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MULTIPLE BINDING OF D-TUBOCURARINE TO ACETYLCHOLINESTERASE

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Abstract—The binding of D-tubocurarine (TC) to acetylcholinesterase (AChE) was studied using different methods of enzyme kinetics. The main results are as follows. TC reversibly inhibits the hydrolysis of different substrates of AChE with three different inhibition constants (K_{i1} = $7.0 \pm 0.8 \times 10^{-5}$ M, $K_{i2} = 3.1 \pm 1.0 \times 10^{-4}$ M, and $K_{i3} = 4.2 \pm 0.5 \times 10^{-3}$ M). Reference inhibitors tetramethylammonium (TMA), tetraethylammonium (TEA), and decamethonium (C-10) inhibit the hydrolysis of different substrates with constants, which are the same for each individual inhibitor. These three inhibitors compete with TC in the inhibition of enzymatic hydrolysis of acetylthiocholine (ASCh); all three of them affect the noncompetitive component of the inhibition of the hydrolysis of ASCh by TC, which arises from the binding of TC to the peripheral anionic site of AChE, but TEA and C-10 affect also the competitive component of this inhibition, which arises from the binding of TC at the catalytic anionic site. TC partially inhibits the methanesulfonylation of AChE; dissociation constant for TC in this process is $K_A = 4.5 \times 10^{-4}$ M. All our results lead to the conclusion that TC binds to three regions on the active surface of AChE. The first region is at the peripheral anionic site; the other two regions are situated in the vicinity of the catalytic anionic site and the esteratic site.

Active surface of AChE* (acetylcholine hydrolase, EC 3.1.1.7) consists of the active center and one or more peripheral anionic sites (cf. [1, 2]). The structure and role of both parts of the active centre, the esteratic and the catalytic anionic site, have already been well established (cf. [3, 4]), but the number of peripheral anionic sites, their arrangement, structure, and physiological role are still poorly understood. Accordingly, in some instances the binding of a ligand to the active surface of AChE is not sufficiently elucidated. This is also true of some pharmacologically interesting ligands, among which TC, a neuromuscular blocking agent and also a reversible inhibitor of AChE, seems to be especially interesting. It is shown that TC binds to AChE to more than one binding site (cf. [5–9]). However, the number of binding sites for TC, their location on the enzyme, and their interrelations are not clear. Consequently, further studies of the binding of TC to AChE seemed worth undertaking, in order to elucidate in some detail the binding of TC to AChE, and to provide additional data on the active surface of this enzyme. The following investigations were carried out in our laboratory: (i) inhibition of the hydrolysis of various substrates of AChE by TC, TMA, TEA, and C-10; (ii) competition between these inhibitors in the inhibition of ASCh hydrolysis; and (iii) influence of TC on methanesulfonylation of AChE. The results of these investigations are reported and discussed below.

MATERIALS AND METHODS

1. *Inhibition of the enzymatic hydrolysis of various substrates of AChE by TC, TMA, TEA and C-10.* The inhibition of the enzymatic hydrolysis of ten substrates of AChE by TC was investigated. The substrates used were ASCh, BCh, AMCh, iPA, IxA, 2NA, pNPhA, IPhA, EA and MA, respectively. Besides, the inhibition of the hydrolysis of ASCh, pNPhA and EA by TMA, TEA and C-10 was also studied. The listed substrates differ considerably in structure, length, charge, hydrophobicity and flexibility of their molecules. It was proposed that structurally different substrates bind into the active centre of AChE in different ways, although they probably all react with the same active serine —OH group in the esteratic site of the enzyme (cf. [10, 11]). According to this, these substrates were used as probes to monitor the binding of TC and other inhibitors to the sites in or in the vicinity of active centre of AChE.

For each inhibitor the mode of inhibition of AChE, the corresponding inhibition constants and Hill coefficient were determined in the presence of each substrate. This was done using the graphical methods of Lineweaver and Burk [12], Dixon [13], Cleland [14] and Loftfield and Eigner [15]. In cases of partially competitive inhibition‡ both constants, K_i and K_{ii} [16], were determined. The values of K_i were first estimated from the limiting slopes of the corresponding Dixon [13] and secondary plots [14] at

* Abbreviations used: AChE, acetylcholinesterase; AMCh, acetyl-β-methylcholine; ASCh, acetylthiocholine; BCh, butyrylcholine; C-10, decamethonium; EA, ethylacetate; iPA, i-pentylacetate; IPhA, indophenylacetate; IxA, indoxylacetate; MA, methylacetate; MSF, methanesulfonylfluoride; 2NA, 2-naphthylacetate; pNPhA, p-nitrophenylacetate; TC, D-tubocurarine; TEA, tetraethylammonium; TMA, tetramethylammonium.

† Types of inhibition are defined according to Dixon and Webb [18].

low concentrations of inhibitors [16], and then the values of K_i and K_{ii} were calculated by means of the HYPRPLT computer program [16, 17].

To analyze the experimental data obtained from the inhibition of the hydrolysis of ASCh by TC, which were not in accordance with classical inhibition schemes which take into account only one binding site for inhibitor on the enzyme [18, 19], the inhibition scheme (Scheme 1) was constructed, which takes into account the binding of inhibitor to the enzyme at two sites—the catalytic and the peripheral anionic sites:

$$\begin{array}{ccccc} & & \overset{K_I}{} & \overset{K_s}{} & \overset{k_1}{} \\ EI & \rightleftharpoons & I + E + S & \rightleftharpoons & ES \rightarrow E + P \\ + & & + & & + \\ I & & I & & I \\ \Updownarrow & K_i' & \Updownarrow K_i & & \Updownarrow K_{ii} \\ & & & \overset{k_2}{} & \\ EII^* & \rightleftharpoons & I + EI^* + S & \rightleftharpoons & ESI^* \rightarrow EI^* + P \end{array}$$

Scheme 1

where E is enzyme (AChE); S substrate (ASCh); P product; I inhibitor (TC) – I^* is inhibitor bound to the peripheral site; ES, EI, EI^*, ESI^* and EII^* the corresponding complexes between enzyme, substrate and inhibitor, K_s, K_I, K_i', K_i and K_{ii} the corresponding equilibrium constants; and k_1 and k_2 the rate constants.

