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Effects of some polymethylene bis(hydroxyethyl) dimethylammonium compounds on acetylcholine synthesis

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Summary

1. The effects of some polymethylene bis(hydroxyethyl)dimethylammonium compounds have been studied on acetylcholine (ACh) synthesis and as substrates for choline acetyltransferase (ChAc).

2. The decamethylene analogue (C_{10}-dichol) inhibited ACh synthesis by mitochondrial (P_2) fractions of guinea-pig cerebral cortex suspended in Tris buffer but had no effect on ACh synthesis by P_2 fractions when the membranes surrounding the ChAc enzyme were broken down by homogenization in Triton X-100.

3. C_{10}-Dichol was acetylated by ChAc almost to the same extent as choline. The initial rate of acetylation, at a concentration of 10^{-3}M, was more rapid than for choline; however, the apparent Michaelis-Menten constant for C_{10}-dichol was greater than the K_m for choline, showing a lower affinity for the ChAc enzyme.

4. All the dicholine compounds were acetylated to some extent by ChAc and the rate of acetylation increased with an increase in the length of the methylene chain between the two quaternary nitrogen atoms in each dicholine molecule.

5. The rate of acetylation of the dicholine compounds paralleled the activity of these analogues at the prejunctional site at the neuromuscular junction. The possibility is suggested that part of the pharmacological activity of these compounds may be due to their incorporation into cholinergic nerve endings followed by acetylation by ChAc before subsequent release as a false transmitter.

Introduction

Many choline analogues influence cholinergic transmission by interfering with the acetylation of choline in the nerve endings (Bowman, Hemsworth & Rand, 1967). The most active hemicholinium compound, HC-3 (Schueler, 1960) prevents the synthesis of acetylcholine (ACh) by organized brain tissue but is without effect after disruption of the subcellular particles (MacIntosh, Birks & Sastry, 1956). Triethylcholine (TEC) also inhibits ACh synthesis (Bowman & Hemsworth, 1965a) and it has been suggested that both HC-3 (Gardiner, 1961) and TEC (Bull & Hemsworth, 1965) exert their *in vivo* effect by inhibiting the uptake of choline into the nerve ending.

Several polymethylene bis(hydroxyethyl)dimethylammonium (dicholine) salts also produce a blockade of neuromuscular transmission (Barlow & Zoller, 1962; Bowman & Hemsworth, 1965b; Bowman *et al.*, 1967) which showed characteristics of block produced by inhibition of ACh synthesis. The decamethylene analogue (C_{10}-dichol) was the most potent bis-quaternary ammonium in the dicholine series of compounds and produced an initial blockade of neuromuscular transmission which showed the characteristics of block by depolarization and a secondary longer lasting block with the characteristics of block produced by inhibition of ACh synthesis. More recently, Hemsworth, Darmer & Bosmann (1971) showed that these dicholines inhibited the uptake of choline-^{14}C into synaptosomes and synaptic vesicles, and C_{10}-dichol was the most active compound in this respect. Several workers have demonstrated the ability of choline acetyltransferase (ChAc) to acetylate various analogues of choline (Dauterman & Mehrotra, 1963; Hemsworth & Morris, 1964; Hemsworth & Smith, 1970a and b), and Burgen, Burke & Desbarats-Schonbaum (1956) investigated the ability of ChAc from rat brain to acetylate 3 dicholine compounds; they found that only the decamethylene analogue (C_{10}-dichol) was acetylated.

This report investigates further the mechanism of action of these dicholines on acetylcholine synthesis and demonstrates the ability of ChAc to enzymically acetylate the compounds.

Methods

Particle isolation

Synaptosomal fractions of guinea-pig brain were prepared by the method of Whittaker, Michaelson & Kirkland (1964), as described previously (Bosmann & Hemsworth, 1970). The fractions of brain particles were freshly prepared and experiments were performed immediately after fractionation and isolation. All fractionations were carried out at 0–4° C.

