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Potentiation of acetylcholinesterase by a series of quaternary ammonium compounds

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Eleven spiran, and two non-spiran, quaternary ammonium compounds have been examined and some of these compounds found to potentiate the hydrolysis of acetylcholine by acetylcholinesterase at high substrate concentrations. The experiments were made in three reaction media, each differing in its ionic composition. The results obtained in these media differ both quantitatively and qualitatively. The mechanism of the potentiation is discussed in terms of the effect of quaternary ammonium compounds on the deacetylation step of acetylcholine hydrolysis.

IN the presence of certain compounds, acetylcholinesterase hydrolyses some substrates at a faster rate than it does in the absence of these compounds. The earlier work was concerned mainly with the potentiation of acetylcholinesterase by both monovalent (Glick, 1941; Mendel & Rudney, 1945; Myers 1952) and divalent (Nachmansohn, 1940; Myers, 1952; Meer, 1953) inorganic cations. Wide discrepancies exist among these results, and Smallman & Wolfe (1954) were the first to recognize that the potentiation by inorganic salts was not estimated comparably by the manometric and titrimetric methods of determining enzyme activity. The differences were mainly a result of the different ionic composition of the reaction media employed in the two methods. The results of Smallman & Wolfe demonstrated that the potentiation of the hydrolytic activity of acetylcholinesterase by inorganic ions was greater when the control activity was estimated in a medium of low inorganic ion concentration than in one with a high ionic concentration. The presence of relatively high concentrations of inorganic ions is inevitable in the manometric method of analysis, but very low concentrations may be used in the titrimetric method.

Some organic molecules have also been shown to potentiate the cholinesterases, e.g. acetylcholinesterase is potentiated by certain aliphatic alcohols (Todrick, Fellowes & Rutland, 1951) and by tetraethylammonium and hexamethonium salts (Kensler & Elsner, 1951). In all of the work so far cited, potentiation was obtained at substrate concentrations higher than optimum, and only with substrates causing substrate inhibition.

We have examined the activities of eleven spiran, and two non-spiran, quaternary ammonium compounds in media with and without added inorganic ions. Spiran quaternary ammonium compounds (spirans) have the characteristic of being relatively rigid, and their size and the stereochemistry of substituents can be controlled (Thomas, 1961a, b).

Experimental

CHEMICAL

The general method used for the synthesis of the spirans was to react an α-ω-dihaloalkane (1 mole equiv.) with a cyclic amine (2 mole equiv.)

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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 and amine hydrohalide. Two methods were used for the reaction: *Method A*, the reactants were refluxed in chloroform for a suitable time (6–24 hr); *Method B*, a solution of reactants in absolute ethanol or methanol was autoclaved ($\frac{1}{2}$–1 hr) at 125°. The spiran was then isolated by one of two methods.

Method (*a*). The reaction solution was distilled to dryness under reduced pressure on a water bath, the solid residue dissolved in water, sodium hydroxide (1 mole equiv.) added and the solution distilled to dryness as before. The resulting solid was extracted with chloroform in a Soxhlet extractor. The product either crystallized from chloroform or was obtained by reducing the volume of chloroform and adding dry ethyl methyl ketone.

Method (*b*). The reaction mixture was distilled to dryness under reduced pressure on a water bath and the spiran isolated by repeated crystallization of the residue from absolute ethanol or methanol.

Commercial samples of tetraethylammonium iodide (TEA, Hopkin & Williams) tetramethylammonium iodide (TMA, British Drug Houses) and acetylcholine perchlorate (British Drug Houses) were used.

All the quaternary compounds are hygroscopic, and were dried over P_2O_5 under reduced pressure overnight before use.

2,2'-Di-iododiethyl ether. 2,2'-Di-iododiethyl ether was prepared from 2,2'-dichlorodiethyl ether by the Finkelstein reaction according to the method of Gibson & Johnson (1930), b.p. 80–84°/3·5 mm.

A list of spirans prepared is given in Table 1 together with analytical data and physical constants.

