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Tremorine and oxotremorine effects on acetylcholinesterase and choline acetylase from rat brain

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INJECTION of Tremorine (1, 4-dipyrrolidino-2-butyne) or its active metabolite oxotremorine (1-(2-pyrrolidono) - 4-pyrrolidino-2-butyne), has recently been shown to raise the level of acetylcholine (AcCh) in the brain of rats by as much as 40%.[1,2] The timecourse of the increase corresponds roughly to that of the tremor produced by the drugs. The mechanism of the effect on brain AcCh is not clear. However, two possibilities are obvious; inhibition of the enzymic hydrolysis of AcCh and activation of AcCh synthesis. The first possibility has been considered less likely, since tremorine appeared to be a very weak cholinesterase inhibitor.[1,3,4] The second possibility is favoured by the fact that tremorine has been reported to activate choline acetylase (ChAc).[5]

When testing the effects of drugs on the activity of enzymes *in vitro* it should be borne in mind that the effect might depend on the degree of substrate saturation of the enzyme. This is illustrated by the influence of imidazole on alcohol dehydrogenase activity. The inhibitory or stimulating effect of imidazole on the enzyme activity depends on the coenzyme and substrate concentrations.[6] In the present paper it was decided to investigate the effects of tremorine and oxotremorine on ChAc and acetylcholinesterase (AcChE) at both optimal and suboptimal substrate concentrations.

The AcChE activity in rat brain homogenate

The AcChE activity in rat brain homogenate was determined by a titrigraphic method using acetyl-β-methylcholine (MeCh) as substrate.[7] The substrate concentration and pH were kept constant by means of continuous addition of equal amounts of MeCh and NaOH at equal rates. AcChE was obtained by homogenizing the weighed whole rat brain in 0·1 M CO_2-free KCl at pH 8·0 (40 mg wet weight per ml).

ChAc activity

The ChAc activity was determined in an acetyl-CoA generating system by a radiometric method.[8]

$$\text{acetate - 1 - }^{14}\text{C} + \text{ATP} + \text{CoA} \xrightleftharpoons{\text{acetyl- CoA synthetase}} \text{acetyl-}^{14}\text{C -CoA} + \text{AMP} + \text{pyrophosphate}$$

$$\text{acetyl - }^{14}\text{C - CoA} + \text{choline} \xrightleftharpoons{\text{ChAc}} \text{AcCh - }^{14}\text{C} + \text{CoA}$$

Acetyl-CoA synthetase was partly purified from baker's yeast[8] and ChAc was prepared from rat brain and homogenized in cold acetone (–30°). The dry powder thus obtained was extracted with 0·02 M phosphate buffer pH 7·1 [8] and the clear extract was used in the experiments.

The rate of enzymic hydrolysis of MeCh by brain AcChE was studied at different submaximal substrate concentrations with and without oxotremorine present. A Lineweaver–Burk plot of the data obtained showed that the reaction followed the Michaelis–Menten equation (Fig. 1). It can be seen that oxotremorine is a competitive inhibitor of AcChE. The dissociation constant of the inhibitor-enzyme complex was found to be $1{\cdot}5 \times 10^{-4}$ M. The corresponding figure for eserin calculated from data found in the literature is 5×10^{-8} M[10]. Intraperitoneal injection of 0·2 mg/kg eserin produces a 40% increase in the AcCh content of rat brain.[11] The same increase is produced by 1 mg/kg of oxotremorine, and thus it seems unlikely that this increase in brain AcCh caused by oxotremorine is due to its weak anticholinesterase properties. Also the characteristic symptoms produced by oxotremorine differ from those obtained with cholinesterase inhibitors.

To enable the study of the effects of the drugs on ChAc activity at suboptimal substrate concentrations, it was necessary to determine the conditions under which the formation of acetyl-CoA is rate-limiting in AcCh biosynthesis. As acetyl-CoA is generated enzymatically, the steady state concentration of this substrate is unknown. Therefore it was necessary to study the activity of ChAc as a function of acetyl-CoA synthetase activity. It can be seen from Fig. 2 that in the presence of excess

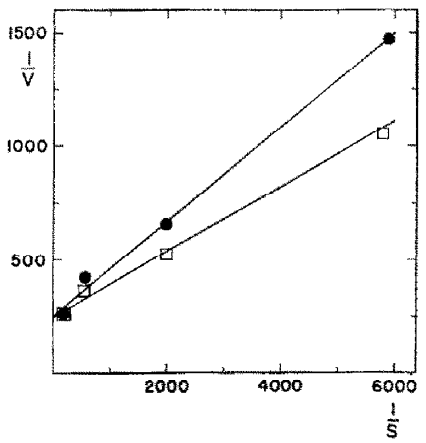

FIG. 1. Lineweaver–Burk plot of acetylcholinesterase activity without oxotremorine □ and in the presence of 10^{-4}M oxotremorine ●.