Two types of synaptosomal fractions were used. One fraction was suspended in 0.1 M Tris buffer at pH 7·6 (1:5 v/v dilution) and 10 μl of this suspension was used in the incubation. A second fraction was suspended in 0·1% Triton X-100 (1:5 v/v dilution) and homogenized, thirty strokes, with a Ten Broeck homogenizer; 10 μl of this suspension was used for the incubation procedure. In general, each fraction contained between 10 and 30 mg protein per ml of synaptosomal fraction.

ChAc enzyme

Fresh rat or guinea-pig cerebral cortices were homogenized in 0·1% Triton X-100 containing 200 mM KCl (Potter, Glover & Saelens, 1968) in a Virtis homogenizer. The homogenate was centrifuged at 45,000 *g* for 1 h and the supernatant fractionated with ammonium sulphate. The fraction precipitating between 20 and 30% (w/v) ammonium sulphate was collected by centrifugation, dissolved in 0·1% Triton X-100 containing 200 mM KCl and dialysed to remove the ammonium sulphate. The enzyme prepared in this manner was used immediately or was stored at −20° C. Before use the stored enzyme was passed through a Sephadex G25 column to remove any choline.

Incubation system

ChAc activity was determined by a modification of the procedure described by McCaman & Hunt (1965). Where acetylation of the dicholine compounds was to be determined 2 μl ChAc or synaptosomal fraction were placed in a plastic microfuge tube (Beckman) together with 2 μl dicholine compound and 2 μl water. Twenty microlitres of a buffer substrate solution containing acetyl-l-^{14}C-Co A (30 mCi/mmol), $5{\cdot}0 \times 10^{-5}$M; $MgSO_4$, 5×10^{-3}M; NaCl, 3×10^{-2}M; physostigmine sulphate, 2×10^{-4}M; disodium EDTA, 10^{-4}M; albumin, 0·05 mg/ml; and potassium phosphate, (pH 7·7) $1{\cdot}5 \times 10^{-2}$M, were then added to the microfuge tube, which was incubated for the required period of time. Incubations were carried out at 37° C unless stated otherwise. The rates of acetylation of these compounds were compared with the rates of acetylation of choline when 2 μl choline was substituted for the dicholine compounds in the incubation system.

When inhibition of acetylcholine synthesis was being determined, 2 μl of synaptosomal fraction were incubated with 2 μl dicholine compound and 2 μl choline together with 20 μl of the buffer substrate solution for 1 h at 37° C.

Isolation of acetylated product

After incubation the microfuge tube was placed in ice and 2 μl of a solution containing 50% TCA and choline 2×10^{-1}M was added to precipitate the enzyme. The tubes were then spun in a microfuge and 15 μl of the supernatant were removed and added to 50 μl ammonium reineckate solution (1:3 dilution of a saturated solution in 0·5 N HCl) and allowed to precipitate for 15 minutes. This precipitated solution was centrifuged and the precipitate washed with 50 μl 0·2 N HCl. After recentrifugation the precipitate was dissolved in 100 μl acetone and transferred to a Whatman glass fibre paper, and the radioactivity was determined by counting in a liquid scintillation counter (Nuclear Chicago).

Protein

Total protein was determined by the method of Lowry, Rosebrough, Farr & Randall (1951).

Materials. Acetyl-l-^{14}C-coenzyme A (30 mCi/mmol) was purchased from New England Nuclear Corporation, Boston, Mass.

Results

Effect on ACh synthesis

The effect of C_{10} dicholine on the inhibition of ACh synthesis by the mitochondrial fraction of rat brain is shown in Table 1. When the mitochondrial fraction was suspended in 0·1 M Tris buffer (pH 7·6), C_{10}-dichol, 10^{-3}M, produced a 26% inhibition of ACh synthesis, whereas 10^{-6}M C_{10}-dichol had no effect. When the mitochondrial fraction was extracted with 0·1% Triton X-100 before incubation, the same concentrations of C_{10}-dichol produced no inhibition (Table 1). Increasing the choline concentration from 5×10^{-3}M to 5×10^{-2}M in the Tris incubation system decreased the inhibition of ACh synthesis by 10^{-3}M C_{10}-dichol. Concentrations of C_{10}-dichol above 10^{-2}M in both Tris and Triton X-100 fractions caused an increase

in the c.p.m. ^{14}C acetylated product above control values. This effect was probably due to acetylation of both the C_{10}-dichol and the choline. It is possible that in the incubations where the concentration of C_{10}-dichol was 10^{-6}–10^{-3}M, acetylation of both C_{10}-dichol and choline occurs; however, the extent of acetylation of this compound in the presence of choline was not determined in these experiments.

