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1,2-Disubstituted cyclohexanes as substrates of acetylcholinesterase and muscarinic agents. A re-investigation

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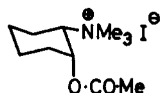
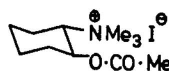
The previously reported high rates of acetylcholinesterase-catalysed hydrolysis of (±)-*cis*- and (±)-*trans*-2-dimethylaminocyclohexyl acetate methiodide (Baldridge, McCarville & Friess, 1955) have been re-investigated, the enantiomers of each geometrical isomer being used. The *cis*-enantiomers were inactive as substrates and the *trans*-enantiomers were hydrolysed at a very slow rate. These results are confirmed by studies on muscarinic tissue (guinea-pig ileum) in which only a weak stimulatory response was given with the *trans*-enantiomers (the *cis*-enantiomers were inactive). With each of the test systems used, the acetylcholine receptor showed an inversion of the "normal" configurational specificity although the enantiomer potency ratio was small, a phenomenon which has been observed in previous reports of inversion of configurational specificity.

Comparative studies of synthetic substrates or inhibitors of the enzyme acetylcholinesterase with their cholinomimetic or cholinolytic activity at the muscarinic receptor have suggested that a close structural similarity exists between the active site of the enzyme and of the muscarinic receptor. Both receptor areas show a marked, and similar, stereoselectivity when interacting with potent asymmetric molecules; for example L(+)-acetyl-β-methylcholine iodide (Beckett, Harper & Clitherow, 1963) and *S*-(−)-3-acetoxyquinuclidine methiodide (Robinson, Belleau & Cox, 1969) act as substrates for the enzyme and are potent muscarinic agents, whereas their enantiomers are inactive. Similarly, L-(+)-*cis*-2-methyl-4-dimethylaminomethyl-1,3-dioxalanmethiodide is the most potent inhibitor of the enzyme and the most potent muscarinic agents of the enantiomeric 2,4-disubstituted 1,3-dioxalans (Belleau & Lacasse, 1964; Belleau & Puranen, 1963; Belleau & Lavoie, 1968). [For additional comparative studies see Belleau (1965), Smissman, Nelson & others (1966), Robinson, Belleau & Cox (1969)].

Within this present context, no detailed enzyme kinetic studies (or pharmacological studies) have been performed on (±)-*cis*-(I) and (±)-*trans*-2-dimethylaminocyclohexyl acetate methiodide (II), compounds which are reported to be excellent





(I) (II)

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substrates for electric-eel acetylcholinesterase (Baldridge, McCarville & Friess, 1955). Studies of the acetylcholinesterase-catalysed hydrolysis of the latter two substrates have shown that, at their respective optimum substrate concentrations, these compounds are hydrolysed at a slightly faster rate than acetylcholine (at its optimum substrate concentration), the (±)-*cis*-isomer being the slightly better substrate. This phenomenon has been attributed to a difference in the functional-group separations in the two compounds (N^+ to acyl-*O* group separation reported as 2·9–3·5Å for the *trans*-isomer and 2·5–2·9Å for the *cis*-isomer, this latter distance more closely approximating to the optimal functional-group separation). However, studies of the conformation of 1,2-disubstituted cyclohexane derivatives make such a view untenable, as, provided the bulky trimethylammonium group does not introduce ring distortion in one of the isomers, the functional group separation should be identical for each isomer (Fig. 1) (approximately 2·9Å from Drieding models constructed by the present authors).

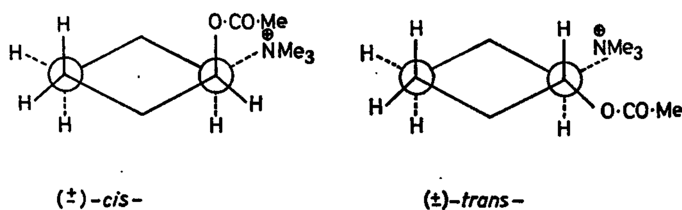


FIG. 1.

