkylation ("aging") of the alkyl-phosphorylated enzyme (3, 7, 8). Until now no direct specific method has been available to determine the activity of the target enzyme of chronic neurotoxic organophosphorus compounds. Johnson (9) described a differential assay combining carboxylesterase inhibition by a non-neurotoxic and a neurotoxic organophosphate to test the target enzyme of neurotoxic organophosphorus substances in chicken brain. This enzyme is defined as Paraoxon[1]-insensitive and Mipafox-sensitive and is referred to as NTE (10). However, purification and enzymological characterization of NTE has not yet been achieved.

By applying the concept of Poulsen and Aldridge (11) of structural analogy between inhibitors and substrates of NTE, Johnson improved the differential test for NTE activity by the use of PV as substrate. Figure 1 shows the structural similarity between PV and CBDP, the active metabolite of the classic neurotoxic organophosphorus substance triorthocresyl phosphate (12). Using the differential assay, only about 10% of total carboxylesterase activity in chicken brain tested with PV can be shown to be NTE activity (13). Thus far little is known about the remaining 90% of PV-hydrolyzing activity with respect to heterogeneity, substrates, and reactivity toward neurotoxic and non-neurotoxic organophosphorus inhibitors.

In recent years many atypical effects of organophosphorus compounds [e.g., on central nervous system (14) and electroencephalograms (15), myocardium (16), muscle spindle function (17), and embryonic development (18)] have been described which cannot be associated with inhibition of either acetylcholinesterase or NTE. Therefore it is of importance to investigate the organo-

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[1] The abbreviations and trade names used are: Paraoxon, *O,O*-diethyl *O*-4-nitrophenyl phosphate; Mipafox, *N,N'*-diisopropylphosphorodiamidic fluoride; NTE, neurotoxic esterase; PV, phenyl valerate; CBDP, cresyl benzodioxophosphorin oxide; DFP, *O,O*-diisopropylphosphofluoridate; PIB, phosphate incubation buffer; TIB, Tris incubation buffer; PVase, PV-hydrolyzing carboxylesterases.

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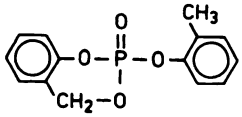
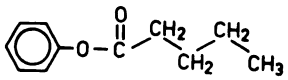


PHENYLSALIGENINPHOSPHATE (CBDP)



PHENYLVALERATE (PV)

FIG. 1. *Structure of the neurotoxic carboxylesterase inhibitor CBDP (metabolite of triorthocresyl phosphate) as a model for synthetic substrates of NTE [modified from Poulsen and Aldridge (11)]*

phosphate sensitivity of PV-hydrolyzing non-NTE carboxylesterases in the central nervous system.

Domestic fowl is the best-suited species for the experimental study of organophosphorus ester-induced delayed neuropathy (19). In the present study the detection and characterization of different PV-hydrolyzing carboxylesterases were carried out by applying iterative elimination of exponential functions on organophosphorus inhibition curves (20). PV-hydrolyzing isoenzymes were characterized with respect to their reactivity toward the organophosphorus inhibitors Paraoxon, DFP, and Mipafox. Paraoxon is a potent anticholinesterase agent, but it fails to produce delayed neurotoxic effects *in vivo*. DFP, an example of so-called nerve gases, is known to cause both acute cholinergic and delayed neurotoxic symptoms. Mipafox predominantly causes neurotoxic effects.

MATERIALS AND METHODS

Preparation of tissue homogenates. One-year-old female Warrensex hens were killed by carotid dissection. Brains were freed from connective tissue and immediately chilled to 0°. A brain mash was prepared, shock-frozen in single portions of 200 mg, and stored at −40°. A portion of the brain mash (200 mg) was homogenized with a Potter-Elvejhem homogenizer in 0.6 ml of ice-cold PIB consisting of 0.2 M NaH_2PO_4/Na_2HPO_4 (pH 7), 50 mM NaCl, and 1 mM Na-EDTA. Homogenates were diluted with PIB to suspensions containing 10 mg of brain per milliliter (acetylcholinesterase determination). For the determination of carboxylesterase activity, brain mash was homogenized in TIB consisting of 50 mM Tris-HCl (pH 8) and 0.2 mM Na-EDTA. Homogenates were diluted with the same buffer to a suspension of 8.3 mg of brain per milliliter.

