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INTERACTIONS OF SOME PARASYMPATHOLYTICS AND STRUCTURALLY RELATED DRUGS WITH ACETYLCHOLINESTERASES: KINETIC STUDIES

ULF-W. WIEGAND*, HELMUT KUHNEN and ROLF HALLER

Pharmazeutisches Institut der Universität Freiburg i. Br. D-78 Freiburg i. Br., West Germany and Fraunhofer-Gesellschaft, Institut für Aerobiologie, D-5948 Grafschaft/Schmallenberg, West Germany

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Abstract—The interactions of the parasympatholytics adiphenine, adiphenine H, propantheline and the structurally related compounds diphenhydramine, D-propoxyphene and methadone with acetylcholinesterases were studied by means of kinetic measurements. Acetylcholinesterase (AChE) was used as membrane-bound enzyme from bovine red cells and in a solubilized form from *Electrophorus electricus*. All the drugs studied are inhibitors of AChE. Differences between the membrane bound and the solubilized form can be deduced from different inhibitory mechanisms. A mixed competitive–non-competitive mechanism is characteristic for the membrane-bound enzyme, while a predominantly non-competitive mechanism is involved in the case of the solubilized enzyme. The negative cooperative behaviour of AChE is not changed by these inhibitors. A "two-site" hypothesis for the binding of the investigated drugs is proposed.

In the course of our studies on the binding properties of parasympatholytics and structurally related compounds [1, 2] to biopolymers, we also examined the interactions of these drugs with AChE†. The present paper describes the effect of these drugs (see Fig. 1) on the catalytic properties of AChE. To date only neuromuscular and ganglionic blocking agents [3–6], as well as atropine [7], have been used for kinetic measurements with AChE. For the experiments reported here, AChE was used in the form of the enzyme bound to erythrocyte membranes as well as the solubilized form obtained from the electric organs of *Electrophorus electricus*. Although both enzymes are thought to be similar to a great extent in their kinetic behaviour, Berman [8] has shown that they have a different number of subunits in addition to a different amino acid composition. In the light of these structural differences, we have investigated the kinetic behaviour of several drugs (see Fig. 1) with respect to both the membrane-bound and the solubilized form of the enzyme.

MATERIALS AND METHODS

Drugs used in this investigation are shown in Fig. 1. Adiphenine, adiphenine H and propantheline have distinct parasympatholytic properties, their spasmolytic effects being attributed to a neurotropic as well as to a musculotropic reaction. Diphenhydramine, D-propoxyphene, and methadone are not, strictly speaking, parasympatholytics, but were included in these investigations because of their structural similarities. It should be noted that their basic chemical structure shows a certain similarity to acetylcholine, the natural substrate of the enzyme.

Bovine erythrocyte ghosts were prepared according to the method of Dodge [9] and Burger [10] by adding 5 mM calcium chloride at pH 7.8. AChE from *Electrophorus electricus* (Worthington, Freehold, N.J.) was the solubilized enzyme.

Kinetic data were obtained from the pH-stat method by simultaneous addition of titrant and substrate [11] (modified Kombititrator 3 D (Metrohm)). In this modification the original syringe system is replaced by two synchronized pistons. The acetic acid formed by decomposition of the substrate is neutralised by addition of NaOH from one syringe. Decomposed substrate is replaced by adding ACh through the second syringe so that the ACh concentration remains constant. In this way AChE activity was determined quite accurately even with substrate concentrations as low as 3 μM. For these measurements the erythrocyte ghosts were suspended in isotonic Tyrode solution (137 mM NaCl, 5.4 mM KCl, 1.6 mM $CaCl_2$) and their enzymatic activity was determined at pH 7.4 and 37° with acetylcholine chloride as substrate. The reaction volume was 10 ml, the incubation period 5 min. The inhibitory effect of the compounds was independent of the incubation period.

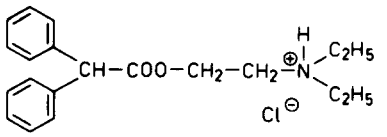
The experimental conditions of the assay of the solubilized enzyme were the same as described for the membrane-bound enzyme. For adiphenine H, the incubation period was 10 min. In the case of adiphenine, however, more reproducible results were obtained after 15 min incubation.

RESULTS AND DISCUSSION

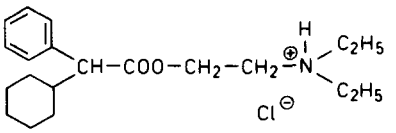
The linear/logarithmic plot and the Hofstee plot shown in Fig. 2 illustrate the reaction of adiphenines with AChE from bovine red cells. The Hofstee plot was chosen instead of the more common plot according to Lineweaver–Burk, because it is inconvenient

* To whom requests for reprints should be addressed. Present address: Institut für Molekularbiologie und Biophysik, Eidgenössische Technische Hochschule Zürich, CH-8049 Zürich, Switzerland.