ENZYMIC

Purified bovine erythrocyte acetylcholinesterase (Nutritional Biochemical Corp.) was used. The enzymic activity was determined by the pH stat method with a Radiometer (Copenhagen) Titrator (TTTlc) attached to a magnetic valve, which regulated the flow of titrant (sodium hydroxide 0·02 or 0·01N) into a 100 ml beaker immersed in a thermostatically controlled water bath at 37°. The solution was stirred continuously and nitrogen bubbled through it. A stock enzyme solution was made by dissolving 20 mg of enzyme in 50 ml of a dilute phosphate buffer [Na_2HPO_4 (M/30)—500 ml, KH_2PO_4 (M/15) to pH 7·4 at 37° (about 200 ml)].

The compounds were studied in three reaction media: *Medium A* contained, in addition to the phosphate buffer, NaCl (0·1M) and $MgCl_2$ (0·04M); *Medium B* contained the phosphate buffer only, with no added ions*; *Medium C* contained no phosphate buffer or inorganic ions. When the hydrolysis was examined in this medium the method of analysis was different from the one described above. A fully automated recording titrimetric method was used as reported by Thomas & Roufogalis (1967).

* The enzyme preparation contains 30·7 mg NaCl, 100 mg gelatin and 5 ml M sodium phosphate per 20,000 "units".

TABLE 1. SPIRAN QUATERNARY AMMONIUM COMPOUNDS PREPARED

No.	Molecular formula	Cyclic amine	α-ω-Dihalo-alkane	Method (see exptl.)	Reflux time (hr)	Solvent for recryst.	m.p.[g]	Analysis							
								Calc.				Found			
								C	H	N	X[h]	C	H	N	X[h]
I	$C_{10}H_{20}NBr$	Piperidine	1,5-Dibromo-pentane	Aa	12	Chloroform[a]	304(d.)	51·3	8·6	6·0	34·1	51·3	8·5	6·1	33·9
II	$C_{12}H_{24}NBr$	*cis*-2,6-Dimethyl[f] piperidine	1,5-Dibromo-pentane	Ba	—	Ethylmethyl ketone	248–249	55·0	9·2	5·3	30·5	54·5	9·0	5·5	30·6
III	$C_{11}H_{22}NBr$	*cis*-2,6-Dimethyl piperidine	1,4-Dibromo butane	Aa	6	Ethylmethyl ketone-chloroform	269–270	53·2	8·9	5·6	32·2	53·1	8·9	5·5	32·2
IV	$C_9H_{18}NBr$	Piperidine	1,4-Dibromo-butane	Aa	15	Chloroform-ethylmethyl ketone	248–250	49·1	8·2	6·4	36·3	48·7	8·3	6·3	36·8
V	$C_8H_{16}NBr$	Pyrrolidine	1,4-Dibromo-butane	Aa	24	Ethylmethyl[b] ketone	250–252	46·6	7·8	6·8	38·8	46·4	8·2	6·9	38·4
VI	$C_9H_{18}ONBr$	Morpholine	1,5-Dibromo-pentane	Ba	—	Chloroform[a]	271–273	45·8	7·7	5·9	33·8	45·4	7·6	5·8	33·9
VII	$C_8H_{16}O_2NI$	Morpholine	2,2′-Diiodo-diethyl ether	Ab[c]	6	Ethanol	275–276	33·7	5·7	4·9	44·5	33·4	5·7	5·0	44·0
VIII	$C_{11}H_{22}ONBr$	2,6-Dimethyl[d,e] morpholine	1,5-Dibromo-pentane	Aa	19	Chloroform	314(d.)	50·0	8·4	5·3	30·3	49·8	8·3	5·6	30·7
IX	$C_{10}H_{20}ONBr$	2,6-Dimethyl morpholine	1,4-Dibromo-butane	Aa	17	Chloroform-ethylmethyl ketone	325	48·0	8·1	5·6	32·0	48·1	8·1	5·9	32·1
X	$C_{10}H_{20}O_2NI$	2,6-Dimethyl morpholine	2,2′-Diiodo-diethyl ether	Ab	6	Ethanol	282–283	38·4	6·4	4·5		38·1	6·4	4·5	
XI	$C_{11}H_{22}ONI$	*cis*-2,6-Dimethyl piperidine	2,2′-Diiodo-diethyl ether	Aa	10	Chloroform	237	42·5	7·1	4·5		42·4	7·1	4·6	

(*a*) Crystallized out during Soxhlet extraction. (*b*) This compound is hygroscopic and was handled in a dry box. (*c*) This compound is unstable to prolonged heating and over P_2O_5/90°C. (*d*) Oxygen is numbered position one. (*e*) Isomers not separated. (*f*) See reference. (*g*) Melting points are uncorrected. (*h*) X = I or Br as appropriate.

