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A Theoretical and Experimental Study of the Semirigid Cholinergic Agonist 3-Acetoxyquinuclidine

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SUMMARY

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The interaction of the two enantiomers of 3-acetoxyquinuclidine (3-AcQ) and of its *N*-methyl derivative with the cholinergic receptor and with cholinesterases was investigated experimentally in systems which respond to acetylcholine (ACh). Molecular structural factors which are conducive to the well-defined ACh-like activity were studied by quantum mechanical methods. The flexible moiety in the semirigid structure of 3-AcQ was shown to permit molecular rearrangements, as required for receptor activation, and to adopt an ACh-like conformation in the energetically preferred geometry. The interaction pharmacophore of the active species, defined by the electrostatic potential fields generated in their surroundings, revealed a reactivity pattern identical with that of ACh. The interaction pharmacophore of the *N*-methyl derivative of 3-AcQ was shown to be much less compatible with the requirements for ACh-like activity. The structural correlation between the ACh-like pattern of the drugs and the biological activity of related psychotropic-anticholinergic amino esters is discussed.

INTRODUCTION

The activity of both agonists and competitive antagonists of acetylcholine is considered to be based on a specific interaction of some functional groups with subsites of the receptor macromolecule (1). The structural relationship between the natural neurotransmitter ACh[6] and semirigid agonist molecules should therefore be of special interest for the mapping of these subsites on the receptor. Studies of the

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[6] The abbreviations used are: ACh, acetylcholine; BuCh, butyrylcholine; 3-AcQ, 3-acetoxyquinuclidine; 3-BuQ, 3-butyroxyquinculidine; SCF-MO, self consistent field molecular-orbitals; INDO, intermediate neglect of differential overlap; CNDO, complete neglect of differential overlap; LCAO-MO, linear combination of atomic orbitals-molecular orbitals.

structure-activity relationships of active molecules (2) and experimental and theoretical approaches to the investigation of the molecular conformation of such compounds (3–7) provide powerful tools for comparative analysis of the structural factors involved in drug-receptor interactions. The semirigid structure of 3-acetoxyquinuclidine, which embodies all the functional groups of ACh, presents an interesting case in which some of these groups are held at fixed relative positions (Fig. 1.) In view of experimental evidence for its ACh-like activity (8, 9) it is pertinent to compare the conformation adopted by the flexible parts of this molecule with that of the more flexible natural neurotransmitter ACh and to evaluate the possible interaction patterns of the two molecules with the receptor. The reactivity of the molecular species studied here and the characteristics of their interaction with the receptor are discussed in terms of the electrostatic potential fields generated by these molecules in their surrounding space. These fields are describable as potential maps and have been considered to provide an "interaction pharmacophore" (1, 10) for a class of cholinergic antagonists. Comparative analysis of such electrostatic potential maps obtained for ACh and the agonists studied here leads to rationalization of the so far unexplained differences between the ACh-like activity of (+)-3-AcQ and its *N*-methyl derivative. The conclusions reached on this basis are compatible with other findings from structure-activity studies and predict similar relationships between the protonated and *N*-methylated forms of other heterocyclic agonists of ACh (11).

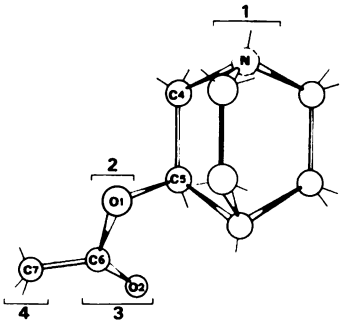


FIG. 1. *Acetylcholine-like functional groups in semirigid structure of 3-acetoxyquinuclidine*

1, "cationic head" surrounding the nitrogen atom; *2*, ester oxygen; *3*, carbonyl group; *4*, methyl group.

In direct relation to this quantum mechanical analysis of the reactivity patterns, we report the results of a detailed experimental investigation of the ACh-like activity of 3-AcQ. The ACh-like activity of the two enantiomers of 3-AcQ and of its *N*-methyl derivative was studied through the contraction responses of (a) guinea pig ileum, a smooth muscle preparation, and (b) the pupil of the intact eye of the mouse. The interaction of the compounds with cholinesterases was studied *in vitro* with acetylcholinesterase and butyrylcholinesterase.

METHODS AND PROCEDURES

Computation Methods

The conformation mapping of 3-AcQ was obtained from all-valence electron SCF-MO calculations, using the INDO approximation. This represents a standard procedure for semiempirical LCAO-MO calculation, which has the advantage of being amply described and documented (12), thus providing a good basis for a comparative study. The computations were performed with the CNINDO program (13), which was modified slightly to avoid divergence in the iterations (14).

The reactivity pattern of the molecules was studied from maps of the electrostatic potential generated by the species in their surroundings. The values of the potential $\phi(\bar{R}_0)$ at each point R_0 include the contributions from all the nuclei and from the electron charge distribution,

$$\phi(\bar{R}_0) = -\int \frac{\rho_1(\bar{r})}{|\bar{r} - \bar{R}_0|}\, d\bar{r} + \sum_{\alpha} \frac{Z\alpha}{|\bar{R}_\alpha - \bar{R}_0|} \qquad (1)$$

where $Z\alpha$ represents the charge of nucleus α [the "effective charge" in the case of INDO calculations (12)] and the charge distribution function $\rho_1(r)$ is defined as

$$\rho_1(r) = N\int \psi^*(r_1, r_2, \cdots r_n)\, \psi(r_1, r_2, r_3, \cdots r_n)\, dr_2, dr_3 \cdots dr_n \qquad (2)$$

The computations were carried out using

an analytical method described elsewhere (15) by which the potentials are obtained from a solution of the Poisson equation

$$\nabla^2\phi(\bar{R}_0) = -4\pi\rho(\bar{R}_0) \qquad (3)$$

which holds for the molecular potentials $\phi(\bar{R}_0)$.

When charge transfer and polarization terms are neglected, the value of the interaction energy of a molecular system with an attacking proton is obtained directly from Eq. 1. In fact, all maps of electrostatic potentials presented here are given in units of kilocalories per mole and represent that interaction energy. While the energy values are approximate, the maps of electrostatic potentials define, to the first order, the pattern of interaction energies "seen" by a proton approaching the molecule. Positive areas represent forbidden regions, while the most negative potentials indicate the most probable protonation sites. A hypothetical negative point charge which does not undergo exchange with the molecular electron distribution would interact with the molecule according to the same pattern, but with opposite signs (16). The predictive power of conclusions based on the pattern of electrostatic potentials is well established and documented for protonation reactions (17–19). The effects of short-range interactions, such as polarization, charge transfer, and geometrical deformations, were analyzed by direct calculations *ab initio* (20). These showed that none of the perturbations can reverse the predictions made for protonation of the peptide bond on the basis of electrostatic potentials, pertaining to the directions and distances of molecule-proton interactions. These predictions would be identical whether the potentials are calculated *ab initio* (21) or from INDO wave functions.[7]

Moreover, elaborately studied examples of interactions of water molecules with a variety of saturated, unsaturated, and heterocyclic small molecules, analyzed through electrostatic potentials and point charges (19, 22, 23), have shown that ionic type associations will follow very closely the locations and distances predictable from the electrostatic potential maps of the interacting molecules. Predictions made from electrostatic potential maps obtained by the method used here have also been shown to retain their validity upon interaction with the polarizing and hydrogen-bonding species HF, indicating again that the effects of short-range interactions may modify significantly the values of the interaction energy but that the geometrical pattern of the active regions remains essentially constant (24).

On this basis the potential maps generated by ACh are considered to represent the characteristic pattern by which the molecule interacts with the receptor, as generated by its active structural conformation. Analysis of the ability of certain rigid molecules to generate such a potential pattern has shown it to have predictive power for the activity or inactivity of ACh antagonists (1, 10). We therefore propose to consider this characteristic pattern of electrostatic potentials to represent the cholinergic "interaction pharmacophore," which confers activity on the molecule (25).