The effect of extraction of the P_2 fraction with Triton X-100 was similar to the effect of ether treatment (Bull & Hemsworth, 1965) and freezing and thawing (Hebb, 1963), and Table 2 illustrates this effect. The P_2 mitochondrial fraction was suspended in 0·1 M Tris (pH 7·6) and some of this fraction incubated to determine ACh synthesis. A portion of the P_2 in Tris was resuspended in an equal volume of 0·2% Triton X-100 and homogenized with thirty strokes of a Ten Broeck homogenizer. This P_2 fraction in a final concentration of 0·1% Triton X-100 was then incubated to determine ACh synthesis. Treatment of the P_2 fraction with Triton X-100 increased the enzyme activity 2-fold, and this effect is probably due to the breaking of membranes surrounding the ChAc.

These results illustrate that C_{10}-dichol inhibits ACh synthesis when the membrane surrounding the enzyme ChAc is intact but has no effect when the membranes are broken down. This effect of C_{10}-dichol is similar to that described for TEC (Bull & Hemsworth, 1965) and suggests that C_{10}-dichol is acting by preventing transport of choline across the synaptosomal (P_2) membrane to the site of acetylation by ChAc.

Acetylation of dicholines

C_{10}-Dichol was substituted for choline in the incubation system. Table 3 shows the acetylation of both compounds expressed as (c.p.m. of ^{14}C acetylated product/mg protein)/hour, and the results show that C_{10}-dichol was acetylated almost to the same extent as choline. Table 3 also shows that the enzyme preparation contained only

TABLE 1. *Effect of C_{10}-dichol on ACh synthesis by mitochondrial (P_2) fractions of guinea-pig brain*

		P_2 in Tris		P_2 in Triton X-100	
	Final conc. (M)	ACh synthesized (nmol/mg protein)/h	% of control	ACh synthesized (nmol/mg protein)/h	% of control
Control		8·22	100	15·17	100
C_{10}-dichol	10^{-3}	6·07	74	15·01	99
,,	10^{-4}	6·98	85	15·09	100
,,	10^{-5}	7·72	94	15·49	102
,,	10^{-6}	8·30	101	14·80	98

Final concentrations of choline, 5×10^{-3}, and ^{14}C-acetyl-CoA, $3{\cdot}8\times10^{-5}$M. Incubation was carried out at 37° C for 1 hour. The incubation system was as described in **Methods**. Data are the mean of four experiments. ACh synthesized was calculated from the c.p.m. ^{14}C acetylated material.

TABLE 2. *Synthesis of ACh by mitochondrial (P_2) fractions of guinea-pig cortex in* 0·1 M *Tris and* 0·1% *Triton X*-100

Final choline conc.	P_2 in 0·1 M Tris, pH 7·6 ACh synthesized (nmol/mg protein)/h	P_2 in 0·1 M Tris, pH 7·6, and homogenized with an equal volume of 0·2% Triton X-100 ACh synthesized (nmol/mg protein)/h	$\left(\frac{\text{Activity of enzyme } P_2 \text{ in Triton X-100}}{P_2 \text{ in Tris}}\right)$
5×10^{-3}M	6·86	13·42	1·96
5×10^{-5}M	4·96	9·96	2·01

The incubation was carried out at 37° C for 1 h with the system as described in **Methods**. Final concentration of ^{14}C-acetyl-CoA, $3{\cdot}8\times10^{-5}$M. Data are the mean of three experiments.

a small amount of endogenous choline ; when no choline substrate was added only 3% ACh was synthesized compared with the amount of ACh synthesized when 10^{-4}M choline was used as substrate. From a graph of the velocity of reaction as a function of the concentration for choline it can be calculated that the concentration of endogenous choline in the ChAc enzyme preparation is 2×10^{-6}M and this amount of endogenous choline would not interfere with the dicholine enzyme substrate reaction.

