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MOLECULAR ASPECTS OF THE INTERACTIONS OF HALOGENOACETYLCHOLINES WITH CHOLINESTERASE*

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SUMMARY

The kinetics of hydrolyses of fluoro-, chloro-, bromo-, and iodoacetylcholines by cholinesterase (acylcholine acyl-hydrolase, EC 3.1.1.8) were studied using manometric techniques. The activity pS curves were suggestive that high substrate concentrations of halogenoacetylcholines inhibited the enzyme. All of them exhibited a pS optimum at about 1.75–2.0. Acetylcholine did not exhibit a pS optimum between pS values 1.5 to 3.0. All halogenoacetylcholines had higher K_m, v_{max} and relative rates of enzymic hydrolysis (pS 2.75–1.50) than those of acetylcholine. There was a linear relationship between pK_a of fluoro-, chloro-, bromo- and iodoacetic acids and pK_m for the cholinesterase hydrolyses of their choline esters. Iodoacetylcholine and sodium iodoacetate were the most effective of the halogen compounds to depress the fluorescence of enzyme proteins, acetylcholinesterase, chymotrypsin and the cholinesterase preparation. Iodoacetate was known to alkylate many enzyme proteins. However, preincubation of cholinesterase with iodoacetate or other halogenoacetates did not depress its activity. Choline and sodium fluoroacetate were weak inhibitors of the enzymic hydrolysis of fluoroacetylcholine. However, the descending limb in the activity pS curve of fluoroacetylcholine could not be explained completely by the products of hydrolysis.

There were no significant differences between the enzymic rates of hydrolyses of 2-dimethylaminoethyl halogenoacetates and the corresponding halogenoacetylcholines.

INTRODUCTION

Studies on the interactions of various substrates and inhibitors with cholinesterase (acylcholine acyl-hydrolase, EC 3.1.1.8) and acetylcholinesterase (acetylcholine hydrolase, EC 3.1.1.7) have greatly contributed to the understanding of the

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similarities and differences between these two enzymes[2,3]. At the surface of both enzymes, there is an esteratic site which is required for the general process of hydrolysis. A basic group in the esteratic site interacts with the electrophilic group of choline esters (carboxyl carbon of acetylcholine) or organophosphorus inhibitors (P atom). The nature of the electrophilic group of the substrates and the inhibitors can be modified by introducing suitable electron-attracting atoms and groups into their molecules[3,4].

A large number of organophosphorus inhibitors were known, in which the electron density of the P atom was modified by the introduction of halogen atoms in the molecule[4]. However, no systematic investigations were reported on the substrates in which the electron density of the carboxyl carbon was varied by the introduction of halogens. The replacement of one of the hydrogens in the acetyl group of acetylcholine by fluorine, chlorine, bromine and iodine changes the electron density of the carboxyl carbon in a well defined manner. Therefore, we have synthesized fluoro-, chloro-, bromo-, and iodoacetylcholines and studied the kinetics of their hydrolysis by serum cholinesterase. Very little was known about the cholinesterase hydrolysis of halogenoacetylcholines. GLICK[5] reported that bromoacetylcholine chloride was hydrolyzed by horse serum at a rate 2–3 times faster than that of acetylcholine. BUSCH *et al.*[6] suggested that the limited duration of the cholinergic effects of fluoroacetylcholine *in vivo* was due to its rapid hydrolysis by cholinesterases.

The enzymic hydrolysis of halogenoacetylcholines by acetylcholinesterase from bovine erythrocytes was described elsewhere[7,8]. It was shown that the enzymic hydrolysis of fluoroacetylcholine (p*S* 2.0) was equal to that of acetylcholine. The enzymic rates of hydrolysis of chloro-, bromo-, and iodoacetylcholines were considerably lower than that of acetylcholine or fluoroacetylcholine. It was suggested that the steric effects due to the halogenation of the acetyl group played a significant role in acetylcholinesterase hydrolysis of halogenoacetylcholines.

MATERIALS AND METHODS

The synthetic methods for the preparation of halogenoacetylcholines and 2-dimethylaminoethyl halogenoacetates were described elsewhere[8]. All halogenoacetylcholines were perchlorates except iodoacetylcholine which was an iodide. All 2-dimethylamino halogenoacetates were hydrochlorides, and were highly hygroscopic. Only hydrochlorides of 2-dimethylaminoethyl fluoroacetate and -chloroacetate could be purified to be included in this investigation. The perchlorates of acetylcholine, propionylcholine and butyrylcholine were obtained from Nutritional Biochemical Corp., Cleveland, Ohio.

Enzymes

Cholinesterase was prepared commercially (Worthington Biochemical Corp. Freehold, N.J.) from horse serum by a modified STRELITZ's procedure[9], as a stable lyophilized powder containing 6 units/mg. One unit was equal to 1 μmole of acetylcholine hydrolyzed per min. The enzyme solutions were prepared in Krebs bicarbonate buffer containing 1.0% albumin for kinetic studies. No albumin was added for fluorescence studies.

Iodoacetates interact with many types of proteins and enzymes. In order to detect the differences in the interaction of iodoacetylcholine or other halogenoacetyl-

cholines and acetylcholine, with proteins and enzymes, we studied the influence of these cholinesters and their products of hydrolysis on α-chymotrypsin, acetylcholinesterase and cholinesterase. Crystalline α-chymotrypsin (45 units/mg protein) was obtained from Nutritional Biochemical Corp., Cleveland, Ohio. One unit of chymotrypsin activity was equivalent to 1 μmole of benzoyl-L-tyrosine ethyl ester hydrolyzed per min at pH 7.8 and 25°, as determined by HUMMEL's method[10]. Crystalline acetylcholinesterase (specific activity[11]: 750 mmoles of acetylcholine hydrolyzed per mg of protein per h) from electric tissue was supplied by Dr. A. L. BAKER of Worthington Biochemical Corp., Freehold, N.J.

