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ACETYLCHOLINESTERASE* HYDROLYSIS OF HALOGEN SUBSTITUTED ACETYLCHOLINES†

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Abstract—The fluoro-, chloro-, dichloro-, trichloro-, bromo- and iodoacetylcholines were synthesized to study the relative roles of: (1) the electronic characteristics of carbonyl keto function, and (2) the configuration and the associated steric effects of the halogenated acyl group of cholinesters on acetylcholinesterase (AChE)–substrate interaction. The reaction kinetics of the hydrolysis by AChE (from bovine erythrocytes) were studied by standard manometric techniques. Although haloacetic acids are stronger acids than acetic acid, their cholinesters exhibit lower affinities ($K_m \times 10^{-3}$ M: fluoroacetylcholine, 4·11; chloroacetylcholine, 6·42; bromoacetylcholine, 8·18; iodoacetylcholine, 6·62; acetylcholine, 1·04) and lower rates of hydrolysis (V_{max}, μl CO_2/hr: fluoroacetylcholine, 309; chloroacetylcholine, 170; bromoacetylcholine, 192; iodoacetylcholine, 211; acetylcholine, 358) than those of acetylcholine (ACh). The Van der Waals' radius of C—R (where R=H in ACh and F, Cl, Br or I in halogen-substituted acetylcholines) gives an indication of the space required for the acyl group at a position adjacent to the esteratic site during AChE–substrate interaction. The initial rates of hydrolysis of cholinesters at their pS-optima could be arranged in the following order: acetylcholine=fluoroacetylcholine>propionylcholine>chloroacetylcholine=bromoacetylcholine=iodoacetylcholine>butyrylcholine. At low substrate concentrations (pS 3·0 to 2·5), the rate of hydrolysis of ACh was higher than that of fluoroacetylcholine. The Van der Waals' radius of C—R and K_m increase from ACh to butyrylcholine. The rate of hydrolysis of dichloroacetylcholine is about $\frac{1}{5}$ of that of ACh. The enzymic hydrolysis of trichloroacetylcholine is not significant. Therefore, the steric effects due to halogenation of the acyl group play a significant role in AChE hydrolysis of halogen-substituted acetylcholines.

STUDIES on the interaction of acetylcholinesterase (AChE) with substrates and inhibitors have greatly contributed to the understanding of active sites and its mode of action.[1–3] At the enzyme surface, there is an esteratic site, which is required for the general process of ester hydrolysis. The esteratic site comprises two subgroups, a basic and an acidic group. The basic group interacts with the electrophilic group (carbonyl carbon) of acetylcholine (ACh). The acidic group is believed to interact with the oxygen atom of the alcohol residue of the substrate. The anionic site at the

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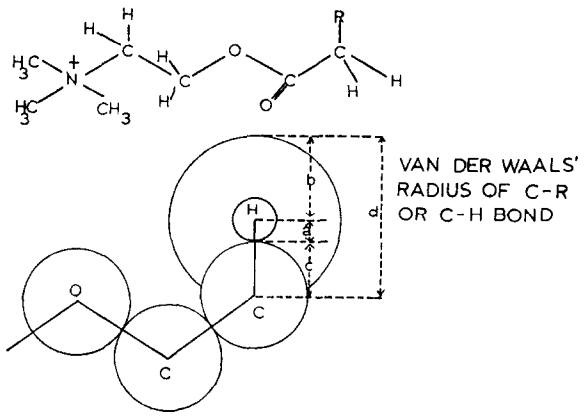
surface of AChE is required for the binding of positively charged substrates and inhibitors.