Figure 1 shows the time course for the rate of acetylation of C_{10}-dichol and choline using ChAc from guinea-pig brain. C_{10}-Dichol was initially acetylated at a more rapid rate than choline. The rate of acetylation of choline was linear for 20 min, whereas the rate of acetylation of C_{10}-dichol was only linear over a period of 10 minutes.

Figure 2 shows a graph of the velocity of reaction as a function of the substrate concentration for C_{10}-dichol and illustrates a typical hyperbolic curve for an enzyme

TABLE 3. *Acetylation of choline and C_{10}-dichol by ChAc from guinea-pig cortex*

Substrate	Final conc. (M)	(c.p.m. of ^{14}C-acetylated product $\times 10^{-6}$/mg protein)/h	% Acetylation with reference to choline at same concentration
Choline	10^{-3}	1·48	
	10^{-4}	0·602	
C_{10}-Dichol	10^{-3}	1·39	94
	10^{-4}	0·543	90
None	—	0·018	

The final concentration of ^{14}C-acetyl-CoA in the incubation system was $3{\cdot}8 \times 10^{-5}$M. Incubation was carried out at 37° C for 1 hour. The incubation system was as described in **Methods**. Data are the means of four experiments.

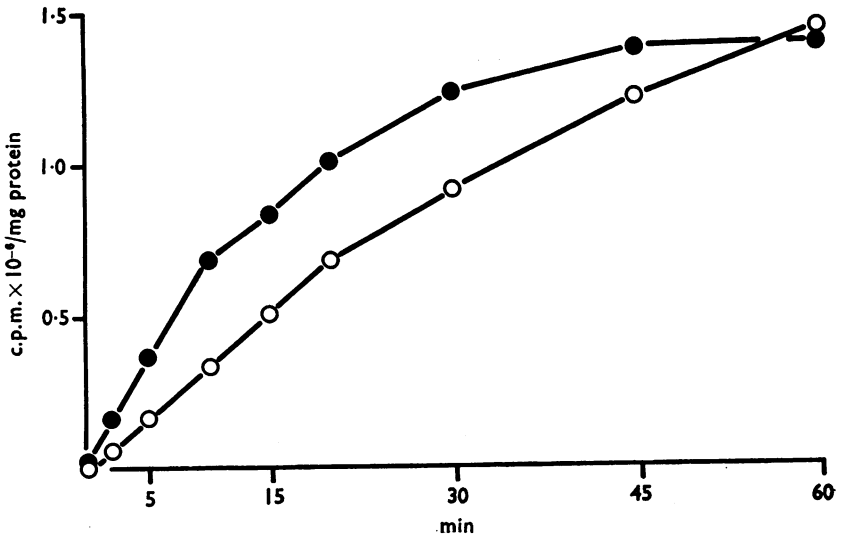

FIG. 1. Time curve for the acetylation of choline (○——○) and C_{10}-dichol (●——●) at a final substrate concentration of 10^{-3}M by ChAc from guinea-pig brain. The final concentration of ^{14}C-acetyl-CoA in the incubation system was $3{\cdot}8 \times 10^{-5}$M. Incubation was carried out at 37° C. Complete system as given in **Methods**.

substrate reaction. At substrate concentrations higher than those shown in Fig. 2, both choline and C_{10}-dichol exhibited substrate inhibition. This effect has been reported previously for both choline and other substrates (Hemsworth & Morris, 1964; Hemsworth & Smith, 1970a). Figure 3 shows the rates of acetylation of choline and C_{10}-dichol over a wide range of concentrations of substrate from 10^{-6} to 10^{-1}M and illustrates the substrate inhibition which occurs.

