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Review Article

The possible role of conformational isomerism in the biological actions of acetylcholine

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SITES OF ACTION OF ACETYLCHOLINE

SINCE acetylcholine is involved as chemical mediator at synapses between neuron and neuron, between neuron and muscle cell, and between neuron and secretory cell, apparently generating essentially similar bioelectric potentials in all three types of post-synaptic cell (Nachmansohn, 1959), it plays a fundamental role in a number of distinct physiological situations. Thus acetylcholine is involved in the transmission of nerve impulses at both the sympathetic and parasympathetic ganglionic synapses (Kibjakow, 1933; Feldberg & Gaddum, 1934; Feldberg & Vartianen, 1935; Grundfest, 1957), at the synapse between motor nerve and voluntary muscle (Dale, Feldberg & Vogt, 1936; Brown, Dale & Feldberg, 1936; Brown, 1937), at the synapses between autonomic nerves and certain exocrine glands (Dale & Feldberg, 1934; Hurley, Shelley & Koelle, 1953; Goodman & Gilman, 1955a) and at the synapse between smooth muscle and those autonomic nerves which, in consequence, have been termed cholinergic (Loewi, 1921; Chang & Gaddum, 1933; Dale & Feldberg, 1934; Gaddum, 1936) and which correspond to the parasympathetic nervous system. Acetylcholine has long been known to be involved in the release of adrenaline and noradrenaline from the modified ganglion cells constituting the adrenal medulla (Feldberg & Minz, 1933; Feldberg, Minz & Tsudzimura, 1934) and more recently it has been shown to be implicated also in autonomic adrenergic transmission (Burn, 1961; Burn & Froede, 1963; Burn, Rand & Wien, 1963). It may also play a role at the termination of sensory nerves (Brown & Gray, 1948; Douglas & Gray, 1953; Davis, 1961; Koelle, 1961, 1962), while new emphasis has recently been given (Nachmansohn, 1959, 1961, 1962) to older views (Nachmansohn, 1946; Lorente de Nó, 1949; Hodgkin, 1951; Toman, 1952; Eccles, 1953; Tasaki, 1953) that it may participate in the conduction of nerve impulses along axons, although the conclusions concerning its involvement in this situation have been contested (Ritchie & Armett, 1963; Triggle, 1965). The occurrence of acetylcholine within the central nervous system suggests even further physiological significance for this compound but its exact functions in this location are still inconclusively established (e.g. Crossland, 1960), although it is known to be concerned in the release of antidiuretic hormone from the neurohypophysis (Pickford, 1945, 1947; Verney, 1947; Duke, Pickford & Watt, 1950; Harris, 1951; Jewell, 1953) and to have a transmitter role at the Renshaw cells in the spinal cord (Eccles, Eccles & Fatt, 1956). Indeed cortical synapses have been postulated to possess an acetylcholine receptor similar to that of the neuromuscular junction (Feher, Klitina & Molnar,

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1965). Further speculations about the biological significance of acetylcholine are to be found in the various comprehensive reviews concerned with drugs which exert their effects by mimicking or antagonizing the neurohormone (*inter alia* Goodman & Gilman, 1955b; Del Castillo & Katz, 1956; Bovet, Bovet-Nitti & Marini-Bettolo, 1959; Nachmansohn, 1959; Cavallito & Gray, 1960; Crossland, 1960; Waser, 1960, 1961a; Bowman, 1962; Koelle, 1962; Stenlake, 1963; Barlow, 1964; Triggle, 1965).

SELECTIVITY OF ACTION OF MIMETICS AND ANTAGONISTS OF ACETYLCHOLINE

Despite the diversity of sites of action of acetylcholine, with some exceptions, at normal dose levels, drugs which mimic or antagonize the actions of acetylcholine, do so at a limited number of sites only. This has given rise to the established pharmacological classification into nicotinic or muscarinic agents for mimetics and neuromuscular blocking agents, ganglion blocking agents or antispasmodic drugs for antagonists. In part, this relative selectivity to site of action may have its origin in transport and permeability factors, the absence of lipid barriers at the neuromuscular synapse (Couteaux, 1947) adequately explaining the ready access to this site of polyonium salts which are normally unable to penetrate such barriers (Brodie & Hogben, 1957; Walsh & Deal, 1959; Rosenberg & Ehrenpreis, 1961a,b; Rosenberg & Podleski, 1962). Certainly the central actions of neuromuscular blocking agents on intrathecal, intracisternal or intraventricular injection (Wislicki, 1958) help to demonstrate the role of the blood brain barrier where these compounds are administered by other routes, while studies with nerve fibre preparations lacking protective lipids show these agents to be capable of exhibiting activity at yet other sites not affected in the intact animal (Rosenberg & Ehrenpreis, 1961a,b; Dettbarn, 1960).

Consideration of permeability factors alone, however, fails to provide a full explanation as to why purely muscarine-like and atropine-like drugs show little or no action at nicotinic sites, since absence of a permeability barrier at the neuromuscular junction can hardly be invoked to explain absence of activity on grounds of a denial of access to these drugs. Similarly, it is difficult to see, from permeability considerations, why local anaesthetics which appear to occupy certain acetylcholine receptors (Goodman & Gilman, 1955c) exhibit no activity at normal muscarinic or nicotinic receptors. Moreover, the recent demonstrations (Takeshige & Volle, 1963; Jones, 1963) that ganglia appear to contain both nicotinic and muscarinic receptors would also seem to strengthen the view that factors other than permeability effects are involved.

POSSIBLE ROLE OF CONFORMATIONAL ISOMERISM IN THE PHYSIOLOGICAL ACTIONS OF ACETYLCHOLINE

That the conformational flexibility of the acetylcholine molecule could make possible the existence of several distinct types of acetylcholine receptor, which would provide a fundamental basis for the observed selectivities of different groups of acetylcholine mimetics and acetylcholine

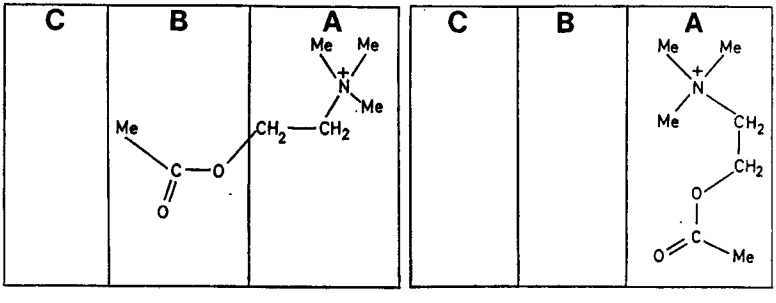
antagonists, has been implied by several workers (Schueler, 1956; Kennard, 1960; Archer, Lands & Lewis, 1962). Indeed, such a situation would not be inconsistent with the somewhat different pictures of the muscarinic and nicotinic receptors emerging from other considerations (*inter alia* Waser, 1961a, 1962; Cavallito, 1962; Triggle & Belleau, 1962; Beckett, Harper & Clitherow, 1963; Belleau & Puranen, 1963; Bebbington & Brimblecombe, 1965), especially since nicotinic activity, in marked contrast to muscarinic activity (Hardeggar & Lohse, 1957; Gyermek & Unna, 1958, 1960; Beckett, Harper & others, 1961; Belleau & Puranen, 1963), does not appear to be greatly influenced by changes in stereochemistry. This is illustrated by the high nicotinic potency present in both enantiomorphs of compounds such as muscarone (Waser, 1961a), lactoylcholine (Rama-Sastry & Auditore, 1963) and nicotine or nornicotine (Hicks, Brücke & Heuber, 1935; Hicks, MacKay & Sinclair, 1947), and by the retention of nicotinic activity in acylcholines where the acid radical has considerable steric bulk (Bergel, 1951; Akcasu, Sinha & West, 1952). Again the conformational flexibility of the acetylcholine molecule could rationalize any differences between the muscarinic and nicotinic receptors on the one hand and the acetylcholinesterase surface (Friess & Baldridge, 1956; Nachmansohn & Wilson, 1959; Krupka & Laidler, 1961; Thomas, 1961; Belleau & Lacasse, 1963; Turpajev, Nistratova & Putintseva, 1963; Wilson, 1963) on the other, although the direct nicotinic action of such typical anticholinesterase drugs as neostigmine and edrophonium (Lewis, 1962) must be taken into account. The hypothesis advanced by Roepke (1937) of the identity of acetylcholinesterase and the cholinergic receptor has since met with both support and opposition and the pertinent evidence has recently been assessed (Holmstedt, 1963; Werner & Kuperman, 1963; Belleau, 1964).