Using rapid equilibrium treatment (cf. [19]), the following rate equation for Scheme 1 was derived:

$$v = \frac{V \cdot \dfrac{1 + (k_2/k_1) \cdot (I)/K_{ii}}{1 + (I)/K_{ii}}}{1 + \dfrac{K_s}{(S)} \cdot \dfrac{1 + (I)/K_i + (I)/K_I + (I)^2/(K_I \cdot K_i')}{1 + (I)/K_{ii}}} \quad (1)$$

from which also the equation for slope (Eqn 2) and vertical intercept (Eqn 3) of the lines from Lineweaver and Burk plot were derived:

$$\text{Slope} = \frac{K_s \cdot [1 + (I)/K_i + (I)/K_I + (I)^2/(K_I \cdot K_i')]}{V \cdot [1 + (k_2/k_1) \cdot (I)/K_{ii}]} \quad (2)$$

$$\text{Intercept} = \frac{1 + (I)/K_{ii}}{V \cdot [1 + (k_2/k_1) \cdot (I)/K_{ii}]} \quad (3)$$

Equations 1–3 were used for analyses of experimental data as described later (see Results and Discussion).

Experiments were done at 25° and pH 8.0 in redistilled water. NaCl was added to all solutions to a final ionic strength of 0.2 M. The activity of AChE was followed titrimetrically with 0.01 M NaOH. Since pNPhA is subjected to a rather fast spontaneous hydrolysis at pH 8.0 [20], the solution of this substrate was always prepared at pH 5.5 and was corrected to pH 8.0 immediately before measurement; approximately 2% of pNPhA, hydrolysed during the pH correction was taken into account in analyses of experimental data. To improve the solubility of 2NA and IPhA, methanol was added to the reaction mixture of these substrates to a final concentration of 2%; methanol reduced the activity of AChE for less than 5%.

2. *Competition between TC and TMA, TEA and C-10, respectively, in the inhibition of the enzymatic hydrolysis of ASCh.* The inhibition of the enzymatic hydrolysis of ASCh by TC was followed in the presence of TMA, TEA and C-10, respectively. The aim of this part of the work was to determine whether or not TMA, TEA and C-10 compete with TC and, consequently, to find out if these ligands inhibit the hydrolysis of ASCh by binding to the same site(s) on the enzyme. The initial rate of the enzymatic hydrolysis of ASCh in various concentrations was followed in the presence of inhibitor pairs TC–TMA, TC–TEA and TC–C-10, respectively. The concentration of TC was varied, while the concentration of TMA, TEA and C-10, respectively, was kept constant; three different fixed concentrations of TMA, TEA and C-10 were always used. The obtained data were analysed graphically according to Lineweaver and Burk [12], Dixon [13], Cleland [14] and Eisenthal and Cornish-Bowden [21].

The experiments were done at 25° and pH 8.0 in the universal buffer solution of Britten and Robinson [22], with the ionic strength of 0.2 M, obtained by addition of NaCl. The activity of AChE was followed by the method of Ellman *et al.* [23].

3. *Influence of TC on methanesulfonylation of AChE.* Methanesulfonylation of AChE in the presence and absence of TC was investigated. Since MSF is a small molecule which binds only to the esteratic site of the enzyme [24, 25], the investigation of the influence of TC on methanesulfonylation can lead to new data on the binding of TC to or close to the esteratic site. Methanesulfonylation of AChE in the presence of a reversible ligand A (in our case TC) follows the equation [26]:

$$\ln \frac{(E)}{(E)_0} = -\frac{k + k_A \cdot \dfrac{(A)}{K_A}}{1 + \dfrac{(A)}{K_A}} \cdot (I) \cdot t = -k_a \cdot (I) \cdot t \quad (4)$$

where (E) is the concentration of uninhibited AChE, $(E)_0$ the total concentration of AChE, k the second-order rate constant for methanesulfonylation, (I) the concentration of MSF, t time, k_A the second-order rate constant for methanesulfonylation of AChE–ligand A complex, (A) the concentration of ligand A, K_A the dissociation constant for the binding of ligand A to AChE, and k_a the second-order rate constant for methane-sulfonylation of AChE in the presence of ligand A in concentration (A).

It follows from Eqn (4) that [27]

$$\frac{k_a - k_A}{k - k_a} = K_A \frac{1}{(A)} \quad (5)$$

The enzyme was incubated for various lengths of time with MSF in various concentrations, with added TC or without it, and the remaining activity of AChE determined according to Ellman *et al.* [23]. The values of k, k_a and k_A were determined as described elsewhere [25, 27]. From these data, the dissociation constant for the binding of TC to AChE, K_A, was determined graphically by means of Eqn (5). Experi-

ments were done at 25° in the universal buffer solution of Britten and Robinson [22]. The pH was 8.4, which is the optimal value for methanesulfonylation of AChE at 25° [25]. The ionic strength was adjusted to 0.2 M with NaCl.

4. Materials. The enzyme used was AChE from the electric organ of *Electrophorus electricus*, purchased from Worthington, NJ. ASCh iodide, BCh iodide, pNPhA, TMA iodide, TEA iodide, and 5,5-dithiobis-nitrobenzoic acid (Ellman's reagent) were from BDH Biochemicals Ltd (Poole, U.K.); TC chloride, IxA, IPA, 2NA, EA and MA were from Fluka AG (Buchs, Switzerland); AMCh chloride was from Savory & Moore Ltd, (London, U.K.); IPhA and C-10 iodide were from Koch-Light Ltd (Colnbrook Bucks, U.K.); and MSF was from Eastman Organic Chemicals (Rochester, U.S.A.).