PROCEDURE

The quaternary ammonium compounds were incubated in the relevant media with 5·0 ml of the enzyme solution (stored at 4°) for 15 min at pH 7·4 and 37°. The volume of this mixture was (50–x) ml, where x is the volume of substrate solution. The substrate (x ml) was then added and the pH adjusted immediately to 7·4. The concentration of substrate used was one which preliminary investigations had shown to give near optimum potentiation with the compounds. This arbitrarily chosen concentration was different for each medium, but was kept constant for each particular medium throughout the investigation. The titrator was started, and the volume of sodium hydroxide consumed recorded every 2·5 min (or every 5 min) during a 15 min period of hydrolysis. The volume of sodium hydroxide used was plotted against time in min, and from the resulting graph the volume of sodium hydroxide consumed during 15 min was obtained and used as a measure of the rate of hydrolysis (when medium C was used the rate was the slope of the initial linear portion of the recorded plot). Since the experiments were made mainly at high substrate concentrations, the plots showed a linear relation.

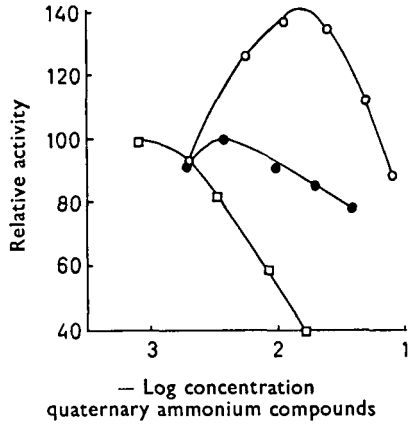

FIG. 1. Effect of quaternary ammonium compounds on the hydrolysis of acetylcholine in medium A. Acetylcholine concentration $3{\cdot}4 \times 10^{-2}$M. Activity in the absence of the compounds is arbitrarily taken as 100. Concentrations of quaternary ammonium compounds is given as g-mole/litre. ○–○, Tetraethylammonium iodide (TEA). □–□, *cis*-2,6-Dimethyl-1,1′-spirobipiperidinium bromide (II). ●–●, 1,1′-Spirobipiperidinium bromide (V).

Results

Figs 1 and 2 show representative examples of the results obtained in media A and B. In Table 2 are listed the I 50 (concentration causing 50% inhibition), the maximum potentiation obtained in each medium, and the concentration of compound which produced this maximum potentiation. I 50 values were determined at substrate concentrations at which potentiation did not occur.

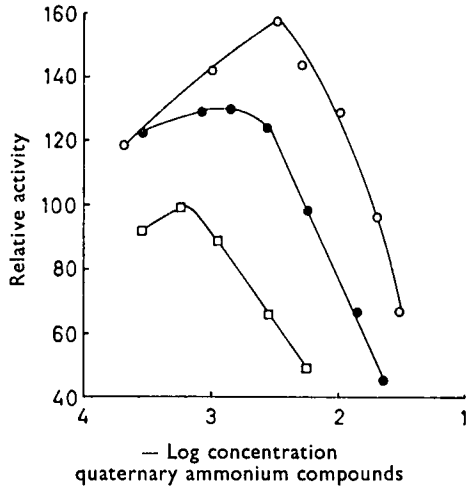

FIG. 2. Effect of quaternary ammonium compounds on the hydrolysis of acetylcholine in medium B. Acetylcholine concentration $3{\cdot}4 \times 10^{-3}$M. Activity in the absence of the compounds is arbitrarily taken as 100. Concentration of quaternary ammonium compounds given as g-mole/litre. ○–○, Tetraethylammonium iodide (TEA), ●–●, *cis*-2,6-Dimethyl-1,1′-spirobipiperidinium bromide (III) □–□, 1,1′-Spirobipiperidinium bromide (I).

