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THE CHOLINERGIC EFFECTS AND RATES OF HYDROLYSIS OF CONFORMATIONALLY RIGID ANALOGS OF ACETYLCHOLINE[1]

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ABSTRACT

CHIOU, C. Y., J. P. LONG, J. G. CANNON AND P. D. ARMSTRONG: The cholinergic effects and rates of hydrolysis of conformationally rigid analogs of acetylcholine. J. Pharmacol. Exp. Ther. **166:** 243–248, 1969. 2-Acetoxy cyclopropyl trimethylammonium iodides (ACTM) are conformationally rigid analogs of acetylcholine (ACh) with transoid and cisoid conformations. The (+)-*trans*-ACTM had strong muscarinic activities on dog blood pressure and guinea-pig ileum preparations, suggesting that the transoid form of ACTM was associated with its muscarinic activities. The (+),(−)-*cis*-ACTM was expected to have strong nicotinic activities owing to its cisoid conformation. However, it had negligible nicotinic activity on frog rectus abdominis muscle, presumably due to the 1,3-interaction of the methylene group of cyclopropane ring with the carbonyl oxygen which is believed to be required for nicotinic activities. The potency ratios of muscarinic activities between (+)- and (−)-*trans*-ACTM were very close to those between L(+)- and D(−)-acetyl-β-methylcholine. The muscarinic activities of ACh and (+)-*trans*-ACTM on dog blood pressure were markedly potentiated by neostigmine (41-fold and 23-fold, respectively), but that of (−)-*trans*-ACTM was poorly potentiated (3-fold). The studies on enzymatic hydrolysis of *trans*-ACTM by acetylcholinesterase and cholinesterase revealed that the relative rates of hydrolysis of (+)- and (−)-*trans*-ACTM by acetylcholinesterase were 96 and 59% that of ACh. With the isomers the hydrolysis rates by cholinesterase were 61 and 34% in relation to acetylcholine. These results indicate that the biologic activity of (+)-*trans*-ACTM is potentiated by neostigmine more than that of (−)-*trans*-ACTM because the former is a better substrate for the cholinesterases.

Despite intensive studies on the molecular features of acetylcholine (ACh), the possible biologic importance of conformational isomerism remains uncertain. For the elucidation of this problem, the compounds reported have been 1) structurally as close to ACh as possible and 2) conformationally as rigid as possible (Martin-Smith *et al.,* 1967). In the present work 2-acetoxy cyclopropyl trimethylammonium iodides (ACTM) were selected to meet the requirements stated above because these compounds have a cyclopropane ring in place of the choline moiety and are considered to be the smallest chemical structure among ACh derivatives capable of conferring conformational rigidity. The transoid and cisoid conformers of ACTM are shown in figure 1. The cholinergic effects and the cholinesterase hydrolysis of ACTM are reported in the present investigation. The relationships of conformational variations and the role of the unsubstituted methylene group of the cyclopropane ring of ACTM to nicotinic and muscarinic effects are also discussed. The potentiation by neostigmine of the muscarinic activities of (+)- and (−)-*trans*-ACTM on dog blood pressure is correlated with their rates of enzymatic hydrolysis by acetylcholinesterase (AChE) and cholinesterase (ChE).

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METHODS. *Dog blood pressure preparations.* Mongrel dogs of either sex, weighing 9 to 13 kg, were anesthetized with 15 mg/kg of thiopental sodium and 250 mg/kg of barbital sodium administered i.v. The trachea was cannulated, and the vagi were sectioned. Throughout the experiments the dogs were artificially ventilated with a Palmer respirator. The right femoral arterial pressure was measured with a Statham pressure transducer (P23AA) and recorded on an Offner Dynograph (type RS). All compounds were injected *via* a polyethylene catheter in-