Unfortunately detailed consideration of the molecular features of the many drugs which act as mimetics or antagonists of acetylcholine sheds little light on the possible biological importance of conformational isomerism in the neurohormone for three main reasons.

(i) *Lack of suitable rigid molecules.* Most drug molecules known to mimic or antagonize the biological actions of acetylcholine are themselves conformationally flexible and there is no way of establishing for a given case which one of several possibilities is in fact operative. In some instances a drug may be capable of precise mimicry of only a single biologically-significant conformer of acetylcholine on account of unfavourable bonded or non-bonded interactions within the drug molecule. In other instances, although itself capable of mimicking more than one biologically-significant conformer of acetylcholine, a drug molecule may not be able to attach itself to certain acetylcholine receptors due to interaction between these receptors and molecular units present in the drug molecule but absent in acetylcholine. Again a wide spectrum of ease of conformational interconversion can be expected for different drugs which could underlie observed differences in relative selectivity with respect to site of action.

(ii) *Difficulty in assessing the relative significance of conformational*

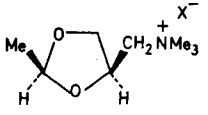
phenomena in mimetics and antagonists of acetylcholine. Even if certain conformational deductions were to prove feasible for drugs interfering with the normal course of events at acetylcholine receptors, the absence of any *a priori* distinction between the molecular features leading to drug receptor interaction (affinity) (Ariëns & Simonis, 1964) and those determining elicitation of a positive biological response (intrinsic activity) makes it difficult to assess the relative significance of conformational phenomena in mimetics and antagonists of acetylcholine. However, in the case of antagonists, it would appear that shielding of the receptor, rather than an exact fit with it, could be the significant factor. Thus many antispasmodic drugs possess somewhat bulky molecules, while increase in the size of the cationic substituents of depolarizing neuromuscular blocking agents (which would be expected to sterically hinder approach to the receptor) is accompanied by a change to non-depolarizing activity. Recently, the hypothesis has been advanced (Belleau & Lacasse, 1964; Belleau, 1964) that affinity may be ascribed to hydrophobic forces serving to transfer a drug from the aqueous phase to non-polar sites on the receptor protein whilst intrinsic activity can be related to the particular conformational perturbation in the tertiary structure of the receptor protein induced through interaction with the drug molecule. Agonistic activity is then correlated with the induction of a unique specific conformational perturbation and antagonistic activity with the induction of any one of a number of non-specific conformational perturbations incapable of inducing biological response. The phenomenon of partial agonism is rationalized by assuming that equilibrium mixtures of receptor protein transformed by the specific and non-specific conformational perturbations are produced by the drug. An extension of this hypothesis has led to the view that the muscarinic receptor is constituted by three non-polar



Nicotinic Receptor		Muscarinic Receptor	
Compartment	*Nature*	*Compartment*	*Nature*
A	Non-polar	A	Non-polar
B	Polar	B	Non-polar
C	Non-polar	C	Non-polar

FIG. 1. Schematic representation of compartmentalization of receptor surfaces (adapted from Belleau, 1965).

compartments whereas the nicotinic receptor is envisaged to be composed of a polar or semi-polar compartment adjacent to two non-polar compartments (Fig. 1), and further that the cholinergic receptor may be an enzyme whose role is to catalyse phosphoryl group transfer (Belleau, 1965). It is to be noted, however, that in the 1,3-dioxolane analogues (e.g. *1*) of acetylcholine, upon which certain of these deductions (Belleau & Lacasse, 1964) concerning the conformation adopted by acetylcholine at the muscarinic receptor and during complex formation with acetylcholinesterase are based, only the equivalent of the $MeCOOCH_2$-fragment of the acetylcholine molecule is locked in a rigid conformation. Free rotation about the remaining bonds in the 1,3-dioxolanes and in acetylcholine itself still permits conformational heterogeneity (*vide infra*).



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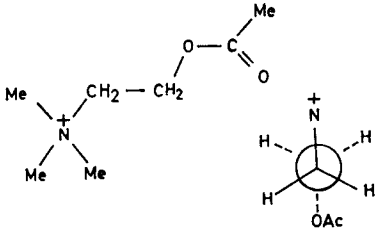
(iii) *Difficulties in determining the focal point of conformational isomerism.* The activity displayed by a compound such as the tetramethylammonium cation, which formally represents a portion of the acetylcholine molecule divorced from conformational significance with respect to the acetoxyethyl moiety, indicates that conformational isomerism within this latter radical cannot exert a crucial influence at all acetylcholine receptors. This raises serious doubts about its exact significance at other centres although the stereochemical requirements of the anionic site in the receptor appear to be reasonably specific (Kellet & Hite, 1965).

In view of these factors, therefore, it would seem necessary to look elsewhere for evidence bearing on the possible role of conformational isomerism in the biological actions of acetylcholine.

THE CONFORMATION OF ACETYLCHOLINE

X-ray studies on crystalline acetylcholine bromide (Sörum, 1959) were originally interpreted to indicate that in the solid state the molecules of acetylcholine coexisted in two separate favoured conformations—the fully staggered conformation (*2*) and the quasi-ring conformation (*3*). The validity of this interpretation was later criticized (Dunitz, 1963) and a reinvestigation of the crystal structure of acetylcholine bromide (Canepa, Pauling & Sörum, 1966) has shown the sole presence of a quasi-ring form (*4*) in which the interatomic angles are such that the methyl group (C–1) seems to form a bent hydrogen bond through one of its hydrogen atoms to the acyloxy oxygen atom (O–1). The structure of acetylcholine in the crystal lattice is thus very similar to those of choline chloride (*5*) (Senko & Templeton, 1960) and L(+)-muscarine iodide (*6*) (Jellinek, 1957). In the latter the stability of the quasi-ring conformation has been attributed to C–H - - - - O bonding (Sutor, 1963).



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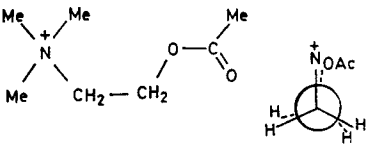

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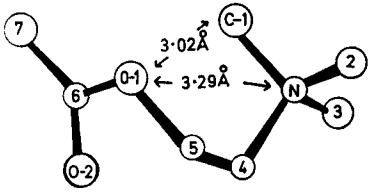

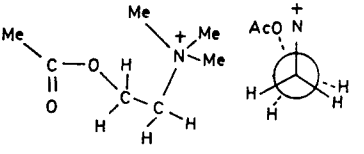

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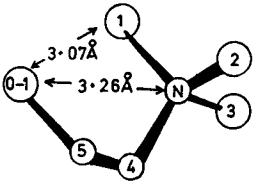

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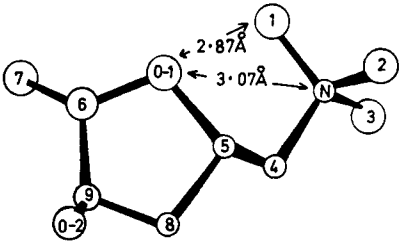

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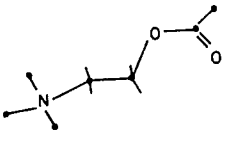
Although the existence of the extended conformation (*2*) of acetylcholine bromide in the solid state has been disproved, this by no means excludes the existence of a fully staggered conformation in other situations. Thus, in the extended conformation (*2*), non-bonded interactions are minimal and at ordinary temperatures it might be expected to be the favoured conformation of the molecule. Conformational isomerism in acetylcholine is dependent upon free rotations about the C–C and C–O bonds of the choline fragment and of the infinite number of conformers possible, nine separate well-defined conformations for acetylcholine must be considered. These are the fully staggered conformation (*2*) and four pairs of identical skewed forms (*2a, b, c, d*). However, the cyclic conformation *2a* may be regarded as being extremely unfavoured from a consideration of steric requirements and interaction energies (Gill, 1965). On the basis of intrachain stereochemical interactions, duly corrected for the dipole interaction between the positive charge of the quaternary nitrogen atom and the ester function, the relative probabilities of the



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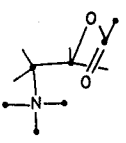

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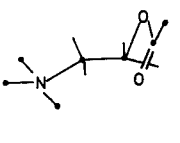

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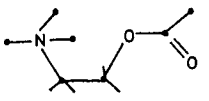
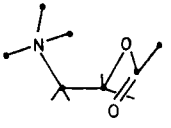


2c (≡ 4)