Discussion

It is apparent from Table 2 that potentiation is influenced by the reaction medium used. The values obtained were some 20–60% higher in the media with no added inorganic ions (media B and C) than in medium A. Some of the compounds (IV, V and VI) which did not potentiate in medium A did so, though weakly, in media B and C.

INHIBITOR POTENCY AND POTENTIATION

There is an inverse relationship between I 50 and ability to potentiate among the series. The compounds which are the strongest inhibitors potentiate weakly or not at all, whereas those that are weaker inhibitors are the better potentiators. The exception is tetramethylammonium, which, although one of the weaker inhibitors, does not potentiate in medium B. It appears possible that this relation is a function of some physicochemical property of these quaternary ammonium compounds.

MECHANISM OF POTENTIATION

There is an inverse relationship between I 50 and ability to potentiate concentrations. This can be seen by comparison of the plots in Fig. 3 with those of Figs 1 and 2. Fig. 3 illustrates the effects of two of the compounds when examined at optimum substrate concentration. The hydrolysis rate does not rise above that of the control at any concentration of the compounds. Figs 1 and 2 show the results obtained above optimum substrate concentration. Under these conditions the hydrolysis

TABLE 2. POTENTIATION AND INHIBITION OF ACETYLCHOLINESTERASE BY QUATERNARY AMMONIUM COMPOUNDS. INHIBITION GIVEN AS I 50 IN G-MOLE/LITRE. POTENTIATION GIVEN AS PERCENTAGE INCREASE IN RATE OF HYDROLYSIS OF ACETYLCHOLINE

Compound number	Compound	I 50 × 10^3 Medium A [S] = 3·4 × 10^{-3}M	Maximum potentiation (%) and concentration of quaternary ammonium compound which produce this potentiation in g-mole/litre × 10^3		
			Medium A [S] = 3·4 × 10^{-2}M	Medium B [S] = 3·4 × 10^{-3}M	Medium C [S] = 2·34 × 10^{-3}M
I	1,1′-Spirobipiperidinium bromide	3·80	0%	0%	—
II	*cis*-2,6-Dimethyl-1,1′-spirobipiperidinium bromide	1·70	0%	—	—
III	*cis*-2,6-Dimethylspiro(piperidine-1,1′-pyrrolidinium) bromide	11·2	26% (6·2)	31% (1·59)	52% (1·3)
IV	Spiro(piperidine-1,1′-pyrrolidinium) bromide	8·41	—	0%	11% (5·0)
V	1,1′-Spirobipyrrolidinium bromide	7·25	0%	0%	19% (0·11)
VI	Spiro(piperidine-1,4′-morpholinium) bromide	26·4	0%	17% (4·68)	—
VII	4,4′-Spirobimorpholinium iodide	71[a]	[b]	37% (25)	—
VIII	2,6-Dimethylspiro(morpholine-4,1′-piperidinium) bromide	1·26	—	0%	—
IX	2,6-Dimethylspiro(morpholine-4,1′-pyrrolidinium) bromide	3·16	—	0%	—
X	2,6-Dimethyl-4,4′-spirobimorpholinium iodide	9·24	—	8·5% (0·10)	—
XI	*cis*-2,6-Dimethylspiro(piperidine-1,4′-morpholinium) iodide	20·0	—	25% (2·0)	—
XII	Tetraethylammonium iodide	21·2	40% (15)	57·5% (3·2)	76% (2·3)
XIII	Tetramethylammonium iodide	31·0	0%	0%	—

(*a*) Concentration producing 34·5% inhibition. (*b*) Concentration needed too high.

