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INHIBITION OF CHOLINESTERASES OF RAT DIAPHRAGM MUSCLE BY ORGANOPHOSPHATES AND SPONTANEOUS RECOVERY OF ENZYME ACTIVITY *IN VITRO**

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Abstract—Pretreatment of the isolated rat diaphragm with organophosphorus compounds, diisopropyl phosphorofluoridate (DFP), paraoxon or phospholine, resulted in inhibition of cholinesterase activity. Spontaneous recovery occurred after washing in Locke's solution for various periods of time (15–180 min). The rate of reactivation was fastest after inhibition with paraoxon and slowest after DFP and was significantly faster for butyrylcholinesterase than for acetylcholinesterase. Similar experiments when performed with the peripheral nerve of lobster, a tissue high in cholinesterase, showed no spontaneous recovery of enzyme activity, even after much longer periods of washing.

THE MODE of recovery of cholinesterases (ChE) following reaction with organophosphorus inhibitors has been studied in great detail.[1,2] Two reactions occur simultaneously *in vivo:* (a) regeneration of the inhibited enzyme by spontaneous hydrolysis of the enzyme–inhibitor complex, and (b) synthesis of new enzyme. Spontaneous recovery, however, and chemical reactivation by nucleophilic agents such as nicotinhydroxamic acid methiodide and pyridine-2-aldoxime methiodide (2 PAM) can only be observed as long as no "aging" has taken place. This is a change in the substituents of the attached phosphoryl group which makes the P—O bond insensitive to the attack of these reactivators.[3] Spontaneous hydrolysis of the enzyme–inhibitor complex largely depends on the stability of the chemical bonds between enzyme and inhibitor, which is determined by the dialkoxy substituents attached to the phosphorus.[1]

In previous studies of the recovery of ChE in diaphragms of rats[4] and mice[5] long intervals of time following the application of organophosphorus inhibitors were measured. In short term experiments[5] the procedure used seemed to be doubtful.[6]

Recently the spontaneous recovery of neuromuscular transmission in the diaphragm and gastrocnemius muscle of rats poisoned with high doses of various organophosphorus ChE inhibitors has been studied.[7] After complete respiratory paralysis, functional recovery of spontaneous respiration was observed within 2·5–7·5 hr depending on the inhibitor administered. Measurements of ChE were not performed. Reapplication of the ChE inhibitor usually reproduced the initial neuromuscular blockade, suggesting that reactivation of ChE had occurred.[7–10] Similar experiments have been

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reported in dogs,[11] where functional recovery of neuromuscular transmission and adequate respiration following soman poisoning was found 2–3·5 hr after the drug application. This, however, was accompanied by no or very little return of ChE as judged by histochemical enzyme activity determination.[11] The observations in the rat[7] suggested that the rat diaphragm might be a suitable preparation to study biochemically the recovery of ChE over short periods of time. Rapid rates of spontaneous reactivation have recently been described in isolated electroplax of the electrical eel.[12] The more recent studies on recovery of ChE activity in diaphragm are based on functional recovery[7,11] or on histochemistry.[11,13] To obtain more complete information about the spontaneous return of the enzyme activity, we have studied, with biochemical methods, the inhibition and rate of ChE reactivation following phosphorylation with a variety of so-called "irreversible" inhibitors of ChE. The inhibitors used (diisopropylfluorophosphate, paraoxon, phospholine and its tertiary derivative) are well known organophosphorus compounds which have been widely used in pharmacological research.[1,2] Furthermore, we have studied rates of recovery for the two types of enzyme, acetylcholinesterase (acetylcholine acetylhydrolase EC 3.1.1.7, AChE) and butyrylcholinesterase (acylcholine acylhydrolase EC 3.1.1.8, BuChE), found in the rat diaphragm preparation. In addition, a few experiments were undertaken to compare these rates of recovery at the neuromuscular junction with those of peripheral nerve fibers from the lobster, a tissue rich in AChE.[14,15]

A study of the phenomenon of spontaneous reactivation is of importance, since the inhibition of ChE activity has been used to observe and/or potentiate the effects of ACh in a variety of preparations. Any change in the degree of inhibition could thus lead to erroneous conclusions concerning the action of ACh on any particular preparation.