Automatic titration was performed by Radiometer automatic titrator; absorption of light was measured by Opton PMQ3 spectrophotometer; and computer programs were run on a Digital PDP-11/34 computer and on a Commodore microcomputer.

RESULTS AND DISCUSSION

1. Inhibition of the hydrolysis of various substrates of AChE by TC, TMA, TEA and C-10

It was found that TC inhibits the hydrolysis of all used substrates. The experimental data (diagrams not shown) for the inhibition of hydrolysis of BCh, AMCh, iPA, IxA, pNPhA, 2NA, IPhA, EA and MA by TC are all in accordance with classical inhibition schemes for pure or partially competitive inhibition [18, 19]. Results obtained with these nine substrates are summarized in Table 1. The inhibition pattern for the inhibition of the hydrolysis of ASCh by TC, however, is quite different. Therefore, the results obtained with ASCh will be presented and discussed separately.

Table 1. Characteristic data for the inhibition of enzymatic hydrolysis of different substrates of acetylcholinesterase by tubocurarine

Substrate	K_m^* (mol l^{-1})	Type of inhibition	K_i (mol l^{-1})	K_{ii}‡ (mol l^{-1})	$\Delta\Delta G$¶ (kJ mol^{-1})	n_H**	n_H††
BCh	1.2×10^{-4}	partially competitive	6.6×10^{-5}† 6.3×10^{-5}§ 6.6×10^{-5}‖	2.7×10^{-4}	3.5	0.75	0.78
AMCh	1.4×10^{-3}	partially competitive	7.2×10^{-5}† 7.7×10^{-5}§ 8.1×10^{-5}‖	4.3×10^{-4}	3.9	0.76	0.78
iPA	7.4×10^{-3}	partially competitive	8.6×10^{-5}† 7.5×10^{-5}§ 5.5×10^{-5}‖	1.5×10^{-4}	2.5	0.51	0.73
IxA	1.4×10^{-2}	partially competitive	6.6×10^{-5}† 6.8×10^{-5}§ 7.0×10^{-5}‖	5.2×10^{-4}	4.9	0.66	0.73
2NA	1.2×10^{-2}	partially competitive	3.0×10^{-4}† 3.8×10^{-4}§ 1.8×10^{-4}‖	3.3×10^{-4}	1.6	0.79	0.78
pNPhA	1.3×10^{-3}	partially competitive	4.3×10^{-4}† 4.9×10^{-4}§ 2.5×10^{-4}‖	5.0×10^{-4}	1.7	0.70	0.71
IPhA	4.1×10^{-4}	competitive	2.1×10^{-4}† 2.0×10^{-4}§			0.85	0.83
EA	0.7	competitive	4.3×10^{-3}† 5.0×10^{-3}§			0.75	0.62
MA	1.1	competitive	3.8×10^{-3}† 3.8×10^{-3}§			1.02	0.62

K_m is Michaelis constant, K_i and K_{ii} inhibition constants, $\Delta\Delta G$ difference between the free energy of binding of tubocurarine to acetylcholinesterase and to acetylcholinesterase–substrate complex, respectively, and n_H Hill coefficient.

* From Lineweaver–Burk plot [12].

† From secondary plot of slopes of lines from Lineweaver–Burk plot [12] against concentration of tubocurarine [14].

‡ From secondary plot of intersections of lines from Lineweaver–Burk plot [12] against concentration of tubocurarine [14].

§ From Dixon plot [13].

‖ Obtained by means of HYPRPLT computer program [17].

¶ Calculated from $\Delta\Delta G = -RT \ln K_i + RT \ln K_{ii}$; K_i and K_{ii} obtained by means of HYPRPLT computer program [17] were used in calculations.

** From plot according to Loftfield and Eigner [15].

†† From Hill equation [36]; see text for calculation procedure.

It should be mentioned first that a minor part of constants, shown in Table 1, has already been determined by other authors; the values available in the literature are identical or very close to our values (compare the values of K_m for BCh, AMCh, iPA, IxA, pNPhA, and EA from Table 1 with the corresponding ones from refs 28–31, 11, 10 and 30, respectively).

As can be seen from Table 1, the values of inhibition constants for TC, determined in the presence of different substrates, are not the same. They can be arranged in three groups:

(i) The first group for the inhibition of the hydrolysis of BCh, AMCh, iPA and IxA; the mean value of all K_i in this group is $7.0 \pm 0.8 \times 10^{-5}$ M (N = 12) and will be symbolized in text by K_{i1}.

(ii) The second group for the inhibition of the hydrolysis of 2NA, pNPhA and IPhA; the mean value of all K_i in this group (K_{i2}) is $3.1 \pm 1.0 \times 10^{-4}$ M (N = 8).

(iii) The third group for the inhibition of the hydrolysis of EA and MA; the mean value of all K_i in this group (K_{i3}) is $4.2 \pm 0.5 \times 10^{-3}$ M (N = 4).

The values of K_i within each group lie in the narrow range (Table 1), but the mean values for K_i of each group (K_{i1}–K_{i3}) are significantly different. Two explanations of these results come into mind: either TC inhibits the hydrolysis of substrates by binding to three different sites on the active surface of AChE, or TC inhibits the hydrolysis of substrates by binding to only one binding site, but different substrates induce different conformations of the enzyme molecule and in this way modify the affinity of TC for this site.

As already mentioned, the inhibition of the hydrolysis of ASCh by TC is quite different from that obtained with other substrates. The inhibition is of mixed type (TC affects apparent Michaelis constant and maximal velocity of ASCh hydrolysis) and is characterized by linear Lineweaver–Burk plot, linear replot of slopes of Lineweaver–Burk lines versus concentration of TC, hyperbolical replot of intercepts of Lineweaver–Burk lines versus concentration of TC and hyperbolical Dixon plot (both replots are shown in Fig. 1). As found by computer simulation of the above listed plots, using the corresponding equations from literature [18, 19], the inhibition pattern is not in accordance with any classical inhibition scheme for the mixed inhibition, which takes into account only one binding site for inhibitor. By the same computer simulation procedure, but using Eqns 1–3, relevant for Scheme 1 (see Methods) it was found that the inhibition of the hydrolysis of ASCh by TC is qualitatively in accordance with Scheme 1.

