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Esters of choline and β-trimethylammonio-propionic acid: geometrical isomerism and anti-acetylcholinesterase activity

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The preparation of the choline esters of *cis*- and *trans*-4-t-butylcyclohexanecarboxylic acid is reported. The similar inhibitory potency displayed by these isomers towards the acetylcholinesterase catalysed hydrolysis of acetylcholine is explained on the basis of the binding of a thermodynamically unstable conformation of the *cis*-isomer to the active site. Similar studies employing the β-trimethylammonio-propionate esters of *cis*- and *trans*-4-t-butylcyclohexanol suggest that the "reverse esters" do not bind to the active site in an identical manner to the acylcholines.

Acylcholines have been used in the past to differentiate the cholinesterase enzymes, acetylcholine being a better substrate than butyrylcholine for acetylcholinesterase, whereas the order of activity is reversed for pseudocholinesterase (Glick, 1938, 1941; Nachmansohn & Rothenburg, 1945). The acyl group in such compounds is probably binding to the enzyme surface over and to the side of the esteratic site (see also Ariens & Simonis, 1967) and it thus becomes important to attempt to define the stereochemical requirements for binding to this area. Previous studies using optically asymmetric acylcholines have shown that the enzyme exerts some degree of stereoselectivity when these compounds act as substrates e.g. L-(+)-lactoylcholine is hydrolysed at a rate approximately four times faster than D-(−)-lactoylcholine at their respective optimum substrate concentrations by the enzyme acetylcholinesterase (Auditore & Sastry, 1964), whereas reduced stereoselectivity is shown by pseudocholinesterase (Sastry & White, 1968). There appears however to have been no attempts to employ cyclic compounds devoid of optical asymmetry to study further the geometrical requirements for binding of acylcholines to these enzymes.

It was thus decided to prepare some isomeric acylcholines and to study the effect of geometrical isomerism on inhibitory activity.

The studies of the conformation of 4-t-butylcyclohexane derivatives (for review see Eliel, Allinger & others, 1965) suggest that the *cis*- and *trans*-isomers of 4-t-butylcyclohexanoic acid would provide suitable acyl groups for such studies.

Additionally, Bass, Schueler & others (1950) have shown that the reversed ester of acetylcholine (i.e. methyl β-dimethylaminopropionate methiodide) is a potent cholinomimetic on tissue preparations and is a weak inhibitor of the enzyme acetylcholinesterase. Thus using the *cis*- and *trans*-isomers of 4-t-butylcyclohexanol it is possible additionally to consider the geometrical requirements for inhibitory activity among the reversed esters of acetylcholine.

The compounds shown in Table 1 have therefore been prepared and their anticholinesterase activity determined.

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The preparation of the above esters was accomplished by means of standard synthetic methods, but the stereochemistry or conformational homogeneity of products, or both, was checked whenever possible, by the use of gas-phase chromatography, infrared spectroscopy, and nuclear magnetic resonance (c.f. later section).

Hydrogenation of *p*-t-butylbenzoic acid over platinum oxide at room temperature yields 4-t-butylcyclohexanoic acid enriched in the *cis*-isomer, whereas hydrogenation of the sodium salt over Raney nickel at elevated temperatures and pressure yields a product enriched in the *trans*-isomer (Bekkum, Kleis & others, 1962). The individual geometrical isomers were separated by formation of the thiourea occlusion complex of the *trans*-4-t-butylcyclohexanoic acid (Bekkum, Verkade & Wepster, 1959). The conformational homogeneity of the pure *trans*- and pure *cis*-acids so isolated was checked by gas-phase chromatography of the methyl esters (prepared by means of diazomethane) (Cavell, Chapman & Johnson, 1960), when each sample showed a single peak, with a retention time different from that of its isomer.

The *cis*- and *trans*-4-t-butylcyclohexanoic acids were separately esterified, via the acid chloride, with dimethylaminoethanol. Gas-phase chromatography of the resultant esters showed the "trans" ester contained approximately 5% of the *cis*-isomer and the "cis" ester to be a mixture of the *cis*- and *trans*-isomers in the ratio of approximately 3:1. Eliel & Gerber (1965) have previously suggested that thermal equilibration of 4-t-butylcyclohexanoyl chlorides does take place, presumably via a series of equilibrium reactions as shown in Fig. 1. The results reported here provide further evidence of such an equilibration.