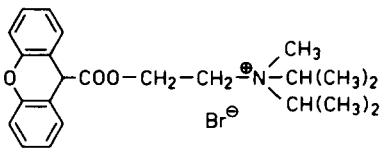
† Abbreviations used: AChE, acetylcholine acetyl-hydrolase, EC 3.1.1.7; ACh, acetylcholine.



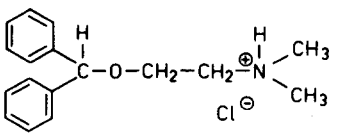
Adiphenine · HCl



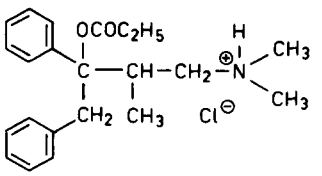
Adiphenine H · HCl



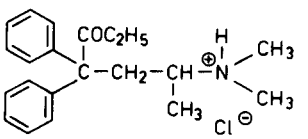
Propantheline · Br



Diphenhydramine · HCl



Propoxyphene · HCl



Methadone · HCl

Fig. 1. Structure of the drugs used in the kinetic studies.

to plot all measured points in the examined range of the substrate concentration as double reciprocals.

The sigmoid bell-shaped curve of the normal activity in the linear/logarithmic plot clearly shows that the kinetic reaction cannot be explained completely by Michaelis–Menten kinetics [11–14]. The deviation from the usual hyperbola of the left branches of the bell shaped curves leads to the conclusion that there are several interacting binding sites, which implies that the enzyme has allosteric properties. This fact is indicated in the Hofstee plot by the clear deviation from the usual straight lines. For the kinetic properties of the enzymes in reaction with the compounds studied in these investigations, only the left branches of the bell shaped curves were considered. The right branches are in the range of substrate inhibition which is commonly found with these enzymes [5, 7, 13]. Substrate inhibition is not prevented by these parasympatholytic drugs as is the case with some pyridinium derivatives [11, 12]. The shape of the normal activity curve suggests the existence of a negative cooperative effect. The double logarithmic plot according to Hill [15] gives a curve for the normal activity which deviates increasingly from linearity with decreasing substrate concentration. Up to a substrate concentration of about 3.0×10^{-4} M the Hill coefficient, n_H, is 1.07. It decreases to $n_H = 0.75$ for acetylcholine concentrations $> 3.0 \times 10^{-5}$ M and finally has a value of 0.45 in the substrate range from 3.0×10^{-6} to 3.0×10^{-5} M (see Fig. 4). According to the model of Koshland [16], Hill coefficients less than unity characterize a negative cooperativity. Furthermore the index of cooperativity, R_S, is greater than 81:

$$R_s = (S_{0.9})/(S_{0.1}) = 187.5.$$

Even though these data suggest the existence of a negative cooperative effect, this possibility has to be examined in more detail. Since the enzyme in the structure bound [5,13] as well as in the soluble form consists only of one species of esterase [4, 6, 7, 17, 20, 21] a negative cooperativity due to the presence of more than one enzyme species can not be assumed. In the solubilized state the AChE may exist in several subunits which are able to dissociate and reaggregate. This, however, has no influence on the kinetic properties and the catalytic activity of the

