The Influence of pH on the Muscarinic Action of Oxotremorine, Arecoline, Pilocarpine, and Their Quaternary Ammonium Analogs

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SUMMARY

Oxotremorine, arecoline, pilocarpine, and their methiodides have been assayed for muscarinic activity on isolated guinea pig ileal segments using bethanechol as a reference compound over the pH range 6.2–8.7.

The relative activity of the tertiary amines decreased with increasing pH, while that of oxotremorine and arecoline methiodides was unaffected by pH. Pilocarpine methiodide was pharmacologically inert. The data were interpreted quantitatively using an iterative nonlinear regression procedure to fit an equation derived on the assumption that only the ionized forms of the compounds were active. A satisfactory fit for the five active compounds was obtained as judged by analyses of variance. The procedure also yielded estimates of the dissociation constants of the amines, which were close to those obtained by direct titration, although small significant differences were found.

All three tertiary amines were more active than the corresponding quaternary ammonium compounds, and the reasons for this anomaly are discussed. Evidence is presented to indicate that pilocarpine methiodide is 1,3-dimethyl substituted on the imidazole ring, and methylation of pilocarpine therefore yields a product which is not sterically analogous to the other two methiodides.

INTRODUCTION

Most potent muscarinic agents are quaternary ammonium compounds, and their tertiary amine analogs possess little or no activity of this type (1–3). Pilocarpine, arecoline, and oxotremorine are exceptions to this generalization, since all three are tertiary amines with powerful muscarinic activity. The methiodide of pilocarpine has been reported to lack this action (4), and arecoline methiodide is less active than the parent compound (5–7). Oxotremorine methiodide has not previously been described.

The importance of a cationic group in determining muscarinic activity is well known; it appears probable that the three tertiary amines referred to above owe their activity to the positively charged protonated form. Since they are weak bases and are only partially ionized in the biologically practicable pH range, a quantitative study of the influence of pH upon their muscarinic activity might provide evidence upon which this question could be decided. In the present report, the results of such a study are presented, and are shown to be consistent with the postulate that only the positively charged protonated form of the amines possesses significant muscarinic activity. A preliminary account of this work has already been published (8). A recent publication by Burgen (9) reports a study of the influence of pH on muscarinic activity of arecoline which is consistent with the results reported here.

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METHODS

Muscarinic activity was estimated by four-point parallel line assays on isolated strips of guinea pig ileum, bethanechol being used as a reference standard. Male guinea pigs weighing 300–500 g were killed by a blow on the head, and ileal segments were prepared in the usual way. The segment was immersed in 40 ml of modified Krebs solution, buffered with 10 mM Tris or *N*-cyanoethyl Tris, depending upon the pH to be used in each particular experiment, and maintained saturated with 100% oxygen. The temperature was maintained at 37° by the device described by Biers and Jenden (10). Isometric contractions were measured with a Statham model G7a-1-2500 strain gauge and recorded on a potentiometric recorder.

Each preparation was used for the assay of a single compound against bethanechol at a single pH, after first allowing 1 hr for equilibration. Activity of the two drugs was compared at 50% of maximum contraction by linear interpolation.

Dissociation constants of pilocarpine, arecoline, oxotremorine, Tris, and *N*-cyanoethyl Tris were determined by stepwise titration of 0.2 mmole of each base in 40 ml of 0.9% sodium chloride with 0.1 N HCl, a radiometer pHM 4B pH meter being used. Temperature was maintained at 37° during the titration. The logarithm of the ratio of hydrochloride and free base was plotted against the pH, and the pK was taken as the intercept of the straight line obtained. Three replicate titrations were made with each compound; in every case the values obtained were within 0.02 pH units of the mean.

Oxotremorine was prepared by the method described by Cho *et al.* (11); arecoline methiodide was synthesized as described by Willstätter (12); pilocarpine methiodide was prepared and crystallized by the method described by Wojciechowski and Ecanow (31).[1]

Pilocarpine hydrobromide and arecoline hydrobromide were purchased from Horton and Converse; bethanechol chloride was supplied through the courtesy of Merck Sharp and Dohme Research Laboratories.