MATERIALS AND METHODS

Animals. (1) Sprague–Dawley rats of both sexes weighing 150–250 g were used. (2) Lobsters (*Homarus americanus*) were obtained from Hines & Smart in East Boston, Mass., and kept in a refrigerated sea water aquarium.

Dissection of tissues. (1) Rat diaphragms were dissected by rejecting the central tendineous part (*pars tendinea*) and the dorsal crura. Only the remaining muscular part (*pars muscularis*) was used, and no further attempt was made to isolate that part of the diaphragm known to contain the neuromuscular endplates.[16] The diaphragms were collected in Locke's solution with the following composition (mM): NaCr, 154; KCl, 5·5; $CaCl_2$, 2·2; dextrose, 5·0. This solution was buffered with 5 mM tris, and the pH was adjusted to 7·5 with HCl. (2) Lobster walking leg nerves were collected in artificial sea water with the following composition (mM): NaCl, 410; KCl, 10; $CaCl_2$, 8; $MgSO_4$, 25; $MgCl_2$, 25; and $NaHCO_3$, 2·4 with a pH of 7·6.

Cholinesterase inhibitors. The ChE inhibitors used were diisopropyl phosphorofluoridate (DFP) from Merck & Co. ("Fluoropryl"), diethyl *p*-nitrophenyl phosphate (paraoxon) from Merck & Co., Rahway, N.J., *O,O*-diethyl-*S*-(2-trimethyl ethylammonium) phosphorothioate methiodide (quaternary phospholine, 217 MI) from Ayerst Laboratories, N.Y., and 2-diethoxyphosphinylthioethyldimethylamine acid oxalate (tertiary phospholine, 217 AO) also from Ayerst Laboratories.

Cholinesterase activity determination. ChE activity was determined at room temperature (22–25°) using a colorimetric procedure.[17] The substrates used were acetyl-

choline iodide (ACh), acetyl-DL-β-methylcholine bromide (MeCh) and butyrylcholine iodide (BuCh), all from Sigma Chemical Corporation, St. Louis, Mo.

To be certain that ChE activity determined corresponded to the actual level of enzyme activity, a known amount of AChE (commercially available red blood cell AChE) was added to paraoxon-treated hemidiaphragms prior to homogenization. After homogenization, no reduction in the activity of the added known amount of AChE was observed. The same observation was made when control diaphragms and paraoxon-treated diaphragms were homogenized together.

The substrates were dissolved in Locke's solution for the rat experiments buffered with 0·1 M tris and HCl to pH 7·5 or in sea water buffered in the same way, in the lobster experiments.

Pretreatment and washing procedure for tissues. The diaphragms were preincubated for at least 30 min in Locke's solution containing the chosen inhibitor. They were then blotted on filter paper, transferred to a beaker placed on a magnetic plate, and washed at room temperature under constant stirring in a large volume of drug-free Locke's solution. The washing fluid was exchanged according to a fixed time schedule, and the washing times ranged from 5 to 180 min. During the first 30 min, the fluid was exchanged every 5 min and thereafter every 15 min.

Washing in the presence of inhibitors of protein synthesis. In some experiments, chloramphenicol or cycloheximide (both from Sigma Chemical Co., St. Louis, Mo.) in concentrations ranging from 5 to 500 mg/l. of Locke's solution was added to the washing fluid.

Pretreated diaphragms, which were rinsed off for just 1 min to remove excess inhibitor from the surface, were used to determine the degree of ChE inhibition at the end of the preincubation period.

Untreated diaphragms were used as controls in every experiment. In many cases, the diaphragms were split into two hemidiaphragms, each of which contained sufficiently high ChE activity for analysis under the experimental conditions.

Similar handling was applied to the lobster nerve samples except that sea water was used throughout.