Although there was very little interindividual variation of total carboxylesterase activity (mean, 7474 mU · g^{-1}; SD, 231 mU · g^{-1}) and total cholinesterase activity (mean, 17.4 units · g^{-1}; SD, 1.0 unit · g^{-1}) in hen brain (n = 6), all experiments were performed using individual hen brains and no pooled material was used.

Cholinesterase assay. Cholinesterase activity was determined by a modification of the method of Ellman *et al.* (21) as described previously (20).

Carboxylesterase assay. Phenol, liberated by enzymatic hydrolysis of PV, was determined according to the method of Gottlieb and Marsh (22). The enzyme assay was carried out using a modification of the method of Johnson (9). A portion (0.1 ml) of the diluted hen brain suspension, containing 0.83 mg of brain tissue, was preincubated with 0.1 ml of water for 5 min at 25°. The enzymatic reaction was started by adding 0.2 ml of substrate solution [4 mM PV and Triton X-100 (0.3 g/liter) in TIB]. The incubation time was 15 min at 25°. The enzyme reaction was stopped by adding 0.2 ml of ice-cold perchloric acid (0.33 M). After a 4-min centrifugation at 600 × g the test tubes were stored in ice until the final color reaction was started. A 0.4-ml sample of the clear supernatant was transferred to a second test tube containing 0.2 ml of 4-aminoantipyrine solution [1.2 mM in 0.5 M Tris-HCl buffer (pH 9)]. An aqueous solution (0.1 ml) of $K_3Fe(CN)_6$ was added. After a 5-min incubation at room temperature the absorbance at 510 nm was determined in a Zeiss PM 4 photometer. A blank was run under identical conditions receiving the brain suspension (in TIB) after the addition of perchloric acid. The molar extinction coefficient of the phenol-color complex was 13,900 (liters · $mole^{-1}$ · cm^{-1}). One unit of carboxylesterase activity was defined as that amount of enzyme hydrolyzing 1 μmole of PV per minute. The variation coefficient (percentage standard deviation) for the measurement of carboxylesterase activity was 1.8%.

Inhibition experiments. Inhibition of acetylcholinesterase was performed by incubating 0.05 ml of brain suspension in PIB (equivalent to 0.5 mg of brain tissue) with 0.05 ml of aqueous organophosphate solution for 60 min at 25°. Inhibition was stopped by adding 0.5 ml of substrate solution in PIB, and residual activity was measured using the cholinesterase assay. Organophosphorus inhibition kinetics of carboxylesterase activity were measured by incubating 0.1 ml of brain suspension in TIB (0.83 mg of brain tissue) with 0.1 ml of aqueous inhibitor solution for 40 min at 25°. Inhibition was stopped by adding 0.2 ml of substrate solution in TIB, and residual activity was determined. Inhibitor concentrations varied from 10 pM to 1 mM (inhibition of cholinesterases) and from 1 nM to 1 mM (carboxylesterase inhibition), respectively. Stock solutions (0.1 M in absolute ethanol) of inhibitors Paraoxon, DFP, and Mipafox were stored at 4°. The inhibitor concentration of the stock solutions was controlled daily by a photometric Paraoxon assay and fluorimetric DFP and Mipafox assays. Different dilutions of organophosphorus inhibitors were prepared immediately before the start of inhibition experiments.

Sequential inhibition experiments. Sequential inhibition experiments using two different organophosphorus compounds were performed in two steps. Partial inhibition with the first organophosphate was stopped by adding a 10-fold volume which contained the second inhibitor in various concentrations. After a fixed inhibition time, enzyme activity was determined as described above and inhibition kinetics with the second inhibitor were determined.

Analysis of inhibition kinetics. Organophosphorus inhibition kinetics of carboxylesterases and cholinesterases were analyzed using a Sharp computer PC 1211. Second-order inhibition rate constants k_2 (liters · $mole^{-1}$ · min^{-1}) were determined by plotting the logarithm of residual activity (ln A) versus the inhibitor concentration (c). From the slope

$$m = \frac{\Delta \ln A}{\Delta c}$$

of the straight line obtained, k_2 can be calculated according to the formula

$$k_2 = \frac{-m}{t}$$

t being the inhibition time in minutes.

