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On the mechanism of potentiation of the activity of acetylcholinesterase by some quaternary ammonium compounds

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Tetraethylammonium iodide (TEA) increases the maximum velocity of acetylcholine hydrolysis nearly 2-fold whereas it shows purely competitive kinetics when the dipropyl-analogue of acetylcholine (2-acetoxyethyldi-n-propylmethylammonium iodide) is the substrate. Unlike acetylcholine, the dipropyl-analogue does not have deacetylation as its rate determining step and this is further evidence that quaternary ammonium compounds potentiate acetylcholinesterase by accelerating the deacetylation step. The effects of 22 quaternary ammonium compounds on the rate of hydrolysis of acetylcholine, phenyl acetate and dimethylcarbamylacetylcholinesterase have been examined. The acceleration of deacylation by quaternary ammonium compounds was found to have a high degree of structural specificity. Possible mechanisms for this phenomenon are discussed in the light of the structure-action results.

Previously, Roufogalis & Thomas (1968 a, b, c) showed that some quaternary ammonium compounds and inorganic ions potentiate the hydrolytic activity of acetylcholinesterase under certain conditions. They suggested that the potentiation was by accelerating the rate of deacetylation in the hydrolytic sequence. We now provide further evidence for this mechanism and suggest a possible explanation for the acceleration of deactylation.

Belleau (1967) examined the free energy of binding of an homologous series of quaternary ammonium compounds onto acetylcholinesterase. He retained as constant the trimethylammonium head and investigated the effect of increasing chain length on the thermodynamics of binding. We have systematically altered the structure of the quaternary ammonium head, and investigated the effects of these changes on both potentiation of acetylcholinesterase and on the free energy of binding. Spiran quaternary ammonium compounds have relatively rigid structures and have proved to be useful for this purpose, since very small structural variations around the quaternary nitrogen produced more or less absolute effects on the consequences of binding of these compounds to the enzyme. The consequences of binding were determined by measuring the effect of the compounds on the deacylation step in the hydrolysis sequence.

EXPERIMENTAL

Chemistry

The synthesis of compounds I to XI (Table 2) has been previously described (Roufogalis & Thomas, 1968a). Two main methods were used for the synthesis of compounds XII to XII (Table 2).

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Method A. An α,ω-dihaloalkane (1 mol equiv) was reacted with amine (2 mo equiv) in a suitable solvent. The total concentration of reactants was kept below 5% (w/v) of the total volume of reaction mixture to promote an intramolecular cyclization, giving the required spiran quaternary ammonium halide or *NN*-dialkylcycloalkylammonium halide and amine hydrohalide. The reaction was carried out by one of two methods: (*a*) reactants were refluxed in chloroform for a suitable time (6–24 h); or (*b*) a solution of reactants in methanol was autoclaved for 50 min at 125°. The reaction solution from either *a* or *b* was distilled to dryness under reduced pressure on a water bath, the solid residue dissolved in water, sodium hydroxide (1 mol equiv) added, and the solution distilled to dryness under reduced pressure on a water bath. The resulting solid was extracted with chloroform in a Soxhlet extractor, and then purified by repeated crystallization from a suitable solvent (mixture).

Method B. The secondary amine (1 mol equiv) was refluxed with an alkyl halide (2 mol equiv) in a suitable solvent. The solution was evaporated to dryness on a water bath, the solid residue dissolved in water, sodium hydroxide (1 mol equiv) added and the resulting free amine extracted with ether, either with three separate portions or continuously. The ether was dried over anhydrous sodium sulphate and distilled on a water bath. The remaining liquid was distilled under reduced pressure in an oil bath. The resulting tertiary amine was added to a small volume of dried ethyl methyl ketone (25–100 ml) and the alkyl halide (2 mol equiv) added. The solution was either allowed to stand for 2–7 days, or refluxed for 6 h. If the solid separated out on cooling it was filtered, otherwise the ethyl methyl ketone was evaporated to dryness on a water bath. The solid was purified by repeated crystallizations.

A list of the quaternary ammonium compounds XII to XII is given in Table 1, together with analytical data, physical constants, and methods of preparation.

2-*Acetoxyethyldi-n-propylmethylammonium iodide* was prepared according to Roufogalis & Thomas (1968c).

The following compounds were used as received: tetramethylammonium iodide, tetra-n-propylammonium iodide, tetra-n-butylammonium iodide, acetylcholine perchlorate (BDH) and tetraethylammonium iodide (Hopkin and Williams).