Preparation of oxotremorine methiodide. A solution of oxotremorine in methanol was refluxed with excess methyl iodide for 2 hr; the solvent was then removed at reduced pressure. The residual methiodide oil was allowed to stand in the freezer for 2 weeks, after which a trace of crystalline 1-(2-pyrrolidone)-4-trimethylammonium-2-butyne iodide[2] was added to provide a nucleus for crystallization. The entire oil crystallized within 24 hr, and after several recrystallizations from an ethyl acetate–ethanol mixture the resulting material melted at 93–94°.

Analytical data

Calculated for $C_{13}H_{21}N_2OI$:	C, 44.84; H, 6.08; N, 8.05
Found:	C, 44.67; H, 6.06; N, 8.26

RESULTS

Figure 1 summarizes the data obtained with oxotremorine, arecoline, and pilocarpine over the pH range 6.2–8.7. Activity relative to bethanechol clearly decreases as the pH is increased; a horizontal asymptote is approached at a lower pH. It is also apparent from this figure that the pH at which the decline in activity begins is different for the three amines, as might be expected from their differing dissociation constants.

In contrast the quaternary N-metho salts of oxotremorine and arecoline showed a relative activity which was independent of pH over the range 6.6–8.7 (Fig. 2). In confirmation of an earlier report (4), pilocarpine methiodide showed no significant muscarinic

[1] The melting point of the recrystallized product was consistently lower (79–81°) than that reported by Wojciechowski and Ecanow (13) (122–124°). Elemental analysis and equivalent weight agreed with pilocarpine methiodide, and the optical rotation agreed with the figure reported by Wojciechowski and Ecanow for this compound. There appears to be little doubt of the identity of the product, and the different melting point may perhaps be due to a polymorphic crystalline form.

[2] We are indebted to Dr. Alan Bebbington, Chemical Defence Establishment, Porton Down, Wiltshire, England, for a sample of this compound.

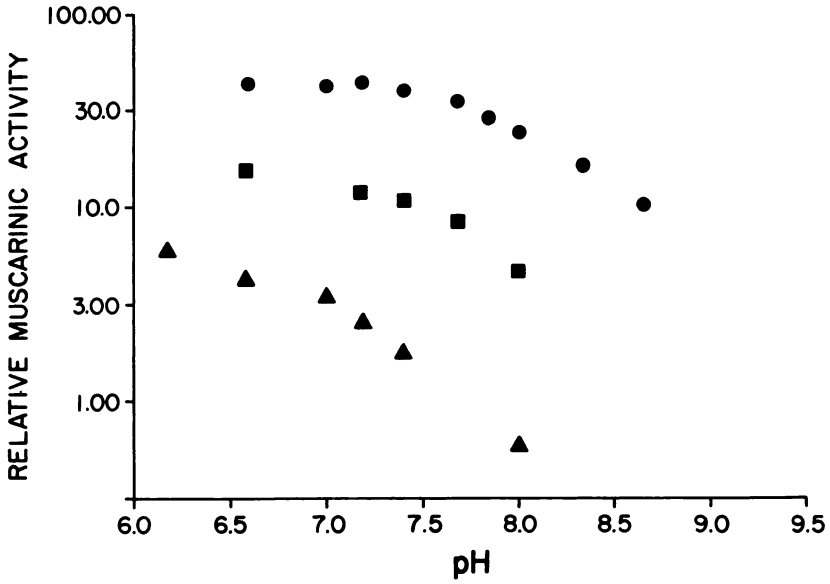


FIG. 1. *Muscarinic activity of oxotremorine* (●), *arecoline* (■), *and pilocarpine* (▲) *relative to bethanechol as a function of pH*

Relative muscarinic activity refers to the ratio of molar concentrations of bethanechol and the test compound which produce the same response. Each point represents the mean of at least three experiments.

activity; a probable explanation of this inactivity is presented below.