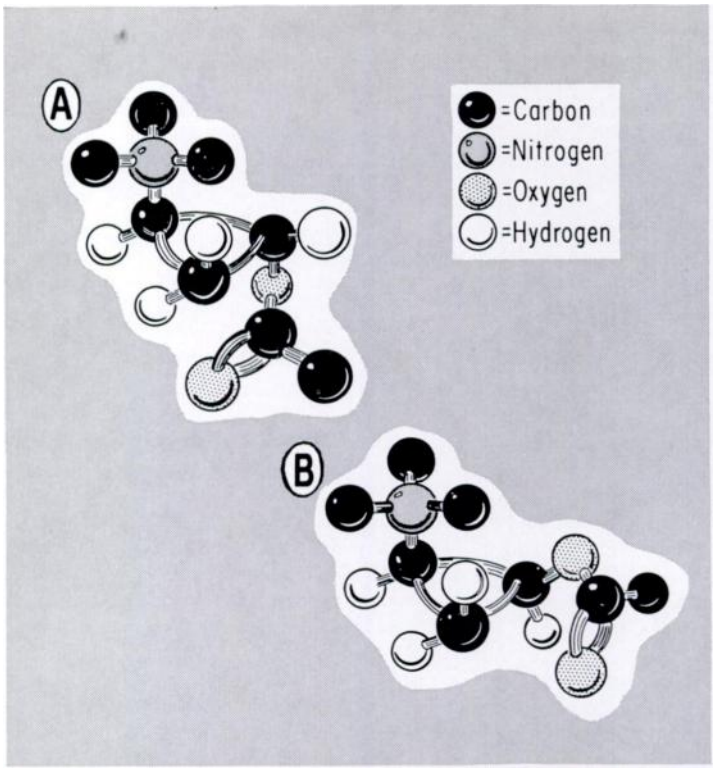

FIG. 1. The transoid (A) and cisoid (B) conformations of 2-acetoxy cyclopropyl trimethylammonium (ACTM). The (+)- and (−)-isomers of either transoid or cisoid ACTM constitute two mirror images which cannot be superimposed.

serted into the left femoral vein and were rapidly washed in with about 2 ml of isotonic saline. In all cases the total volume of the solutions injected was kept constant at 3 ml.

The doses used in the bioassay were varied by 4-fold intervals: 0.1 μg/kg and 0.4 μg/kg for (+)-*trans*-ACTM, 20 μg/kg and 80 μg/kg for (−)-*trans*-ACTM and 0.4 μg/kg and 1.6 μg/kg for ACh. The degree of potentiation of muscarinic activity of these compounds by 50 μg/kg of neostigmine was studied. Before addition of neostigmine, the same dose levels as stated above were used. After neostigmine, 0.008 μg/kg and 0.032 μg/kg of (+)-*trans*-ACTM, 8 μg/kg and 32 μg/kg of (−)-*trans*-ACTM, and 0.016 μg/kg and 0.064 μg/kg of ACh were used. The administrations of doses and drugs were randomized, and all criteria for a valid parallel-line bioassay were met.

Frog rectus abdominis muscle preparation. The rectus abdominis muscle was obtained from *Rana pipiens* as described by Burn (1952). The muscle was threaded at both ends and was superfused with frog Ringer's solution (NaCl, 6.43; KCl, 0.30; $CaCl_2 \cdot 2H_2O$, 0.23; $NaHCO_3$, 0.36; glucose, 0.71 g/liter); this was oxygenated with 95% O_2–5% CO_2 at room temperature. The rate of flow of superfusion fluid was 3 to 4 ml/min and was maintained by a Holter motor pump (type RD 45). Drug solutions were injected into the stream of superfusion fluid in volumes of not more than 0.1 ml. The rectus muscle was placed at an initial tension of 1 g. The tension developed by contraction of the muscle was measured in grams on a Statham transducer (type GT-03) and recorded on an Offner Dynograph recorder (type RS). Solutions of the compounds were prepared in distilled water. The concentrations of drugs used in the bioassay were spaced by 4-fold intervals: 0.4 μg and 1.6 μg for ACh, 40 μg and 160 μg for (+)-*trans*-ACTM, 200 μg and 800 μg for (−)-*trans*-ACTM and 100 μg and 400 μg for (+),(−)-*cis*-ACTM. The administrations of drugs and doses were randomized.

Guinea-pig ileum preparations. Guinea pigs weighing 300 to 500 g were stunned by a blow on the head. The terminal portion of the ileum, approximately 3 cm in length, was used. An initial tension of 1 g was placed on the tissue. The methods of injection of drugs and recording

of the contractions were as described in the last section. The ilea were superfused with Krebs–bicarbonate solution (NaCl, 6.93; KCl, 0.354; $CaCl_2 \cdot 2H_2O$, 0.373; KH_2PO_4, 0.163; $MgSO_4$, 0.143; $NaHCO_3$, 2.09; glucose, 1.80 g/liter). The concentrations of drugs used in the bioassay were spaced by 4-fold intervals: 0.001 μg and 0.004 μg for ACh and 10 μg and 40 μg for (+), (−)-*cis*-ACTM. The administration of drugs and doses were randomized. The relative potencies and 95% confidence limits of (+)- and (−)-*trans*-ACTM were cited from Armstrong *et al.* (1968).