ChAc is a two substrate enzyme and each substrate affects the affinity of the enzyme for the other, but at a constant concentration of acetyl-CoA an apparent

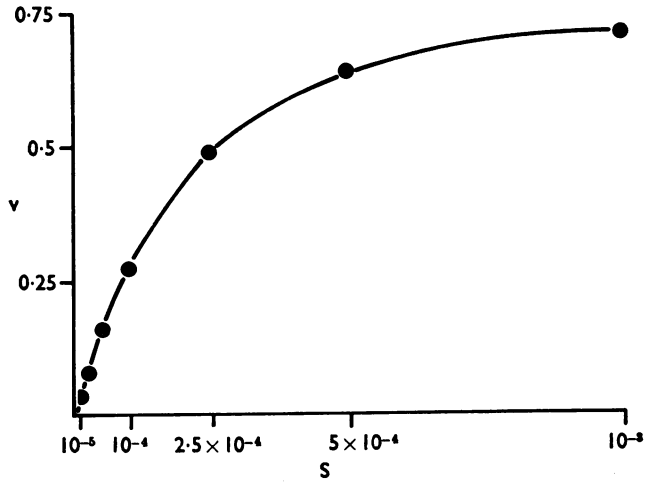

FIG. 2. Rate of acetylation of C_{10}-dichol by ChAc from guinea-pig cerebral cortex. (v), Velocity of reaction ((c.p.m.$\times 10^{-6}$ of ^{14}C-acetylated product/mg protein)/10 min); (S), molar concentration of C_{10}-dichol substrate. To obtain initial rates of acetylation incubations were carried out for 10 min at 37° C. Final concentration of ^{14}C-acetyl-CoA was $3{\cdot}8\times10^{-5}$M. Complete incubation system as given in **Methods**.

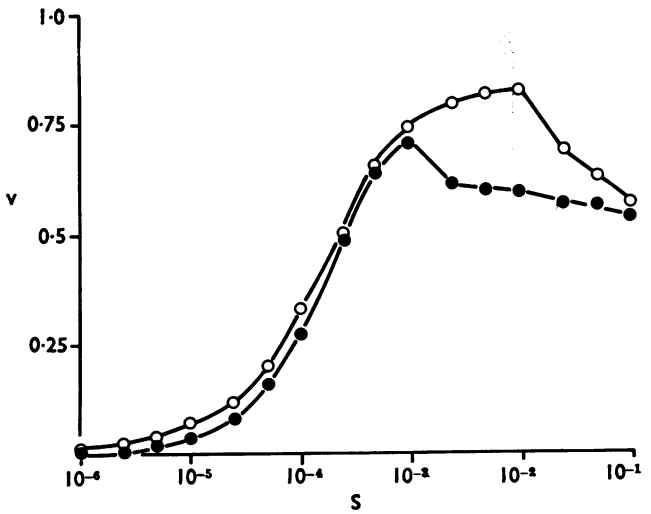

FIG. 3. Rate of acetylation of choline (○——○) and C_{10}-dichol (●——●) by ChAc from guinea-pig cerebral cortex. (S), Molar concentrations of substances; (v), velocity of reaction ((c.p.m.$\times 10^{-6}$ of ^{14}C-acetylated product/mg protein)/10 min). Incubation was for 10 min at 37° C. Final concentration of ^{14}C-acetyl CoA was $3{\cdot}8\times10^{-5}$M. Complete incubation system as given in **Methods**.

Michaelis-Menten constant can be determined. The apparent Michaelis-Menten constants for choline and C_{10}-dichol were derived from Lineweaver & Burk (1934) graphs (Fig. 4). The apparent K_m for choline was similar to that obtained by previous workers (Hemsworth & Smith, 1970a and b); C_{10}-dichol had a higher apparent K_m, showing a lower affinity for ChAc than choline. C_{10}-Dichol has two sites available for acetylation, and it is not known to what extent either or both groupings take place in the enzyme-substrate reaction.