RESULTS

Rat diaphragms

Studies on inhibition and reactivation. (1) Paraoxon. After establishing the minimal concentration of each inhibitor needed to inhibit completely the ChE of rat diaphragm *in vitro*, the reactivation experiments were begun. Following 30 min preincubation in 5×10^{-5} M paraoxon, any hydrolysis of 5×10^{-3} M ACh was abolished. There was no measurable return of activity within 5 hr if the preparation was rinsed off only briefly and then incubated in the substrate solution (ACh 5×10^{-3} M, MeCh 2×10^{-2} M, or BuCh 1×10^{-2} M). However, samples that were washed in Locke's solution showed a gradual reappearance of the enzyme activity. After 30-min washing time, the hydrolysis of ACh was clearly measurable, and the reactivation of ChE activity proceeded with increasing duration of the washing time in an almost linear fashion reaching 35 per cent of control within 3 hr (Fig. 1). This curve shows the combined recovery of both AChE and BuChE.

When the paraoxon concentration was increased to 1×10^{-3} M, reactivation by washing was measurable as well, but the amount of enzyme activity reappearing was

lower and amounted to only 16 per cent in 3 hr. With this concentration no activity was determined prior to 60 min of washing (Table 1).

(2) Phospholine. The only other inhibitor which showed a significant rate of reactivation by washing was 217 AO, the tertiary derivative of phospholine (Table 1).

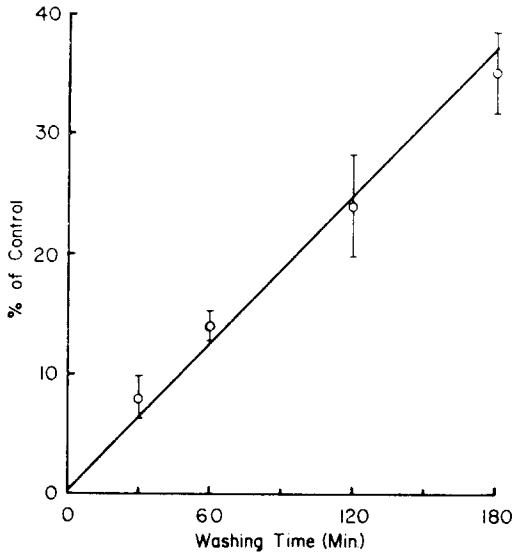

FIG. 1. Time course of recovery of ChE activity in isolated rat diaphragms after inhibition with paraoxon. Hemidiaphragms were preincubated in 5×10^{-5} M paraoxon for 30 min. Then they were washed for various periods as indicated and ChE was determined at room temperature with the procedure of Hestrin,[17] using 5×10^{-3} M ACh as the substrate. Values represent the mean and vertical bars the standard deviation of at least six determinations expressed as per cent of control. For further details see Methods.

Recovery of enzyme activity after inhibition with 1×10^{-4} M phospholine was only slight. No significant change in the recovery was seen after the initial 15-min washing period. This effect, however, was significant, since without washing no enzyme recovery was observed.

(3) Diisopropyl phosphorofluoridate. Reactivation after DFP pretreatment (Table 1) in the concentrations of 1×10^{-3}, 1×10^{-4} and 5×10^{-5} M (fully inhibitory concentration *in vitro*) was slow, though measurable, compared to paraoxon and did not amount to more than 10 per cent after 3-hr washing time. Due to the small increases in activity, it was not possible to distinguish any differences among the rates of recovery from the various concentrations of DFP.

Recovery of AChE and BuChE. In order to characterize more closely the type of ChE being reactivated, the hydrolysis of the specific substrates for AChE (MeCh) and for BuChE (BuCh) were examined.

(1) Control diaphragms. Substrate concentration–activity relationship: intact hemidiaphragms were incubated with increasing concentrations of ACh, MeCh and BuCh ranging from 1×10^{-3} to 5×10^{-2} M. The amount of substrate hydrolyzed increased in an almost linear fashion for all three substrates used as the concentration of choline