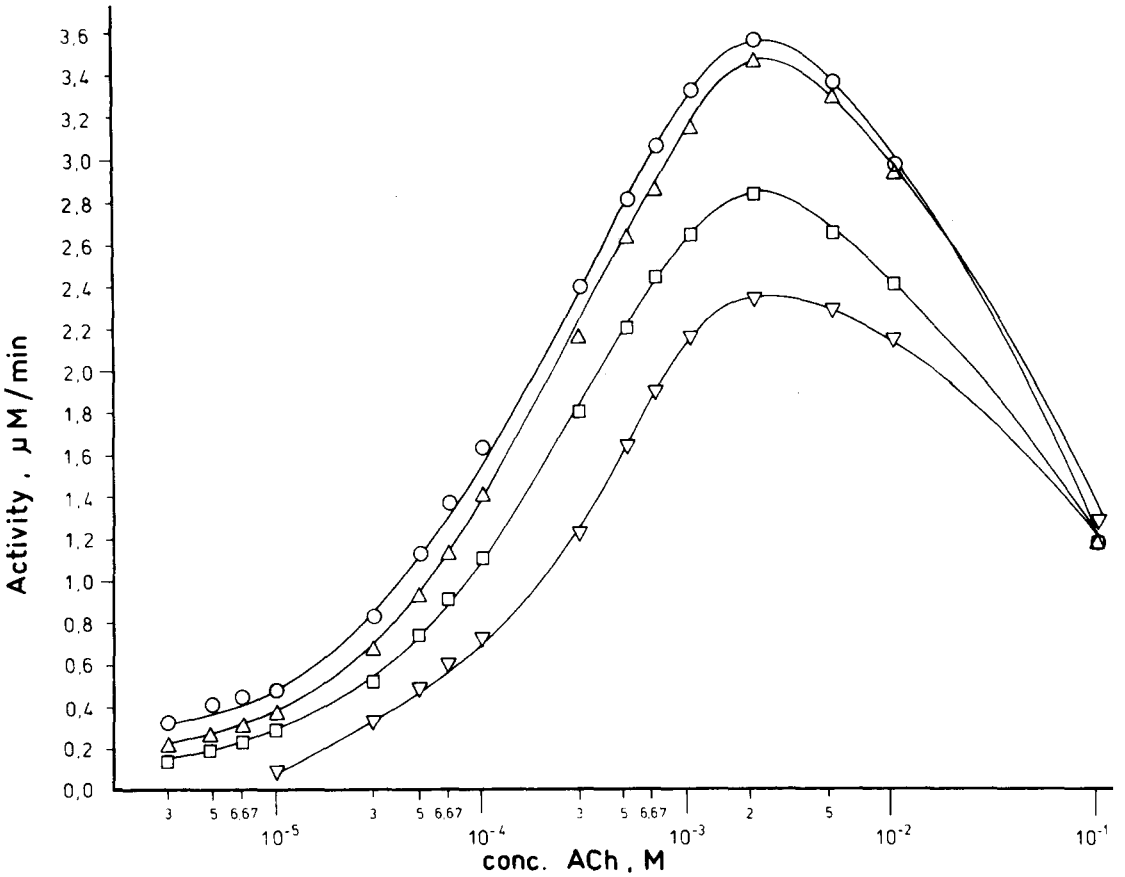


Fig. 2a. Influence of various concentrations of adiphenine on the activity of AChE from bovine erythrocytes: pH-stat method, pH 7.4, 37°, substrate acetylcholine chloride. Each mean represents the average of six different experiments. The calculated S.E.M. does not exceed the size of the symbols, and is therefore not indicated. Normal activity, ○. Adiphenine concentrations: △ 1.0×10^{-4} M, □ 2.0×10^{-4} M, ▽ 1.0×10^{-3} M.

