Biochemical Pharmacology, Vol. 32, No. 11, pp. 1693–1699, 1983.
Printed in Great Britain.

0006-2952/83 $3.00 + .00

BRAIN CHOLINESTERASES

DIFFERENTIATION OF TARGET ENZYMES FOR TOXIC ORGANOPHOSPHORUS COMPOUNDS

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(*Received* 10 *September* 1982; *accepted* 17 *November* 1982)

Abstract—Cholinesterases in hen brain were characterized with respect to inhibition kinetics and substrate specificity. Three organophosphorus inhibitors were used: diethyl *p*-nitrophenyl phosphate (Paraoxon, E 600), di-isopropylphosphorofluoridate (DFP), and *N,N'*-di-isopropylphosphorodiamidic fluoride (Mipafox®). The kinetics of irreversible cholinesterase inhibition were studied using two substrates, acetylthiocholine and butyrylthiocholine. The inhibition curves were analysed by the method of iterative elimination of exponential functions. Final classification of the different enzymes was done by combining two inhibitors in sequential inhibition expts. Six cholinesterases were shown to hydrolyse choline esters in hen brain, one was identified as acetylcholinesterase (EC 3.1.1.7) and one as cholinesterase (EC 3.1.1.8). Four enzymes can be classified as intermediate type cholinesterases according to their substrate specificity and to their inhibition constants. The possible role of different brain cholinesterases for the development of atypical symptoms following organophosphate intoxication is discussed.

Acetylcholinesterase, AChE (EC 3.1.1.7) is mainly found in nerve tissue; its role in synaptic transmission is well established.

Nothing is known about any physiological function of cholinesterase, ChE (EC 3.1.1.8), a more unspecific choline ester hydrolysing enzyme present in serum and liver. It can be distinguished from acetylcholinesterase by means of substrate specificity and by organophosphate inhibition [1, 2]. Acute symptoms in organophosphate intoxications in man and animal are caused by irreversible covalent inhibition of acetylcholinesterase.

For a long time inhibition of unspecific cholinesterase was thought to be the underlying mechanism of delayed neurotoxicity [3], but nowadays by the work of Johnson and co-workers it is well established that neurotoxic esterase (NTE), a particular carboxylesterase (EC 3.1.1.1) of the central nervous system, is the primary target enzyme [5] of organophosphorus compounds with delayed neurotoxic effects [4]. However, some atypical clinical effects of organophosphorus compounds, such as cardiac failure [6, 7], persisting EEG-impairment [8, 9] and muscle necrosis [10, 11], cannot be related to inhibition of either AChE or NTE.

Since multiple forms of acetylcholinesterase [12, 13] and isoenzymes of cholinesterase [14–16] have been described, it seems to be possible that inhibition of choline ester hydrolysing target enzymes other than 'classic' AChE may account for atypical toxic effects of organophosphorus compounds.

Acute and chronic symptoms of organophosphate intoxication in domestic fowl closely resemble intoxication in man [17]. Therefore, the present study refers to inhibition kinetics of choline ester hydrolysing enzymes in hen brain. Three compounds have been used for inhibition of choline ester hydrolysis in brain homogenates: Paraoxon is known to cause only acute cholinergic symptoms, DFP predominantly leads to acute cholinergic and additionally to delayed neurotoxic symptoms, and Mipafox® mainly causes delayed neurotoxicity [18].

MATERIALS AND METHODS

Materials. The following substrates, inhibitors and reagents were used: *N,N'*-di-isopropylphosphorodiamidic fluoride (Mipafox®) from Bayer AG, Leverkusen, F.R.G; di-isopropylphosphorofluoridate (DFP) from Fluka AG, Buchs, Switzerland; diethyl *p*-nitrophenyl phosphate (Paraoxon, E 600), 5,5'-dithiobis-(2-nitrobenzoic acid), = DTNB, butyrylthiocholine iodide (BuSCh) from Serva Feinbiochemica, Heidelberg, F.R.G; acetylcholinesterase (AChE, bovine erythrocytes) from Mann Research Laboratories, NY, U.S.A. All other reagents were of analytical grade and purchased from local suppliers.