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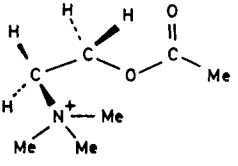

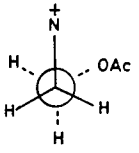

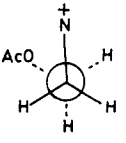

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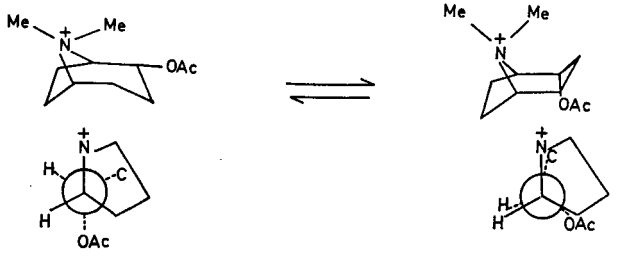
four forms *2*, *2b*, *2c* and *2d* of acetylcholine in solution have been calculated as 1:0·55:0·08:0·02 (Gill, 1965). Despite these considerations however, acetylcholine has been concluded to exist in aqueous solution as a quasi-ring form akin to *3* or *4* (Canepa, 1965). Indeed a nuclear magnetic resonance study of acetylcholine in deuterium oxide has been interpreted as providing evidence that the mean conformation of acetylcholine in aqueous solution is best represented as in *7* (Culvenor & Ham, 1966). This conformation is similar to that pertaining in the crystal lattice (Canepa & others, 1966) in that the $\overset{+}{N}$–C–C–O system is in a *gauche* arrangement but differs (compare *4*) in that the CH_2–O–CO–Me grouping has the normal conformer populations of a primary ester. This does not preclude the possibility that both the extended form and the quasi-ring form of acetylcholine may separately be of biological significance, as has been conjectured by other workers (Schueler, 1956; Kennard, 1960; Archer & others, 1962; Smissman, Nelson & others, 1966), since the receptor may not necessarily complex with the energetically most favoured form of a given molecule (Gill, 1965). Furthermore, if the receptor surfaces are in compartments as envisaged by Belleau (1965) and if the muscarinic and nicotinic receptors differ solely in the polarity of the central compartment (B in Fig. 1) then the proposed polar nature of this compartment in the nicotinic receptor may well accommodate the polar acyloxy function of the extended conformation (*2*) of acetylcholine. It may be significant that effective nicotinic drugs frequently include a polar group at three to four interatomic distances away from the quaternary

nitrogen atom (Rossum, 1962). On the other hand, the central compartment of the muscarinic receptor is considered to be non-polar and would thus be unable to accommodate a polar function. Therefore, the quasi-ring form of acetylcholine (e.g. *4*) could well be involved at the muscarinic receptor.

THE POSSIBLE PHYSIOLOGICAL INVOLVEMENT OF ACETYLCHOLINE CONFORMERS

Archer & others (1962) have advanced the hypothesis that nicotinic activity depends on conformation *4* of acetylcholine and muscarinic activity on conformation *2* since the L- and (±)-2α-acetoxytropane methiodides (*8*) possessed comparable weak muscarinic activity (absent in the L-2β-acetoxytropane methodide) while L-2β-acetoxytropane



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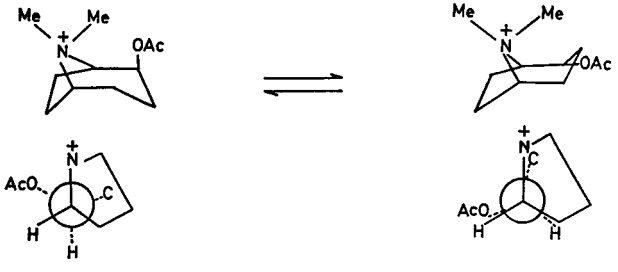

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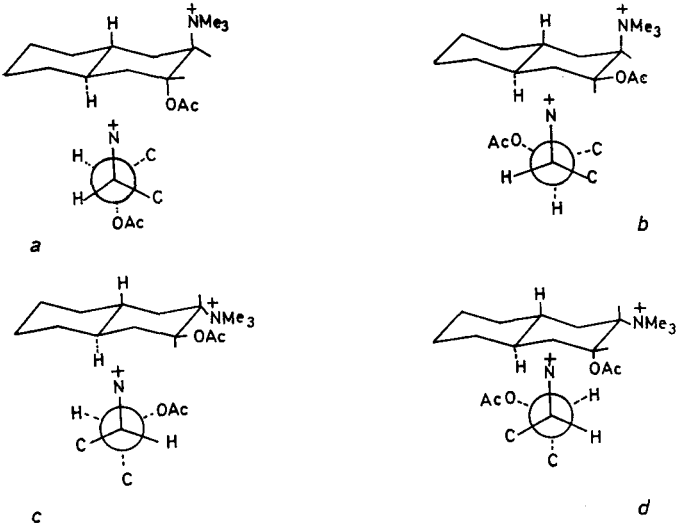
L-Forms depicted

methiodide (*9*) possessed stronger nicotinic activity than the L- and (±)-2α-compounds. Smissman & others (1966) also consider from a study of the isomeric 3-trimethylammonium-2-acetoxy-*trans*-decalin halides (*10a-d*) and the isomeric α,β-dimethylacetylcholine halides (*11a* and *b*) that muscarinic activity depends on conformation *2* of the acetylcholine molecule, but the activities of the compounds were very weak. Unfortunately only the racemic forms of the various isomers were tested and nicotinic activities were not recorded. These hypotheses would also



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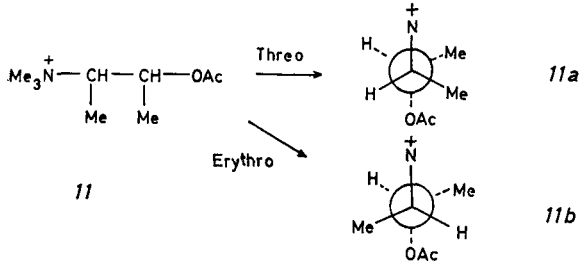
(One enantiomorph only of each compound is shown)

accommodate the pronounced muscarinic properties of arecoline (*12*) which can assume a conformation in which the acyloxy oxygen atom and the tertiary nitrogen atom are spatially disposed in a manner somewhat similar to the corresponding functional groups in conformation *2* of acetylcholine. Arecoline, however, also exhibits significant nicotine-like actions (Goodman & Gilman, 1955). It is to be noted that the muscarinic properties of (+)-pilocarpine (*13*) are difficult to accommodate on the



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11a

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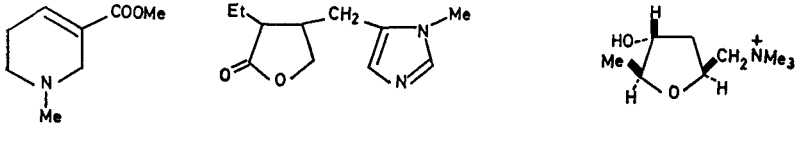
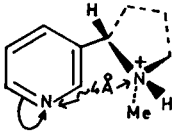
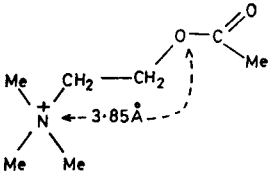


12 *13* *14*

basis of the above ideas. It is of course possible that some, if not all, of these discrepancies arise from the compound concerned inducing acetylcholine release and not exerting a direct action in its own right.