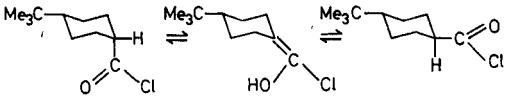


Fig. 1

The retention times on gas-phase chromatography of the *cis*- and *trans*-2-dimethylaminoethyl 4-t-butylcyclohexanoates were sufficiently different to allow a separation on a preparative scale and each purified product was then quaternized with methyl iodide.

The *cis*- and *trans*-isomers of 4-t-butylcyclohexanol were separated by column chromatography (Winstein & Holness, 1955) of a commercial sample of 4-t-butylcyclohexanol (approximately 75% *trans*- and 25% *cis*-isomer). The identity and conformational homogeneity of each product was checked by comparison with published infrared (Eliel & Ro, 1957; Eliel & Rerich, 1960) nmr, (Eliel & Gianni, 1962) and gas-phase chromatographic data (Eliel & Ro, 1957; Roberts 1965). The products were found to be homogeneous by each method.

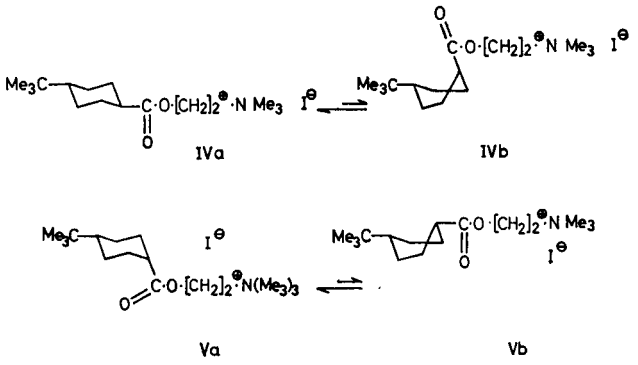
The above alcohols were separately esterified by an ester interchange reaction (Johnson, Paton & Farquharson, 1961) with ethyl β-dimethylaminopropionate. Gas-phase chromatography of the product esters showed the "trans" isomer contained less than 0·5% of "*cis*"-isomer, whereas the "cis" isomer had equilibrated under the reaction conditions and now contained 12% of the "*trans*" isomer. The retention times of the two isomers however were again sufficiently different to allow a separation of the two isomers by means of preparative-scale gas-phase chromatography before they were separately quaternized with methyl iodide.

DISCUSSION

From a comparison of the structures of the acylcholine derivatives with that of acetylcholine, it would be reasonable to assume that the acyl group will interact with an area of the enzyme surface which is close to, but outside, the "active site".

The chemical and physical properties of this area are presently unknown but comparison of the K_I value of compound III with those of compounds IV and V (a lowering of the value of K_I being taken as indicative of stronger binding of the inhibitor to the enzyme) would suggest that this area is hydrophobic in nature, the addition of the t-butyl group having increased the activity by a factor of 10 approximately.

The geometrical requirements for binding to this area are difficult to assess, there being little difference in the activities here reported for compounds IV and V. Although the bulky t-butyl group will act as a "lone handle" to prevent the chair-boat-chair interconversion possible in the parent cyclohexane derivative (III), these compounds in solution could exist in a chair-boat equilibrium conformation.†



Although such a boat conformation is the thermodynamically less stable conformation, this does not preclude such a conformation becoming bound preferentially to the receptor surface if the difference in the free energy of binding of the two conformations Va and Vb exceeds the conformational free energy difference between these two

† The choline moiety could theoretically adopt an infinite number of conformations in solution (for review of possible conformations of acetylcholine c.f. Martin-Smith, Smail & Stenlake, 1967). If the choline moiety in compound V adopts a cyclic conformation (Fig. 2), as suggested by Stenlake for acetylcholine (although no evidence currently exists to substantiate this postulate), the non-bonded interactions due to the bulky axial substituent would cause the equilibrium to be established even more in favour of a boat conformation. However, since the side-chain is common to compounds III, IV, and V, it is possible that this moiety is bound similarly to the "active site" in all the compounds considered, and the problem of the actual conformation of the choline moiety can be neglected in the present discussion.