Preparation of tissues. Female Warren-sex hens, 1-year-old, were supplied by a local breeder. They were fed a standard diet (Schräder Standard Legemehl). Animals were killed by bleeding after pharyngeal dissection of the carotid arteries. The brain from each single animal was removed immediately, chilled to 0° and freed from connective tissue. A crude homogenate was prepared with a scalpel. Single portions (200 mg) of the crude brain homogenate were shock-frozen and stored at −40°.

Dilution of homogenates. Dilution of homogenates, inhibition expts and determination of cholinesterase activity were performed in phosphate incu-

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bation buffer (PIB) consisting of NaH_2PO_4/Na_2HPO_4, pH 7.0 (0.2 M), NaCl (50 mM) and Na-EDTA (1 mM). Brain (200 mg) was homogenized in 0.6 ml of cold PIB using a Potter-Elvejhem homogenizer. Homogenates were diluted with the same buffer to suspensions containing 10 mg brain per ml for acetylcholinesterase determinations and 100 mg brain per ml for measurement of cholinesterase activity.

Cholinesterase assay. Diluted brain suspensions (50 μl) containing 0.5 mg brain tissue for AChE- and 5 mg brain tissue for ChE-determination were preincubated with 50 μl H_2O for 5 min at 25°. The enzyme reaction was started by adding 0.5 ml substrate solution (1.4 mM ASCh or 30 mM BuSCh in PIB). Incubation time was 25 min at 25°. The enzyme reaction was stopped by adding 0.5 ml ice-cold perchloric acid (0.33 M), followed by centrifugation (4 min at 600 *g*). Clear supernatant (0.55 ml) was transferred to a freshly prepared mix of 0.5 ml Na_2HPO_4 (0.5 M) and 50 μl DTNB solution (10 mM DTNB dissolved in 0.1 M phosphate buffer, pH 7.0). The absorbance at 412 nm due to the immediately formed thionitrobenzoic anion [19] was determined in a Zeiss photometer PM 4. A blank was run under identical conditions using a homogenate previously inhibited by DFP (50 μM) for 60 min at 25°. One unit (1 U) of enzyme activity hydrolysed 1 μmole substrate per min. The variation coefficient (percentage S.D.) for the measurement of ASCh- and BuSCh-hydrolysing activity in hen brain is 1.2–3.5%.

Enzyme inhibition. Organophosphate inhibition kinetics of cholinesterase activity were measured by incubating 50 μl brain suspension (0.5 mg brain for AChE- and 5 mg brain for ChE-inhibition expts) with 50 μl aqueous organophosphate solutions for 60 min at 25°. Inhibitor concentrations varied from 10 pM to 1 mM. Inhibitor stock solutions (0.1 M in ethanol) were stored at 4°. Different concentrations of inhibitors were prepared from stock solutions immediately before the start of inhibition. Inhibition was stopped by adding 0.5 ml substrate solution. Residual enzyme activity was measured as described above. Second-order velocity constants, k_2 ($M^{-1} \cdot min^{-1}$) of organophosphate inhibition were calculated by plotting log residual activity (log *A*) vs organophosphate concentration (*c*). The slope of the straight line obtained is

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

giving

$$k_2 = \frac{-m \cdot 2.303}{t}$$

t being the inhibition time.

If two or more cholinesterases with different inhibition constants are present, no straight line is obtained in the log activity vs organophosphate concentration plot [20]. If such a heterogeneity of cholinesterase activity was found in the inhibition plots, the different activities (mU/g brain) and the corresponding second-order rate constants, k_2 ($M^{-1} \cdot min^{-1}$) of organophosphate inhibition were determined by iterative elimination of exponential functions beginning with the slowest reacting activity component [16, 21, 22]. The identity of different activity components was established by sequentially inhibiting the enzymes with two different organophosphate inhibitors. Using inhibition rate constants of the different activity components (cf. Tables 1 and 2) experimental conditions were calculated for a selective inhibition of fast reacting components leaving active the components of interest. Inhibition with the first organophosphate was stopped by adding the second inhibitor in a 10-fold vol. Inhibition kinetics of remaining activity components for the reaction with the second organophosphate were determined as described above. Activity components equivalent to only 1% of total activity can be identified and their inhibition rate constants for the reaction with organophosphorus compounds can be exactly measured (cf. Tables 1 and 2). Although there is little inter-individual variation with respect to total activity, activity components and to inhibition rate constants, all expts in the present paper were completely performed on individual hen brains, no pooled brain material was used.