Enzymic analyses

Acetylcholinesterase was a purified bovine erythrocyte preparation (Nutritional Biochemicals Corporation). Enzymic analyses were made by the pH stat method. A Radiometer titrator (TTTIc) equipped with recorder (SBR2c), expanded scale (pHA 630) and syringe burette (0·5 ml) with sodium hydroxide solution (0·01N, CO_2 free) as titrant, were used. The solutions were stirred mechanically throughout and dry nitrogen passed over the surface. Corrections for changes in pH caused by stirring of low ionic strength media were made (Roufogalis & Thomas, 1968b).

Procedure for determining acetylcholine hydrolysis at high substrate concentrations. The enzyme solution was made by dissolving 10 mg of acetylcholinesterase in 100 ml of glass distilled water. It was stored at 4°. Fresh solutions were made daily.

The quaternary ammonium compounds were incubated for 15 min at pH 7·4 and 37° in a medium containing glass-distilled water and 3·0 ml of the enzyme solution in a jacketed glass vessel. The volume of the incubation mixture was (20-x) ml, where x

Table 1. *Preparation of quaternary ammonium compounds XII to XXII, their physical constants and analytical data.* All compounds had C, H, N analyses within the usual limits

Compound	Amine	Alkyl halide	Method (see text)	Solvent and time of reflux	Solvent for recrystallization	m.p.[1] (°C)
XII	Piperidine	1,4-Dibromopentane[3]	Aa	Chloroform 6 h	Ethyl methyl ketone	265
XIII	2,5-Dimethyl pyrrolidine[4,5]	1,5-Dibromopentane	Ab	Methanol	Chloroform	242–243
XIV	2-Methylpiperidine	1,4-Dibromobutane	Aa	Chloroform 12 h	Chloroform–ethyl methyl ketone	285–286
XV	2-Methylpiperidine	1,5-Dibromopentane	Aa	Chloroform 12 h	Chloroform	247[3]
	Piperidine	Iodoethane	B[7]	70% Ethanol 6 h	Absolute[6] ethanol	214–216[6]
XVI	*N*-Ethylpiperidine	Bromoethane	B[7]	Ethyl methyl ketone	Ethyl methyl ketone	274–275
XVII	Morpholine	Iodoethane	[8]	—	Absolute[9] ethanol–ethyl methyl ketone	246–247
XVIII	Diethylamine	1,4-Dibromobutane	Aa	Chloroform 12 h	Chloroform–ethyl methyl ketone	286
XIX	Pyrrolidine	1,4-Diiodopentane[3]	Aa	Chloroform 4 h	Ethyl methyl ketone[2]	280–281
XX	Diethylamine	1,4-Dibromopentane[3]	Aa	Chloroform 8 h	Chloroform–ethyl methyl ketone	276–277
XXI	*trans*-2,6-Dimethylpiperidine[10]	1,4-Dibromobutane	Ab	Methanol	Chloroform–ethyl methyl ketone	265–266
	Diethylamine	2-chloroethanol	B	70% Ethanol	—	b.p.[11] 160–161
XXII	2-Diethylaminoethanol	2-Iodo ethanol	B	Ethyl methyl ketone	Absolute ethanol	230–231

[1] Uncorrected m.p.
[2] Soxhlet extraction method was used for recrystallization.
[3] Prepared by J. Thomas and D. Hawley.
[4] *cis* and *trans* isomers not separated.
[5] This compound is 90–95% one isomer.
[6] Compound was isolated as *N*-ethylpiperidine and the solvent for crystallization, m.p. and analysis figures refer to the hydrobromide derivative.
[7] Repeated attempts to prepare this compound by method A (diethylamine and 1,5-dibromopentane) were unsuccessful.
[8] *NN*-Diethylmorpholinium iodide was prepared by refluxing morpholine (8·7 g) and ethyliodide (16·0 g) in methanol (100 ml) for 6 h, then sodium hydroxide (4 g) was added and stirred until dissolved. Ethyl iodide (16·0 g) was added and reaction refluxed for a further 6 h. Product worked up as in method Ba.
[9] Attempts to use absolute ethanol-chloroform were repeatedly unsuccessful.
[10] *trans* isomers separated from *cis*/*trans* mixture with a Loenco preparative GLC.
[11] Boiling point refers to 2-diethylaminoethanol, lit. b.p. 163°.