A number of studies have described the effects of modifications in the quaternary group and the substituents on the electrophilic carbons of cholinesters on their activities.[2-6] However, no detailed investigations have been reported on AChE hydrolysis of halogen-substituted acetylcholines (HACh). The replacement of one of the hydrogens by fluorine, chlorine, bromine and iodine would change the electronic characteristics of the carbonyl keto function quantitatively in a well defined manner.



d = a + b + c
c = Covalent radius of carbon = 0.77Å

R	Covalent radius a	Van der Waals' radius b	d
H	0·30	1·20	2·27
F	0·64	1·35	2·76
Cl	0·99	1·80	3·56
Br	1·14	1·95	3·86
I	1·33	2·15	4·25

FIG. 1. Structural similarities between acetylcholine (ACh) and halogen-substituted acetylcholines (HACh). Calculation of Van der Waals' radius of C—H or C—R (R = halogen) bond. The Van der Waals' radius of C—R bond gives an estimate of the steric volume necessary adjacent to the esteratic site to accomodate the alkyl group during the formation of enzyme–substrate complex. The covalent radii and Van der Waals' radii are from the Prentice-Hall framework molecular orbital models.

Further, due to the increase in the atomic size from fluorine to iodine, HACh are convenient substrates for the study of the steric effects of the acyl group on AChE hydrolysis. Steric hindrance due to halogenation, with or without electronic involvement, has been demonstrated in several organic reactions.[7, 8] Therefore, we have synthesized fluoro-, chloro-, (dichloro-, trichloro-), bromo-, and iodoacetylcholines (Fig. 1) and studied the kinetics of their hydrolysis by AChE to evaluate the relative roles of 1) the electronic characteristics of the carbonyl keto function, and 2) the configuration and the associated steric effects of the acyl group on AChE–substrate interaction.

MATERIALS AND METHODS

Substrates and inhibitors. All substrates used are perchlorates, except iodoacetylcholine (IACh) which is synthesized as an iodide. The halide salts of halogen-substituted acetylcholines (HACh) are highly hygroscopic, and it is difficult to purify them satisfactorily by crystallization.[9, 10] The perchlorates of cholinesters are usually nonhygroscopic stable salts and can readily be purified by fractional crystallization.[11] The iodide of IACh was synthesized due to the low solubility of its perchlorate in water.

The perchlorates of choline, dichloroacetylcholine (Cl_2ACh) and trichloroacetylcholine (Cl_3ACh) were synthesized according to the methods described in the literature.[12] The perchlorates of fluoroacetylcholine (FACh), chloroacetylcholine (ClACh) and bromoacetylcholine (BrACh) and IACh iodide were synthesized by the methods described below. The perchlorates of ACh* and propionylcholine† were obtained from commercial sources.

Fluoroacetylcholine perchlorate. A mixture of 15·3 g (0·075 mole) choline perchlorate, 7·02 g (0·09 mole) fluoroacetic acid, 0·43 g (0·003 mole) of 70% $HClO_4$, 1·0 g *p*-toluene sulfonic acid, and 300 ml benzene was refluxed for 4–5 hr with vigorous stirring in a flask equipped with an azeotropic receiver. When the theoretical amount of water of esterification was collected in the azeotropic receiver, the reaction mixture was cooled, and the colorless precipitate was allowed to settle. The benzene was decanted and the precipitate was recrystallized from acetone–ethyl acetate. The final product (17·5 g, yield 88·5 per cent) was colorless needles melting at 89·5 to 91°. *Anal.*‡ Calcd. for $C_7H_{15}ClFNO_6$: C, 31·89; H, 5·73; Cl, 13·45; N, 5·31. Found: C, 31·86; H, 5·70; Cl, 13·38; N, 5·24.

Chloroacetylcholine perchlorate. To 11·2 g (0·075 mole) choline chloride placed in an Erlenmeyer flask cooled in ice water, 10·17 g (0·09 mole) chloroacetyl chloride was added with constant stirring. The reaction product (a viscous mass) was dissolved in 15 ml of ice-cold absolute ethanol and 11·25 ml of 70% perchloric acid was added. A white crystalline precipitate (16 g, yield 76 per cent) separated, which was filtered and recrystallized from acetone–ethyl acetate. The final product was colorless crystals melting at 90·5 to 92·5°. *Anal.* Calcd. for $C_7H_{15}Cl_2NO_6$: C, 30·02; H, 5·39; Cl, 24·96; N, 5·00. Found: C, 30·04; H, 5·42; Cl, 25·23; N, 5·14.