Kinetics of the hydrolysis of the substrates

The volume of CO_2 liberated from Krebs–Ringer bicarbonate buffer by the acid formed during the hydrolysis of the ester was measured by manometric methods using Rotary Warburg apparatus. The buffer contained $2.3 \cdot 10^{-2}$ M $NaHCO_3$, $7.5 \cdot 10^{-2}$ M KCl, $7.5 \cdot 10^{-2}$ M NaCl and $4 \cdot 10^{-2}$ M $MgCl_2 \cdot 6H_2O$, and its pH was 7.5 at 37° when measured using a thermostated capillary glass electrode and a thermostated calomel electrode. Further details of the apparatus and preparation of the solutions were described elsewhere[8,12,13].

The total volume of the reactants was 3.0 ml in a 15-ml flask. The main compartment contained 2.5 ml of the buffer and 0.2 ml of the enzyme. The substrate (0.3 ml) was placed in the side arm. The air in the reaction vessels was displaced with 5% CO_2 and 95% N_2 and the contents were preincubated for 15 min unless otherwise specified. The manometers were read every 2 min during the first 10 min and every 5 min during 10–30 min. The initial linear velocities were determined for at least seven substrate concentrations between pS values 1.5 to 3.0, and the activity–pS curves were constructed. The kinetic constants, K_m and v_{max} were determined graphically from Lineweaver–Burk plots, which were drawn from initial linear velocities.

Influence of halogeno-acetylcholines and sodium halogenoacetates on the fluorescence of α-chymotrypsin, acetylcholinesterase and cholinesterase

α-Chymotrypsin contains a tryptophyl nucleus and exhibits a characteristic fluorescence spectrum with an emission peak at 345 mμ upon excitation at 285 mμ (ref. 14) (Fig. 4). Crystalline acetylcholinesterase gave similar fluorescence spectrum. The fluorescence spectrum of the cholinesterase preparation was similar to that of chymotrypsin and acetylcholinesterase (Fig. 4). The fluorescence of cholinesterase preparation was probably due to the tryptophyl nucleus because in proteins containing tryptophan, the fluorescence of this residue alone was observed[14].

Carboxymethylation of >NH and –SH (and possibly –OH) groups occurs with halogenoacetates especially iodoacetate[15,16]. Further, some cholinesters may exhibit non-specific interactions with acetylcholinesterase, chymotrypsin or cholinesterase. These differences in the interaction of halogenoacetylcholines or halogenoacetates with these enzymes might produce significant changes in the environment of tryptophyl residues. Therefore, we studied the effects of halogenoacetylcholines on the fluorescence characteristics of α-chymotrypsin, acetylcholinesterase and the cholinesterase preparation. Acetylcholinesterase (20 units, 1.6 μg of protein) or chymotrypsin (0.25 unit, 51 μg of protein) or the cholinesterase preparation (0.18 unit, 30 μg of protein) and the substance (10^{-2} M) were incubated at 37° in 3.0 ml of Krebs bicar-

bonate buffer for 30 min and the fluorescence was recorded using Ferrand spectrofluorometer.

RESULTS

Initial linear velocities

The rates of evolution of CO_2 were linear (for pS values 1.5–3.0) during 0–30 min with acetylcholine, propionylcholine or butyrylcholine as substrates (Fig. 1C). The rates of hydrolyses of fluoro-, chloro-, bromo-, and iodoacetylcholines were linear during 0–10 min and were significantly depressed during 10–30 min (Figs. 1 A–D, see also Table IV).

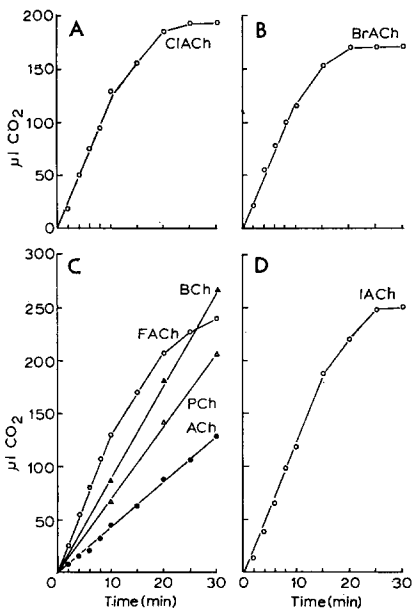


Fig. 1. Evolution of CO_2 as a function of time from various substrates during their hydrolyses by cholinesterase. A, chloroacetylcholine (ClACh). B, Bromoacetylcholine (BrACh). C, Fluoroacetylcholine (FACh), acetylcholine (ACh), propionylcholine (PCh), and butyrylcholine (BCh). D, Iodoacetylcholine (IACh). pS of substrate: 2.00; enzyme: 0.2 ml (0.2 units).

Activity–pS curves of acetylcholine and halogenoacetylcholines

The activity–pS curves were constructed for the initial linear velocities during 0–10 min and mean reaction velocities during 10–20 min (Fig. 2). The activity–pS curves of acetylcholine for both reaction periods coincided with one another suggesting that the rates of hydrolysis of acetylcholine were linear at all substrate concentrations during 0–20 min. With fluoro-, chloro-, bromo-, and iodoacetylcholines, the activity–pS curves for reaction period 10–20 min were at a lower level than the corresponding activity–pS curves for 0–10 min. This indicated that the rates of hydrolysis of these four substrates were depressed during 10–20 min at substrate concentrations lower than 10^{-2} M (see Fig. 2 and Table IV).