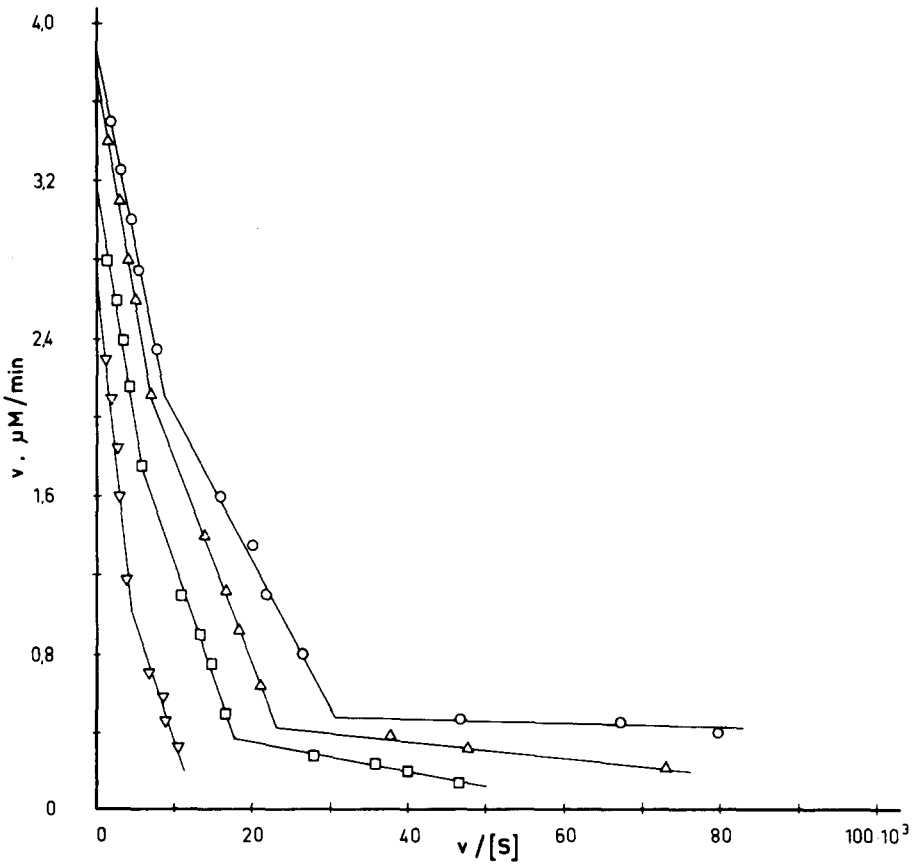


Fig. 2b. Hofstee plot of the experimental data from Fig. 2a. Each inhibitor concentration yields three different V_{max} according to the "quasi straight" sections of the left branch of the sigmoidal curve from Fig. 2a. Normal activity, ○. Adiphenine concentrations: △ 1.0×10^{-4} M, □ 2.0×10^{-4} M, ▽ 1.0×10^{-3} M.

enzyme. The n_H-values show a significant deviation from 1 only at substrate concentrations less than 3.0×10^{-5} M. Data in this concentration range are, under normal assay conditions, not accurate enough to deduce sigmoidal kinetics. These methodical difficulties can be neglected in the present case, however, since a constant substrate concentration was used. The lowest substrate concentration employed by most of the authors (cf. e.g. Wombacher [5] and Roufogalis [17]) are in the range of 3.0 to 5.0×10^{-5} M. The experimental proof of a negative cooperativity has therefore been very difficult.

The results given in Figs. 2 and 3 and Table 1 show that all these drugs are inhibitors of AChE. The mechanism of inhibition can be deduced from the linear/logarithmic plot and from the plots according to Hofstee and Dixon: a weak mixed competitive–noncompetitive inhibition. These drugs cannot, how-