Bowman *et al.* (1967) showed that the C_5- and C_6-dicholines also had a prejunctional action; C_4-dichol produced a postjunctional non-depolarizing block, and C_3-

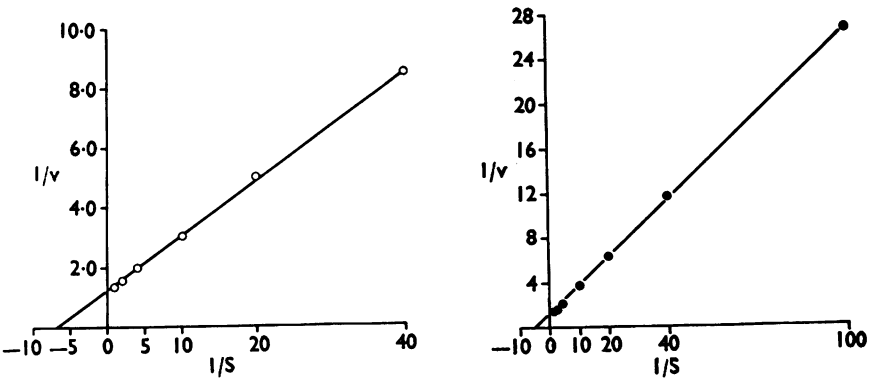

FIG. 4. Derivation of Michaelis-Menten constants by the method of Lineweaver & Burk (1934). The incubation conditions were as described in the text. (s), Concentration of substrate, mM; (v), velocity of reaction ((c.p.m. $\times 10^{-6}$ of ^{14}C-acetylated product/mg protein)/10 min). ○, Choline; ●, C_{10}-dichol. The apparent Michaelis-Menten constants (K_m) (where the concentration of ^{14}C-acetyl-CoA in the incubation system was $3{\cdot}8 \times 10^{-5}$M) were: choline $1{\cdot}5 \times 10^{-4}$M; C_{10}-dichol $2{\cdot}4 \times 10^{-4}$M.

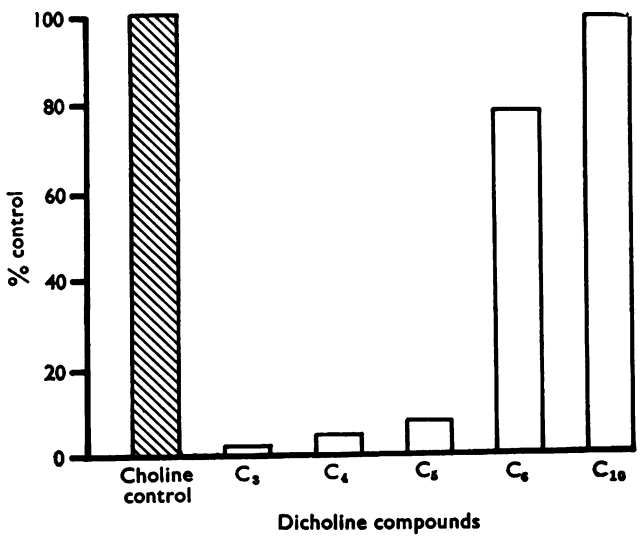

FIG. 5. Rate of acetylation of choline and some dicholine compounds by ChAc from guinea-pig cerebral cortex. The incubation was carried out for 1 h at 37° C. The final substrate concentration of choline and each dicholine compound was 10^{-3}M. Complete incubation system as described in the **Methods.** Dicholine compounds are C_3, tri-; C_4, tetra-; C_5, penta-; C_6, hexa-; and C_{10}, decamethylene bis(hydroxyethyl)dimethylammonium.

dichol was inactive as a neuromuscular blocking drug. Hemsworth *et al.* (1971) demonstrated that all these dicholines inhibited the incorporation of choline-^{14}C into synaptosomal and synaptic vesicle fractions. C_{10}-Dichol was the most effective analogue in this respect; however, C_3-dichol was more active than C_6-dichol. It seemed of interest to investigate the acetylation of these compounds by ChAc. Figure 5 shows the results obtained. C_{10}-Dichol is acetylated almost to the same extent as choline (see also Fig. 1 and Table 3). C_6-Dichol was acetylated 78% in comparison with choline. The remaining dicholine compounds were acetylated less than 10%. C_5-Dichol was acetylated 7·6%, C_4-dichol 4·8%, and C_3-dichol 2·1%, demonstrating that a shortening of the methylene chain length between the two quaternary nitrogens in each dicholine molecule caused a decrease in the rate of acetylation.

