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Regulation of Membrane-Bound Acetylcholinesterase Activity by Bisquaternary Nitrogen Compounds

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SUMMARY

The inhibitory effect of bisquaternary compounds on erythrocyte membrane-bound acetylcholinesterase (acetylcholine acetyl-hydrolase, EC 3.1.1.7) was investigated by kinetic methods. The membranes were obtained by osmotic hemolysis of human erythrocytes. A special photometric method was used for measurements, which made it possible to record the enzyme reaction in the steady-state phase. In contrast to former investigations, in which the reaction medium contained Na^+ and Mg^{2+} ions at rather high concentrations, these ions were omitted in the present experiments.

Kinetic analysis of the results yielded the following types of inhibitory mechanisms: *d*-tubocurarine and gallamine, partially competitive–partially noncompetitive; succinylbischoline, partially competitive–noncompetitive ("mixed inhibition"). The noncompetitive portion of the mechanism of all compounds investigated is interpreted in the following way. *d*-Tubocurarine, gallamine, and succinylbischoline are bound to a site distinct from the active site of acetylcholinesterase. This site, called the "regulatory site," does not show catalytic activity and contains at least two negative charges. The presence of noncompetitive inhibition suggests that the binding of these effectors to the regulatory site must be combined with a conformational change, which affects the enzyme reaction rate negatively in the case of *d*-tubocurarine, gallamine, and succinylbischoline. The phenomenon of substrate concentration-dependent activation and inhibition, which is observed with the effector pentamethonium, is explicable only if the existence of a ternary enzyme-substrate-inhibitor (*ESI*) complex is accepted. This means that the reaction mechanism is expected to be partially noncompetitive in this case too, and consequently pentamethonium has to be designated as an allosteric effector. The regulatory site, whose existence is proved by the presence of the noncompetitive part of the reaction mechanism, could be identical with the cholinergic receptor defined by pharmacological experiments.

INTRODUCTION

The structure and catalytic properties of isolated acetylcholinesterase (acetylcholine acetyl-hydrolase, EC 3.1.1.7) vary dramatically with ionic strength [cf. Changeux (1)]. Some time earlier, Wolf (2) had shown that divalent cations such as Mg^{2+} and Ca^{2+} have pronounced inhibitory or activating effects on acetylcholinesterase activity, depending on the substrate concentration. Consequently, an analytical kinetic treatment of this enzyme reaction has to consider the effect of these ions. For example, it is possible that in the presence of sufficiently high Mg^{2+} concentrations kinetic behavior may be simulated that obscures the real

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properties of the enzyme. In many cases cations (especially Mg^{2+}) affect the kinetic properties of regulatory enzymes in much the same ways as do the organic effectors [cf. Atkinson (3)]. It should be noted that in previous experiments the reaction medium usually contained Na^+ and Mg^{2+} ions [e.g., Krupka (4)].

In addition, in most experiments the concentration range of substrate and effector has been too narrow. This might explain why former investigators were able to describe the kinetic behavior of acetylcholinesterase by ordinary or slightly modified Michaelis-Menten (5) kinetics. For the majority of enzymes, however, Michaelis-Menten or Briggs-Haldane kinetics (6) is inadequate. Therefore the computed constants are often "apparent" and their numerical values have no simple interpretation, and hence are of little value in deducing molecular mechanisms [cf. Botts and Morales (7)].

With respect to these considerations and with the aim of obtaining further insight into the kinetic properties of acetylcholinesterase, we have investigated the curare-like compounds, pentamethonium and tetramethylammonium iodide, by steady-state kinetics, omitting the conventional additions of Na^+ and Mg^{2+} and measuring the reaction rate over a wide concentration range of substrate and effector. Moreover, care was taken to ensure that all measurements were carried out within the steady-state phase of the reaction.

MATERIALS AND METHODS

Human erythrocyte ghosts were prepared according to the method of Dodge *et al.* (8). Salts were removed by repeated dialysis against 100 volumes of 50 mM Tris hydrochloride, pH 7.8, and finally against twice-redistilled water. Subsequently the ghosts were lyophilized. The specific activity of batch I was 0.25 unit/mg of dry material, and that of batch II was 0.48 unit/mg of dry material.[2] The difference between the two batches is due to the use of different pH values during the preparation (batch I, pH 7.4; batch II, pH 7.8). The dried ghosts contained the following amounts of ions, estimated by flame photometry: sodium = 0.013% of dry material; potassium $\leqq$ 0.01%; magnesium $\leqq$ 0.02%; calcium $\leqq$ 0.07%; iron $\leqq$ 0.008% ($\pm$20%).

Chemicals. Phenol red and Tris (2-amino-2-hydroxymethyl-1,3-propandiol) (Merck, Germany); acetylcholine iodide, pentamethonium, succinylbischoline dichloride, tetramethylammonium iodide, and decamethonium (Fluka, Switzerland); *d*-tubocurarine chloride (Schuchardt, Germany); gallamine triethiodide (K & K Laboratories, U. S. A.). The water was twice redistilled in a quartz apparatus.

Assay method. The reaction rate was measured by a photometric method according to Grégoire *et al.* (9) with the following modifications. The test solution contained the following in a final volume of 10.0 ml: 0.8 mM Tris hydrochloride buffer, 52 μg of phenol red, and 0.8 mg of enzyme preparation. Substrate concentrations were varied from 0.02 to 10 mM. The effector concentrations are indicated in the figure legends. The ionic strength used in those experiments whose results were submitted to kinetic interpretation was between 0.0002 and 0.002. All components were dissolved in twice-redistilled water. After the addition of substrate, the reaction was followed at 546 nm only within the initial phase of zero reaction order, using an Eppendorf photometer combined with a recorder. In order to maintain the temperature at 30°, the cuvette holder was connected with a thermostat.

RESULTS AND DISCUSSION

Kinetic analysis of the steady state is based on a reaction scheme proposed by various investigators, including Botts (10), Botts and Morales (7), Laidler (11), and Ohlenbusch (12).