Radiometer titration method for cholinesterase activity. The acetic acid formed during the hydrolysis of esters was titrated with 0.05 N NaOH on a Radiometer titrator type TTT1c and titrigraph type SBR2c. One milliliter of potassium hydrogen phthalate (0.3676 g/200 ml; 1 ml = 9 μmol of NaOH) was titrated with 0.05 N NaOH under N_2 gas. From this titration curve, the ordinate of the titrigraph chart was calibrated directly in micromoles of NaOH used, which is equivalent to micromoles of acetic acid liberated from esters by cholinesterases. The values were expressed in micromoles per hour per unit of enzyme. The enzyme used was prepared 1.0 U/ml in Krebs–bicarbonate solution without $NaHCO_3$ (7.5×10^{-3} M NaCl, 7.5×10^{-3} M KCl and 4×10^{-3} M $MgCl_2 \cdot 6H_2O$). The substrate solutions were prepared with the same solution in a concentration of 5.6×10^{-3} M. The total volume of the reactants was 1 ml. The reaction vessel contained 0.8 ml of the enzyme at pH 7.0. The substrate (0.2 ml) was added through the sample hole, and the reaction mixture was titrated at pH 7.0 at 38°C for 10 min. The air in the reaction vessel was replaced by N_2 gas.

Drugs used. The drugs used in this study were ACh bromide, atropine sulfate, neostigmine methyl sulfate and *d*-tubocurarine chloride. The (+)-*trans*-ACTM, (−)-*trans*-ACTM and (+), (−)-*cis*-ACTM iodides were synthesized by Armstrong *et al.* (1968). All doses of drugs used refer to the salt form. AChE and ChE were obtained from Nutritional Biochemicals Corporation with specific activities of 1000 U/mg of protein and 4 U/mg of protein, respectively.

Statistical analysis. The relative potencies and the degree of potentiation of the compounds were calculated from a four-point parallel-line bioassay as described by Finney (1955). Paired observations were evaluated with Student's *t* test (Snedecor, 1956). A probability value of .05 or less was considered to be significant.

RESULTS. *The muscarinic activities of ACTM on dog blood pressure.* The response elicited by ACTM was a fall in blood pressure that was immediate in onset and of brief duration. The relative potencies and the 95% confidence limits of (+)- and (−)-*trans*-ACTM are shown in table 1. The (+)-*trans*-ACTM was 4.7 times more potent than ACh, whereas (−)-*trans*-ACTM was only 1/45 as potent as ACh. Direct comparison of (+)- and (−)-*trans*-ACTM showed that the former was 192 times more active than the latter (the 95% confidence limits were 105–385). The depressor effects of (+)- and (−)-*trans*-ACTM and ACh were completely blocked by 2 mg/kg of atropine sulfate.

The muscarinic activities of ACTM on guinea-pig ileum. Table 1 shows the relative muscarinic activities of (+)- and (−)-*trans*-ACTM and (+),(−)-*cis*-ACTM on guinea-pig ilea. The (+),(−)-*cis*-ACTM was 1/10,000 as active as ACh. There was no significant difference in muscarinic activity on guinea-pig ileum between ACh and (+)-*trans*-ACTM. The (−)-*trans*-

TABLE 1

The relative muscarinic activities of (+)- *and* (−)-*trans-ACTM*[a] *and* (+),(−)-*cis-ACTM with respect to acetylcholine (ACh)*

Compound	Dog Blood Pressure			Guinea-Pig Ileum		
	No. of animals	Relative potency	95% Confidence limits	No. of animals	Relative potency	95% Confidence limits
ACh		1			1	
(+)-*trans*-ACTM	10	4.70	3.21–9.79	10	1.13[b]	0.81–1.46
(−)-*trans*-ACTM	10	0.023	0.021–0.025	10	0.0022[b]	0.0019–0.0025
(+),(−)-*cis*-ACTM				5	0.00010	0.00004–0.00029

[a] ACTM, 2-acetoxy cyclopropyl trimethylammonium iodide.
[b] Cited from Armstrong *et al.* (1968).

ACTM was about 1/800 as active as ACh (Armstrong *et al.*, 1968).

