A Steady-State Kinetic Model of Butyrylcholinesterase from Horse Plasma

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(Received 11 March 1974)

The steady-state kinetics of the butyrylcholinesterase-catalysed hydrolysis of butyrylthiocholine and thiophenyl acetate were shown to deviate from Michaelis–Menten kinetics. The ‘best’ empirical rate law was selected by fitting different rate equations to the experimental data by non-linear regression methods. The results were analysed in view of two alternative interpretations: (1) the reaction is catalysed by a mixture of enzymes, or (2) the activity is due to a single enzyme displaying deviations from Michaelis–Menten kinetics. It was concluded that the second alternative applies, and this conclusion was further supported by experiments involving simultaneous hydrolysis of alternative thiol ester substrates (butyrylthiocholine/thiophenyl acetate) as well as alternative thiol ester and oxygen ester substrates (butyrylthiocholine/phenyl acetate; thiophenyl acetate/butyrylcholine; acetylthiocholine/phenyl acetate). On the basis of the conclusion that a single enzyme is responsible for the activity, a molecular model is proposed. This model involves an acylated enzyme, and implies binding to the enzyme of one acyl group and one ester molecule, but not two ester molecules at the same time. Thus butyrylcholinesterase, which is structurally a tetramer, behaves functionally as a co-operative dimer, an interpretation in accordance with available data from active-site titrations.

The steady-state kinetics of the cholinesterases have been subjected to intensive studies during the last decades, but the use of impure enzyme preparations has been a great obstacle in the assessment of satisfactory models of the molecular mechanisms of the enzyme-catalysed reactions. The deviations from the Michaelis–Menten equation observed in the kinetics of butyrylcholinesterase (EC 3.1.1.8) have been given different explanations: (1) the reaction is catalysed by a mixture of enzymes, or (2) the activity is due to a single enzyme displaying deviations from Michaelis–Menten kinetics. The first of these possible explanations is supported by the existence of multiple enzyme forms (see Main, 1969; La Motta & Woronick, 1971; Augustinsson, 1973), whereas the second explanation is founded on structural data, which show that the enzyme is a tetramer (Main *et al.*, 1972).

The aim of the present investigation was to exclude rigorously one of the above-mentioned alternative explanations of the kinetics of butyrylcholinesterase and put forward a simple molecular model of the mechanism in terms of the alternative chosen. The strategy used to select the best mathematical model of the experimental kinetic data was to fit, by non-linear regression methods, alternative rate equations corresponding to reasonable reaction mechanisms. The discrimination between the mathematical models has been based largely on statistical criteria (Bartfai & Mannervik, 1972). This method is considered to be more objective than the usual procedure, which to a

larger extent bases the choice of the rate law on the inspection of proper plots of the data (Cleland, 1967).

Materials and Methods

Enzyme and substrates

Butyrylcholinesterase from horse serum was a partly purified preparation, free from other esterase activities, and was obtained from Organon, Oss., The Netherlands. The enzyme was dissolved in 50 mM-Tris–HCl buffer, pH 7.2.

The choline and thiocholine esters, in the form of iodide salts, were obtained from Sigma Chemical Co., St. Louis, Mo., U.S.A. Phenyl acetate and thiophenyl acetate were synthesized as described by Augustinsson *et al.* (1972) or were purchased from Fluka A.G., Buchs, Switzerland (phenyl acetate) and Polysciences Inc., Warrington, Pa., U.S.A. (thiophenyl acetate) respectively.

Other reagents

5,5′-Dithiobis-(2-nitrobenzoic acid) was obtained from Sigma and was dissolved in 50 mM-Tris–HCl buffer (pH 7.2) to give a 0.25 mM solution. Other chemicals used were standard commercial products and all solutions were made up in twice-deionized water.

Assay of esterase activity

Esterase activity was measured by a spectrophotometric method based on a technique first described by

Ellman *et al.* (1961) for cholinesterases, which was recently modified for the quantitative assay of other esterase activities as well (Augustinsson *et al.*, 1972). Activity measurements were made at 30°C with a Beckman DB-G spectrophotometer by using a Contron 3012 recorder to monitor the increase of absorbance at 412nm. The results shown in Fig. 1(a) were obtained by using an Aminco-Chance dual-wavelength spectrophotometer (highest sensitivity: 0.05 absorbance unit for full-scale deflexion).