TABLE 1. REACTIVATION OF ORGANOPHOSPHORUS-INHIBITED RAT DIAPHRAGM ChE BY WASHING*

Inhibitor (M)	Enzyme activity expressed as % of control activity Washing time (min)					
	5	15	30	60	120	180
Paraoxon 1×10^{-3}				5·4 (3·7–4·9) N = 3	10·5 (9·9–11·1) N = 3	16·3 (14–18·6) N = 3
Paraoxon 5×10^{-5}			8·0 (5·6–13·3) N = 9	14·2 (10·7–16·9) N = 9	23·8 (18·8–29·6) N = 9	35·2 (32·1–38·7) N = 6
Tert. phospholine 5×10^{-5}	3·3 (2–4·6) N = 6	7·1 (6·5–7·7) N = 6	10·2 (8·5–12) N = 6	11·5 (9·2–13·8) N = 6	10·4 (7·3–13·7) N = 6	25·2 (16·7–37·5) N = 6
Quat. phospholine 1×10^{-4}		4·9 (3·9–6·4) N = 3	5·2 (3·6–6·3) N = 3	7·2 (6–9·1) N = 3	7·7 (6·1–10·1) N = 3	6·8 (5·4–9·6) N = 3
DFP 1×10^{-4}			10·2 (8·1–13·0) N = 3	11·7 (7·2–18·4) N = 3	5·6 (4·3–7·9) N = 3	6·3 (2·5–13·8) N = 3

* Isolated hemidiaphragms were first preincubated for 30 min with the inhibitors of ChE indicated in the left column. Following this period they were washed in Locke's solution, pH 7·5, for various times. Then ChE activity was determined with the Hestrin[17] procedure. Values are expressed as mean percentage of activity recovered (in parentheses range of the values) for the number of samples indicated as compared to control. Substrate was ACh 5×10^{-3} M. For further details see Methods.

ester was increased (Fig. 2). When potential barriers for the penetration of the quaternary choline esters were reduced by homogenization of the tissue, the rate of hydrolysis of ACh was increased 2- to 4-fold. The differences were more pronounced at the low substrate concentration of 1×10^{-3} M (4-fold) than at 1×10^{-2} M and 5×10^{-2} M when they were only 2-fold (Fig. 2). At equal substrate concentrations of 1×10^{-2} M or 5×10^{-3} M intact hemidiaphragms hydrolyzed MeCh at about half the rate of ACh and hydrolyzed BuCh at about 30 per cent of the rate of MeCh (Fig. 2, Table 2).

(2) Organophosphate pretreated diaphragms. Following the preincubation period, all samples were washed for 2 hr with Locke's solution prior to the incubation in the substrate solution. The results are summarized in Table 2 and expressed as micromoles of substrate hydrolyzed per hemidiaphragm.

The recovery observed following inhibition by 5×10^{-5} M paraoxon seemed to a large extent to be due to a reactivation of BuChE. BuCh was hydrolyzed to 71 per cent of the control value, while the MeCh was hydrolyzed at a rate of only 17 per cent of its original activity. Since ACh is hydrolyzed by both BuChE and AChE the recovery was 27 per cent within the 2-hr washing period. Spontaneous hydrolysis under our experimental conditions is negligible. No recovery of enzyme activity was seen without washing. Thus, any observed hydrolysis after washing indicates enzymic reactivation.

Exposure to DFP, which is known to inhibit BuChE more effectively than AChE,[19,20] seemed to be followed by a slightly higher reactivation of the MeCh-hydrolyzing enzyme than that of BuCh (7·2 vs. 4·4 per cent of the respective controls). However, as

has been pointed out in the previous section, the total recovery within the period of observation was low when compared to paraoxon.

No differences in the rate of recovery were found with any one of the three substrates when diaphragms exposed to 1×10^{-4} M quaternary phospholine were used. In each case 10 per cent of the original activity reappeared during the washing period.