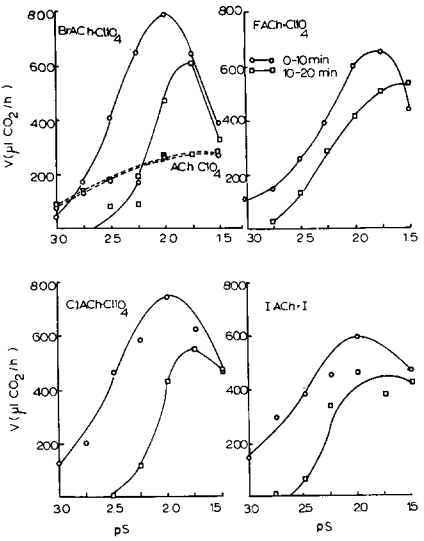


Fig. 2. Activity–pS curves for cholinesterase hydrolyses of acetylcholine (ACh), fluoroacetylcholine (FACh), chloroacetylcholine (ClACh) and iodoacetylcholine (IACh). Cholinesterase: 0.2 ml (0.18 units). Each point was a mean from three values. The differences between the enzymic rates of hydrolyses of acetylcholine during the reaction periods 0–10 min and 10–20 min were not significant ($P > 0.05$) at any pS value. The differences between the enzymic rates of hydrolyses of any one of the four halogenoacetylcholines during the reaction periods 0–10 and 10–20 min were significant ($P < 0.05$) at pS values higher than 2.00. The initial rate of hydrolysis of fluoroacetylcholine at pS 1.5 was significantly ($P < 0.05$) lower than that at 1.75. The initial rates of hydrolyses of chloro-, bromo-, and iodo-acetylcholines at pS 1.75 were significantly ($P < 0.05$) lower than their corresponding rates of hydrolyses at pS 2.0. Initial linear velocities were calculated from the rates of hydrolyses during 0–10 min.

The activity–pS curves of halogenoacetylcholines exhibited pS optima for both reaction periods 0–10 min and 10–20 min. The pS optimum for fluoroacetylcholine was about 1.75, and for chloro-, bromo-, and iodoacetylcholines was about 2.0 during 0–10 min. During 10–20 min, the pS optima moved to higher substrate concentrations or lower pS values with halogenoacetylcholines*. These results were suggestive that (a) high substrate concentrations of halogenoacetylcholines inhibited the enzyme and (b) the substrate inhibition became more apparant even at pS values higher than the pS optima (2.00) when the enzyme was incubated with these substrates for 10 min.

The activity–pS curve of acetylcholine did not exhibit a pS optimum. There was no evidence for the substrate inhibition even when the enzyme was incubated with acetylcholine for 10 min at high substrate concentrations.

Affinities of acetylcholine and monohalogenoacetylcholines

Monohalogenoacetylcholines had higher K_m's and lower affinities than those of acetylcholine (Table I). They had higher v_{max} (both observed and calculated) than those of acetylcholine.

* The shift of the pS optimum of the substrate to a higher substrate concentration was generally observed under steady-state conditions in the presence of a competitive inhibitor[17–19]. In the present situation halogenoacetylcholines did seem to act both as substrates and inhibitors. Inhibition was more evident after 10 min of incubation.

TABLE I

K_m AND v_{max} OF HALOGENOACETYLCHOLINES AND ACETYLCHOLINE

*Substrate**	*Y (moles/l)***	*v_{max} (µl/h)*	
		Calculated	*Observed*
Acetylcholine	3.8 ± 0.3	337 ± 33	248 ± 36
Iodoacetylcholine iodide	5.3 ± 0.4	937 ± 30	621 ± 68
Bromoacetylcholine	6.5 ± 0.3	1392 ± 144	828 ± 91
Chloroacetylcholine	9.0 ± 0.6	1574 ± 463	754 ± 42
Fluoroacetylcholine	13.0 ± 1.9	1407 ± 296	652 ± 62

* All are perchlorates, unless otherwise specified. All values are means from 3 experiments.
** $K_m = Y \times 10^{-3}$

Relative rates of hydrolyses

At pS optima, the initial linear rates of the enzymic hydrolyses of monohalogenoacetylcholines were about twice as high as those of acetylcholine (Table II). There were no significant differences between the rates of hydrolyses of the four halogenated compounds at their pS optima (2.0). However, at low substrate concentrations (pS 2.75), the enzymic rate of hydrolysis of iodoacetylcholine was about 1.5 times faster than those of fluoro-, chloro-, and bromoacetylcholines. One would anticipate that the enzymic inhibition caused by iodoacetylcholine at pS 2.75 was less than at pS 2.0.

Relationship between pK_m's for cholinesterase hydrolyses of monohalogenoacetylcholines and pK_a's of monohalogenoacetic acids

Regarding the influence of halogen atoms, the following consideration did show that monohalogenoacetylcholines followed a simple rule on cholinesterase. According

TABLE II

RELATIVE RATES OF HYDROLYSIS OF HALOGENOACETYLCHOLINES AND ACETYLCHOLINE

*Substrates**	*Relative rate of enzymic hydrolysis*		*Relative rate of non-enzymic hydrolysis pS 2.00*	*pK_a of the acid in acyl group*	*Van der Waals' radius of C–R†*
	*pS 2.75***	*pS 2.00****			
Acetylcholine	1.00 ± 0.22	1.00 ± 0.07	1.0 ± 0.2	4.75	2.27
Iodoacetylcholine	2.60 ± 0.04	1.84 ± 0.15	16.5 ± 2.1	2.91	4.25
Bromoacetylcholine	1.56 ± 0.28	2.04 ± 0.19	18.4 ± 2.4	2.85	3.86
Chloroacetylcholine	1.73 ± 0.41	2.05 ± 0.25	18.5 ± 2.3	2.85	3.56
Fluoroacetylcholine	1.59 ± 0.16	2.16 ± 0.16	18.5 ± 1.7	2.66	2.75
Dichloroacetylcholine	not significant	not significant	308 ± 11	1.30	—
Trichloroacetylcholine	not significant	not significant	403 ± 7	0.70	—

* All are perchlorates except iodoacetylcholine which is an iodide. All are means from three experiments unless otherwise specified.
** The hydrolytic rate of iodoacetylcholine was significantly different from others at $P < 0.01$.
*** Each value is a mean of six experiments. The hydrolytic rates of all halogenoacetylcholines were significantly higher than that of acetylcholine at $P < 0.01$.
† $(CH_3)_3N^+CH_2CH_2OCOCH_2R$ where R = hydrogen or halogen. The Van der Waals' radius of C–R bond gives an estimate of the steric volume necessary adjacent to the esteratic site to accommodate the alkyl group during the formation of enzyme–substrate complex. These were calculated by CHIOU AND SASTRY[8].

