THE IMPORTANCE OF STEREOISOMERISM IN MUSCARINIC ACTIVITY

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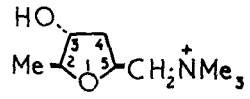
The muscarinic activities and rates of hydrolysis by acetylcholinesterase of the optical isomers of the acetyl-α- and acetyl-β-methylcholines of known configuration have been determined. The results have been correlated and the possible characteristics of muscarinic receptors outlined.

In a recent review, Waser (1961) has emphasised the importance of stereochemical factors in muscarinic activity. L(+)-Muscarine(I) (Hardeggar and Lohse, 1957) is 200 to 800 times more active than the D(−)-isomer. (±)-Epimuscarine, (±)-allomuscarine and (±)-epiallomuscarine, which are diastereoisomers of (±)-muscarine, have 1/300th, 1/150th and 1/100th respectively the activity of (±)-muscarine (Gyermek and Unna, 1958; Waser, 1958). On the other hand, (−)-muscarone(II) is only about twice as active as the racemic form and three times as active as its enantiomorph. The diastereoisomer of (±)-muscarone, (±)-allomuscarone(III) (Me and $CH_2 \cdot N^+Me_3$ *trans*), has about the same muscarinic potency as (+)-muscarone. The muscarinic activity of (+)-acetyl-β-methylcholine-(IV) was shown by Major and Bonnett (1935) and Major and Cline (1936)



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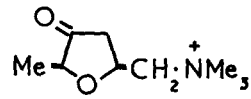

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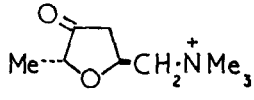

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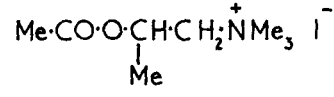

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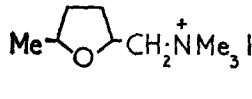

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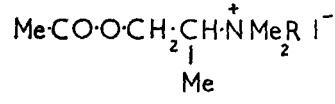

VI

to be about 100 times that of its enantiomorph. In muscarine, a *cis* arrangement of the 2–Me and 5–$CH_2 \cdot N^+Me_3$ groups with a 3–OH group in

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a position *trans* to these appears to be necessary for muscarinic activity. Any variation in the arrangement or orientation of these three groups or the omission of one or more groups from the tetrahydrofuran ring markedly reduces activity. The importance of stereochemical factors in muscarinic activity in some but not all active molecules prompted us to investigate the muscarinic activities of 5-methyltetrahydrofurfuryltrimethylammonium iodide (V), the racemic and optically active forms of acetyl-α- (VI, R = Me) and acetyl-β-methylcholine(IV) iodides and 2-dimethylaminopropyl acetate ethiodide (VI, R=Et) with a view to providing further information about the stereochemical requirements for muscarinic activity.

Experimental Methods

Pharmacological

Guinea-pig ileum. Strips of ileum from freshly killed guinea-pigs were set up in Tyrode-Ringer at 32°. The spasmogenic activities of the test compounds were compared with that of acetylcholine chloride in 4-point assays of Latin square design. The spasmogenic action of these compounds was found to be unaffected by concentrations of hexamethonium bromide, 1×10^{-5}, and to be blocked by atropine sulphate, 1×10^{-8}.

Cat blood pressure. The vasodepressor activity of the test compounds was compared with that of acetylcholine chloride in 4-point assays of Latin square design, using cats anaesthetised with 80 mg./kg. chloralose. (Full experimental details will be published by E. Lesser.)

Enzymic Studies

Technique. All estimations were made using the Warburg manometric technique described by Ammon (1933) and modified by Augustinsson (1957) and Umbreit and others (1957).