Experimental Procedure

Chemicals. The resolution of (±)-3-quinuclidinol (Aldrich) was carried out according to Kalir *et al.* (26) for the (+) isomer, and according to Starnbach and Kaiser (27) for the (−) isomer. The optical rotation $[\alpha]_D^{25}$ of the two isomers determined in 1 N HCl, $c=2.0$, was +46° and −46°, respectively. Acylation of these amino alcohols with either acetic or butyric anhydride was performed in pyridine according to Robinson *et al.* (28). Although the acylation of 3-quinuclidinol is stereospecific, Robinson *et al.* (28) reported that the product of acylation of (−)-quinuclidinol had $[\alpha]$ +28.5°; in our experiments the value of $[\alpha]$ was −16.3°. We did not find such discrepancies between enantiomer pairs. The apparent discrepancy could be due to the nature of the solvent in which $[\alpha]$ was measured. It is known that optical rotation may be pH-dependent; i.e., it may depend on the state of ionization of the compound. Robinson *et al.* (28) measured optical rotation in ethanol solution whereas our measurements were carried out in aqueous 1 N

[7] H. Weinstein, unpublished observations.

HCl. Thus the positive value of [α] measured by Robinson *et al.* may have misled them to assume that the AcQ obtained by acylation of (−)3-quinuclidinol was the (+) enantiomer. This would also explain the negative [α] value of the derivative obtained by these authors upon methylation; the derivative has a positively charged quaternary nitrogen and hence its optical rotation will be independent of the nature of the solvent. Indeed, the values of [α] for the *N*-methiodide derivatives of the two enantiomers of 3-AcQ obtained by Robinson *et al.* (28) are in agreement with our values. Hydrochloride and methiodide salts of the amino esters were prepared in anhydrous ether. The derivatives thus obtained are given in Table 1. Dimethylaminoethyl acetate was prepared according to published procedures (30, 31), and phencyclidine [1-(1-phenylcyclohexyl) piperidine], according to Kalir *et al.* (32). Arecoline, ACh, BuCh, and atropine were obtained from Aldrich Chemical Company. Isopropyl methylphosphonofluoridate (sarin) was a gift from Dr. H. Lider.

Enzymes. A hemolysate of fresh human red blood cells was used to test for acetylcholinesterase activity. This batch was free of any butyrylcholinesterase activity. Butyrylcholinesterase (horse serum) was obtained from Worthington.

Methods. Acetylcholinesterase activity was measured with a Radiometer pH-stat under nitrogen at pH 7.4 and 37° by a method utilizing two titrants (5 mM), according to Roufogalis and Thomas (33). The assay mixture (22.5–27.0 ml) contained acetylcholine (0.1–5 mM), 0.2 M NaCl, and 0.5 unit of enzyme. Butyrylcholinesterase activity was determined similarly: the 4.0-ml assay mixture contained the substrate (1.8–37.5 mM), phosphate buffer (5 mM), and 0.6–1.0 enzyme unit, with 0.02 N NaOH as a titrant. The rate of butyrylcholinesterase phosphorylation with sarin, in the presence of the substrate, was followed titrimetrically according to Main and Dautermann (34) and Volkova (35). The reaction mixture (10.7 ml) contained phosphate buffer (5 mM), enzyme (1.0 unit), sarin (0.2 μM), and various concentrations of the substrates.

The ACh-like activity of the drugs was evaluated in guinea pig ileum, using five or six pieces for each drug (36).

The activity of the drugs was evaluated by local application to a mouse eye which had been treated with the mild mydriatic phencyclidine as described by Treister *et al.* (37). Thus 20-sec contact with 10 mM phencyclidine (HCl) (pH 8.0, 0.1 M phosphate buffer) produced full mydriasis within 2 min. Three minutes after phency-

TABLE 1

Characterization and comparative optical rotation values of acetoxyquinuclidine derivatives

(+)- and (−)-3-Quinuclidinol were resolved according to Kalir *et al.* (26) and Starnbach and Kaiser (27), respectively. Acylation of the amino alcohol was carried out according to Robinson *et al.* (28). Hydrochlorides and methiodide salts of the amino esters were prepared in anhydrous ether.

Compound	b.p. (mm Hg) or m.p. (°C)	$[\alpha]_D^{25}$	Concentration	Solvent	Ref.
			g/100 ml		
(±)-3-AcQ	118–120				
(±)-3-AcQ·CH_3I	165				
(−)-3-AcQ	175	+28.5°	2.9	Ethanol	28
		+24.1°	2.5	H_2O	29
		−16.3°	1.5	1 N HCl	This report
(+)-3-AcQ	175	−10.7°	2.9	Ethanol	28
		+14.3°	1.5	1 N HCl	This report
(−)-3-AcQ·CH_3I	201	−11°	2.03	H_2O	28
		−11.5°	1.5	1 N HCl	This report
(+)-3-AcQ·CH_3I	201	+11°	1.9	H_2O	28
		+11.5°	1.5	1 N HCl	This report

clidine application, 1 drop of the test solution was applied to the same eye, and the pupil diameter was measured with binoculars (Nikon, × 20) every 30 sec for 10 min. During the observation period the eye was kept under strong illumination. Every drug at each concentration was tested on six mice. The results represent average values ± standard deviations.

RESULTS

Results of Computations

Conformation. Since the atomic sequence of the functional groups in the molecule of 3-AcQ is identical with that of ACh, the conformation may be specified in terms of the same dihedral angles used for the natural neurotransmitter (6): τ_0 (O_2—C_6—O_1—C_5), τ_1 (C_6—O_1—C_5—C_4), τ_2 (O_1—C_5—C_4—N), and τ_3 (C_5—C_4—N—C_3) (Fig. 1). It is now established both theoretically (6, 7, 38) and experimentally (3, 4, 39) that the energetically preferred values of τ_0 and τ_3 in ACh should be 0° and 180°, respectively. The cholinergic pharmacophore is therefore characterized, in ACh-like molecules, by the values of the two additional dihedral angles. From a detailed comparative study of the crystal structure of ACh-like agonists, Baker *et al.* (4) found the average values of these angles to be $\tau_1 = 180° \pm 35°$ and $\tau_2 = +73°$ to +137°. The usefulness of such crystallographically obtained values as criteria for ACh-like activity is limited to species with comparable structural frames forming the dihedral angles, and they should not be considered without analyzing other requirements which have been proved pertinent to ACh-like behavior [e.g., molecular regions which mimic some functional groups of ACh (5, 10) or certain flexibility requirements (24)].

In 3-AcQ the rotation angle τ_2 (O_1—C_5—C_4—N) may be considered to be fixed at a value around 120°, corresponding to a nearly tetrahedral hydridization of the carbon atoms in the piperidine moiety.[8] This fixes one of the basic distances in the pharmacore (5) presented by this molecule at the receptor. The other elements, consisting of the N—C_6, N—C_7, and N—O_2 (carbonyl) distances, depend on the value of the dihedral angle τ_1 (C_6—O_1—C_5—C_4), which has to be specified from the minimal energy conformation. The results obtained from INDO calculations of the energy of 3-AcQH$^+$ as a function of τ_1 are shown in Fig. 2. Minima are observed for τ_1 values around 60°, 165°, and −35° (325°). The molecular conformations corresponding to these values are given in Fig. 3, which also lists the net atomic charges. It becomes evident from an analysis of the conformations, using space-filling Corey-Pauling-Koltum models, that the unusual geometry corresponding to $\tau = -35°$ is unrealistic and has to be disregarded. The very steep minimum in the energy profile in Fig. 2 indicates that this conformation should be very unfavorable thermodynamically (38). Its appearance on the INDO energy surface may be attributed to the well-characterized "connectivity" property of INDO (43, 44), by which nonbonded interactions allow neighboring atoms an exaggerated approach.