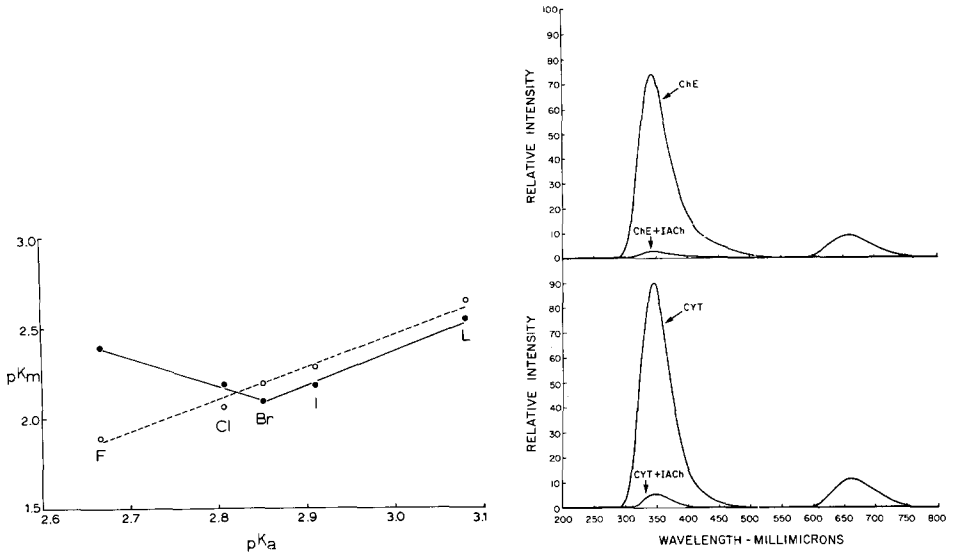


Fig. 3. Relationships between pK_m's for the hydrolyses of monohalogeno-acetylcholines by cholinesterase (ChE) or acetylcholinesterase (AChE) and pK_a's of monohalogeno-acids. ○— —○, relationship with cholinesterase; ●—●, relationship with acetylcholinesterase. F, fluoroacetylcholine or fluoroacetic acid; Cl, chloroacetylcholine or chloroacetic acid; Br, bromoacetylcholine or bromoacetic acid; I, iodoacetylcholine or iodoacetic acid; L, lactoylcholine or lactic acid. Lactoylcholine with strong electrophilic carboxyl carbon obeyed the same relationships. K_m values for lactoylcholine were reported by SASTRY AND WHITE[13]. K_m values of monohalogeno-acetylcholines for acetylcholinesterase were determined by CHIOU AND SASTRY[8]. While there was a linear relationship between pK_m's and pK_a's with cholinesterase, there was a minimum corresponding to pK_m of bromoacetylcholine for acetylcholinesterase.

Fig. 4. Fluorescence spectra of cholinesterase (ChE) and α-Chymotrypsin (CYT) with and without iodoacetylcholine (IACh) in the medium. Top, Cholinesterase. Bottom, α-Chymotrypsin. Acetylcholinesterase gave a similar fluorescence spectrum. α-Chymotrypsin and acetylcholinesterase were crystalline enzymes and their fluorescence spectra represented their chemical characteristics. The cholinesterase preparation contained other proteins. It was evident that iodocompounds interacted with the enzyme-proteins as well as non-enzyme proteins (see also Table III).

to the procedure of HAMMETT[20], a linear relationship should be expected between the dissociation constants of the halogenoacetic acids and the equilibrium constants of the cholinesterase hydrolyses of halogenoacetylcholines. We selected for comparison the Michaelis–Menten constants neglecting the dissociation constants of the esteratic site[21].

The pK_m's for cholinesterase hydrolyses of halogenoacetylcholines and pK_a's of halogenoacetic acids did show a linear proportionality* (Fig. 3) as required by the following equation.

$$pK_m = A \cdot pK_a + B \text{ (A and B are constants)}$$

There was no such linear relationship between pK_m's for acetylcholinesterase hydrolyses of halogenoacetylcholines and pK_a's of halogenoacetic acids.

* The linear relationship is remarkable because the HAMMETT equation is not usually applicable to aliphatic compounds. Similar linear relationship was reported by BERGMANN AND SHIMONI[22] between pK_m's for the liver esterase hydrolyses of alkyl halogenoacetates and pK_a's of halogenoacetic acids.

Influence of monohalogenoacetylcholines and sodium halogenoacetates on the fluorescence of α-chymotrypsin, acetylcholinesterase and the cholinesterase preparation

The perchlorates and iodides of acetylcholine and choline did not depress the fluorescence of α-chymotrypsin, acetylcholinesterase and the cholinesterase preparation (Table III). Iodoacetylcholine was the most effective to depress the fluorescence of all three enzymes (Fig. 4, Table III). Similarly, sodium iodoacetate was the most effective of the four monohalogenoacetates in depressing the fluorescence of all three enzymes. Therefore, these observations indicated that iodoacetylcholine and sodium iodoacetate did change the environment of tryptophyl residues. The depression of fluorescence might be due to one or more of the following reasons: (1) alkylation of reactive groups (>NH, –OH, –SH) of enzymes (2) conformational changes of the enzymes with masking of tryptophyl residues or (3) non-specific interactions. Further experiments to clarify the depression of the fluorescence of tryptophyl residues by sodium iodoacetate are in progress. However, these "non-specific interactions" of iodoacetate did not change the activity of the cholinesterase preparation (see also Table V).