Thus, before one can adequately discuss any difference in biological activity between the *cis*- and *trans*-isomers (whether interacting with the enzyme or at the muscarinic receptor), it is necessary to know the absolute configuration and biological activity of the individual enantiomers in each pair. The resolution and determination of the absolute configuration of the *cis*- and *trans*-2-dimethylaminocyclohexyl acetate methiodide has recently been reported (Kay & Robinson, 1969; Robinson, 1970) and the present paper reports the results of studies with the resolved enantiomers as substrates for the enzyme acetylcholinesterase and as stimulants of the muscarinic receptor.

EXPERIMENTAL

Substrates of acetylcholinesterase

Bovine erythrocyte acetylcholinesterase (Sigma Chemicals, London) was used throughout this work. The rates of hydrolysis were measured by the pH-stat method (Alles & Hawes, 1940), with an automatic titrator (type TTT1c) equipped with a recorder (SBR2c) and syringe burette (SBU1a) (Radiometer, Copenhagen).

All incubations were carried out in a total volume of 25 ml of enzyme solution previously made 0·04M in $MgCl_2$ and 0·05M in NaCl, in jacketted vessels at $25^+ \pm 0{\cdot}1°$ and the pH maintained at 7·4 by the addition of 0·01N NaOH solution. A CO_2-free nitrogen atmosphere was maintained throughout the experiments. The velocity of the reaction was calculated from the average slope of the recording during the second and third minutes of the incubation.

The results were plotted as Lineweaver–Burk plots (Lineweaver & Burk, 1934) and the K_m value for each substrate calculated from the gradient of the resultant plots.

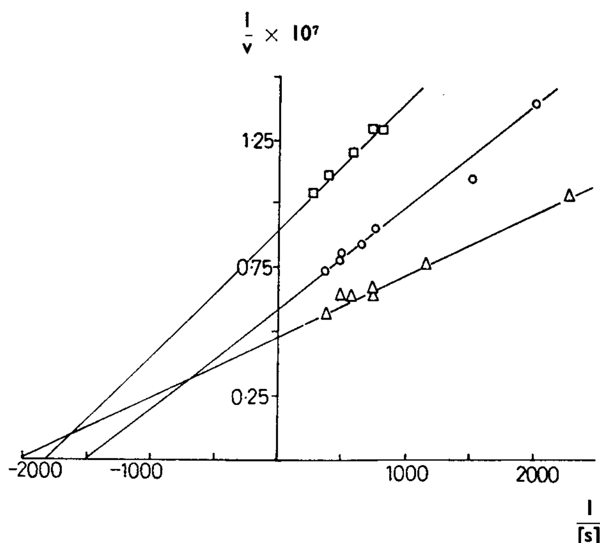


FIG. 2. Lineweaver-Burk plot of acetylcholinesterase-catalysed hydrolysis of (±)-,(1*S*, 2*S*)-(+)- and (1*R*,2*R*)-(−)-*trans*-2-dimethylaminocylohexyl acetate methiodide.
—○—○— (±). —△—△— (1*R*,2*R*)-(−)-. —□—□— (1*S*,2*S*)-(+)-.

Acetylcholine perchlorate (BDH) was used to standardize the enzyme preparation, which was found to have a K_m value of 5.5×10^{-4} and a V_{max} of 2.67×10^{-6} mol min^{-1}. The results obtained with the synthetic substrates are shown in Fig. 2 and the computed results in Table 1.

Table 1. *K_m and V_{max} values obtained with synthetic substrates*

Substrate	$K_m \times 10^4$	$V_{max} \times 10^7$ mol min^{-1}
Acetylcholine	5·5	26·7
(±)-*trans*-2-Dimethylaminocyclohexyl acetate methiodide	6·8	1·7
(1*S*,2*S*)-(+)-*trans*-enantiomer	5·7	1·11
(1*R*,2*R*)-(−)-*trans*-enantiomer	4·9	2·1
(±)-*cis*-2-Dimethylaminocyclohexyl acetate methiodide	Inactive at concentrations $6·5 \times 10^{-4}$M to $1·4 \times 10^{-2}$M	

Muscarinic activity-isolated guinea-pig ileum preparation

Drugs. Acetylcholine chloride (BDH), atropine sulphate (BDH), hexamethonium bromide (Vegolysen) (M & B), histamine acid phosphate (BDH), 5-hydroxytryptamine creatinine sulphate (Koch-Light), mepyramine maleate (M & B), methysergide bimaleate (Sandoz) and nicotine hydrogen tartrate (BDH).