is the volume of the substrate solution. After 15 min incubation the substrate was added and the titrator started. The pH was maintained at 7·4 and the temperature at 37°. At very low substrate concentrations the reaction volume was increased to 40 ml (the amount of enzyme was also doubled to maintain a constant concentration). The resulting plots of sodium hydroxide against time were linear for at least 2·5 min and

Table 2. *The effect of quaternary ammonium compounds on the hydrolysis of acetylcholine, phenyl acetate and dimethylcarbamyl-acetylcholinesterase at low ionic strength.* "Maximum potentiation" has been defined in Methods. "V/Vo" gives the ratio of the maximum velocity in the presence of compound to that in its absence, at ionic strength less than 0·005. "*a*k3′/k3′" gives the ratio of the apparent rate of decarbamylation in the presence of compound to that in its absence, at ionic strength less than 0·005

	Compound	Maximum potentiation (Acetylcholine hydrolysis)	V/Vo (Phenyl acetate hydrolysis)	*a*k3′/k3′ (decarbamylation)
	Tetramethylammonium iodide	—	1·27 ± 0·03	1·25
	Tetraethylammonium iodide	76 ± 5%	1·94 ± 0·34	3·95
	Tetrapropylammonium iodide	—	0·62 ± 0·03[1] 0·43 ± 0·02[2]	1·3
	Tetrabutylammonium iodide	—	—	1·1
I	1,1′-Spirobipiperidinium bromide	—	—	2·7
II	*cis*-2,6-Dimethylspirobipiperidinium bromide	—	1·12 ± 0·05	1·2
III	*cis*-2,6-Dimethylspiro-(piperidine-1,1′-pyrrolidinium) bromide	52 ± 2%	2·02 ± 0·12	9·6
IV	Spiro(piperidine-1,1′-pyrrolidinium) bromide	11 ± 3%	—	0·94
V	1,1′-Spirobipyrrolidinium bromide	19 ± 4%	—	1·2
VI	Spiro(piperidine-1,4′-morpholinium) bromide	—	—	4·2
VIII	2,6-Dimethylspiro(morpholine-4,1′-piperidinium) bromide	—	1·32 ± 0·45	—
XII	2-Methylspiro(pyrrolidine-1,1′-piperidinium) bromide	0%	1·17 ± 0·28	2·55
XIII	2,5-Dimethylspiro(pyrrolidine-1,1′-piperidinium) bromide	—	1·10 ± 0·09	1·23 ± 0·27
XIV	2-Methylspiro(piperidine-1,1′-pyrrolidinium) bromide	—	—	3·5
XV	2-Methyl-1,1′-spirobipiperidinium bromide	9 ± 1%	—	2·8
XVI	1,1-Diethylpiperidinium bromide	—	—	0·93
XVII	1,1-Diethylmorpholinium iodide	24 ± 1%	—	2·1
XVIII	1,1-Diethylpyrrolidinium bromide	33 ± 2%	2·02 ± 0·38	—
XIX	2-Methylspirobipyrrolidinium iodide	11 ± 1%	—	—
XX	1,1-Diethyl-2-methylpyrrolidinium bromide	56 ± 4%	2·26 ± 0·42	—
XXI	*trans*-2,6-Dimethylspiro-(piperidine-1,1′-pyrrolidinium) bromide	50 ± 2%	—	—
XXII	Di(2-hydroxyethyl)-diethylammonium iodide	—	0·92 ± 0·06	71

[1] This result was obtained at pH 8·4. Concentration of TPA $2{\cdot}21 \times 10^{-6}$M.
[2] This result was obtained at pH 8·4. Concentration of TPA $1{\cdot}11 \times 10^{-5}$M.

the amount of substrate consumed during this time was less than 20% of the total available. All rates were corrected for non-enzymic hydrolysis.

Final estimates of "maximum potentiation" (Table 2) are the average of two independent determinations. The effect of quaternary ammonium compounds on acetylcholine hydrolysis at ionic strengths less than 0·005 was obtained by:

(*a*) varying the concentration of compound over a 100-fold range at an acetylcholine concentration of $2{\cdot}34 \times 10^{-3}$M (8-fold higher than the optimum substrate concn):

(*b*) varying the concentration of acetylcholine over a 100-fold range in the presence

of a concentration of compound found to give "maximum potentiation" in (*a*) above, and again obtaining the maximum potentiation at [S] = $2{\cdot}34 \times 10^{-3}$M.