Acetylcholinesterase source. This consisted of bovine erythrocyte stromata prepared by a modification of the method of Cohen and Warringa (1953) and Warringa and Cohen (1955). The freeze-dried enzyme preparation (approx. 75 mg.) was used to prepare an homogenate (100 ml.) in buffer solution consisting of NaCl, 0·117M; $NaHCO_3$, 0·034M; $MgCl_2 \cdot 6H_2O$, 0·001M buffered to pH 7·4 by the passage of a gas mixture, CO_2, 5 per cent; N_2, 95 per cent, for 20 min. and preserved with chloroform 0·1 per cent w/v.

Assay of esterase activity of acetylcholinesterase homogenates. The procedure used was essentially that described by Augustinsson (1957). Assays of esterase activity were done at 37° using acetylcholine iodide (AchI) at its optimum concentration ([S]opt) and 1·5 ml. of the homogenate, prepared as described, in a final volume of 3 ml. buffer solution. The concentration of enzyme in the homogenate was chosen such that the amount of CO_2 released during 30 min. under the conditions described was 80–130 μl. Homogenates having esterase activities of this order were used in all investigations made on the relevant esters.

Amount of acetylcholinesterase homogenate used. 1·5 ml. in all determinations.

Substrates. The following esters were used: acetylcholine iodide, (±)-, L-(+)- and D-(−)-acetyl-β-methylcholine iodide and (±)-, L-(−)- and D-(+)-acetyl-α-methylcholine iodide. The preparation of these has been described (Beckett, Harper and Clitherow, 1962).

Calculations. In the determinations of [S]opt. and the relative rates of hydrolysis of the relevant esters, the volume of CO_2 (μl.) released due to enzymic activity was plotted against time in min. The extrapolated 30 min. value used as the unit expressing cholinesterase activity was symbolised as $b_{30}^{1\cdot5}$. Each manometric determination was always done in duplicate.

Measurement of optimum substrate concentrations. Estimations of the optimum substrate concentrations of the relevant esters were made at 37°. The concentration ([S]) of the substrates used in these determinations were within the range 1×10^{-3}M and $3\cdot3 \times 10^{-2}$M and the estimations were made using an acetylcholinesterase homogenate, previously assayed with AchI as described.

Substrate solutions. Solutions of a suitable strength were prepared in freshly prepared and gassed buffer solution immediately before use.

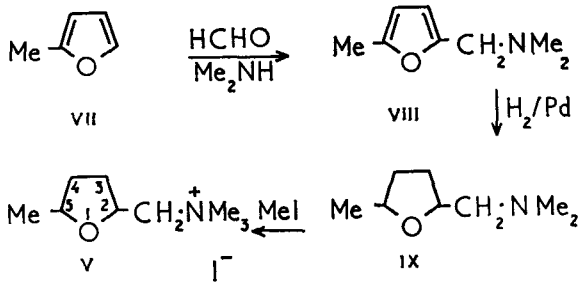
Determination of [S]opt. [S]opt. was determined for each substrate by plotting the value of $b_{30}^{1\cdot5}$ against the corresponding −log[S] (or pS). At low substrate concentrations, $b_{30}^{1\cdot5}$ values obtained by the extrapolation procedure were not accurate. More accurate results were obtained using a method of calculation involving the use of first order kinetics.

Absence of other esterases in the acetylcholinesterase preparation. The absence of the most likely other esterase contaminant (pseudocholinesterase) was established by measuring the degree of enzymic hydrolysis of acetyl-β-methylcholine (selective substrate for acetylcholinesterase, Augustinsson, 1957) in the absence and presence of the inhibitors Nu 1250 and Nu 683 (selective inhibitors of acetylcholinesterase and pseudocholinesterase respectively), inhibitor concentrations used being 10^{-7} and 10^{-8}M respectively (Hawkins and Gunter, 1946; Hawkins and Mendel, 1949).