Guinea-pig ileum (3 cm lengths) was suspended in Krebs solution (10 ml) maintained at 36 ± 0·2° and gassed with 5% carbon dioxide in oxygen. Isotonic contractions of the ileum were recorded on a kymograph with a magnification of 8 to 1 and a load on the tissue of 500 mg. Agonist drugs were added to the tissue bath on a 3-min dose cycle. Antagonist drugs, when present, were allowed to equilibrate with the ileum for 15 min before addition of any agonist drug. All drug concentrations referred to in the text are expressed as the final bath concentration (mol).

RESULTS

(±)-*cis*-2-Dimethylaminocyclohexyl acetate methiodide was inactive as a muscarinic agent at all the concentrations used. Dose-response curves on the guinea-pig ileum were obtained for acetylcholine, (±)-*trans*- and (1*R*,2*R*)-(−)-*trans*-2-dimethylaminocyclohexyl acetate methiodide (Fig. 3 a, b and c respectively). However, the (1*S*,2*S*)-(+)-*trans*-enantiomer (Fig. 3d) showed only a low activity and a concentration of 8 $\times$ 10^{-4} mol produced a contraction which was less than 50% of the acetylcholine maximum. Of the three effective agonists, acetylcholine was the most potent (1·3 $\times$ 10^{-8} to 1·6 $\times$ 10^{-6} mol). The (1*R*,2*R*)-(−)-*trans*-2-dimethylaminocyclohexyl acetate methiodide (1·5 $\times$ 10^{-6} to 4·0 $\times$ 10^{-4} mol) in the example shown, was four times more potent than the (±)-*trans*-2-dimethylaminocyclohexyl acetate methiodide (6·1 $\times$ 10^{-6} to 1·6 $\times$ 10^{-3} mol).

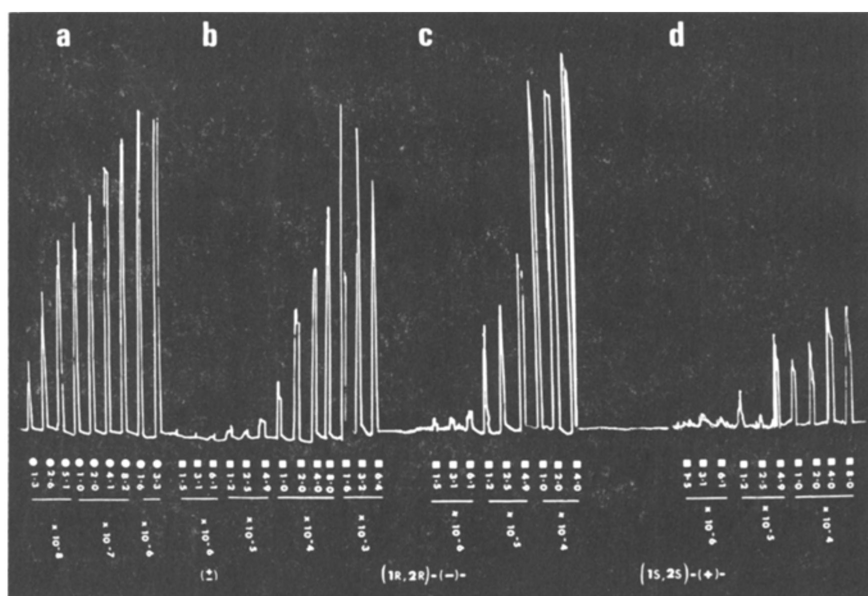


FIG. 3. Dose-response curves on the guinea-pig ileum. The (1*S*,2*S*)-(+)-*trans*-enantiomer has only a low activity, 8 $\times$ 10^{-4} mol producing a contraction less than 50% of the acetylcholine maximum. ●, Acetylcholine; ■, *trans*-2-dimethylaminocyclohexyl acetate.

The effect of atropine on the contraction of the guinea-pig ileum produced by either acetylcholine, (±)-compound or the (1*R*,2*R*)-(−)-*trans*-enantiomer is shown in Fig. 4. Atropine in a concentration of 1 $\times$ 10^{-9} mol had a similar inhibitory effect on the contractions produced by all three agonists.