[2] One unit is defined ["Enzyme Nomenclature: Recommendations (1964) of the International Union of Biochemistry," Elsevier, Amsterdam] as that amount which will catalyze the transformation of 1 μmole of substrate per minute under optimum conditions (in the present case 30°, pH 8.0, 4 mM acetylcholine).

$$\begin{array}{ccccc} & I & & I & \\ & + & & + & \\ S + E & \overset{K_s}{\rightleftharpoons} & ES & \xrightarrow{k_2} & E + P \\ {\scriptstyle K_i}\uparrow\downarrow & & \uparrow\downarrow{\scriptstyle \alpha K_i} & & \\ S + EI & \underset{\alpha K_s}{\rightleftharpoons} & ESI & \xrightarrow{\beta k_2} & EI + P \end{array}$$

This scheme and the kinetics derived from it do not include the substrate inhibition typical of acetylcholinesterase at high substrate concentrations.

The terminology used in this paper is accommodated to that of Webb (13). The symbols are defined as follows.

E = free enzyme
$[E]_t$ = total enzyme concentration
S = substrate
P = reaction products
I = inhibitor
k = reaction rate constant
K = equilibrium constant
$\frac{k_{-1} + k_2}{k_1} = K_m$ = Michaelis constant
$\frac{k_{-1}}{k_1} = K_s$ = equilibrium constant of the reaction $E + S \rightleftharpoons ES$
$\frac{k'_{-1}}{k_1'} = \alpha K_s$ = equilibrium constant of the reaction $EI + S \rightleftharpoons ESI$
$\frac{k_{-i}}{k_i} = K_i$ = equilibrium constant of the reaction $E + I \rightleftharpoons EI$
$\frac{k'_{-i}}{k_i'} = \alpha K_i$ = equilibrium constant of the reaction $ES + I \rightleftharpoons ESI$
v = reaction rate in presence of any substrate concentration and absence of inhibitor
v_i = reaction rate in presence of any substrate and inhibitor concentration
$V_{max} = k_2[E]_t$ = limiting value of reaction rate at infinite substrate concentration in absence of inhibitor
V^I_{max} = limiting value of reaction rate at infinite substrate concentration and a given inhibitor concentration

Reaction rate equation for $\beta = 0$. From the scheme presented above, the following simplified expression is derived, if $\beta = 0$.

$$v_i = \frac{V_{max}}{K_m(1 + [I]/K_i) + [S](1 + [I]/\alpha K_i)} \quad (1)$$

which in the conventional double-reciprocal form becomes

$$\frac{1}{v_i} = \frac{1}{V_{max}}\left(1 + \frac{[I]}{\alpha K_i}\right) + \frac{1}{V_{max}}\left(1 + \frac{[I]}{K_i}\right)\frac{K_m}{[S]} \quad (2)$$

Equation 2 is applied in the case of succinylbischoline.

Reaction rate equation for $\beta \neq 0$, but not much greater than 1. From the same scheme, the following complete equation is derived.

$$v_i = \frac{V_{max}\alpha K_i[S] + V_{max}(\beta k_2/k_i)[S] + \beta V_{max}[S][I]}{K_m\alpha K_i + K_m(\beta k_2/k_i) + (\beta k_2/k_1)[I] + \alpha K_i[S] + (\beta k_2/k_i)[S] + K_m\alpha[I] + K_m(\beta k_2/k_i)[I]/K_i + (\beta k_2/k_1)[I]^2/K_i + [S][I]} \quad (3)$$

α = characterizes change in constants K_s and K_i induced by binding of I and S, respectively, to E
β = characterizes ratio of rate constants of the reactions $ESI \rightarrow EI + P$ and $ES \rightarrow E + P$

In discussing the effects of the inhibitors studied in the present investigation it is sufficient to consider β values between 0 and 1, and β not much greater than 1. Assuming the presence of a complete equilibrium among E, ES, EI, and ESI, the following conditions must be realized: $k_2 \ll k_{-1}$, $k_2 \ll k_i'$, $\beta k_2 \ll k'_{-i}$, and $\beta k_2 \ll k'_{-1}$. Because

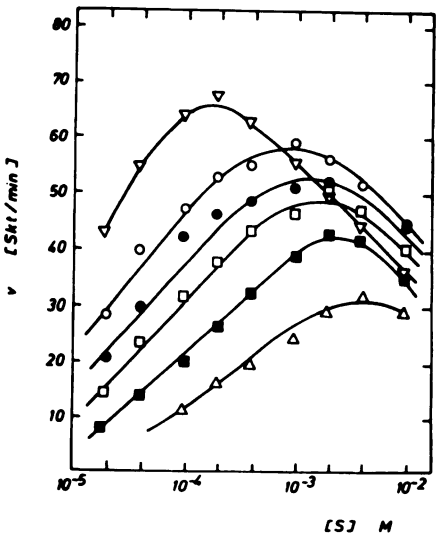

FIG. 1. *Activity-[S] curves for enzymatic hydrolysis of acetylcholine by acetylcholinesterase in the presence of various concentrations of succinylbischoline*

○——○, 0.5 μM; ●——●, 1 μM; □——□, 2 μM; ■——■, 5 μM; △——△, 10 μM; ▽——▽, control.

$k_2 \ll k_i'$ then $\beta k_2 \ll k_i'$ if β is not much greater than 1 (see above).

On account of these considerations Eq. 3 may be simplified to

$$v_i = V_{max}\left(\frac{\alpha K_i + \beta[I]}{\alpha K_i + [I]}\right)\frac{[S]}{(K_m + \beta k_2 k_i[I]/\alpha k_{-i}k_1)\,[(\alpha K_i + \alpha[I])/(\alpha K_i + [I])] + [S]} \quad (4)$$

In the case $\beta = 0$, Eq. 4 is reduced to Eq. 1.

