Hydrolysis of Choline Esters by Rabbit Liver Oligomeric and Monomeric Carboxylesterases (EC 3.1.1.1)*

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A. Russell Main‡ and Robert S. Rush

From the Department of Biochemistry, North Carolina State University, Raleigh, North Carolina 27650

The velocity (v) against substrate concentration [S] relationships for the hydrolysis of butyrylthiocholine (BuSCh) by electrophoretically pure oligomeric (oCE) and monomeric (mCE) rabbit liver carboxylesterases were sigmoidal, as was that of mCE with butyrylcholine. Reactions which were barely detectable at 1 mM BuSCh were readily measurable at 100 mM, since the oCE activity had then increased 1,400 times. With 1 mM substrates used routinely, the phenylthiobutyrylrate (pSBu) to BuSCh activity ratios were about 260,000 for oCE and 1,400 for mCE. As the BuSCh concentration increased and the velocities approached their theoretical maximum, the pSBu/BuSCh activity ratio of the oCE decreased from 260,000 to 139 or by a factor of 2,000. Similarly, the mCE ratio decreased to 12 or by a factor of 80. The oCE was a typical mammalian carboxylesterase and consequently its ability to hydrolyze BuSCh was unexpected and the possibility of cholineesterase contamination had to be considered. However, a number of criteria including the unique sigmoidal v against [S] plot indicated that oCE itself hydrolyzed BuSCh. The v against [S] relationships characterize the hydrolysis of ethyl butyrate, m-(n-heptanoyloxy)benzoate, and pSBu by oCE and mCE were also determined and none followed Michaelis-Menten kinetics. Inhibition, activation, and the sigmoidal behavior were interpreted by assuming the presence of a modifier site, in addition to the active site, to which a second molecule of substrate could bind. An equation was derived from the reaction scheme describing this concept. Theoretical curves based on this equation were fitted to the experimental points using the derived equation. Fits were obtained for three different types of curve corresponding to each of the three types of behavior.

The activity of purified carboxylesterase preparations toward choline esters has frequently been tested (1), but the results have almost invariably been negative, and there are no authenticated reports of choline ester hydrolysis by carboxylesterases. Thus, in a recent review Junge and Krisch (2) listed choline esters among the substrates not hydrolyzed by carboxylesterases.

In apparent contradiction, the present paper reports results which clearly indicate that highly purified preparations of two different carboxylesterases from rabbit liver do catalyze the hydrolysis of choline esters at significant rates. One of the carboxylesterases is an oligomer, probably a trimer (3), and appeared to be closely related to the carboxylesterases from the livers of a number of other animals such as pig (4-7), horse (8, 9), rat (10, 11), and human (12). The other was a monomeric carboxylesterase which was purified for the first time by Miller et al. (3).

The hydrolysis of substrates by mammalian liver carboxylesterases is often complex and has frequently been characterized either by inhibition (13, 14) or activation (15) in the presence of excess substrate. The hydrolysis of choline esters by the rabbit liver carboxylesterases appeared to involve an added degree of complexity in that the velocity versus substrate concentration relationships were sigmoidal. Barker and Jencks (15) and Stoops et al. (16, 17) have proposed that carboxylesterases contain an 'activator' or 'modifier' site in addition to the active site, and they have used this concept to explain either activation or inhibition. In the present paper, the sigmoidal v versus [S] relationship has also been interpreted by assuming the presence of a modifier site, and it is shown that both activation and inhibition by excess substrate and the observed sigmoidal behavior can be interpreted by a single scheme and equation based on the concept of a modifier site.

A variety of evidence, including the amino acid sequence around the active serines (18, 19), has indicated that carboxylesterases and cholinesterases may be closely related, and Augustinsson (20) has suggested that they may share a common ancestor. The significance of the present results to this relationship is discussed within the context of the properties of the two monomeric butyrylcholinesterases which were also purified from rabbit liver (21).

**EXPERIMENTAL PROCEDURES**

**RESULTS AND DISCUSSION**

Hydrolysis of BuSCh and BuCh by Rabbit Liver oCE and mCE—As would have been expected from the literature re-

1 Portions of this paper (including Figs. 1 through 4 and Tables I and II) are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are available from the Journal of Biological Chemistry, 9650 Rockville Pike, Bethesda, Md. 20014. Request Document 79M-1953, site author(s), and include a check or money order for $1.50 per set of photocopies. Full size photocopies are also included in the microfilm edition of the Journal that is available from Waverly Press.