If a group of N esterases with overlapping substrate specificity is inhibited by a certain organophosphorus compound, no straight line is obtained in the plot of ln A versus inhibitor concentration (or versus inhibition time, respectively), because of the superposition of N different inhibition curves characterized by N different inhibition rate constants:

$$\ln(A) = \ln(A_0^1 \cdot e^{-k_2^1 \cdot t \cdot c} + \cdots + A_0^N \cdot e^{-k_2^N \cdot t \cdot c}) \quad (1)$$

The slope s of the inhibition curve (1) in a semilogarithmic plot of residual activity in relation to inhibitor concentration is given by

$$s = \frac{d(\ln A)}{dc} = \frac{\sum_{i=1}^{N} k_2^i \cdot t \cdot A_0^i \cdot e^{-k_2^i \cdot t \cdot c}}{\sum_{i=1}^{N} A_0^i \cdot e^{-k_2^i \cdot t \cdot c}} \quad (2)$$

Raising the concentration of inhibitor, according to

$$\lim_{c \to \infty} \frac{d(\ln A)}{dc} = -k_2^N \cdot t \quad (3)$$

the slope s of the inhibition curve in a semilogarithmic plot only is determined by k_2^N, the second-order inhibition rate constant of the most slowly reacting activity component. The corresponding linear part of the inhibition curve, when extrapolated to the ordinate, gives the partial activity A_0^N of the most slowly reacting enzyme. Its activity

$$A^N = A_0^N \cdot e^{-k_2^N \cdot t \cdot c} \quad (4)$$

can be subtracted from total activity A for all inhibitor concentrations used, giving

$$A' = A - A^N \quad (5)$$

The same procedure may be applied iteratively on the residual activity A' until a straight line relationship is obtained between the logarithm of residual activity and the concentration of inhibitor (iterative elimination of exponential functions).

Reactivity of acetylcholinesterase and NTE with organophosphorus inhibitors. The time $t_{1/2}$ needed for 50% inhibition of an esterase activity at a given temperature is

$$t_{1/2} = \frac{\ln 2}{k_2 \cdot c} \quad (6)$$

where k_2 is the inhibition rate constant (second-order) and c is the concentration of inhibitor. If Eq. 6 is expressed in logarithms, it follows:

$$\log t_{1/2} = \log\left(\frac{\ln 2}{k_2 \cdot c}\right) \quad (7)$$

or

$$\log t_{1/2} = -\log c - (0.159 + \log k_2) \quad (7a)$$

In a plot of log $t_{1/2}$ versus log c (cf. Fig. 3), a straight line is obtained.

Materials. Inhibitors were supplied as follows: Mipafox by Bayer AG, Leverkusen, Germany; DFP from Fluka AG, Switzerland; Paraoxon from Serva Feinbiochemica, Heidelberg, Germany. Acetylthiocholine iodide was obtained from E. Merck, Darmstadt, Germany. PV was synthesized as described by Johnson (9). Additional reagents were purchased from the following suppliers: Titriplex III, Tris; potassium hexacyanoferrate, phenol, valeryl chloride, petroleum ether (50–70°), and perchloric acid from E. Merck, Darmstadt, Germany; 4-aminoantipyrine, Triton X-100, dimethylformamide, and 5,5′-dithiobis-(2-nitrobenzoic acid) from Serva Feinbiochemica, Heidelberg.

RESULTS

Organophosphorus inhibition of hydrolytic enzymes can be kinetically described as an irreversible bimolecular reaction leading to alkyl-phosphorylated serine hydroxyls at the enzymes' active centers. Inhibition follows first-order kinetics if the inhibitor is in molar excess (23). The parameter best suited to describe the reaction is the second-order velocity constant, or bimolecular rate constant, k_2 (liters · mole^{-1} · min^{-1}) (24).

The appropriate method of comparing inhibitory effects of different organophosphorus compounds on a certain enzyme is to plot enzyme activity versus log inhibitor concentration. If only one enzyme is inhibited by a single inhibitor, a characteristic sigmoidal curving is obtained (20). The inflection point of the sigmoidal curve yields the molar concentration (I_{50}) of the organophosphorus compound needed to cause 50% inhibition within the inhibition time t (in minutes) and at the temperature used. The time- and concentration-independent parameter k_2 (second-order inhibition constant) can be calculated from I_{50} according to the formula

$$k_2 = \frac{\ln 2}{I_{50} \cdot t}$$

Total carboxylesterase activity in chicken brain, determined with PV as substrate, was inhibited by the organophosphorus compounds Paraoxon, DFP, and Mipafox. The nonsigmoidal inhibition curves shown in Fig. 2 confirm the enzymological heterogeneity of PV-hydrolyzing carboxylesterases. For the bulk of PV-hydrolyzing carboxylesterase activity, obviously DFP is the most reactive inhibitor, while a considerable amount of activity seems to be relatively insensitive to Mipafox inhibition.

