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KINETIC ANALYSIS OF DIFFERENCES IN BRAIN ACETYLCHOLINESTERASE FROM FISH OR MAMMALIAN SOURCES*

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Abstract—A kinetic analysis of the interactions of gold fish and rat brain AChE (acetylcholine acetylhydrolase, EC 3.1.1.7) with acetylthiocholine substrate and sulfonyl fluoride inhibitors revealed several differences between these enzymes. The fish brain AChE had a K_m of 250 μM while the mammalian brain AChE had a K_m of 55 μM under the same assay conditions. In addition, the mammalian brain enzyme reacted with methanesulfonyl fluoride at more than three times the rate at which the fish enzyme reacted, and fish AChE did not react with phenylmethanesulfonyl fluoride at a measurable rate while mammalian AChE was found to react rapidly. Leptocurares were found to accelerate methanesulfonylation of both enzymes; however, they inhibited phenylmethanesulfonylation. These results suggest that fish and mammalian AChE may have topographic differences in the vicinity of the anionic portion of the active site.

Some esters of methanesulfonic acid have been shown to irreversibly inhibit AChE (acetylcholine acetylhydrolase, EC 3.1.1.7) by sulfonylating the active site serine hydroxyl group [1]. In addition, since the rates at which sulfonyl fluorides react with this enzyme are strongly influenced by binding and orientation [2], the sulfonyl fluorides provide sensitive molecular probes of the active site of AChE. In fact, methanesulfonyl fluoride has been used successfully to study interaction topographies of curare-induced conformational changes in AChE [3]. In view of the sensitivity of the sulfonyl fluorides as molecular probes of the active site of AChE, it was of great interest that Turini *et al.* [4] reported that phenylmethanesulfonyl fluoride would react with AChE from human erythrocytes, but would not react with AChE from electric eel as first reported by Fahrney and Gold [2]. This suggested that there might be a difference between the active sites of fish and mammalian AChE that would allow phenylmethanesulfonyl fluoride to react with mammalian, but not fish, enzyme. There are other possible differences, however, that confound the interpretation of this apparent difference. Specifically, human AChE was studied in membrane-bound form while the eel enzyme had been purified. It could be that removing the naturally membrane-bound enzyme from the membrane was the cause of the observed difference. In addition, the tissue of the erythrocyte has a different embryological origin than the electric organ and the difference in enzyme might have been due to tissue, rather than species, differences. The purpose of the present study was to determine the relative reactivities of methanesulfonyl fluoride and phenylmethanesulfonyl fluoride toward AChE from the same tissue, using the same preparation, from fish and mammalian sources and, in addition, to compare the kinetic effects of curares on sulfonylation in these two enzymes.

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MATERIALS AND METHODS

Assay of AChE. A simple brain homogenate was selected for study because the homogenate contains essentially all of the total assayable AChE in the brain as the natural membrane-bound enzyme [5]. Although purification of the enzyme might simplify the interpretation of molecular interactions, it causes the enzyme to be studied under highly artificial conditions, whereas a simple homogenate has the advantage of minimizing the disturbance of the enzyme from its natural state. Insofar as coupling in the membrane may be important for subunit arrangment or conformational changes required for enzyme regulation under natural conditions, the enzyme was not removed from the membrane for further purification. Both rats and fish were killed by decapitation and the brains were removed within 3 min and immediately cooled in 0° phosphate (Na) buffer, pH 7.0, containing 0.02 M $MgCl_2$. Brains from 300-g male albino rats were homogenized as 20% w/v and diluted 1:10 in the same buffer. Brains from 5–7-g gold fish were homogenized as above except, because of the smaller size, as 2% w/v but without further 1:10 dilution. The homogenates were stored at 0° without freezing and were diluted 1:30 into the assay system as a source of AChE activity. The final dilution from whole brain was, therefore 1:1500 in most experiments. Whole blood contained in the homogenate (estimated from hemoglobin content) diluted to the final assay concentration was less than 4×10^{-5}

g/ml. Cholinesterase activity of whole blood diluted to this concentration with either acetylthiocholine or butyrylthiocholine substrates was negligible. In some experiments the fish brain homogenate was diluted 1:5 as an additional dilution to obtain a level of activity equivalent to that obtained in the mammalian brain homogenate. In some experiments, which included potent reversible inhibitors of AChE activity such as succinylcholine and decamethonium, higher concentrations of enzyme were used to obtain enough activity for reliable measurements. AChE activity was assayed by the method of Ellman *et al.* [6] at pH 7.0, 25°, using a model 55 Coleman spectrophotometer as described elsewhere [7] except that the assay buffer contained 0.02 M $MgCl_2$ to replicate the conditions of Belleau *et al.* [3]. Even though AChE has optimum activity at about pH 8 [8], the assays were conducted at pH 7.0 to approximate *in vivo* conditions. All assays were initiated by the addition of 0.1 ml of enzyme preparation into 3.0 ml of total assay medium [7] and were performed in triplicate.