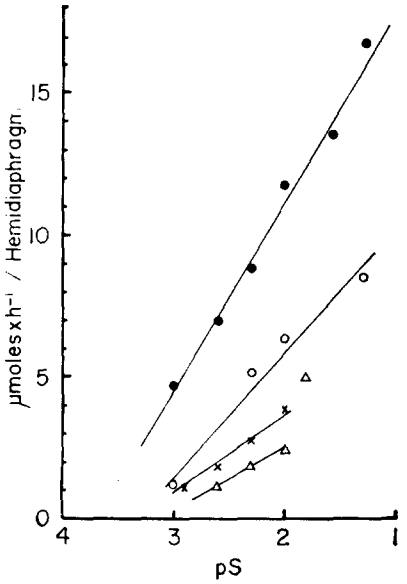

FIG. 2. Hydrolysis of various choline esters by ChE from rat diaphragm. Hemidiaphragms were incubated with increasing substrate concentrations and the hydrolysis of the choline esters determined with the colorimetric procedure of Hestrin.[17] Intact hemidiaphragms were used for BuCh △—△ and MeCh +—+, while intact ○—○ and homogenized ●—● were compared using ACh. The actual concentrations of MeCh in the incubation medium were twice as high as indicated in the graph. MeCh is commercially available only as the DL-racemate, but only the D-form of the compound is hydrolyzed by AChE.[18] Each point represents the mean value of at least three determinations for BuCh and MeCh, and four or more determinations for ACh.

Inhibitors of protein synthesis. In an attempt to differentiate spontaneous recovery *in vitro* from a possible contribution due to *de novo* synthesis of enzyme protein, diaphragms were washed as usual, but the washing fluid now contained chloramphenicol or cycloheximide in concentrations varying between 5 and 500 µg/ml. The compounds when used *in vitro* at these concentrations had an inhibitory effect on ChE activity of control diaphragms; thus the data did not allow us to draw any conclusions as to the mechanism of the observed ChE reactivation.

Lobster walking leg nerves

Studies on inhibition and reactivation. Completely different results were obtained with peripheral nerves of the lobster under experimental conditions quite similar to those applied to rat diaphragms. There seemed to be some reappearance of enzymatic activity as measured by the hydrolysis of ACh, but the recovery slowed to a very low rate as washing time was increased (Table 3). The rates of reappearance of enzymatic activity

TABLE 2. RECOVERY OF CHOLINESTERASES IN RAT DIAPHRAGM FOLLOWING PRETREATMENT WITH VARIOUS ORGANOPHOSPHORUS INHIBITORS AND WASHING FOR 2 hr AS DETERMINED BY THE USE OF SPECIFIC SUBSTRATES*†

Inhibitor (M)	Enzyme activity					
	ACh‡	% of control	MeCh‡	% of control	BuCh‡	% of control
Control	7·61 ± 1·02 (11)		3·78 ± 0·61 (10)		1·99 ± 0·29 (13)	
Paraoxon 5×10^{-5} M	2·09 ± 0·55 (11)	27	0·64 ± 0·27 (13)	17	1·43 ± 0·36 (13)	71
Control	5·64 ± 0·63 (4)		2·76 ± 0·16 (4)		1·93 ± 0·19 (4)	
DFP 1×10^{-4} M	0·232 ± 0·081 (6)	4·1	0·20 ± 0·09 (8)	7·2	0·087 ± 0·028 (8)	4·4
Control	6·02 ± 0·63 (4)		3·17 ± 0·13 (4)		1·97 ± 0·28 (4)	
Quat. phospholine 1×10^{-4} M	0·662 ± 0·097 (6)	10·9	0·322 ± 0·087 (8)	10·1	0·212 ± 0·046 (8)	10·8

* Substrate concentration, 1×10^{-2} M.

† Data are expressed as micromoles of substrate hydrolyzed at room temperature (22–25°). Enzyme activity was measured with the colorimetric procedure of Hestrin.[17] Values show hydrolysis per hemidiaphragm (mean ± S.D.) and the number of samples is indicated in parentheses. The results of the inhibitor-treated samples are also given in terms of per cent of activity returning compared to the control value.

‡ No spontaneous substrate hydrolysis was observed during the assay for enzyme activity.