The low energy barrier observed between the other two minima corresponds to the flat regions observed in the potential surface calculated by Beveridge and Radna (6, 38) for ACh at dihedral angle values of $\tau_1 = 90$–210° and $\tau_2 = 40$–60°. The identical methods and procedures used for this calculation of ACh and for the study of 3-AcQ presented here form a common basis for comparison of both the conformational results and the corresponding charge distributions. A comparison of the distance patterns between key atoms and groups in the two molecules is given in Table 2 for the related configurations [(60°, 180°) in ACh and (165°) in 3-AcQ] in an energy variation range of 0.5 kcal/mole. Likewise, an identical charge distribution pattern from the two molecules is suggested by a comparison of the net atomic charges in the related configurations, as shown in Figs. 3 and 4. Moreover, it is shown in Fig. 3a that both the minimum energy conformation and the net atomic charges remain almost identical for the

[8] Crystallographic studies have yielded a variety of values for this torsional angle in different quinuclidine structures: 125.7° (40), 111.8° (41), and 107.7° (42).

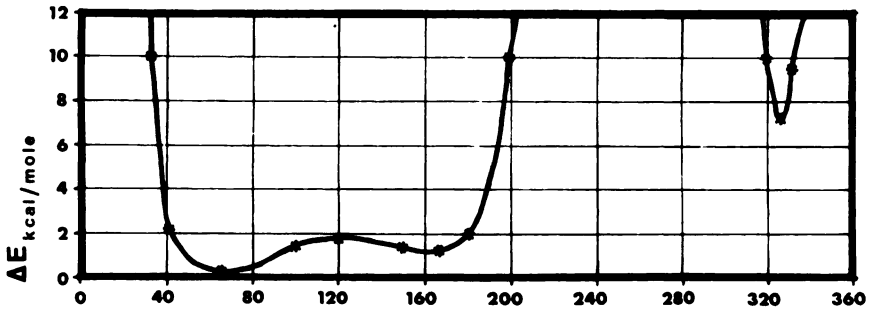


FIG. 2. *Variation of total energy with* τ_1 (C_6—O_1—C_5—C_4) *at fixed* τ_2 (O_1—C_5—C_4—N) = 120°

TABLE 2

Comparison of interatomic distance patterns in related configurations of 3-acetoxyquinuclidine (I) and acetylcholine (II)

Intervals are for configurations corresponding to energy differences of less than 0.5 kcal/mole near the corresponding local minimum of each molecule.

Structure	Molecule	N	N \ C_4—C_5	C_4 \ C_5—O
		A	*A*	*A*
—O⁄	I (135–180°)	3.46	2.42	1.46
	II (40–60°, 180°)	3.15 ± 0.15	2.52	1.50
=O	I	4.95 ± 0.10	4.00 ± 0.05	2.59
	II	5.02 ± 0.02	4.18	2.67
H \ H—C_7—C_6 / H	I	5.55 ± 0.15	4.80 ± 0.10	3.81
	II	5.35 ± 0.15	4.86	3.75
O=C_6⁄ O	I	4.55 ± 0.05	3.65 ± 0.03	2.37
	II	4.38 ± 0.08	3.71	2.38

related quaternary species *N*-methyl-3-acetoxyquinuclidinium.

Interaction pharmacophore. The analysis of the cholinergic interaction pharmacophore, based on the pattern of potentials generated by ACh suggested that not all the functional groups in the active molecular structure might interact simultaneously with the receptor (10). A sequence is proposed in which the trimethylammonium cationic group interacts first with the anionic subsite of the receptor and thereby activates the negative region surrounding the ester oxygen. Around this atom a region of negative potential is formed, which may then interact with the corresponding subsite. It has been shown (10) that this region of negative potentials (corresponding to contributions from the electronic charge distribution in Eq. 1 which exceed the positive contributions from the nuclei) develops in the vicinity of the ester oxygen atom only when the interaction of the molecule with an anionic site

FIG. 3. *Molecular conformation and net atomic charges* ($\times 10^2$) *of 3-AcQ at local energy minima* τ_1 = 165° (a); 60° (b); 325° (c). Underlined in Fig. 3a are the net charges for the *N*-methyl derivative of 3-AcQ.