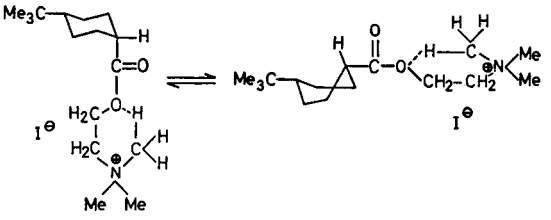


Fig. 2

conformations. Thus a possible explanation of the similar inhibitory potencies of compounds IV and V may lie in a suggestion that the active conformations bound to the receptor area are conformations IVa and Vb respectively. It should be noted here that the "chair" and "boat" conformation are the extremes of an infinite number of conformations of the ring and thus a "twist conformation" of compound V becoming bound to the receptor area is not precluded.

Similar considerations are difficult when applied to the "reversed ester" derivatives. The parent compound (II), although reported to be a potent cholinomimetic (in many tissues equal in potency to acetylcholine), is a weak inhibitor of the enzyme acetylcholinesterase (Bass & others, 1950). It is uncertain at this stage whether the "reversed ester" is binding over the esteratic site of the muscarinic receptor in a manner similar to that of acetylcholine. Schueler, Keasling & Featherstone (1951) have reported that the β-methyl analogue (methyl α-methyl-β-dimethylaminopropionate methobromide) has only about $1/10^4$ of the muscarinic activity of compound II. Comparison of these results with the muscarinic activities reported for acetyl-β-methylcholine (Beckett, Harper & Clitherow, 1963) might suggest that different modes of binding of the drug to this receptor are operative in the cases of acetylcholine and methyl β-dimethylaminopropionate methiodide.

Comparison of the results reported here for the inhibition of acetylcholinesterase by the "reversed esters" and the acylcholine derivatives might also suggest that the two series of compounds are not binding to the enzyme receptor area in identical manners. Although an increase in the lipophilicity of the alkyl-oxygen function produces an increase in the inhibitory potency (compare compounds II, VI, and VII), this increase is not as marked as with acylcholine derivatives (compounds III and IV). A more detailed comparison of the geometrical requirements for binding among these compounds cannot be attempted here, however, since there is a variation in the kinetics of inhibition shown by compound (VIII) at different concentrations.

EXPERIMENTAL

All melting points are uncorrected. Infrared spectra were recorded using a Perkin Elmer Model 237 grating infrared spectrophotometer and nmr spectra were recorded using a Varian A60 spectrometer with tetramethylsilane as internal standard. Gas-phase chromatographic separations were performed by means of a Wilkens Aerograph autoprep Model 705 with flame ionization detector and the following columns *Method A.* 6 ft $\times$ $\frac{1}{4}$ in stainless steel column containing 4% SE30 adsorbed onto Silanized Chromasorb W. *Method B.* 20 ft $\times$ $\frac{1}{4}$ in stainless steel column containing 20% SE30 adsorbed onto Silanized Chromasorb W. *Method C.* 6 ft $\times$ $\frac{1}{4}$ in stainless steel containing Tide detergent (36–60 mesh).

cis-4-*t-Butylcyclohexanoic acid.* Reduction of *p*-t-butylbenzoic acid (12·4 g) dissolved in glacial acetic acid (150 ml) over platinum oxide (1·5 g) at room temperature and atmospheric pressure gave a mixture of isomers (12·67 g) containing predominantly the *cis*-isomer (Bekkum & others, 1962).