RESULTS

Acetylthiocholine hydrolysing activity in hen brain is 17.4 U per g brain ($n = 6$; S.D.: 1.0 U · g^{-1}; range:

Table 1. Acetylthiocholine hydrolysing activity components in hen brain (total activity 17387 mU/g brain)

Inhibitor	Activity components [mU/g brain]	Inhibition rate constants, k_2 [$M^{-1} \cdot min^{-1}$]
Paraoxon	209	5.1×10^4
	661	1.1×10^6
	16,517	6.4×10^6
Mipafox	609	3.5×10^1
	15,908	3.3×10^2
	209	1.6×10^5
	661	1.4×10^7
DFP	16,517	1.4×10^5
	209	1.5×10^6
	661	6.3×10^7

Activity components and corresponding inhibition rate constants, k_2, were obtained by iterative elimination of exponential functions.

Table 2. Butyrylthiocholine hydrolysing activity components in hen brain (total activity 1159 mU/g brain)

Inhibitor	Activity components (mU/g brain)	Inhibition rate constants, k_2 ($M^{-1} \cdot min^{-1}$)
Paraoxon	143	3.9×10^2
	51	5.1×10^4
	846	1.1×10^6
	119	6.4×10^6
Mipafox	125	3.5×10^1
	137	3.3×10^2
	51	1.6×10^5
	846	1.4×10^7
DFP	143	2.3×10^3
	119	1.4×10^5
	51	1.5×10^6
	846	6.3×10^7

Activity components and corresponding inhibition rate constants, k_2, were obtained by iterative elimination of exponential functions.

15.9–18.7 $U \cdot g^{-1}$). It can be totally inhibited by Paraoxon, DFP and Mipafox. To compare the different inhibition curves a semi-logarithmic plot of activity vs log inhibitor concentration is shown in Fig. 1. Paraoxon is the most potent inhibitor for total hen brain acetylthiocholine hydrolysis, while Mipafox is only effective at concentrations of more than 10 μM. The deviations from conventional sigmoidal covalent inhibition curves shown in Fig. 1 are reproducible and indicate heterogeneity of cholinesterase activity in hen brain.

As confirmed with purified bovine erythrocyte acetylcholinesterase, Paraoxon, DFP and Mipafox inhibition follows first-order kinetics in the presence of excess inhibitor. In semi-logarithmic plots of log activity vs inhibitor concentration at fixed inhibition time and of log activity vs inhibition time at fixed inhibitor concentrations purified acetylcholinesterase did show a linear decay of log activity over the whole range of inhibitor concentrations and of inhibition times, respectively [22].

If inhibition data of hen brain cholinesterase activity (cf. Fig. 1) are plotted in the same way, deviations from linearity are observed with Paraoxon (Fig. 2A), DFP and Mipafox. At high concentrations of all three organophosphorus inhibitors a linear decay of activity indicates that in this range only one single enzyme, namely the slowest reacting activity, is determining inhibition velocity (see Fig. 2A for Paraoxon inhibition). The original uninhibited activity (at $c = 0$) of this slow reacting enzyme can be evaluated by extrapolating the linear part of the inhibition curve to the ordinate. As shown in Fig. 2A, the slow reacting activity with Paraoxon is 209 mU per g brain. With DFP and Mipafox slow reacting activity components are 16,517 and 609 mU, respectively (Table 1).