The reaction mixture was composed of 3.0ml of 50mM-Tris-HCl buffer (pH7.2), containing 0.25mM-5,5'-dithiobis-(2-nitrobenzoate), 0.02ml of substrate solution, and 0.05ml of the enzyme solution, added in that order. The spontaneous hydrolysis of the substrates was negligible under the experimental conditions used.

It was claimed (Brownson & Watts, 1973) that 5,5'-dithiobis-(2-nitrobenzoate), used in the assay system, is responsible for an activation of butyrylcholinesterase. However, experiments in this institute (Augustinsson & Eriksson, 1974) showed that no effect of 5,5'-dithiobis-(2-nitrobenzoate) on butyrylcholinesterase can be detected when the activity was measured by either the spectrophotometric or the Warburg (Augustinsson, 1957) technique. In fact, these experiments suggest that a side reaction had perturbed the data of Brownson & Watts (1973). Thus it is concluded that the deviations from Michaelis-Menten kinetics treated in the present

paper arise from the enzymic properties of butyrylcholinesterase.

Several 5,5'-dithiobis-(2-nitrobenzoate) concentrations were tested to ascertain that the enzymic reaction was rate-limiting, and at all substrate concentrations used, 0.25mM-5,5'-dithiobis-(2-nitrobenzoate) fulfilled this requirement.

The reaction velocity was expressed in μmol of substrate hydrolysed/min.

Data treatment

The mathematical models were fitted to the experimental data by a Gauss-Newton non-linear regression program (University of California, Biomedical Programs, BMDP-3R). The results were evaluated by means of discrimination criteria (Bartfai & Mannervik, 1972; Mannervik & Bartfai, 1973), with special reference to the comparison of the sum of squares (Q^2) of the alternative models:

$$Q_j^2 = \left[\sum_{i=1}^n (v_i - \hat{v}_{ij})^2 \right] / (n - p_j) \\ = \left[\sum_{i=1}^n q_{ij}^2 \right] / (n - p_j) \quad (1)$$

where v_i is the measured velocity obtained in the i th experimental point, $\hat{v}_{ij}$ is the velocity calculated

Fig. 1. Effect of (a) butyrylthiocholine and (b) thiophenyl acetate on the initial velocity

The amount of enzyme used in the reaction systems of the two experiments was (a) 8.3 and (b) 16.7 μg . The enzyme solutions in these experiments were prepared from a different batch as compared with those used in Figs. 3 and 4. For further details see the Materials and Methods section.

according to the j th model, n is the number of experimental points, p_j is the number of parameters included in model j , and q_{ij} is the i th residual given by the j th model. The choice of the best mathematical model was also facilitated by examination of residual plots.

Several sets of initial values of the parameters were used in the regression to confirm that the global minimum of the sum of squares had been found for each model. The standard errors cited are asymptotic values calculated under the assumption of linearity of the models in the vicinity of the solution vector in the parameter space.

Results

Single-substrate data

Fig. 1 indicates that butyrylcholinesterase does not obey the Michaelis–Menten equation when butyrylthiocholine (Fig. 1a) or thiophenyl acetate (Fig. 1b) serves as the substrate. To confirm that the deviations from linearity shown in Fig. 1 are statistically significant and to select the best mathematical model for the description of the data, the equations shown in Tables 1 and 2 were used in a non-linear regression analysis. Higher-power terms are required in the rate equations

to describe the deviations from Michaelis–Menten kinetics, and successively more complicated rate laws were considered until the fit to the data could not be improved. The mathematical models used were limited to those which correspond to realistic kinetic mechanisms, e.g. the equation for an enzyme with two kinetically non-identical sites (IV), of which one may give a dead-end complex with the substrate (II) or serve as a non-catalytic regulatory site (III) (Cleland, 1967). Some of the models fitted to the data could have been dismissed by inspection of plots, but since the two substrates gave different curve shapes, it was decided to fit all equations considered to both data sets. In this approach, examination of the parameter values of an inappropriate model demonstrates how the model degenerates to a simpler rate law, whereas the sum of squares gives an objective quantitative measure of the goodness of fit.