However, the fact that L(+)-muscarine (*14*) in the solid state exists in a quasi-ring conformation (Jellinek, 1957) which corresponds closely with the quasi-ring conformer (*4*) of acetylcholine (Canepa & others, 1966) might suggest that this conformation (*4*) rather than the fully-staggered conformation (*2*) of acetylcholine is more likely to be involved at the muscarinic sites. In addition, should the molecules of both acetylcholine and nicotine have a two point attachment at the nicotinic receptor,





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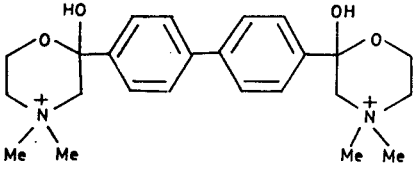

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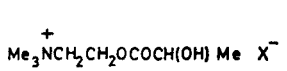
evidence is available that conformation *2* of the acetylcholine molecule may be the nicotinic conformation since examination of molecular models of nicotine shows that in one of the two conformations in which the pyridine and pyrrolidine rings are almost co-planar (*15*) the internitrogen distance of *ca* 4Å closely corresponds with the distance in *2* between the nitrogen atom and the acyloxy oxygen atom. Since the pyrrolidine nitrogen atom of nicotine is generally assumed to be in cationic form at physiological pH (Barlow & Dobson, 1955; Gillis & Lewis, 1956) and the pyridine nitrogen atom can be regarded as being somewhat analogous electronically to the acetoxy oxygen atom in acetylcholine, the purely nicotinic actions of nicotine are fully rationalized in terms of a receptor accepting conformation *2* of acetylcholine, provided both molecules share a common two point attachment involving the atoms just indicated. It is perhaps significant that the hemicholiniums (e.g. *16*) (Schueler, 1955), which possess morpholine rings in which the nitrogen and oxygen atoms are constrained in a steric relationship akin to that pertaining in conformation (*4*) of acetylcholine, do not act at the myoneural synaptic receptors in the same manner as other muscle relaxants, but exert their effect by

preventing the synthesis of acetylcholine (Reitzel & Long, 1959). This could perhaps be taken as evidence that conformation (*4*) is not accepted by the nicotinic receptors. Similarly a number of morpholine and isoxazolidine compounds (Eugster, Haffliger & others, 1958) exhibit high muscarinic activity (Waser, 1961a) although in this case the situation is complicated by the simultaneous presence of a degree of nicotinic activity. Again a series of morpholinium compounds were two to three times less potent than the corresponding piperidinium compounds as neuromuscular blocking agents (Donahoe, Seiwald & others, 1957, 1961).

That a three point or multipoint attachment is not involved in the production of an acetylcholine-like response at the nicotinic receptors would seem to be strongly indicated by the high nicotinic activity of both enantiomorphs of lactoylcholine (*17*), muscarone (*18*), nicotine (*19*, R = Me), and nornicotine (*19*, R = H). This clearly demonstrates that the three point projected asymmetry associated with optical isomerism (compare Beckett, 1959) is of little significance at nicotinic sites. Moreover it is impossible for the muscarones to achieve a three point correspondence with any conformation of acetylcholine as is apparent from inspection of molecular models.



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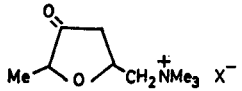

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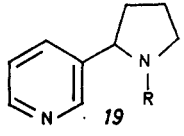

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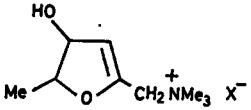

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Conformational isomerism in the acetylcholine molecule may indeed be an important factor in determining its ease of access to a sterically protected anionic receptor where only a one point attachment is involved, but the operation of such a one point attachment does not provide an interpretation of nicotinic activity in other molecules, through the absence of a second point of reference. However the muscarinic receptor, in contrast to the nicotinic receptor, is obviously highly stereoselective as evidenced by the pronounced differences in activity of L(+)- and D(−)-muscarine (Hardeggar & Lohse, 1957; Gyermek & Unna, 1958), of the enantiomorphs of acetyl-β-methylcholine (Beckett & others, 1961, 1963), of 4,5-dehydromuscarine (*20*) (Gyermek & Unna, 1960), and *cis*-2-methyl-4-trimethylammonium-methyl-1,3-dioxolane iodide (*1*) (Belleau & Puranen, 1963; Belleau & Lacasse, 1964), and by the marked effect of the size of the acyl group of the acylcholines upon affinity for the muscarinic receptor (Chang & Gaddum, 1933; Rossum, 1963). The stereoselectivity of the muscarinic site has been further emphasized in various attempts to give a pictorial representation of the receptor (Beckett & others, 1961, 1963; Waser, 1961a; Belleau & Puranen, 1963) which in no way invalidate conformational considerations.

CONFORMATIONALLY RIGID ANALOGUES OF ACETYLCHOLINE

It would appear, in the light of the preceding discussion, that a comprehensive study of conformationally rigid acetylcholine-like molecules in which the quaternary nitrogen atom and the acetoxy group are spatially held in mimicry of *2* and *4* with respect to muscarinic activity, is more likely to yield definite information about the role of conformational isomerism in determining the biological actions of acetylcholine than is any study of nicotinic activity, although the situation is complicated by the possibility that two acetylcholine molecules might react with one receptor (Turpajev & others, 1963). The appropriate absence of nicotinic activity might give supporting evidence provided that unfavourable solubility, permeability, transport or biotransformation phenomena do not prevent such drugs reaching the receptors, and provided that Gill's (1959) contention that conformationally rigid molecules will prove inactive is unfounded.

Few such rigid molecules have so far been examined. The 2-acetoxytropane methiodides (*8* and *9*) examined by Archer & others (1962) are conformationally non-rigid and the four racemic isomeric 2-acetoxy-3-trimethylammonium-*trans*-decalin halides (*10a-d*), although conformationally restricted, can exist in a variety of double boat and boat-chair conformations as well as in the double chair conformations shown. Baldridge, McCarville & Friess (1955) have prepared and tested the racemic forms of the stereoisomeric *cis* and *trans* 2-acetoxycyclohexyltrimethylammonium iodides (*21* and *22* respectively). Fig. 2 shows the theoretically possible extreme conformations of the *cis*-cyclohexane derivative (*21*) and Fig. 3 those of the *trans* isomer (*22*). It is to be noted that in both the *cis* and *trans* isomers of 1-acetoxy-2-trimethylammonium cyclohexane there is a form in which there is a $+60°$ projection angle between the quaternary nitrogen and the acetoxy oxygen atom with the $\overset{+}{N}$–O intergroup distance being 2·94 Å. Unfortunately compounds *21* and *22*, although tested for their ability to function as substrates for acetylcholinesterase and for their activity on the kitten phrenic nerve diaphragm preparation (Standaert & Friess, 1960), have not been investigated for muscarinic actions (S. L. Friess, personal communication). Schueler (1956) prepared and tested the two cyclic analogues *23* (which is optically inactive) and *24* (as the racemate). Compounds *23* and *24* appear to exhibit comparable muscarinic activity but are less potent than acetylcholine. The theoretically possible extreme conformations of the *NN*-dimethyl-3-oxomorpholinium ion (*23*) and of the *NN*-dimethyl-3-acetoxypiperidinium ion (*24*) are shown in Figs 4 and 5 respectively. Since none of the compounds *21–24* approximate to true rigidity they are therefore of little value in delineating the possible role of conformational isomerism at the different sites of action of acetylcholine. The racemic *cis* and *trans* 2-acetoxycyclopentyltrimethylammonium iodides (*25* and *26* respectively) (Friess & Baldridge, 1956) are, however, more nearly rigid, as the cyclopentane ring permits of only limited conformational isomerism (Pitzer & Donath, 1959; Brutcher, Roberts & others, 1959; McCullough, Pennington & others, 1959). Fig. 6 shows an approximate Newman

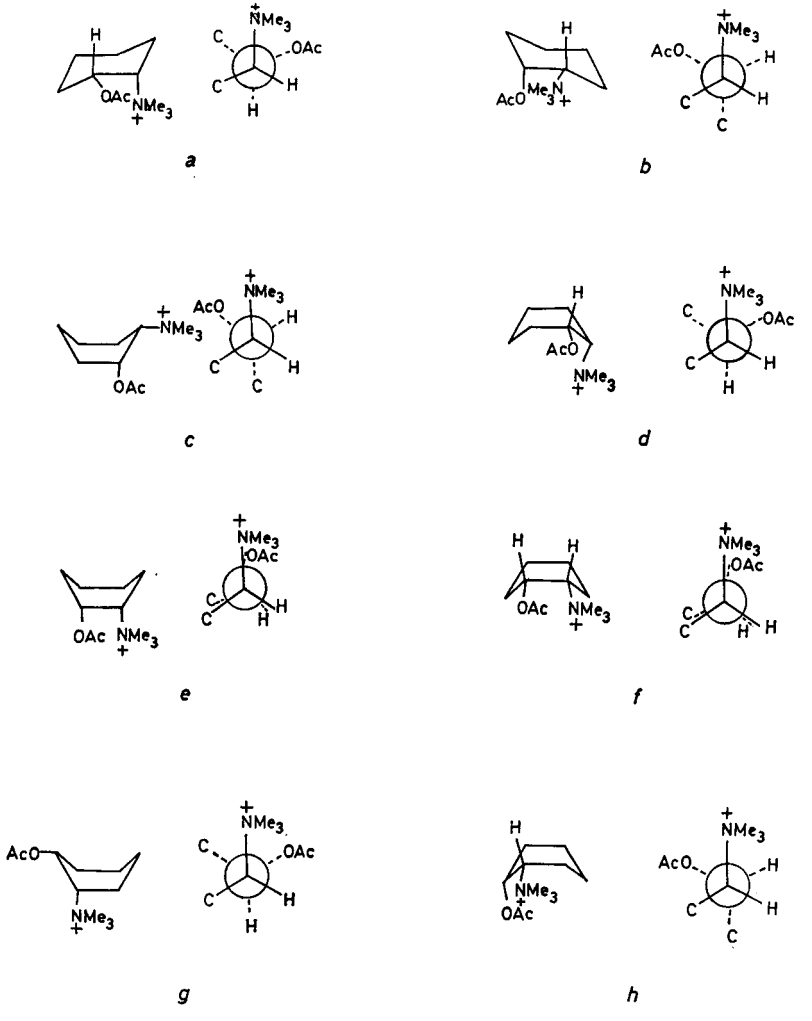

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FIG. 2. Theoretically possible extreme conformations of *cis*-1-acetoxy-2-trimethylammonium cyclohexane. (One optical enantiomorph only is shown.)