Hexamethonium, in a concentration of 9·9 $\times$ 10^{-5} mol, which produced marked inhibition of the nicotine-induced contraction, was without effect on the contraction produced by the *trans*-racemate.

Mepyramine (concentration 3·5 $\times$ 10^{-5} mol) produced marked inhibition of the histamine-induced response and methysergide (1·1 $\times$ 10^{-6} mol) reduced the 5-hydroxytryptamine-induced response of the guinea-pig ileum, but these inhibitors were without effect on the contraction produced by (±)-*trans*-2-dimethylaminocyclohexyl acetate methiodide.

A maximum contraction of the ileum produced by acetylcholine ($5{\cdot}1 \times 10^{-5}$ mol was not modified by any of the antagonists used.

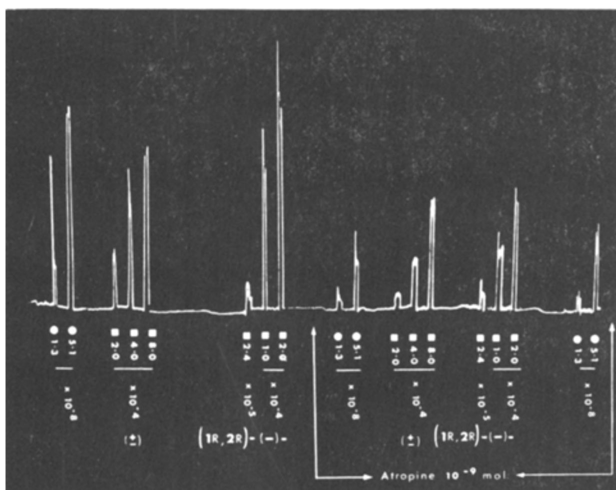


FIG. 4. The effect of atropine on the contraction of the guinea-pig ileum produced by either acetylcholine, (±)-compound or the (1*R*,2*R*)-(−)-*trans*-enantiomer. Atropine 1×10^{-9} mol had a similar effect on contractions produced by all three agonists. ●, Acetylcholine; ■, *trans*-2-dimethylaminocyclohexyl acetate methiodide.

DISCUSSION

The first and most striking observation to be made of the present work is that the acetylcholinesterase-catalysed hydrolysis rates of (±)-*cis*- and (±)-*trans*-2-dimethylaminocyclohexyl acetate methiodide are very different from previous reports (Baldridge & others, 1955). In the present work the (±)-*cis*-isomer was not hydrolysed by the enzyme and the (±)-*trans*-isomer was a very poor substrate for the enzyme, whereas it was previously reported that both the geometrical isomers were hydrolysed at a velocity greater than that of acetylcholine at their respective optimum substrate concentrations.

There are however, certain differences in the experimental conditions employed in the present work which should be noted:

(i) Previous work (Baldridge & others, 1955) was performed on acetylcholinesterase which had been extracted from the brain tissue of electric eel (*Electrophorus electricus*). The enzyme used in the present work was a commercial enzyme isolated from ox erythrocytes and although the two enzyme preparations are considered to be very similar (K_m values for the two enzyme preparations with acetylcholine as substrate, are identical within experimental error), the present authors know of no reports where the two enzyme preparations have been subjected to rigorous comparative studies with synthetic substrates and inhibitors of preferably a rigid/semi-rigid structure.

(ii) The medium used in the present experimental work differs in the concentrations of magnesium chloride and sodium chloride from that reported by Baldridge & others (1955). It has previously been reported (Friess, Wilson & Cabib, 1954) that the activity of electric-eel acetylcholinesterase is dependent upon the concentration of magnesium ions and more recently Changeux (1966) has shown that the