Mechanism of inhibition by *succinylbischoline*. The results for the inhibitor succinylbischoline can be discussed employing Eq. 2. In both the Lineweaver-Burk (14) and Dixon (15) plots, these results yield straight lines (Figs. 4 and 5).

From the Lineweaver-Burk plot the following $1/v$ intercepts are obtained:

$$\lim_{[S]\to\infty}\left(\frac{1}{v_i}\right) = \frac{1}{V_{max}}\left(1 + \frac{[I]}{\alpha K_i}\right) \quad (5)$$

In order to estimate $1/V_{max}$ and $1/\alpha K_i$, these intercepts are plotted against $[I]$ (Fig. 6). The slope of the Lineweaver-Burk lines

$$\frac{d(1/v_i)}{d(1/[S])} = \frac{K_m}{V_{max}}\left(1 + \frac{[I]}{K_i}\right) \quad (6)$$

can be used for the estimation of the K_i and α values. For the Dixon plots, Eq. 2 has to be transformed into the following expression:

$$\frac{1}{v_i} = \frac{1}{V_{max}}\left(1 + \frac{K_m}{[S]}\right) + \frac{1}{V_{max}}\left(\frac{1}{\alpha} + \frac{K_m}{[S]}\right)\frac{[I]}{K_i} \quad (7)$$

From the Dixon plot the following $1/v_i$ intercepts are obtained:

$$\lim_{[I]\to 0}\left(\frac{1}{v_i}\right) = \frac{1}{V_{max}}\left(1 + \frac{K_m}{[S]}\right) \quad (8)$$

The slope of the straight lines is expressed as

$$\frac{d(1/v_i)}{d[I]} = \frac{1}{V_{max}}\left(\frac{1}{\alpha} + \frac{K_m}{[S]}\right)\frac{1}{K_i} \quad (9)$$

The plot of the Dixon $1/v_i$ intercepts as a function of the reciprocal substrate concentration leads to a straight line, from whose slope K_m and V_{max} can be estimated (Fig. 7):

$$\frac{d\left(\lim_{[I]\to 0}(1/v_i)\right)}{d(1/[S])} = \frac{K_m}{V_{max}} \quad (10)$$

The intercept of this line yields V_{max} :

$$\lim_{\substack{[I]\to 0\\ [S]\to\infty}}\left(\frac{1}{v_i}\right) = \frac{1}{V_{max}} \quad (11)$$

The shift of the optimum substrate concentration to higher values in the presence of increased inhibitor concentrations characterizes phenomenologically the competitive part of the "mixed inhibition." In contrast, the increase in $1/V_{max}$ values resulting from the increase in inhibitor concentrations indicates the simultaneous presence of noncompetitive inhibition. The extremely low value of $\alpha = 6.9$ emphasizes the preponderance of the noncompetitive part of the succinylbischoline inhibition mechanism. Purely noncompetitive inhibition is characterized by $K_i' = K_i$, which

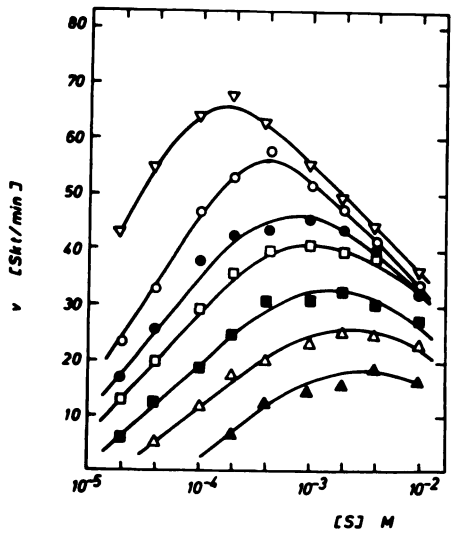

FIG. 2. *Activity–[S] curves for enzymatic hydrolysis of acetylcholine by acetylcholinesterase in the presence of various concentrations of d-tubocurarine*

○——○, 1 μM; ●——●, 2 μM; □——□, 5 μM; ■——■, 10 μM; △——△, 20 μM; ▲——▲, 100 μM; ▽——▽, control.

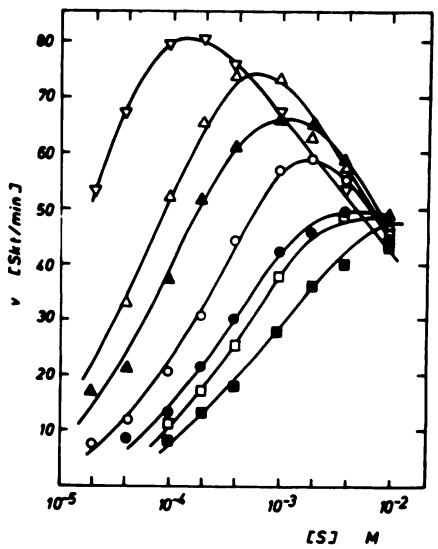

FIG. 3. *Activity–[S] curves for enzymatic hydrolysis of acetylcholine by acetylcholinesterase in the presence of various concentrations of gallamine*

▽——▽, control; △——△, 1 μM; ▲——▲, 2 μM; ○——○, 5 μM; ●——●, 10 μM; □——□, 20 μM; ■——■, 50 μM.

means that $\alpha = 1$ ["simple noncompetitive inhibition" according to Laidler (16)].

In the case of purely competitive inhibition ($\alpha = \infty$), the substrate and inhibitor compete for the active site. In contrast to this, two binding sites for acetylcholine and succinylbischoline are required on account of the low value of α. Although K_i' is not exactly equal to K_i, the inhibition by succinylbischoline has to be characterized as noncompetitive.

Mechanism of inhibition by d-tubocurarine and gallamine. The results of the succinylbischoline experiments show straight lines on both Lineweaver-Burk and Dixon plots (Figs. 4 and 5) within the concentration range tested. In addition, the Lineweaver-Burk plots for *d*-tubocurarine and gallamine are linear (Figs. 8 and 9), whereas the Dixon plots (Figs. 10 and 11) are nonlinear. As shown in the following discussion, the effect of the two inhibitors can be described by assuming a partially competitive and partially noncompetitive mechanism according to Eq. 4. Figures 10 and 11 show that the values calculated by applying Eq. 4 are in agreement with the experimental values.