Kinetic studies with substrate. Michaelis constants (K_m values) for acetylthiocholine substrate under the assay conditions described above were calculated by the statistical method of Wilkinson [9] using initial velocities obtained over a substrate range from 25 to 500 μM. In these experiments, cuvettes containing the assay medium were equilibrated to 25.0° with stirring for 5 min. After the addition of the enzyme, 90 sec was allowed for mixing and then the change in absorbance was monitored for 2 min.

Sulfonylation studies. One-ml aliquots of the enzyme preparation were placed in 12 × 75 mm tubes with stirring and were equilibrated to 25.0° in 10 min. If a ligand was to be included in the sulfonylation reaction, it was added at the beginning of the equilibration. At the end of 10 min, a 0.1-ml aliquot of the enzyme preparation was removed and assayed at 0.5 mM substrate as a measure of activity present before the sulfonylation reaction was begun. The sulfonylation reaction was initiated by rapid addition of 0.1 ml of a solution of sulfonyl fluoride in ethanol. The ethanol had no effect on the enzyme activity. Immediately after the addition of the sulfonyl fluoride and at specific times thereafter, 0.1-ml aliquots of the reaction mixture were removed and assayed at 0.5 mM substrate for 2 min for the determination of residual activity. Since the sulfonylation studies were performed under conditions which gave first-order kinetics, the enzyme activity in the aliquot of the reaction mixture removed and quenched immediately after the addition of the sulfonyl fluoride was taken as the zero time value of 100 per cent activity for each experiment.

Linear reaction rates between enzyme and substrate in the assay medium indicated that the sulfonylation reaction was adequately quenched by the 1:30 dilution into the assay medium and by the protection of the active site by the presence of 0.5 mM substrate. All sulfonyl fluoride experiments were replicated at least three times and the control conditions (no modifiers present during sulfonylation) were replicated at least five times to provide accurate rate constant determination.

Materials. Acetylthiocholine bromide, butyrylthiocholine chloride, and the 5′,5′, dithio-bis-(2-nitrobenzoic acid) required for the assay were purchased from Sigma Chemical Co. (P.O. Box 14508, St. Louis, MO 63178). Phenylmethanesulfonyl fluoride was obtained from CalBiochem (P.O. Box 12087, San Diego, CA 92112) and methanesulfonyl fluoride was obtained from Aldrich (940 W. Saint Paul Ave., Milwaukee, WI 53233). Tetramethyl ammonium hydroxide, succinylcholine chloride, hexamethonium bromide, decamethonium bromide, *d*-tubocurarine chloride, and acetyl-β-methyl choline bromide were all obtained from Sigma Chemical Co.

RESULTS

Kinetic studies with substrate. The Michaelis constant (K_m) for AChE in the rat brain homogenate, with acetylthiocholine substrate under the assay conditions described, was 55 μM with a standard error of 2.4 μM. This K_m is virtually identical to the K_m reported earlier, which was determined under essentially the same assay conditions with AChE solubilized from rat brain by Triton X-100 in a partially purified preparation [10]. The K_m for AChE in the fish brain homogenate with identical substrate and assay conditions was 250 μM with a standard error of K_m of 16 μM. This difference in K_m was replicated in three separate experiments using different homogenates in each experiment.