The nonsigmoidal inhibition curves in Fig. 2 result from superposition of different PV-hydrolyzing enzyme activities, which are inhibited with different velocities by the same inhibitor. The inhibition curves can be analyzed by iterative elimination of exponential functions giving components of activity and their corresponding velocity constants for the reaction with the inhibitor.

Kinetic analysis of the Paraoxon inhibition curve yielded six activity components (Table 1). The second-order inhibition rate constants varied from 10^1 to 10^7 (liters · mole^{-1} · min^{-1}). Inhibition rate constants obtained by DFP inhibition also varied (by as much as 6 orders of magnitude), but it should be emphasized that the six activity components listed in Table 2 are not identical with those resulting from analysis of Paraoxon inhibition.

As shown in Fig. 2 and Table 3, the main fraction of PV-hydrolyzing carboxylesterase activity is inhibited by Mipafox with a very low inhibition rate constant. Only four activity components could be differentiated by kinetic analysis of the Mipafox inhibition curve.

Sequential inhibition experiments combining two different organophosphorus inhibitors (24) were used to establish identity or nonidentity of the different fractions of carboxylesterase activity listed in Tables 1–3. For instance, by inhibiting hen brain carboxylesterases with 1.25×10^{-4} M Paraoxon for 40 min at 25°, 92% of the Paraoxon-reactive activity component P_1 (cf. Table 1) was still active. By analysis of the inhibition kinetics of this activity fraction with DFP as second inhibitor, P_1 was differentiated into three DFP-reactive enzymes with inhibition rate constants of 1.0×10^1 (270 mU · g^{-1}), 1.2×10^4 (176 mU · g^{-1}), and 1.6×10^5 (658 mU · g^{-1}). When control experiments were performed under identical conditions using Mipafox instead of DFP, identical enzyme activities were obtained, the rate constants for the reaction with Mipafox being 6.7×10^0 (210 mU · g^{-1}), 1.1×10^4 (176 mU · g^{-1}), and 1.3×10^3 (658 mU · g^{-1}). An activity component behaving as a single enzyme in the reaction with a certain organophosphorus inhibitor obviously may show kinetic heterogeneity for the reaction with a second inhibitor in sequential inhibition experiments, indicating that the corresponding activity has to be attributed to different PV-hydrolyzing enzymes.

By combining different organophosphorus inhibitors in sequential inhibition experiments it was established that 11 carboxylesterases were taking part in the hydrol-

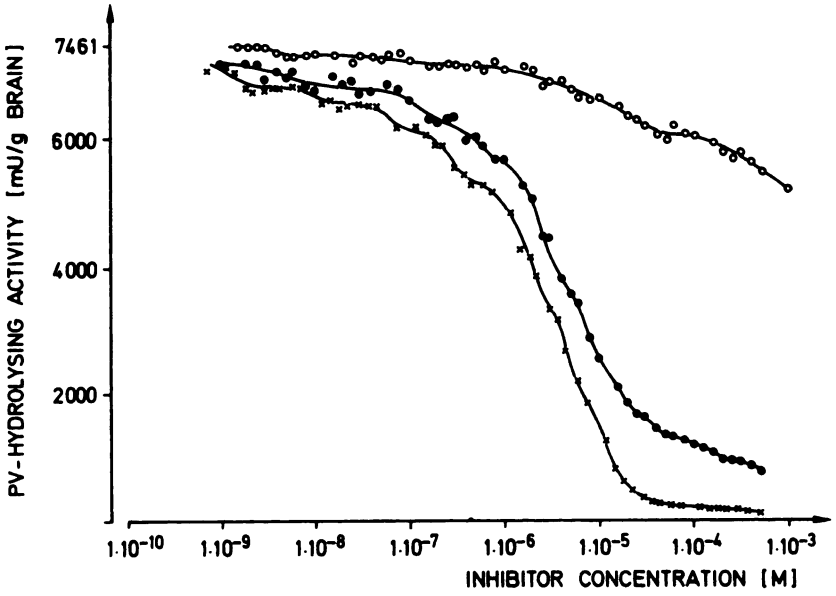


FIG. 2. *Organophosphorus inhibition of PV hydrolysis in chicken brain homogenate*
Total uninhibited activity was 7461 mU/g of brain. Inhibition by Paraoxon (●), DFP (×), and Mipafox (○) for 40 min at 25°.