In the next step of investigations the inhibition was quantitatively analyzed and the relevant kinetic constants of the system were determined, as follows. The value of K_{ii} and the ratio k_1/k_2 were obtained from the hyperbolic replot of intercepts of Lineweaver–Burk lines versus concentration of TC (Fig. 1b), using HYPRPLT computer program [17]—compare Eqn 3 with the corresponding equation in HYPRPLT program. Other three constants (K_I, K_i, and K_i') can not be obtained directly either graphically or mathematically as independent constants. However, predicting one of them, the other two constants can be calculated from Eqn 2 and the experimental data from Fig. 1a. Assuming that the value of K_I is identical to one of three values of inhibition constants of TC ($K_{il} - K_{i3}$, see above) obtained with other substrates—not an unreasonable assumption—each of these three constants was taken as K_I and the corresponding values of K_i and K_i' were calculated. For each set of constants obtained in this way, the theoretical values of intercepts of Lineweaver–Burk lines as a function of TC concentration were calculated, using Eqn 2. Finally, all three sets of theoretical values of intercepts were compared to the experimental data in Fig. 1a. Two of these sets were far from fitting the experimental data; the third set, however, obtained with the value of $K_I = K_{i2} =$

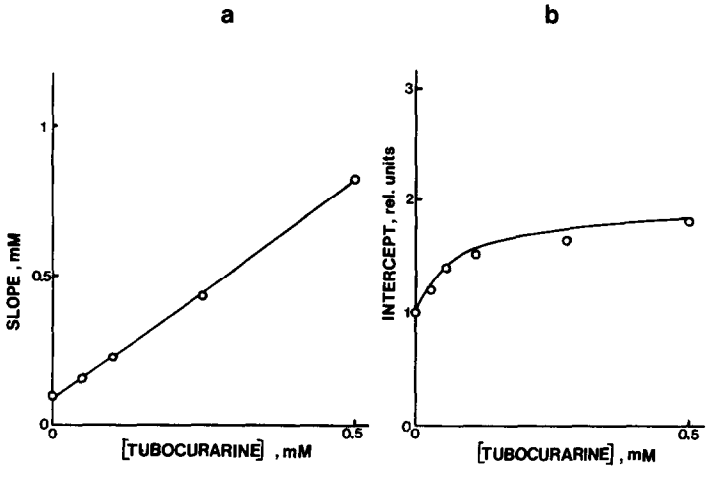

Fig. 1. Inhibition of the enzymatic hydrolysis of acetylthiocholine by D-tubocurarine: replots of slopes (Fig. 1a) and vertical intercepts (Fig. 1b) of Lineweaver–Burk lines versus concentration of D-tubocurarine. Points are experimental; each point represents the mean value of three determinations. Curves are theoretical, calculated by means of Eqn (2) (Fig. 1a) and Eqn (3) (Fig. 1b), using the corresponding constants from Table 2.

Table 2. Characteristic constants for the inhibition of enzymatic hydrolysis of acetylthiocholine by D-tubocurarine

Substrate	K_m* (mol l^{-1})	K_I† (mol l^{-1})	K_i† (mol l^{-1})	K_i'† (mol l^{-1})	K_{ii}‡ (mol l^{-1})	k_1/k_2‡	n_K§	n_H‖
ASCh	1.2×10^{-4}	3.1×10^{-4}	4.5×10^{-5}	1.2×10^{-5}	3.5×10^{-5}	1.89	0.88	0.83

K_m is Michaelis constant; K_I, K_i, K_i', and K_{ii} inhibition constants (see Scheme 1); k_1 and k_2 rate constants (see Scheme 1) and n_H Hill coefficient.

* From Lineweaver–Burk plot [12].

† Determined from Eqn (2) and data in Fig. 1a (see text).

‡ Obtained by means of HYPRPLT computer program [17].

§ From plot according to Loftfield and Eigner [15].

‖ From Hill equation [36]; see text for calculating procedure.

3.1×10^{-4} M, fitted the experimental data very well (see Fig. 1). The corresponding calculated constants are listed in Table 2.

Good fitting of the theoretical curves to the experimental data is a strong indication that the inhibition of the hydrolysis of ASCh by TC proceeds according to Scheme 1. In this process TC inhibits by binding to two different sites, to the catalytic anionic site and to the peripheral anionic site, with the corresponding constants $K_I = 3.1 \times 10^{-4}$ M and $K_i = 4.5 \times 10^{-5}$ M. It can be seen from Tables 1 and 2 that the value of K_i is very close to the values of the inhibition constants of TC obtained with BCh, AMCh, iPA and IxA; the value of K_I corresponds to the values of inhibition constants of TC, obtained with 2NA, pNPhA and IPhA. The identity of constants is generally an indication for the identity of binding sites. Having this in mind, it can be concluded that TC inhibits the hydrolysis of ASCh noncompetitively from the same binding site, from which it inhibits the hydrolysis of BCh, AMCh, iPA and IxA (i.e. peripheral anionic site), and that TC inhibits the hydrolysis of ASCh competitively from the site from which it inhibits the hydrolysis of 2NA, IPhA and pNPhA (i.e. catalytic anionic site). The results of the experiments of the inhibition by TC of the enzymatic hydrolysis of ASCh thus corroborate the idea of TC inhibiting the hydrolysis of substrates by binding to three different sites on AChE.