2 The abbreviations used are BuSCh, butyrylthiocholine; BuCh, butyrylcholine; pSBu, phenylthiobutyrate; mCE, m-(n-heptanoyloxy)benzoic acid; oCE, oligomeric carboxylesterase; mCE, monomeric carboxylesterase; mBuChE and II, monomeric butyrylcholinesterase from rabbit liver; oBuCE, oligomeric butyrylcholinesterase from the sera of human, horse, and rabbit; amiton, diethyl S-(2-diethylaminoethyl)phosphorothiolate.
ports, the rabbit liver oCE did not at first appear to hydrolyze the 1 mM butyrylthiocholine used for routine assays. However, when the BuSCh concentrations were increased, the corresponding rates of hydrolysis increased dramatically. For example, the rate with 1 mM BuSCh of 0.0007 μmol min⁻¹ mg⁻¹ was barely detectable and was at least 260,000 times less than the rate with 1 mM phenylthiobutyrate. However, at 10 mM BuSCh the rate had increased 75-fold and with 100 mM BuSCh it had increased by 1,400-fold. A rate which was barely detectable at 1 mM BuSCh could then readily be measured at higher concentrations.

It seemed possible that this low activity might be from contaminating mBuChE I (21) or possibly from the mCE (3). To test this possibility, the effect of increasing BuSCh concentrations on the velocities of the oCE, mCE, and mBuChE I were compared. To aid in the comparisons, the velocities were normalized with respect to the maximum velocities determined experimentally since the actual velocities of these esterases toward BuSCh varied so widely. The comparison is shown in Fig. 1A. v against [S] plots of both the oCE and mCE were clearly sigmoidal, while that of the mBuChE I was a rectangular hyperbola. Moreover, the BuSCh concentration at which oCE reached half its maximum was 30 mM and was almost 20 times higher than that characterizing mBuChE I under comparable conditions. The different shapes of the curves and the widely different BuSCh concentrations at one-half maximum v was consistent with the conclusion that the hydrolysis of BuSCh by the electrophoretically pure oCE preparation used was a characteristic of oCE itself and not due to contaminating mBuChE I. The observation that 10⁻⁵ M eserine did not affect the hydrolysis of BuSCh by the oCE preparation (Table II) is consistent with this conclusion. Parallel arguments also indicate that the oCE hydrolysis of BuSCh was not from contaminating mCE.

The mCE activity with 1 mM BuSCh was 160 times greater than the comparable oCE activity, but it was still 1000 times less than the activity toward the 1 mM phenylthiobutyrate used routinely to monitor mCE and oCE activity. Although much lower than the φSBu activity, the BuSCh activity with mCE could readily be measured under routine conditions and, as with the oCE, it was at first attributed to contaminating mBuChE I with which mCE was closely associated during purification (21). However, the sigmoidal v against [S] relationship was quite different from the rectangular hyperbola characterizing mBuChE I (Fig. 1A), thus indicating that the hydrolysis of BuSCh by the mCE preparation was a characteristic of the mCE itself and was not from mBuChE I contamination.

To examine further the possibility of mBuChE I contamination, the progress of mCE inhibition by the tertiary amine organophosphate, amiton, was compared with that of mBuChE I, and the results are shown in Fig. 2B. Amiton inhibited mBuChE I about 4 times faster, on the average, than it did mCE, and the rate of mCE inhibition leveled off well above that of mBuChE I. Moreover, the rate of mCE inhibition measured with φSBu substrate was the same as that measured by BuSCh, again clearly indicating that the hydrolysis of BuSCh was due to mCE and not to contaminating mBuChE I.

In addition to BuSCh, mCE also hydrolyzed butryrylcholine and again the v versus [S] plots were sigmoidal (Fig. 1A). Although oCE appeared to hydrolyze BuCh, the activity was very low and the progress curves were not linear. With few exceptions, the velocities used in the analysis of v against [S] relationships were obtained from progress curves which were linear over the better part of the measured course of the reaction. With BuSCh, it was necessary to use a titrimetric pH-stat method (24) to obtain linear curves, some of which are shown in Fig. 1, B and C. The spectrophotometric method of Ellman et al. (23) tended to give non-linear curves, as shown for example in Fig. 2A and described under "Experimental Procedures."