TABLE 3. RECOVERY OF CHOLINESTERASES IN LOBSTER WALKING LEG NERVE FOLLOWING PRETREATMENT WITH PARAOXON AND WASHING FOR VARIOUS LENGTHS OF TIME*

Inhibitor (M)	Enzyme activity expressed as % of control activity Washing time (min)				
	10	30	60	120	180
5×10^{-5}					6·3 (2·2–9·6) N = 7
1×10^{-3}	4·3 (4·1–4·6) N = 9	4·8 (4·4–5·2) N = 6	5·5 (5·3–5·6) N = 9	7·8 (5–9·8) N = 9	7·1 (4·7–9·6) N = 5

* Nerve bundles from the walking leg of the lobster were preincubated for 30 min with paraoxon in the concentrations specified. Then the nerves were washed for the times indicated in artificial sea water. Following this washing period, the hydrolysis of 5×10^{-3} M ACh was determined with the procedure of Hestrin.[17] Enzyme activity is expressed as per cent of activity returning compared to control following complete inhibition. Values represent means and in parentheses the range of the number of experiments is indicated.

were variable and no differences were seen in the amount recovered following 5×10^{-5} or 1×10^{-3} M paraoxon. In some preparations, the activity obtained after only 10 min of washing was approximately the same as that measured after 3 hr of washing.

DISCUSSION

When intact rat hemidiaphragms were exposed to the generally "irreversible" organophosphorus inhibitors of ChE, the ability to hydrolyze choline esters returned after washing the tissue. Diaphragms treated with paraoxon showed the highest rate of recovery. The rate of reactivation was almost linearly dependent upon the length of the washing time, an indication that this rate was dose dependent. At equal washing times, the amount reactivated after exposure to 5×10^{-5} M paraoxon was much larger than that obtained following 1×10^{-3} M. Tertiary phospholine inhibited enzyme recovered to 25 per cent after 3 hr washing, while the quaternary analogue reached only 7 per cent. Both phospholines as well as paraoxon form a diethoxy phosphorylated enzyme; however, in addition to the interaction with the esteratic site by phosphorylation, the quaternary phospholine (217 MI) probably binds by coulombic forces to the anionic site of the enzyme. This second binding site, which results in a tighter fixation of the inhibitor, may explain the much lower reactivation obtained during 3 hr of washing after 217 MI compared to the tertiary base 217 AO (Table 1).

DFP-inhibited enzyme, resulting in a diisopropyl-phosphorylated enzyme, showed the poorest recovery. The findings are in agreement with the observations on purified enzyme[21,22] and on rabbit erythrocyte AChE in which the order of the reversal rate was methyl,-ethyl,-isopropyl-phosphoryl enzyme.[23] The lack of any progress in the reactivation of DFP-inhibited enzyme beyond 60 min is probably due to "aging", a process which has been studied in the diisopropyl-phosphorylated enzyme and is complete within a few hours depending on the type and source of the cholinester-

ase.[3,6,24] In electroplax, this reaction does not seem to occur as indicated by the slowly progressing spontaneous reactivation of ChE.[12]

The amount of ChE recovered *in vitro* is much larger than that estimated by Denz histochemically.[4] This is probably due to our washing procedure and to the fact that the biochemical determination of ChE, is more sensitive and accurate. The actual percentage of reactivated enzyme present in the tissue at the end of the 3-hr washing period may be still increased during the subsequent incubation in substrate. This takes another 2–4 hr under our experimental conditions, and reactivation could proceed during this time. In the case of DFP, however, this delay may contribute to the "aging" process and thus stop the initial phase of slow spontaneous hydrolysis of the enzyme–inhibitor complex. This observation in the rat diaphragm differs from the electroplax where a continuing reactivation has been observed.[12] The rapidity of the reactivation *in vitro* of rat diaphragm ChE suggests that spontaneous recovery rather than removal of supposedly extracellular free paraoxon by chloroform is the reason for the rapid reactivation of cholinesterase activity as was observed in mouse diaphragm.[5] Observations in our laboratory do not support the assumption that there is free reactive paraoxon in the brain* even 30 min after injection. Brain tissue and diaphragms from rats killed by paraoxon when homogenized in the presence of a standardized amount of known ChE activity did not inhibit this preparation.