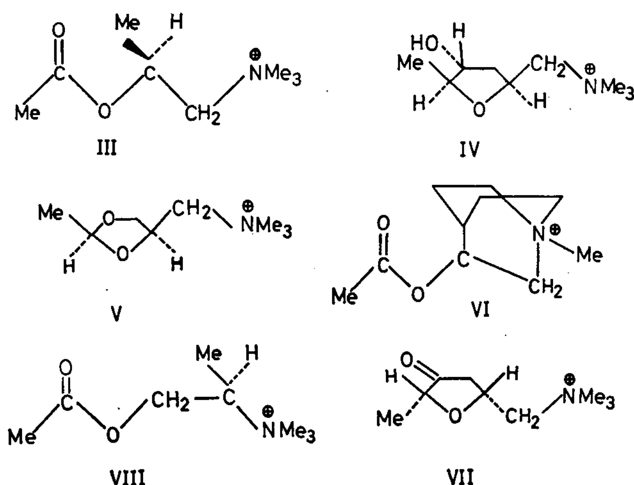
degree of inhibition of acetylcholinesterase (isolated from *Torpedo marmorata*) by certain inhibitors can be decreased up to 250 times by the addition of magnesium ions. These differences in activity of the enzyme and inhibitor-sensitivity of the enzyme to varying concentrations of magnesium ion have been ascribed to reversible aggregation-deaggregation phenomena with higher polymeric forms of the enzyme predominating at low ionic strength (Changeux, 1966). However, assuming that acetylcholinesterase isolated from different sources is identical, it would be surprising if a small change in magnesium ion concentration could change a compound from behaving as an extremely good substrate (±-*cis*-isomer) (Baldridge & others, 1955) to having no activity as a substrate whatsoever, especially when acetylcholine was hydrolysed rapidly under both conditions.

The active site of the enzyme does show some slight degree of stereoselectivity towards the enantiomers of *trans*-2-dimethylaminocyclohexyl acetate methiodide, the (1*R*,2*R*)-(−)-enantiomer being a slightly better substrate than the (±)-compound and (1*S*,2*S*)-(+)-enantiomer.

If it is accepted that there is a structural similarity between the enzyme "active site" and the muscarinic receptor (and considerable evidence has accumulated recently to substantiate this hypothesis, see Belleau & Puranen, 1963, Belleau & Lacasse, 1964, Belleau, 1965, Belleau & Lavoie, 1968, Robinson & others, 1969), then a similar pattern of activities should emerge when the above compounds are tested as muscarinic agents. The (±)-*cis*-2-dimethylaminocyclohexyl acetate methiodide was, in fact, found to be inactive as a muscarinic agent and the (±)-*trans*-isomer to be only weakly active.

A similar pattern of stereoselectivity to that displayed by the enzyme is also shown by the muscarinic receptor, the (1*R*,2*R*)-(−)-*trans*-enantiomer being about four times more potent than the racemate, and the (1*S*,2*S*)-(+)-*trans*-enantiomer being unable to elicit a maximal contraction of the guinea-pig ileum preparation even at a concentration of 8×10^{-4} mol. Thus, the enzyme-catalysed hydrolysis rate studies are substantiated by the low activity displayed by the above compounds towards muscarinic tissue and by the similar pattern of stereoselectivity shown within the two test systems. The only previous report of pharmacological studies with these compounds has shown that (±)-*cis*-2-dimethylaminocyclohexyl acetate methiodide, at a concentration 1000 times that of acetylcholine, was unable to elicit a response from the frog rectus preparation (Fellman & Fujita, 1962). The absence of any nicotinic activity in either the *cis*- or *trans*-isomers has been demonstrated in the present work employing guinea-pig ileum tissue.

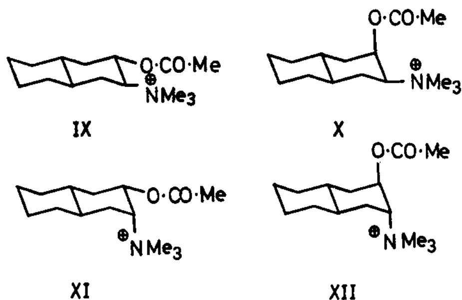
A second important feature of the present work comes from a more detailed consideration of the stereoselectivity of the enzyme and muscarinic receptor towards the enantiomers of *trans*-2-dimethylaminocyclohexyl acetate methiodide. As can be seen from Table 1 and from the pharmacological results, the more active enantiomer has the (1*R*,2*R*)-*trans*-configuration. Previous work has shown that, of compounds displaying a high degree of stereoselectivity at either the acetylcholinesterase "active site" or on the muscarinic receptor, the enantiomer having the L(*S*)-configuration is the most active, e.g., L(*S*)-(+)-acetyl-β-methylcholine (III) (Beckett & others, 1963), L(2*S*,3*R*,5*S*)-(+)-muscarine (IV) (Hardegger & Lohse, 1957, Gyermek & Unna, 1958), L(2*S*,4*R*)-(+)-*cis*-2-methyl-4-dimethylaminomethyl-1,3-dioxalanmethohalide (V) (Belleau & Puranen, 1963, Belleau & Lacasse, 1964), *S*-(−)-3-acetoxyquinuclidine methiodide (VI) (Robinson & others, 1969).