In Fig. 2 the Paraoxon inhibition curve of total

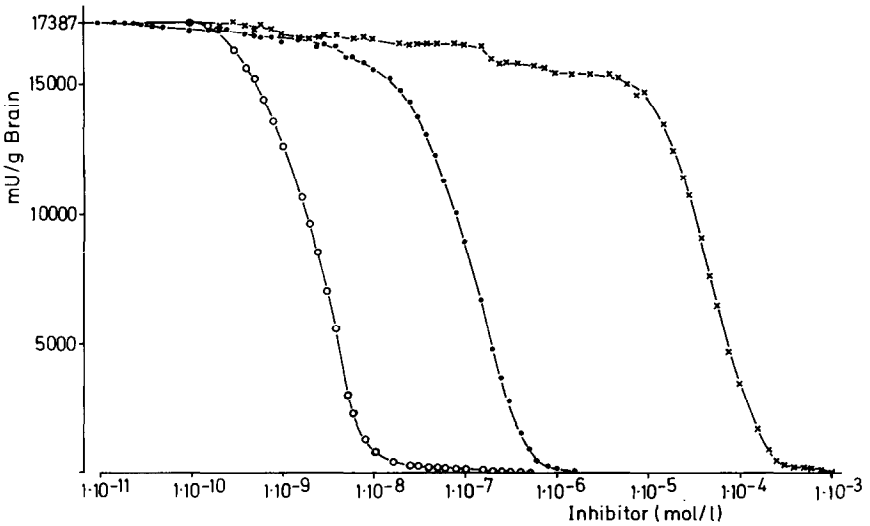

Fig. 1. Organophosphate inhibition of acetylthiocholine hydrolysis in hen brain homogenate. Inhibition for 60 min at 25° by Paraoxon (○), DFP (●), and Mipafox (×).