The estimation of the most important constants, α, β, and K_i, may be shown briefly. [For further information concerning the additional constants and a critical discussion of the kinetics applied to the problems of this investigation, see Wombacher (17).]

It is evident that the equilibrium concentration of the *ESI* complex is comparatively low in the presence of low inhibitor and high substrate concentrations, so that, especially if β is very low, the contribution of the *ESI* complex to enzymatic catalysis can be neglected. Therefore, in the Dixon plots, sections of curves are obtained that may be considered approximately as straight lines. After extrapolation they intersect at one point with the coordinates:

$$\left(-K_i, \frac{1 - 1/\alpha}{V_{max}(1 - \beta/\alpha)}\right) \qquad (12)$$

In the case of gallamine a K_i value of 0.5 μM is obtained (Fig. 12); the corresponding value for *d*-tubocurarine is 0.65 μM. V_{max} and K_m are determined using the intercepts of the Dixon plot, which are plotted against

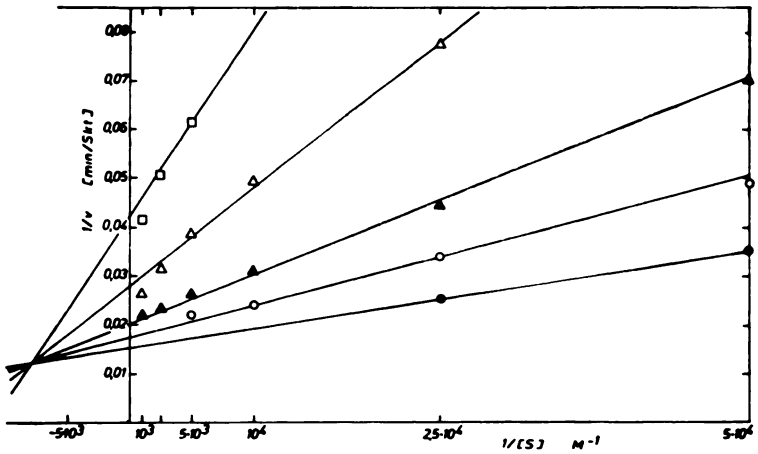

FIG. 4. *Lineweaver-Burk plot*

The curves represent the following concentrations of succinylbischoline: ●——●, 0.5 μM; ○——○, 1 μM; ▲——▲, 2 μM; △——△, 5 μM; □——□, 10 μM.

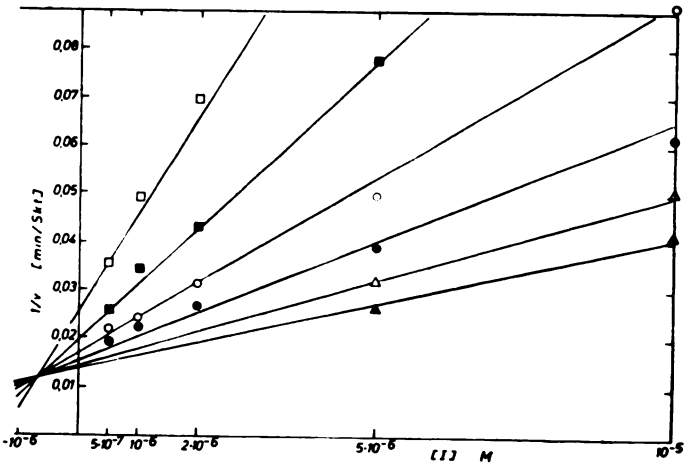

FIG. 5. *Dixon plot*

The curves represent the following concentrations of the substrate acetylcholine: □——□, 20 μM; ■——■, 40 μM; ○——○, 100 μM; ●——●, 200 μM; △——△, 400 μM; ▲——▲, 1 mM.

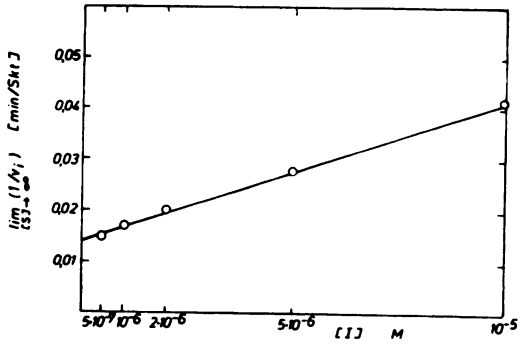

FIG. 6. *1/v intercepts of Lineweaver-Burk plot for succinylbischoline, plotted as a function of inhibitor concentration*

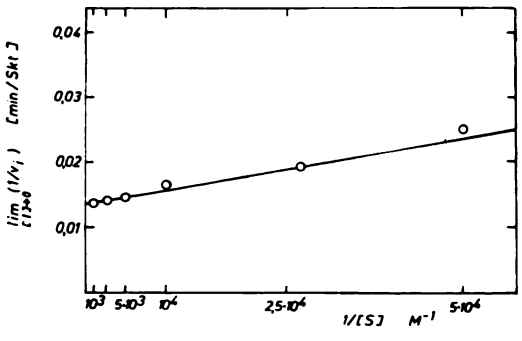

FIG. 7. *1/v intercepts of Dixon plot for succinylbischoline, plotted as a function of 1/[S]*

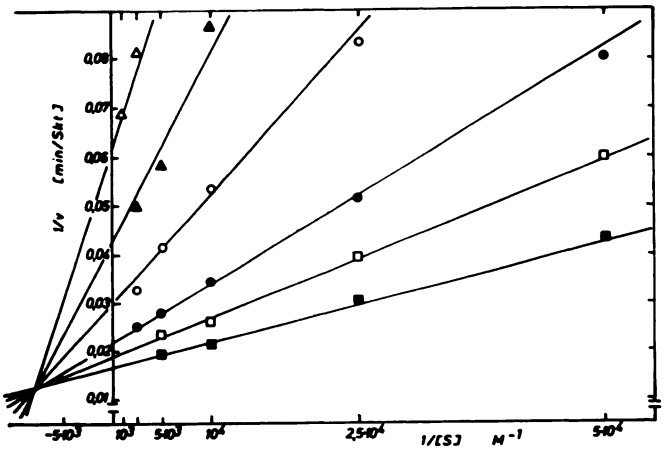

FIG. 8. *Lineweaver-Burk plot*

The curves represent the following concentrations of *d*-tubocurarine: ■——■, 1 μM; □——□, 2 μM; ●——●, 5 μM; ○——○, 10 μM; ▲——▲, 20 μM; △——△, 100 μM.