In view of the large difference in K_m and the unpurified nature of the enzyme preparation, one explanation could be that either the fish or the rat brain contained significant cholinesterase activity from butyrylcholinesterase (EC 3.1.1.8) while the other preparation did not. A mixture of enzyme activity in one preparation and not in the other would result in an apparent difference in K_m. To test this possibility, activity toward 0.5 mM butyrylthiocholine was determined from both the fish and rat AChE preparations. The fish AChE preparation showed virtually no activity with butyrylthiocholine substrate while the rat AChE preparation showed less than 5 per cent of the activity with 0.5 mM butyrylthiocholine substrate than was observed with 0.5 mM acetylthiocholine. The finding that whole rat brain homogenate shows less than 5 per cent activity toward butyrylthiocholine as compared to acetylthiocholine confirms an earlier report by Ellman *et al.* [6], who reported 3 per cent under similar assay conditions. The finding that neither preparation showed significant activity toward butyrylthiocholine substrate makes an explanation of the large difference in K_m on the basis of a mixture of enzymes unsatisfactory. The activity observed in both preparations was specific to acetylthiocholine substrate.

As a further study of enzyme specificity, affinity toward acetyl-β-methyl choline was determined. Insofar as acetyl-β-methyl choline is hydrolyzed only by AChE and not by butyrylcholinesterase, which has a very low affinity for this substrate [8], hydrolysis of and affinity for acetyl-β-methyl choline would support the argument that the enzyme studied in both fish and mammalian samples was AChE. In the assay system used in this research, the hydrolysis of acetyl-β-methyl choline would appear as pure competitive inhibition of the hydrolysis of acetylthiocholine when

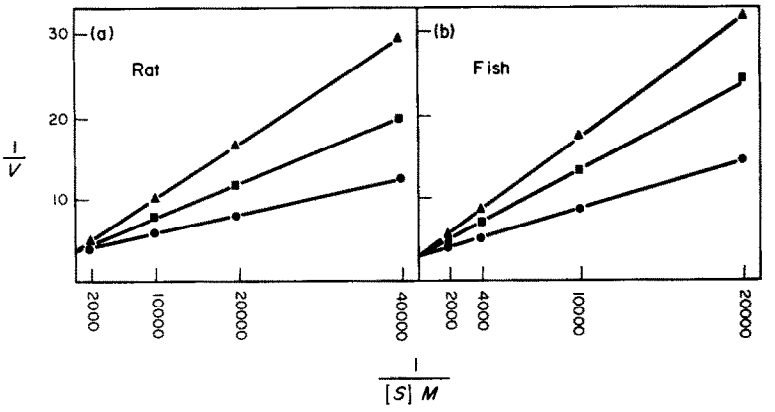

Fig. 1. Double reciprocal plot of the inhibition of acetylthiocholine hydrolysis by AChE caused by the inclusion of acetyl-β-methyl choline substrate. The control condition (no inhibitor) is shown by circles, 0.5 mM acetyl-β-methyl choline by squares, and 1.0 mM acetyl-β-methyl choline by triangles. Apparent K_m values for rat brain AChE (A) are 55, 107 and 171 μM for the control, 0.5, and 1.0 mM acetyl-β-methyl choline conditions respectively. V_{max} values are 100, 96 and 105 per cent for the control, 0.5 mM and 1.0 mM acetyl-β-methyl choline conditions respectively. The apparent K_i for acetyl-β-methyl choline computed from these data is 0.5239 + 0.031 mM. Apparent K_m values for fish brain AChE (B) are 250, 435 and 507 μM for the control, 0.5 and 1.0 mM acetyl-β-methyl choline conditions respectively. V_{max} values are 100, 103 and 91 per cent for the control, 0.5 and 1.0 mM acetyl-β-methyl choline conditions respectively. The apparent K_i for acetyl-β-methyl choline computed from these data is 0.6535 ± 0.044 mM.

both substrates were included in the assay system. The results from these experiments are shown as double reciprocal plots in Fig. 1, where acetyl-β-methyl choline is analyzed as an inhibitor of acetylthiocholine hydrolysis. As expected in the case of AChE, acetyl-β-methyl choline shows pure competitive inhibition. These results argue against a mixture of AChE with other esterases as an explanation of the apparent difference between fish and mammalian AChE.

One unexpected result was that fish brain homogenate contained approximately five times the amount of AChE activity per gram of wet brain weight as compared to the mammalian brain homogenate. The fish brain contained 3.25×10^{-5} moles/g/min activity (standard error of 7×10^{-9} moles/g/min) while the mammalian brain contained only 6.57×10^{-6} moles/g/min (standard error of 2.2×10^{-8} moles/g/min).