Comparison of the sums of squares in Table 1 shows that model IV offers the best description of the kinetics of the hydrolysis of butyrylthiocholine. The data (cf. Fig. 1a) are scattered at velocities below $0.1 \mu\text{mol/min}$, indicating the limitations of the spectrophotometric method used, but model IV was the best model irrespective of whether the velocity values lower than $0.1 \mu\text{mol/min}$ were included or excluded

Table 1. Model-fitting by non-linear regression to kinetic data obtained when butyrylthiocholine served as substrate

The data are presented in Fig. 1(a) ($n = 91$). Units of the parameters are expressed in their appropriate dimensions by using $\mu\text{mol/min}$ and mM as basic units of velocity and concentration.

Model	Parameter values ($\pm$ S.E.)		Q^2 value ($\mu\text{mol}^2/\text{min}^2$)
I $v = \frac{V[A]}{K + [A]}$	$V = 1.31 \pm 0.02$	$K = 0.31 \pm 0.02$	0.003413
II $v = \frac{V[A]}{K_1 + [A] + K_2[A]^2}$	$V = 1.31 \pm 0.02$	$K_1 = 0.31 \pm 0.02$ $K_2 = 10^{-6*}$	0.003453
III $v = \frac{V[A]^2}{K_1 + K_2[A] + [A]^2}$	$V = 1.31 \pm 0.02$	$K_1 = 10^{-6*}$ $K_2 = 0.31 \pm 0.02$	0.003493
IV $v = \frac{V_1[A] + V_2[A]^2}{1 + K_1[A] + K_2[A]^2}$	$V_1 = 9.57 \pm 0.27$ $V_2 = 15.43 \pm 1.63$	$K_1 = 16.28 \pm 0.90$ $K_2 = 10.79 \pm 1.17$	0.000138
V $v = \frac{V_1[A]}{K_1 + [A]} + \frac{V_2[A]}{K_2 + [A]}$	$V_1 = 0.85 \pm 0.02$ $V_2 = 2.13 \pm 0.13$	$K_1 = 0.39 \pm 0.01$ $K_2 = 0.064 \pm 0.004$	0.000138
VI $v = \frac{V_1[A] + V_2[A]^2}{1 + K_1[A] + K_2[A]^2 + K_3[A]^3}$	$V_1 = 9.49 \pm 0.28$ $V_2 = 14.24 \pm 1.91$	$K_1 = 15.86 \pm 0.96$ $K_2 = 9.83 \pm 1.44$ $K_3 = 0.007 \pm 0.007$	0.000138
VII $v = \frac{V_1[A] + V_2[A]^2 + V_3[A]^3}{1 + K_1[A] + K_2[A]^2 + K_3[A]^3}$	$V_1 = 9.49 \pm 0.27$ $V_2 = 14.24 \pm 1.93$ $V_3 = 0.0066^\dagger$	$K_1 = 15.86 \pm 0.97$ $K_2 = 9.83 \pm 1.44$ $K_3 = 0.012 \pm 0.007$	0.000139

* Lower limit of this parameter in the regression.

† This parameter value was found at the beginning of the iteration; therefore no standard error was obtained.

from the regression analysis. It should be noted that the substrate concentrations cover a 40000- or a 2000-fold range in the two cases. Weighting of the data, under assumption of constant relative error, also confirmed the choice of model IV. On the basis of statistical criteria (Bartfai & Mannervik, 1972) we conclude that the best description of the present data is given by model IV.

The same models were fitted to the data obtained with thiophenyl acetate as the substrate (Table 2), and here too model IV proved to be the best one.

Each of the equations can be interpreted in terms of different physical models or reaction schemes but at this stage of analysis the equations only serve to establish by statistical criteria the best empirical rate law; an interpretation in terms of a chemical mechanism is attempted, according to the strategy used, only when the proper rate law has been selected. For both substrates model IV was found to give the smallest sum of squares (Q^2) of all models tested, which had no redundant parameter values and lacked any apparent correlation between residuals and substrate concentration or velocity. To exemplify the residual analysis, the plots of residuals versus predicted velocity corresponding to models I and IV are

shown in Fig. 2. The residuals of model I exhibit a sigmoidal correlation (Fig. 2a), whereas the residuals of model IV do not show any functional dependence on the velocity or substrate concentration (Fig. 2b). When butyrylthiocholine was used as the substrate, model V, which is a restricted form of model IV (applicable in some cases when two enzymes act on the same substrate; see the Appendix), gave the same Q^2 value as model IV.