Bromoacetylcholine perchlorate. The compound was synthesized according to the procedure described in the preparation of ClACh from 18·41 g (0·1 mole) choline bromide and 24·23 g (0·12 mole) bromoacetylbromide. The product was obtained in 88 per cent yield (28·6 g). Upon recrystallization from an acetone–ethyl acetate system, colorless needles melting at 103·5 to 104·5° were obtained. *Anal.* Calcd. for $C_7H_{15}BrClNO_6$: C, 25·90; H, 4·66; N, 4·32. Found: C, 26·43; H, 4·68; N, 4·41.

2-Dimethylaminoethyl chloroacetate hydrochloride. To an ice-cold solution of 50·3 ml (0·5 mole) 2-dimethylaminoethanol in 400 ml anhydrous benzene, 45·3 ml (0·6 mole) chloroacetyl chloride was added slowly with vigorous stirring. The temperature was kept below 30° during the reaction. After the addition of chloroacetyl chloride, the reaction mixture was stirred for 30 min at the laboratory temperature. The precipitate that formed at the end of the reaction was washed with chloroform–ether

* Nutritional Biochemicals Corp., Cleveland, Ohio.

† K and K Laboratories, Plainview, N.Y.

‡ All analyses by International Chemical and Nuclear Corp. City of Industry, Calif.

and recrystallized twice from isopropanol. The final product (57·8 g, yield 57 per cent) was hygroscopic white crystals melting at 126–128°. *Anal.* Calcd. for $C_6H_{13}Cl_2NO_2$: C, 35·66; H, 6·48; Cl, 35·09; N, 6·93. Found: C, 35·50; H, 6·66; Cl, 35·04; N, 6·80.

Iodoacetylcholine iodide. 2-Dimethylaminoethyl chloroacetate hydrochloride (46·5 g, 0·2302 mole) dissolved in 400 ml chloroform and 66·7 ml of saturated potassium carbonate solution were thoroughly mixed and the layers were allowed to separate. The chloroform layer was dried over sodium sulfate and treated with 65·37 g (0·4604 mole) methyl iodide at room temperature for 3 days. An oily product separated, which solidified into a white cake upon treatment with acetone. The product was obtained in a 73 per cent yield (51·9 g). Upon recrystallization from a methanol–ethanol solvent system, colorless crystals melting at 146–148° were obtained. *Anal.* Calcd. for $C_7H_{15}I_2NO_2$: C, 21·07; H, 3·79; I, 63·61; N, 3·51. Found: C, 21·16; H, 3·89; I, 63·56; N, 3·60.

Enzymes. AChE (1,000 units/mg protein) was prepared commercially* from bovine erythrocytes. One unit of AChE activity is equivalent to the disappearance of 1 μmole ACh ($2·7 \times 10^{-3}$ M) per min at 25°, as determined by Hestrin's reaction.[13] The solution of the enzyme was prepared in Krebs bicarbonate buffer containing 1% albumin for kinetic studies. No albumin was added for the fluorescence studies.

To supplement the fluorescence studies with AChE, crystalline α-chymotrypsin (45 units/mg protein) was obtained from commercial sources.* One unit of chymotrypsin activity is equivalent to 1 μmole benzoyl-L-tyrosine ethyl ester hydrolyzed per min at pH 7·8 and 25°, as determined by Hummel's method.[14]

Kinetics of the hydrolysis of the substrates. The volume of CO_2 liberated from a bicarbonate buffer by the acid formed during the hydrolysis of the ester was measured at 37° by the Warburg manometric method. The Krebs–Ringer bicarbonate buffer, which consisted of $2·3 \times 10^{-2}$ M $NaHCO_3$, $7·5 \times 10^{-2}$ M KCl, $7·5 \times 10^{-2}$ M NaCl and 4×10^{-2} M $MgCl_2.6\ H_2O$, was prepared according to the methods described previously.[15] The pH of this buffer was found to be 7·5 at 37° when measured according to the methods described by Siggaard-Andersen using a thermostated capillary glass electrode and a thermostated calomel electrode.[16]

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. The manometers were read at suitable intervals during the reaction period 0–60 min. The activity pS curves were constructed for the reaction period 0–20 min, during which the rate of evolution of CO_2 was constant. At least 8 substrate concentrations between the pS values 1·00 and 3·00 were used for each compound. K_m and V_{max} were determined graphically from a Lineweaver-Burk plot of 1/V against 1/S at low substrate concentrations.