ysis of PV in chicken brain. The activity of the PVases (milliunits per gram of brain) and the inhibition rate constants are listed in Table 4.

The identification of PV-hydrolyzing isoenzymes was reproduced with a high degree of accuracy on individual hen brains. While total PV-hydrolyzing activity tested in six hens showed a mean of 7475 $mU \cdot g^{-1}$ (range, 7079–7765 $mU \cdot g^{-1}$) and a standard deviation of 231 $mU \cdot g^{-1}$, the activity of isoenzyme PVase II showed a mean of 160 $mU \cdot g^{-1}$ (range, 77–276 $mU \cdot g^{-1}$) and a standard deviation of 72 $mU \cdot g^{-1}$. PV-hydrolyzing activity of PVase III was determined to be 724 $mU \cdot g^{-1}$ (mean), the standard deviation being 116 $mU \cdot g^{-1}$ (range, 550–886 $mU \cdot g^{-1}$).

Some of the carboxylesterases listed in Table 4 are of special interest. PVase I exhibited a very low sensitivity toward inhibition by all three organophosphorus inhibitors used. PVases II and III showed low reactivity with the non-neurotoxic inhibitor Paraoxon but were well inhibited by the neurotoxic agents DFP and Mipafox. Thus they are the only PV-hydrolyzing carboxylesterase isoenzymes fulfilling the criteria established by Johnson (10) for NTE. These NTE isoenzymes ought to be named NTE_A (PVase II) and NTE_B (PVase III). The main PV-hydrolyzing carboxylesterase is PVase V, which is responsible for about 40% of total PV hydrolysis in chicken brain. This enzyme is well inhibited by Paraoxon and DFP but shows a very low inhibition rate constant for the reaction with Mipafox.

PVase XI was present in chicken brain with an activity of 361 $mU \cdot g^{-1}$ and showed comparatively high inhibition velocities with all three organophosphorus compounds used.

To compare the reactivity of hen brain NTEs (NTE_A and NTE_B) toward organophosphorus inhibitors with that of the target enzyme for organophosphorus compounds with acute cholinergic symptoms, the inhibition rate constants of acetylcholinesterase were determined for the reaction with Paraoxon ($k_2 = 6.4 \times 10^6$ liters · $mole^{-1} \cdot min^{-1}$), DFP ($k_2 = 1.4 \times 10^5$), and Mipafox ($k_2 = 3.3 \times 10^2$). Using the inhibition rate constants, a direct comparison of acute and chronic organophosphorus tox-

TABLE 1

Components of PV-hydrolyzing activity in chicken brain obtained by analysis of Paraoxon inhibition curve

Activity components and corresponding inhibition rate constants were obtained by iterative elimination of exponential functions applied on a plot of log activity versus Paraoxon concentration (data shown in Fig. 2). Activity components are numbered and listed according to increasing velocity constants of inhibition

Activity component	P_1	P_2	P_3	P_4	P_5	P_6
Carboxylesterase activity (mU/g brain)	1044	581	3224	1484	767	361
Inhibition rate constant (liters · $mole^{-1} \cdot min^{-1}$)	$1.2 \cdot 10^1$	$2.7 \cdot 10^2$	$2.9 \cdot 10^3$	$8.8 \cdot 10^3$	$1.7 \cdot 10^5$	$1.4 \cdot 10^7$

TABLE 2

Components of PV-hydrolyzing activity in chicken brain obtained by analysis of DFP inhibition curve

Activity components and corresponding inhibition rate constants were obtained by iterative elimination of exponential functions applied on a plot of log activity versus DFP concentration (data shown in Fig. 2). Activity components are numbered and listed according to increasing velocity constants of inhibition.