Interpretation of the Kinetic Plots Characterizing the Hydrolysis of BuSCh by oCE and mCE and of BuCh by mCE—The sigmoidal v against [S] plots shown in Fig. 1A indicated that the hydrolysis of BuSCh by mCE and oCE and of BuCh by mCE did not follow Michaelis-Menten kinetics. To aid in their interpretation, the results were plotted by the u/[Eₜ] against v/[Eₜ][S] relationship recommended by Hofstee (27) and extensively used by others (15, 16). The results by the Hofstee plot (Fig. 3, A and B) showed hook-shaped curves which are unlike those typically reported for carboxylesterases. For example, the plots reported for the hydrolysis of mC; by pig liver oCE showed activation at high substrate concentrations (15) and consisted of two linear regions forming a curve resembling an open L (i.e.   .). By drawing lines through the linear regions, values of Kₚ₁ and Vₜₘₐₓ in the region of high substrate concentration (H) and Kₚ₂ and Vₜₘₐₓ in the low (L) region were obtained. The hook-shaped curves of Fig. 3, A and B, obviously were not amenable to this treatment, but it seemed reasonable to assume that a second site, such as the activator site proposed by Barker and Jencks (15) or the corresponding modifier site proposed by Stoops et al. (16, 17) to explain both activation and inhibition, could be used to interpret our results.

Since carboxylesterases appear to react by the two-step scheme characterizing serine hydrolases, it seemed possible that the second site might be the site occupied by the aryl or alkyl leaving group of the substrate which is unoccupied following acylation of the esterase, as illustrated in Scheme I under "Supplementary Reaction Schemes and Equation." The problem with Scheme I is that secondary substrate binding affects only the deacylation, kₚ, controlled step, whereas the low velocities associated with thiocholine ester hydrolysis by oCE and mCE clearly indicates that these reactions are acylation or kₚ controlled. Consequently Scheme I does not appear to be relevant to the present results.

The alternative Scheme II shown under "Supplementary Reaction Schemes and Equation" involves binding to a second or modifier site (ms) which is not part of the active site, as well as to the empty aryl or alkyl pocket on the active site. Since kₚ and βₖₚ of Scheme II (β = α > 1) are much greater than kₓ and akₓ, Scheme II reduces to Scheme III.

\[
\begin{align*}
E + S & \xrightarrow{K_a} ES \\
K_m & \xrightarrow{+S} K_m' \\
SE + S & \xrightarrow{ak_x} SE \\
E + P & \xrightarrow{k_p} E + P
\end{align*}
\]

(III)

where E is enzyme, ES is the complex with S bound to the active site, SE is the complex with S bound to the modifier site, and SES is the complex with S bound to both sites. The equilibrium constants governing the formation of ES, SE, and SES are Kₛ, Kₚₛ, and Kₚₛ, respectively. The rate constants governing the acylation step, which is rate-limiting, are kₓ and akₓ. The effect of occupation of the modifier site is measured by α.

Equation 2 below was derived from Scheme III.

\[
v = \frac{V[A[S]]/K_m + 1}{1 + K_a[S] + k_p/[E]K_m + K_p/K_m}
\]

(2)

Equations can be derived from Scheme III in terms of any of the three or four equilibrium constants. Equation 2 was de-
rived in terms of $K_a$, $K_{ma}$, and $K_{ma}$. The fourth constant, $K'_{s}$, can, if needed, be calculated from the relationship, $K_s/K'_{s} = K_{ma}/K_{ma}$, from which $K'_{s} = K_{ma}K_s/K_{ma}$.

Theoretical lines were fitted to the experimental points by iterative selection of the constant coefficients of Equation 2; $a$, $V$, $K_{s}$, $K_{a}$, and $K_{ma}$. The values of the coefficients giving the theoretical curves shown in Figs. 3 and 4 are given in Table I as $K_{ma}$, $K_s$, $K_{ma}/K_{ma}$, and $K_{cat}$, where $K_{cat} = V/[E_0]$, $v$ is the total enzyme concentration.

The theoretical curves were not fitted statistically and the constant coefficients defining these curves (Table I) are therefore provisional. However, the fit of the theoretical curves to the experimental points appears adequate to allow the conclusion that Equation 2 does provide a reasonable interpretation of the sigmoidal $v$ against $[S]$ relationship, characterizing the hydrolysis of BuSch by oCE and mCE and the hydrolysis of BuCh by mCE.

The reactions of the rabbit liver oCE and mCE with some commonly employed substrates were also studied. Among the substrates which have previously been used with mammalian carboxylesterases are: methyl butyrate (9, 11), ethyl butyrate (4, 13, 28), phenyl butyrate (10, 29), and mC7: (14, 15). For purposes of comparison, the reactions of rabbit liver oCE and mCE were therefore studied with an aliphatic ester, ethyl butyrate, an aromatic ester, $p$SBu; and with mC7. In addition, the acyl specificities of the oCE and mCE were determined with phenyl thioacetate, propionate, and butyrate.

