INHIBITORY EFFECT OF ISOPROPYL METHYL PHOSPHONOFLUORIDATE ON TISSUE CHOLINESTERASES OF MOUSE, RAT, AND GUINEA PIG *in vitro*

J. PATOČKA

J. E. Purkyně Military Research Institute for Postgraduate Training, Hradec Králové

Received November 9th, 1970

The inhibitory effect of isopropyl methyl phosphonofluoridate on cholinesterases of brain, liver, heart, and lung homogenates of mouse, rat, and guinea pig *in vitro* was studied. The interaction was characterized as allosteric inhibition and described in terms of I_{50}-constants, Hill coefficient values, and size of bimolecular rate constants.

The animal cholinesterases represent a population of enzymes which cleave choline esters and can be roughly classified as acetycholinesterase and butyrylcholinesterase[1]. The former enzyme occurs above all in the nervous system of animals where it plays a vital role in the cholinergic transfer, the latter enzyme is contained predominantly in blood plasma and its physiologic part has not been elucidated in full[2].

Cholinesterases of various origin differ in their affinity for substrates and inhibitors. They belong to the group of the so-called serine enzymes and are irreversibly inhibited by organophosphorus compounds and certain carbamates and sulfonates[3,4]. Some of these compounds show a high affinity for cholinesterases, like, *e.g.* diisopropylphosphorofluoridate (DFP), tetraethyl pyrophosphate (TEPP), and, among the carbamates, physostigmine or prostigmine[4,5]. To organophosphorus compounds with a high affinity for cholinesterases belongs also isopropyl methyl phosphonofluoridate (IMPF), which was used in this study[3,5]. All the compounds mentioned are biologically very active and highly toxic[3–5] due to their high affinity for cholinesterases. This paper describes the interaction *in vitro* of isopropyl methyl phosphonofluoridate with certain tissue cholinesterases from most commonly used laboratory animals, *i.e.* mouse, rat, and guinea pig.

EXPERIMENTAL

Biological material. Individual tissues — brain, heart, lungs, and liver — were obtained from mice, white rats, and guinea pigs of both sex after sacrificing the animals by scissure of the carotid arteries. The organs were excised, washed with physiological saline, three times repeatedly frozen (−35°C) and thawed, and then homogenized (Ultra-Turrax, Janke-Kunkel) to 10% homogenates in veronal–phosphate buffer at pH 8·0, isotonized by sodium chloride[6]. The homogenates were stored frozen (−35°C) and thawed immediately before use.

Measurement of enzymatic activity. The activity of cholinesterases in homogenates was measured by the electrometric method[6] using a semiautomatic equipment with direct recording[7]. The reaction mixture contained 18 ml of veronal–phosphate buffer at pH 8·0, isotonized by sodium chloride[6], 1 ml of homogenate, and 1 ml of distilled water, when the activity of the intact sample was measured, or 1 ml of aqueous solution of isopropyl methyl phosphonofluoridate of known molar concentration when measuring the activity of the inhibited sample. The measurement was started by the addition of 1 ml of $1 \cdot 10^{-2}$ M acetylcholine iodide. For the measurement of the activity of heart and liver cholinesterase, propionyl and butyryl-choline iodides at the same concentration were also used. All substrates were products of Lachema, Brno. The measurement of enzymatic activity was carried out at 25°C.

Inhibition of tissue cholinesterases by isopropyl methyl phosphonofluoridate. The reaction mixture, which contained 18 ml of veronal–phosphate buffer, 1 ml of the corresponding tissue homogenate, and 1 ml of the aqueous solution of isopropyl methyl phosphonofluoridate was incubated 30 min at 25°C. The prolongation of the incubation period did not lead to a significant increase in the degree of inhibition. The substrate solution (1 ml) was added afterwards and the residual activity was measured.

Calculation of kinetic constants. The affinity constants I_{50} and their negative common logarithms (pI_{50}) were calculated from the probit-logarithmic transformation of the dependence of per cent of inhibition on molar concentration of isopropyl methyl phosphonofluoridate according to the corresponding program in Minsk-22 computer (ref.[7]). The size of Hill coefficients was calculated as the slope of the straight lines expressing the Hill plot of log $(v_i/v_0 - v_i)$ *versus*

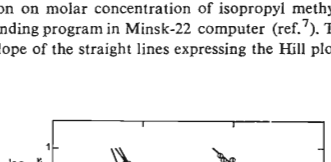

FIG. 1

Dependence of Inhibition of Tissue Cholinesterases of Mouse on Concentration of Isopropyl Methyl Phosphonofluoridate in Hill Plot

○ Lungs, ● brain, ⊙ heart, ◎ liver. The enzyme was incubated 30 min with the inhibitor at 25°C and pH 8·0. The enzymatic activity was measured with acetylcholine as substrate. Propionylcholine and butyrylcholine were also used as substrates for the enzymes from liver and lungs. Each point of the graph represents the average of two measurements.

log [I], where v_i is the initial rate of the inhibited reaction, v_0 the initial rate of the noninhibited reaction, and [I] the molar concentration of the inhibitor. The interpolation of the lines through the experimental points was carried out numerically by the method of regression analysis in the computer. Each experimental point represents the average of two measurements. The bimolecular inhibition rate constants (k_i) were calculated from Aldridge's formula[8] $k_i = 1/t\,[\mathrm{I}] \cdot \ln(v_0/v_i)$, where t is the time for which the enzyme had been preincubated with the inhibitor. The meaning of the remaining symbols is the same as in the preceding case. The value of k_i was calculated also in the computer.