The data presented in Fig. 1 are clearly consistent in general form with the hypothesis that the muscarinic activity of these amines is proportional to the concentration in ionized form. A more exact estimate of the precision with which the data fit this hypothesis may be obtained. The equilibrium between the free base and hydrogen ions may be represented by the equation

$$B + H^+ \leftrightarrows BH^+$$

and the concentration of protonated amine (U) is related to the total amine concentration (U_0) by the relation

$$U = U_0 \frac{[H^+]}{K + [H^+]} \quad (1)$$

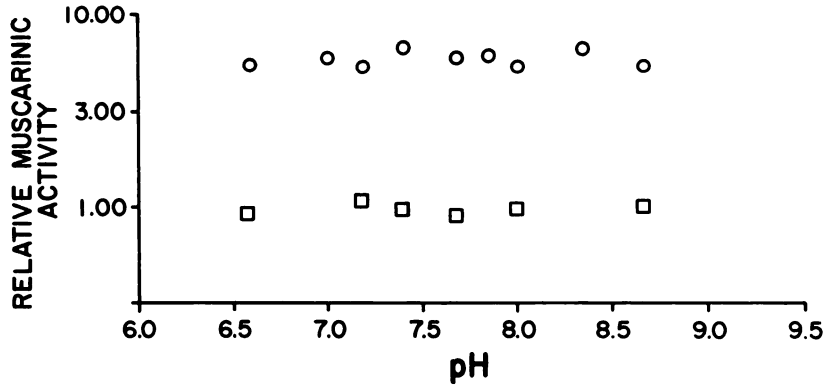


FIG. 2. *Muscarinic activity of oxotremorine methiodide* (○) *and arecoline methiodide* (□) *relative to bethanechol as function of pH*

Relative muscarinic activity refers to the ratio of molar concentrations of bathanechol and the test compound which produce the same response. Each point represents the mean of at least three experiments.

If the muscarinic activity of the amine is attributable entirely to the ionized fraction, and the protonated amine has a pharmacologic activity (Y_0) relative to bethanechol, the activity of the amine (Y) as a function of the hydrogen ion concentration is given by the equation

$$Y = Y_0 \frac{[H^+]}{K + [H^+]} \quad (2)$$

It might be anticipated from the assay procedure used that the error of the estimated relative potency is proportional to the mean; inspection of the data confirmed this relationship. Approximately uniform variance in the dependent variable was obtained by a logarithmic transformation. A statistical test of the hypothesis was now made by fitting the data to the nonlinear regression equation

$$\log Y = \log Y_0 - \log\left(1 + \frac{K}{[H^+]}\right) \quad (3)$$

in which $[H^+]$ is the independent variable. The significance of deviations from the regression equation was assessed by comparing the variance estimate from differences within sets of replicate observations with the variance estimate obtained from differences between the mean of each set of replicates and the regression line.

The regression equation (Eq. 3) was fitted to the experimental data by the Gauss-Newton method (14), an iterative least squares procedure in which provisional parameter estimates are used to achieve a quasi-linearization of the regression equation in the vicinity of these estimates. Linear regression analysis leads to more accurate estimates of the parameters, and the process is repeated until convergence is obtained.

Table 1 summarizes analyses of variance for the data on oxotremorine, arecoline, pilocarpine, oxotremorine methiodide, and arecoline methiodide when analyzed in this

TABLE 1

Analysis of variance for regression of data according to Eq. 3

Source	Sum of squares	D.F.	Mean square	*F*	*P*
Oxotremorine					
Regression	1.14833	1	1.14833	298.3	$<<0.001$
Deviations from regression	0.01482	7	0.00212	0.551	>0.5
Between replicates	0.08865	23	0.00385	—	—
Total	1.25180	31			
Arecoline					
Regression	0.44556	1	0.44556	232.8	$<<0.001$
Deviations from regression	0.00444	3	0.00148	0.932	>0.5
Between replicates	0.01906	10	0.00191	—	—
Total	0.46900	14			
Pilocarpine					
Regression	1.93923	1	1.93923	362.5	$<<0.001$
Deviations from regression	0.01212	4	0.00303	0.566	>0.5
Between replicates	0.06415	12	0.00535	—	—
Total	2.01550	17			
Oxotremorine methiodide					
Regression	0.00035	1	0.00035	0.080	>0.5
Deviations from regression	0.03425	7	0.00489	1.082	>0.2
Between replicates	0.08144	18	0.00452	—	—
Total	0.11604	26			
Arecoline methiodide					
Regression	0.00014	1	0.00014	0.028	>0.5
Deviations from regression	0.00693	4	0.00173	0.349	>0.5
Between replicates	0.05943	12	0.00495	—	—
Total	0.06647	17			

TABLE 2

Summary of estimates of pK and muscarinic activity of cationic species relative to bethanechol, obtained from regression analysis described in text

Figures in parenthesis are 95% confidence limits. Direct estimates of pK by titration are included for comparison.