Sulfonylation studies. Figure 2 shows representative plots of the time course for irreversible inhibition of fish and rat brain AChE by 6×10^{-4} M methanesulfonyl fluoride. These data are plotted in accordance with the first-order rate law and, in accordance with this law, these plots are linear for more than 90 per cent inactivation of the enzyme. Pseudo-first-order rate constants can also be determined from these data and are 0.1216 min^{-1} for rat brain AChE and 0.0325 min^{-1} for fish brain AChE. These rates show that rat brain AChE is 3.74 times more reactive with 6×10^{-4} M methanesulfonyl fluoride than fish brain AChE. These figures also show the effects of alkyltrimethylammonium ion ligands on the rate of sulfonylation of these enzymes. Belleau *et al.* [3] have observed that leptocurares (polymethoniums of the decamethonium series, including succinylcholine) accelerate methanesulfonylation of erythrocyte AChE, with the greatest acceleration in the presence of succinylcholine and less acceleration in the presence of decamethonium, tetramethonium and hexamethonium in that order. In addition, Belleau *et al.* [3] observed that pachycurares (e.g. *d*-tubocurarine and gallamine) protect against

Table 1. Effects of lepto- and pachycurares on the rates of methanesulfonylation of fish and mammalian brain AChE*

Ligand	Ligand concn (M)	Methanesulfonylation acceleration		Phenylmethanesulfonylation acceleration
		Fish AChE	Mammalian AChE	Mammalian AchE
Tetramethonium	1×10^{-2}	4.14	5.38	2.09
Hexamethonium	6.5×10^{-1}	2.74	2.18	0.37
Decamethonium	5×10^{-5}	6.68	8.35	0.50
Succinylcholine	9×10^{-3}	30.30	21.20	0.54
d-Tubocurarine	5×10^{-3}	0.73	0.69	0.53

*Acceleration or inhibition values represent the ratios of the initial slopes shown in Figs. 2 and 3. Values greater than 1.0 indicate acceleration while values less than 1.0 indicate inhibition. Since the slopes are determined from a 100 per cent value obtained in the presence of the ligands listed above, the direct inhibitory effects of these compounds on AChE activity have been compensated for automatically. Ligand concentrations used in these experiments are after Belleau *et al.* [3].

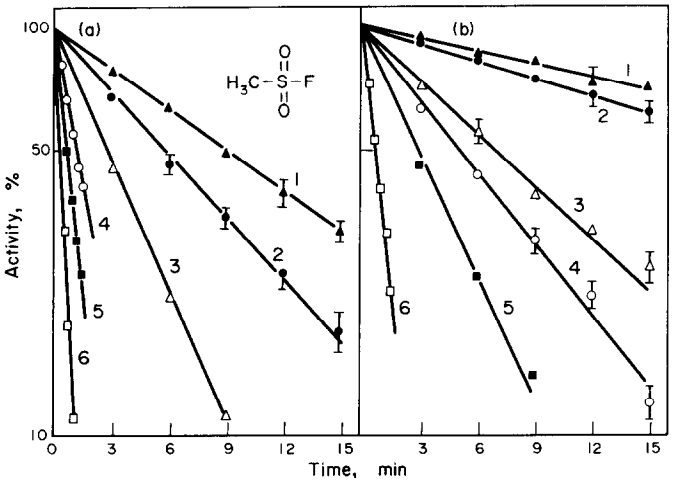

Fig. 2. Progressive irreversible inhibition of AChE from rat brain (a) and fish grain (b) by 6×10^{-4} M methanesulfonyl fluoride. The numbered lines represent inhibition by methanesulfonyl fluoride in the presence of various modifiers which are as follows: line 1 (filled triangles) 5×10^{-3} M *d*-turbocurarine; line 2 (filled circles) control condition (no modifier); line 3 (open triangles) 6.5×10^{-1} M hexamethonium; line 4 (open circles) 10^{-2} M tetramethonium; line 5 (filled squares) 5×10^{-5} M decamethonium; and line 6 (open squares) 9×10^{-3} M succinylcholine. The pseudo-first-order rate constant for rat brain AChE for the control condition is 0.1216 min^{-1} while the second-order rate constant calculated by dividing the pseudo-first-order rate constant by the concentration of the sulfonylfluoride is 202.63 M^{-1} min^{-1}. The pseudo-first-order rate constant for the fish brain AChE for the control condition is 0.0325 min^{-1} while the second-order rate constant is 54.18 M^{-1} min^{-1}. Actual values of acceleration or inhibition caused by the modifiers are shown in Table 1.