(1956) projection formula for one of the optical enantiomorphs of each of the *cis* (*25*) and *trans* (*26*) cyclopentane compounds. In these compounds none of the possible conformers with their partial eclipsing can be expected to correspond exactly to *2* although *25* is very close to *4*. Unfortunately compound *25* has not given as much information as could have been desired since it was prepared solely as the racemate and, moreover, was tested only for its ability to suffer hydrolysis by acetylcholinesterase when it was found to be more effectively hydrolysed than the corresponding *trans* racemate (*26*) (Friess & Baldridge, 1956). To secure

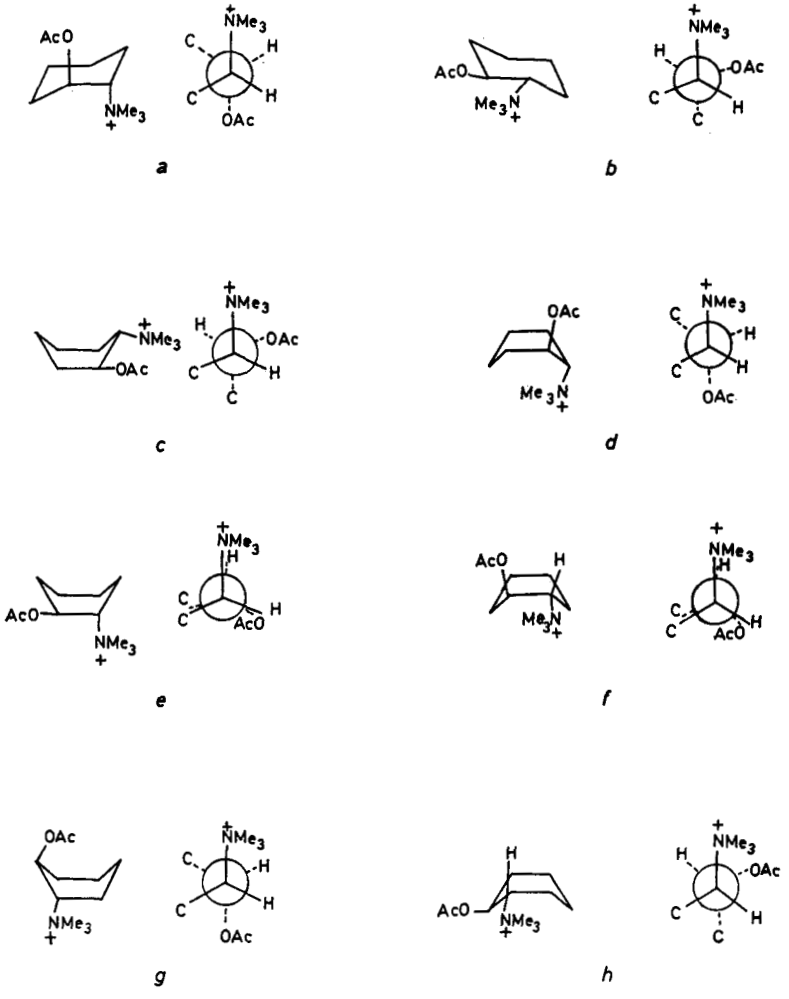

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FIG. 3. Theoretically possible extreme conformations of *trans*-1-acetoxy-2-trimethylammonium cyclohexane. (One optical enantiomorph only is shown.)

meaningful information compounds *25* and *26* should be subjected to pharmacological screening at both nicotinic and muscarinic sites, in the form of each separate pure enantiomorph.

THE POSSIBLE INVOLVEMENT OF INTRAMOLECULAR NC–H - - - - O HYDROGEN BONDING IN DETERMINING THE MUSCARINIC ACTIVITY OF ACETYLCHOLINE AND OTHER COMPOUNDS

Further support that *2* may be the nicotinic conformation and *4* the muscarinic conformation of acetylcholine could be advanced if it could be established conclusively that the stability of the quasi-ring conformations

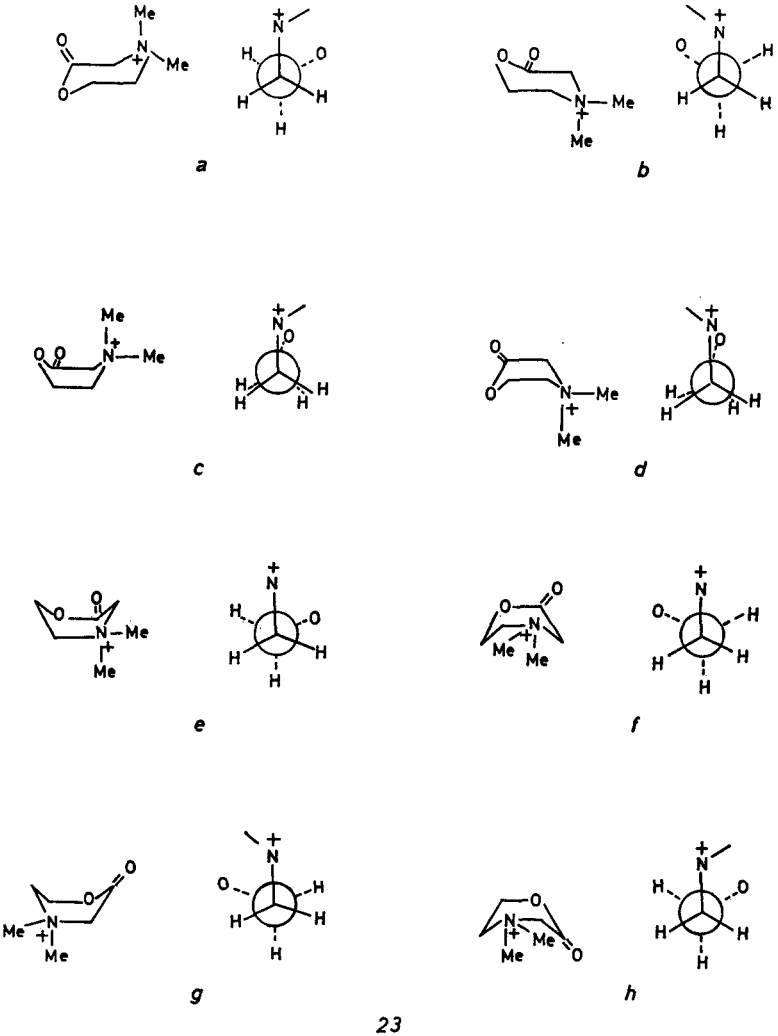

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FIG. 4. Theoretically possible extreme conformations of the *NN*-dimethyl-3-oxomorpholium ion.

of acetylcholine (*4*) and muscarine (*6*) has its origin either in intramolecular coulombic attraction between the quaternary nitrogen and ether oxygen atoms or in intramolecular NC–H - - - - O bonding as suggested by Sutor (1962, 1963) since factors tending to reduce the electron density on the ether oxygen atom (or its equivalent)—and hence also to inhibit hydrogen bonding—should then tend to favour nicotinic properties, whilst factors tending to increase electron density at this point, and so favour quasi-ring formation, should tend to favour muscarinic activity.