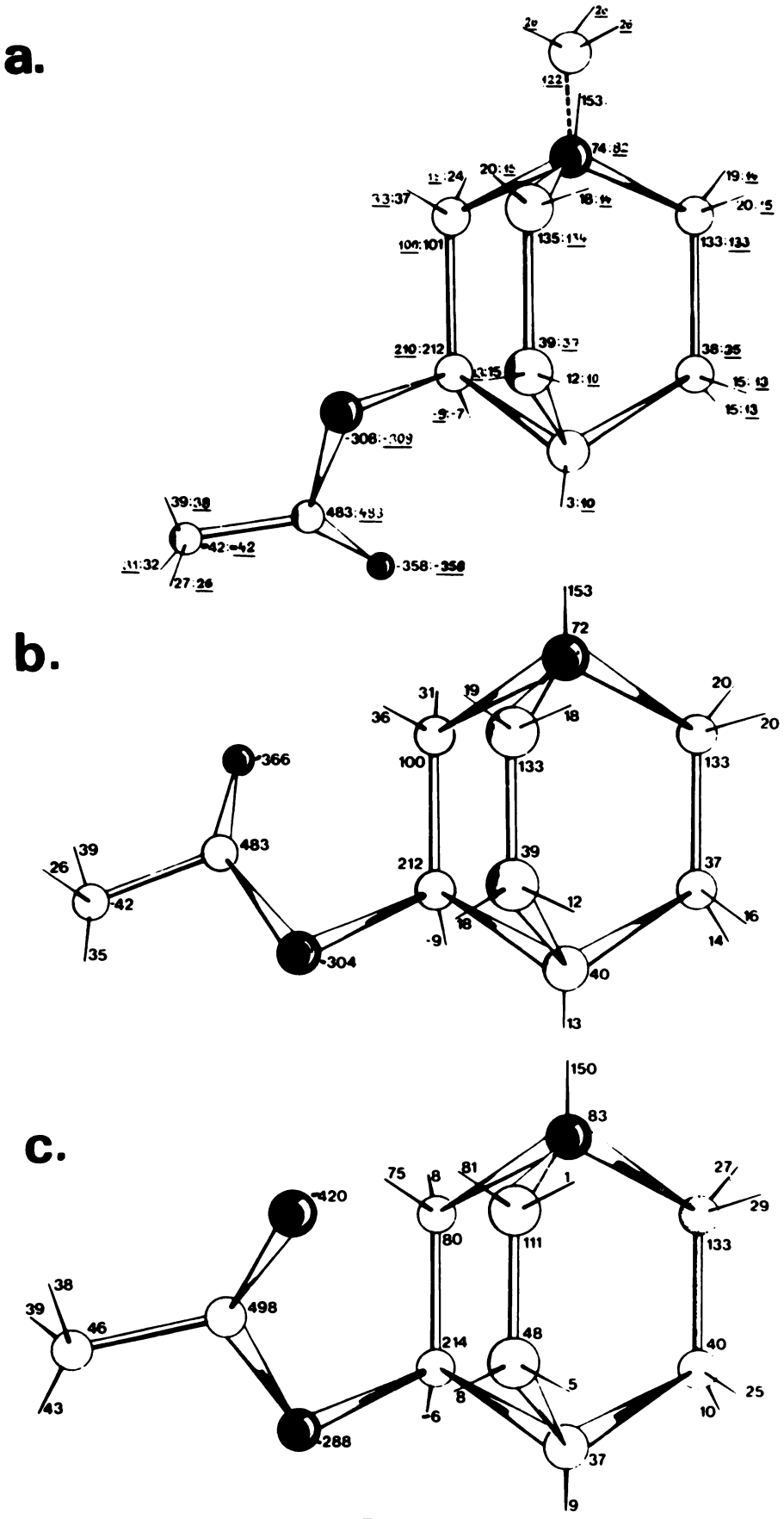


FIG. 3

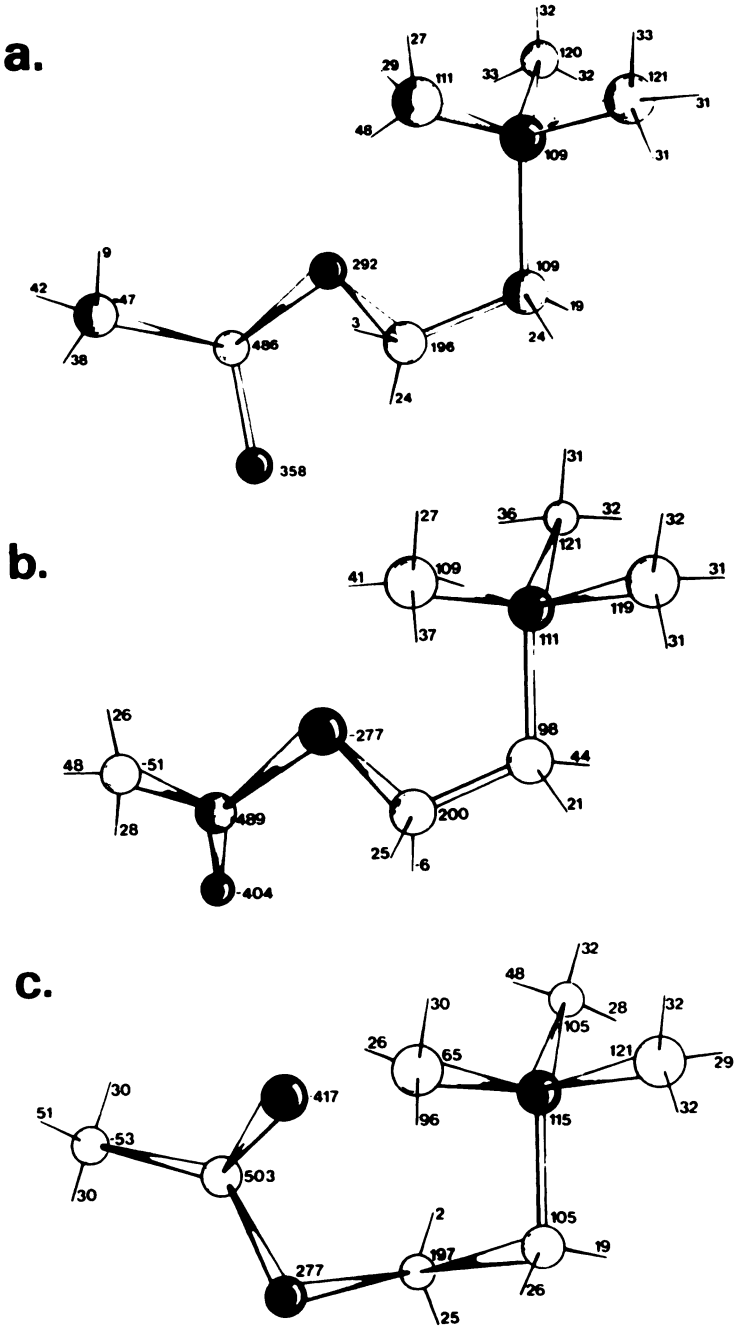


FIG. 4. *Molecular conformation and net atomic charges* ($\times 10^3$) *of ACh at three of its local energy minima, relative to those of 3-AcQ*

a. (40°, 180°). b. (59°, 50°). c. (50°, 270°).

in the receptor is taken into consideration. The map of the positively charged isolated ACh molecule shows the surrounding potential to be entirely positive (with the exception of a small region near the carbonyl oxygen atom O_2), although negative net charges appear on several atoms (Fig. 4a). The interaction of ACh with an an-

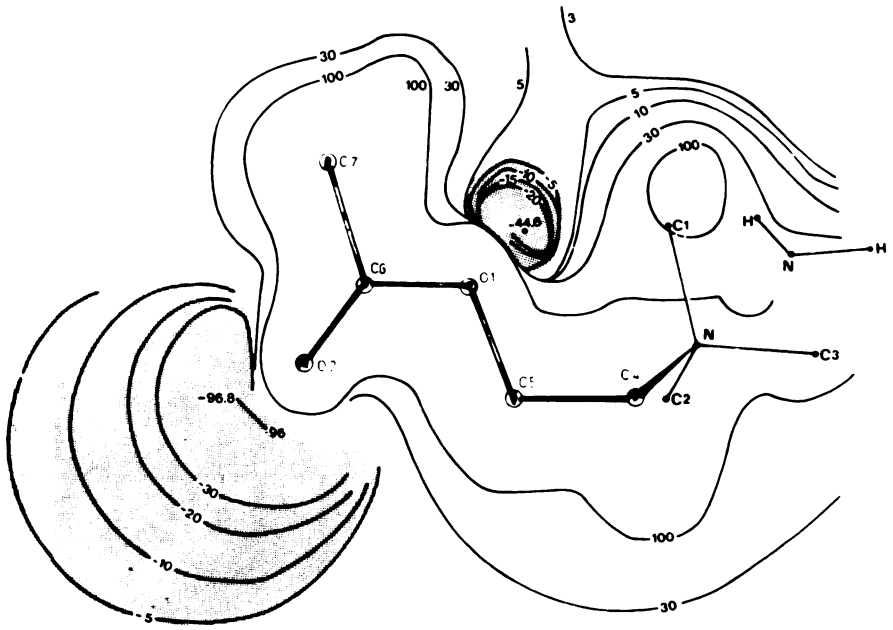


FIG. 5. *Interaction pharmacophore of ACh represented by electrostatic potential map generated by ACh in interaction with a simulated anionic site (NH_2^-)*

Shaded areas represent regions of negative potential, attractive to positively charged groups.

ionic subsite is simulated by letting a negative group (e.g., $HCOO^-$, OH^-, or NH_2^-) approach the trimethylammonium cationic head. The resulting map of potentials for ACh is shown in Fig. 5. The map was calculated for the ACh–anionic group complex, with ACh in one of its energetically preferred conformations (60°, 180°), which is considered to represent the biologically relevant geometry (6, 38). The very similar interaction pharmacophore of 3-AcQ (protonated) is represented in Fig. 6 by the electrostatic potentials calculated in the plane including C_5, O_1, C_6, and C_7. The optimal position of the anionic group $HCOO^-$ was obtained from energy mapping, in which the —NH—O distance was varied while keeping constant the conformation of the 3-AcQ species, the H···O—H angle (104°), and the O···H distance (0.94 A).[9] The INDO method was used for all energy calculations.

For the *N*-methyl derivative the potential map was also calculated in the presence of an anionic group. Two positions of an approaching OH^- were chosen above the methyl group, as shown schematically in Fig. 7 (*a* and *b*), and the optimal distance was obtained by energy mapping. Of the two, position *a* was found to be preferred energetically at either distance and was used in the calculation of the potentials. Because of the rigidity of the piperidine frame, the position of interaction between the cationic moiety and the anionic site is the main factor in the orientation of the other functional groups toward the receptor. A third position, *c*, was therefore probed (Fig. 7). This position was found to be favored energetically by about 4 kcal/mole, as compared to position *a*. The general pattern (shape and position) of the potentials generated by *N*-methyl-3-acetoxyquinuclidinium (Fig. 8), however, was found to be insensitive to the relative positions (*a*, *b*, *c*) and the nature of the anionic group (OH^-, $HCOO^-$, NH_2^-).

Experimental Results

The ACh-like (muscarinic) activity recorded for the various enantiomers of 3-

[9] The assumption that the preferred conformation of the cation 3-AcQH$^+$ also corresponds to a preferred conformation in the complex with the anionic site has been confirmed by complete energy mapping for the supermolecule.