Discussion

The results presented here demonstrate that the polymethylene bis(hydroxyethyl) dimethylammonium salts can affect acetylcholine synthesis by two actions, an inhibition of choline transport to the intracellular sites of its acetylation and by the compounds themselves being acetylated by the enzyme ChAc.

The action of C_{10}-dichol in reducing ACh synthesis by mitochondrial fractions of guinea-pig brain is similar to the experiments described by Bowman & Hemsworth (1965b) although these workers, using 10^{-2}M C_{10}-dichol, obtained a greater percentage inhibition of ACh synthesis. In these experiments ACh synthesis was determined by measuring ^{14}C-acetylated product and it is likely that when C_{10}-dichol and choline are present in the incubation mixture both ^{14}C-acetyl C_{10}-dichol and ^{14}C-acetylcholine are formed. Bowman & Hemsworth (1965b) estimated ACh synthesis by a biological assay procedure using the dorsal leech muscle. Acetyl C_{10}-dichol is very much less active as a nicotinic agonist than acetylcholine (Barlow, 1955) and therefore the small quantitative differences in these results compared with those of Bowman & Hemsworth (1965b) can be explained by the different methods used for estimating ACh synthesis.

The effects of C_{10}-dichol on ACh synthesis by the fractions treated with Tris and Triton are similar to the effect of TEC on the fractions treated with ether and those not treated with ether (Bull & Hemsworth, 1965). My results show that C_{10}-dichol has an action similar to that of TEC in blocking choline transport.

The neuromuscular blocking properties of the dicholine compounds have previously been described. Barlow & Zoller (1962) showed a marked postjunctional action, and Barlow (1955) demonstrated the weak anticholinesterase activity of some of these compounds *in vitro*. Bowman & Hemsworth (1965b) showed that in addition to a postjunctional neuromuscular blocking action, C_{10}-dichol also showed characteristics of a prejunctional effect and my experiments show that C_{10}-dichol is acetylated by ChAc.

Burgen *et al.* (1956) found C_{10}-dichol was acetylated by ChAc, but these workers did not show any acetylation of the C_3- and C_5-dicholines. The rates of acetylation of these substrates were determined by the disappearance of acetyl-CoA during the incubation and this method is not as sensitive as the radiochemical method used in these experiments where a small acetylation of both C_3- and C_5-dichol was obtained. Also Burgen *et al.* (1956) compared the acetylation rates of these dicholine com-

pounds with rates for choline at only one concentration of substrate. Hemsworth & Smith (1970a) demonstrated the importance of using a wide range of substrate concentrations and of calculating apparent K_m values when comparing the rates of acetylation of different substrates for ChAc. My results also show that because of the substrate inhibition which occurs at concentrations above 10^{-2}M it is possible to get different rates of acetylation with reference to acetylation of choline if only one substrate concentration is used (see Fig. 3).

These experiments show that all these dicholine compounds can be acetylated by ChAc although C_{10}-dichol has a higher rate of acetylation than the other dicholine derivatives. The greater rate of acetylation with increasing methylene chain length parallels the activity of these compounds at the prejunctional site of the neuromuscular junction (Bowman & Hemsworth, 1965b; Bowman *et al.*, 1967). It is possible that *in vivo* these compounds may be incorporated into the nerve ending and subsequently released as false inactive neurotransmitters. The non-specificity of enzymes involved in the synthesis of both the cholinergic and adrenergic transmitters has led to a hypothesis of false transmitter release. Muscholl & Maître (1963) were the first workers to demonstrate that a false transmitter substance could be released from adrenergic nerve endings and since that time several phenylethylamine derivatives (for example octopamine, metaraminol) have been shown to have a similar action (Kopin, 1968). Bowman & Rand (1961) suggested that at cholinergic synapses TEC might be incorporated into nerve endings and subsequently released as a false transmitter. Hemsworth & Bosmann (unpublished observation) have recently shown that ^{3}H-TEC is incorporated into isolated synaptosomes and synaptic vesicles from guinea-pig cortex and therefore acetylation of TEC and subsequent release may contribute to its pharmacological effect. It may be that a similar situation exists with these dicholine compounds.

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