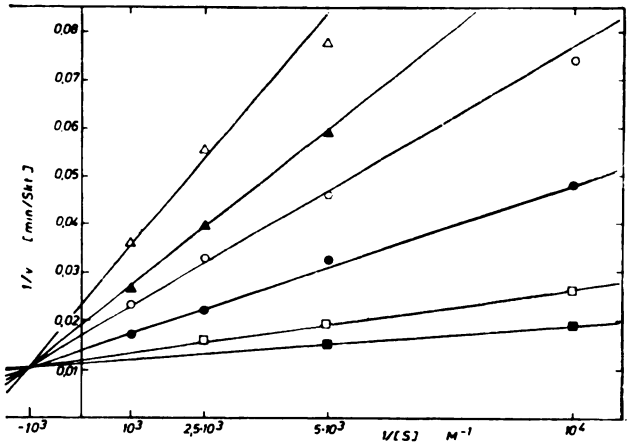

FIG. 9. *Lineweaver-Burk plot*

The curves represent the following concentration of gallamine: ■——■, 1 μM; □——□, 2 μM; ●——●, 5 μM; ○——○, 10 μM; ▲——▲, 20 μM; △——△, 50 μM.

$1/[S]$. From Fig. 13 a K_m value of 32 μM is obtained.

The estimation of α and β is more difficult than the estimation of the K_m and V_{max} values. Equation 4 is transformed into

$$\frac{1}{v_i} = \frac{1}{V_{max}}\left(\frac{1 + [I]/\alpha K_i}{1 + \beta[I]/\alpha K_i}\right) + \frac{K_m + k_2/k_1(\beta[I]/\alpha K_i)}{V_{max}} \cdot \left(\frac{1 + [I]/K_i}{1 + \beta[I]/\alpha K_i}\right)\frac{1}{[S]} \quad (13)$$

As the $\frac{1}{[S]}$ − free term is identical with the intercepts of the Lineweaver-Burk plot, it may be written

$$\frac{1}{\hat{V}^I_{max}} = \frac{1}{V_{max}}\left(\frac{1 + [I]/\alpha K_i}{1 + \beta[I]/\alpha K_i}\right) \quad (14)$$

and, further,

$$\frac{V_{max}}{\hat{V}^I_{max}} = \frac{1 + [I]/\alpha K_i}{1 + \beta[I]/\alpha K_i} \quad (15)$$

The terms α and β can be calculated using

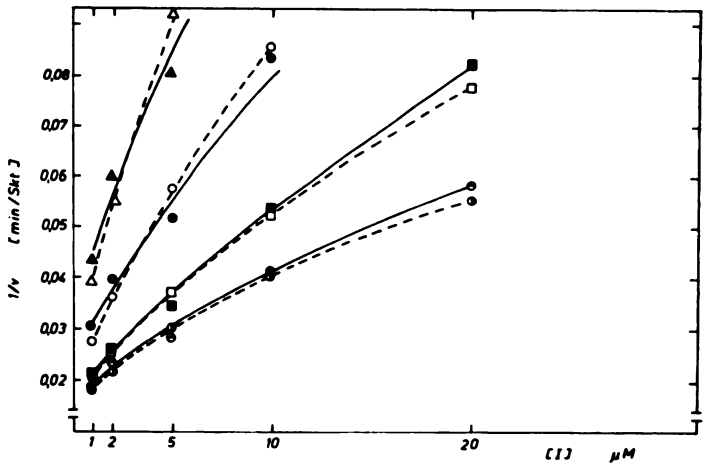

FIG. 10. *Dixon plot*

The curves represent the following concentrations of the substrate acetylcholine: ▲——▲, 20 μM; ●——●, 40 μM; ■——■, 100 μM; ◓——◓, 200 μM. The dashed lines represent the theoretical curves according to Eq. 4.

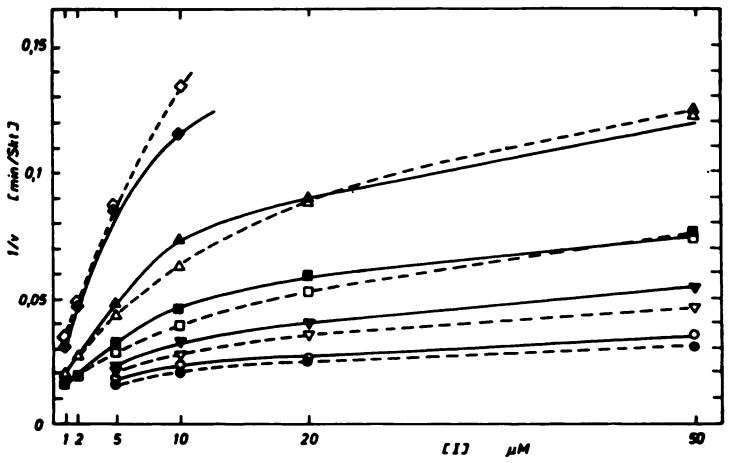

FIG. 11. *Dixon plot*

The curves represent the following concentrations of the substrate acetylcholine: ◆——◆, 40 μM; ▲——▲, 100 μM; ◙——◙, 200 μM; ▼——▼, 400 μM; ○——○, 1 mM. The dashed lines represent the theoretical curves according to Eq. 4.