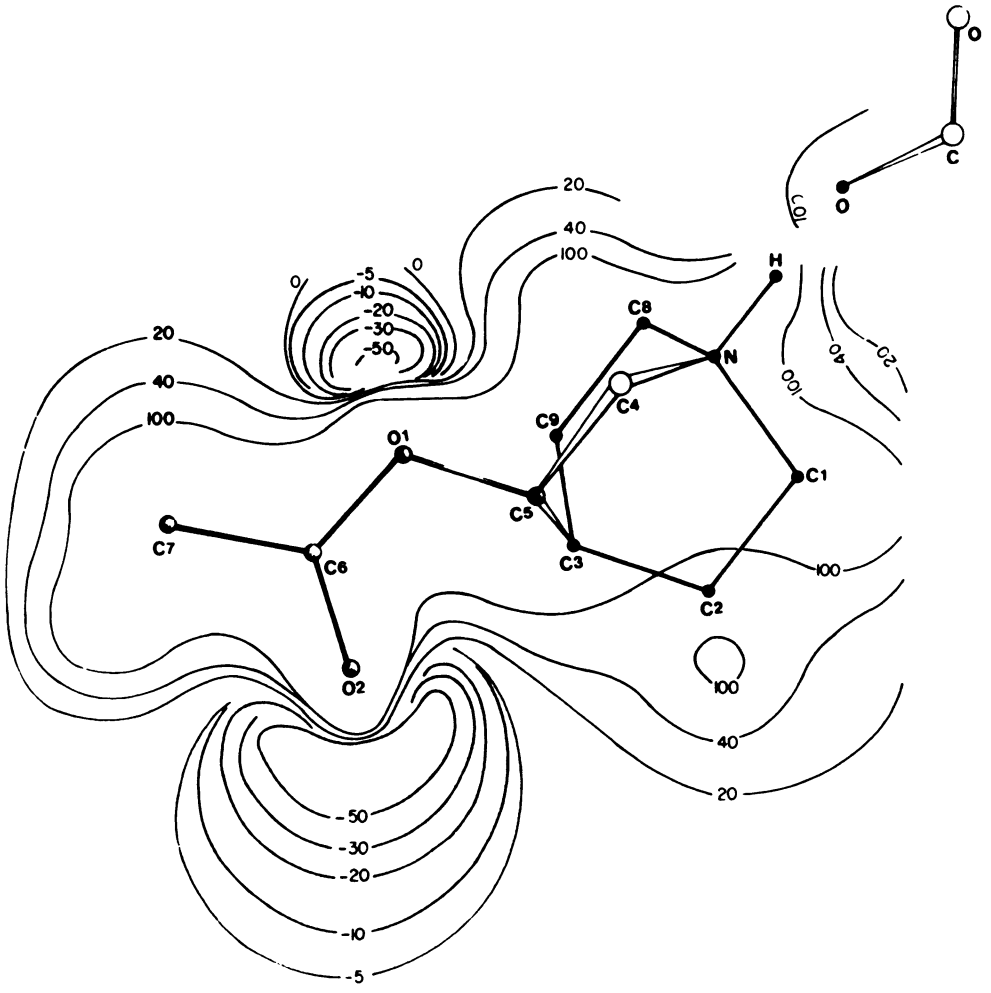
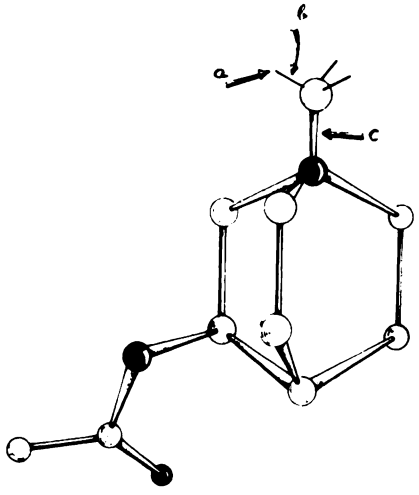


FIG. 6. *Interaction pharmacophore of 3-AcQ*
The anionic site is simulated by $HCOO^-$.



AcQ and its *N*-methyl derivative on the quinea pig ileum is summarized in Table 3. The (+) enantiomer (HCl salt) was found to be the strongest agonist and was blocked by 0.03 μM atropine. The dose-response curves obtained with each enantiomer of the HCl salts of the studied compounds were found to be parallel to that of ACh and to reach the same maximum response value (Fig. 9 shows such curves for the racemic mixtures).

The most active enantiomer, (+)-3-AcQ HCl, also possessed the highest miotic potency on mouse eyes. For comparison, Fig.

FIG. 7. *Channels of approach for minimization of interaction energies with simulated anionic site*

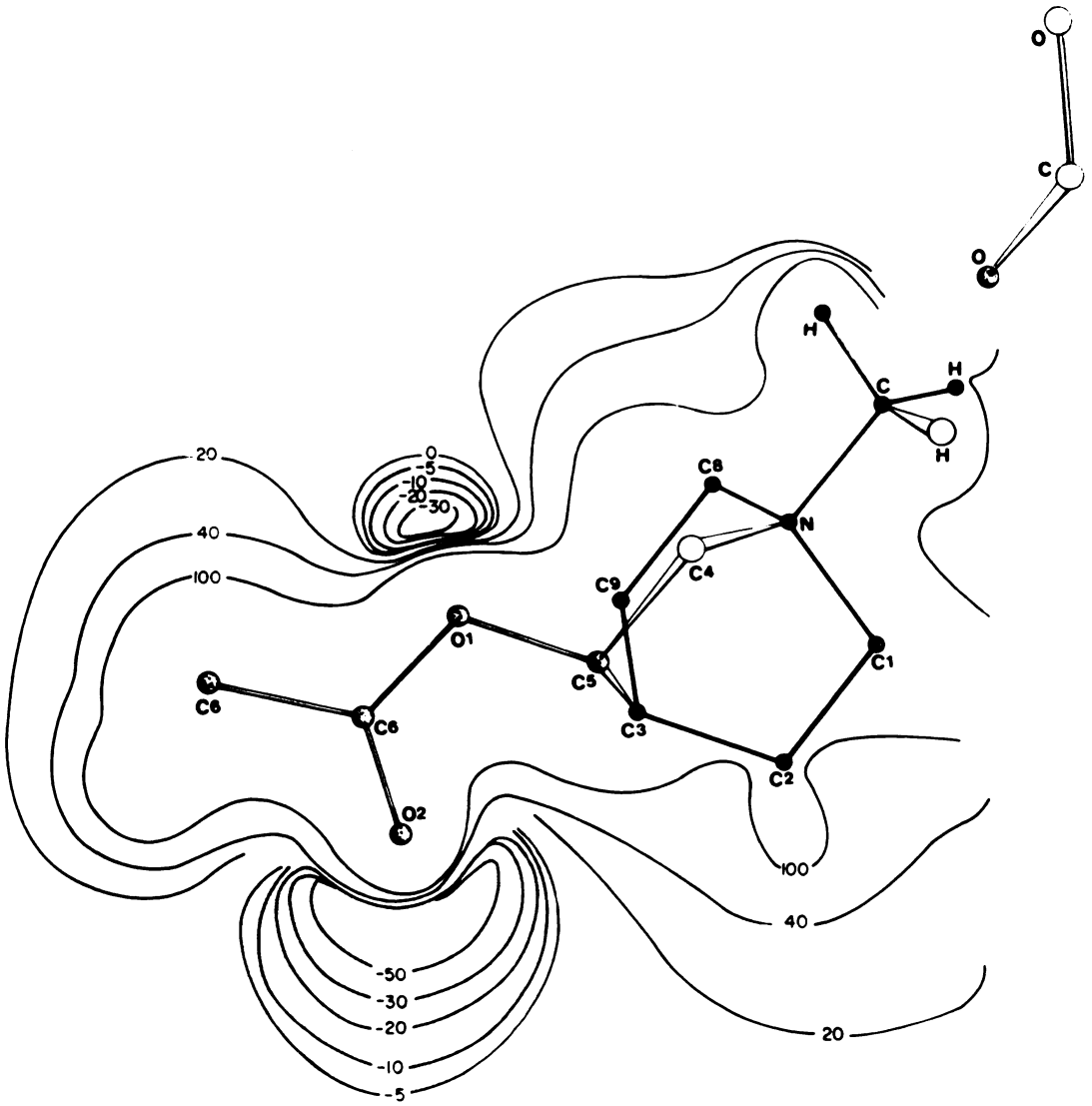


FIG. 8. *Interaction pharmacophore of N-methyl-3-acetoxquinuclidine*

10 shows the sequence of miotic potency of some known muscarinic agonists: oxotremorine > (+)-3-AcQ > 2-acetoxy-1-dimethylaminoethanol. The (−) isomer of 3-AcQ (HCl), as well as both isomers of its *N*-methyl derivative and ACh, could not reverse the mydriasis caused by phencyclidine (Table 4). Correlation between reversal of mydriasis and muscarinic potency was demonstrated recently (37). The inability of the *N*-methyl derivatives as well as that of ACh itself to antagonize the mydriasis induced by phencyclidine is probably a consequence of the inability of drugs possessing a quaternary nitrogen moiety to penetrate rapidly the biological media leading to the receptor site in the intact eye (11, 37).