The nicotinic activities of ACTM on frog rectus abdominis muscle. The effect of ACTM on the frog rectus abdominis muscle was contraction, which was abolished by 5×10^{-5} M *d*-tubocurarine chloride. As indicated in table 2, ACh was 77 times and 357 times more active than (+)- and (−)-*trans*-ACTM, respectively. A direct comparison of the (+)- and (−)-*trans*-ACTM was made, and the (+)-*trans*-ACTM was found to be 4.6 times more active (the 95% confidence limits were 3.3–6.5). The (+),(−)-*cis*-ACTM was about 1/250 as active as ACh.

Potentiation of muscarinic activities of ACTM by neostigmine on dog blood pressure. As indicated in table 3, the muscarinic activities of ACh and (+)-*trans*-ACTM were potentiated 41-fold and 23-fold by 50 μg/kg of neostigmine, indicating that both compounds were good substrates for cholinesterases. The activity of (−)-*trans*-ACTM was potentiated 3-fold only, suggesting that it was a poor substrate for cholinesterases.

The enzymic hydrolysis of trans-ACTM by cholinesterases. The relative rates of hydrolysis of ACh and *trans*-ACTM at the substrate concentration of 5.6×10^{-3} M are shown in table 4. The results indicated that both ACh and (+)-*trans*-ACTM were good substrates for AChE, whereas (−)-*trans*-ACTM was a poorer substrate than (+)-*trans*-ACTM for AChE. The rate of hydrolysis was measured at substrate concentrations of 1.8×10^{-3} M, 1×10^{-3} M, 5.6×10^{-4} M, 3.2×10^{-4} M, 1.8×10^{-4} M and 1×10^{-4} M. The substrate concentration-activity curves obtained were bell-shaped with optimum rates of hydrolysis at a substrate con-

TABLE 2

The relative nicotinic activity of (+)- and (−)-trans-ACTM[a] and (+),(−)-cis-ACTM with respect to acetylcholine (ACh) on frog rectus abdominis muscle

Compound	No. of Animals	Relative Potency	95% Confidence Limits
ACh		1	
(+)-*trans*-ACTM	10	0.013	0.008–0.021
(−)-*trans*-ACTM	10	0.0028	0.0018–0.0046
(+),(−)-*cis*-ACTM	5	0.0042	0.0039–0.0047

[a] ACTM, 2-acetoxy cyclopropyl trimethylammonium iodide.

TABLE 3

The degree of potentiation of muscarinic activities of (+)- and (−)-trans-ACTM[a] and acetylcholine (ACh) by neostigmine[b] on dog blood pressure

Compound	No. of Animals	Degree of Potentiation	95% Confidence Limits
ACh		41-fold	22–100
(+)-*trans*-ACTM	7	23-fold	11–79
(−)-*trans*-ACTM	7	2.8-fold	1.6–5.4

[a] ACTM, 2-acetoxy cyclopropyl trimethylammonium iodide.

[b] 50 μg/kg of neostigmine methyl sulfate.

TABLE 4

The rates of hydrolysis of (+)- and (−)-trans-ACTM[a] and acetylcholine (ACh) by AChE[b] and ChE[c]

Substrate	AChE			ChE		
	No. of expts.	Rate of hydrolysis (mean ± S.E.)	Relative rate of hydrolysis (mean ± S.E.)	No. of expts.	Rate of hydrolysis (mean ± S.E.)	Relative rate of hydrolysis (mean ± S.E.)
		μmol/hr/U of enzyme	%		*μmol/hr/U of enzyme*	%
ACh	5	9.2 ± 0.5	99.8 ± 5.4	5	33.8 ± 0.6	100.3 ± 1.8
(+)-*trans*-ACTM	5	8.8 ± 0.4	96.0 ± 4.4[d]	5	20.5 ± 0.2	60.8 ± 0.7[e]
(−)-*trans*-ACTM	5	6.2 ± 0.9	58.9 ± 7.9[e]	5	11.3 ± 0.3	33.5 ± 0.8[e]

[a] ACTM, 2-acetoxy cyclopropyl trimethylammonium iodide at 5.62×10^{-3} M.

[b] AChE, acetylcholinesterase, 1 U/ml.

[c] ChE, cholinesterase, 1 U/ml.

[d] $P > .05$ compared with acetylcholine.

[e] $P < .05$ compared with acetylcholine.

centration of 5.6×10^{-3} M for ACh as well as for (+)- and (−)-*trans*-ACTM, indicating that a high substrate concentration of (+)- and (−)-*trans*-ACTM inhibits AChE. The relative rates of hydrolysis of (+)- and (−)-*trans*-ACTM by ChE were 61 and 34% of that of ACh. The substrate concentration-activity curves showed no inhibition of ChE by high substrate concentrations.