RESULTS

The dependence of the inhibition of mouse, rat, and guinea pig tissue cholinesterases on the concentration of isopropyl methyl phosphonofluoridate in the Hill plot is shown in Figs 1–3. Except for enzymes from mouse and guinea pig liver, the course

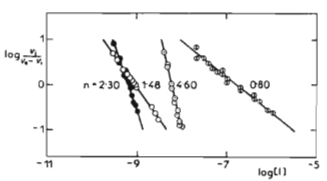

FIG. 2

Dependence of Inhibiton of Tissue Cholinesterases of Rat on Concentration of Isopropyl Methyl Phosphonofluoridate in Hill Plot

○ Lungs, ● brain, ⊘ heart, ⓘ liver. For details see legend to Fig. 1.

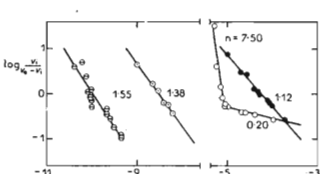

FIG. 3

Dependence of Inhibition of Tissue Cholinesterases of Guinea Pig on Concentration of Isopropyl Methyl Phosphonofluoridate in Hill Plot

● Lungs, ⊖ brain, ⊘ heart, ○ liver. For details see legend to Fig. 1.

of this plot is linear for all the remaining enzymes. The corresponding Hill coefficients together with the pI_{50}-values are summarized in Table I.

The results show that the affinity of isopropyl methyl phosphonofluoridate for cholinesterases from the animals studied is very similar for the individual organs. An exception represents the enzyme from guinea pig brain and lungs. The enzyme from the brain shows an affinity for the inhibitor which is by one order higher than the affinity of the same enzyme from mouse or rat. By contrast, the enzyme from guinea pig lungs is very little sensitive to isopropyl methyl phosphonofluoridate and can thus be described as a resistant type.

A comparison of the bimolecular rate constants k_i, summarized in Table II, points to similar conclusions.

TABLE I

Inhibition of Tissue Cholinesterases of Mouse, Rat and Guinea Pig by Isopropyl Methyl Phosphonofluoridate Size of pI_{50} and Hill coefficients (n)

Measured at 25°C and pH 8·0. The pI_{50}-values are given as average ± confidence limit for $P = 0{\cdot}95$.

Tissue	Mouse		Rat		Guinea pig	
	pI_{50}	n	pI_{50}	n	pI_{50}	n
Brain	8·93 ± 0·03	1·60	9·21 ± 0·02	2·30	10·00 ± 0·07	1·55
Heart	8·86 ± 0·02	1·36	8·21 ± 0·01	4·60	8·52 ± 0·03	1·38
Liver	6·24[a]	0·85—5·0	6·67 ± 0·04	0·80	6·11[a]	0·2—7·5
Lungs	9·29 ± 0·02	2·18	9·05 ± 0·01	1·48	4·25 ± 0·01	1·12

[a] In view of the nonlinear profile of the regression function the confidence limit could not be calculated.

TABLE II

Bimolecular Rate Constants of Inhibition of Tissue Cholinesterases of Mouse, Rat, and Guinea Pig by Isopropyl Methyl Phosphonofluoridate

Measured at 25°C and pH 8·0. The values are given as average ± confidence limit for $P = 0{\cdot}95$. Each constant was calculated from the results of at least 10 measurements.

Tissue	k_i, l . mol^{-1} min^{-1}		
	mouse	rat	guinea pig
Brain	$1{\cdot}84 \pm 0{\cdot}21 \cdot 10^7$	$3{\cdot}58 \pm 0{\cdot}86 \cdot 10^7$	$2{\cdot}23 \pm 0{\cdot}39 \cdot 10^8$
Heart	$1{\cdot}56 \pm 0{\cdot}12 \cdot 10^7$	$4{\cdot}84 \pm 1{\cdot}41 \cdot 10^6$	$7{\cdot}25 \pm 0{\cdot}67 \cdot 10^6$
Liver	$3{\cdot}98 \pm 0{\cdot}64 \cdot 10^4$	$1{\cdot}36 \pm 0{\cdot}31 \cdot 10^5$	$3{\cdot}00 \pm 0{\cdot}71 \cdot 10^3$
Lungs	$4{\cdot}80 \pm 0{\cdot}58 \cdot 10^7$	$2{\cdot}47 \pm 0{\cdot}12 \cdot 10^7$	$3{\cdot}93 \pm 0{\cdot}39 \cdot 10^2$