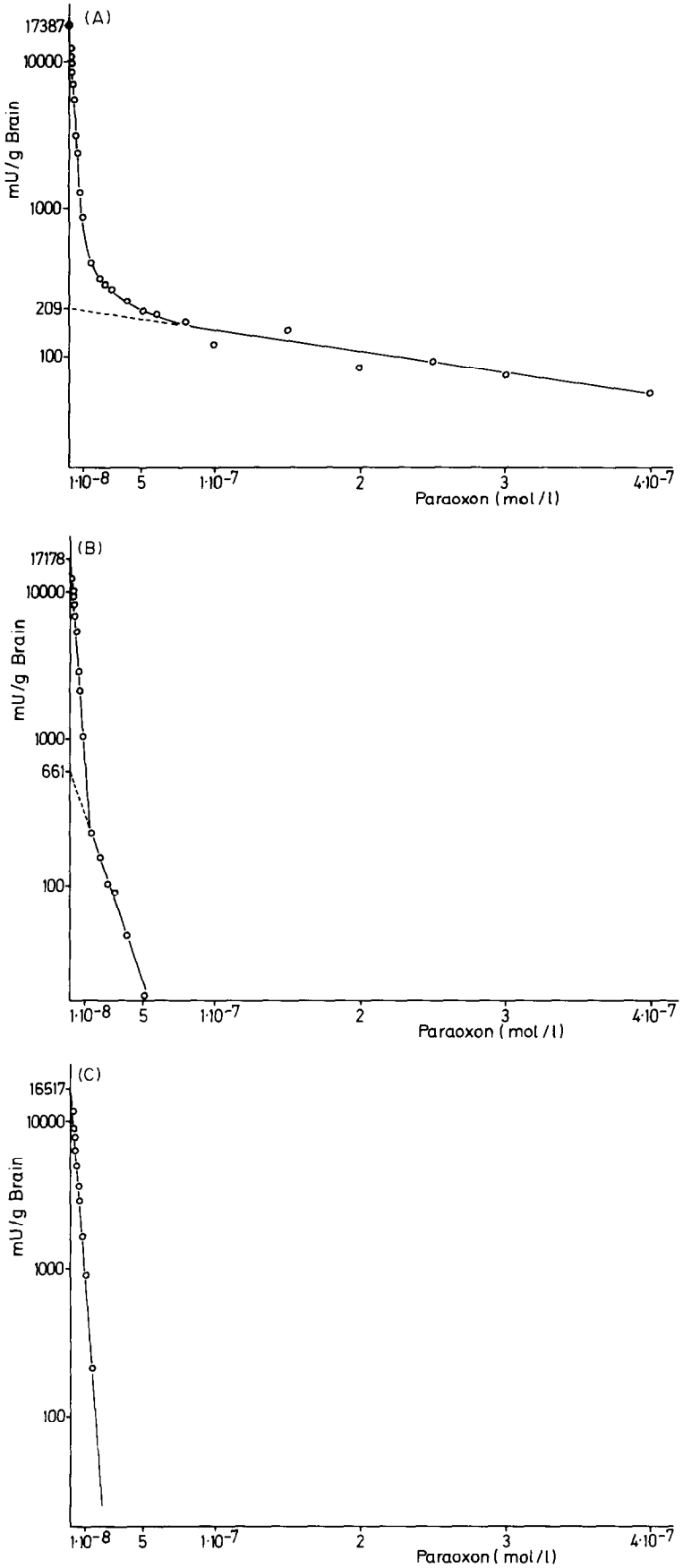

Fig. 2. Iterative elimination applied on the Paraoxon inhibition curve of ASCh-hydrolysis in hen brain. (A) Extrapolation of the slowest reacting activity component (209 mU, $k_2 = 5.1 \times 10^4$). (B) Residual activity after slowest reacting activity component has been subtracted. Extrapolation of second activity component (661 mU, $k_2 = 1.1 \times 10^6$). (C) Third activity component (16,517 mU, $k_2 = 6.4 \times 10^6$) calculated by subtraction of second activity component from total activity in plot (B).

hen brain cholinesterases with ASCh as substrate (cf. Fig. 1) is analysed by iterative elimination of exponential functions [22]. Three activity components are obtained: 209 mU with a second-order rate constant of 5.1×10^4, 661 mU with $k_2 = 1.1 \times 10^6$, and 16,517 mU with $k_2 = 6.4 \times 10^6$.

The same analytical procedure applied on DFP and Mipafox inhibition curves gives three activity components reacting with DFP and four components, which are inhibited by Mipafox. Their second-order velocity constants are listed in Table 1.

Activity components and bimolecular inhibition rate constants can be determined with great precision (variation coefficient for activity components less than 5%, for inhibition rate constants less than 10%). There are only slight differences between individual hens concerning both cholinesterase activity components and inhibition rate constants. The slow Paraoxon-reactive activity component (cf. Table 1) is about 1% of total ASCh-hydrolysing activity. In six different animals it showed a mean activity of 198 $mU \cdot g^{-1}$ (range: 177–225 $mU \cdot g^{-1}$; S.D.: 21 $mU \cdot g$). The corresponding inhibition rate constants for the reaction with Paraoxon varied from 3.7×10^4 to $5.7 \times 10^4\ M^{-1} \cdot min^{-1}$ ($n = 6$; mean: $4.9 \times 10^4\ M^{-1} \cdot min^{-1}$; S.D.: $0.7 \times 10^4\ M^{-1} \cdot min^{-1}$).

Identical inhibition expts with Paraoxon, DFP and Mipafox were performed using butyrylthiocholine (BuSCh) as substrate. Figure 3 shows that BuSCh-hydrolysing activity in hen brain is only 6.6% that of ASCh hydrolysis. Heterogeneity of BuSCh-hydrolysing activity in hen brain homogenate is obvious in these expts too. In contrast to the inhibition curves obtained with acetylthiocholine as substrate (Fig. 1), the bulk of butyrylthiocholine-hydrolysing activity shows the fastest reaction with DFP, followed by Mipafox and Paraoxon. The second-order rate constants for the inhibition of butyrylthiocholine hydrolysis in hen brain homogenate were calculated by iterative elimination of the inhibition curves, they are listed in Table 2. Four activity components can be distinguished by inhibition with Paraoxon, DFP and Mipafox. As compared with the data found with acetylthiocholine (Table 1), additional slow reacting components are found with Paraoxon and DFP using butyrylthiocholine as substrate. The activity of these components, with ASCh as substrate (0.5% of total activity), could be determined after a selective inhibition of the fast reacting components (99.5% of total) with 1.5×10^{-6} M DFP for 10 min at 25° using brain suspension in a 10-fold concentration (100 mg brain per ml).