Activity component	D_1	D_2	D_3	D_4	D_5	D_6
Carboxylesterase activity (mU/g brain)	210	1154	3790	421	1119	767
Inhibition rate constant (liters · $mole^{-1} \cdot min^{-1}$)	$1.0 \cdot 10^1$	$1.0 \cdot 10^3$	$3.5 \cdot 10^3$	$1.2 \cdot 10^4$	$1.6 \cdot 10^5$	$9.6 \cdot 10^6$

TABLE 3

Components of PV-hydrolyzing activity in chicken brain obtained by analysis of Mipafox inhibition curve

Activity components and corresponding inhibition rate constants were obtained by iterative elimination of exponential functions applied on a plot of log activity versus Mipafox concentration (data shown in Fig. 2). Activity components are numbered and listed according to increasing velocity constants of inhibition.

Activity component	M_1	M_2	M_3	M_4
Carboxylesterase activity (mU/g brain)	6266	658	176	361
Inhibition rate constant (liters·mole^{-1}·min^{-1})	$6.7 \cdot 10^0$	$1.3 \cdot 10^3$	$1.1 \cdot 10^4$	$3.1 \cdot 10^5$

icity could be achieved by plotting the logarithm of the time $t_{1/2}$ needed for 50% enzyme inhibition versus the logarithm of inhibitor concentration for both target enzymes acetylcholinesterase and NTE (Fig. 3).

DISCUSSION

Organophosphorus compounds are potent inhibitors of different classes of hydrolytic enzymes, as, for instance peptidases (25), carboxylesterases (1, 11), and cholinesterases (5). Since for most of these enzymes no specific substrate is known, organophosphorus inhibitors have been used for their differentiation and characterization. Aldridge (26) first classified A-esterases and B-esterases, and Johnson (10), in developing his NTE test, divided PV-hydrolyzing carboxylesterases into organophosphate-insensitive and organophosphate-sensitive enzymes.

Main (27) first applied a kinetic analytical method described by Brown and Fletscher (28) to multiple enzyme forms. A formal presentation of iterative elimination of exponential functions recently has been published, including a practical demonstration of the reliability and the resolution limits of the method by the use of artificial mixtures of different cholinesterases (20). The method is of special interest for the differentiation of enzymes with overlapping substrate specificity, which are difficult to separate and for which no specific substrates or inhibitors are known.

Applying the Johnson criteria (10) to PV-hydrolyzing carboxylesterases, two isoenzymes must be classified as neurotoxic esterases (NTE_A and NTE_B), bringing up 2.4% and 8.8%, respectively, of total PV-hydrolyzing activity in hen brain. Until now nothing could be said about their individual involvement in the genesis of delayed neurotoxic signs *in vivo*.

Enzymatic heterogeneity also has been demonstrated for cholinesterases in chicken brain (24).

Acute and chronic hazards of organophosphorus compounds can be evaluated and may be compared if the time needed for 50% inhibition ($t_{1/2}$) of acetylcholinesterase and NTE is plotted versus the concentration c of inhibitor in a double-logarithmic plot:

$$\log(t_{1/2}) = -\log c - (0.159 + \log k_2)$$

In each case a straight line is obtained (see Materials and Methods). The graphs shown in Fig. 3 prove the basic fact, that irreversible covalent organophosphorus inhibition of an esterase can be achieved either by the use of very high inhibitor concentrations for short inhibition times or by inhibitory action of low inhibitor concentrations for a very long time of inhibition. Therefore, a no-effect concentration cannot be defined for any organophosphorus inhibitor.

From Fig. 3A it is quite obvious that, within reasonable inhibition times, NTE may be inhibited by Paraoxon only if the inhibitor is present in extremely high concentrations. *In vivo* these Paraoxon concentrations would inhibit total acetylcholinesterase activity within seconds. In case of high-dose Paraoxon intoxication, experimental animals would die of cholinergic crisis long before delayed neuropathy could develop. In this context it is of interest that De Jager and co-workers (29) recently reported a case of attempted suicide by an extremely high dose of parathion. While under therapy at an intensive care unit and after 7 weeks in a deep coma the patient showed typical signs of delayed neuropathy.