In order to determine whether the products of hydrolysis (sodium haloacetates) and weak substrates (Cl_2ACh and Cl_3ACh) would inhibit AChE, the enzyme was preincubated with one of these substances (10^{-2} M) before ACh was added and the evolution of carbon dioxide was recorded. It was found that none of these substances would inhibit AChE significantly.

* Nutritional Biochemicals Corp., Cleveland, Ohio.

Influence of HACh and sodium haloacetates on the fluorescence of chymotrypsin and AChE. It has been shown that, in proteins containing tryptophan, the fluorescence of this residue alone will be observed.[17] Chymotrypsin contains a tryptophyl nucleus and is shown to exhibit a characteristic fluorescence spectrum.[17] It has been observed by us that chymotrypsin as well as AChE exhibits an emission peak at 345 mμ upon excitation at 285 mμ, and the fluorophore is a tryptophyl nucleus. Further, it is known that carboxymethylation of >NH and −SH occurs with haloacetates, especially with iodoacetate.[18, 19] The alkylation of chymotrypsin or AChE with HACh should produce significant changes in the environment of tryptophyl residues. Therefore, we have studied the effects of HACh on the fluorescence characteristics of AChE and chymotrypsin. AChE (6·2 units, 6·2 μg protein) or chymotrypsin (0·25 unit, 51 μg protein) and the substance (10^{-2} M) were incubated at 37° in 3·0 ml Krebs bicarbonate buffer for 30 min and the fluorescence was recorded with a Ferrand spectrofluorometer.

RESULTS

Rates of AChE hydrolysis of HACh and ACh at various reaction times. The rate of evolution of CO_2 was linear during 0–60 min with ACh and propionylcholine as substrates (Fig. 2). There were no significant differences between the rates of hydrolysis of ACh iodide and ACh perchlorate. The rate of hydrolysis of FACh was depressed

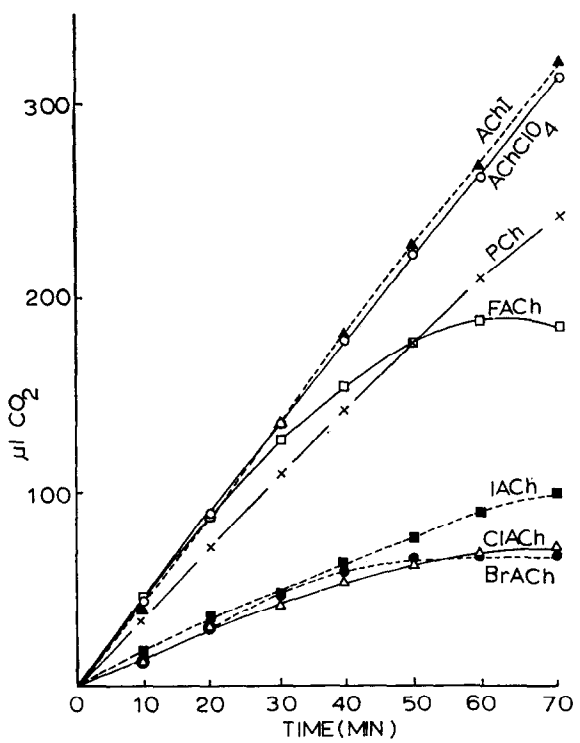


FIG. 2. Pattern of the AChE hydrolysis of acetylcholine (ACh), proprionylcholine (PCh) and haloacetylcholines (FACh, ClACh, BrACh and IACh) as a factor of time. AChE, 2·1 units/ml; pS of substrates, 2·25. Each point is a mean of 3 values.

after 20 min of interaction. The rate of hydrolysis of IACh was not depressed in the first 50 min.