Comparative infrared studies (Fellman & Fujita, 1962, 1963, 1965, 1966)

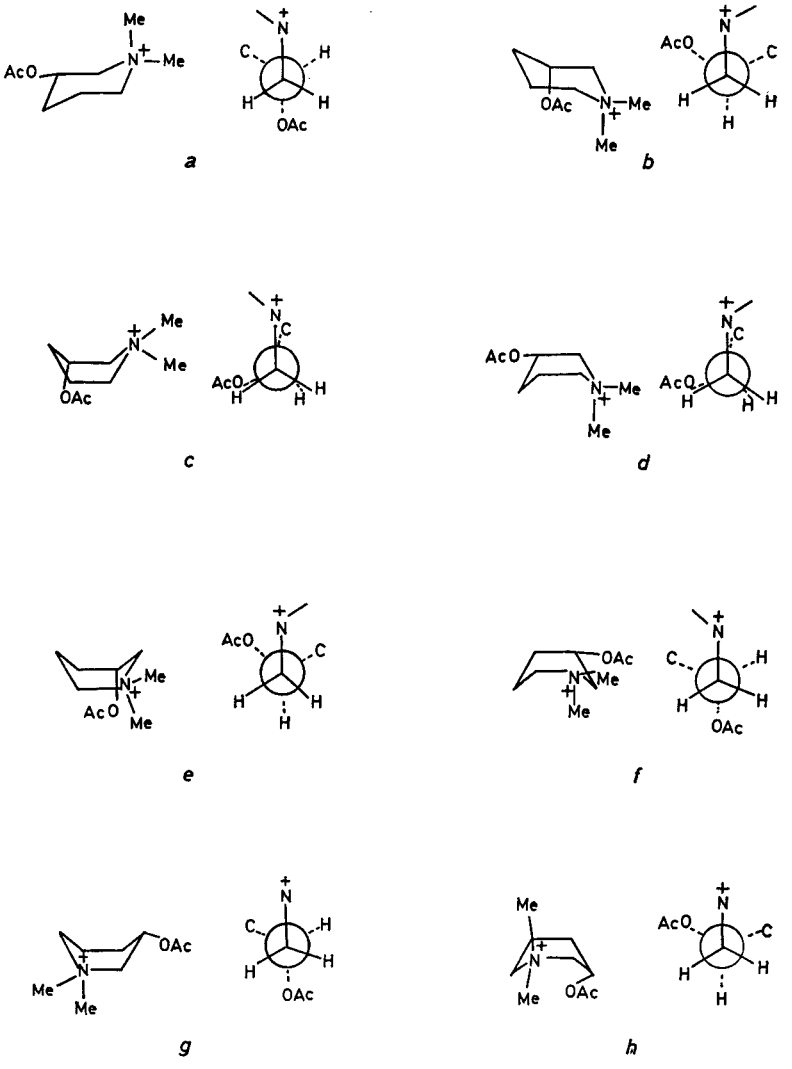

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FIG. 5. Theoretically possible extreme conformations of the *NN*-dimethyl-3-acetoxypiperidinium ion. (One optical enantiomorph only is shown.)

and kinetic investigations showing a high electrophilicity for the ester carbonyl carbon atom in acetylcholine (Butterworth, Eley & Stone, 1953; Fellman & Fujita, 1962) have been interpreted in terms of the influence of the inductive effect from the quaternary ammonium group (*27*) although this interpretation has been challenged (Canepa & Mooney, 1965). At the same time the later results of Fellman & Fujita (1963, 1965, 1966), while disproving the existence of the previously postulated conformation

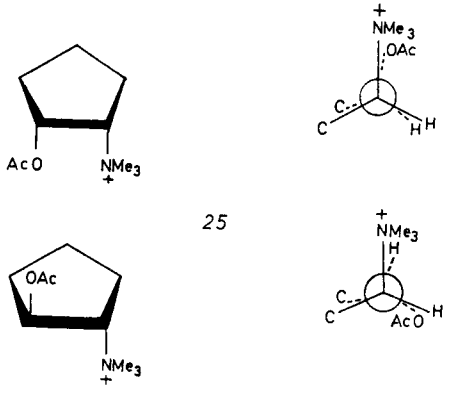
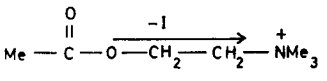


FIG. 6. Newman projection formulae of the *cis*- and *trans*-1-acetoxy-2-trimethylammonium cyclopentones. (One optical enantiomorph only is shown.)

(*28*) (Fellman & Fujita, 1962) which would result in a decreased carbonyl double bond character with consequent lowering of the stretching frequency (Jones & Sandorfy, 1956), would not be incompatible with the existence in solution of conformation *29* of acetylcholine, provided the stability of this conformation does result from intramolecular NC–H - - - - O bonding (Fellman & Fujita, 1966; Martin-Smith, Smail & Stenlake, 1967). In *29* electron withdrawal from the carbonyl group occurs *via* the acyloxy oxygen atom which would serve to depress the permanent polarization of the carbonyl group with consequent rise in its absorption frequency (Henbest & Lovell, 1957; West, Korst & Johnson, 1960; Bruice & Fife, 1962; Biggins, Cairns & others, 1963).

The proposed NC–H - - - - O bonding (e.g. *29* and *30*) could perhaps best be rationalized in terms of the inductive effects shown in *29*. The



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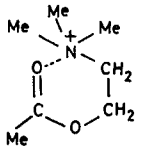

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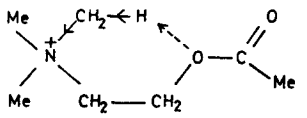

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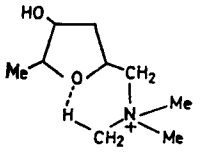

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primary C–N inductive effect is unexceptional and is even paralleled in amines, other than quaternary salts, where electronegativity differences

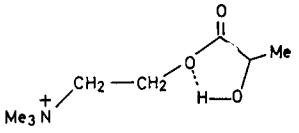
alone are operating, e.g. the increase in nucleophilicity of the nitrogen atom on replacement of the hydrogen atoms in the ammonia molecule by one and then two methyl groups (Sykes, 1961). The weakening of the NC–H bond responsible for the hydrogen bonding would also seem to be a normal phenomenon in *N*-methyl compounds, as is perhaps indicated by the low *N*-methyl C–H stretching frequency in the infrared spectrum of these compounds (Cross, 1960) and by analogy with ylide formation (Cope, LeBel & others, 1961). Relevant to the proposed N–C–H - - - - O hydrogen bonding are the results of an nmr study of *o*-fluoro-*NN*-dimethylbenzamide and *o*-fluoro-*N*-cyclohexyl-*N*-methylbenzamide (Lewin 1964) which would not be incompatible with N–C–H - - - - F hydrogen bonding.

Since sulphur and selenium are less electronegative than oxygen (Pauling, 1944), and do not as readily enter hydrogen bonding or possess the same high point electron charge density as does oxygen, it is instructive to compare the acetylcholine-like properties of the sulphur and selenium isosteres of such compounds as acetylcholine, acetyl-β-methylcholine and muscarine, in which quasi-ring conformations analogous to *29* and *30* are less likely to be favoured, with those of their prototypes. Indeed acetylthiocholine and acetylselenocholine are reported to be weaker muscarinic agents and stronger nicotinic agents than acetylcholine (Renshaw, Dreisbach & others, 1938; Günther & Mautner, 1963) whilst thiomuscarine gives rise to a decrease in muscarinic activity without the production of any marked nicotinic properties (Waser, 1961a). In the latter case steric hindrance similar to that which must pertain for acetyl-β-methylcholine would readily explain the absence of nicotinic potency, although anomalies exist in acetyl-β-methylthiocholine (Renshaw & others, 1938) and in thiomuscarone (Waser, 1961a,b) which show nicotinic activity.

These considerations might thus be taken as suggesting that conformation *2* could be the nicotinic conformer of acetylcholine, a view reinforced by the virtual absence of muscarinic activity in the (+) and (−)-lactoylcholines (*17*) (Rama-Sastry & Auditore, 1963) where hydrogen bonding from the hydroxyl group to the acyloxy oxygen atom (*31*) would stabilize the extended conformer. Further instances of expected destabilization of quasi-ring conformations akin to *4* may be sought in compounds where the thermodynamically favoured six-membered ring present in *29* is not possible or where the electron density on the acyloxy oxygen atom is decreased. The former situation is encountered in higher homologues of acetylcholine possessing more than two methylene groups between the trimethylammonium group and the acetoxy function, and indeed such compounds show markedly reduced muscarinic properties (Barlow, 1964). With respect to the second situation it is interesting to note that stress has been laid by other workers (Beckett & others, 1961, 1963) on the importance of the ether oxygen of the muscarine molecule in terms of a primary binding site in the receptor, whereas the function of this atom with its high electron density could in fact be more concerned in conformational stabilization. Decreased electron density on this atom with

consequent increase in favourability of an open chain conformer would be expected in benzoylcholine, where the benzene ring can act as an electron sink, and in methanesulphonylcholine (*32*) and in fact these compounds show little or no muscarinic activity (Akcasu & others, 1952; Eckhardt & Schueler, 1963). However, the concurrent operation of steric factors cannot be overlooked, as is clear from the pronounced nicotinic properties of trimethylacetylcholine (*33*) (Bergel, 1951) where the inductive effect is in the opposite direction.