One single enzyme should exhibit identical inhibition rate constants independent of the substrate used to test its activity. An activity component of 209 $mU \cdot g^{-1}$ with ASCh can be detected by inhibition expts with all three organophosphates (Table 1). Since a BuSCh-hydrolysing activity component of 51 $mU \cdot g^{-1}$ with identical inhibition rate constants is listed in Table 2, these activities can easily be attributed to one single choline ester hydrolysing enzyme, which is listed as cholinesterase I in Table 3. In the same way, activity components of 661 $mU \cdot g^{-1}$ with ASCh (Table 1) and of 846 $mU \cdot g^{-1}$ with BuSCh (Table 2) were related to cholinesterase II of Table 3. The identity or non-identity of the remaining activity components in Tables 1 and 2 was established by two-step inhibition expts combining two different organophosphorus inhibitors (sequential inhibition expts). For instance, after 60 min incubation with 1×10^{-4} M Mipafox at 25° one out of four Mipafox-reactive BuSCh-hydrolysing activity components (cf. Table 2) is still active: 125 $mU \cdot g^{-1}$ ($k_2 = 3.5 \times 10^1$ for the reaction with Mipafox). This activity was inhibited by a second organophosphorus compound (DFP or Paraoxon) and the inhibition curves were analysed by iterative elimination. Two different activity components were detected by DFP inhibition (111 $mU \cdot g^{-1}$ with $k_2 = 2.3 \times 10^3$ and 14 $mU \cdot g^{-1}$ with $k_2 = 1.4 \times 10^5$) as well as by Paraoxon inhibition (111 $mU \cdot g^{-1}$ with

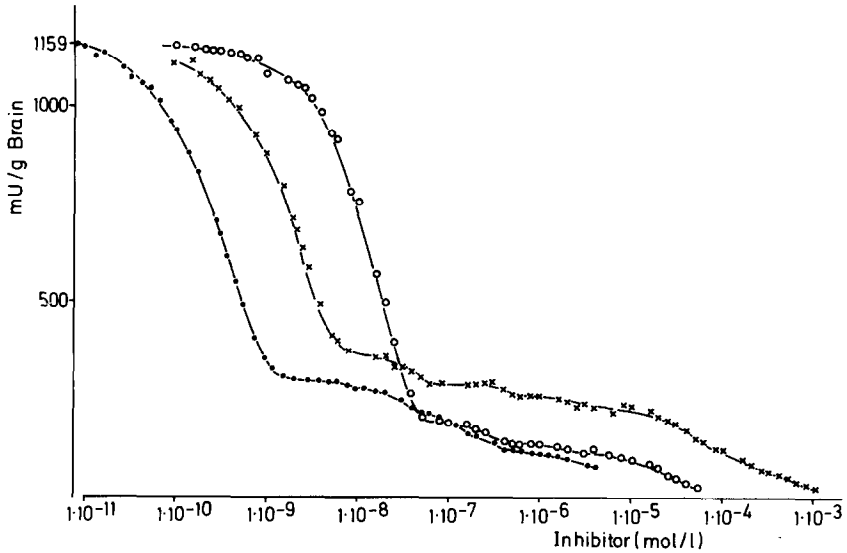

Fig. 3. Organophosphate inhibition of butyrylthiocholine hydrolysis in hen brain homogenate. Inhibition for 60 min at 25° by Paraoxon (○), DFP (●) and Mipafox (×).

Table 3. Substrate specificity and inhibition rate constants of cholinesterases in hen brain

Cholinesterase	Activities (mU/g)		Inhibition rate constants, k_2 ($M^{-1} \cdot min^{-1}$)		
	ASCh	BuSCh	Paraoxon	DFP	Mipafox
I	209	51	5.1×10^4	1.5×10^6	1.6×10^5
II	661	846	1.1×10^6	6.3×10^7	1.4×10^7
III	609	14	6.4×10^6	1.4×10^5	3.5×10^1
IV	15,908	105	6.4×10^6	1.4×10^5	3.3×10^2
V	54	111	3.9×10^2	2.3×10^3	3.5×10^1
VI	38	32	3.9×10^2	2.3×10^3	3.3×10^2

$k_2 = 3.9 \times 10^2$ and $14\ mU \cdot g^{-1}$ with $k_2 = 6.4 \times 10^6$). Therefore, in sequential inhibition expts activity components behaving as a single enzyme in the reaction with a certain organophosphorus compound (cf. Tables 1 and 2) may show kinetic heterogeneity in the reaction with a second inhibitor, indicating that their activity has to be attributed to two different choline ester hydrolysing enzymes (e.g. cholinesterases III and V of Table 3). Applying sequential inhibition expts with different combinations of inhibitors, the final classification of choline ester hydrolysing enzymes was achieved, step by step, by combining the results of all inhibition expts with both substrates (Table 3).