Activity–pS curves of HACh and ACh. The activity–pS curves (Fig. 3) were constructed for the reaction period 0–20 min, during which the rate of evolution of CO_2 was constant. The activity–pS curves of ACh as well as of HACh were bell-shaped, indicating that all of these substances would inhibit AChE at high substrate concentrations. The pS-optimum was about 2·25 for all of them.

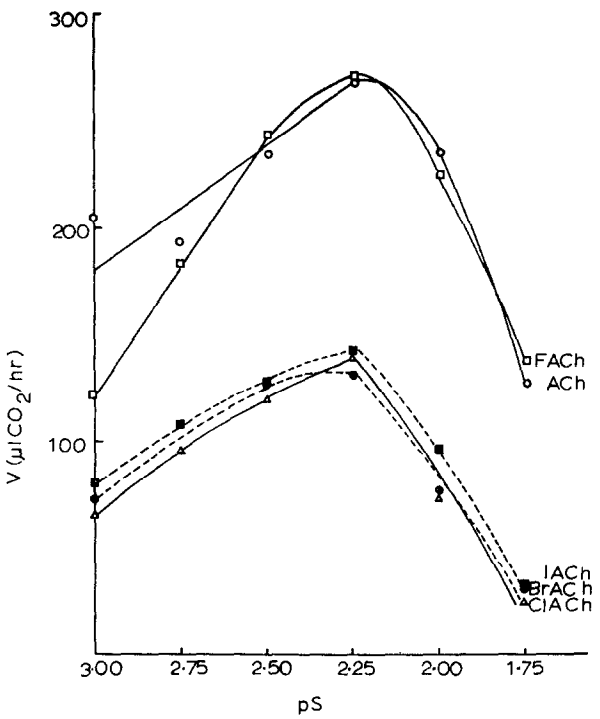


FIG. 3. Activity–pS curves for the enzymatic hydrolysis of acetylcholine (ACh) and haloacetylcholines (FACh, ClACh, BrACh and IACh). AChE, 2·1 units/ml. Each point is a mean of 6 values. The difference between the rates of hydrolysis of FACh and ACh at pS 3·0 is significant at $P < 0.01$. The differences between the rates of hydrolysis of FACh and ACh are not significant ($P > 0.05$) between pS values 2·5 and 1·75. The differences between the rates of hydrolysis of FACh (or ACh) and ClACh (or BrACh or IACh) are significant ($P < 0.001$) at all pS values studied. There are no significant differences ($P > 0.05$) between the rates of hydrolysis of ClACh, BrACh and IACh at all pS values.

At high substrate concentrations, the activity–pS curve of FACh coincides with that of ACh. However, at low substrate concentrations, the hydrolysis of ACh was faster than that of FACh. There are no major differences in the shape of the activity–pS curves of ClACh, BrACh and IACh, and all of them occur clumped together, far below those of ACh or FACh.

Affinities of ACh and HACh to AChE. There were no significant differences between the affinities of the perchlorate and iodide salts of ACh to AChE (Table 1). HACh had higher K_m values and lower affinities than ACh. The affinity of FACh was lower than

TABLE 1. K_m AND V_{max} OF ACh AND HAChs FOR HYDROLYSIS BY AChE

Substrate*	K_m (1×10^{-3} M)†	V_{max} calculated (μl CO_2/hr)†	V_{max} found† (μl CO_2/hr)
ACh·I	1·04 ± 0·21	358 ± 27	270 ± 10
ACh·ClO_4	0·99 ± 0·14	340 ± 23	281 ± 7
FACh·ClO_4	4·11 ± 1·17	309 ± 68	270 ± 7
ClACh·ClO_4	6·42 ± 1·61	170 ± 17	131 ± 10
BrACh·ClO_4	8·18 ± 0·52	192 ± 25	126 ± 9
IACh·I	6·62 ± 1·60	211 ± 6	138 ± 2

* The substrate concentrations have pS values higher than 2·5.
† All values are means ± S.E. of three determinations.

that of ACh, but its V_{max} was equal to that of ACh. There were no significant differences among ClACh, BrACh and IACh in their values for K_m and V_{max}.