While α-chymotrypsin and acetylcholinesterase were crystalline enzymes, the cholinesterase preparation contained other proteins. Iodoacetylcholine and sodium iodoacetate had similar influence on the fluorescence of all three enzyme preparations. Therefore, one could anticipate that iodoacetylcholine and sodium iodoacetate depressed the fluorescence of all three enzymes by similar mechanisms.

A part of the fluorescence of the cholinesterase preparation might be due to the proteins other than cholinesterase. Even then, it was possible that the iodocompounds reacted with cholinesterase as well as non-enzyme proteins because the concentration of the iodocompounds was considerably higher than the total (enzyme + non-enzyme) protein concentration.

Cholinesterase hydrolysis of 2-dimethylaminoethyl halogenoacetates

The rates of hydrolyses of 2-dimethylaminoethyl fluoroacetate, 2-dimethylaminoethyl chloroacetate and acetylcholine were studied simultaneously in six experiments at pS 2.00. The relative rates of the enzymic hydrolyses of 2-dimethylaminoethyl fluoroacetate (1.7 ± 0.4) and 2-dimethylaminoethyl chloroacetate (1.3 ± 0.3) were higher than that of acetylcholine (1.0 ± 0.02). They were not significantly lower than the rates of the enzymic hydrolyses of their quaternary derivatives.

The hydrochlorides of 2-dimethylaminoethyl fluoroacetate and 2-dimethylaminoethyl chloroacetate were hygroscopic and were difficult to handle in weighing and preparation of their solutions. Therefore, their rates of hydrolyses were approximate. The hydrochlorides of 2-dimethylaminoethyl bromoacetate and 2-dimethylaminoethyl iodoacetate were not included in the present study because they were highly hygroscopic and could not be crystallized to obtain reasonably pure samples.

Spontaneous hydrolysis of dichloroacetylcholine and trichloroacetylcholine

The non-enzymatic rates of hydrolyses of dichloro- and trichloroacetylcholines were about 300 and 400 times higher than that of acetylcholine. Therefore, the rates of their enzymatic hydrolyses could not be determined with reasonable accuracy. There were no significant differences between the rates of hydrolyses of dichloroacetyl-

TABLE III

INFLUENCE OF HALOGENOACETYLCHOLINES AND HALOGENOACETATES ON THE FLUORESCENCE OF CHYMOTRYPSIN, ACETYLCHOLINESTERASE AND THE CHOLINESTERASE PREPARATION

Comparison groups	*Compound with which the enzyme was incubated*	*Relative fluorescence intensity* ± S.E.*			*Relative activities to depress fluorescence*			*Relative chemical activities*
		Chymotrypsin 100 ± 2.8	*Acetylcholinesterase 100 ± 2.0*	*Cholinesterase 100 ± 2.8*	*Chymotrypsin*	*Acetylcholinesterase*	*Cholinesterase*	
1	Acetylcholine perchlorate	91.9 ± 2.5	105.8 ± 1.0	93.0 ± 1.1	N.S.	N.S.	N.S.***	—
	Acetylcholine iodide	105.9 ± 2.8	95.8 ± 0.6	97.7 ± 6.7	N.S.	N.S.	N.S.	—
	Choline perchlorate	100 ± 4.3	101.3 ± 1.0	—	N.S.	N.S.	—	—
	Choline iodide	—	101.7 ± 2.5	98.3 ± 5.4	—	N.S.	N.S.	—
2	Fluoroacetylcholine perchlorate	91.8 ± 1.9	109.5 ± 2.6	93.2 ± 2.2	N.S.	N.S.	N.S.	—
	Chloroacetylcholine perchlorate	91.0 ± 3.8	101.5 ± 1.6	100.8 ± 2.8	N.S.	N.S.	N.S.	—
	Bromoacetylcholine perchlorate	83.3 ± 2.2	96.0 ± 0.3	95.7 ± 3.0	19	4	5 (N.S.)	—
	Iodoacetylcholine iodide	11.4 ± 0.9	9.0 ± 1.0	16.0 ± 1.2	100	95	95	—
3	Sodium fluoroacetate	94.0 ± 3.6	—	96.2 ± 1.5	N.S.	—	N.S.	N.S.
	Sodium chloroacetate	107.5 ± 5.7	103.6 ± 1.4	103.3 ± 2.3	N.S.	N.S.	N.S.	N.S.
	Sodium bromoacetate	92.0 ± 2.8	90.4 ± 1.4	96.6 ± 6.3	N.S.	10	N.S.	69**
	Sodium iodoacetate	10.8 ± 0.6	6.4 ± 0.2	11.9 ± 1.0	100	100	100	100**

* Excitation maximum 285 mμ. Emission maximum 345 mμ. All values are means from five experiments.
** Relative chemical reactivities of monohalogenoacetates with thioglycolate[16].
*** N.S.: not significant at $P = 0.05$.

choline* (or trichloroacetylcholine**) with and without the enzyme in the reaction medium.

Influence of choline and sodium fluoroacetate on the cholinesterase hydrolysis of acetylcholine

Choline, sodium acetate or an equimolar mixture of choline and sodium acetate (each $4 \cdot 10^{-3}$ M) did not alter significantly the cholinesterase hydrolysis (Fig. 5) of acetylcholine (10^{-2} M). Similarly, sodium fluoroacetate or a mixture of choline and sodium fluoroacetate (each $4 \cdot 10^{-3}$ M) did not depress the enzymic hydrolyses of acetylcholine. All sodium monohalogenoacetates did not depress the enzymic hydrolysis of acetylcholine even at a concentration of 10^{-2} M, which was equal to the substrate concentration (Table IV).