Considering further the binding of TC to three sites on AChE, it seems that such binding would result in inhibition with three different K_i for TC only if also the used substrates bound into the active centre of AChE in three different ways. It is well known that BCh, AMCh and iPA, when bound to AChE, interact with the esteratic and catalytic anionic site (cf. [32]); the hydrolysis of these substrates (and also IxA) is inhibited by TC with the same inhibition constant K_{i1} (see p. 2290). The hydrolysis of 2NA, pNPhA and IPhA is inhibited with another constant K_{i2}. These substrates incorporate in the alcoholic moiety of their molecules an aromatic group. It was suggested that such substrates bind to the esteratic site and also to another site, which is complementary to aromatic structures, but is not identical with the catalytic anionic site [33]. As shown above, the inhibition by TC of ASCh hydrolysis is produced by binding of TC to two sites; the binding to the first site is characterized by K_{i1} and to the second one by K_{i2}. It is generally accepted that ASCh binds into the esteratic and the catalytic anionic site, as do also BCh, AMCh and iPA. However, there must be some differences between ASCh and BCh, AMCh, and iPA, respectively, in the binding into the active centre of the enzyme, since ASCh in inhibition by TC fills two molecules of TC, but BCh, AMCh and iPA, respectively, fill only one of them; these differences have been noticed also by other authors (cf, [32]). Finally, the hydrolysis of EA and MA is inhibited by TC with the third inhibition constant K_{i3}; EA and MA are small substrates which probably bind only to the esteratic site of AChE [10, 11, 30]. Evidently, different ways of binding of substrates to the enzyme match well three inhibition constants (K_{i1}–K_{i3}) for TC, obtained in the presence of these substrates.

It can be seen from Tables 1 and 2 that the values of the Hill coefficient (n_H) for TC, obtained by the procedure of Loftfield and Eigner [15] in the presence of different substrates, are in all cases but one, less than one. Such values of n_H suggest multiple binding of TC to AChE [34]. The values of n_H for TC were obtained also by another procedure, as follows. Three different inhibition constants of TC (K_{i1}–K_{i3}) were used in Adair equation [34, 35] to calculate the fractional saturation of AChE with TC as a function of TC concentration. These data were then used in the original Hill equation and Hill plot [36], to determine the "theoretical" n_H for the binding of TC to AChE for all those concentrations of TC, which were previously used in determination of n_H by the method of Loftfield and Eigner [15]. As can be seen in Tables 1 and 2, the values of both kinds of n_H are in most cases in good accordance with each other, what is an additional support to the idea of TC binding to three different sites on AChE.

The results of the inhibition of the enzymatic hydrolysis of ASCh, pNPhA and EA by TMA, TEA and C-10, respectively, are summarized in Table 3. As shown, TMA, TEA and C-10 inhibit the hydrolysis of all three substrates with the constants, which are approximately the same for each individual inhibitor. The values of all Hill coefficients are around one. These results are quite different from those obtained with the inhibition of hydrolysis of ASCh, pNPhA and EA by TC; as already discussed, the hydrolysis of these three substrates is inhibited by TC with three significantly different inhibition constants K_{i1}–K_{i3}. One possible explanation of such inhibition by TC would be the modification of the affinity of TC for the enzyme, induced by different substrates through conformational changes of the

Table 3. Characteristic data for the inhibition of enzymatic hydrolysis of acetylthiocholine, p-nitrophenylacetate, and ethylacetate by tetramethylammonium, tetraethylammonium, and decamethonium. (K_i is inhibition constant, and n_H Hill coefficient)

	Inhibitor TMA			Inhibitor TEA			Inhibitor C-10		
Substrate	Type of inhibition	K_i/mol l^{-1}	n_H‖	Type of inhibition	K_i/mol l^{-1}	n_H‖	Type of inhibition	K_i/mol l^{-1}	n_H‖
ASCh	Competitive	5.5×10^{-4}* 5.6×10^{-4}‡	0.98	Competitive	2.0×10^{-4}* 2.4×10^{-4}‡	0.90	Mixed¶	2.6×10^{-6}* 8.6×10^{-6}† 3.0×10^{-6}‡	1.10
pNPhA	Partially competitive	6.7×10^{-4}* 6.9×10^{-4}‡ 5.2×10^{-4}§	0.90	Competitive	2.3×10^{-4}* 2.5×10^{-4}‡	1.03	Competitive	8.1×10^{-6}* 8.5×10^{-6}‡	0.95
EA	Partially competitive	1.0×10^{-3}* 9.0×10^{-4}‡ 7.9×10^{-4}§	0.94	Partially competitive	6.0×10^{-4}* 6.6×10^{-4}‡ 4.5×10^{-4}§	0.93	Partially competitive	1.0×10^{-5}* 9.8×10^{-6}‡ 8.1×10^{-6}§	0.92
Mean value of K_i	$7.1 \pm 1.6 \times 10^{-4}$			$3.8 \pm 1.8 \times 10^{-4}$			$7.3 \pm 2.7 \times 10^{-6}$		

* From secondary plot of slopes of lines from Lineweaver–Burk plot [12] versus concentration of tubocurarine [14].
† From secondary plot of intersections of lines from Lineweaver–Burk plot [12] versus concentration of tubocurarine yn[14].
‡ From Dixon plot [13].
§ Obtained by means of HYPRPLT computer program [17].
‖ From the plot according to Loftfield and Eigner [15].
¶ Mixed inhibition of the partially competitive–noncompetitive type (Ib + IIa, see ref. 18).

enzyme molecule. If this were true, one would expect that not only TC, but also other inhibitors inhibit the hydrolysis of these substrates with different inhibition constants. However, with TMA, TEA and C-10 this is evidently not the case. Therefore, the results of the inhibition of hydrolysis of ASCh, pNPhA and EA by TMA, TEA and C-10, respectively, corroborate the binding of TC to three different binding sites on AChE.

