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REVERSIBLE INHIBITION OF ELECTRIC-ORGAN CHOLINESTERASE BY CURARE AND CURARE-LIKE AGENTS

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SUMMARY

The inhibitory effect *in vitro* was determined of *d*-tubocurare and other curare-like agents on the cholinesterase activity of the electric organ of *Electrophorus electricus* L. The observed inhibition was competitive and reversible. The reversible inhibition with *d*-tubocurare was studied, *in vitro*, in the presence of an anionic polyelectrolyte also extracted from the electric organ. Complete reversion of the enzyme activity was thus obtained.

INTRODUCTION

In the course of our studies[1,2] on the interaction of curarizing quaternary ammonium bases with extracts of electric-organ homogenates, the effect of these bases on the organ's cholinesterase activity was investigated *in vitro*. The data in this communication are related to the inhibitory effect on cholinesterase of *d*-tubocurare, its dimethyl derivative, and other quaternary ammonium bases. The reversible effect on this inhibition of the acidic polysaccharides (Sf_3) from the electric organ[2] will also be described.

Very little information on the effect of curare and curare-like compounds on cholinesterase is to be found in the literature, although WILSON[3] made an extended study of the activity of other "onium" compounds, and KRUPKA AND LAIDLER[4] on the enzyme kinetics, with different substrates, in presence of carbachol and succinylcholine which are depolarizing agents. Our main interest was the evidence of an inhibitory effect on the enzyme by those substances which have been used by us in affinity studies of the so-called "acetylcholine receptor"[2].

MATERIALS AND METHODS

Purified cholinesterase from the electric organ, which is generally described as acetylcholinesterase (EC 3.1.1.7.), a true cholinesterase, still has a small hydrolitic activity towards acetylmethacholine and butyrilcholine. The enzyme preparation used for these experiments was kindly supplied by Dr. A. B. HARGREAVES, of this Institute, in a fair stage of purification through a procedure which involves calcium phosphate-gel precipitation, magnesium and ammonium sulfate fractionation and column chromatography[5]. This sample was rechromatographed on a DEAE-cellulose column at pH 7.0

and the enzyme eluted with a gradient concentration of magnesium sulfate, instead of sodium chloride, increasing the enzyme activity. The most active fraction thus obtained was used for the kinetic studies. It was observed that increased enzyme activity favored the inhibitory effect of *d*-tubocurare and dimethyl-*d*-tubocurare. So far, no evidence of an homogeneous monodisperse preparation, or of a single component on paper electrophoresis has been obtained with cholinesterase of the electric organ, although esteratic activity was attained with the above procedure (5–15 g of acetylcholine hydrolysed per hour per mg of protein). Other authors[3,6], who claimed a still higher activity of this enzyme, have been unable to give, with precision, any of the usual characteristic physico-chemical constants for cholinesterase (diffusion constant, isoelectric point, sedimentation constant, mol. wt., etc.) as are described for many other purified macromolecules. However, some information on the structure of split products from another type of cholinesterase[7] has been given.

The naturally occurring alkaloid *d*-tubocurare possesses the curine type of skeleton (with two coclaurine units linked unsymmetrically) and four ionizable groups, that is, two onium and two phenolic hydroxyls. It has an intermediate blocking strength on mammals and on the electric eel when compared with its dimethyl derivative, dimethyl-*d*-tubocurare, and the synthetic curare-like gallamine triethyliodide of which we have been measuring the affinity to macromolecules from electric-tissue extracts[1,2]. Comparative inhibition studies were also made with other onium compounds, such as decomethonium, succinylcholine and carbachol which have acetylcholine-like properties.

The activity of cholinesterase was measured by an electrometric procedure, described by HARGREAVES[5], with 20 ml of a $0.55 \cdot 10^{-2}$ M acetylcholine solution in 0.01 M sodium acetate as substrate. After the enzyme addition, the substrate which had been taken to pH 8.3 was maintained at this pH for optimum activity with a 0.01 N NaOH solution added from a burette graduated to 0.01 ml. The amount of hydroxide used was measured at constant intervals (3 min) for a period of 9 min and corresponded to the acetic acid formed from acetylcholine. Specific activity was calculated as the amount of acetylcholine in μmoles hydrolyzed per hour per mg of protein. An E.E.L. direct-reading pH meter, model 23 A, was used for the electrometric titration.