Another set of properties relating 3-AcQ to ACh is revealed by their characteristic interactions with enzymes of the cholinergic system. The (−) isomer was found to be a rather good substrate for both acetylcholinesterase and butyrylcholinesterase (Table 5). The characteristic substrate inhibition observed for the enzyme-substrate pair acetylcholinesterase-acetylcholine (45) was also observed for acetylcholinester-

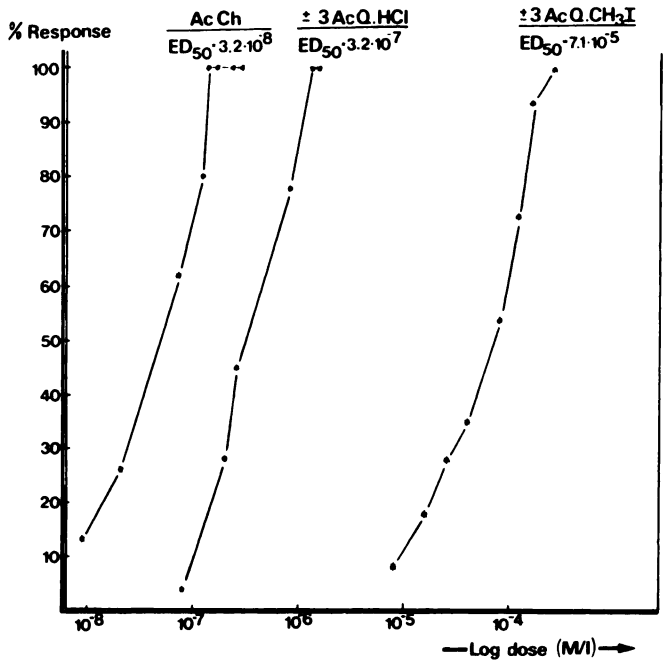
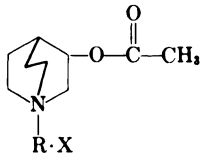


FIG. 9. *Dose-response curves of ACh, (±)-3-AcQ, and (±)-3-AcQ·CH₃I in perfused, isolated guinea pig ileum*

TABLE 3

Muscarinic activity of 3-AcQ derivatives in isolated smooth muscle

The ACh-like activity of the amino esters was determined in guinea pig ileum (36).



Amino alcohol	R	X	ED_{50}	Equipotent molar ratio
			μM	
(±)	H	Cl	0.7	20 ± 5
(+)	H	Cl	0.15	5 ± 3
(−)	H	Cl	3	100
(±)	CH_3	I	70	>100
(+)	CH_3	I	20	>100
(−)	CH_3	I	80	>100
Acetylcholine			0.03–0.06	1.0

ase and 3-acetoxyquinuclidine. By analogy with the choline esters, (±)-3-BuQ was found to have higher V_{max} and lower K_m values than (±)-3-AcQ (Table 5). The (+) isomer of 3-AcQ was not hydrolyzed by acetylcholinesterase or butyrylcholinesterase but was found to be a very weak inhibitor of these enzymes (Table 5). However, (+)-3-BuQ HCl is hydrolyzed, although slowly, by butyrylcholinesterase.[10]

The ability of the 3-quinuclidinol esters studied here to lower the rate of phosphorylation of butyrylcholinesterase by an organophosphate compound is an additional criterion for their ACh-like properties. Specific substrates are considered to lengthen the half-life ($t_{1/2}$) of the enzyme by protecting its active site against the attack of the organophosphate. $t_{1/2}$ values of butyrylcholinesterase modification by sarin, in the presence of the protecting choline or 3-quinuclidinol esters, were calculated from straight lines describing log percentage of residual enzymatic activity vs. time. The slopes of the straight lines obtained by plotting $t_{1/2}$ values vs. substrate concentration are inversely proportional to the K_m values of all four substrates (Table 6). This again suggests a similar interaction with the active site of the enzyme (Fig. 11).

[10] M. Rechavi, S. Maayani, and M. Sokolovsky, unpublished observations.

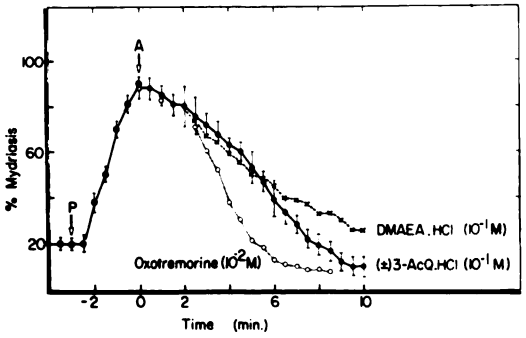


FIG. 10. *Comparison of miotic potencies in mouse eyes of muscarinic agonists oxotremorine, (±)-3-AcQ, and dimethylaminoethyl acetate DMAEA, tertiary derivative of ACh*

The ability of these drugs to reverse mydriasis caused by treatment with phencyclidine at time *P* is shown by the extent and slope of the time curve, starting at their administration time (*A*) and monitored until the lowest possible percentage of mydriasis was achieved. See the text for details.

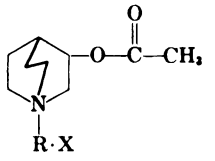
DISCUSSION

The experimental results definitely show (+)-3-AcQ HCl to be a more active muscarinic agonist than its (−) enantiomer. Robinson *et al.* (28) found their (−)-3AcQ CH_3I to be more active than the (+) enantiomer. The methiodide (+) enantiomer seemed to be somewhat more active in our experiments even though the activity ratio was much closer to unity here than in the nonmethylated species. How-

TABLE 4

Antagonistic activity of 3-AcQ derivatives to mydriasis induced by phencyclidine in mouse eyes

Mydriasis developed after local application of 10 mM phencyclidine (HCl) (pH 8.0, 0.1 M phosphate buffer). Induction of miosis in the same intact, phencyclidine-treated eye was carried out similarly (see the text for details).



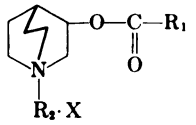
Amino alcohol	R	X	Reversal of mydriasis	
			Concentration	Time
			mM	*min*
(±)	H	Cl	200	5
	H	Cl	20	10
	H	Cl	2	>15
(+)	H	Cl	200	4[a]
	H	Cl	20	7
	H	Cl	2	>15
(−)	H	Cl	200	>15
(±)	CH_3	I	200	>15

[a] Acute miosis developed after 4 min.

TABLE 5

3-Quinuclidinol esters as substrates for cholinesterases

Cholinesterase activity was measured titrimetrically at pH 7.4 and 37°.