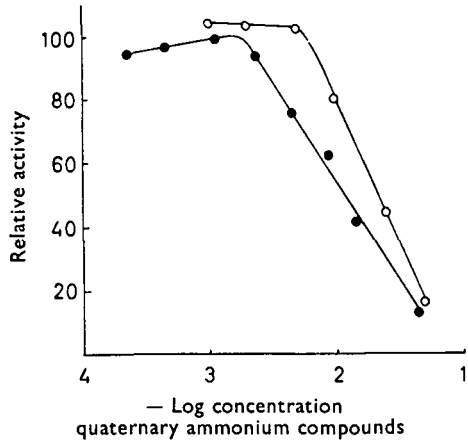

FIG. 3. Effect of quaternary ammonium compounds on the hydrolysis of acetylcholine in medium A. Acetylcholine concentration $3{\cdot}4 \times 10^{-3}$M. Activity in the absence of these compounds arbitrarily taken as 100. Concentration of quaternary ammonium compounds given as g-mole/litre. ○–○, Tetraethylammonium iodide (TEA). ●–●, *cis*-2,6-Dimethylspiro(piperidine-1,1′-pyrrolidinium) bromide (III).

rate in the presence of these (and some of the other) compounds is greater than the control hydrolysis rate in their absence. Potentiation occurs at low concentrations of the quaternary ammonium compounds, but at higher concentrations the hydrolysis rate is inhibited.

Acetylcholinesterase is inhibited by high concentrations of acetylcholine; this is generally considered to be due to the binding of a second molecule of acetylcholine on an enzyme substrate complex (Zeller & Bissegger, 1943; Bergmann, Wilson & Nachmansohn, 1950; Wilson & Cabib, 1956) to form a ternary complex. The presence of this second molecule inhibits hydrolysis. It follows that the most likely explanation for the observed potentiation at above optimum substrate concentrations

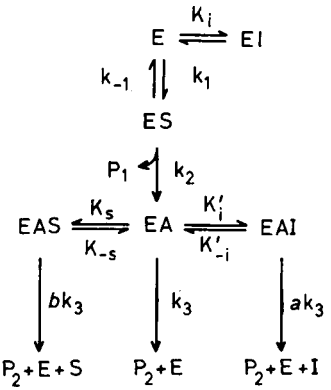

FIG. 4. Reaction scheme for substrate inhibition proposed by Krupka & Laidler in the presence of inhibitor.

is that the small quaternary ammonium compounds are bound to the binary complex, and so protect the enzyme against substrate inhibition. If this is so, then an explanation has to be found for the fact that only some of the compounds potentiate, and that the compounds potentiate at low concentrations but inhibit at high concentrations.

A general mechanism for the interaction of acetylcholine and inhibitors with acetylcholinesterase has been proposed by Krupka & Laidler (1961) (Fig. 4). In this scheme the substrate, S, can combine with the free enzyme, E, or with the acetyl enzyme, EA, but not with the Michaelis complex, ES, which does not have a free anionic site available for interaction. EAS, once formed, will break down to products at b times the rate of EA. The deacetylation of EA is considered to be the rate-determining step in acetylcholine hydrolysis (Wilson & Cabib, 1956). The value of b is generally considered to be 0·1 (Krupka, 1964).

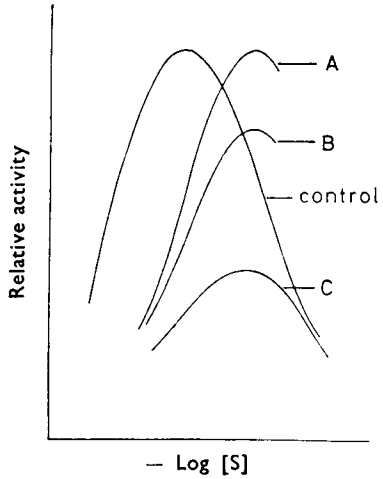

FIG. 5. Three classes of behaviour predicted by Krupka from the scheme in Fig. 4 (see discussion).

Likewise, a cationic compound, I, may react with the free enzyme to form the binary complex EI, or with the acetyl enzyme to give EAI. This ternary complex then may or may not deacetylate to give products, the rate of deacetylation being ak_3. Krupka (1965) considers that the value of a can vary between 0 and 1. When I completely blocks the deacetylation step $a = 0$, and if I has no effect on the deacetylation rate $a = 1$; a can also have intermediate values.

The reaction scheme in Fig. 4 shows that I can compete with S for interaction with EA*. If I blocks the deacetylation less than S, then, in the presence of I and at substrate concentrations where EAS can form, an apparent increase in enzyme activity would be predicted. This is

* That the compounds under consideration interact with EA is indicated by the non-competitive component in inhibition of acetylcholinesterase by tetramethylammonium and tetraethylammonium (Krupka, 1965).

what has, in fact, been observed to various degrees among the compounds under investigation (Table 2). The inhibition at high I concentrations is probably due to the high proportion of enzyme bound as EI.