The appearance of nicotinic as well as muscarinic activity in the muscarones (e.g. *34*) can perhaps also be attributed to an increased favourability of the extended conformation as compared to the situation in muscarine and it will be interesting to learn whether an X-ray study of crystalline



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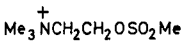

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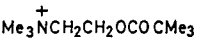

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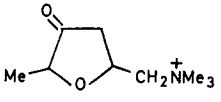

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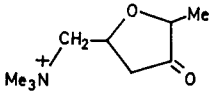

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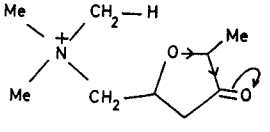

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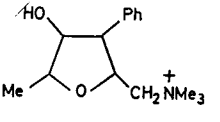

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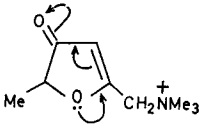

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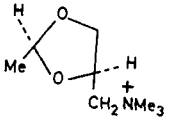
muscarone iodide will demonstrate the existence of such a conformation (*34a*) rather than a quasi-ring conformation (*6*) as is characteristic of muscarine iodide (Jellinek, 1957). If indeed stabilization of the quasi-ring conformation of muscarine is due to intramolecular NC–H - - - - O hydrogen bonding (Sutor, 1962, 1963) then the polarization of the oxo group in muscarone could perhaps destabilize the quasi-ring form through

relayed inductive effects (*35*) but complexities are introduced by the possibility that muscarone could interact with the receptor in its enol form (Waser, 1961a). Similar relayed inductive effects might explain the dual muscarinic and nicotinic properties of 3-phenylmuscarine (*36*) in which a delicate balance between the extended and quasi-ring conformers can readily be envisaged, but once again such electronic considerations cannot be divorced from steric arguments (Waser, 1961a; Beckett & others, 1963; Belleau & Puranen, 1963). With 4,5-dehydromuscarine (*20*) resonance between the π-electrons of the double bond and the p-electrons of the furan oxygen would serve to lower electron density on the latter, again explaining the dual nicotinic and muscarinic properties in terms of virtually equal favourability of an extended conformer and a quasi-ring conformer. The extended conjugation present in 4,5-dehydromuscarone (*37*) would presumably have a similar effect.

On the other hand, factors tending to increase the electron density of the acetoxy oxygen atom of acetylcholine should favour the quasi-ring conformation (*4*) if it is due to coulombic attractions or hydrogen bonding of the types discussed. Such a situation would be expected to pertain in choline ethyl ether (Simonart, 1932) and β-methylcholine ethyl ether (Holton & Ing, 1949) which are potent muscarinic agents. This is in contrast to the aryl ethers of choline in which the electron density on the ether oxygen will be decreased and which are well established as nicotinic agents (Hey, 1949, 1952; Fukui, Chikayoshi & Akira, 1960). The pronounced muscarinic activity of certain ketals (Fourneau, Bovet & others, 1944; Triggle & Belleau, 1962) could also result from the possibility of forming six-membered quasi-rings similar to *4*. Thus, in the 1,3-dioxolane series (Belleau & Lacasse, 1964) where the most active compound was L(+)-*cis*-2-methyl-5-trimethylammonium-methyl-1,3-dioxolane iodide (*38*) which contains only the equivalent of the $MeCOOCH_2$-fragment of acetylcholine locked in rigid conformation, the stereochemical and configurational specificity of this compound is still in accord with the possible existence of the quasi-ring conformation (38*a*).

Sekul & Holland (1961a,b; 1963a,b) have suggested that the activity of muscarinic compounds depends upon a fractional positive charge in the position corresponding approximately to the acetoxy oxygen atom of acetylcholine whereas nicotinic activity requires a fractional negative charge on the carbonyl oxygen atom. This hypothesis with regard to muscarinic activity is certainly in agreement with the concept of NC–H - - - - O hydrogen bonding since, although hydrogen bonding is favoured by increased electron density on the ether oxygen atom, a fractional positive charge could well arise on this atom after the hydrogen bond has formed. In support of their contention Sekul & Holland (1961a,b; 1963a,b) advance the facts that methoxymethylcholine ether (*39*) and propargylcholine ether (*40*) are twice as active as muscarinic agents as the propenyl (*41*) and propyl (*42*) analogues. However, interpretation of these results suffers from the disadvantage that there is no proof that the methoxyl group of *39* and the acetylenic group of *40* are in fact acting as electron withdrawing substituents. A consideration of the primary and

secondary inductive effects in the four choline ethers allows an alternative interpretation of these results. The $-CH_2CH_2\overset{+}{N}Me_3$ moiety is common to all four ethers and therefore its influence on the comparative electron density on the oxygen atom of the ethers may reasonably be ignored. The other primary inductive effects in methoxymethylcholine ether are shown in *39a* and the secondary inductive effects in *39b*. Although no quantitative assessment of electronic effects is possible, the electron density



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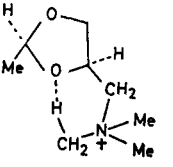

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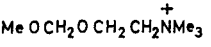

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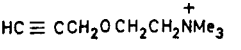

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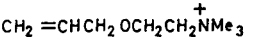

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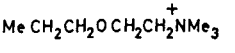

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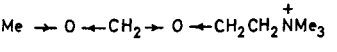
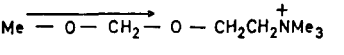
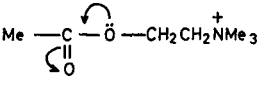


39a



39b



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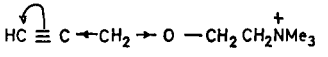

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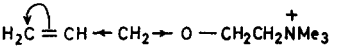

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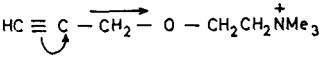

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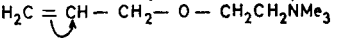

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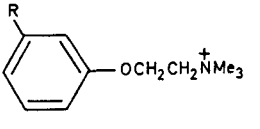


43

on the ether oxygen of methoxymethylcholine ether would *a priori* be expected to be greater than that on the acetoxy oxygen atom of acetylcholine for two main reasons.

(i) Electron withdrawal from the "ether" oxygen is greater in acetylcholine than in methoxymethylcholine ether due to the stronger polarization of the carbonyl group (*39c*) as compared to the weak inductive effect of the methoxymethylene function in *39a*.

(ii) The secondary inductive effect (*39b*) partially restores electron density on the ether oxygen atom of the choline ether.

The lower muscarinic activity of methoxymethylcholine ether as compared to acetylcholine may be rationalized on the basis of the differing stereochemical requirements of the methoxymethylene and acetyl groups respectively.

The primary inductive effects in the propargyl- and propenyl-choline ethers are shown in *40a* and *41a* respectively and the respective secondary inductive effects in *40b* and *41b*. In both of these compounds the secondary inductive effects probably outweigh the primary inductive effects as is perhaps evidenced by the anti-Markownikoff addition of hydrogen halides to allylic alcohols (e.g. Finar, 1959). Once again the secondary inductive effect aids in the restoration of electron density on the ether oxygen atom. The greater muscarinic activity of the propargyl ether (*40*) when compared to the propenyl analogue (*41*) may be on account of the greater mobility of electrons in the acetylenic system.

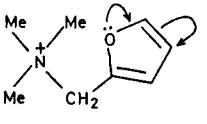
In the propyl ether (*42*) the primary and secondary inductive effects operate in the same direction. This compound would thus be expected to exhibit muscarinic properties (on the basis of NC–H - - - - O hydrogen bond formation) but perhaps weaker than those of *39*, *40* and *41* due to the relatively small inductive effect associated with alkyl groups as shown, for example, by certain infrared studies (Flett, 1957; Stone & Thompson, 1957; Brown, Eglinton & Martin-Smith, 1962).