A stock solution, $1 \cdot 10^{-2}$ M of *d*-tubocurare, was used for the inhibition studies. Two kinds of measurements were made: one with constant acetylcholine concentration and increasing amounts of *d*-tubocurare, and the other with constant *d*-tubocurare concentration and increasing amounts of acetylcholine. For the latter determination, a concentration of *d*-tubocurare was chosen that would inhibit, with the usual substrate concentration, 50 % of the enzyme's activity. With a fairly active enzyme preparation (specific activity 15 000 μmoles acetylcholine per hour per mg protein), this was observed even with $1 \cdot 10^{-6}$ moles of *d*-tubocurare per 20 ml substrate medium as described above. Compared with an eserine sulfate solution that will inhibit the enzyme to the same extent, the potency of *d*-tubocurare is lower by a factor of 10. In contrast with eserine inhibition, *d*-tubocurare inhibition can be reversed by addition of a polyanionic substance such as the acidic polysaccharide, Sf_3, prepared from the electric-organ extracts[2]. This effect has some similarity with that described by CHEYMOL[8] in his review of substances which modify curarizing activity and is also of interest in connection with the biological role of acidic polysaccharide in the conductive tissue of the electric eel, as will be discussed.

The AUGUSTINSSON AND NACHMANSOHN[9] equation was generally used for the analysis of cholinesterase inhibition with the drugs assayed; *i.e.*

$$\frac{v}{v'} = 1 + \frac{[I]\,K_s}{K_i(S + K_s)}$$

The plot of v/v' (v is the reaction velocity without inhibitor and v' with inhibitor) against $[I]$ the inhibitor concentration, permitted the calculation of K_i (dissociation constant of the enzyme – inhibitor complex) from the slope of the line, $K_s/K_i\ (S+K_s)$, with constant enzyme and substrate concentrations. K_s, the dissociation constant of the enzyme – substrate complex, was determined as K_m with the LINEWEAVER AND BURK reciprocal plot[10] following the argument of WILSON[3] for electric-organ acetylcholinesterase. The inhibition of cholinesterase by *d*-tubocurare with constant enzyme and inhibitor concentration, and different substrate concentration was analysed by the last method. Competitive inhibition was obtained, and a good agreement was found with the two equations. The K_m value, for acetylcholine as substrate, in the concentration range of our analysis, was of the order of $4.5 \cdot 10^{-4}$ M.

Measurements were also made of inhibition by a tertiary base, *d*-isochondodendrine, a chondodendron alkaloid with the two coclaurine units joined symmetrically, and no curare-like action. This alkaloid also proved to be as good an inhibitor of cholinesterase as *d*-tubocurare, showing that possession of inhibitory properties, in this case, was not a special feature of the onium group. Both *d*-tubocurare and *d*-isochondodendrine contain two phenolic hydroxyls which are ionizable and apt to form hydrogen bonds. Comparative results of the AUGUSTINSSON–NACHMANSOHN plot for eserine, prostigmine, *d*-tubocurare, *d*-isochondodendrine, dimethyl-*d*-tubocurare and

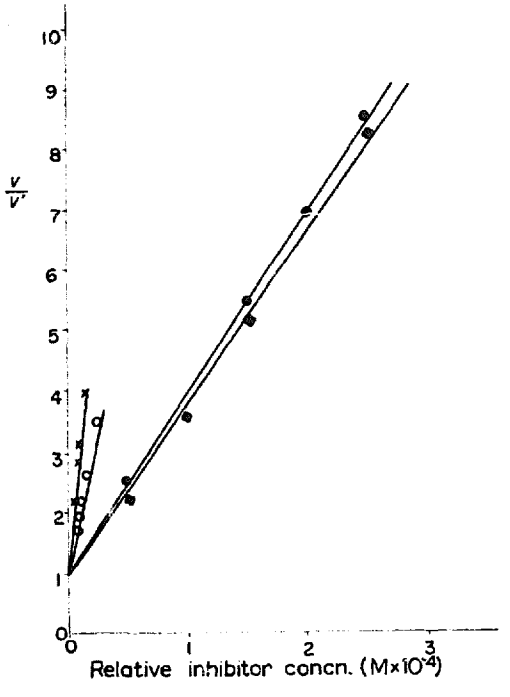

Fig. 1. Inhibition of cholinesterase by *d*-tubocurare, *d*-isochondodendrine, eserine and prostigmine as a function of inhibitor concentration; AUGUSTINSSON AND NACHMANSOHN plot[9]. v, velocity in absence and v' in presence of inhibitor, expressed as μmoles of acetylcholine hydrolysed per hour per mg of protein. ×—×, eserine; O—O, prostigmine; ●—●, *d*-tubocurare; ■—■, *d*-isochondodendrine.