Eq. 15, which characterizes the dependence on inhibitor concentration of the ratio of the maximum reaction rate in the absence and presence of the inhibitor. From this equation β can be eliminated, as

$$\lim_{[I]\to\infty} \frac{V_{max}}{\hat{V}^I_{max}} = \frac{1}{\beta} \tag{16}$$

For the determination of α, the first derivative of Eq. 15 is taken.

$$\frac{d(V_{max}/\hat{V}^I_{max})}{d[I]} = \frac{1}{K_i}\left[\frac{(1/\alpha) - (\beta/\alpha)}{[1 + (\beta/\alpha)([I]/K_i)]^2}\right] \tag{17}$$

Thus, the determination of α is possible using the maximum slope of Eq. 15:

$$\lim_{[I]\to 0} \frac{d(V_{max}/\hat{V}^I_{max})}{d[I]} = \left(\frac{1-\beta}{K_i}\right)\frac{1}{\alpha} \tag{18}$$

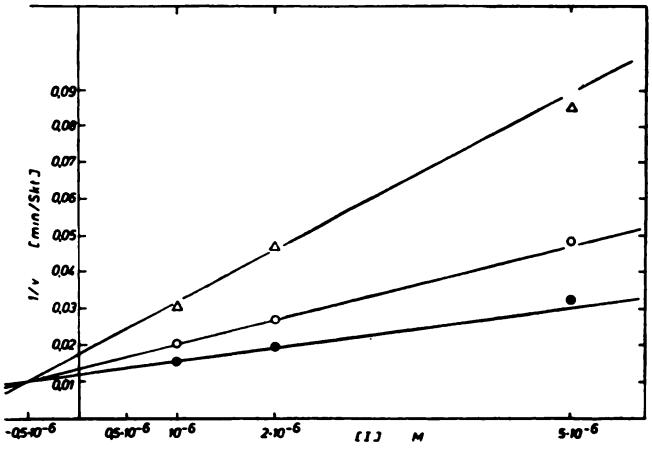

FIG. 12. *Approximate linear portion of Dixon plot for gallamine*
The curves represent the following concentrations of the substrate acetylcholine: △——△, 40 μM; ○——○, 100 μM; ●——●, 200 μM.

According to Fig. 14, in the case of gallamine, $\alpha = 16$ and $\beta = 0.42$; the corresponding values for *d*-tubocurarine are 7 and 0.22.

Finally, the question of the blocking of one reaction path, e.g., by steric hindrance by the inhibitors *d*-tubocurarine and gallamine, may be discussed. This possibility arises not only because of the size of these molecules, but also because of the presence of the $[I]^2$ term in the denominator of the reaction rate equation (Eq. 4). This question may be resolved on the basis of the following consideration. In the case of steric hindrance of enzymatic catalysis by the inhibitors *d*-tubocurarine and gallamine, the reaction rate would fall to zero in the presence of high inhibitor concentrations, since all enzyme would be "trapped" in the form of the *EI* complex and would be unable to react with substrate. However, experiments show that high concentrations of *d*-tubocurarine and gallamine yield a finite value for the reaction rate, the limiting value βV_{max}. Hence it follows that there is no blockage of a reaction path in the present case.

Succinylbischoline as well as *d*-tubocurarine and gallamine show, in addition to the competitive component, noncompetitive inhibition. This noncompetitive part is characterized by a decrease in V_{max}, which cannot be abolished by an increase in substrate concentration. In view of this indica-

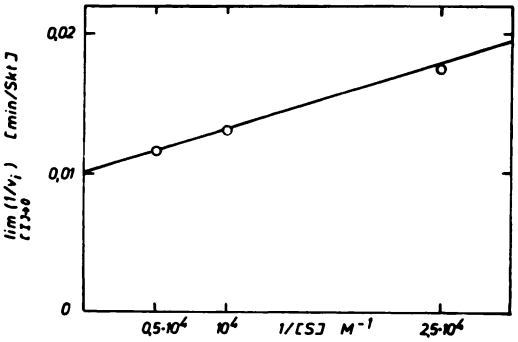

FIG. 13. $1/v$ *intercepts of Dixon plot for gallamine (Fig. 12), plotted as a function of* $1/[S]$

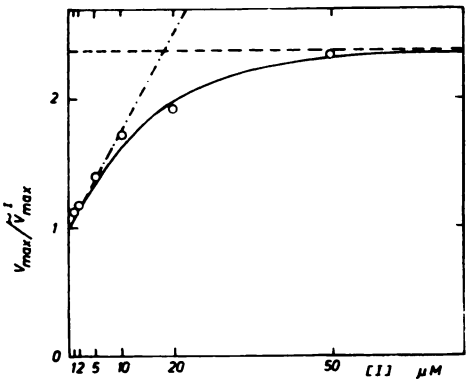

FIG. 14. V_{max}/V^I_{max} *plotted as a function of inhibitor concentration (gallamine)*
This plot describes a graphical method for determining the value of α and β from the highest and lowest slopes of the curve.

TABLE 1

Summary of kinetic constants

Inhibitor	K_i	α	β
	μM		
Succinylbischoline	0.75	6.9	0
d-Tubocurarine	0.65	7.0	0.22
Gallamine	0.50	16.0	0.42

tion of noncompetitive inhibition, the kinetic behavior can be described only if a ternary *ESI* complex is accepted. In contrast to the inhibition by succinylbischoline, this *ESI* complex participates in the enzymatic catalysis, which is represented by the β values for *d*-tubocurarine and gallamine (see Table 1).