DISCUSSION. It is reasonable to assume that the flexible ACh molecule has different conformations and thus is capable of fitting to the different types of ACh receptors. It has been suggested that the cisoid form of ACh is associated with its nicotinic activity and the transoid form with muscarinic activity (Schueler, 1956; Archer *et al.*, 1962; Smissman *et al.*, 1966). Based on this hypothesis, it would be expected that the transoid form of ACTM (fig. 1A) would elicit mainly muscarinic responses and the cisoid form (fig. 1B) mainly nicotinic responses. The results indicate that this is true for muscarinic responses (table 1) but not for nicotinic responses (table 2). Therefore, some factors other than *cis-trans* isomerism must be involved in determining nicotinic activity.

Structurally, ACTM is similar to acetyl methylcholine and resembles a hybrid of acetyl-α-methylcholine (A-α-MCh) and acetyl-β-methylcholine (A-β-MCh), both of which have been synthesized and studied by Simonart (1932) and Major and Bonnett (1935). The predominant muscarinic activity of A-β-MCh is presumably due to the 1,3-interaction of the β-methyl group with the carbonyl oxygen, which is required for the nicotinic activity (Sekul and Holland, 1961a,b; Sekul *et al.*, 1963; Coleman *et al.*, 1965; Triggle, 1965), whereas the predominant nicotinic activity of A-α-MCh is probably due to the 1,3-interaction of the α-methyl group with the ether oxygen which is required for the muscarinic activity (Ing *et al.*, 1952; Waser, 1961; Beckett *et al.*, 1961; Triggle, 1965). As shown in figure 1, the methylene group of the cyclopropane ring of ACTM would interact with the carbonyl oxygen but not with the ether oxygen. Therefore, the methylene group abolishes the nicotinic activity of ACTM. Accordingly, ACTM is structurally similar to A-β-MCh but not A-α-MCh. The studies on cholinergic effects of ACTM in the present work support this conclusion because (+)-*trans*-ACTM has strong muscarinic activity (table 1) but very weak nicotinic activity (table 2). In other words, (+)-*trans*-ACTM has predominant muscarinic activity (table 1) owing to its transoid conformation, which is favorable for proper fitting with the muscarinic receptor. In addition, the 1,3-interaction of the methylene group of *trans*-ACTM with the carbonyl oxygen eliminates the nicotinic activity. The *cis*-ACTM is not a muscarinic stimulant due to its cisoid conformation (table 1), nor is it a nicotinic stimulant due to the 1,3-interaction of the methylene group with the carbonyl oxygen (table 2). The opposite hypothesis suggesting that the transoid form of ACh favors nicotinic activities and the cisoid form muscarinic activities (Jellineck, 1957; Canepa *et al.*, 1966) is unlikely because in the present study (+)-*trans*-ACTM had strong muscarinic activities.

It is interesting to note that there is about a 250-fold difference in the muscarinic activities between L(+)- and D(−)-A-β-MCh on guinea-pig ileum and cat blood pressure (Beckett *et al.*, 1961; Beckett *et al.*, 1963). It has been suggested that the difference in activity is due to the β-methyl group in D(−)-A-β-MCh, which prevents its proper interaction with the muscarinic receptor. This hypothesis is further supported in the present work, as there is a similar difference in muscarinic activities between (+)- and (−)-*trans*-ACTM (192-fold and 330-fold differences in activities on dog blood pressure and guinea-pig ileum, respectively).

The studies on the enzymic hydrolysis of ACTM by AChE and ChE reveal that (+)-*trans*-ACTM is a good substrate for AChE since it is hydrolyzed by AChE as fast as ACh. (−)-*Trans*-ACTM is a poorer substrate for AChE since its relative rate of hydrolysis is 59% that of ACh. The relative rates of hydrolysis of (+)- and (−)-*trans*-ACTM by ChE are 61 and 34% that of ACh (table 4). These results explain the observation that the muscarinic activities of ACh and (+)- and (−)-*trans*-ACTM are potentiated by neostigmine 41-fold, 23-fold and 3-fold, respectively (table 3). In other words, the muscarinic activity of (+)-*trans*-ACTM is potentiated by neostigmine more than that of (−)-*trans*-ACTM because the former is a better substrate for the cho-

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