The *trans*-isomer was removed as the thiourea occlusion complex (Bekkum & others, 1959) and the *cis*-enriched methanolic filtrate poured into water (300 ml) and extracted with hexane (3 $\times$ 200 ml). The combined organic extracts were dried ($MgSO_4$), filtered and evaporated to yield a white solid (8·5 g), m.p. 112–116°. The crude product was purified by sublimation and a material, m.p. 120–121°, was obtained

(2·7 g) (lit. m.p. 116°, Stolow, 1959). Found: C, 72·0; H, 10·5. Calc. for $C_{11}H_{20}O_2$: C, 71·7; H, 10·8%. pK_a = 7·17; 7·25 in 50% ethanol at 25° (lit. pK_a = 6·78; Bekkum & others, 1961).

trans-4-*t-Butylcyclohexanoic acid.* Reduction of *p*-t-butylbenzoic acid (35·6 g) in aqueous sodium hydroxide solution (160 ml, 5% w/v) over Raney nickel at 200° and 100 atmospheres (Bekkum & others, 1962) gave a mixture of isomers containing predominantly the *trans*-isomer. The *trans*-isomer was separated as the thiourea occlusion complex (Bekkum & others, 1959), and the complex decomposed to give *trans*-4-t-butylcyclohexanoic acid (18·0 g), m.p. 173–175° (lit m.p. 176–177°, Stolow, 1959; Bekkum, Verkade & Wepster, 1961). Found: C, 71·3; H, 10·6%. pK_a 6·65; 6·63 in 50% ethanol at 25° (lit. pK_a=6·28; Bekkum & others, 1961).

Methyl cis-4-*t-butylcyclohexanoate. cis*-4-t-Butylcyclohexanoic acid (50 mg) was dissolved in ether (2 ml) and an ethereal solution of diazomethane added until the solution was a persistent yellow colour. After standing overnight at room temperature, the ether was removed and the residue micro-distilled at 10 mm pressure and at a bath temperature of 120° (Cavell & others, 1960).

On gas-phase chromatography (method A, using N_2 as carrier gas at 50 ml/min; injector temperature 220°; column temperature 145°; detector temperature 230°; collector temperature 220°), the product showed a single peak, retention time 2·6 min.

Methyl trans-4-*t-butylcyclohexanoate.* This ester was prepared as for methyl *cis*-4-t-butylcyclohexanoate, but using *trans*-4-t-butylcyclohexanoic acid (100 mg). On gas-phase chromatography (conditions as for methyl *cis*-ester), the material showed only one peak, retention time 3·2 min.

2-*Dimethylaminoethyl* cis-4-*t-butylcyclohexanoate. cis*-4-t-Butylcyclohexanoic acid (1·5 g, m.p. 120–121°) was added to thionyl chloride (10 g) and the mixture allowed to stand at room temperature overnight. The excess of thionyl chloride was removed under reduced pressure at a temperature not exceeding 50° to leave an oily product, which was dissolved in dry benzene (15 ml) and added dropwise during 10 min to 2-dimethylaminoethanol (0·83 g) in benzene (10 ml). The mixture was then heated at a temperature not exceeding 95° for 30 min, cooled and basified with sodium hydroxide solution. The benzene layer was separated and the aqueous phase extracted with ether (3 × 100 ml). The combined organic extracts were dried ($MgSO_4$), filtered and the solvents distilled off. The oily residue was fractionally distilled under reduced pressure, and the fraction, b.p. $_9$ = 168°, collected (1·68 g); ν_{max} (liquid film) 1740 cm^{-1} (C = O), *picrate* (from ethanol), m.p. 155–157°. Found C, 53·4; H, 6·4. $C_{22}H_{32}N_4O_9$ requires C, 53·2; H, 6·45%.

Gas-phase chromatography of the product ester (method B, using N_2 as carrier gas at 30 ml/min; injector temperature 260°, column temperature 230°; detector temperature 260°; collector temperature 290°), showed the product to contain both *cis*- and *trans*-isomers in the ratio of 3:1 (retention times; *cis*-isomer, 15·3 min, *trans*-isomer, 18·3 min). Preparative-scale gas-phase chromatography of the product allowed the isolation of the chromatographically homogeneous *cis*-isomer (0·71 g.).

2-*Dimethylaminoethyl* trans-4-*t-butylcyclohexanoate.* This compound was prepared in a manner similar to that used for the preparation of the *cis*-isomer, but starting with *trans*-4-t-butylcyclohexanoic acid (1·8 g. m.p. 172–175°). The 2-dimethylaminoethyl *trans*-4-t-butylcyclohexanoate was obtained as an oil, b.p. $_{11}$ = 166° (1·52 g); ν_{max} (liquid film) 1741 cm^{-1} (C = O), *picrate* (from ethanol), m.p. 158–159°. Found: C, 53·4; H, 6·0%.