Alternative-substrates data

Experiments were carried out in the presence of both butyrylthiocholine and thiophenyl acetate in an attempt to find out whether the activities obtained with the two substrates are due to a single enzymic species or to more than one enzyme form. The observed velocity was independent of the presence of thiophenyl acetate at high butyrylthiocholine concentrations (Fig. 3), which shows that under saturating conditions the latter compound eliminates the contribution of thiophenyl acetate to the activity. However, excess of thiophenyl acetate did not extinguish the hydrolysis of butyrylthiocholine in the concentration range examined.

In the experiments in which both thiol esters were

Table 2. Model fitting by non-linear regression to kinetic data obtained when thiophenyl acetate served as substrate

The data are presented in Fig. 1(b) ($n = 19$). Units of the parameters are expressed in their appropriate dimensions by using $\mu\text{mol/min}$ and mM as basic units of velocity and concentration.

Model	Parameter values ($\pm$ S.E.)		Q^2 value ($\mu\text{mol}^2/\text{min}^2$)
I $v = \frac{V[A]}{K + [A]}$	$V = 3.99 \pm 0.20$	$K = 3.24 \pm 0.36$	0.0117
II $v = \frac{V[A]}{K_1 + [A] + K_2[A]^2}$	$V = 8.99 \pm 2.18$	$K_1 = 8.99 \pm 2.58$ $K_2 = 0.138 \pm 0.057$	0.0039
III $v = \frac{V[A]^2}{K_1 + K_2[A] + [A]^2}$	$V = 3.27 \pm 0.10$	$K_1 = 1.04 \pm 0.14$ $K_2 = 1.42 \pm 0.22$	0.0026
IV $v = \frac{V_1[A] + V_2[A]^2}{1 + K_1[A] + K_2[A]^2}$	$V_1 = 0.592 \pm 0.079$ $V_2 = 0.828 \pm 0.225$	$K_1 = 0.282 \pm 0.099$ $K_2 = 0.279 \pm 0.070$	0.0008
V $v = \frac{V_1[A]}{K_1 + [A]} + \frac{V_2[A]}{K_2 + [A]}$	$V_1 = 1.03^\dagger$ $V_2 = 3.24^\dagger$	$K_1 = 2.90 \pm 0.21$ $K_2 = 3.24 \pm 0.52$	0.0142
VI $v = \frac{V_1[A] + V_2[A]^2}{1 + K_1[A] + K_2[A]^2 + K_3[A]^3}$	$V_1 = 0.576 \pm 0.113$ $V_2 = 0.952 \pm 0.566$	$K_1 = 0.375 \pm 0.378$ $K_2 = 0.301 \pm 0.123$ $K_3 = 0.001 \pm 0.004$	0.0010
VII $v = \frac{V_1[A] + V_2[A]^2 + V_3[A]^3}{1 + K_1[A] + K_2[A]^2 + K_3[A]^3}$	$V_1 = 0.576 \pm 0.118$ $V_2 = 0.952 \pm 0.591$ $V_3 = 10^{-5*}$	$K_1 = 0.375 \pm 0.395$ $K_2 = 0.301 \pm 0.128$ $K_3 = 0.001 \pm 0.005$	0.0009

* Lower limit of this parameter in the regression.

† This parameter value was found at the beginning of the iteration; therefore no standard error was obtained. Model V cannot afford a good fit, because the values of K_1 and K_2 of model IV violate relation (1) given in the Appendix.

Fig. 2. Plots of residuals versus predicted velocities obtained by fitting the experimental data of Fig. 1(a) to (a) model I and (b) model IV

Note that model I is highly unsatisfactory (in spite of reasonable parameter values; cf. Table 1) owing to the sigmoidal correlation of the residuals with the predicted velocity.