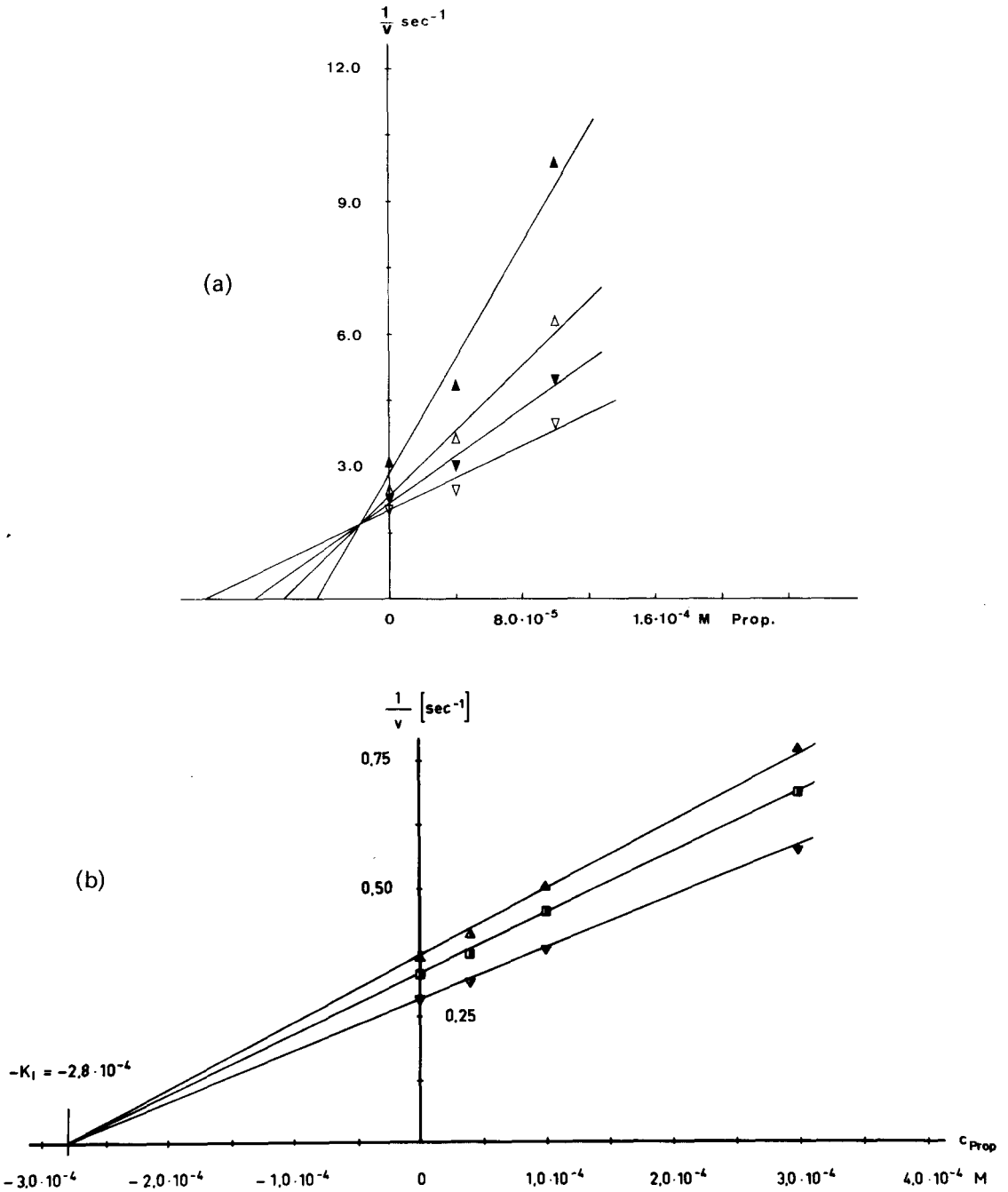


Fig. 3. Dixon plots for the inhibition of AChE from bovine erythrocytes by propantheline. (a) acetylcholine concentrations: ▲ 3.0×10^{-6} M, △ 5.0×10^{-6} M, ▼ 6.67×10^{-6} M, ▽ 1.0×10^{-5} M. (b) acetylcholine concentrations: ▲ 5.0×10^{-4} M, ◨ 6.67×10^{-4} M, ▼ 2.0×10^{-3} M.

Table 1. K_I-values of the studied drugs

Inhibitor	K_I^a (M)	K_I^b (M)
Adiphenine	7.2×10^{-4}	5.7×10^{-4}
Adiphenine H	3.0×10^{-4}	1.5×10^{-4}
Propantheline	2.8×10^{-4}	4.4×10^{-4}
Diphenhydramine	6.3×10^{-3}	3.3×10^{-3}
D-Propoxyphene	2.2×10^{-3}	1.1×10^{-3}
Methadone	1.2×10^{-3}	1.0×10^{-3}

K_I^a, AChE from bovine erythrocytes; K_I^b, AChE from *Electrophorus electricus*. The K_I values were determined from Dixon plots (as shown in Fig. 3). The ACh concentrations were as high as indicated in the graph.

ever, relieve the inhibition by excess of substrate. It is interesting to note that most of these compounds also show a competitive–noncompetitive antagonism at the muscarinic receptor towards acetyl-β-methylcholine [18].

The mixed mechanism can be seen to a great extent in the Hofstee plots (see Fig. 2b). The competitive mechanism prevails at low substrate concentrations (3.0×10^{-6}–1.0×10^{-5} M). The rearward extrapolation of the pseudolinear sections intersect at one point on the ordinate. At higher substrate concentrations (3.0×10^{-4}–2.0×10^{-3} M) the straight lines run approximately parallel, i.e. they have the same K_m-values, but different V_{max}-values. Therefore a noncompetitive inhibition prevails under these conditions. In the medium concentration range (3.0×10^{-5}–1.0×10^{-4} M acetylcholine), there exists a transition between the two mechanisms. The difference among the studied drugs becomes obvious in this range of substrate concentration, where one or the other type of inhibition prevails depending upon the compound.

The nature of the inhibitory effect depends not only on the substrate but also on the inhibitor concentration: at higher acetylcholine concentrations (3.0×10^{-4}–2.0×10^{-3} M) an increase in the inhibitor concentration causes a decrease in the degree of the noncompetitive inhibition. The great similarity in the reaction of all examined inhibitors towards the erythrocyte AChE suggests that these drugs are bound to the enzyme in a similar manner. It can be assumed that the quaternary nitrogen atom and the aryl groups are involved, a mechanism which we should like to call a "two-site" hypothesis according to the concept developed by Wombacher [5] in regard to neuromuscular and ganglionic blocking agents.

The structure of the active sites of AChE suggests also that the inhibitors of this enzyme, especially those bearing structural similarity to the substrate, are primarily bound to one of the anionic centres