Examples of the resulting plots from (a) and (b) above may be found in Figs 2 and 7(b) in Roufogalis & Thomas (1968a).

The procedures for determining the effect of quaternary ammonium compounds on phenyl acetate hydrolysis were described by Roufogalis & Thomas (1968b), then for the kinetics of acetylcholine and 2-acetoxyethyldi-n-propylmethylammonium iodide hydrolysis at below optimum substrate concentrations by Roufogalis & Thomas (1968c) and those for the decarbamylation of dimethylcarbamylacetylcholinesterase by Roufogalis & Thomas (1969).

RESULTS AND DISCUSSION

That some quaternary ammonium compounds potentiate acetylcholinesterase by accelerating the deacetylation step in the hydrolysis sequence has been concluded from the following evidence. First, in experiments on the rate of hydrolysis *vs* substrate (acetylcholine) concentration, the presence of compounds such as tetraethylammonium iodide (TEA) causes the optimum substrate concentration to be shifted towards a higher substrate concentration, and the optimum rate of hydrolysis in the presence of these compounds is greater than the optimum rate in their absence (Roufogalis & Thomas, 1968a).

Second, the maximum velocity of phenyl acetate hydrolysis in the presence of, for example, TEA is increased (about 2-fold) over the maximum velocity obtained in the absence of such cations. Since the maximum velocity of phenyl acetate hydrolysis is directly dependent on the rate determining deacetylation step in the hydrolysis sequence, it follows that compounds such as TEA are accelerating the deacetylation step in the hydrolysis sequence (Roufogalis & Thomas, 1968b).

Third, the first-order rate of decarbamylation of dimethylcarbamyl-acetylcholinesterase is accelerated by TEA and some other quaternary ammonium compounds (Roufogalis & Thomas, 1969). Decarbamylation is considered to be analogous in many ways to deacetylation of the acetyl-enzyme and this system serves as a model for the deacetylation reaction.

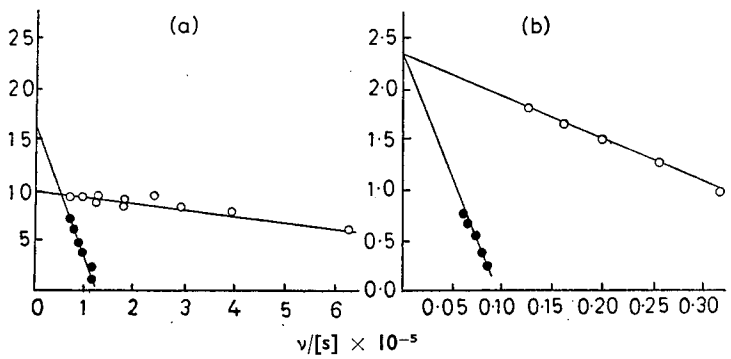


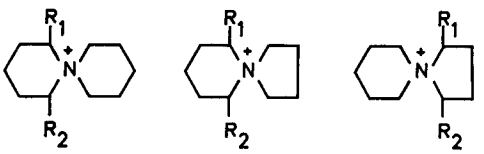
FIG. 1. Effect of TEA on the hydrolysis of (a) acetylcholine and (b) 2-acetoxyethyldi-n-propylmethylammonium iodide. The maximum velocity for the hydrolysis of acetylcholine has been arbitrarily set at 10·0, and represents a maximum velocity of approximately 20 μm/min. Substrate concentrations studied are in the range $1\text{–}15 \times 10^{-5}$M. Ionic strength in the control plots is less than 0·005. ○—○ Control. ●—● A, TEA, $1{\cdot}22 \times 10^{-3}$M. ●—● B, TEA, $4{\cdot}87 \times 10^{-4}$M.

As further evidence we have now examined the kinetics of the effect of TEA on the hydrolysis of acetylcholine, a substrate considered to have deacetylation as the rate-determining step in the enzyme-catalysed hydrolysis (Wilson & Cabib, 1956). The result is shown in Fig. 1a and it can be seen that at low substrate concentrations TEA inhibits acetylcholine hydrolysis, but the maximum velocity of hydrolysis of acetylcholine in the presence of TEA is increased 1·6-fold over that of the control. In Fig. 1b is shown the effect of TEA on the hydrolysis of 2-acetoxyethyldi-n-propylmethylammonium iodide. Comparison of the control plots of Fig. 1a and b reveals that the maximum velocity for the hydrolysis of dipropyl compound is 4·3 times slower than that for acetylcholine hydrolysis. Since both substrates are acetate esters, it follows that deacetylation, a step common to the hydrolysis of both substrates, cannot be the rate-determining step in the hydrolysis of the dipropyl compound. If quaternary ammonium compounds are acting by accelerating deacetylation, TEA would not be expected to increase the maximum velocity of the dipropyl compound, since deacetylation is not rate-determining in the hydrolysis of this substrate. Purely competitive kinetics might be expected in this case (Krupka & Laidler, 1961a). This is what is observed in Fig. 1b, which further supports the proposed mechanism.