Effects of sodium haloacetates on the hydrolysis of ACh. The sodium salts of fluoro-, chloro-, bromo- and iodoacetates did not influence the rate of hydrolysis of ACh by AChE (Table 2). This suggests that inhibition observed with high substrate concentrations of HACh was not due to the accumulation of the corresponding sodium haloacetates in the reaction medium. Further, the carboxylmethylation with iodoacetate (see Table 4) did not influence the enzymic activity of AChE.

TABLE 2. INFLUENCE OF VARIOUS HALOGENATED COMPOUNDS ON THE RATE OF HYDROLYSIS OF ACh BY AChE

Compound* added to incubation mixture of AChE and ACh	Relative rate of hydrolysis† (rate of hydrolysis of ACh without any compound: 100 ± 2·7)
Fluoroacetate	99·9 ± 4·6
Chloroacetate	108·5 ± 4·8
Bromoacetate	101·6 ± 4·8
Iodoacetate	98·9 ± 1·4
Dichloroacetate	97·1 ± 1·8
Trichloroacetate	100·7 ± 1·0
Dichloroacetylcholine	103·5 ± 0·6
Trichloroacetylcholine	104·8 ± 0·3

* The enzyme was preincubated with the compound for 15 min before ACh was added. pS of ACh, 2·25; pS of added compounds, 2·0. All acetates are sodium salts; all cholinesters are perchlorates.
† Each value is a mean ± S.E. from 3 experiments. Differences between various means are not significant ($P > 0·05$).

Effects of Cl_3ACh and Cl_2ACh on AChE hydrolysis of ACh. The lack of hydrolysis of Cl_3ACh or very little hydrolysis of Cl_2ACh (Table 3) might be due 1) to the steric hindrance experienced by these molecules to form enzyme–substrate complex, or 2) to the stability of acylated enzyme (dichloroacetyl- or trichloroacetyl-AChE). Preincubation of AChE with Cl_2ACh or Cl_3ACh did not depress the hydrolysis of ACh (Table 2) indicating that steric hindrance is a major influencing factor in the formation of enzyme–substrate with Cl_3ACh and Cl_2ACh. These experiements did not exclude the possibility that the alkyl chain of Cl_2ACh or Cl_3ACh would not bind

TABLE 3. RELATIONSHIPS BETWEEN AChE HYDROLYSIS, VAN DER WAALS' RADIUS OF C—R BOND AND ELECTROPHILIC CHARACTER OF THE CARBONYL GROUP OF HALOGEN-SUBSTITUTED ACETYLCHOLINES

Substrate*	Relative activity of hydrolysis (%)†		Van der Waals' radius of C—R bond (Å)‡	pKa of acids in the acyl component
	Enzymic	Nonenzymic		
ACh·I§	100·0 ± 7·1		2·27	4·75
ACh·ClO$_4$	97·3 ± 1·6	1·0 ± 0·18	2·27	4·75
FACh·ClO$_4$	104·9 ± 7·5	18·5 ± 1·7	2·76	2·66
PCh·ClO$_4$	72·2 ± 7·6		3·12	4·86
ClACh·ClO$_4$	32·3 ± 4·2	18·5 ± 2·4	3·56	2·85
BrACh·ClO$_4$	32·3 ± 7·4	18·4 ± 2·4	3·86	2·85
IACh·I	39·2 ± 7·1	16·5 ± 2·1	4·25	2·91
Cl$_2$ACh·ClO$_4$‖	19·4 ± 4·1	307·6 ± 11·3		1·30
BCh·ClO$_4$	7·7 ± 2·6		4·31	4·83
Cl$_3$ACh·ClO$_4$‖	not significant	403·1 ± 6·5		0·70

* pS = 2·00.