Measurement of the relative rates of hydrolyis of the relevent compounds. The $b_{30}^{1\cdot5}$ value obtained for each compound at its optimum concentration was compared with that of AchI using an assayed acetylcholinesterase homogenate. The rate of hydrolysis of each compound was calculated as a percentage of that of AchI (= 100 per cent). Several series of determinations were made using acetylcholinesterase homogenates of slightly differing strengths and the results were found to be in close agreement. The average percentage rates of hydrolysis of each compound are recorded in Table II (for full experimental details, see Clitherow, 1961).

Chemistry

5-Methyltetrahydrofurfuryltrimethylammonium iodide (V) was prepared by the following synthetic route:



5-*Methylfurfuryldimethylamine* (*VIII*). 2-Methylfuran was converted to 5-methylfurfuryldimethylamine by the method of Eliel and Peckham (1950). The free base had b.p. 85–86° at 49–53 mm. (calc. for $C_8H_{13}NO$ equiv., 139. Found: equiv., 144). The picrate (from ethanol) had m.p. 116·5–117·5°. (Calc. for $C_{14}H_{16}N_4O_7$ equiv., 368. Found: equiv., 366.) (Eliel and Peckham, 1950, quote m.p. 116–116·5°.)

5-*Methyltetrahydrofurfuryltrimethylammonium iodide* (*V*). 5-Methylfurfuryldimethylamine (13·5 g.) in acetic acid (140 ml.) was hydrogenated in the presence of palladium (10 per cent) on charcoal (6 g.). The solution was filtered, the acetic acid evaporated under reduced pressure and the residue made alkaline with a saturated solution of sodium hydroxide. Excess anhydrous Na_2CO_3 was added and the solid extracted with ether. The ethereal extracts were dried (anhyd. Na_2SO_4), the ether removed by evaporation under reduced pressure and the residual liquid distilled to give a fraction b.p. 86–89° at 54–55 mm. having an equivalent weight of 152. This basic fraction consisted mainly of 5-methyltetrahydrofurfuryldimethylamine and gave a *picrate* (from ethanol) m.p. 117·5–118·5°. (Found: C, 45·4; H, 5·6; N, 15·0 per cent; equiv., 373. $C_{14}H_{20}N_4O_8$ requires C, 45·2; H, 5·4; N, 15·1 per cent; equiv., 372.) Catalytic hydrogenation would be expected to give predominantly the *cis* isomer with probably a small proportion of the *trans* form (Eugster, 1960). A gas chromatogram (column, stationary phase: liquid paraffin and polyethylene glycol; inert support, Celite; column length, 6 ft.; column temperature, 94°; bridge current, 100 mA; carrier gas, N_2; flow rate, 2 litres/hr.; pressure: inlet 715 mm., outlet 285 mm. Hg) showed two closely aligned peaks indicating the presence of *cis* and *trans* isomers in the ratio of about 4 : 1.

A solution of the base (2 g.) in acetone (10 ml.) was treated with methyl iodide (2 ml.) at room temperature and the solid which separated was crystallised from ethanol-ether to give *cis*-5-*methyltetrahydrofurfuryltrimethylammonium iodide* (3 g.), m.p. 154–155°. (Found: C, 38·4; H, 7·1; N, 4·9 per cent; equiv., 285. $C_9H_{20}INO$ requires C, 37·5; H, 7·1; N, 4·9 per cent; equiv., 285.) The allocation of this configuration is based on the fact that the compound was isolated in high yield (80 per cent) and that the isomeric mixture had a well defined melting-point.

Acetylcholine analogues. The preparation of these has already been described (Beckett, Harper and Clitherow, 1962).

RESULTS

The muscarinic potencies of the relevant compounds are shown in Table I.