Inhibition by other quaternary nitrogen compounds. In the last section quaternary compounds such as succinylbischoline, *d*-tubocurarine, and gallamine were shown to act as very potent inhibitors of acetylcholinesterase. Consequently it is rather surprising that pentamethonium, likewise a bisquaternary nitrogen compound, not only inhibits but also activates acetylcholinesterase, depending on the substrate concentration (see Fig. 15). In contrast, decamethonium, a homologous compound, inhibits very powerfully over the total range of substrate concentration (Fig. 16). The kinetic behavior of the effectors pentamethonium, decamethonium, and tetramethylammonium iodide (Fig. 17) cannot be described by Eq. 4, which is based on an equilibrium analysis. However, a consideration should be discussed that may explain the transformation from inhibition to activation and integrate this behavior into the kinetic picture of acetylcholinesterase obtained so far. Wolf (2), investigating the effects of Mg^{2+} and Ca^{2+} ions on acetylcholinesterase, found inhibition by these ions in the presence of low and activation in the presence of high substrate concentrations.[3] The dependence of activation and inhibition on substrate concentration, which is also observed in the case of pentamethonium and, to a lesser extent, with tetramethylammonium iodide,

[3] This fact prompted us to omit Mg^{2+} and Ca^{2+} from our reaction media.

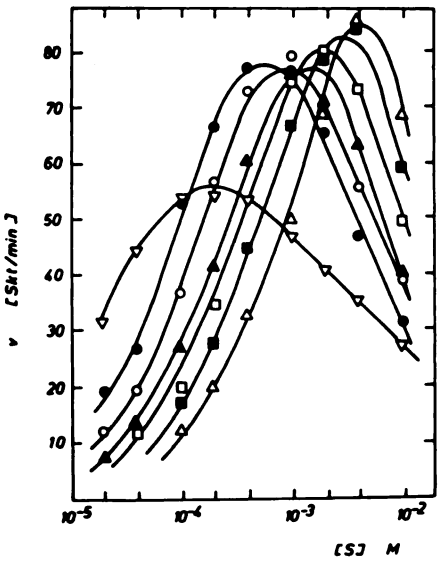

FIG. 15. *Activity–[S] curves for enzymatic hydrolysis of acetylcholine by acetylcholinesterase in the presence of various concentrations of pentamethonium*

●——●, 0.05 mM; ○——○, 0.1 mM; ▲——▲, 0.2 mM; □——□, 0.5 mM; ■——■, 1 mM; △——△, 2 mM; ▽——▽, control.

can be explained in the following way. The basis of this explanation is given by a theoretical study of Laidler (11) concerning exact steady-state equations derived from a general scheme, which is also used by us. The general case of the action of an effector is given by an inequality, in which the reaction rate in the presence of effector is compared with the reaction rate in its absence. After simplification and the adoption of our terminology, we obtain, in the case of activation,

$$\beta > \frac{\alpha + [S]/K_m}{1 + [S]/K_m} \qquad (19)$$

However, if the two sides are equal, there is no net activation even though the reaction may proceed in part via the ternary enzyme complex. If the left-hand side is greater than the right-hand one, activation results; if the left-hand side is smaller, inhibition occurs. Furthermore, the inequality (Eq. 19) shows that there may even be a change-over from inhibition to activation, and vice versa, when the substrate concentration is

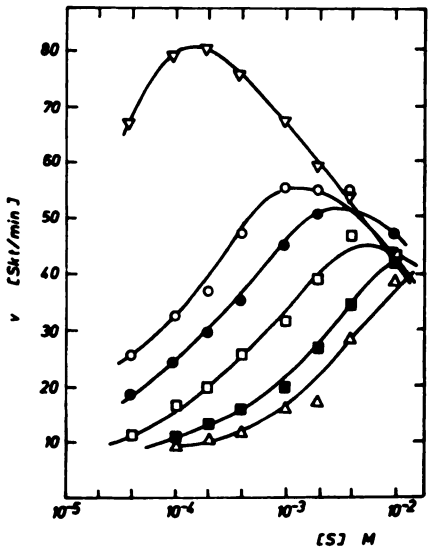

FIG. 16. *Activity-[S] curves for enzymatic hydrolysis of acetylcholine by acetylcholinesterase in the presence of various concentrations of decamethonium*

○——○, 0.05 μM; ●——●, 0.1 μM; □——□, 0.2 μM; ■——■, 0.5 μM; △——△, 1 μM; ▽——▽, control.

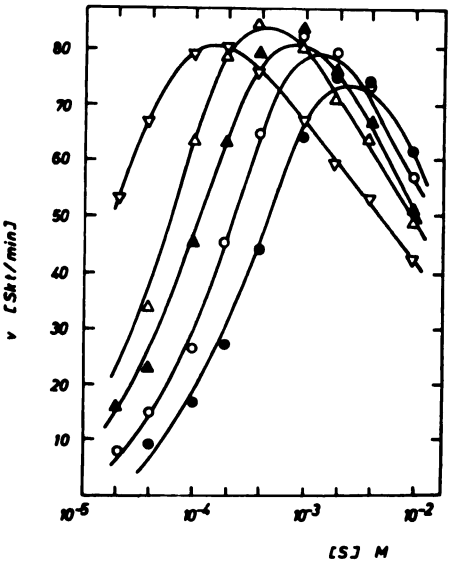

FIG. 17. *Activity-[S] curves for enzymatic hydrolysis of acetylcholine by acetylcholinesterase in the presence of various concentrations of tetramethylammonium iodide*

△——△, 1 mM; ▲——▲, 2 mM; ○——○, 5 mM; ●——●, 10 mM, ▽——▽, control.

varied. Likewise it can be seen from this inequality (Eq. 19) that the same degree of activation or inhibition is given only in the case of a "simple noncompetitive" mechanism (i.e., $\alpha = 1$). Thus, variation of the substrate concentration can change the degree of activation or inhibition, and even a transformation from activation to inhibition, or vice versa, is possible. This phenomenon is associated with the existence of a ternary enzyme complex. However, if "simple noncompetitive" inhibition is present or β is equal to zero, this phenomenon does not occur.