Structure-activity relations

In an attempt to elucidate the mechanism by which some quaternary ammonium compounds accelerate deacetylation when bound, presumably to the acetylenzyme (Krupka & Laidler, 1961b; Roufogalis & Thomas 1968 a, b), the structure-activity relations among 22 quaternary ammonium compounds have been investigated. Three different systems have been used, the hydrolysis of acetylcholine at above optimum substrate concentrations, the hydrolysis of phenyl acetate, and the decarbamylation of dimethylcarbamyl-acetylcholinesterase. The results (Table 2) are discussed below.

Fig. 2 shows the effects of eight representative compounds on the rate of decarbamylation. The number after each compound is the number of times that compound



I $R_1=R_2=H$,	2·7	IV $R_1=R_2=H$,	0·94	IV $R_1=R_2=H$,	0·94
XV $R_1=H$; $R_2=Me$,	2·8	XIV $R_1=H$; $R_2=Me$,	3·5	XII $R_1=H$, $R_2=Me$,	2·6
II $R_1=R_2=Me$,	1·2	III $R_1=R_2=Me$,	9·6	XIII $R_1=R_2=Me$,	1·2

FIG.2. Effect of some spiran quaternary ammonium compounds on the decarbamylation of dimethylcarbamyl-acetylcholinesterase. The number after each compound represents the number of times the reaction is accelerated by the compounds; a value of 1 represents no effect and values greater than 1 the amount of acceleration. The decarbamylation has been studied in the absence of added inorganic ions (see methods).

accelerates this reaction. In the middle column, as methyl groups are added to the piperidine ring of compound IV, in the position α to the nitrogen, there is a progressive increase in accelerator potency, from no effect with compound IV to 10-fold acceleration with compound III. However increase in branching does not always lead to an increase in accelerator potency, as seen in the first and third columns. In these cases progressive addition of methyl groups to the piperidine ring of compound I and the

pyrrolidine ring of compound IV does not lead to an increase in accelerator potency, since in both series the most highly branched members are inactive as accelerators of the decarbamylation reaction. Thus the structure-activity relations are subject to highly specific steric control. This is well illustrated in the third row, where it is seen that contraction of the unsubstituted piperidine ring of compound II (inactive as accelerator) by one carbon, to give compound III increases accelerator potency 10-fold. When the methyl groups of compound III are transferred to the pyrrolidine ring, as in compound XIII, the compound becomes inactive as an accelerator. The *cis*- and *trans*-isomers of 2,6-dimethylspiro(piperidine-1,1′-pyrrolidinium) bromide have the same accelerator potency (Table 2, compounds III and XXI respectively). Tetraethylammonium is a potent accelerator of deacetylation and decarbamylation, but *NN*-diethylpiperidinium bromide (XVI) is inactive as an accelerator of decarbamylation (Table 2). On the other hand, *NN*-diethylpyrrolidinium bromide (XVIII) is a fairly potent accelerator of deacetylation and branching with one methyl group on the pyrrolidinium ring (compound XX) increases accelerator potency even further (Table 2).

It appeared possible that the compounds which do not potentiate acetylcholine hydrolysis were inhibiting deacetylation. However, the compounds which do not potentiate acetylcholine or phenyl acetate hydrolysis i.e. (TMA, II, VIII, XII, XIII, and XXII) do not block deacetylation (Roufogalis, 1968). The one exception is tetrapropylammonium iodide (TPA); this compound non-competitively decreases the maximum velocity of phenyl acetate hydrolysis, probably by blocking deacetylation (Krupka, 1965, Roufogalis & Thomas, 1968b).