TABLE I

MUSCARINIC POTENCIES OF SOME ACETYLCHOLINE ANALOGUES AND THEIR OPTICAL ISOMERS (ACH = ACETYLCHOLINE)

Compound	Isomer	Guinea-pig ileum: Muscarinic activities. Mol. of drug = 1 mol. ACH. Mean limits P = 0·05		Ratio of activities of isomers (+)/(−)	Cat blood pressure: Muscarinic activities. Mol. of drug = 1 mol. ACH. Mean limits P = 0·05		Ratio of activities of isomers (+)/(−)
Acetyl-β-methylcholine iodide ..	(±)	1·58	1·35–1·91	240	0·97	0·76–1·22	280
	L-(+)-	1·01	0·94–1·10		0·71	0·54–0·93	
	D-(−)-	240	208–277		202	156–261	
Acetyl-α-methylcholine iodide ..	(±)	49	35–71	8	36	30–43	6
	L-(−)-	232	204–264		143	121–170	
	D-(+)-	28	22–35		25	21–29	
2-Dimethylaminopropyl acetate iodide	(±)	264	243–286	12	191	128–286	5
	L-(−)-	1,980	1,695–2,310		446	344–576	
	D-(+)-	170	156–186		91	69–114	
5-Methyltetrahydrofurfuryltrimethylammonium iodide	Mainly *cis*	20	18–21				

The rates of hydrolysis of acetylcholine and the racemic and optically active forms of the acetyl-α- and acetyl-β-methylcholine iodides at their optimum concentrations at 37° by acetylcholinesterase are shown in Table II.

TABLE II

RATES OF HYDROLYSIS OF (+), (−) AND (±) ACETYL-α- AND ACETYL-β-METHYLCHOLINE IODIDES COMPARED WITH ACETYLCHOLINE IODIDE (ACH) AT THEIR OPTIMUM CONCENTRATIONS AT 37° BY A STANDARD ACETYLCHOLINESTERASE HOMOGENATE

Compound	Isomer	[S]opt × 10^3M	Rates of hydrolysis compared with Ach (= 100 per cent)
Acetylcholine iodide		4·9	100
Acetyl-α-methylcholine iodide..	L-(−)-	6·7	97·4
	(±)	6·3	91·7
	D-(+)-	6·7	78·0
Acetyl-β-methylcholine iodide	L-(+)-	10·0	54·5
	(±)-	18·0	46·2
	D-(−)-	—	weak inhibition

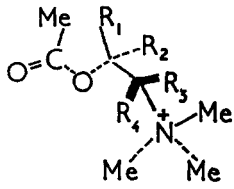
DISCUSSION

The intensity of muscarinic activity of cholinergically active compounds will, to some extent, be dependent upon their susceptibility to cholinesterase attack as well as their reaction with muscarinic receptors. Muscarine and muscarine-like compounds have been found to be weak inhibitors of acetylcholinesterase (Witkop, Durant and Friess, 1959)

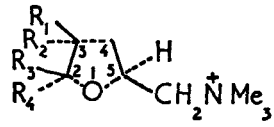
and their muscarinic activity may, therefore, be regarded as a measure of their interaction with a muscarinic receptor. The isomers of acetyl-α- and acetyl-β-methylcholine are, however, substrates or antagonists of acetylcholinesterase and susceptibility to cholinesterase attack must be taken into account when considering optimum structural requirements for fit at muscarinic receptors.

The absolute configuration of the (+)-isomers of acetyl-α-methylcholine (X; $R_1=R_2=R_3=H$, $R_4=Me$) (Beckett and others, 1961) and acetyl-β-methylcholine (X; $R_1=Me$, $R_2=R_3=R_4=H$) (Ellenbroek and van Rossum, 1960; Beckett, Harper, Clitherow and Lesser, 1961) have recently been determined and the configurational identity of L-(+)-acetyl-β-methylcholine with L-(+)-muscarine (XI; $R_1=R_4=H$, $R_2=OH$, $R_3=Me$) about C(5) established.