TABLE IV

DIFFERENCE IN THE CHOLINESTERASE HYDROLYSIS OF HALOGENOACETYLCHOLINES BETWEEN THE REACTION PERIODS 0–10 min AND 10–20 min

The number in the brackets indicates the number of experiments.

Expt. No.	*Substrate**	*µl of CO_2 evolved per h at pS 2.00*		*Reliability of difference between means M_1 and M_2 P value*	*% Depression during 10–20 min***
		0–10 min $M_1 \pm$ S.E.	*10–20 min $M_2 \pm$ S.E.*		
1	Fluoroacetylcholine	497 ± 52 (6)	361 ± 24 (6)	<0.05	27.4
2	Chloroacetylcholine	560 ± 51 (6)	398 ± 37 (6)	<0.05	29.0
3	Bromoacetylcholine	576 ± 42 (6)	400 ± 29 (6)	<0.01	30.6
4	Iodoacetylcholine	485 ± 43 (6)	418 ± 25 (6)	<0.05	14.2
5	Acetylcholine	231 ± 14 (12)	241 ± 12 (12)	<0.05	No significant difference

* All are perchlorates except iodoacetylcholine which is an iodide.

** About 10–12% of this depression was due to decrease in the concentrations of fluoro-, chloro-, and bromoacetylcholines after 10 min of interaction. About 3% of this depression was due to the decrease in the concentration of iodoacetylcholine.

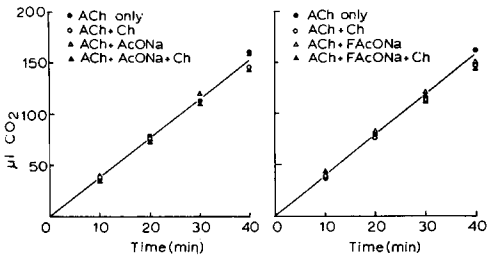
Influence of choline and sodium fluoroacetate on the cholinesterase hydrolysis of fluoroacetylcholine

Choline or sodium fluoroacetate alone ($4 \cdot 10^{-3}$ M)*** did not depress significantly the cholinesterase hydrolysis of fluoroacetylcholine (10^{-2} M). However, an equimolar mixture of choline and sodium fluoroacetate (each $4 \cdot 10^{-3}$ M) depressed by about 5–6% the cholinesterase hydrolysis of fluoroacetylcholine (Fig. 6). Therefore, the depression of the rate of evolution of CO_2 as a function of time could be explained partially by product inhibition. However, this did not explain completely the descending limb in the cholinesterase hydrolysis of fluoroacetylcholine.

* Total hydrolysis (enzymic + non-enzymic): 445 ± 12 µl of CO_2 per 10 min. Non-enzymic hydrolysis: 446 ± 16 µl of CO_2 per 10 min.

** Total hydrolysis: 585 ± 4 µl of CO_2 per 5 min. Non-enzymic hydrolysis: 585 ± 9 µl of CO_2 per 10 min.

*** The concentration of choline or sodium fluoroacetate formed in the reaction mixture due to the enzymic and non-enzymic hydrolysis of fluoroacetylcholine (10^{-2} M) in 30 min was about $4 \cdot 10^{-3}$ M. Choline at a concentration of 10^{-2} M inhibited significantly the cholinesterase hydrolysis of acetylcholine.



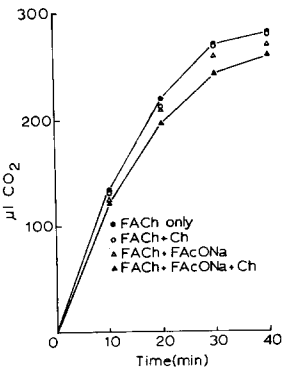


Fig. 5. Influence of choline (Ch), sodium acetate (AcONa) and sodium fluoroacetate (FAcONa) (with or without choline) on the hydrolysis of acetylcholine (ACh) by cholinesterase. Concentrations: acetylcholine 10^{-2} M; choline $4 \cdot 10^{-3}$ M; sodium fluoroacetate $4 \cdot 10^{-3}$ M. The enzymic hydrolysis of acetylcholine was not depressed by sodium acetate, choline and an equimolar mixture of sodium acetate and choline ($P > 0.05$). Similarly, the enzymic hydrolysis of acetylcholine was not depressed significantly by choline, sodium fluoroacetate or a mixture of choline and sodium fluoroacetate ($P > 0.05$).

Fig. 6. Inhibition of cholinesterase hydrolysis of fluoroacetylcholine (FACh) by an equimolar mixture of choline (Ch) and sodium fluoroacetate (FAcONa). Sodium fluoroacetate or choline alone did not inhibit significantly ($P > 0.05$) the enzymic hydrolysis of fluoroacetylcholine. An equimolar mixture of sodium fluoroacetate and choline inhibited significantly ($P < 0.05$) the enzymic hydrolysis of fluoroacetylcholine. Concentrations: fluoroacetylcholine 10^{-2} M; choline $4 \cdot 10^{-3}$ M; sodium fluoroacetate $4 \cdot 10^{-3}$ M.