Compound	Relative muscarinic activity of cation	pK (from biological data)	pK (titration)
Oxotremorine	46.7 (43.7–49.9)	8.12 (8.07–8.18)	7.91
Arecoline	16.7 (14.4–19.4)	7.64 (7.52–7.82)	7.72
Pilocarpine	6.48 (5.37–7.81)	6.99 (6.87–7.16)	6.85
Oxotremorine methiodide	4.64 (4.51–4.78)	(>9.31)	—
Arecoline methiodide	0.964 (0.894–1.04)	(>9.45)	—

way. The regression is highly significant in the case of the three tertiary amines while the two quaternary salts showed no significant regression on hydrogen ion concentration. None of the compounds gave a significant variance component for deviations from regression, indicating that the form of the regression equation satisfactorily represented the data in every case.

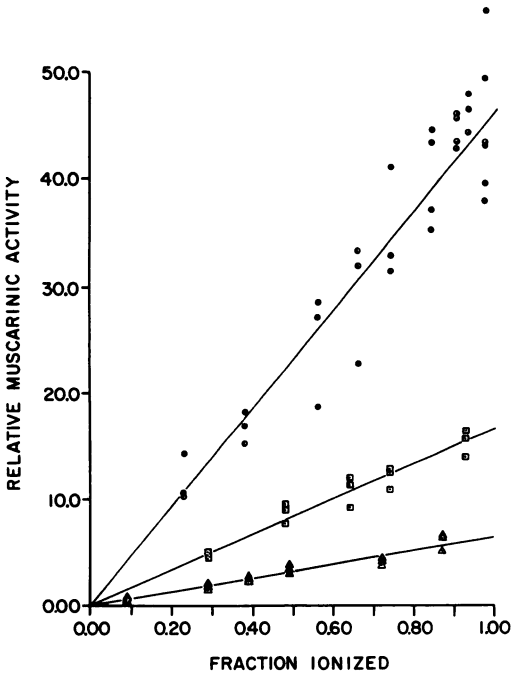


FIG. 3. *Muscarinic activity of oxotremorine* (⊙), *arecoline* (⊡), *and pilocarpine* (△) *plotted against the fraction ionized at the pH used in each experiment*

Relative muscarinic activity refers to the ratio of molar concentrations of bethanechol and the test compound which produce the same response. Each point represents a separate experiment.

The regression analysis provided estimates of the dissociation constant of each compound and of the pharmacologic activity of the cation relative to bethanechol. Table 2 summarizes these estimates and their confidence intervals, and includes for comparison the dissociation constants determined by direct titration of the amines in isotonic NaCl solution at 37°.

Agreement between the two pK estimates for each compound is sufficiently close to justify confidence in the working hypothesis, although in the case of oxotremorine and pilocarpine the differences are statistically significant. The possible reasons for this discrepancy are discussed later.

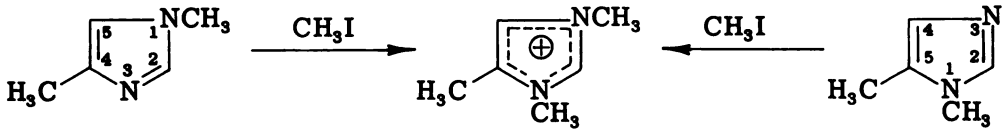
The fit between the experimental data obtained for the three amines with Eq. 3 is illustrated graphically in Fig. 3, in which the pharmacologic activity relative to bethanechol (Y) is plotted on the ordinate against the proportion of drug in protonated form, (U/U_0) as calculated from Eq. 1, using the pK estimates obtained from the regression analysis.