L-(+)-Muscarine is about 200–800 times more active than its enantiomorph and 2·5 times more potent than the (±)-form. The muscarinic activity is considerably reduced in those diastereoisomers of muscarine in which the Me, $CH_2{\cdot}N^+Me_3$ and OH groups are inverted, e.g. (±)-epi- (XI; $R_2=R_4=H$, $R_1=OH$, $R_3=Me$), (±)-allo- (XI; $R_2=R_3=H$, $R_1=OH$, $R_4=Me$) and (±)-epiallo-muscarine (XI; $R_1=R_3=H$, $R_2=OH$, $R_4=Me$) are about 1/300th, 1/150th and 1/100th respectively the activity



X

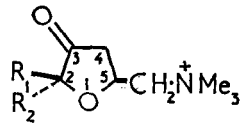


XI

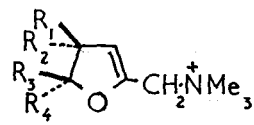
of (±)-muscarine. The muscarine type compounds have rigid structures in which the spatial arrangements of the substituent groups on the tetrahydrofuran ring are known. These compounds were used as models for a consideration of the most probable surface for presentation to a receptor. A diagramatic representation of the proposed muscarinic receptor has been made (Fig. 1) (Beckett and others, 1961) in which the quaternary N group and the ether oxygen, lying almost on a plane, form the two main centres for drug-receptor association while the –OH group is considered to act as a secondary site of association. In Fig. 1, the distance between the binding sites correspond approximately to the distances between the active centres in the muscarine molecule (Jellinek, 1957). The importance of the ether oxygen as a primary binding site is illustrated by the high muscarinic potencies of choline-ethyl ether and β-methylcholine-ethyl ether (Simonart, 1932). Molecular models would appear to indicate that if attachment of L-(+)-muscarine to the receptor takes place mainly by the quaternary nitrogen and ether oxygen, then one surface of the molecule, i.e. that which bears the *cis* arranged Me and $CH_2{\cdot}N^+Me_3$ groups, would not present a surface complementary to that of the receptor site.

It might be expected that due to steric factors, D-(−)-muscarine, (±)-2-methylmuscarine (XI; R_1=H, R_2=OH, R_3=R_4=Me) and the diastereoisomers of (±)-muscarine would not be able to present a surface complementary to that of the depicted receptor. Other analogues of muscarine (XI; R_1=H, R_2=OH, R_3 or R_4=Pr; and XI; R_1=OH, R_2=H, R_3 or R_4=Pr; and XI; R_1=H, R_2=OH, R_3 or R_4=Bu) also show reduced muscarinic activity and this may be attributed to steric factors associated with the propyl and butyl groups in the 2-position of the muscarine molecule. The importance of having one methyl group in the 2-position and a correctly orientated 3-OH group is exemplified by the relatively low activities of the *trans* and *cis* forms of (±)-desmethylmuscarine (*trans*; XI; R_2=OH, R_1=R_3=R_4=H) (*cis*; XI; R_1=OH, R_2=R_3=R_4=H) which have respectively about 1/100th and 1/1000th the muscarinic activity of acetylcholine. The importance of the –OH group and its orientation *trans* to the $CH_2{\cdot}N^+Me_3$ group is exemplified by the fact that (±)-tetrahydrofurfuryl trimethylammonium (XI; R_1=R_2=R_3=R_4=H) has only about 1/100th to 1/300th the activity of acetylcholine. A similar pattern of configurational dependence for activity is observed in the series-(±)-muscarine (XI; R_1=R_4=H, R_2=OH, R_3=Me), (±)-5-methyltetrahydrofurfuryltrimethylammonium (V) and (±)-epimuscarine (XI; R_2=R_4=H, R_1=OH, R_3=Me) which have about 4/5th, 1/20th and 1/300th the activity of acetylcholine respectively.