† All values are means ± S.E. for 6 determinations and are calculated from the initial rates of hydrolysis. pH of the reaction medium 7·5 at 37°.

‡ Calculations are shown in Fig. 1.

§ With all substrates except Cl$_2$ACh·ClO$_4$ and Cl$_3$ACh·ClO$_4$, the initial linear rate (μl CO_2/20 min.) for total hydrolysis (enzymic + nonenzymic) is significantly different from the nonenzymic rate of hydrolysis at $P < 0·001$; both rates of hydrolysis were measured simultaneously.

‖ With Cl$_2$ACh·ClO$_4$ as substrate, the initial linear rate (424 ± 3 μl CO_2/10 min) for total hydrolysis (enzymic + nonenzymic) is significantly different from the nonenzymic rate of hydrolysis (413 ± 3 μl CO_2/10 min) at $P < 0·05$; both rates of hydrolysis were measured simultaneously. With Cl$_3$ACh·ClO$_4$ as substrate, the initial rate for total hydrolysis (413 ± 4 μl CO_2/10 min) does not differ significantly from nonenzymic rate of hydrolysis (413 ± 3 μl CO_2/10 min). Due to the fast nonenzymic rate of hydrolysis, these observations are valid only for qualitative interpretation to a limited extent. For the same reasons, their K_m and V_{max} could not be determined.

TABLE 4. EFFECTS OF VARIOUS HALOGENATED COMPOUNDS ON THE FLUORESCENCE OF ACETYLCHOLINESTERASE AND CHYMOTRYPSIN

Substance incubated with AChE* or chymotrypsin	Relative fluorescence intensity† (% ± S.E.)	
	AChE = 100 ± 1·5‡	Chymotrypsin = 100 ± 2·8‡
Acetylcholine iodide	90·3 ± 0·5	
Acetylcholine perchlorate	93·6 ± 1·4	91·9 ± 2·5
Fluoroacetylcholine	88·4 ± 3·1	91·8 ± 1·9
Chloroacetylcholine	87·4 ± 0·6	91·0 ± 3·8
Bromoacetylcholine	78·9 ± 2·0	83·3 ± 2·2
Iodoacetylcholine	5·7 ± 0·2	11·4 ± 0·9
Sodium iodoacetate	1·4 ± 0·2	10·8 ± 0·6
Dichloroacetylcholine	91·0 ± 1·7	89·1 ± 2·5
Sodium dichloracetate	92·6 ± 2·5	95·1 ± 3·8
Trichloroacetylcholine	85·3 ± 2·8	89·7 ± 9·1
Sodium trichloracetate	89·1 ± 2·5	93·9 ± 2·3
Choline iodide	96·6 ± 2·2	
Choline perchlorate	101·3 ± 1·0	100 ± 4·3

* AChE, 2·1 units/ml; substance, 10^{-2} M; period of incubation, 30 min; reaction medium, Krebs bicarbonate buffer at 37°. All substances are perchlorates unless otherwise stated.

† Excitation maximum, 285 mμ; emission maximum, 345 mμ.

‡ All values are means from 4 observations.

at nonspecific sites on the enzyme protein by hydrophobic bonds.[20] However, no changes in the enzyme activity or its fluorescence characteristics were observed during the interaction with AChE (Tables 2 and 4).

Influence of HACh on the fluorescence of AChE. Among all cholinesters, IACh was most effective in quenching the fluorescence of AChE (Table 4). Similarly, carboxymethylation with iodoacetate quenches the fluorescence of AChE. BrACh was about 15–16 times less effective than IACh as an alkylating agent. There were no significant differences among FACh, ClACh, Cl_2ACh, Cl_3ACh and ACh in quenching the fluorescent intensity of AChE or chymotrypsin.

DISCUSSION

It is clearly evident from the results reported in this communication that halogenation of the acyl component of ACh influences significantly the hydrolysis by AChE. At least three physicochemical factors should be evaluated in considering HACh as substrates to AChE: 1) the influence of an α-halogen atom on the electrophilic nature of the carbonyl group, 2) the lability of the halogen atom, and 3) the steric effects of halogenation.