Gas-phase chromatography of the product ester (method B, conditions as for *cis*-isomer) showed the product to contain less than 2% of the *cis*-isomer. A small sample was purified by preparative-scale gas-phase chromatography (method B) and converted to the methiodide, m.p. 227° (c.f. preparation of 2-dimethylaminoethyl *trans*-4-t-butylcyclohexanoate methiodide).

2-*Dimethylaminoethyl* cis-4-*t-butylcyclohexanoate methiodide* (V). To 2-dimethylaminoethyl *cis*-4-t-butylcyclohexanoate (0·70 g, purified by gas-phase chromatography) in ether (20 ml) was added methyl iodide (10 g). The resulting solid product (1·07 g) was collected and recrystallized from absolute ethanol to yield 0·72 g of product as white crystalline plates, m.p. 188°. Found: C, 48·65; H, 8·1. $C_{16}H_{32}INO_2$ requires C, 48·4; H, 8·1%.

2-*Dimethylaminoethyl* trans-4-*t-butylcyclohexanoate methiodide* (IV). This material was made in a manner similar to that used for the preparation of the *cis*-isomer, but using 2-dimethylaminoethyl *trans*-4-t-butylcyclohexanoate (0·8 g, containing approximately 2% of the *cis*-isomer). After recrystallization from absolute ethanol, 1·0 g, of white crystalline plates, m.p. 227°, were obtained. (c.f. preparation of above material using tertiary amino-ester purified by gas-phase chromatography). Found: C, 48·3; H, 8·0%.

2-*Dimethylaminoethyl cyclohexanoate.* This was prepared in a manner similar to that used for the preparation of 2-dimethylaminoethyl *cis*-4-t-butylcyclohexanoate but using cyclohexanoic acid (4·0 g) and thionyl chloride (10 g). The crude cyclohexanoyl chloride (4·3 g) was reacted, in benzene, with 2-dimethylaminoethanol (3·0 g) to yield 2-dimethylaminoethyl cyclohexanoate (3·4 g), b.p. $_{0\cdot1}$ = 78°; ν_{max} (liquid film) 1740 cm^{-1} (C = O). Equivalent weight, found: 201·1; Calculated for $C_{11}H_{12}NO_2$ = 199.

2-*Dimethylaminoethyl cyclohexanoate methiodide* (III). Prepared in a manner similar to that used for the preparation of 2-dimethylaminoethyl *cis*-4-t-butylcyclohexanoate methiodide but starting with 2-dimethylaminoethyl cyclohexanoate (2·26 g). The product was recrystallized from ethanol to yield white plate-like crystals, m.p. 173–174°. Found: C, 42·7; H, 7·2. $C_{12}H_{24}INO_2$ requires C, 42·2; H, 7·0%; $C_{12}H_{124}INO_2{\cdot}\frac{1}{2}C_2H_5OH$ requires C, 42·9; H, 7·4%.

Separation of cis- *and* trans-4-*t-butylcyclohexanol.* 4-t-Butylcyclohexanol (29·5 g) (commercial mixture of isomers) was placed on an activated alumina column (1080 g, Peter Spence, Type H, 100/200 mesh.), using pentane. The column was eluted with pentane, followed by pentane containing increasing amounts of ether. Fractions, each of 200 ml, were collected.

cis-4-t-Butylcyclohexanol (5·9 g) was eluted in fractions 217–281 (pentane 60%, ether 40%). Fractions 312 to exhaustion (pentane 60%, ether 40%) gave *trans*-4-t-butylcyclohexanol (17·42 g).

The products were identified by the following properties: m.p. *cis*-isomer 82–83° (lit. m.p. 81–82°, Winstein & Holness, 1955; 82–82·5°, Stork & White 1956; 82·5–83·5° Eliel & Ro, 1957), *trans*-isomer 79–80° (lit m.p. 78–79°, Winstein & Holness, 1955; 81–82°, Eliel & Ro, 1957).