All our results, discussed so far, point to the binding of TC to three different binding sites on the enzyme. From the first site (i.e. peripheral anionic site), characterized by K_{i1}, TC inhibits the hydrolysis of BCh, AMCh, iPA, IxA and ASCh, respectively; from the second site (i.e. catalytic anionic site or near), characterized by K_{i2}, it inhibits the hydrolysis of 2NA, pNPhA, IPhA and also ASCh; and from the third site (near the esteratic site), characterized by K_{i3}, it inhibits the hydrolysis of EA and MA. The simplest mechanism of inhibition, according to these results, would be sterical interference between TC and substrates on three different regions of the active surface of AChE. In the cases of partially competitive inhibition of the hydrolysis of substrates by TC, some insight into degree of the possible sterical interference between TC and substrates can be obtained from the values of the inhibition constants K_i and K_{ii} (Table 1), which show the affinity of TC for the free enzyme and for the enzyme–substrate complex, respectively [16]. From these constants, the free energy of binding of TC to the enzyme and to the enzyme–substrate complexes were calculated ($\Delta G = -RT\ln K$); the differences between these values ($\Delta\Delta G$) are shown in Table 1. The values of $\Delta\Delta G$ are small, and are in all cases less than 5 kJ/mol which roughly corresponds to the hydrophobic binding of one $-CH_3$ group [37]. This indicates that the interference between TC and substrate is small, what is also expected, since the inhibition is only partially competitive. It can be seen from Table 1 that the values of $\Delta\Delta G$ in the series of substrates iPA, BCh, AMCh and IxA increase from iPA to IxA. This can be related to the increasing bulkiness of the alcoholic moiety of these substrates, indicating that steric hindrance between TC and iPA, BCh, AMCh and IxA, respectively, occurs in the vicinity of the catalytic anionic site, where the alcoholic moiety of these substrates is bound.

2. Competition between TC and TMA, TEA and C-10, respectively, in the inhibition of ASCh hydrolysis

The influence of TMA on the inhibition of the hydrolysis of ASCh by TC is shown in Figs 2–4. TMA reduces this inhibition (Fig. 2). However, with increasing concentration of TC, the influence of TMA decreases. This indicates that TC competes with TMA. Figures 3 and 4 show that TMA increase relative V^i (apparent maximal rate of ASCh hydrolysis in the presence of TC), and does not influence relative K_m^i (apparent Michaelis constant for ASCh in the presence of TC). From analogous diagrams (not shown) it was found that TC competes also with TEA and C-10; however, TEA and C-10 not only increase relative V^i, but also decrease relative K_m^i.

As shown, TC inhibits the hydrolysis of ASCh by binding to the peripheral and catalytic anionic site (see Scheme 1). On the other hand, it was found that TMA, TEA, and C-10 bind to the catalytic anionic site, but TEA and C-10 also to the peripheral anionic site (cf. [5–7, 38]). It is, however, highly improbable that TMA, a structural analog of TEA and C-10, would not also bind to the peripheral anionic site. We believe, therefore, that all three ligands (TMA, TEA, and C-10) bind both to the catalytic and to

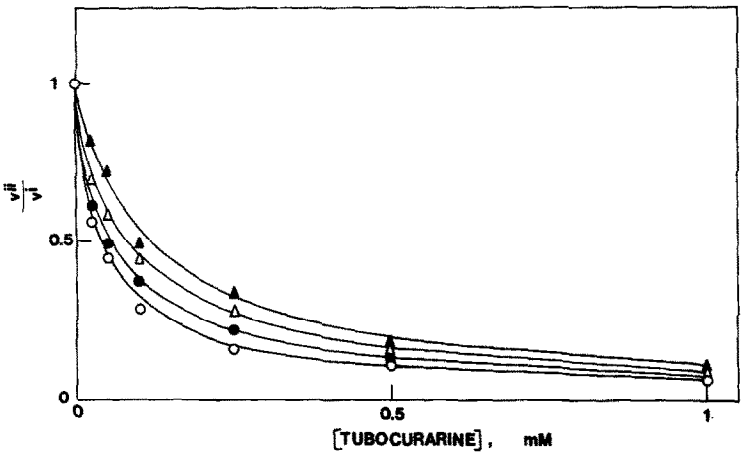

Fig. 2.

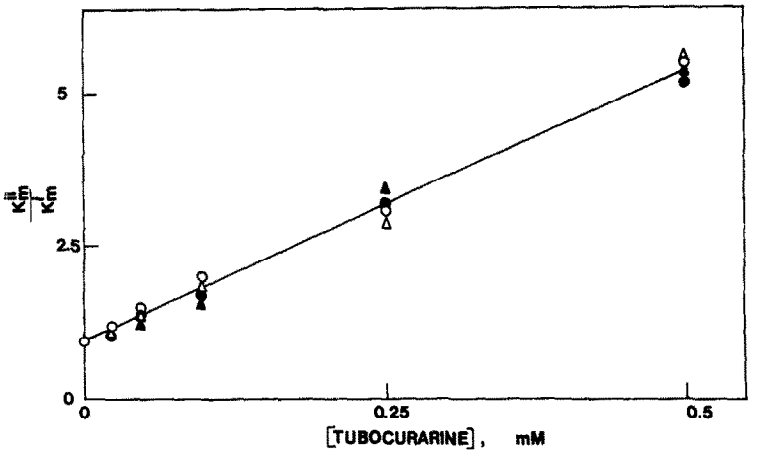

Fig. 3.

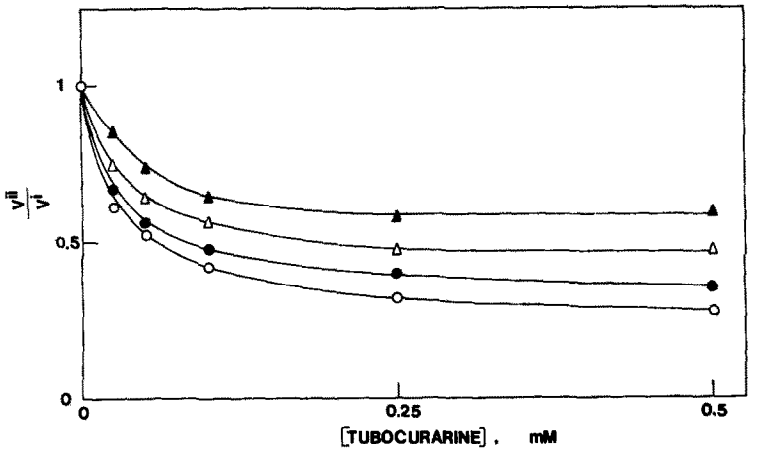

Fig. 4.

