INHIBITION OF ACETYLCHOLINESTERASE BY CETYLTRIMETHYLAMMONIUM BROMIDE

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SUMMARY

Employing the Warburg manometric method of assay, the effect of cetyltrimethylammonium bromide on acetylcholinesterase activity was studied. Cetyltrimethylammonium bromide was found to possess anticholinesterase properties; kinetic analysis together with the results from dialysis experiments show that the inhibition is mixed, there being both reversible and irreversible aspects of the total inhibition; the extent of the irreversible inhibition increases with increasing time of incubation of the enzyme with cetyltrimethylammonium bromide. It is concluded that it is unlikely that the inhibition of acetylcholinesterase by cetyltrimethylammonium bromide underlies the previously observed *in vivo* blockade of impulse conduction in whole nerve.

INTRODUCTION

It has been demonstrated that nerves which were appropriately treated with CTMB are rendered especially susceptible to reversible conduction block by a variety of externally applied lipid-insoluble quaternary ammonium ions[1]. If the conditioning exposure to CTMB is too long, however, then complete irreversible conduction block of whole nerve occurs due to the CTMB itself; exposure to CTMB for shorter periods or to lower concentrations produces partial block (*i.e.* total block of a portion of the constituent fibers in a nerve trunk) which is also irreversible[2]. Several questions arise: What is the action of CTMB on nerves which renders them so susceptible to reversible block by curare, acetylcholine, prostigmine, etc.? What is the cause of the irreversible conduction block produced by CTMB itself? Are the two phenomena separate and distinct or do they simply represent two aspects of a continuum of action of CTMB?

This paper deals with an examination of the effect of CTMB on acetylcholinesterase in order to assess one possible mechanism of CTMB-induced block of impulse conduction in nerve. It is reasonable to suspect that CTMB might possess anticholinesterase activity, since many quaternary ammonium compounds are inhibitors of the enzyme. Certainly if CTMB is not an anti-cholinesterase, then the mechanism whereby it produces conduction block cannot be due to inhibition of this enzyme.

Abbreviations: CTMB, cetyltrimethylammonium bromide; DFP, diisopropylfluorophosphate.

If, on the other hand, CTMB is an anticholinesterase, the mechanism of block could be the inhibition of the enzyme, if CTMB is an irreversible inhibitor. It is unlikely, however, that the mechanism involves inhibition of the enzyme if CTMB is a reversible inhibitor, for conduction block produced by CTMB is irreversible.

METHODS

Employing the Warburg manometric technique of AMMON[3], the rate of hydrolysis of acetylcholine was determined by measurement of the CO_2 evolved from a bicarbonate buffer at 37°. Readings were taken every 15 min after gassing with 95 % N_2 and 5 % CO_2 and thermal equilibration. The fluid volume of each flask was 3 ml and the final reaction mixtures comprised NaCl (0.13 M), $MgCl_2$ (0.04 M), $NaHCO_3$ (0.025 M), gelatin (0.1 %, w/v), and 1.25 units of a preparation of acetylcholinesterase derived from bovine erythrocytes (Nutritional Biochemicals Corp.). Acetylcholine chloride (Merck) was used as the substrate, and its final flask concentration was varied from $4.59 \cdot 10^{-3}$ to $1.15 \cdot 10^{-3}$ M. Final CTMB concentrations were varied from 10^{-4} to $5 \cdot 10^{-5}$ M.

RESULTS AND DISCUSSION

Table I shows some of the results in terms of per cent inhibition; similar data on two other anticholinesterases have been included for comparison. It is apparent that CTMB inhibits acetylcholinesterase, though its potency on a molar basis is far less than for such agents as prostigmine, DFP, and a variety of other tertiary and quaternary ammonium and organophosphorus compounds.

TABLE I

INHIBITION OF ACETYLCHOLINESTERASE

Inhibitor	*Concn.* ($M \times 10^{-6}$)	*Acetylcholine concn.* ($M \times 10^{-3}$)	*Inhibition* (%)
CTMB	5.0	4.59	29
CTMB	6.0	4.59	34
CTMB	6.0	1.84	51
CTMB	9.0	4.59	68
CTMB	9.0	1.84	75
CTMB	10.0	3.06	84
Prostigmine*	0.1	3.30	50
DFP*	0.125	3.30	50

* From AUGUSTINSSON AND NACHMANSOHN[4] employing acetylcholineesterase prepared from the electric organ of *Electrophorus electricus*.

Fig. 1 is an analysis of the kinetic data by the procedure of LINEWEAVER AND BURK[5]. As indicated by the convergent trend of the three curves at the ordinate, the relative degree of inhibition decreases as the ratio of acetylcholine/inhibitor increases. However, the maximum velocity (from the $1/V$ intercept) is less for the CTMB curves than for the uninhibited reaction, and it is least for the higher concentration of CTMB ($9 \cdot 10^{-5}$ M). Since the three curves do not give the same maximum velocity (converge to the same $1/V$ intercept) it appears that the inhibition of acetyl-

cholinesterase by CTMB is not a case of simple reversible inhibition. An extrapolation of the uninhibited and inhibited curves to the $1/S$ intercept reveals that they do not converge there either. According to kinetic analysis of enzyme inhibition[6], this latter point (failure of all three curves to converge at $-1/K_m$) means that the inhibition of acetylcholinesterase by CTMB is not a case of simple irreversible inhibition. Thus the kinetic analysis, utilizing the technique of LINEWEAVER AND BURK, indicates that the inhibition studied is neither simply reversible nor simply irreversible.