Infrared spectra (CS_2 solution): *cis*-isomer, characteristic peaks at 1010 and 960 cm^{-1}; *trans*-isomer, characteristic peaks at 1060 and 980 cm^{-1} (lit. *cis*-isomer 1010 and 951 cm^{-1} and *trans*-isomer 980 cm^{-1} in CS_2 solution; Eliel & Rerich, 1960; Eliel & Ro, 1957).

Nmr (CCl_4 solution). *cis*-isomer, 6·10 τ (1 proton-multiplet-equatorial C_1 proton) $W_{\frac{1}{2}} = 6$ Hz *trans*-isomer 6·48–6·80 τ (broad, 1 proton-multiplet-axial C_1 proton) ($W_{\frac{1}{2}}$ = approx. 16 Hz) (lit. *cis*-isomer 6·1 τ, $W_{\frac{1}{2}} = 7$ Hz, *trans*-isomer 6·63 τ, $W_{\frac{1}{2}} =$ 22 Hz, Lemieux, Kulling & others, 1958; Eliel & Gianni, 1962).

Gas-phase chromatography (method C, N_2 as carrier gas at 30 ml/min; injector temperature 180°; column temperature 160°; detector temperature 185°. Retention time: *cis*-isomer 3·12 min, *trans*-isomer 4·4 min.

Ethyl β-dimethylaminopropionate. Dimethylamine (152·2 ml; 103·5 g; 2·3 mole) and ethyl acrylate (250 ml; 230 g; 2·3 mole) were mixed and the mixture allowed to stand at room temperaure for four days. The reaction mixture was fractionally distilled, the ethyl β-dimethylaminopropionate, b.p.$_{21}$ = 73°, being collected. The product was redistilled (b.p.$_{24}$ = 74°); yield 147 g (lit b.p.$_{12}$ = 56–57°, Adamson, 1949), ν_{max} (liquid film) 1747 cm^{-1} (C = O). Equiv. wt, found: 144·5; 145·1; calculated for $C_7H_{15}NO_2 = 145$.

trans-4-*t-Butylcyclohexyl β-dimethylaminopropionate.* Ethyl β-dimethylaminopropionate (4·83 g) and *trans*-4-t-butylcyclohexanol (5·2 g) were dissolved in xylene (30 ml) and the mixture distilled through a fractionating column until the temperature at which the distillate passed over reached 136°. Sodium (0·1 g) was then added to the residual reaction mixture and the ethanol distilled off. This required about 20 min, by which time the temperature recorded at the head of the fractionating column had again risen to 136°. The reaction mixture was heated for a further hour, cooled, an equal volume of water added and the mixture acidified with dilute HCl and then extracted with ether (3 × 150 ml). The aqueous phase was made alkaline with ammonia solution and extracted with ether (3 × 150 ml). The ether extracts were dried ($MgSO_4$), filtered and the solvent distilled to leave an oil, which was fractionally distilled, the *trans*-4-t-butylcyclohexyl β-dimethylaminopropionate, b.p.$_{0·1}$ = 108–109°, being collected (2·37 g), ν_{max} (liquid film) 1740 cm^{-1} (C = O). Equiv. wt, found: 263; 265; Calculated for $C_{15}H_{29}NO_2 = 255$. *Picrate*, an oil. Gas-phase chromatography (method B) showed the presence of less than 0·5% of *cis*-isomer (retention time; *cis*-isomer, 15·2 min; *trans*-isomer, 18·2 min) and the product was considered to be sufficiently pure for the preparation of the methiodide.

cis-4-*t-Butylcyclohexyl β-dimethylaminopropionate.* This compound was prepared in a manner similar to that used for the preparation of the *trans*-isomer, but using *cis*-4-t-butylcyclohexanol (2·6 g) and ethyl β-dimethylaminopropionate (2·4 g). The product was fractionally distilled, the fraction, b.p.$_{0·1}$ = 104°, being collected; yield 1·04 g. ν_{max} (liquid film) 1745 cm^{-1} (C = O). Gas-phase chromatography (method B) showed the product to contain approximately 12% of the *trans*-isomer. The product was then purified by gas-phase chromatography (preparative scale), whereupon 0·42 g of pure material was obtained. *Picrate* (from ethanol), m.p. 145–147°. Found: C, 52·8; H, 6·35. $C_{22}H_{32}N_4O_9$ requires C, 53·2; H, 6·45%.