Therefore the subdivision of hen brain carboxylesterases into Paraoxon-susceptible and Paraoxon-resistant enzymes, as, for instance NTE (7), should not be maintained. All of the 11 carboxylesterases listed in Table 4 can be inhibited by Paraoxon. However, second-order rate constants for Paraoxon inhibition of PVases I, II, and III are 5 orders of magnitude lower than those of acetylcholinesterase inhibition by Paraoxon. In the case of Mipafox inhibition of acetylcholinesterase and NTE (Fig. 3B), as compared with Paraoxon inhibition (Fig. 3A), an inversion of the inhibition lines in the double-logarithmic plot is obtained, explaining the toxicological fact that, *in vivo*, Mipafox predominantly will cause delayed neurotoxic symptoms (4).

The inhibition rate constants presented in Table 4 for the PV-hydrolyzing carboxylesterases of chicken brain can be used to evaluate the specificity and sensitivity of the differential assay for NTE activity developed by Johnson (9). For hen brain it can be shown that up to 92% of total NTE activity will be measured by the use of the differential assay and that only less than 1% of the

TABLE 4

PV-hydrolyzing carboxylesterases of chicken brain

The identity or nonidentity of different activity components (cf. Tables 1–3) and their relationship to different PVases was established by two-step inhibition experiments combining two organophosphorus esterase inhibitors in a sequential inhibition (see Materials and Methods).

PVase	Activity	Inhibition rate constant k_2		
		Paraoxon	DFP	Mipafox
	mU/g brain	*liters·mole^{-1}·min^{-1}*		
I	210	$1.2 \cdot 10^1$	$1.0 \cdot 10^1$	$6.7 \cdot 10^0$
II	176	$1.2 \cdot 10^1$	$1.2 \cdot 10^4$	$1.1 \cdot 10^4$
III	658	$1.2 \cdot 10^1$	$1.6 \cdot 10^5$	$1.3 \cdot 10^3$
IV	581	$2.7 \cdot 10^2$	$1.0 \cdot 10^3$	$6.7 \cdot 10^0$
V	2879	$2.9 \cdot 10^3$	$3.5 \cdot 10^3$	$6.7 \cdot 10^0$
VI	245	$2.9 \cdot 10^3$	$1.2 \cdot 10^4$	$6.7 \cdot 10^0$
VII	100	$2.9 \cdot 10^3$	$1.6 \cdot 10^5$	$6.7 \cdot 10^0$
VIII	573	$8.8 \cdot 10^3$	$1.0 \cdot 10^3$	$6.7 \cdot 10^0$
IX	911	$8.8 \cdot 10^3$	$3.5 \cdot 10^3$	$6.7 \cdot 10^0$
X	767	$1.7 \cdot 10^5$	$9.6 \cdot 10^6$	$6.7 \cdot 10^0$
XI	361	$1.4 \cdot 10^7$	$1.6 \cdot 10^5$	$3.1 \cdot 10^5$