The main acetylthiocholine-hydrolysing activity, CHE IV, can be classified as acetylcholinesterase (EC 3.1.1.7). The most active butyrylthiocholine-hydrolysing enzyme, CHE II, shows all characteristics of cholinesterase (EC 3.1.1.8). Enzymes CHE I, III, V and VI have to be classified as 'intermediate type cholinesterases' [23].

DISCUSSION

According to classic definition acetylcholinesterases (EC 3.1.1.7) hydrolyse acetylcholine at a high rate, while butyrylcholine is practically not attacked [1]. Acetylcholinesterase is well inhibited by DFP, while Mipafox is much less effective [2].

Unspecific cholinesterases (EC 3.1.1.8) hydrolyse butyrylcholine faster than acetylcholine [1]. They are inhibited by DFP and Mipafox with nearly identical rates and about 2–5 orders of magnitude faster than acetylcholinesterase [2]. Applying these criteria on the choline ester hydrolysing enzymes in hen brain, cholinesterase IV is classified as acetylcholinesterase and cholinesterase II as unspecific butyrylcholinesterase. Hen brain cholinesterases I, III, V and VI show characteristics of both types of esterases. They should be classified as 'intermediate type cholinesterases' [23]. Multiple forms of cholinesterases or cholinesterase isoenzymes have been found in many vertebrate species by chromatographic [14, 24, 25], electrophoretic [15, 26, 27] and ultracentrifugal [28, 29] methods. Only few data exist concerning kinetic characteristics of cholinesterase and acetylcholinesterase isoenzymes [15, 16, 30].

Serum cholinesterases from man and horse were analysed by Main [16] using organophosphate inhibition kinetics. Since in these experiments variations in cholinesterase concentration or temperature of incubation changed the relative concentrations of cholinesterase isoenzymes, isoenzymes were thought to be interconvertible. No interconversion of hen brain cholinesterase isoenzymes could be detected in our experiments. A lot of data exists concerning the appearance and developmental changes of cholinesterase activity in embryonic brain tissue [27–29] and in cell cultures of chicken muscle cells [31]. These data refer to total cholinesterase activity and to molecular weight changes as shown by polyacrylamide electrophoresis and ultracentrifugal analysis. In the present study inhibition kinetics of cholinesterases with organophosphorus compounds are analysed according to a method first applied on kinetics of spontaneous hydrolysis of different esters [21]. Analysis and characterization of different cholinesterases in hen brain homogenate in the present study could only be achieved because a very sensitive and reproducible activity test has been developed and because inhibition was performed at fixed inhibition times with up to 50 different inhibitor concentrations [22].

The main advantage of the kinetic analytical method is that it can be applied on crude tissue homogenates without any preparative solubilization or purification steps.

Although the second-order rate constants of organophosphate inhibition of cholinesterases in hen brain vary up to six orders of magnitude, it should be emphasized that none of the inhibitors tested is specific for any of the six cholinesterases (Table 3).

Acute cholinergic symptoms following organophosphate intoxications in man and animals are well investigated and understood on a molecular level.

Delayed neurotoxic symptoms in man and certain animal species following intoxication by triorthocresyl phosphate (TOCP), DFP or Mipafox have been investigated on an enzymological basis and are the result of inhibition and dealkylation of neurotoxic esterase [33, 34].

In contrast, clinical observations concerning atypical organophosphorus effects, for instance persisting EEG-changes [8, 9], acute and chronic cardiac failure [6, 7], muscle necrosis [10, 11] and persisting impairment of muscle spindle function [32], cannot be explained up to now. Since some of the cholinesterase isoenzymes in hen brain react with organophosphorus inhibitors as fast or even faster than 'classic' acetylcholinesterase does, it well may be, that inhibition of one of these enzymes does account for some of the atypical organophosphorus effects in vertebrates.

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