Cyclohexyl β-dimethylaminopropionate. This compound was prepared in a manner similar to that used in the preparation of *trans*-4-t-butylcyclohexyl β-dimethylaminopropionate, but using cyclohexanol (3·34 g) and ethyl β-dimethylaminopropionate (4·83 g). The product was fractionally distilled, the fraction, b.p.$_{11}$ = 126°, being collected; yield 3·59 g. Equiv. wt, found: 206·5; calculated for $C_{11}H_{21}NO_2 = 199$.

trans-4-*t-Butylcyclohexyl β-dimethylaminopropionate methiodide* (VII). To *trans*-4-t-butylcyclohexyl β-dimethylaminopropionate (1·71 g) (containing less than 0·5% of the *cis*-isomer) in ether (20 ml) was added methyl iodide (10 g). The resulting solid

was filtered and recrystallized from absolute ethanol to yield white crystals, m.p. 192–196°. Found; C, 48·5; H, 8·2 $C_{16}H_{32}INO_2$ requires C, 48·4; H, 8·1%. Nmr (in deuterated DMSO) 5·58–5·92 τ (broad 1 proton multiplet—axial C_1 proton).

cis-4-*t-Butylcyclohexyl β-dimethylaminopropionate methiodide* (VIII). This was prepared in a manner similar to that used for the preparation of the *trans*-isomer, but using *cis*-4-t-butylcyclohexyl β-dimethylaminopropionate (0·37 g, purified by gas-phase chromatography method B). Recrystallization gave a product, m.p. 200–202°, as a white crystalline material. Found: C, 47·6; H, 8·0. $C_{16}H_{32}INO_2$ requires C, 48·4; H, 8·1%; $C_{16}H_{32}INO_2,\frac{1}{2}H_2O$ requires C, 47·3; H, 8·1%. Nmr (in deuterated DMSO) 5·02 τ (1 proton multiplet-equatorial C_1 proton).

Cyclohexyl β-dimethylaminopropionate methiodide (VI). This compound was prepared in a manner similar to that for the *trans*-isomer, but using cyclohexyl β-dimethylaminopropionate (2·16 g). The product was recrystallized from absolute ethanol to give plates, m.p. 146°. Found: C, 42·8; H, 7·4. $C_{12}H_{24}INO_2$ requires C, 42·2; H, 7·0%; $C_{12}H_{24}INO_2,\frac{1}{2}C_2H_5OH$ requires C, 42·85; H, 7·4%.

Methyl β-dimethylaminopropionate methiodide (II). Ethyl β-dimethylaminopropionate (20 g) was dissolved in conc. HCl (30 ml) and refluxed overnight. The solution was evaporated to dryness and the residue dissolved in dry methanol. The solution was saturated with dry HCl gas and then allowed to stand overnight. The reaction mixture was poured into water, the solution made alkaline with solid Na_2CO_3 and extracted with chloroform (3 × 100 ml). The extracts were dried ($MgSO_4$), filtered and the solvent distilled to leave a liquid, which was fractionally distilled, the methyl β-dimethylaminopropionate, b.p. 148–152°, being collected (lit. b.p. 151·5–154°, Halverstadt, Hardie & Williams, 1959). The ester (5 g) was dissolved in methanol (10 ml) and methyl iodide (10 g) added. The precipitated solid was collected and recrystallized from absolute ethanol to yield a product, m.p. 198–200° (lit. m.p. 194–195° Halverstadt & others, 1959). Found: C, 31·1; H, 6·0. $C_7H_{16}NIO_2$ requires C, 30·8; H, 5·9%.

ENZYMIC STUDIES

Bovine erythrocyte acetylcholinesterase (Sigma Chemicals) was used throughout the work.