Isomer	R_1	R_2	X	Acetylcholinesterase		Butyrylcholinesterase	
				K_m	V_{max}	K_m	V_{max}
				M	*μmole/ml/min*	*M*	*μmole/mg/min*
(±)	CH_3	H	Cl	5×10^{-4}	1×10^{-2}	2.2×10^{-3}	5.5×10^{-2}
(−)	CH_3	H	Cl	5×10^{-4}	1×10^{-2}	2×10^{-3}	5.7×10^{-2}
(+)	CH_3	H	Cl	Inhibitor		Inhibitor	
(±)	CH_3	CH_3	I	5×10^{-4}	3.3×10^{-2}	1.1×10^{-3}	40×10^{-2}
(−)	CH_3	CH_3	I	4.3×10^{-4}	3.5×10^{-2}	1×10^{-3}	45×10^{-2}
(+)	CH_3	CH_3	I	Inhibitor		Inhibitor	
(±)	C_3H_7	H	Cl			1.5×10^{-3}	31×10^{-2}
	C_3H_7	CH_3	I			1.3×10^{-3}	220×10^{-2}
ACh (ClO_4)						2×10^{-3}	100×10^{-2}
BuCH (I)						1×10^{-3}	240×10^{-2}

TABLE 6

Affinity and protective activity of several butyrylcholinesterase substrates

K_m values were determined according to Lineweaver and Burk. $t_{1/2}$ values were calculated from linear plots of log percentage residual butyrylcholinesterase activity vs. time in the presence of both substrates and sarin.

Substrate	K_m	Slope ($t_{1/2}$/[S])	K_m (Ac)/K_m (Bu)	Slope (Bu)/slope (Ac)
	mM			
3-BuQ	1.3	33	1.4	1.6
3-AcQ	1.8	21		
BuCh	1.0	350	2	1.9
ACh	2.0	183		

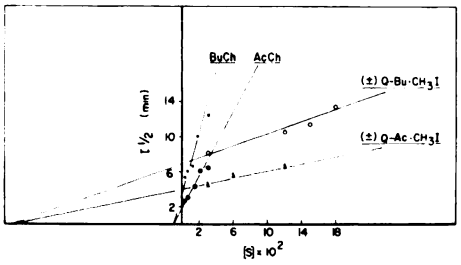


FIG. 11. *Protective action of specific substrates against phosphorylation of active site of butyrylcholinesterase*

Half-life values ($t_{1/2}$) of butyrylcholinesterase modification by sarin in the presence of butyrylcholine (BuCh), acetylcholine (AcCh), (±)-3-butyroxyquinuclidine methiodide [(±) Q-Bu·CH_3I], and (±)-3-acetoxyquinuclidine methiodide [(±) Q-Ac·CH_3I] as substrates are shown as a function of substrate concentration [S]. See the text for details.

ever, because of the rather high concentrations at which any of the isomers of the *N*-methyl derivative showed activity (more than 10 μM), this result is less reliable than in the case of 3-AcQ HCl. This apparent discrepancy in the biological results obtained with the (+) and (−) enantiomers of 3-AcQ HCl should be considered in view of the conditions under which the optical rotation values were determined, as reported in the previous section and summarized in Table 1.

The ACh-like activity of (+)-3-AcQ, as revealed by the experimental results reported here, may be considered to be a consequence of the similarity of the interaction patterns of the two molecules with the cholinergic receptor. This similarity pertains to the requirements for the two steps which are considered to be related to ACh-like activity (24, 42, 46): receptor recognition and receptor activation. The comparison of the interaction pharmacophores generated by the active (+)-3-AcQ (HCl) with those of ACh emphasizes the ability of these molecules to interact with the same subsites in the receptor. The characterization of the second step may be related to the flexibility in the spatial orientation of certain groups in the two molecules, as revealed by the similarity in their calculated conformational energy profiles (Fig. 2) and (38).

The mechanism of cholinergic receptor activation has been related to the "intrinsic activity" of ACh-like agonists (46) in the activation stage, in which a conformational rearrangement has been considered to occur in the drug-receptor complex following the preliminary receptor-recognition phase. Indeed, some molecules which possess the elements necessary for a direct interaction with the receptor, but where these are incorporated in a rigid structural frame, have proved to be antagonists rather than agonists of ACh (1, 10, 45). The present study of the semirigid agonist (+)-3-AcQ shows that flexibility around only the dihedral angle τ_1 (C_6—O_1—C_5—C_4) seems to be enough to ensure activation. This situation is analogous to the activation of the receptor by the potent agonist (+)-*trans*-2(*S*)-acetoxycyclopropyl-1(*S*)-trimethylammonium [equipotent molar ratio = 0.9 (47,48)], in which the same dihedral angle should be involved in the rearrangement of the drug-receptor complex. The N—O_1 distance in this agonist remains fixed by the cyclopropyl ring (4), as in 3-AcQ. Likewise, compounds which may be considered related structurally to 3-AcQ, but in which the rotation around the O_1—C_5 bond is precluded, have been found to lose agonistic properties and to turn into antagonists of ACh[11]. These findings indicate than a certain flexibility is a structural requirement for agonistic activ-

[11] S. Cohen, unpublished observations.

ity (24). It must be stressed, however, that in certain classes of potent semirigid agonists [including muscarine and muscarone (5, 49) or the dioxolanes (50)] the conformational flexibility results from rotations around bonds other than O_1—C_5. An investigation of such differences in the reorientation angles leads to some conclusions on the mechanism of interaction with the receptor, which will be described in a forthcoming report.

Energy mapping for the interaction of 3-AcQ (cation) with the approaching negative group shows that the distance between the anionic site and the region of negative potential near the ester oxygen (approximately 5 A) is compatible with that evaluated for ACh. For the *N*-methyl derivative, however, this distance is larger by at least 0.75 A in any of the interaction positions shown in Fig. 7. This difference may seriously hinder the agonistic activity of the compound, by lowering the number of "successful bindings" which lead to activation. From the numerical values of the interaction energies with the anionic group, it also appears that the complex of 3-AcQ with the receptor would be strongly preferred to that achieved by the *N*-methyl derivative. These effects are expected to lead to a decrease in the relative potency of the quaternary derivative.

The functional groups which form the ACh-like moiety in the agonist 3-AcQ are also identifiable in a series of potent anticholinergic amino esters such as atropine, scopolamine, and, obviously, 3-quinuclidinyl benzilate. Since these compounds have also been found to be active in the central nervous system and to bind to the muscarinic receptor in brain homogenates (51) and in peripheral preparations (52), a relationship between their anticholinergic and psychotomimetic properties should be considered (for some early suggestions, see ref. 53). The ACh moiety being almost rigid in these molecules, the anticholinergic amino esters might be expected to present at the receptor an interaction pharmacophore which closely resembles the one calculated here for the agonist 3-AcQ. Indeed, structural changes in the ACh-like moiety have been found to influence strongly the anticholinergic properties of these molecules (54). These effects were accompanied by a marked attenuation of their psychotropic activity (53). It has been shown, on the other hand, that some additional structural fragments, such as the hydroxyl group and the phenyl rings, also influence the antagonistic properties of these drugs. The direct interaction of the psychotomimetic-anticholinergics with the cholinergic receptor (51, 52) may therefore be attributed to their ACh-like interaction pharmacophores, while the specific biological response—anticholinergic and psychotropic should be related to factors which influence the subsequent stages and prevent the receptor activation mechanism (1).

APPENDIX

The molecular electrostatic potential maps presented in this work were calculated from the density matrix $\mathcal{D}$, in which only the elements $(\mathcal{D})_{ij}$ were retained, where i and j are atomic orbitals on the same atom, in accordance with the basic assumptions of INDO. However, since "deorthogonalization" of molecular orbitals obtained from INDO has been suggested (55), we compared the potentials obtained with and without deorthogonalization for a variety of model molecular systems.[12] At the suggestion of the Editorial Board, we include this APPENDIX with some examples of such comparisons. The theoretical basis for the deorthogonalization procedures used in the literature is not entirely clear, since it is based on the mere assumption that the relationship between a Slater type basis and the INDO atomic basis is given by the Löwdin symmetric orthogonalization (56). The numerical results obtained for the potential after deorthogonalization of INDO and CNDO wave functions have been shown, however, to be closer to the ones obtained from minimal-basis SCF wave functions (55, 57). In most cases the qualitative differences in the electrostatic potentials obtained from deorthogonalized and non-

[12] The calculations were performed in collaboration with Dr. C. L. Johnson, Department of Pharmacology, Mount Sinai School of Medicine.