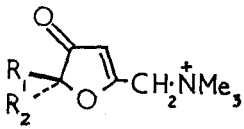
(±)-Muscarone (XII; R_1=Me, R_2=H) is about six times more potent than (±)-muscarine. This may be due to the greater polarity of the keto group which may facilitate binding to site 3 of the receptor and to the



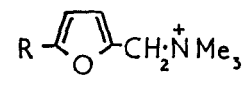
XII



XIII



XIV



XV

greater planarity of the molecule which may permit a closer drug-receptor association. The greater degree of planarity might explain why (−)-muscarone is only three times more active than its enantiomorph and about twice as active as the (±)-form, in distinct contrast to larger differences in activity between the less planar (+) and (−)-muscarine isomers. An anomaly appears to exist concerning the relationship between configuration and activity of the muscarone isomers. The more active (−)-muscarone is reported to have the D- configuration at C(5) (Waser, 1962), i.e. the inverse configuration to (+)-muscarine at this centre, but the chemical evidence is not unequivocal.

Neither isomer of (±)-allomuscarone (XII; R_1=H, R_2=Me) which has about 1/2 the activity of (±)-muscarone, would be expected to present a surface complementary to the depicted receptor. (±)-Allomuscarone is a more planar molecule than (±)-allo- and (±)-epiallomuscarine [from which it may be derived chemically (Eugster and others, 1958a, b, c)]. The increased planarity in (±)-allomuscarone would diminish the steric effect of the 2-methyl group and allow a closer drug-receptor association than in the case of the above mentioned precursors.

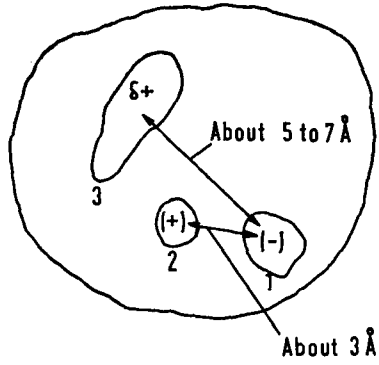


FIG. 1. Diagramatic representation of muscarinic receptors.

1. Anionic cavity negatively charged to accommodate quaternary nitrogen. 2. Positively charged point accommodating ether linkage of muscarine or ester linkage of acetylcholine and its analogues. 3. (+) Charged area to accommodate OH of muscarine, C = O of acetylcholine and its analogues or double bond of furan analogues of muscarine.

The importance of planarity in muscarinic activity is further exemplified by the high activities of (±)-4,5-dehydromuscarine (XIII; R_1=R_4=H, R_2=OH, R_3=Me), (±)-4,5-dehydro-epimuscarine (XIII; R_2=R_4=H, R_1=OH, R_3=Me), (±)-4,5-dehydromuscarone (XIV; R_1=Me, R_2=H) [which may exist in equilibrium with the enolic form (Waser, 1962)] and 5-methylfurfuryltrimethylammonium (XV; R=Me) which have respectively 1, 1/2, 5·3 and 1/3 to 1 times the activity of acetylcholine (Gyermek and Unna, 1958, 1960a, b; Ing, Kordik, Tudor-Williams, 1952).

The importance of the methyl group in the 2-position of muscarine (or the 5-position of furan analogues) and the steric implications of this group have been stressed. It is not unreasonable to suppose that this group contributes to an increased electron availability in the vicinity of the ether oxygen which would facilitate drug-receptor association, e.g. the muscarinic potencies of (±)-desmethylmuscarone (XII; R_1=R_2=H) and furfuryltrimethylammonium (XV; R=H) have respectively only about 1/40th and 1/10th the activity of their methyl analogues (Zwicky, Waser and Eugster, 1959; Armitage and Ing, 1954; Ing and others, 1952).