gallamine triethyliodide are presented in Figs. 1 and 2. K_i values for *d*-tubocurare and the other substances studied are given in Table I. All these experiments were conducted as described for *d*-tubocurare, with constant substrate concentration. From Table I it

TABLE I

VALUES OF K_i CALCULATED WITH THE AUGUSTINSSON AND NACHMANSOHN EQUATION AT CONSTANT SUBSTRATE CONCENTRATIONS

Acetylcholine, $0.55 \cdot 10^{-2}$ M; K_m, $4.5 \cdot 10^{-4}$ M, calculated as described in the text.

Inhibitor	K_i
Eserine	$2.20 \cdot 10^{-7}$
Prostigmine	$5.90 \cdot 10^{-7}$
Decamethonium	$8.90 \cdot 10^{-7}$
d-Tubocurare	$2.52 \cdot 10^{-6}$
d-Isochondodendrine	$2.60 \cdot 10^{-6}$
Succinylcholine	$2.00 \cdot 10^{-5}$
Dimethyl-*d*-tubocurare	$0.80 \cdot 10^{-4}$
Carbachol	$1.01 \cdot 10^{-4}$
Gallamine triethyliodide	$1.08 \cdot 10^{-4}$

can be concluded that the inhibitory effect can be mainly related to structural features but not to curarizing or depolarizing activity. Dimethyl-*d*-tubocurare is about 40 times less effective as an inhibitor than *d*-tubocurare which is not as basic as the former compound or gallamine triethyliodide. Like other cationic inhibitors, described by WILSON[3], these substances may inhibit by occupying the anionic site. Evidence suggesting that the observed inhibition was due to electrostatic interaction of the cations with the anionic site was given by the observation that the purified enzyme was more effectively inhibited by lower concentrations of *d*-tubocurare, for example, than were the crude enzyme preparations. Also the ionic strength of the substrate medium decreased the inhibitory activities. These observations led us to examine the effect of the two polysaccharides purified from electric-organ homogenates, Sf_1 (neutral polysaccharide) and Sf_3 (acidic polysaccharide), as mentioned above, which have marked affinities for the

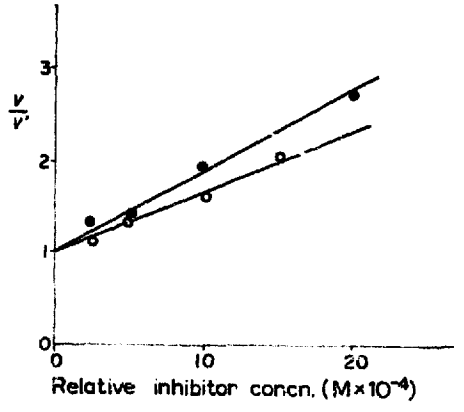

Fig. 2. Inhibition of cholinesterase by dimethyl-*d*-tubocurare and gallamine triethyliodide, as function of inhibitor concentration[9]. Description as in Fig. 1. ●—●, dimethyl *d*-tubocurare; O—O, gallamine triethyliodide.

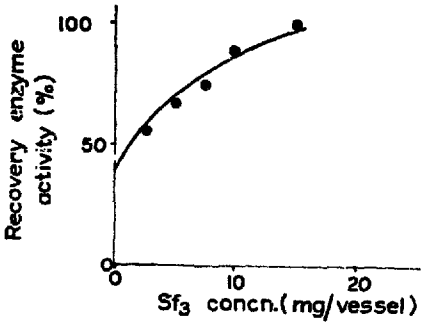

Fig. 3. Reactivation by Sf_3 of cholinesterase inhibited by *d*-tubocurare. Percent recovery of initial enzyme activity, without inhibitor, to which a value of 100 is given, as function of Sf_3 concentration (in mg) in the reaction vessel.

onium bases, but no intrinsic activity. Sf_1 had no effect on the inhibition of cholinesterase by *d*-tubocurare, decamethonium or dimethyl-*d*-tubocurare. Sf_3 certainly contributed anionic sites that displaced these cations from the enzyme, probably by an ion-exchange effect. Fig. 3 shows complete reversion of 60 % cholinesterase inhibition by *d*-tubocurare, with increasing amounts of Sf_3 added to the substrate media, until a constant level was reached.

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