DISCUSSION

The obtained results show that the cholinesterases from individual tissues of the same animal are not identical. This is evidenced both by the difference in affinity (pI_{50}) and rate (k_i) constants and also by differences in the size of the Hill coefficient. The Hill coefficients are in all cases higher than one and the interaction of isopropyl methyl phosphonofluoridate with the investigated cholinesterases can thus be regarded as allosteric inhibition. Eventhough the cholinesterases have not yet appeared on the list of known allosteric enzymes[9], there is evidence of their allosteric behavior[10,11] and the problem has been devoted considerable attention during the past few years[12,13].

The nonlinear profile of the Hill plot and the corresponding change in the Hill coefficient for the interaction of isopropyl methyl phosphonofluoridate with the cholinesterase of mouse and guinea pig liver indicates most likely the presence of two (or more) cholinesterase types in these organs. A similar, broken, profile of the Hill plot has been observed with the inhibition of mouse liver cholinesterase by pinacolyl methyl phosphonofluoridate[14]. Gel filtration of this material afforded two enzyme fractions with cholinesterase activity, which differed in molecular weight, affinity for pinacolyl methyl phosphonofluoridate, and size of Michaelis constant for choline esters[14].

There is very little difference in the affinity of isopropyl methyl phosphonofluoridate for the same type of tissue cholinesterases in individual animals. An exception represent the enzymes from guinea pig where the difference between the lung and the brain enzyme is especially striking. A comparison of the size of I_{50} shows that the enzyme from brain of guinea pig is 58000-times more sensitive to isopropyl methyl phosphonofluoridate than the enzyme from lungs, while the same ratio for mouse is 0·45 and for rat 1·22.

A problem so far unsolved is to what degree the results of our measurements can be affected by the degradation of isopropyl methyl phosphonofluoridate during the 30 min incubation period by the action of phosphoryl phosphatases present in the biological material[15,16]. In view of the low activity of these enzymes, which inactivate organophosphorus compounds, this seems to be rather improbable. Similarly the distribution of these enzymes in the individual tissues of our experimental animals indicates that this factor cannot significantly influence the results. The relatively highest quantity of these enzymes is contained in the liver[17,18] and the cholinesterase from this tissue is likewise less sensitive to isopropyl methyl phosphonofluoridate; however the cholinesterase from guinea pig lungs, which is inactivated only by high concentrations of the inhibitor, contains only a small amount of phosphoryl phosphatases[18]. An additional decrease of the actual concentration of the inhibitor could result from its nonspecific adsorption to the protein components of the homogenate. However, it has already been reported by Heilbronn[19], who studied horse plasma and a purified preparation of cholinesterase from this source, that this influence is negligible.

REFERENCES

1. Alles G. A., Howes R. C.: J. Biol. Chem. *133*, 375 (1940).
2. Svensmark O.: Acta Physiol. Scand. *64*, Suppl. 245 (1965).
3. Koelle G. B. in the book: *Handbuch der Experimentellen Pharmakologie* (O. Eichler, A. Farah, Eds), Vol. 15. Springer, Berlin 1963.
4. O'Brien R. D.: Ann. N. Y. Acad. Sci. *160*, 204 (1969).
5. O'Brien R. D.: *Toxic Phosphorus Esters*. Academic Press, New York and London 1960.
6. Michel H. O.: J. Lab. Clin. Med. *34*, 1564 (1949).
7. Patočka J., Tulach J.: Sborník věd. prací VLVDÚ Hradec Králové *32*, 361 (1968).
8. Aldridge W. N.: Biochem. J. *46*, 451 (1950).
9. Monod J., Wyman J., Changeux J. P.: J. Mol. Biol. *12*, 88 (1965).
10. Patočka J., Tulach J.: This Journal *34*, 3191 (1969).
11. Aldridge W. N., Reiner E.: Biochem. J. *115*, 147 (1969).
12. Kitz R. J., Braswell L. M., Ginsburg S.: Mol. Pharmacol. *6*, 108 (1970).
13. Gridelet J., Foidart J. M., Wins P.: Arch. Intern. Physiol. Biochim. *78*, 253 (1970).
14. Patočka J.: Sborník věd. prací VLVDÚ Hradec Králové *36*, 39 (1968).
15. Mazur A.: J. Biol. Chem. *164*, 271 (1946).
16. Augustinsson K.-B., Heimbürger G.: Acta Chem. Scand. *8*, 753 (1954).
17. Mounter L. A., Dien L. T. H., Chanutin A.: J. Biol. Chem. *215*, 691 (1955).
18. Murphy S. D., Du Bois K. P.: Proc. Soc. Exptl. Biol. Med. *96*, 813 (1957).
19. Heilbronn E.: Biochim. Biophys. Acta *58*, 222 (1962).

Translated by V. Kostka.