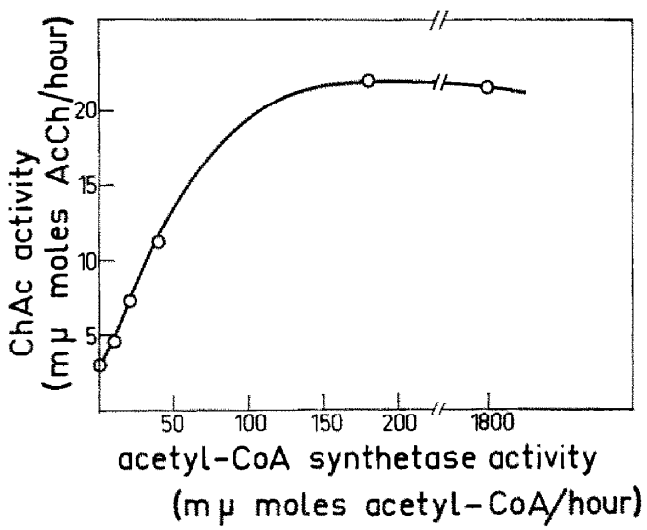

FIG. 2. Relation between the activities of choline acetylase and acetyl-CoA synthetase.

choline, the maximum rate of AcCh formation is reached when more than 0·15 μmoles acetyl-CoA are produced per hour, and half this rate is obtained at 0·04 μmoles acetyl-CoA per hour. The concentration of choline giving half the maximum rate of AcCh formation in the presence of excess acetyl-CoA is taken from the literature.[12]

The experiments used to investigate the effects of tremorine and oxotremorine on ChAc activity under these different conditions are summarized in Table 1. It can be seen that no significant effect on the enzyme activity could be demonstrated at either optimal or suboptimal conditions. However, this does not exclude the possibility that the drugs may affect AcCh biosynthesis *in vivo*, since the synthesis is dependent on several enzyme systems in intact brain tissue.

TABLE 1. EFFECTS OF TREMORINE AND OXOTREMORINE ON ChAc AT OPTIMAL AND SUBOPTIMAL SUBSTRATE CONCENTRATIONS

The following mean values of AcCh formed per mg aceton powder per hour at 37° were obtained for the controls: 20·3* ± 1·4 (mμmoles AcCh ± S.D.) (both substrates at optimal concentrations), 11·1* ± 0·8 (suboptimal choline concentration), 11·5* ± 0·7) suboptimal aceytl-CoA concentration).

Compound investigated	Acetyl-CoA mμmoles/hr	Choline moles/l	% effect on AcCh formation†
tremorine (10^{-2}M)	1800	10^{-2}	− 1
oxotremorine (10^{-2}M)	1800	10^{-2}	+ 2
oxotremorine (10^{-3}M)	1800	10^{-2}	−10
tremorine (10^{-2}M)	40	10^{-2}	− 4
oxotremorine (10^{-2}M)	40	10^{-2}	+13
tremorine (10^{-2}M)	1800	$7{\cdot}5\ 10^{-4}$	+ 1
oxotremorine (10^{-2}M)	1800	$7{\cdot}5\ 10^{-4}$	− 2

* 5 experiments
† mean of 3 experiments

Toxicology Research Unit,
Swedish Medical Research Council,
Karolinska Institutet,
Stockholm 60, and the
Research Institute of National Defence,
Department 1,
Sundbyberg 4, *Sweden.*

B. HOLMSTEDT
G. LUNDGREN
J. SCHUBERTH
A. SUNDWALL

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Oxotremorine was synthesized[9] and kindly supplied by Professor N. Löfgren, University of Stockholm, Sweden. Tremorine was kindly supplied by Dr. G. Everett, Abbott Laboratories, Chicago, U.S.A. CoA (70% purity), acetate-1-^{14}C and ATP were purchased from Sigma, U.S.A. Other chemicals used were commercially available and of analytical grade.