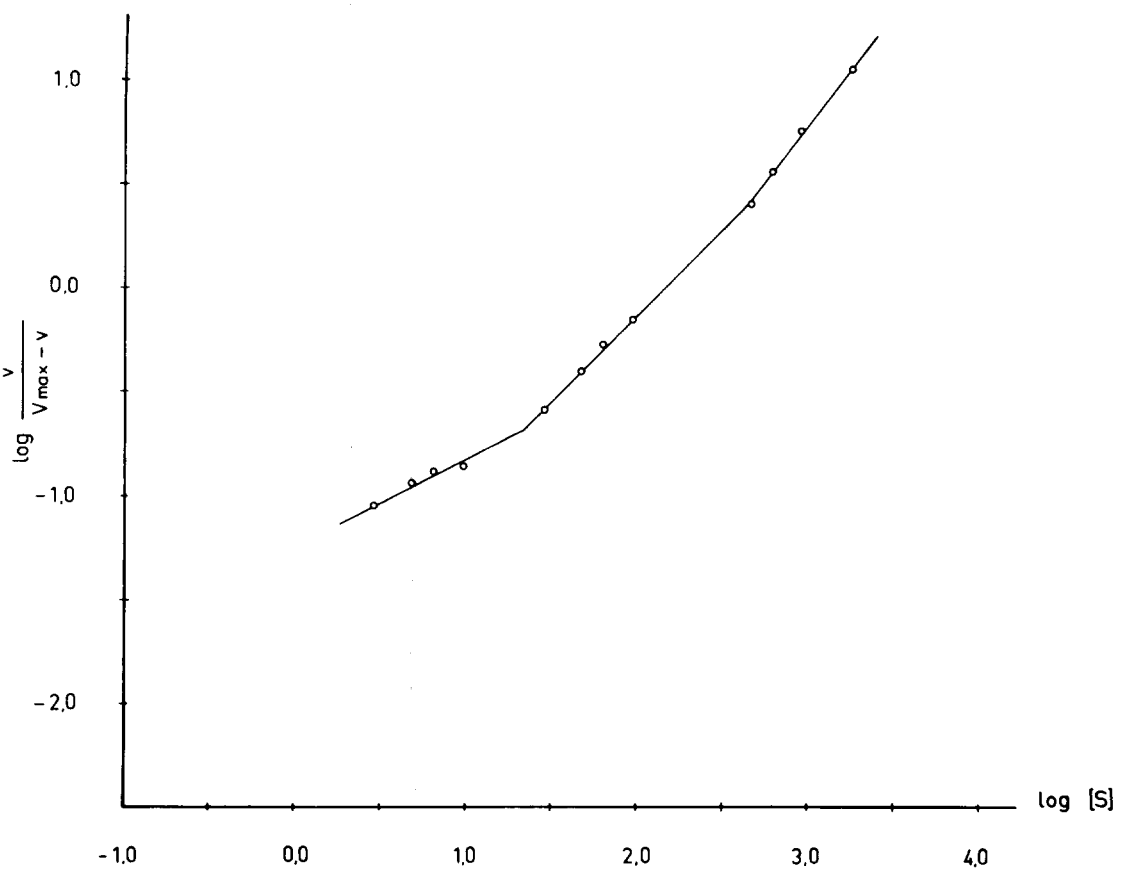


Fig. 4. Hill plot of the normal activity curve from Fig. 2a based on a V_{max}-value of 3.8. According to the three different V_{max} resulting from the Hofstee plot, this presentation shows three different straight lines with different slopes.

by the quaternary nitrogen atoms [5, 19]. Such binding inhibits the reaction between enzyme and substrate. According to the model of Roufogalis [17], this binding may be caused by the α-anionic binding site in the esteratic centre, because only the interaction with this binding site results in an inhibition.

In agreement with the above mentioned two-site hypothesis, the binding of the inhibitors also occurs through the aryl group of these drugs. These binding sites are predominantly hydrophobic and should be located at an appropriate distance from the anionic binding site. Kabachnik [20] assumes a mainly hydrophobic binding site, called E, close to the esteratic centre of the enzyme. This binding site could enable interactions with the aryl groups of the examined drugs for the following reasons. (1) The distance between the α-anionic and the hydrophobic binding site corresponds with the atomic distance in the inhibitor molecules. (2) Activating properties of this centre cannot be assumed, because this centre was deduced from the analysis of inhibitors [20]. (3) According to Kabachnik [20] an isohexyl group is optimally bound by this site of AChE. This group is comparable in its size with the cyclic part of the studied compounds.

The two-site hypothesis explains the partially competitive–noncompetitive inhibition observed: the substrate competitively displaces the inhibitor from the α-anionic binding site with increasing concentration. As a result, the inhibitor is predominantly fixed to the enzyme by the hydrophobic binding site E. Thus at higher acetylcholine concentrations the noncompetitive mechanism becomes more important (see Fig. 3).

Table 1 shows the K_I-values of the drugs studied. The K_I-values were determined graphically by Dixon plots (example shown in Fig. 3) using the high range of acetylcholine concentration as indicated in the graph. The numerical values of the constants show that these drugs are relatively weak inhibitors of acetylcholinesterase in comparison with eserine, for instance.