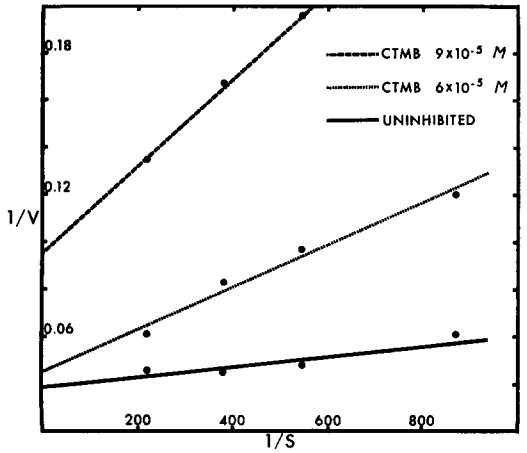

Fig. 1. Inhibition of acetylcholinesterase by CTMB. $1/V$ is the reciprocal of μl of CO_2/15 min (corrected for autolysis). $1/S$ denotes the reciprocal of the molarity of acetylcholine.

Dialysis experiments were conducted in order to amplify upon the apparent simultaneous occurrence of reversible and irreversible inhibition of acetylcholinesterase by CTMB. Four aliquots of equipotent enzyme solution were employed: (a) the uninhibited control, containing no CTMB, (b) the inhibited control, containing 10^{-4} M CTMB and not dialyzed, (c) the short-term incubated experimental, containing 10^{-4} M CTMB and incubated 10 min at room temperature prior to dialysis (15 h), and (d) the long-term incubated experimental, containing 10^{-4} M CTMB and incubated 10 min at room temperature and then 19 h at 10° prior to dialysis (20 h). Data from the manometric analysis of enzyme activity reveal that extensive dialysis restored 78 % of the activity of the enzyme, (c), which had been very briefly exposed to CTMB and 38 % of the activity of aliquot (d) which had been incubated with CTMB for a prolonged period.

The kinetic analysis of the initial experiments together with the results of the dialysis experiments support the conclusion that the inhibition of acetylcholinesterase by CTMB is of the mixed type, there being both reversible and irreversible inhibition occurring simultaneously.

The results, showing a mixed type of inhibition, might have been anticipated from a consideration of the CTMB molecule. The molecule consists of a cationic, quaternarized ammonium "head" and a long-chain, aliphatic cetyl "tail"—found in many surface-active agents possessing cytolytic and germicidal properties[7]. WILSON[8] has demonstrated the reversible, competitive inhibition of acetylcholinesterase by simple alkylated ammonium ions; thus, CTMB might have been expected to exert

a degree of reversible inhibition of the enzyme, as our kinetic analysis suggests and the dialysis experiments verify. On the other hand, the presence of the cetyl group might have led us to predict an element of irreversible inhibition by virtue of the known protein denaturing action of many surface-active agents[9].

Regardless of the intimate details of the irreversible* portion of the inhibition of acetylcholinesterase by CTMB, the pertinent fact is that a substantial degree of the total inhibition can be reversed by dilution or dialysis. This fact seems incompatible with the possibility that the complete, irreversible block of conduction in whole nerve by CTMB, *per se*, could be due solely to inhibition of the endogenous axonal acetylcholinesterase; for the nerve block, if not completely, is nearly irreversible**.

Thus the mechanism whereby CTMB blocks impulse conduction must be something more than the mere inhibition of acetylcholinesterase. We are currently examining, electronmicroscopically, the morphological effects of CTMB, *per se*, on nerves and their myelin investments. It has already been speculated that CTMB (and, presumably, other surface-active agents) disrupts cell membranes in a nerve trunk[2]. It has been suggested that Schwann investments are first attacked and then the neuronal membranes themselves. The first sites of attack are associated with the conditioning of nerves to subsequent block by externally applied lipid-insoluble quaternary ammonium ions, and the attack on the neuronal membranes is associated with the eventual conduction block produced by CTMB itself. Presumably the reversible block by acetylcholine, curare, etc., in CTMB-treated nerves is due to a reversible reaction with the membrane receptors.

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* By irreversible we connote a non-reversibility of the enzyme activity by conventional dilution and dialysis techniques.

** In our experience with nerves (frog sciatic) the block is not reversed after 2 h of washing. However, there is the possibility that there could be a reversal of up to 6% that might go undetected, due to a limitation in our techniques of action potential recording[1,2]. This possible 6% reversal is to be compared with the 35-40% restoration of enzyme activity after dialysis following incubation of the enzyme with CTMB for periods of time far exceeding the times of exposure of whole nerves to CTMB.