The free energy of binding of quaternary ammonium compounds to acetylcholinesterase

The equilibrium constant for the dissociation of quaternary ammonium compounds from acetylcholinesterase can be characterized by K_I. This constant may be used to determine the free energy of binding of a compound to the enzyme from the relation

$$\Delta F_I = -RTL_n \frac{1}{K_I}$$

There is now a substantial body of evidence to indicate that the active site of acetylcholinesterase is non-polar in nature (see Belleau & Lacasse, 1964). The present discussion refers to the effects of the quaternary ammonium compounds on the acetyl-enzyme, and it is also considered to be non-polar.*

Belleau & Lacasse (1964) have pointed out that if an enzyme active site is non-polar, the presence of hydrophobic substituents in a substrate or inhibitor molecule will supply a significant driving force for adsorption onto the enzyme and this driving force has its origins in the entropically unfavourable hydrophobic interactions created by the non-polar substituents in water. It has been calculated from studies on the transfer of amino-acids from water to octanol (Cohn & Edsall, 1943) that the contribution of a methyl or methylene group to hydrophobic transfer is −720 cal. It is possible therefore by determining the ΔF_I of an inhibitor of acetylcholinesterase to calculate a theoretical ΔF_I for a second inhibitor which differs from the first by a

* Results obtained by Krupka (1965) support this assumption. In the series TMA, TEA, TPA, and tetra-n-butylammonium iodide (TBA), the equilibrium constant for the dissociation of these compounds with the free enzyme (K_I) and with the acetylated enzyme (K'_I) were of the same order and the variation of both K_I and K'_I with the structure of the above series of compounds followed a similar pattern.

$-CH_2-$ group. This was the approach used by Belleau & Lacasse (1964) with a series of dioxalan and tetrahydrofuran derivatives and by Belleau (1967) with the alkyltrimethylammonium series. Calculations of this type have now been made on a series of quaternary ammonium compounds and the calculated ΔF_i values compared with the experimentally determined ΔF_i values.

Table 3. *The effect of the addition of four methyl groups to* TMA *and* TEA *on the free energy of binding to free enzyme and acetyl-enzyme*

Compound	ΔFi Obsd[2]	ΔFi Calc	ΔFi Calc-Obsd (Free enzyme)	ΔFi Calc-Obsd[3] (Acetyl-enzyme)	Effect on Deacetyl-ation[1]
TMA	—4940				○
TEA	—5250	—7820	—2570	—2170	+++
TPA	—7180	—8170[4]	—990[4]	—730 [4]	−−

[1] A negative sign indicates compounds block deacetylation and the number of signs indicates the degree of blocking. ○ indicates no effect on deacetylation. + indicates acceleration of deacetylation and the number of signs indicates the degree of acceleration.
[2] Ki values calculated from Roufogalis & Thomas (1968b).
[3] Computed from the results of Krupka (1965).
[4] Calculated from the observed value of ΔFi for TEA so that ΔFi (Calc-Obsd) refers to the effect of the addition of four methyl groups to TEA.

In Table 3 this treatment has been applied to the series, TMA, TEA and TPA. The free energy of binding observed for TMA is −4940 cal. The addition of four $-CH_2-$ groups to produce TEA should increase the free energy of binding by −2880 cal (4×720) to a value of −7820 cal if hydrophobic transfer forces are operating optimally. In fact the ΔF_i observed for the binding of TEA to both free enzyme and the acetylated enzyme is over 2000 cal lower than the ΔF_i calculated. This suggests that the forces determining the difference in enzyme interaction in going from TMA to TEA are not simply hydrophobic and that other factors offset the hydrophobic transfer. TEA is a good potentiator. A similar analysis of the effect of the addition of four $-CH_2-$ groups to TEA to produce TPA shows that there is now a much smaller deviation between the calculated and observed values of ΔF_i for binding of TPA (−860 cal). It is also noted that TPA is not a potentiator but is an inhibitor of the deacetylation reaction. The closeness of the calculated and observed values for the binding of TPA suggests that the γ-methyl groups of TPA interact largely with a non-polar environment of the active site. Small deviations from calculated values of ΔF_i have been interpreted by Belleau & Lacasse (1964) in terms of the difficulty of getting an "ideal solution" of a non-polar group in a non-polar environment of the active site.