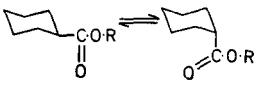
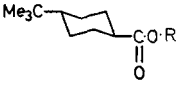
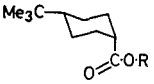
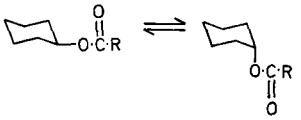
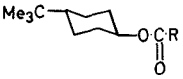
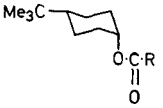
Acetycholine perchlorate (B.D.H.) was used as substrate and the rates of hydrolysis were measured by the pH-stat method, (Alles & Hawes, 1940), using an automatic titrator (type TTTIC), equipped with a recorder (SBR2C) and syringe burette (SBU1A) (Radiometer, Copenhagen).

All incubations were carried out in a total volume of 25 ml of enzyme solution previously made 0·04 M in $MgCl_2$ and 0·05 M in NaCl. The incubations were carried out in jacketted vessels at 25 ± 0·1° and the pH maintained at 7·4 by the addition of 0·02 N NaOH. A CO_2-free nitrogen atmosphere was maintained throughout the experiments.

All inhibitors were pre-incubated with the enzyme for 3 min and the reaction started by the addition of acetylcholine solution. The velocity of the reaction was calculated from the average slope of the recording during the second and third minutes of the incubation. The velocities were all corrected for aqueous hydrolysis.

The K_m value for acetylcholine, under these conditions, was found to be $4{\cdot}45 \times 10^{-4}$. The K_I values for the inhibitors were calculated from Lineweaver-Burk plots (Lineweaver & Burk, 1934), using a four-fold range of substrate concentration and an

Table 1. *Enzyme-inhibitor dissociation constants* (K_1) *of acylcholines and β-trimethylammoniopropionic acid derivatives*

	Compound	Ki	Nature of inhibition
	$R=[CH_2]^{\oplus}N\ Me_3\ I^{\ominus}$		
I	Me·CO·O·R	$K_m = 4{\cdot}45 \times 10^{-4}$ $V_{max} = 2{\cdot}26 \times 10^{-6}$ mole/min	Substrate
II	Me·O·CO·R	$3{\cdot}95 \times 10^{-3}$	Competitive
III		$1{\cdot}8 \times 10^{-3}$	Competitive
IV		$1{\cdot}5 \times 10^{-4}$	Competitive
V		$1{\cdot}9 \times 10^{-4}$	Competitive
VI		$2{\cdot}3 \times 10^{-3}$	Competitive
VII		$1{\cdot}3 \times 10^{-3}$	Competitive
VIII		$1{\cdot}3 \times 10^{-4}$ (low concentrations $1{\cdot}4$–$2{\cdot}0 \times 10^{-4}$ mole) (At higher concentrations shows mixed inhibition).	Competitive

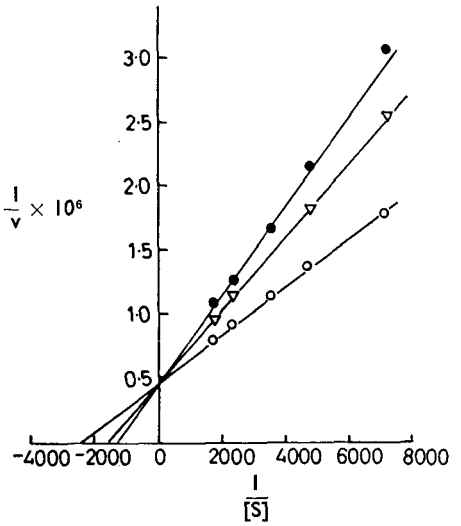


FIG. 3. Competitive Inhibition of acetylcholinesterase by two concentrations of 2-dimethylaminoethyl *cis*-4-t-butylcyclohexanoate methiodide. ●[I] = $1{\cdot}48 \times 10^{-4}$M ▽[I] = $8{\cdot}30 \times 10^{-5}$M. ○[I] = O.

approximately two-fold range of inhibitor concentration. The K_I values were computed from the equation:

$$\text{Gradient} = \frac{K_m\left(1 + \frac{[I]}{Ki}\right)}{V_{max}}.$$

The computed results are shown in Table 1 and a typical Lineweaver-Burk plot obtained for one of the inhibitors in Fig. 3.

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