The characterization of ChE in the whole diaphragm by means of "specific substrates" agrees closely with the values obtained with "specific inhibitors"[4] in that BuCh is split at about 30 per cent of the rate of ACh (Table 2). However, BuCh is hydrolyzed by BuChE more rapidly than ACh. Thus the actual amount of BuChE and its proportionate hydrolysis of ACh is likely to be lower than 30 per cent. The recovery *in vitro* of BuChE after inhibition with paraoxon is much faster than AChE, e.g. 71 vs. 17 per cent, as determined by the hydrolysis of BuCh and MeCh respectively (Table 2). This is in good agreement with the data obtained *in vivo*.[4]

The substrate concentration–activity relationship curve showed linear dependency on the substrate concentration for all three substrates tested. This is surprising since, at least for ACh hydrolysis, one might expect substrate inhibition by excess ACh.[17] The substrate inhibition was seen in crude homogenates of rat diaphragms[25] but was not observed in the present experiments with ACh concentrations as high as 5×10^{-2} M (Fig. 2). At present we have no explanation for the absence of substrate inhibition usually seen in the higher ACh concentration range. It is not due to lack of accuracy of the Hestrin technique,[17] since bell-shaped concentration velocity curves have been obtained when other preparations such as electro-plax[26] or lobster nerve[14] were used. It is possible however that ACh may be hydrolyzed by some other enzyme, such as BuChE, which in spite of its slower rate of hydrolysis could mask the substrate inhibition at these higher concentrations.

Permeability barriers for ACh are probably more obvious at low substrate concentrations, which may explain the 4-fold increase of ACh hydrolysis at 1×10^{-3} M. Similar differences were recently reported between surface and internal ChE activity; the latter is accessible to ACh only after homogenization in rat diaphragm.[27]

Essentially no spontaneous reactivation from paraoxon inhibition was seen with the ChE from lobster nerve. This agrees with previous results in which even longer washing periods did not restore enzyme activity.[28] This finding indicates differences in the

* F. Welsch and W.-D. Dettbarn, unpublished observations.

binding characteristics of organophosphates to ChE of various species and suggests a different attachment to the esteratic site of ChE in the lobster from that in the rat. It may be due partly to the fact that the peripheral nerves of the lobster contain very little BuChE as compared to AChE (< 10%).[14,15] As was determined previously and in the present investigation (Table 2), BuChE was found to be reactivated much faster after paraoxon in the rat diaphragm.

Whether spontaneous reactivation is the sole mechanism underlying the measured rapid recovery of choline ester hydrolyzing enzymes cannot be decided from the experimental results.

Due to the variability of the effect of the classical inhibitors of protein synthesis, chloramphenicol and cycloheximide, on control preparations, these drugs were not useful tools to differentiate *in vitro* whether there was *de novo* synthesis contributing to the over-all recovery measured. Cycloheximide has been used more successfully in experiments *in vivo* as an inhibitor of *de novo* ChE synthesis in retina following DFP[29] and sarin or soman ChE inhibition.[30] There may be some *de novo* synthesis occurring *in vitro* simultaneously with the spontaneous dephosphorylation, and this process could even be stimulated following the exposure to organophosphates. A higher rate of protein labeling with radioactive amino acid precursors has been found in rat brain[31] and lobster nerve[32] after treatment with irreversible ChE inhibitors. A stimulatory effect of soman on enzyme biosynthesis in the rat liver has been reported.[33] This aspect of differentiation between spontaneous reactivation of the old enzyme and *de novo* synthesis of new enzyme in the first few hours following ChE inhibition is now under investigation. Previously it has been concluded that the slow rates of ChE recovery in brain[5,34] and erythrocytes[6] correspond to the physiological protein turnover and coincide with the rate of synthesis of new erythrocytes.[6]

Similar conclusions were also reached in houseflies by Ahmad,[35] where reactivation following malathion occurred in two stages: 40 per cent recovery of ChE in the first 24 hr presumably mainly due to spontaneous dephosphorylation, followed by a slow steady rate which was interpreted as resynthesis. However, the occurrence of an isozyme of ChE has been recently described in rat retina, which had a half-life of only 3 hr.[29] It is difficult to draw any parallels from the rate of ChE recovery observed *in vitro* with the time course of this reaction in the living rat. It has been estimated that 1–2 per cent of the ChE activity would be sufficient to maintain spontaneous respiration.[7] Further studies on the short term recovery in surviving animals will be needed to establish whether the findings *in vitro* are comparable and indicative of the time course of spontaneous reactivation *in vivo*.

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