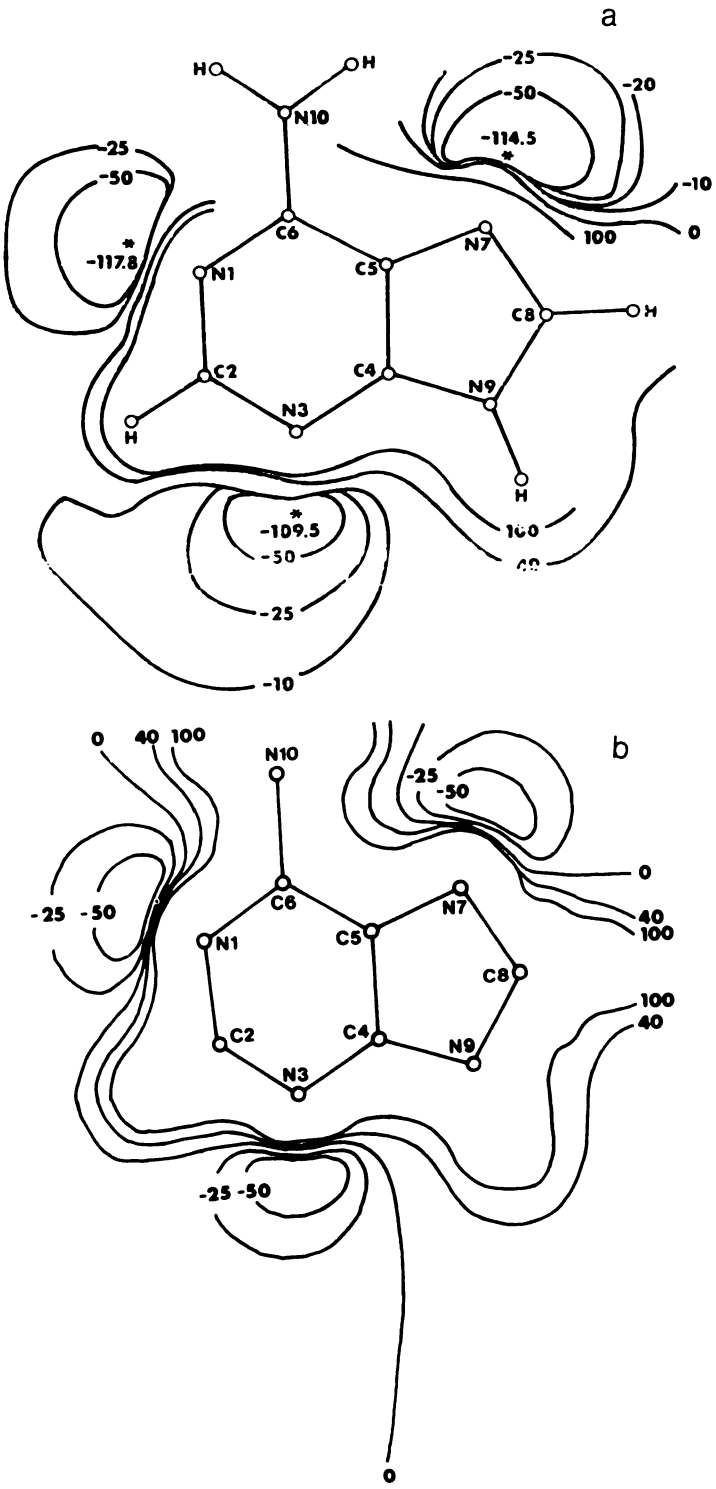

a
H
H
N10
-25
-50
-20
-114.5
-10
100
0
-25
-50
-117.8
C6
N7
N1
C5
C8
H
C2
C4
N9
N3
H
H
-109.5
-50
-25
-10
b
0
40
100
N10
-25
-50
0
-25
-50
C6
N7
40
100
N1
C5
C8
100
40
C2
C4
N9
N3
-25
-50
0

FIG. 12.

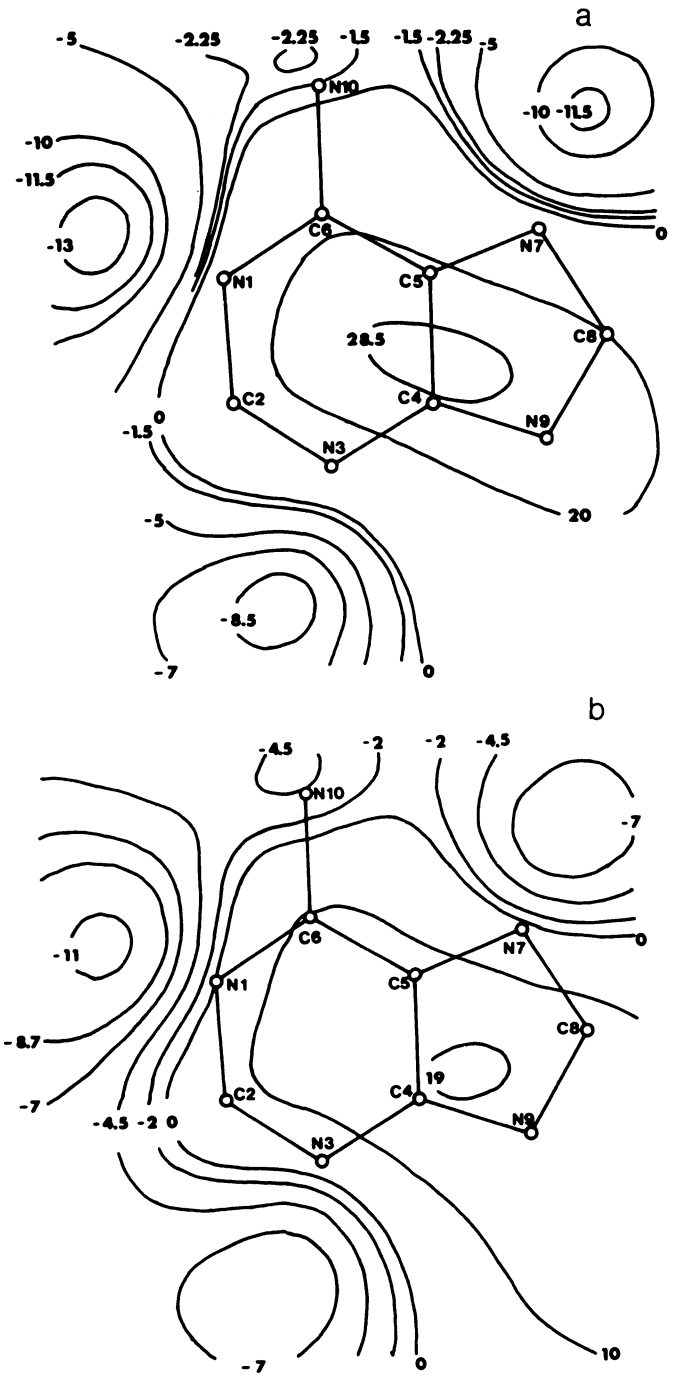


FIG. 13.

deorthogonalized wave functions seem insignificant. The numerical values change and some regions may appear more like local minima when calculated with one approximation than with another, but independent inspection of both types of maps leads to identical qualitative conclusions. The very few cases in which somewhat

more significant differences have been observed (57) are special examples in which lone pairs of neighboring atoms generate a potential pattern which may seem to contain one broad, or two separated minima. This difference may be of some consequence to interaction with a very small reagent, but molecular polarizability and interactions with larger species may modify the pattern enough to make these differences negligible for practical consideration. For the qualitative identification of an active pattern (the "interaction pharmacophore") in molecules having biological activity, and the comparison of unrelated species to establish whether this active pattern is generated in their surroundings, nondeorthogonalized potentials have been found to provide a reliable and economical method of analysis.

Figure 12 shows a comparison between molecular electrostatic potentials calculated from (a) INDO and (b) deorthogonalized INDO wave functions of adenine in the molecular plane. Figure 13 shows the same comparison in a plane 2 A above the molecular plane of adenine. Comparisons leading to the same qualitative results were performed for the formaldehyde, aniline, and nitrobenzene molecules.

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