The binding of these drugs to AChE does not seem to abolish the negative cooperative behaviour of this enzyme. The characteristic curve of the normal activity is barely changed in the linear/logarithmic plot (see Fig. 2a). In the presence of the inhibitors there is an increase in the Hill coefficients, which come close to the value of $n_H = 1$ with increasing substrate concentration. This indicates that the addition of an inhibitor results in a decrease of the negative cooperativity. Consequently, the examined drugs have the same effect as that caused by an increase of the substrate concentration.

Results of the measurements with acetylcholinesterase from *Electrophorus electricus* are shown in Fig.

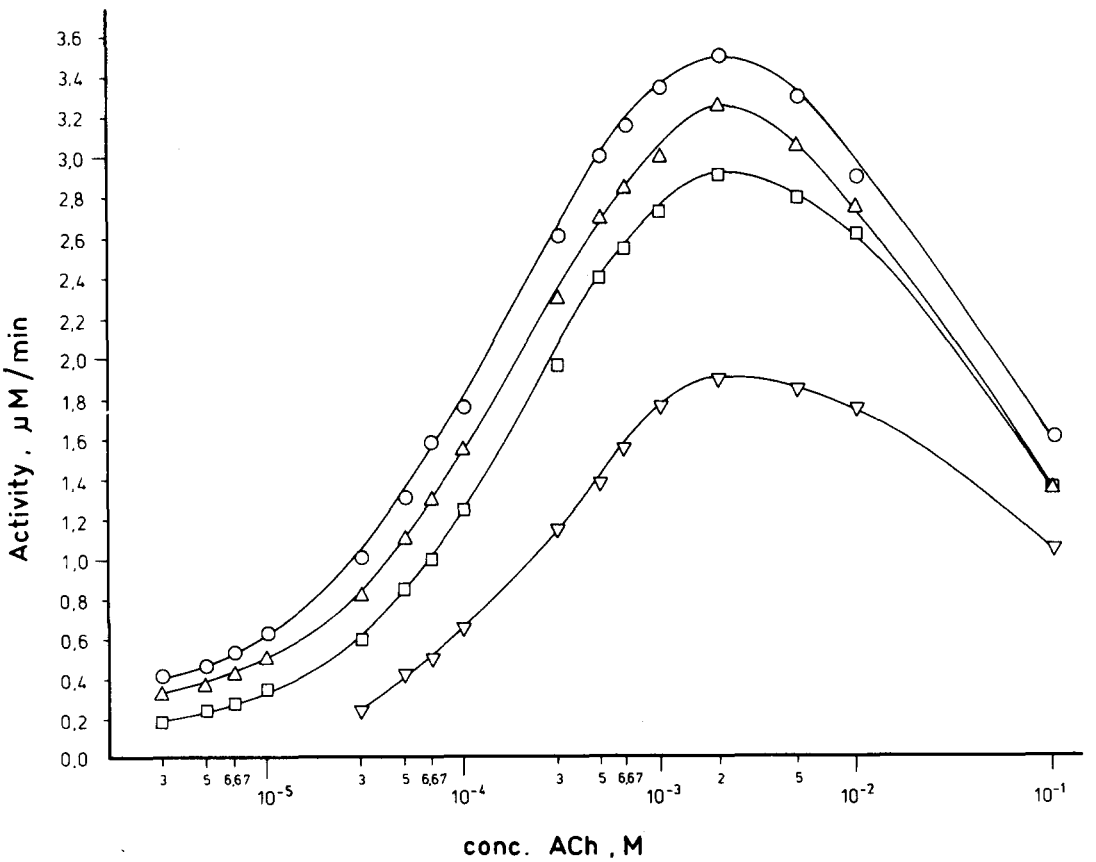


Fig. 5a. Influence of various concentrations of propantheline on the activity of AChE from *Electrophorus electricus*. For experimental details see Fig. 2a. Normal activity, ○. Propantheline concentrations: △ 4.0×10^{-5} M, □ 1.0×10^{-4} M, ▽ 3.0×10^{-4} M.