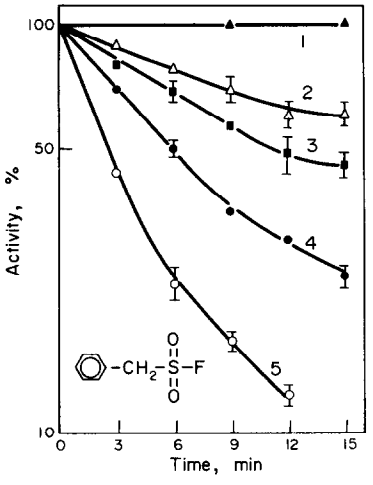

Fig. 3. Progressive irreversible inhibition of rat and fish brain AChE by 7.5×10^{-4} M phenylmethanesulfonyl fluoride. Line 1 (filled triangles) represents the reaction between fish brain AChE and phenylmethanesulfonyl fluoride. Line 2 (open triangles) represents the reaction between rat brain AChE and phenylmethanesulfonyl fluoride in the presence of 6.5×10^{-1} M hexamethonium. Line 3 (filled squares) represents the reaction between rat brain AChE and phenylmethanesulfonyl fluoride in the presence of 5×10^{-3} M *d*-tubocurarine, 5×10^{-5} M decamethonium, and 9×10^{-3} M succinylcholine, all of which produced virtually identical lines. Line 4 (filled circles) is the control condition showing inhibition of rat brain AChE by phenylmethanesulfonyl fluoride without any modifiers present. Line 5 (open circles) shows the inactivation of rat brain AChE in the presence of 10^{-2} M tetramethonium. Rate constants for the reaction between rat brain and phenylmethanesulfonyl fluoride were not determined because the reaction was not followed long enough to exhaust the more reactive form of the enzyme which would be necessary for accurate determination. Actual values of acceleration or inhibition caused by the modifiers are shown in Table 1.

methanesulfonylation. As shown in Fig. 2, the same relationship is observed in both fish and mammalian AChE preparations. The acceleration and inhibition values for the two enzyme preparations are shown in Table 1.

Figure 3 shows the irreversible inhibition of mammalian brain AChE by 7.5×10^{-4} M phenylmethanesulfonyl fluoride and the effects of various curares on the rate of phenylmethanesulfonylation of this enzyme. In addition, Fig. 3 shows that fish brain AChE did not react at a measurable rate with phenylmethanesulfonyl fluoride. In contrast to the acceleration of methanesulfonylation produced by the leptocurares, phenylmethanesulfonylation was inhibited by the leptocurares as well as the pachycurare *d*-tubocurarine. The only acceleration observed was produced by tetramethonium. Actual values of acceleration or inhibition are shown in Table 1. Another difference between phenylmethanesulfonylation and methanesulfonylation was that the plots for the reaction between phenylmethanesulfonyl fluoride and mammalian brain AChE were nonlinear whereas the plots for methanesulfonylation were strictly linear. In order to investigate the phenylmethanesulfonylation reactions, mammalian brain AChE was inactivated by lower concentrations of phenylmethanesulfonyl fluoride and the reaction was followed in more detail, as shown in Fig. 4. One explanation for the nonlinear character of these plots might be that two or more forms of the enzyme are reacting at different rates. The different forms could be either different subsites on the same enzyme molecule which react at different rates with phenylmethanesulfonyl fluoride or a mixture of different isozymes. In either case, the kinetic results would be the same. The data shown in Fig. 4 were analyzed assuming only two forms even though there