The treatment used above has been extended to some representative spiran quaternary ammonium compounds. The results are in Table 4 and the differences between the calculated and observed values for ΔF_i of binding for these compounds are compared with the effects of the compounds on deacetylation. The calculated values of the ΔF_i of binding for the compounds which accelerate deacetylation strongly (III, XI) are about 1500 cal different from the values observed, whereas the differences between the calculated and observed ΔF_i for the compounds which do not accelerate deacetylation (i.e. I, II, XIII, VIII) are much smaller (300 to 900 cal positive or

Table 4. *The free energy of binding of spiran quaternary ammonium compounds with acetylcholinesterase*

Compound	ΔFi Obsd[2]	ΔFi Calc[3]	ΔFi Calc-Obsd	Effect on Deacetylation[1]
IV	—2940			○ to +
I	—3420	—3660	— 240	○ to +
III	—2760	—4380	—1620	+++
II	—3920	—3480	+ 440[4]	○
		—4860	— 940[5]	
XIII	—3500[6]	—4380	— 880	○
VI	—2230			++
VIII	—4110	—3670	+ 440	○
XI[7]	—2400	—3670	—1270	++ to +++

[1] For meaning of signs see Table 3.
[2] ΔFi calculated from I50 values from Roufogalis & Thomas (1968a).
[3] ΔFi (calc) obtained by adding —720 cal per methyl or methylene group to ΔFi (Obsd) for parent compound IV or VI, unless otherwise stated.
[4] Refers to the addition of $-CH_2-$group to compound III.
[5] Refers to the addition of two methyl groups to compound I.
[6] Calculated from Ki from Roufogalis (1968).
[7] 2,6-Dimethylspiro(piperidinium-1,4′-morpholinium) iodide.

negative). In both series the compounds which are potentiators are the ones which show the greatest deviation of calculated to observed ΔF_i values. Precisely what this means in terms of a mechanism of acceleration of deacetylation is difficult to interpret. However it does seem to indicate that acceleration is related to a poor interaction between quaternary ammonium ions and the enzyme. A detailed analysis of the factors which offset the free energy change which results from hydrophobic transfer is difficult because the addition of methyl or methylene groups to the spiran quaternary ammonium compounds will alter parameters in the compounds other than their hydrophobic nature. For example, changes in the overall shape of the molecule, in steric effects due to protruding substituents or changes in the ring size would all affect the interaction with the enzyme and solvent. Coulombic interaction could also be modified as a result of changes in the radius of the cation (Robinson & Stokes, 1965) although this effect is not likely to be as important in the spirans as in the tetra-alkyl-ammonium series. Furthermore, these changes may also be sufficient to alter the site of interaction with the enzyme (O'Brien, 1969). Nevertheless despite these difficulties of interpretation, by applying this treatment to the present series, it can be seen that deviation in ΔF_i observed and calculated is different for potentiators and non-potentiators. To date this is the only property which has correlated with potentiation.

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REFERENCES

BELLEAU, B. (1967). *Ann. N.Y. Acad. Sci.*, **144**, 705–719.

BELLEAU, B. & LACASSE, G. (1964). *J. mednl Chem.*, **7**, 768–775.

COHN, E. S. & EDSALL, J. T. (1943). In *Proteins Amino Acids and Peptides* p. 212. New York: Reinhold.

KRUPKA, R. M. (1965). *Biochemistry*, **4**, 429–435.
KRUPKA, R. M. (1966). *Ibid.*, **5**, 1988–1998.
KRUPKA, R. M. & LAIDLER, K. J. (1961a). *J. Am. chem. Soc.*, **83**, 1454–1458.
KRUPKA, R. M. & LAIDLER, K. J. (1961b). *Ibid.*, **83**, 1448–1454.
O'BRIEN, R. D. (1969). *Biochem. J.*, **113**, 713–719.
ROBINSON, R. A. & STOKES, R. H. (1965). *Electrolyte Solutions* 2nd. Edn. revised, p. 124, London: Butterworths.
ROUFOGALIS, B. D. (1968). Ph.D. Thesis, University of Sydney.
ROUFOGALIS, B. D. & THOMAS, J. (1968a). *J. Pharm. Pharmac.*, **20**, 135–145.
ROUFOGALIS, B. D. & THOMAS, J. (1968b). *Molec. Pharmac.*, **4**, 181–186.
ROUFOGALIS, B. D. & THOMAS, J. (1968c). *Life Sci.*, **70**, 985–992.
ROUFOGALIS, B. D. & THOMAS, J. (1969). *Molec. Pharmac.*, **5**, 286–293.
WILSON, I. B. & CABIB, E. (1956). *J. Am. chem. Soc.*, **78**, 202–207.