The changeover from inhibition to activation caused by the same effector when the substrate concentration is varied is impressive in the case of pentamethonium (Fig. 15). This may be a proof that in the case of pentamethonium a ternary complex is also formed, and consequently a noncompetitive mechanism may be expected too. However, a "simple noncompetitive" mechanism can be excluded, since in this case the degree of activation or inhibition would be the same throughout the entire range of substrate concentration, and a transformation from inhibition to activation would not be possible.

Acetylcholinesterase as an allosteric enzyme. In the case of both noncompetitive and the mixed type of inhibition, it is evident that the effector molecules bind to sites distinct from the active site. Thus the noncompetitive component of the mechanism suggests that the enzyme reaction proceeds by an allosteric mechanism. The occurrence of a noncompetitive component in the mechanisms of all the inhibitors tested suggests that cooperative effects between the active site and an allosteric site, to be discussed below, are also present in the case of acetylcholinesterase. These cooperative effects may be due to conformational change during the enzyme action [see Koshland and Neet (18)].

In order to explain the kinetic behavior of acetylcholinesterase and the effectors investigated, a "two-site" hypothesis is offered. The existence of two ligand-binding sites, i.e., one regulatory site in addition to the familiar active site, is assumed. The active site consists of an anionic site and an esteratic site, according to well-known

models [cf. Wilson (19) and Krupka and Laidler (20)]. The regulatory site contains at least two negative charges and, in contrast to the active site, possesses no catalytic activity. It may be supposed that compounds whose nitrogen atoms are approximately 14 A apart bind preferentially to the negative charges of the regulatory site. This does not mean that the negative charges have a "preformed" distance of 14 A. In agreement with the "induced fit" theory (21–23), it is supposed instead that the effector induces a conformational change at the regulatory site, i.e., that the positive charges of these compounds (*d*-tubocurarine, gallamine, succinylbischoline) fit the two negative charges in a suitable position. On the other hand, the regulatory site should be able to induce a corresponding conformational change in the effector. Under these circumstances the noncompetitive inhibition observed with these compounds can be interpreted as an allosteric effect upon the catalytic center of the acetylcholinesterase molecule. The binding of these compounds to the regulatory site and the associated conformational change lead to a decrease in the catalytic power of the active site, as shown by the decrease in V_{max}. Since a small competitive component exists in addition to the noncompetitive main part of the mechanisms (mixed inhibition), the question arises whether this competitive part of the inhibition can also be explained by allosteric cooperation between the regulatory and the active site. In this case these compounds would have to be bound to the regulatory site, not to the active site. The affinity of the active site for the substrate is decreased as a result of cooperative interaction between the regulatory site and the active site. As the negative effect on the substrate affinity of the active site is explicable by a conformational change of the residues, which are responsible for the binding of substrate, the observed increase of the K_m value is real.

However, competitive inhibition, in the classical sense of direct competition between substrate and these compounds at the active site, cannot be excluded. The existence of a negative charge at the active site suggests that all positively charged compounds may compete directly for the active site. In this case the observed increase in the K_m value would be only apparent.

Consequently the noncompetitive component of inhibition by these compounds has to be interpreted by an allosteric interaction, whereas the competitive component of inhibition may be interpreted as due to allosteric inhibition, which is induced by binding of these compounds at the regulatory site, as well as to a direct competition reaction.

A reaction similar to the competition reaction between substrate and effector at the active site might take place at the negative charges of the regulatory site. The exact number of these charges is still unknown, but it may be large, since the number of negative charges on the acetylcholinesterase molecule is in the region of 300 (24). The negative charges at the regulatory site are binding sites for substrate as well, but their affinity for substrate is considerably lower than that of the active site, and consequently only high concentrations of acetylcholine interfere with the binding of these compounds at the regulatory site. In the absence and, to a lesser extent, in the presence of effector, acetylcholine at high concentrations is bound to the regulatory site, and it is plausible that the substrate acetylcholine also negatively influences the active site and that the familiar substrate inhibition may be interpreted in this way.

The bisquaternary compound pentamethonium, whose nitrogens are linked by five methylene groups, shows activation or inhibition as the substrate concentration is varied. This remarkable effect is also explicable by the proposed hypothesis. The kinetic analysis has shown that a noncompetitive mechanism is present in the case of pentamethonium too. In contrast to compounds such as *d*-tubocurarine, gallamine, succinylbischoline, and decamethonium, the binding of pentamethonium to the regulatory site results in a positive effect, i.e., in activation of the enzyme reaction (increase in the V_{max} values). The binding of pentamethonium to the regulatory site can be explained preferably on the basis of the

"induced fit" theory (21–23). The possible binding of pentamethonium to the regulatory site could be evidence for the view that the two negative charges postulated at the regulatory site are not fixed at a distance of 14 A.

The high affinity of the regulatory site for bisquaternary compounds whose nitrogens have a distance of approximately 14 A [e.g., for gallamine $K_i = 0.5$ μM, which is in good agreement with the value of 0.3 μM found by Changeux (1)] and the conspicuous noncompetitive behavior in the enzyme mechanism raises the question whether the regulatory site defined in our experiments is identical with the cholinergic receptor proposed by pharmacologists. This possibility was first mentioned by Changeux *et al.* (24) At present, this matter cannot be resolved, although the possibility that the active site is identical with the cholinergic receptor can be excluded with certainty.

The kinetic analysis of our results indicates that two distinct sites, different from each other, must exist. They may be situated either on two different proteins, which are associated by their quaternary structure, or on the acetylcholinesterase molecule, with cooperative interactions occurring between them, as suggested by Podleski (25).

In addition, three other investigations support the idea that acetylcholinesterase is an allosteric enzyme, namely, those of Kitz *et al.* (26), Changeux *et al.* (24), and Meunier and Changeux (27). It is quite interesting that our own results, based on membrane-bound acetylcholinesterase, do not differ from those of Changeux and Meunier, who investigated a very pure, soluble, crystalline preparation of the enzyme.

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