Fig. 3. Effect of simultaneous presence of butyrylthiocholine and thiophenyl acetate on the initial velocity

Butyrylthiocholine concentration was varied and thiophenyl acetate was used at fixed concentrations of (○) 3.2, (△) 1.0, and (●) 0.32 mM; the amount of enzyme used in the reaction system was 8.3 μg.

used, the observed velocity was due to the hydrolysis of both substrates. Supplementary information is obtainable by the simultaneous use of an oxygen ester and a thiol ester, because the spectrophotometric assay used records only hydrolysis of the thiol ester. In such experiments, involving alternative substrates, the oxygen ester will nonetheless influence the kinetics by competition with the thiol ester substrate, and the effect is an inhibition of the hydrolysis of

the latter compound. Three such combinations were used, namely, butyrylthiocholine/phenyl acetate, thiophenyl acetate/butyrylcholine and acetylthiocholine/phenyl acetate. The experiments involving the first and second pairs of alternative substrates correspond to the experiment shown in Fig. 3, with the substitution of oxygen for sulphur in one of the thiol esters. The third combination gives additional information by use of substrates having the same acyl moiety. Fig. 4 shows the results of these experiments, and, in accordance with the prediction, the oxygen esters inhibited the hydrolysis of the thiol esters. Three mathematical models, which correspond to alternative physical mechanisms, were fitted to the data (Table 3). Model IV applies if the oxygen ester does not interfere kinetically with the enzyme hydrolysing the thiol ester. Model VIII corresponds to the scheme in Fig. 5(b), with the limitation that no EAB complex is permitted, and model IX was derived for the reaction scheme in Fig. 6(b). The rate equation of the mechanism including EAB in Fig. 5 (model X) has too many parameters to be fitted directly, but subsets of data were examined to evaluate the adequacy of model X (see the Discussion section). Model IX gave the best fit for all of the three combinations of alternative substrates.

Discussion

The non-linearity demonstrated in Fig. 1(a), which was also confirmed by regression analysis, can be given two basically different interpretations. The first explanation is that more than one enzyme acts on the substrate, as earlier proposed for butyrylcholinesterase (Wilson, 1954; Heilbronn, 1958). The second alternative is that the activity is due to a single enzyme, which does not obey the Michaelis-Menten equation (Hardegg, 1958; Augustinsson, 1973). The data obtained with thiophenyl acetate, on the other hand, show a type of non-linearity (cf. Fig. 1b), which can not be explained as the resultant of two Michaelis-Menten equations (model V), owing to the inherent constraint on the parameters ($4K_2 \leq K_1^2$; see the Appendix). According to Table 2, $4K_2 = 1.128$ and $K_1^2 = 0.078$, values incompatible with model V. Therefore the least restrictive conclusion is that at least one enzyme is present, the kinetics of which obey model IV.

Fig. 3 shows that increasing the butyrylthiocholine concentration extinguishes the contribution of thiophenyl acetate hydrolysis to the observed velocity. This result implies that any enzyme species which is active with thiophenyl acetate must also use butyrylthiocholine as a substrate. The simplest assumption is that there is only one enzyme species catalysing the hydrolysis of each substrate. If so, a single enzyme is responsible for the activity obtained with both substrates. This conclusion has strong support from the

Fig. 4. Effect of alternative oxygen ester and thiol ester substrates on the initial velocities

(a) Subset of the experimental data used for Table 3 showing the effect of varying the butyrylthiocholine concentrations in the absence (○) or presence (●) of phenyl acetate (10mM). (b) Subset of the experimental data used for Table 3 showing the effect of varying the thiophenyl acetate concentrations in the absence (○) or presence (●) of butyrylcholine (1mM). (c) Data set used for Table 3 showing the effect of varying the acetylthiocholine concentration in the absence or presence of fixed levels of phenyl acetate. The concentrations of the latter compound were (from top to bottom) zero, 1.0, 5.0, 10, 15 and 20mM. The amount of enzyme used in the reaction systems of different experiments was 8.3, 16.7 and 42μg respectively.

Table 3. Model fitting by non-linear regression to kinetic data obtained from experiments with alternative oxygen ester and thiol ester substrates

Subsets of the data are presented in Fig. 4. A denotes the thiol ester, the hydrolysis of which is measured; n is the number of experimental points. The rate equations were derived according to the rules of Wong & Hanes (1962). Model IV applies if two enzymes are present, one reacting exclusively with the aromatic ester and one only with the choline ester. Model VIII obtains when the only connexion in the reaction scheme between the EA and EB complexes is via the free enzyme E. Model IX corresponds to the scheme in Fig. 6(b).