The rate of cholinesterase hydrolysis of fluoroacetylcholine was depressed during 10–20 min by about 27% at pS 2.0 (Table IV). About 5–6% of this total 27% depression could be explained by product inhibition. About 10–12% was due to the change in the substrate concentration after 10 min of interaction. The reasons for the remaining 9–12% of this depression were not clear in the present study. Similar results were obtained with chloro-, bromo-, and iodoacetylcholines (Table IV). Experiments to

TABLE V

INFLUENCE OF VARIOUS HALOGENO-ACETATES ON THE RATE OF HYDROLYSIS OF ACETYLCHOLINE BY CHOLINESTERASE

The enzyme was preincubated with the compound for 15 min before acetylcholine was added. pS of acetylcholine and added compounds was 2.0.

Compound added to the incubation mixture of cholinesterase and acetylcholine	*Relative rate of hydrolysis* (rate of hydrolysis of acetylcholine without any compound: 100 ± 3.8)*
Sodium fluoroacetate	99.1 ± 4.0
Sodium chloroacetate	105.8 ± 2.5
Sodium bromoacetate	98.8 ± 8.4
Sodium iodoacetate	96.4 ± 2.3

* Each is a mean ± S.E. from three experiments. Differences between various means are not significant ($P > 0.05$).

elucidate the mechanisms of the substrate inhibition of halogenoacetylcholines are in progress.

DISCUSSION

Consideration should be given to different physico-chemical factors in the evaluation of the relative rates of the hydrolyses of halogenoacetylcholines such as: (1) the changes in the electronic characteristics of the carboxyl carbon, (2) the space occupied by the acyl tail, (3) the lability of the halogen atom and the non-specific interactions of the halogenocompounds with the enzyme proteins, (4) the influence of the products of hydrolysis, especially the halogenoacetates on the enzyme activity, and (5) the substrate inhibition, if any.

A comparison of the pK_a values of halogenoacetic acids suggested that the carboxyl carbons in halogenoacetylcholines were more electrophilic than that of acetylcholine (Table II). The non-enzymatic rates of hydrolyses of halogenoacetylcholines were higher than that of acetylcholine. Similarly, their enzymatic rates of hydrolysis were higher than that of acetylcholine.

A direct comparison of halogenoacetylcholines with acetylcholine was not possible, because their activity–pS curves were not parallel. The enzymatic rates of hydrolyses of halogenoacetylcholines were depressed after 10 min of interaction while the enzymatic rate of hydrolysis of acetylcholine was constant for more than 10 min. High substrate concentrations of halogenoacetylcholines inhibited the enzyme, while acetylcholine was not a cholinesterase inhibitor. However, halogenoacetylcholines could be compared with one another because their activity–pS curves were similar (Fig. 2).

All halogenoacetylcholines had higher K_m's and lower affinities to cholinesterase than those of acetylcholine. They had higher v_{max} values than that of acetylcholine. Iodoacetylcholine had the greatest affinity of all halogenoacetylcholines. Accordingly, its rate of enzymatic hydrolysis was faster than those of fluoro-, chloro-, and bromoacetylcholines at a substrate concentration (pS 2.75)* lower than the optimum substrate concentration (pS 2.00).

There was a linear relationship between pK_m for the cholinesterase hydrolysis of halogenoacetylcholines and pK_a of halogenoacetic acids. This relationship could be depicted by the equation: $pK_m = 1.75\ pK_a - 2.75$. There was a good agreement between the observed and calculated pK_m's of halogenoacetylcholines. Even in case of lactoylcholine with a strong electrophilic carboxyl carbon, there was a good agreement between calculated and observed pK_m's. Therefore, in these monohalogenoacetylcholines and lactoylcholine, the electron displacement due to the influence of the α-substituent was the decisive factor. Such linear relationship was reported also by BERGMANN AND SHIMONI[22] for the hydrolysis of alkyl halogenoacetates by liver esterase. However, when halogenoacyl tail was replaced by bulky aromatic rings or alkyl chains which did not contain any electron withdrawing groups, there was no agreement between the observed and calculated pK_m's (Table VI). In these substrates Van der Waals forces did appear to play a decisive role.

The steric effects of halogenation did not seem to play an important role in

* The initial linear velocities at pS values higher than 2.25 ($5.623 \cdot 10^{-3}$ M) were used to determine K_m and v_{max}.

TABLE VI

RELATIONSHIPS BETWEEN pK_a'S OF ACIDS AND pK_m'S FOR THE CHOLINESTERASE HYDROLYSIS OF THEIR CHOLINE ESTERS

The choline esters in Group 1 satisfied the equation, $pK_m = 1.75\ pK_a - 2.75$. They were substrate inhinitors. Choline esters in Group 2 did not satisfy the above equation and they were substrate inhibitors. Choline esters in Group 3 did not satisfy the equation and they were not substrate inhibitors.

Comparison groups	*Choline ester*	*pK_a of the acid*	*pK_m of the ester*		*pS optima if any*	*Ref. for K_m and pS optimum*
			*measured**	*calculated***		
1	Fluoroacetylcholine	2.66	1.89	1.91	1.75	Table I, Fig. 2
	Chloroacetylcholine	2.81	2.05	2.16	2.00	Table I, Fig. 2
	Bromoacetylcholine	2.85	2.19	2.24	2.00	Table I, Fig. 2
	Iodoacetylcholine	2.91	2.28	2.34	2.00	Table I, Fig. 2
	Lactoylcholine	3.08	2.63	2.64	2.00	SASTRY AND WHITE[13]
2	Furoylcholine	3.11	3.00	2.69	2.00	LEVY[26]
	Acetylsalicylcholine	3.42	2.10	3.24	2.00	AUGUSTINSON[27]
	Benzoylcholine	4.19	3.09	4.58	2.00	AUGUSTINSON[27]
3	Acetylcholine	4.75	2.42	5.56	—	SASTRY AND WHITE[13]
	Butyrylcholine	4.81	2.70	5.67	—	AUGUSTINSSON[27]
	Isobutyrylcholine	4.84	2.49	5.72	—	LEVY[26]

* All values were measured on cholinesterase except for acetylsalicylcholine which was measured on liver esterase.