Figs. 2–4. Effect of tetramethylammonium on the inhibition of acetylcholinesterase by D-tubocurarine; substrate is acetylthiocholine. v^i, K_m^i, and V^i are the initial rate of the hydrolysis of 0.25 mM acetylthiocholine, the apparent Michaelis constant, and the apparent maximal rate, respectively, in the presence of tetramethylammonium; v^{ii}, K_m^{ii} and V^{ii} are the same constants in the presence of tetramethylammonium and D-tubocurarine. The values of K_m^i, K_m^{ii}, V^i and V^{ii} were obtained from the corresponding direct linear plots [21]. Concentrations of tetramethylammonium are: zero (○—○), 0.5 mM (●—●), 1 mM (△—△), and 2 mM (▲—▲). Each point represents the mean value of three determinations.

peripheral anionic site, and compete with TC for these two sites.

To analyze the competition between TC and TMA, TEA, and C-10, respectively, the following expressions for K_m^i and V^i were derived from Eqn 1:

$$K_m^i = K_s \cdot \frac{1+(I)/K_i+(I)/K_I+(I)^2/(K_I \cdot K_i')}{1+(I)/K_{ii}} \quad (6)$$

$$V^i = V \cdot \frac{1+(k_2/k_1)\cdot(I)/K_{ii}}{1+(I)/K_{ii}} \quad (7)$$

Since $K_i \cong K_i' \cong K_{ii}$ (see Table 2), Eqn 6 simplifies into

$$K_m^i \cong K_s \cdot (1+(I)/K_I) \quad (8)$$

It is obvious from Eqns (7) and (8) and from Scheme 1 that the influence of a ligand on the binding of TC to the catalytic anionic site of AChE (characterized by K_I) would be reflected in modified K_m^i, and influence on the binding of TC to the peripheral anionic site (characterized by K_{ii}) in modified V^i. Our experimental results, partly shown in Figs 2–4 and described above, show that TMA competes with TC for the peripheral anionic site, but does not interfere with the TC at the catalytic anionic site. TEA and C-10, on the other hand, compete with TC for both sites. These results confirm the binding of TC to or to the vicinity of the peripheral and catalytic anionic site. However, it remains to explain why TC and TMA do not compete for the catalytic anionic site. The reason for this may lie in the differences in microlocation of the binding of TC, TMA and ASCh at the catalytic anionic site of AChE, and in the small size of TMA molecule.

3. *Influence of TC on methanesulfonylation of AChE*

As shown in Fig. 5, TC inhibits methanesulfonylation of AChE. The inhibition is only partial—TC slows down the rate of methanesulfonylation for maximally 50%. This is in good agreement with the findings of other authors [5, 9, 40]. The second-order rate constant for the binding of MSF to AChE-TC complex, k_A, and the dissociation constant for the binding of TC to AChE in this process, K_A, are 1.4 l mol^{-1} sec^{-1} and 4.5×10^{-4} M, respectively. The value of K_A is in good accordance with the value of the inhibition constant for TC as an inhibitor of the enzymatic hydrolysis of 2NA, pNPhA and IPhA (Table 1), and with one of the constants of TC as inhibitor of ASCh hydrolysis (Table 2). It seems, therefore, that TC inhibits methanesulfonylation of AChE and hydrolysis of the above mentioned substrates by binding to the same site on the enzyme. This is surprising. It would be expected that TC inhibits methanesulfonylation from the same site from which it inhibits the hydrolysis of EA and MA, since these two small substrates bind only to the esteratic site [10, 11, 30], as does also MSF [24, 25].

To obtain additional data on the microgeography of binding of MSF, TC, EA and MA to the esteratic site of AChE, the influence of EA and MA on methanesulfonylation of the enzyme was investigated. The results are shown in Fig. 6. It can be seen that EA partly inhibits methanesulfonylation, k_A is 1.6 l mol^{-1} sec^{-1}. The dissociation constant for the binding of EA to AChE in this process, K_A, is 0.7 M (from Eqn 5), which is identical to the value of K_m for this substrate (Table 1). On the other hand, it is also seen that MA does not inhibit methanesulfonylation of AChE, but accelerates this process. The best fitting of the Eqns 4 and 5 to the experimental points for MA from Fig. 6 was obtained with k_A is 4.5 l mol^{-1} sec^{-1} and K_A is 1.0 M, respectively; K_A obtained in this way is very close to the value of K_m for MA (Table 1). Partial inhibition of methanesulfonylation by EA might be a consequence of the partial overlapping of the binding sites for EA and MSF, while the acceleration of methanesulfonylation by MA excludes overlapping of binding sites for MA and MSF. This indicates that EA and MA bind to the margin of the esteratic site of AChE,

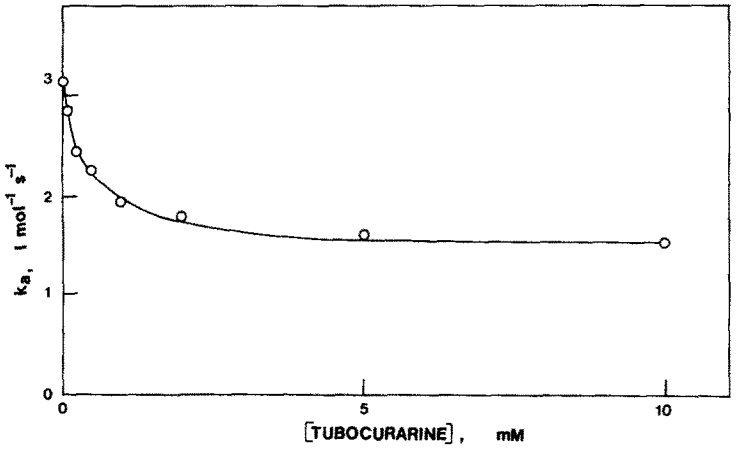

Fig. 5. Effect of D-tubocurarine on methanesulfonylation of acetylcholinesterase. k_a is the second-order rate constant for methanesulfonylation of acetylcholinesterase in the presence of D-tubocurarine. Each point represents the mean value of three determinations. The curve is theoretical, calculated by means of Eq (4), using $k = 3.1$ l mol^{-1} sec^{-1}, $k_A = 1.4$ l mol^{-1} sec^{-1}, and $K_A = 0.45$ mM; see Methods for the meaning of these constants.