Again the results obtained from a series of substituted choline phenyl ethers (*43*, R = I,Br,Cl,F,NH_2,H,NO_2), in which the order of nicotine-like stimulant action (*43*, R = I>NH_2>Br>Cl>F>H>NO_2) was the reverse of that expected by Hey's (1949) prediction (Coleman, Hume & Holland, 1965), are not above unambiguous interpretation. The complete delocalization present in the aromatic system makes it difficult to separate the various polarization and polarizability effects (Ingold, 1953) especially when the substituent is in the 3-position. Furthermore, even if electromeric effects could be disregarded, the influence of purely inductive effects on the ether oxygen atom instigated by substituent R (in *43*) must be practically non-existent being three carbon atoms from the oxygen atom. Further objections to the hypothesis of Sekul & Holland (1961a,b, 1963a,b) have been raised by Barlow (1964).

If the stability of quasi-ring conformations akin to *4* is crucially influenced by quite minor changes in the electron density of the ether oxygen atom it becomes possible to reinterpret the Five-Atom Rule (Ing, 1949; Alles & Knoefel, 1939; Ing, Kordik & Tudor-Williams, 1952). For

instance, as Beckett & others (1961) have proposed, the pronounced differences in muscarinic potency between furfuryltrimethylammonium (*44*) and 5-methylfurfuryltrimethylammonium (*45*) could be explained on the grounds that the inductive effect of the 5-methyl substituent in the latter may induce a crucial restoration of electron density on the furan oxygen (Acheson, 1960) which in the former compound will be depleted through resonance interaction with the furan ring. The net result is restoration of the stability of the quasi-ring conformation.

The effect of the proposed NC–H - - - - O hydrogen bonding is to constrain the acetylcholine molecule in the quasi-ring conformation (*4*)



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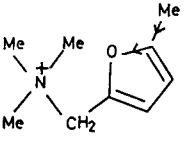

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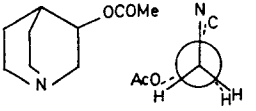

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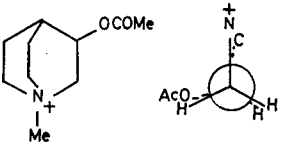

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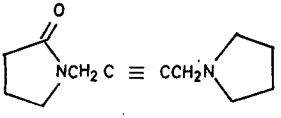

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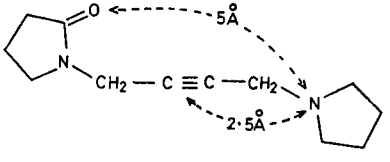

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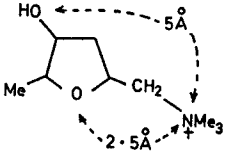

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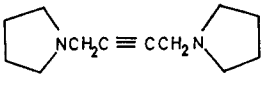
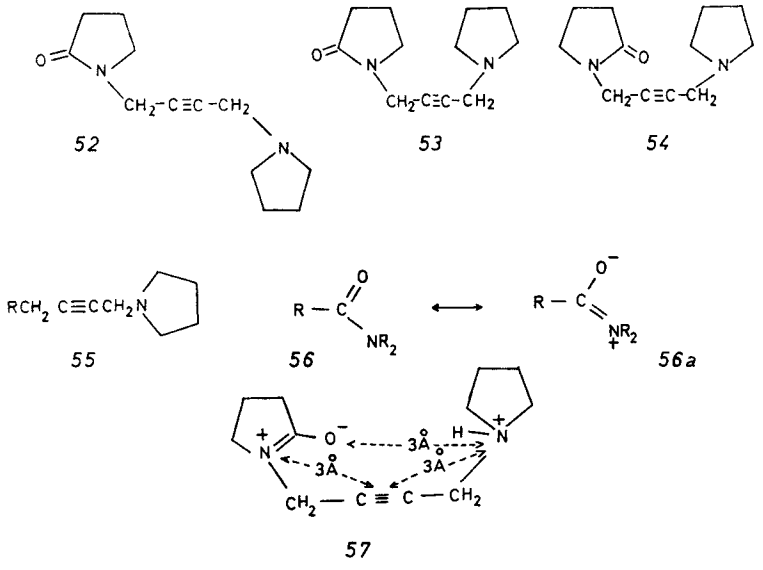


51

in which the N–O acyloxy distance approaches 3·2 Å. This consideration is important with respect to certain quinuclidine derivatives. Thus, 3-acetoxyquinuclidine (*46*) shows high muscarinic activity and almost complete absence of nicotinic properties (Mashkovsky, 1963) and 3-acetoxyquinuclidine methiodide (*47*) has been used to show that the enzyme acetylcholinesterase must favour a transoid conformation of acetylcholine with a similar disposition of the quaternary nitrogen atom and acetoxy group to that which pertains in the rigid quinuclidine salt (Solter, 1965). However, despite this transoid arrangement the N–O acetoxy distance in both *46* and *47* is *ca* 3·4 Å which is in close agreement with the N–O distance in the *gauche* conformer (*4* and *7*) of acetylcholine. In the former the rigid quinuclidine ring system constrains the functional groups in the requisite position whereas in the latter NC–H - - - - O hydrogen bonding might reasonably be supposed to do the same.

The tertiary acetylenic amine, oxotremorine (*48*), has been shown (Cho, Haslett & Jenden, 1962) to be a potent muscarinic agent devoid of nicotinic activity and on the assumption that this compound was acting directly and not via acetylcholine release it was proposed that the high activity could best be explained on the basis of the Koshland (1959) "induced fit" theory. Bebbington & Brimblecombe (1965, 1966) consider from an examination of molecular models that in the planar transoid form (*49*) the distance between possible active centres coincides with that of L(+)-muscarine (*50*). Thus, if the pyrrolidine nitrogen atom is protonated at physiological pH (Cho & others, 1962) then the acetylenic linkage of *49* coincides with the furan oxygen of *50* as a centre of high electron density and the carbonyl oxygen is considered to interact with site 3 (Beckett & others, 1961) of the muscarinic receptor. That the carbonyl group of oxotremorine plays some vital role in its muscarinic activiy would seem to be strongly indicated by the complete absence of any comparable activity in tremorine (*51*) itself (Cho & others, 1962). Although the planar transoid form (*49*) of oxotremorine was chosen by Bebbington & Brimblecombe (1965, 1966) there seems little reason to exclude the other planar transoid form (*52*) or the two planar cisoid forms (*53* and *54*), but in either *52* or *53* the carbonyl oxygen of the pyrrolidone moiety, being some 8 Å from the pyrrolidine nitrogen atom, can no longer be involved in receptor interaction of the type envisaged by these authors. It has further been shown (Bebbington & Brimblecome, 1965, 1966) that within a series of oxotremorine analogues (*55*, R a group containing amide, ester or ketone functions) muscarinic activity was inversely proportional to the infrared frequency of the carbonyl absorption and thus amides, which absorb at lower frequencies, had much greater activity than related esters or ketones. It is well known that the lower frequency of amide carbonyl absorption as compared to esters or ketones is attributable to the greater single bond character of the carbonyl group (Jones & Sandorfy, 1956) and therefore a substantial contribution from structures analogous to *56a* is implied. Application of these arguments to oxotremorine makes it not inconceivable that the molecule exists substantially in the form *57* assuming protonation of the pyrrolidine nitrogen atom at



physiological pH. In this structure there are now the equivalent of two positively charged nitrogen atoms at *ca* 3 Å from two centres of high electron density which again compares favourably with the N–O acetoxy distance in the quasi-ring form of acetylcholine (*4*). Hydrogen bonding between the protonated pyrrolidine nitrogen atom and the negatively charged oxo function of the pyrrolinium group could reasonably be considered as the force serving to constrain the molecule in what, *a priori*, would be considered an unfavoured conformation but what in fact may be a receptor favoured conformation. There is no conformation of oxotremorine complementary to the extended conformation (*2*) of acetylcholine thereby rationalizing the absence of nicotinic activity in the former and, at the same time, lending credence to the view that conformation *2* of acetylcholine is involved at the nicotinic sites.

These arguments seem to indicate that there are at least theoretical grounds for the involvement of conformational isomerism in the biological actions of acetylcholine. The supposition, however, that the fully staggered conformation and the quasi-ring conformation are actually involved at the receptors would be considerably strengthened if it were demonstrated that both are simultaneously present in solution. Studies toward this end have already been instigated (Martin-Smith & others, 1967; Fellman & Fujita, 1966) and future work may provide definitive evidence on the role of conformational isomerism at the cholinergic receptor. Valuable information should also be forthcoming from comparative pharmacological studies with further fully conformationally rigid molecules designed as analogues of various extreme conformations of acetylcholine. Such compounds should be prepared in both enantiomorphic forms, where molecular asymmetry exists, in view of the marked

stereoselectivity of the muscarinic site (Beckett & others, 1961, 1963; Waser, 1961a; Belleau & Puranen, 1963).

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