** Calculated from the equation $pK_m = 1.75\ pK_a - 2.75$.

cholinesterase hydrolysis of halogenoacetylcholines. Iodine atom is the largest of halogen atoms and iodoacetylcholine has the largest Van der Waals' radius for C–R bond (Table II). F atom is the smallest of halogens and it can be substituted for a hydrogen without a major disturbance to the shape of the molecule. However, iodoacetylcholine exhibited greater affinity to cholinesterase than fluoroacetylcholine. In contrast to this finding the steric effects due to the halogenation did play a significant role in acetylcholinesterase hydrolyses of halogenoacetylcholines[8]. Fluoroacetylcholine exhibited greater affinity to acetylcholinesterase than iodoacetylcholine. The increase in the size of the halogen atom increases the Van der Waals' force between the enzyme and the substrate. Benzoylcholine[26] and acetylsalicylcholine[27] with bulky acyl groups were good substrates for cholinesterase. However, further investigations are necessary to clarify as to which one of the following aspects of iodoacetylcholines plays a more decisive role: (1) its greater affinity due to Van der Waals' forces or (2) its lower efficacy to inhibit cholinesterase than other halogenoacetylcholines.

A question might arise whether the covalently bonded halogens could spontaneously dissociate or alkylate the enzyme under the present experimental conditions. Of all compounds studied, iodoacetylcholine or sodium iodoacetate were able to bring about changes in the environment of tryptophyl residues and depress the fluorescence intensity of acetylcholinesterase, α-chymotrypsin and the cholinesterase preparation. Sodium iodoacetate was the most reactive chemically of all monohalogenoacetates (Table III). A number of studies have indicated that I–C bond was the weakest, and

iodine may split off from iodoacetate (or iodoacetylcholine) with the attachment of $-CH_2COO^-$ to –SH or >NH (or –OH) groups of enzymes[15,16]. However, "the non-specific interactions" of sodium iodoacetate or iodoacetylcholine with cholinesterase did not seem to influence the enzymatic hydrolysis of iodoacetylcholine because (1) sodium iodoacetate did not inhibit the enzymic hydrolysis of acetylcholine and (2) the tryptophyl nucleus was probably not a part of the active site of cholinesterase[3].

The mechanism of hydrolysis of cholinesters by acetylcholinesterase was explained by assuming that a labile acylenzyme was formed as an intermediate during hydrolysis[28,29]. Further, the recent investigations suggested that deacetylation of acetyl enzyme was prevented by binding of a molecule of acetylcholine to the acyl-enzyme at the anionic site at high substrate concentrations[30,31]. A question might arise as to whether the deacylation of the halogenoacetyl-enzyme was prevented by binding of a second molecule of the halogenoacetylcholine to cholinesterase. There are a number of ways by which a second molecule of the halogenoacetylcholine could combine with the halogenoacetyl-enzyme: (1) the alkylation of cholinesterase due to the lability of the halogen atom, (2) the anionic site, and (3) the binding of a second molecule of the halogenoacetylcholine to the enzyme due to the negative charge on the halogen atom, (4) the unmasking of a negative site due to the conformational change in the enzyme–substrate complex or the halogenoacetyl-enzyme.

Among halogenoacetylcholines only iodoacetylcholine did seem to interact with the enzyme and change the environment of tryptophyl residues. Treatment of the cholinesterase preparation with sodium iodoacetate did not depress the enzyme activity. All halogenoacetylcholines exhibited the characteristics of weak substrate inhibitors. The weak inhibition of cholinesterase by the products of hydrolysis of fluoroacetylcholine, choline or sodium fluoroacetate did not explain completely the descending limb in the activity–pS curve of fluoroacetylcholine. Similarly, the anionic site did not seem to be involved in the substrate inhibition caused by halogenoacetyl-cholines. Alkyl esters of halogenoacetic acids do not have cationic heads and they were substrate inhibitors of cholinesterase[22,32]. Acetylcholine and butyrylcholine contain cationic heads and they were not substrate inhibitors of cholinesterase.

The effectiveness of halogenoacetylcholines as substrate inhibitors may be related partly to the electrophilic strength of their carboxyl carbons. Besides halogeno-acetylcholines, several other cholinesters exhibited the characteristics of the substrate inhibitors of cholinesterase (Table VI). Halogen acetic acid esters of aliphatic alcohols[22,32] and choline esters of lactic acids[13,23–25], benzoic acid[27,33], acetylsalicylic acid[27] and furoic acid[26] inhibited cholinesterase at high substrate concentrations. The carboxyl carbons in all of these esters are more electrophilic than that of acetylcholine or butyrylcholine. Further, a fair correlation was reported between the capacity to inhibit cholinesterase and the electrophilic strength of the carboxyl carbon of carboxamides derived from piperidine[34]. The inhibitory strength increased with the enhancement of the electrophilic character of the carboxyl carbon. Also, it was shown that in the course of the inhibition of cholinesterase the inhibitor was slowly hydrolyzed, due to the strong binding between the electrophilic carbonyl carbon and the nucleophilic group at the esteratic site[35]. Therefore, strong electrophilic carbonyl or carboxyl carbon was one of the common features of carbamate inhibitors and substrate inhibitors.

Acetylcholinesterase and chymotrypsin undergo conformational changes under varied conditions[36]. Cholinesterase bears certain resemblances to these two enzymes

and such conformational changes may occur during its interactions with substrates and inhibitors. Tertiary changes may take place during the interaction of halogeno-acetylcholines and other substrate inhibitors with unfolding of new basic groups. The interaction of an additional molecule of the substrate at the new basic group of the enzyme–substrate complex may interfere with acylation and deacylation reactions. Further studies to explore the mechanisms of the substrate inhibition of halogeno-acetylcholines are planned.

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