The determination of the absolute configurations of the acetyl-α- and acetyl-β-methylcholine isomers and the configurational identity of the

latter with L(+)-muscarine about C(5) permits consideration of the fit of these compounds at the proposed muscarinic receptor. It is assumed that acetylcholine and the acetyl-α- and acetyl-β-methylcholine isomers adopt conformations similar to that of L(+)-muscarine when acting at the muscarinic receptors. The (+)-isomer of acetyl-β-methylcholine (X; R_1=Me, R_2=R_3=R_4=H) can adopt a conformation complementary to that of the depicted receptor while the (−)-isomer (X; R_1=R_3=R_4=H, R_2=Me) cannot do so. Simonart (1932) showed that the muscarinic activity of (±)-acetyl-α-methylcholine was about 1/20th that of acetylcholine which suggests that the α-methyl group might exert a steric effect on the cationic head thereby preventing a close drug-receptor association. The muscarinic activity of D-(+)-acetyl-α-methylcholine (X; R_1=R_2=R_3=H, R_4=Me) is about 7 times greater than its enantiomorph (X; R_1=R_2=R_4=H, R_3=Me) (Table I). Both isomers of acetyl-α-methylcholine can probably present surfaces complementary to the receptor, the only difference between the two isomers, when in the desired conformation, being in the direction in which the methyl groups project. One would, therefore, expect these isomers to have not greatly dissimilar low potencies.

As indicated previously, the hydrolysis of the optical isomers of acetyl-α- and acetyl-β-methylcholine by acetylcholinesterase is a complicating factor in the interpretation of their relative muscarinic potencies. Since the muscarine-like molecules are not susceptible to cholinesterase hydrolysis and are only weak inhibitors of acetylcholinesterase, their muscarinic potencies may be interpreted as being a measure of their relative interactions at the muscarinic receptors.

The muscarinic potency of L(+)-acetyl-β-methylcholine is about the same as that of acetylcholine whereas its rate of hydrolysis by acetylcholinesterase is about 54 per cent that of acetylcholine (Table II). The slower rate of hydrolysis of L(+)-acetyl-β-methylcholine in the biophase of the muscarinic receptors probably compensates for the deleterious effect of a β-methyl group on the association of this molecule and a muscarinic receptor with the result that acetylcholine and L(+)-acetyl-β-methylcholine have equimuscarinic potencies.

The weak inhibitory action of D(−)-acetyl-β-methylcholine towards acetylcholinesterase (Table II) has been shown by Beckett and others (1961) to reinforce slightly the muscarinic activity of the L(+)-isomer in (±)-acetyl-β-methylcholine.

Acetylcholinesterase hydrolyses D(+)- and L(−)-acetyl-α-methylcholine at 78 per cent and 97 per cent of the rate of acetylcholine respectively (Table II). The L(−)-isomer probably presents a more favourable complementary conformation to the active site of acetylcholinesterase than does its enantiomorph. L(−)-Acetyl-α-methylcholine might also present a more favourable complementary conformation to the muscarinic receptors than the D(+)-isomer but, owing to the faster rate of enzymic hydrolysis and inactivation of the former isomer in the biophase of the muscarinic receptors, the latter isomer would be expected to exert the greater muscarinic activity.

Replacement of a methyl group in the cationic head of acetylcholine by an ethyl group causes a threefold reduction in muscarinic activity (Ing, 1949; Holton and Ing, 1949; Ing and others, 1952). A similar effect was observed in the present investigations in which the muscarinic potencies of D(+)-, L(−)- and (±)-2-dimethylaminopropyl acetate ethiodide were found to be about 1/5th of the corresponding acetyl-α-methylcholine isomers (Table I).

Since (±)-tetrahydrofurfuryltrimethylammonium (XI; $R_1=R_2=R_3=R_4=H$) and (±)-β-methylcholine ethyl-ether have about 1/100th to 1/300th and 1/20th to 1/40th the muscarinic activity of acetylcholine respectively (Simonart, 1932; Fellows and Livingston, 1940), the presence of the 2–Me and 3–OH is important in rigid muscarine-type molecules. Such groups do not appear to be essential if flexibility exists in the molecules, i.e. in acetylcholine-like molecules.

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