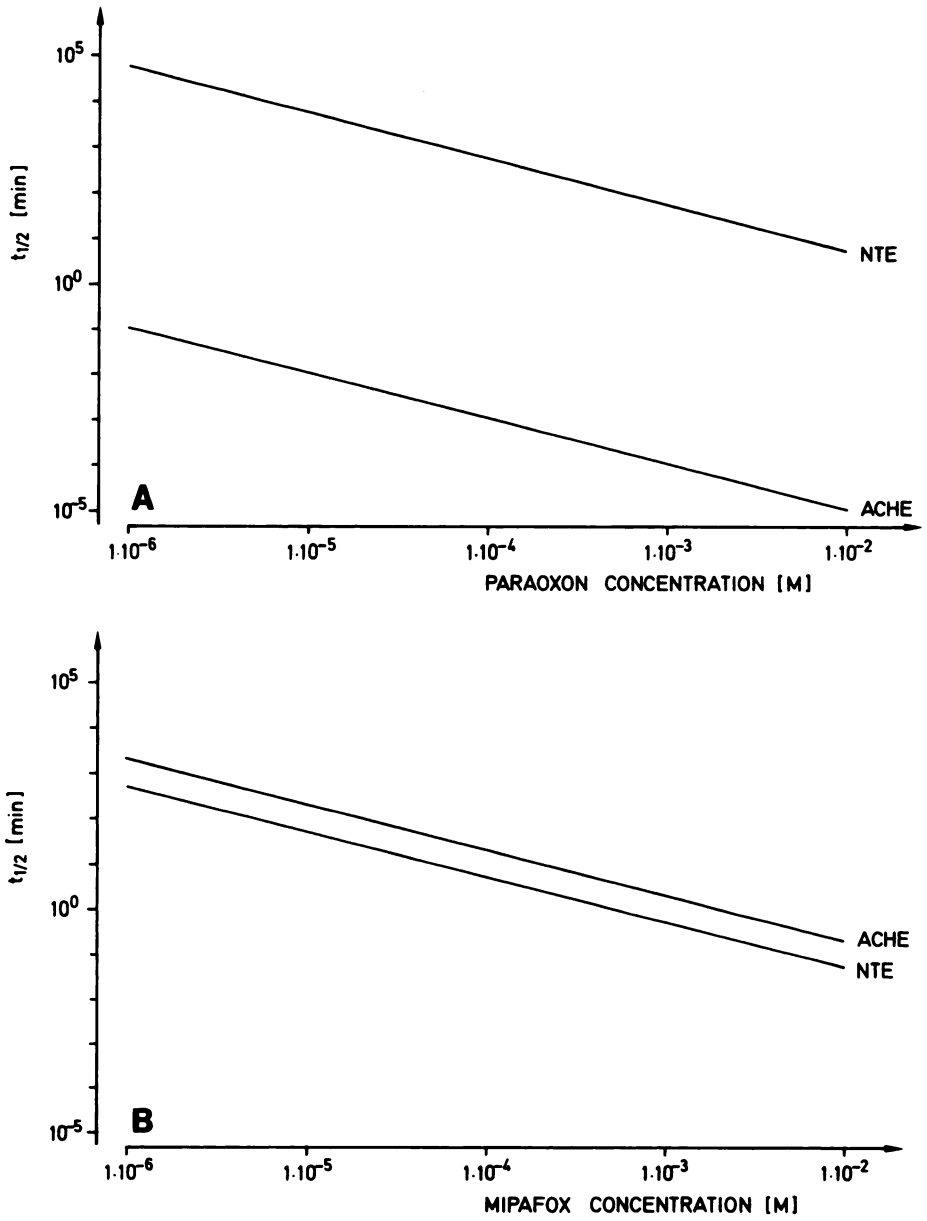


FIG. 3. *Sensitivity of acetylcholinesterase (ACHE) and NTE in chicken brain toward inhibition by a non-neurotoxic (A) and a neurotoxic (B) organophosphorus inhibitor*

Double-logarithmic plot of the time needed for 50% inhibition ($t_{1/2}$) of enzyme activity versus concentration of Paraoxon (A) and Mipafox (B) (see Materials and Methods).

activity detected in the test is due to PV-hydrolyzing carboxylesterases other than NTE. However, if the NTE test is to be applied to other species, experimental conditions must be adapted to the system investigated in order to maintain its excellent sensitivity and specificity.

In most organophosphorus inhibition experiments *in vivo* and *in vitro* the concentration of the Michaelis complex of inhibition is not sufficient to influence the rate of phosphorylation (30). Under these conditions the reaction between an organophosphorus inhibitor and its target enzymes can best be characterized by using bimolecular inhibition rate constants k_2. Main and Iverson (31) first demonstrated for the DFP inhibition of cholinesterases that bimolecular inhibition rate constants are determined by both the Michaelis-type constant of phosphorylation, K_i, and by k_{+2}, the phosphorylation constant (first-order) of the Michaelis complex. Recently similar investigations were performed on Mipafox inhibition of NTE activity in a microsomal preparation from hen brain (32). However, heterogeneity of NTE activity in hen brain was not taken into account and the resulting bimolecular rate constant was about 10-fold that determined in the present paper for NTE_B.

It should be emphasized that some of the non-NTE carboxylesterases show a greater reactivity with neurotoxic and non-neurotoxic organophosphorus inhibitors than do acetylcholinesterase and NTE. PVase XI is extremely sensitive to organophosphorus inhibition. It well may be that inhibition of PVase XI or of a yet unknown organophosphate-hypersensitive hydrolase of

the central nervous system may account for the development of atypical effects of organophosphorus compounds which have been reported in recent years, e.g., changes in regional brain metabolism (14), decreases in brain muscarinic receptor levels (33), long-term effects on electroencephalograms (15), subcellular changes at the motor end-plate (34), cardiac failure (16), impairment of muscle spindle function (17), and abnormalities in embryonic development (18).

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