Some examples of "inversion" of configurational specificity have been reported however, although in such cases the difference in activity between enantiomers is usually very small. For example, D(2*R*,5*R*)-(−)-muscarone (VII) is approximately three times more potent than its enantiomer on muscarinic tissue (Gyermek & Unna, 1960). D(*R*)-(+)-acetyl-α-methylcholine (VIII) is approximately eight times more potent than its enantiomer on muscarinic tissue (guinea-pig ileum) and both enantiomers show similar rates of hydrolysis by the enzyme acetylcholinesterase (Beckett & others, 1963).

These general conclusions are supported within the present work, where, although an inversion of stereoselectivity is shown by both the acetylcholinesterase "active site" and the muscarinic receptor, there is only a small difference in potency between enantiomers. No attempt is made at this time to explain the phenomenon.

Finally, mention should be made of the studies on the isomeric 3-trimethylammonium-2-acetoxy-*trans*-decalin halides (IX to XII), although comparison of the results obtained with the results reported in the present paper is difficult due to the large differences in experimental conditions employed (Smissmann & others, 1966).

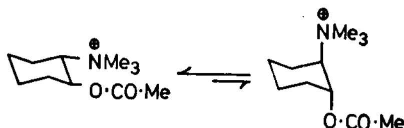


Using electric-eel acetylcholinesterase, it was found that compounds IX and X were inactive as substrates for the enzyme, that compound XI was hydrolysed only very slowly (approx. 1/500th of the rate of acetylcholine under identical conditions) and that compound XII was hydrolysed by the enzyme at a faster rate ($\frac{1}{8}$th of the rate of acetylcholine), all compounds being tested as racemates.

It was thus suggested that a *trans*-diaxial arrangement of the functional groups presents the closest approximation to the conformation of enzyme-bound

acetylcholine (Smissmann & others, 1966). However, the above enzyme-catalysed hydrolyses were performed at only one substrate concentration (9·9 μmol-ml^{-1}) at pH 6·6 and an unrecorded temperature. Repeating the above studies over a wide concentration range and at a pH more closely approximating to physiological pH may well give a very different indication of the conformation required for optimal rates of enzyme catalysed hydrolysis.

Alternatively, it may be suggested from the above results employing *trans*-decalin derivatives, that the *trans*-2-dimethylaminocyclohexyl acetate methiodide enantiomers may be interacting with the active site in the *trans*-diaxial conformation.



However, simple calculations, based on the assumption of the additivity of conformational free-energy values of the substituent groups, suggest that the free-energy difference between the two conformations is approximately 24·3 kJ mol^{-1} (5800 cal mol^{-1}) in favour of the di-equatorial conformation.

The free energy of binding associated with a series of substrates and inhibitors of acetylcholinesterase is essentially constant at 17·57 $\pm$ 2·5 kJ mol^{-1} (4200 $\pm$ 600 cal mol^{-1}) (Belleau & Lavoie, 1968). Thus, since the conformational free energy difference between the two lowest energy conformations of *trans*-2-dimethylamino-cyclohexyl acetate methiodide is not exceeded by the free energy of binding to the enzyme, it would be dangerous, in the absence of accurate experimental data of the free-energy difference between conformations, to postulate the binding of the *trans*-diaxial conformation to the enzyme. Indeed, it would be more reasonable to assume that the presence of the additional carbocyclic ring in the *trans*-decalin series, with the consequent possibility of additional hydrophobic bonding occurring with the enzyme surface, has significantly influenced the stereochemical requirement for binding to the enzyme.

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