Model	Q^2 value(μmol ² /min ²)		
	A = acetylthio- choline B = phenyl acetate ($n = 74$)	A = butyrylthio- choline B = phenyl acetate ($n = 133$)	A = thiophenyl acetate B = butyrylcholine ($n = 158$)
IV $v = \frac{V_1[A] + V_2[A]^2}{1 + K_1[A] + K_2[A]^2}$	3.563	0.345	2.632
VIII $v = \frac{V_1[A] + V_2[A]^2}{1 + K_1[A] + K_2[A]^2 + K_3[B] + K_4[B]^2}$	0.467	0.0718	0.666
IX $v = \frac{V_1[A] + V_2[A]^2 + V_3[A][B]}{K_1 + K_2[A] + K_3[A]^2 + K_4[A][B] + K_5[B] + [B]^2}$	0.191	0.0173	0.135

Fig. 5. Simple reaction schemes for single-substrate (a) and alternative-substrates experiments (b)

E symbolizes the enzyme and A and B denote the alternative substrates. The products of a substrate molecule are released in the step marked with a curved arrow, which leads from the corresponding enzyme-substrate complex. The general rate laws of the steady-state kinetics corresponding to these schemes are models IV and X.

Fig. 6. Reaction schemes involving an intermediate F (e.g. acylated enzyme), which is distinct from the EA (or EB) complex, in single-substrate (a) and alternative-substrates (b) experiments

The products of a substrate (e.g. thiocholine and acetate) are released in two separate reaction steps, which are unidirectional in the diagram, because the product concentrations are zero when initial rates are measured. Triangular loops involving addition of substrate followed by release of products (e.g. $E \rightarrow EA \rightarrow F \rightarrow E$ or $F \rightarrow FB \rightarrow EB \rightarrow F$) are productive patterns in the mechanism. The general rate laws corresponding to the schemes are models IV and IX respectively.

experiments in which one of the substrates was exchanged for the corresponding oxygen ester, because the best mathematical model (IX) of these experiments predicts complete inhibition of the hydrolysis of the thiol ester by excess of the oxygen analogue. This result was obtained with butyrylthiocholine and phenyl acetate as well as with thiophenyl acetate and butyrylcholine. Consequently, any enzyme species active with the thiol esters is able to bind any of the oxygen esters. This finding proves that if more than one enzyme species is present, all are active with both butyrylthiocholine and thiophenyl acetate. We therefore conclude that only a single enzyme is responsible for all activities measured, and that the deviations from Michaelis-Menten kinetics should be interpreted in terms of a single enzyme species rather than a mixture of enzymes.

The finding that no model more complex than model IV can be supported by the regression analysis indicates, according to the structural rules of Wong & Hanes (1962), that only two elementary steps involving the addition of substrate have to be predicted in the scheme of the reaction mechanism. It is evident that more complex reaction schemes can be postulated, but since mechanisms leading to third- or higher-power terms in the rate equation cannot be supported by the results of the present analysis, we have omitted such models from the discussion.

Two simple reaction schemes were examined, which were consistent with rate law IV. One includes consecutive addition of two ester molecules to the enzyme. The release of the dissimilar product molecules originating from a single substrate molecule is depicted as a single step (Fig. 5a). The second scheme (Fig. 6a) involves an additional enzyme form F, which may be identified with the acyl intermediate

of the enzyme, introduced for the description of acetylcholinesterase (Wilson *et al.*, 1950; Krupka & Laidler, 1961). A discrimination between these alternatives can be founded on the results of experiment in which alternative substrates, i.e. thiol esters and oxygen esters, were added simultaneously to the enzyme. Since the hydrolysis of the oxygen ester also deviates from Michaelis-Menten kinetics (Wilson, 1954; Hardegg, 1958; Heilbronn, 1958) it was assumed that kinetic models symmetrical with respect to thiol and oxygen ester substrate should be examined for description of the data.

When a model of the alternative-substrates experiments is derived on the basis of consecutive symmetrical addition of substrates (Fig. 5b) rate equation X obtains:

$$v = \frac{V_1[A] + V_2[A]^2 + V_3[A]^3 + V_4[A][B] + V_5[A]^2[B] + V_6[A][B]^2}{K_1 + K_2[A] + K_3[A]^2 + K_4[A]^3 + K_5[B] + K_6[B]^2 + K_7[B]^3 + K_8[A][B] + K_9[A]^2[B] + [A][B]^2} \quad (X)$$

under conditions when A is the thiol ester and B is the oxygen ester, v is the rate of thiol formation, V_i ($i = 1-6$) and K_j ($j = 1-9$) are kinetic constants.