Structure of Pilocarpine Methiodide

Pilocarpine differs from the other bases studied here in that it contains two nitrogen atoms in the imidazole ring. The additional methyl group in the methiodide might conceivably be substituted on either of these nitrogen atoms. The following evidence indicates a 1,3 substitution for the compound used in this study.

In the simpler case of substituted imid-

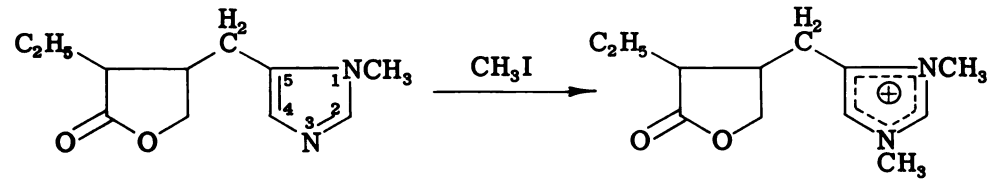
azoles, it has been shown (15) that methylation of 1,4-dimethylimidazole and of 1,5-dimethylimidazole provides the same product, 1,3,5-trimethylimidazolium iodide:



Recent nuclear magnetic resonance (NMR) studies provide further evidence that methylation of 1-methylimidazole yields 1,3- rather than 1,1-dimethylimidazole (16), since the product shows a single N-methyl peak at τ = 5.99 ppm and a single C-H peak at τ = 5.19 ppm for the C-4 and C-5 protons. The single C-H peak indicates that C-4 and C-5 are equivalent, and this would be true only if the substitution of N-1 and N-3 were identical.

The imidazole ring of pilocarpine is unsymmetrical with an alkyl side chain at the 5-position. The N-1 and N-3 are not equivalent and 1,3-dimethyl substitution should be characterized by two different N-methyl peaks in the NMR spectrum. In conformity with this, N-methyl peaks are seen at τ = 6.15 and 6.23 ppm in the NMR spectrum of pilocarpine methiodide.[3]

The pilocarpine methiodide used in the present study therefore contains the 1,3-dimethylimidazolium group and has the following structure:



DISCUSSION

The influence of pH on pharmacologic activity has been of interest to a number of investigators, and several groups of drugs have been studied from this point of view (17–22). Until recently, the only information on muscarinic activity was a brief report by Schild that pilocarpine loses its activity when this is measured at pH 9, whereas acetylcholine does not (23), suggesting that pilocarpine acts in its ionized form. Burgen (9) has recently presented evidence that the activity of arecoline on the guinea pig ileum declines relative to that of carbachol as the pH is elevated, and that ionization of the amine is a satisfactory explanation of this effect. An effect on the state of ionization of the drug is only one of several ways in which pH can change the response of a tissue; comparison with a drug which bears a constant charge has been used to control against the effects of pH on responsiveness of the tissue (9, 18, 21, 22). If an amine owes its pharmacologic activity to the proportion present in the ionized form, then its activity relative to a stable quaternary compound should be a predictable function of the pH. Although experimental data have previously been interpreted by a general comparison with such a theoretical model, the use of nonlinear regression analysis to provide a precise quantitative assessment of the fit between experimental data and the theoretical model allows a greater degree of objectivity in the interpretation of the results. Maximum likelihood estimates and confidence intervals of the pK and activity of the protonated form may also be obtained by this technique.

The data presented in this report are quantitatively consistent with the view that the muscarinic activity of oxotremorine, arecoline, and pilocarpine is entirely attributable to that fraction of the amine which is ionized at the pH used. However, the possibility cannot be excluded that the uncharged form may make a finite contribu-

[3] Kindly obtained in D_2O by Dr. N. S. Bhacca of Varian Associates, Palo Alto, California.

tion to the activity which was not detectable in these experiments.