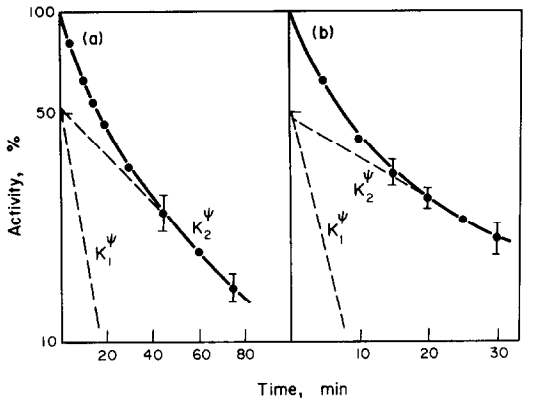

Fig. 4. Progressive irreversible inhibition of rat brain AChE by phenylmethanesulfonyl fluoride. Plot a shows inhibition by 2.5×10^{-4} M phenylmethanesulfonyl fluoride. The pseudo-first-order rate constant calculated for the more reactive form is 0.090 min^{-1} while the corresponding second-order rate constant is 360 M^{-1} min^{-1}. The pseudo-first-order rate constant calculated from the less reactive form from the later portion of the curve after the more reactive form had disappeared is 0.017 min^{-1} while the corresponding second-order rate constant is 67 M^{-1} min^{-1}. The dotted line computed from least-squares linear regression from the later portion of curve a shows that the less reactive form of the enzyme accounts for 52 per cent of the total enzyme activity. Plot b shows inhibition by 5.0×10^{-4} M phenylmethanesulfonyl fluoride. The pseudo-first-order rate constant calculated for the more reactive form is 0.208 min^{-1} while the second-order rate constant is 416 M^{-1} min^{-1}. The pseudo-first-order rate constant for the less reactive form is 0.028 min^{-1} while second-order rate constant is 57 M^{-1} min^{-1}. The dotted line computed from least-squares linear regression from the later portion of curve b shows that the less reactive form of the enzyme accounts for 48 per cent of the total enzyme activity.

may have been more than two different reaction rates present. As shown in Fig. 4, after some time, the curve becomes "linear" because the more reactive enzyme has disappeared. The final slope, therefore, gives the pseudo-first-order rate constant k_2^ψ for the less reactive form. Subtraction of the values along the extrapolated final slope from the observed values gives a second line from which the pseudo-first-order rate constant k_1^ψ can be calculated for the more reactive form. The intercept of the extrapolated line gives the fraction of the enzyme, about 50 per cent, which reacts at the faster rate k_1^ψ. These values are approximations for two reasons: (1) it was assumed that there were only two subspecies of the enzyme and (2) the two subspecies appeared to differ in reactivity only about 6-fold and a greater difference is necessary to achieve good resolution of the rate constants.

DISCUSSION

The finding that phenylmethanesulfonyl fluoride reacts at different rates with two or more forms of the mammalian brain AChE was not expected. These results are, however, consistent with those of Main [11], who found similar kinetic evidence of multiple forms of erythrocyte AChE using organophosphate inhibitors. Insofar as much evidence has accumulated which suggests that AChE from various sources consists of isozymes [12–14], and that AChE from mammalian erythrocytes can be separated into two subspecies in a ratio of approximately 1:1 by affinity chromatography [15], it is not surprising to find that phenylmethanesulfonyl fluoride may react at different rates with different subspecies.

The results obtained with butyrylthiocholine and acetyl-β-methyl choline substrates suggest that virtually all enzyme activity from both sources was due to AChE. The results from the methanesulfonylation experiments which show that these two enzymes are affected in substantially the same manner by the curares studied during methanesulfonylation suggest that the peripheral binding sites occupied by the lepto- and pachycurares as described by Belleau *et al.* [3] are identical in both fish and mammalian brain AChE.

There does, however, appear to be some difference between mammalian and fish AChE insofar as there was approximately a 5-fold difference in K_m between these two sources of enzyme, and methanesulfonyl fluoride reacted with mammalian AChE nearly four times as fast as with fish AChE. The difference was more obvious in the phenylmethanesulfonyl fluoride experiments, where it was found that phenylmethanesulfonyl fluoride did not react at a measurable rate with fish enzyme but reacted rapidly with mammalian enzyme. Inasmuch as the only difference between phenylmethanesulfonyl fluoride and methanesulfonyl fluoride is the phenyl group, it appears that the mammalian enzyme active site can accommodate the phenyl group in such a way that the orientation of the sulfonyl group is suitable for reaction while the active site topography of the fish enzyme is different and the orientation of the sulfonyl group is unsuitable for reaction.

The finding that the leptocurares inhibited the rate of phenylmethanesulfonylation suggests that the phenyl group must be oriented toward the anionic portion of the active site if the orientation of the leptocurares proposed by Belleau *et al.* [3] is correct. For the same reason, it is probably the topography in the vicinity of the anionic portion of the active site that is different in the fish enzyme that prevents phenylmethanesulfonylation. These results, while limited by the fact that they were obtained from kinetic analysis, suggest that the implicit assumption that AChE from all species is the same may not be experimentally correct. Furthermore, generalizations between species concerning pharmacodynamic effects of drugs on AChE may, in some cases, be invalid.

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