The model based on the acyl intermediate gives rise to rate equation IX, when the acyl moieties of the alternative substrates are identical (Fig. 6b):

$$v = \frac{V_1[A] + V_2[A]^2 + V_3[A][B]}{K_1 + K_2[A] + K_3[A]^2 + K_4[A][B] + K_5[B] + [B]^2} \quad (IX)$$

Subsets of data obtained by varying $[A]$ at constant $[B]$ should be described by an equation including third-power terms of $[A]$ (model VII) according to the consecutive substrate addition scheme (Fig. 5), and by an equation containing second-power terms of $[A]$ (model IV) according to the acyl-intermediate mechanism, respectively.

Fitting of model VII to subsets of data, obtained by varying the acetylthiocholine (A) concentration at different fixed concentrations of phenyl acetate (B), gave Q^2 values which were not significantly different from the corresponding Q^2 values obtained with model IV. Examination of the parameter values showed that model VII degenerated to model IV.

This result and the similarities between butyrylcholinesterase and acetylcholinesterase favour the mechanism involving the acylated enzyme as an intermediate (Fig. 6), even if no direct evidence for the existence of such an intermediate of butyrylcholinesterase has been obtained. The corresponding rate equation (model IX) turned out to be the best mathematical model also for the description of the experiments involving the pairs of butyrylthiocholine/phenyl acetate and thiophenyl acetate/butyrylcholine as the alternative substrates, in spite of the fact that the acyl moieties bound to the enzyme are different. A reaction scheme involving alternative substrates,

containing different acyl groups, would be expected to lead to a more complicated rate equation, which however, is not required for the description of the experimental data. Therefore the different acyl-containing enzyme forms seem to be kinetically indistinguishable and representable by the same enzyme form (F) in the reaction scheme. Mechanistically this interpretation implies that an acylated esteratic site of the enzyme signals 'occupied' with the same consequences for the kinetics irrespective of whether the acyl group is acetyl or butyryl. The reaction scheme of Fig. 6 also accounts for the deviations from Michaelis-Menten kinetics noted above for oxygen ester substrates of butyrylcholinesterase.

Our interpretation of the mathematical model (IV) finally chosen is that the kinetically demonstrable

binding may involve one ester molecule and one acyl group, but not two ester molecules per enzyme molecule. The most likely molecular mechanism corresponding to this reaction pattern is that two subunits of the tetrameric butyrylcholinesterase (Main *et al.*, 1972) can operate simultaneously in the catalysis, which is consistent with the finding that two sites per molecule are phosphorylated by di-isopropyl phosphorofluoridate (Main *et al.*, 1972). Fig. 7 shows a reaction scheme, which combines the information obtained from the kinetic studies with the structural data. The enzyme is represented as a functional dimer and an enzyme form corresponding to the doubly

Fig. 7. A reaction scheme of butyrylcholinesterase

The enzyme is depicted as a functional dimer, although it is structurally a tetramer; monomers acylated by the substrate are shaded.

acylated enzyme is included in addition to the corresponding scheme in Fig. 6. This extension of the model will not alter the form of the rate equation (model IV), but has support from the titration experiments with di-isopropyl phosphorofluoridate (Main *et al.*, 1972).

It is also possible to elaborate mechanisms in terms of 'half-site reactivity' (Levitzki *et al.*, 1971) and 'flip-flop' mechanisms (cf. Lazdunski, 1972) which are consistent with model IV. However, more detailed mechanisms can be founded only on more extensive structural and kinetic data.

The model proposed can be formulated in the terms of homotropic co-operative regulation of butyrylcholinesterase by the ester substrate, the effect of which is that the first substrate bound prevents binding of a second substrate molecule. This view is based on the lack of demonstrable $[A]^3$ terms in the best mathematical mode which would be expected if the reaction scheme in Fig. 6(a) were extended with an EAA complex. Once the leaving group of the substrate has been released this inhibition of binding of a substrate molecule is relieved in spite of the fact that the subunit binding the first substrate is still blocked by the remaining acyl group.

The structural similarities of the substrate-binding sites of acetylcholinesterase and butyrylcholinesterase have been discussed (see Augustinsson, 1966). The model proposed in the present paper seems to extend the similarities to homotropic co-operativity, which has earlier been established for acetylcholinesterase (Changeux *et al.*, 1968; Kato *et al.*, 1972; Rosenberry & Bernhard, 1972).