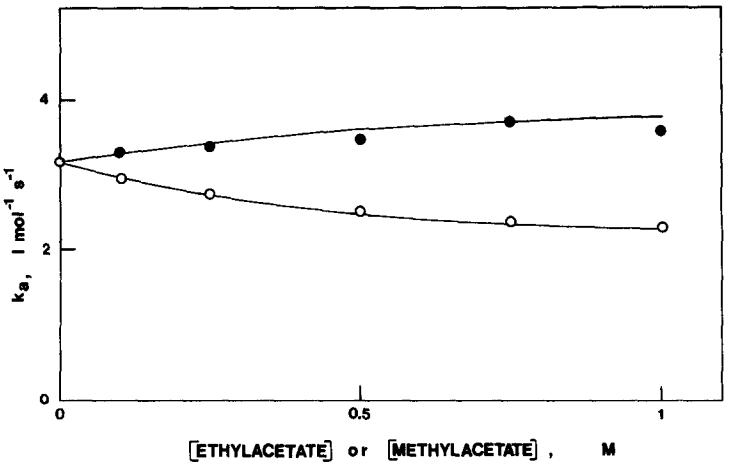

Fig. 6. Effect of ethylacetate and methylacetate, respectively, on the methanesulfonylation of acetylcholinesterase. k_a is the second-order rate constant for methanesulfonylation of acetylcholinesterase in the presence of ethylacetate or methylacetate. Each point (○ for ethylacetate and ● for methylacetate) represents the mean value of three determinations. Curves are theoretical, calculated by means of Eqn (4), using k = 3.1 l mol^{-1} sec^{-1}, k_A = 1.6 l mol^{-1} sec^{-1}, and K_A = 0.7 M for ethylacetate and k = 3.1 l mol^{-1} sec^{-1}, k_A = 4.5 l mol^{-1} sec^{-1}, and K_A = 1.0 M for methylacetate, respectively—see Methods for the meaning of these constants.

from where only larger EA partly blocks the binding of MSF into this site; a similar marginal binding of IPhA to the active centre of AChE has been proposed by Krupka [11]. Since the binding of MSF into the esteratic site of AChE is not identical to the binding of EA and MA to this site, it is possible that TC inhibits methanesulfonylation of AChE from one, and the hydrolysis of EA and MA from another binding site; however, both these sites for TC should be in the vicinity of the esteratic site of the enzyme.

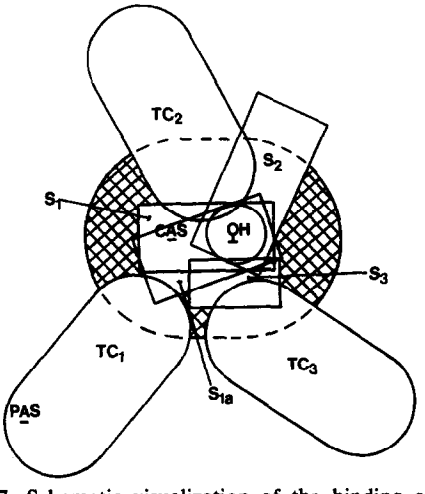

Fig. 7. Schematic visualization of the binding of tubocurarine, different substrates, and methanesulfonylfluoride to the active surface of acetylcholinesterase. The visualization is based on the schematic model of the active surface of acetylcholinesterase, proposed by Krupka [10]; dashed area represents the hydrophobic surface surrounding the active centre, CAS is the catalytic anionic site, $\underset{\perp}{\text{OH}}$ is the serine —OH group in the esteratic site, PAS is a peripheral anionic site. Molecules of tubocurarine are represented as ovals labelled as TC_1, TC_2 and TC_3. Substrates are represented as rectangles: S_1 is acetylthiocholine; S_{1a} is butyrylcholine, acetyl-β-methylcholine, i-pentylacetate and/or indoxylacetate; S_2 is *p*-nitrophenylacetate, 2-naphthylacetate and/or indophenylacetate; and S_3 is ethylacetate and/or methylacetate. Methanesulfonylfluoride is represented as the circle around $\underset{\perp}{\text{OH}}$.

4. *Schematic visualization of the binding of TC to the active surface of AChE*

In Fig. 7 a schematic visualization is offered as a summary of our results.

As shown in Fig. 7, three molecules of TC bind to three regions of the active surface of AChE:

The first region at the peripheral anionic site is characterized by the binding constant for TC $K_{i1} = 7.0 \times 10^{-5}$ M; from this region TC inhibits the hydrolysis of ASCh, BCh, AMCh, iPa and IxA.

The second region in the vicinity of the catalytic anionic site and esteratic site is characterized by $K_{i2} = 3.1 \times 10^{-4}$ M; from this region TC inhibits the hydrolysis of ASCh, 2NA, pNPhA and IPhA and also inhibits methanesulfonylation of AChE.

The third region in the vicinity of esteratic site, but away from the catalytic anionic site, is characterized by $K_{i3} = 4.2 \times 10^{-3}$ M; from this region TC inhibits the hydrolysis of EA and MA.

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