In the case of oxotremorine and pilocarpine a significant difference was found between the pK estimates obtained from the data and those obtained by direct titration. Although many explanations for this discrepancy could be offered, perhaps the simplest is a systematic difference between the pH of the bathing medium and the pH at the receptor at which the drug acts. Normal metabolic processes would be expected to produce a standing hydrogen ion concentration gradient between the interior of the tissue and surrounding medium; in spite of the buffer used in the latter this gradient might be sufficient to cause a significant overestimation of pH at the receptor. This would in turn lead to an overestimate of the pK. Another possible explanation of the discrepancy is a true difference between the effective pK of the drug as it combines with the receptor and its pK in free solution; such a difference might for example be caused by a local change in dielectric constant in the vicinity of a charged protein or interface, or by an inductive effect exerted upon the drug molecule by the receptor. Neither of these proposals provides a convincing explanation of the fact that a discrepancy between the estimated and titrimetically determined pK was observed only with oxotremorine and pilocarpine, satisfactory agreement being obtained in the case of arecoline.

It has already been noted that most potent quaternary muscarinic agents are considerably more active than their tertiary counterparts (1–3), yet in the present series of compounds, the tertiary amines are all more active than their quaternary analogs. This anomaly has already been noted in the case of arecoline (5–7) and pilocarpine (4), but oxotremorine methiodide has not previously been reported on. Similar behavior has been noticed with 1-methyl-3-acetoxymethylpiperidine and some related compounds (24), aceclidine (3-acetoxyquinuclidine) (25), and some tertiary amine analogs of oxotremorine (26). It is difficult to construct a hypothesis which satisfactorily accounts for the enormous variation in relative muscarinic activity in tertiary amines and their quaternary analogs. In most, but not all, tertiary amines showing greater muscarinic activity than their methiodides, the nitrogen atom is contained in a cyclic structure, and in arecoline analogs the double bond is critical for this property (6). Bebbington *et al.* (26) have drawn attention to the polarity of the carbonyl group in determining whether a fixed positive charge is necessary for optimum activity in their compounds, but this factor is of little predictive value in other series.

A hydrogen bond formed between a protonated amine group and a nucleophilic group on the receptor should have a much higher energy than the simple ionic bond which a quaternary ammonium group could make. It might therefore be expected that tertiary amines should in general be more active than their quaternary counterparts, and it is the inactivity of tertiary amines like dimethylaminoethyl acetate which appears anomalous rather than the relatively low activity of arecoline and oxotremorine methiodides. When the dichotomy is considered from this viewpoint, two possible explanations present themselves. First, the highly directional nature of a hydrogen bond between the receptor and a protonated amine group would place much more serious constraints on the "fit" with the remainder of the molecule than would the relatively nondirectional ionic bond: hence a more specific conformation and orientation of the other bonding groups might be required. Second, evidence has been adduced from hydrolysis kinetics that the protonated form of dimethylaminoethyl acetate exists in a cyclic form in which the ammonium group is linked to the carbonyl oxygen by a hydrogen bond (27). The resulting conformational constraint and charge redistribution may preclude interaction with the receptor and account for the low muscarinic activity in comparison to acetylcholine, which does not assume a cyclic form in aqueous solution (27, 28). In agreement with this explanation, a similar study on 3-acetoxyquinuclidine and its methiodide (29) suggested that no such cyclic structures are formed in significant amount; the tertiary amine is considerably

more active in this pair. This promising lead is now being developed by an extended study on muscarinic amino acetates, and preliminary results are in agreement with this working hypothesis.

A different explanation may be advanced for the complete inactivity of pilocarpine methiodide as a muscarinic agent. In arecoline and oxotremorine methiodides, the additional methyl group is attached to the cationic nitrogen atom which presumably interacts with the receptor. Pilocarpine contains two nitrogen atoms, and the charge in both the methiodide and the pilocarpinium ion is distributed throughout the imidazolium ring. Either of the nitrogen atoms or the entire ring may therefore interact with the receptor at its anionic site, and methylation in the 3-position may not be sterically analogous to methylation of the other muscarinic amines studied. In this connection it is of interest that while pilocarpidine (*N*-demethylpilocarpine) retains some pilocarpine-like activity (30), neopilocarpine (3-methylpilocarpidine) is quite inactive (31), suggesting that 3-methyl substitution sterically hinders the drug–receptor interaction.

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