The present investigation of butyrylcholinesterase has been focused on the analysis of kinetic data obtained with one choline and one non-choline ester and on selection of one mathematical model of several alternatives. The aim has been to set the overall framework which can serve as the basis for the formulation of more detailed chemical mechanisms. For the latter purpose a more extensive investigation has to be carried out, particularly to evaluate the role of the quaternary nitrogen atom in the 'natural' substrate. The structural differences between the choline and non-choline esters are reflected by the differences in the kinetics, which are expressed in the curve shapes in Figs. 1(a) and 1(b). The unique structure of the choline esters, which is fundamental for the specific biological activities of the compounds, is also instru-

mental in the expression of the regulatory properties of the cholinesterases.

Mr. Håkan Eriksson contributed to this investigation in its early phase. His continued interest in the work is gratefully acknowledged. We thank Mrs. Inga Andersson for excellent performance of the experimental part and Miss Kerstin Jacobsson for skilful assistance in the computer work. This investigation was supported by the Swedish Natural Science Research Council.

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APPENDIX

Discrimination between Different Interpretations in Terms of Number of Enzyme Species of an Empirical Rate Equation Involving Second-Degree Terms of the Substrate Concentration

Assume that the true empirical rate equation of an enzymic reaction involving one substrate is model IV

$$v = \frac{V_1[A] + V_2[A]^2}{1 + K_1[A] + K_2[A]^2} \quad (\text{IV})$$

where V_1 , V_2 , K_1 and K_2 are composite kinetic constants. Two basically different interpretations of this equation can be proposed in terms of molecular models, i.e. a single enzyme, which does not obey the Michaelis-Menten equation, is present, or two enzymes are acting on the same substrate. The problem is to find out under which conditions a choice can be made between the alternatives given. A related problem, treated previously by several authors (see Cleland, 1970; Laidler & Bunting, 1973), is the shape of the curve resulting from the sum of two Michaelis-Menten equations (model V). In these analyses it has been proved that model V cannot give sigmoid kinetics, and consequently results in a curve concave upward in a plot of v versus $v/[A]$. In the present treatment the numerical relationship between the constants of model IV has been examined in order to find a criterion that can be used for the discrimination between the alternative origins of model IV.

The simplest and most likely case of the alternative involving two enzymes is realized when both enzymes obey the Michaelis-Menten equation but have unequal Michaelis constants

$$v = \frac{V'[A]}{K' + [A]} + \frac{V''[A]}{K'' + [A]} \quad (\text{V})$$

where V' and V'' are (equal or unequal) maximal velocities, and K' and K'' are the Michaelis constants. Alternative, but very unlikely, possibilities are that there are present (1) two enzymes both obeying model IV and having values of K_1 and K_2 that are identical for both enzymes respectively; or (2) two

enzymes, one obeying model IV and one obeying model I, provided that $1/K = \sqrt{K_2}$ and $K_1 = 2\sqrt{K_2}$. Thirdly, it should be noted that in general it is impossible to evaluate the number of enzyme species present, if the denominators of the individual rate equations are identical.

If model IV is produced by a single enzyme, the parameters K_1 and K_2 may assume any positive value. However, if model IV arises according to model V, the values of empirical parameters K_1 and K_2 must fulfil the restriction

$$4K_2 \leq K_1^2 \quad (1)$$

Thus, violation of condition (1) provides strong evidence that model IV results from a single enzyme and not from a mixture of enzymes. However, values satisfying $4K_2 \leq K_1^2$ contain no information for discriminating between the alternatives.

The proof of condition (1) is as follows. Model V can be transformed into

$$v = \frac{(V'K'' + V''K')[A] + (V' + V'')[A]^2}{K'K'' + (K' + K'')[A] + [A]^2} \quad (2)$$

and the parameters K_1 and K_2 of model IV can therefore be expressed as

$$K_1 = (K' + K'')/K'K''$$

and

$$K_2 = 1/K'K''$$

Now

$$\frac{K_1^2}{K_2} = \left(\frac{K' + K''}{K'K''} \right)^2 \cdot K'K'' = \frac{K'^2 + 2K'K'' + K''^2}{K'K''} \geq 4$$

or $4K_2 \leq K_1^2$ (equality when $K_1 = K_2$).

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