Krupka (1963), in a theoretical treatment of the kinetics of this proposed scheme of inhibition, has predicted three classes of inhibitor behaviour, reproduced in Fig. 5. Class A behaviour results when the inhibitor competes with the substrate for acetylenzyme, the inhibitor having no effect on the deacetylation rate when bound. Class B behaviour arises when I partially blocks deacetylation, and class C when I completely blocks the deacetylation. These types of behaviour have been observed experimentally, and Fig. 6 shows the effect of III and TEA over a range of substrate concentrations, in a medium containing NaCl (0·1M) and $MgCl_2$ (0·04M). Under these conditions both III and TEA potentiate the rate of the acetylcholinesterase at high substrate concentrations, but the hydrolysis never rises above the optimum hydrolysis rate of the control. Krupka (1965) has calculated that the ternary complex EAI deacetylated at 0·83 times the rate of EA when I is TEA. TMA does not potentiate in either medium A or B, and the value of a found by Krupka (1965) for TMA in a medium similar to medium A was from 0·42 to 0·65.

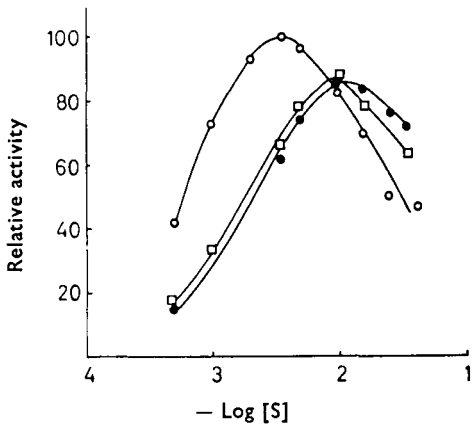

FIG. 6. Effect of quaternary ammonium compounds on the hydrolysis of acetylcholine over a range of substrate concentrations in medium A. Maximum activity of control arbitrarily taken as 100. Substrate concentration is given in g-mole/litre. ○–○, Control. ●–●, Tetraethylammonium iodide (TEA), $1{\cdot}45 \times 10^{-2}$M. □–□, *cis*-2,6-Dimethylspiro(piperidine-1,1′-pyrrolidinium) bromide (III), $6{\cdot}2 \times 10^{-3}$M.

Some of the compounds, in media B and C, where the enzyme has not been already potentiated by added inorganic ions, show a fourth class of behaviour (Fig. 7A, B). Again there is potentiation of the enzyme hydrolysis rate only at high substrate concentrations. But in these media the hydrolysis rises above that of the optimum rate of hydrolysis of the control. A possible explanation for this is that in media B and C the ternary complex EAI deacetylates at a faster rate than EA, that is, a is greater than 1. The possibility of this is being investigated.