A comparison of the pK_a values of halogenated acetic acids suggests that the carbonyl carbons in HACh are more electrophilic than that of ACh (FACh > ClACh > BrACh > IACh). The nonenzymic rate of hydrolysis of HACh are according to the electrophilic nature of their carbonyl carbons (Table 3). Therefore, one would expect that the relative rates of AChE hydrolysis of HACh should be higher than that of ACh. However, all of them were hydrolyzed by AChE at lower rates than that of ACh. At pS-optimum, the rate of hydrolysis of FACh was equal to that of ACh; but at low substrate concentrations, the rate of hydrolsis of ACh was considerably higher than that of FACh. Similarly, ACh exhibited significantly higher affinity to AChE than FACh, because K_m values were determined from the initial rates of hydrolysis at low substrate concentrations. There were no significant differences among ClACh, BrACh and IACh in their relative rates of hydrolysis, K_m or affinities, and V_{max}. Therefore, replacement of one of the hydrogens in the acetyl group with any one of the halogens decreases the affinity to the enzyme, and V_{max} that is equivalent to that of ACh could be obtained only with FACh. Replacement of two hydrogens with chlorines decreased the activity by about 80 per cent. Cl_3ACh was not hydrolyzed by the enzyme.

A question might arise as to whether the covalently bonded halogens could spontaneously dissociate or alkylate the enzyme under the present experimental conditions. Of all compounds studied, IACh or sodium iodoacetate was able to bring about changes in the environment of tryptophyl residues and depress the fluorescence intensity of AChE or chymotrypsin. A number of studies have indicated that the I—C bond was the weakest, and iodine may split off from iodoacetate (or IACh) with the attachment of $—CH_2COO^-$ to —SH or >NH groups of enzymes.[18, 19] However, alkylation of the enzymes did not seem to influence the hydrolysis of IACh because; 1) sodium iodoacetate does not inhibit the enzymic hydrolysis of ACh, and 2) the tryptophyl nucleus is not part of the active site of AChE.[21]

The steric effects of halogenation seem to play an important role in the AChE hydrolysis of HACh. The fluorine atom is the smallest of the halogen atoms, and

replacement of hydrogen with fluorine decreased the affinity. Substitution of a hydrogen with larger halogen atoms, Cl, Br or I, decreased both affinity and V_{max}. Addition of two or three halogens to the acyl group of ACh acts: 1) to shorten interatomic distances and thus increase the force with which the halogens are held to carbon; 2) to increase the steric effects by increasing bulk around the methylene carbon; and 3) to increase the electrophilic nature of the carbonyl carbon. The rate of hydrolysis of Cl_2ACh was about 1/5 of that of ACh. The hydrolysis of Cl_3ACh* was not catalyzed by AChE. Further, the relative rates of hydrolysis of HACh could be arranged in the order of their Van der Waals' radii of C—R bond (Fig. 1, Table 3). Therefore, the space available at the esteratic site of AChE seems to be optimum for ACh, and substitution of even one hydrogen by a halogen atom decreases its affinity for the enzyme.

The recent investigations by Krupka and Laidler,[22] and of Wilson and Alexander[23] suggest that deacetylation of acetyl-AChE is prevented by binding of a molecule of ACh to the acylenzyme at the anionic site at high substrate concentrations. A similar possibility exists for the accumulation of haloacetyl-AChE because: 1) the maximum concentrations of reaction products formed, choline or sodium haloacetates, did not inhibit the enzyme; and 2) carboxymethylation of AChE with iodoacetate did not depress the rate of hydrolysis of ACh. A molecule of HACh may interact with the anionic site of haloacetyl-AChE and prevent its dehaloacylation at high substrate concentrations.

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* High concentrations of CCl_3COOH precipitate proteins in strong acid solutions. AChE was not precipitated because the pH of the reaction mixture was 7·5, and preincubation with Cl_3COONa at pH 7·5 did not depress the activity of AChE.

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