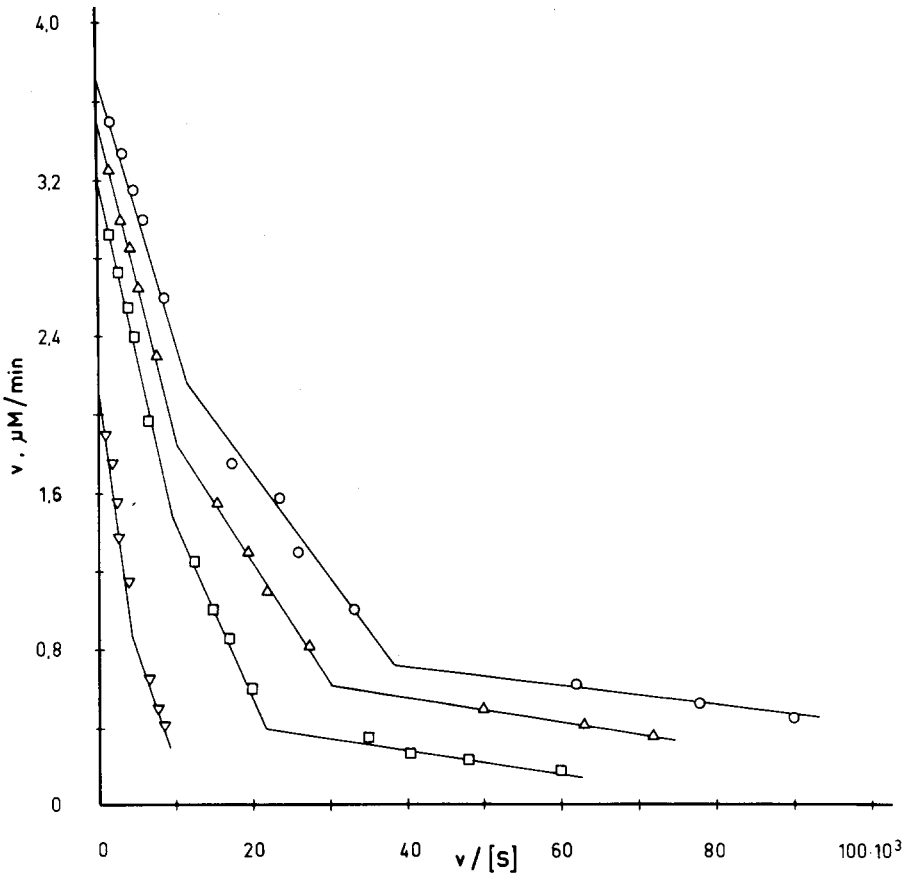


Fig. 5b. Hofstee plot of the experimental data from Fig. 5a. Normal activity, ○. Propantheline concentrations: △ 4.0×10^{-5} M, □ 1.0×10^{-4} M, ▽ 3.0×10^{-4} M.

5 and Table 1. A comparison of the normal activity curves in the linear/logarithmic and the Hofstee plots with those plots of the membrane-bound enzyme suggest that negative cooperativity can also be assumed for the solubilized enzyme. Values of $n_H < 1$ can be computed for low substrate concentrations.

The Hofstee plots show even more clearly than the linear/logarithmic presentations of the experimental data that the action of the inhibitors has changed compared with the membrane-bound enzyme. The competitive inhibition of AChE from erythrocytes at low substrate concentration does not exist for the enzyme of *Electrophorus electricus*. The effect of the drugs on the solubilized enzyme can be described principally as noncompetitive inhibition for the whole range of substrate concentration examined. They do not prevent inhibition due to substrate excess. In this respect they differ from atropine, which relieves the inhibition caused by substrate excess [7, 21].

The model developed above can also be used for the interpretation of the kinetic behaviour of the investigated drugs with solubilized AChE. The mechanism of inhibition at lowest substrate concentration is noncompetitive. Thus it can be assumed that the ionic interactions between the quaternary nitrogen atom and the α-anionic site of the esteratic centre decrease. The amino group ionisation of the inhibitors did not change since the experimental conditions were maintained constant. This observation suggests a change in the ionic properties of the enzymatic binding site. In this context one should bear in mind the results of Berman [8]: a higher turnover number and a greater specific activity of AChE from electric eel, indicating a higher affinity between enzyme and substrate. The binding sites for the aryl groups of the inhibitors are outside of the esteratic centre, so they are not influenced by a change in the affinity of the substrate for the esteratic centre. As a result the noncompetitive inhibition does not change.

The present investigations therefore suggest that there are differences in the kinetic behaviour between the membrane-bound AChE from bovine red cells and the solubilized enzyme from the electric organs of *Electrophorus electricus*. It cannot be decided yet whether the different amino acid composition of the two enzymes or other differences between the membrane-bound and the solubilized form play a greater role in this respect. Kuhnen [11] studied the reaction of bis-pyridinium compounds with AChE from bovine red cells and from *Electrophorus electricus*. He found that these compounds exert the effect of competitive inhibitors in regard to the soluble enzyme, while they show partially a competitive, partially a noncompetitive mechanism in regard to the membrane-bound enzyme.

In a comparative evaluation of the results between solubilized and membrane-bound AChE, one should not overlook the fact that, in the case of the membrane-bound enzyme, the isolated erythrocyte ghosts do not have the same membrane structure as intact erythrocytes. This fact could result in a change of the kinetic behaviour of acetylcholinesterase [22–25].

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