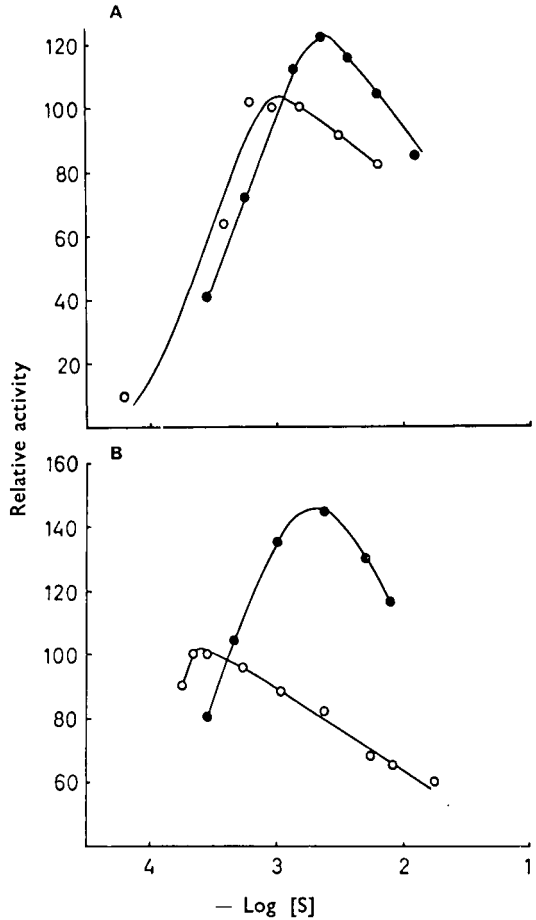

FIG. 7. Effect of quaternary ammonium compounds on the hydrolysis of acetylcholine over a range of substrate concentrations (A) in medium B; (B) in medium C. Maximum activity of control arbitrarily taken to be 100. Substrate concentration given in g-mole/litre. A. ○–○, Control. ●–●, *cis*-2,6-Dimethylspiro(piperidine-1,1′-pyrrolidinium) bromide (III), $1{\cdot}39 \times 10^{-3}$M. B. ○–○, Control. ●–●, Tetraethylammonium iodide (TEA), $2{\cdot}24 \times 10^{-3}$M.

STRUCTURE-ACTIVITY RELATIONS

Potentiation appears to be strongly structure dependent. There is no obvious relationship between potentiation and the size of the molecules, since all the compounds examined are a similar size. Thus the replacement of one methylene group in compound I by one oxygen atom does not significantly alter its size, but the change produces a potentiator VI—and the replacement of two methylene groups of compound I by two oxygen atoms (VII) has an even greater effect. Compound II does not potentiate, but the contraction of the unsubstituted ring by one carbon produces III, a strong potentiator. The features which appear to be

important for potentiator activity are bulk around the positive nitrogen (II, XI and TEA) and the presence of an oxygen atom (VI, VII, X, XI), although the presence of these features does not necessarily lead to potentiators (II, VIII, IX). Freedom of rotation of carbon-carbon, carbon-nitrogen bonds also appears to be important. Whereas TEA is the strongest potentiator of the series, when the rotation of the ethyl groups is restricted by cyclization, as in V, potentiation is greatly diminished. At the present time no satisfactory explanation can be given for the high degree of structural specificity exhibited by the series in relation to ability to potentiate acetylcholinesterase.

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References

Bergmann, F., Wilson, I. B. & Nachmansohn, D. (1950). *Biochim. biophys. Acta*, **6**, 217–224.
Gibson, C. S. & Johnson, J. D. A. (1930). *J. chem. Soc.*, 2525–2530.
Glick, D. (1941). *Nature, Lond.*, **148**, 662–663.
Kensler, C. J. & Elsner, R. W. (1951). *J. Pharmac. exp. Ther.*, **102**, 196–199.
Krupka, R. M. (1963). *Biochemistry*, **2**, 76–82.
Krupka, R. M. (1964). *Ibid.*, **3**, 1749–1754.
Krupka, R. M. (1965). *Ibid.*, **4**, 429–435.
Krupka, R. M. & Laidler, K. J. (1961). *J. Am. chem. Soc.*, **83**, 1448–1454.
Meer, C. van der (1953). *Nature, Lond.*, **171**, 78–79.
Mendel, B. & Rudney, H. (1945). *Science, N.Y.*, **102**, 616–617.
Myers, D. K. (1952). *Archs Biochem. Biophys.*, **37**, 469–487.
Nachmansohn, D. (1940). *Nature, Lond.*, **145**, 513–514.
Smallman, B. N. & Wolfe, L. S. (1954). *Enzymologia*, **17**, 133–144.
Thomas, J. (1961a). *J. mednl pharm. Chem.*, **3**, 45–51.
Thomas, J. (1961b). *Ibid.*, **3**, 309–321.
Thomas, J. & Roufogalis, B. D. (1967). *Molec. Pharmac.*, **3**, 103–107.
Todrick, A., Fellowes, W. P. & Rutland, J. P. (1951). *Biochem. J.*, **48**, 360–368.
Wilson, I. B. & Cabib, E. (1956). *J. Am. chem. Soc.*, **78**, 202–207.
Zeller, E. A. & Bissegger, A. (1943). *Helv. chim. Acta*, **26**, 1619–1630.