The $v/[E_0]$ against $v/[E_0][S]$ plots characterizing the hydrolysis of ethyl butyrate are shown in Fig. 4A. The plot with oCE was hook-shaped while the mCE plot was an open L shape. The theoretical lines fitting both sets of results were calculated using Equation 2 and the constant coefficients ($a$, $K_a$, etc.) given in Table I.

The $v/[E_0]$ against $v/[E_0][S]$ plots characterizing the hydrolysis of mC7 by mCE and oCE are shown in Fig. 4B. As with the hook-shaped and open L curves, theoretical lines were fitted to the inhibition curves using Equation 2, and the constant coefficients ($a$, $K_a$, etc.) are given in Table I.

Thus the hydrolyses of ethyl butyrate, BuSch, BuCh, and mC7: by rabbit liver oCE and mCE were characterized either by hook-shaped, open L-shaped, or inhibitory, $v$ against $v/[S]$ plots. All three of these plots were adequately interpreted by Scheme III, as indicated by the theoretical curves obtained by iterative fitting using Equation 2 and shown in Figs. 3 and 4.

The differences between the hook-shaped, open L-shaped, and inhibitory plots appear then to be quantitative rather than qualitative. Both the L-shaped and the hook-shaped plots reflect activation and the particular shape seems to depend on the degree of activation and on the relative binding of the substrate to the active site as compared with the modifier site. The degree to which the modifier site affects the activity of the oCE and mCE with the various substrates tried can be gauged from the $a$ values and from the ratio $K_a/K_{ma}$ in Table I. The $K_a/K_{ma}$ ratio indicates whether or not the substrate binds to the modifier site on the free enzyme better than it does to the active site. The $K_a/K'_{s}$ and $K_{ma}/K_{ma}$ ratios indicate further whether or not prior occupation of either site will affect binding to the other, as well as giving a measure of this effect. The results in Table I indicate, for example, that prior occupation of either the modifier site or the active site of oCE by BuSch greatly improves secondary binding to both sites. Equation 2 is quite complex and, to paraphrase Barker and Jencks (15) when they were considering an analogous situation, it is too complex for a completely definitive evaluation at this time.

Comparison of the kinetic constants characterizing oCE and mCE in Table I reveals numerous differences. For example, mC7 was the best substrate of those tested for the mCE, but it was a poor substrate for the oCE, which hydrolyzed mC7 at one-tenth the mCE rate. Conversely, ethyl butyrate was an excellent substrate for the oCE, but it was one of the poorer substrates for the mCE. These and other kinetic differences were consistent with the conclusion of Miller et al. (3) that mCE and oCE are quite different esterases. Both oCE and mCE were butyrate-specific as judged by their hydrolysis of the phenylthioesters (Table I). However, the length of the acyl chain would have to be increased to determine optimum specificity.

Comparison of the rabbit liver oCE activities with the activities of the liver oCE's of pig, horse, ox, and sheep indicates that the rabbit oCE is as active as the ox and horse toward $p$Bu or $p$SBu (17), but it is about half as active as the pig and sheep oCE's with these substrates. The rabbit liver oCE activity toward ethyl butyrate ($k_{cat} = 195 \text{ s}^{-1}$) was less than half that of the pig oCE (551 $\text{s}^{-1}$) while the difference with mC7 was even greater (15). The rabbit liver oCE in general appeared to be about as reactive as the ox and horse liver oCE's, but it appeared to be significantly less reactive than the pig and sheep oCE's.

The observation that rabbit liver carboxylesterases hydrolyze choline esters, together with the kinetic properties of this hydrolysis, suggests that carboxylesterases are more closely related to cholinesterases than previous evidence had indicated (20). Inhibition of acetylcholinesterase (EC 3.1.1.8) by acetylcholine is classically attributed to secondary binding to the empty anionic pocket of the acetylated enzyme (30, 31), and activation of serum butyrylcholinesterases can be explained in the same way (32). However, an accumulating body of evidence indicates that acetylcholinesterases (33) and serum butyrylcholinesterases (34) contain an allosteric or modifier site. In the present work the evidence clearly indicates that a modifier site is responsible for the hook-shaped Hofsteet plots characterizing carboxylesterase hydrolysis of choline esters. Further, it is shown that the hook-shaped plot is only one of at least three plots involving activation and inhibition by excess substrate which can be interpreted by assuming a modifier site. Thus, it seems possible that carboxylesterases and cholinesterases share a common catalytic mechanism which includes a modifier site in addition to the active site.

Cholinesterases have been distinguished from carboxylesterases primarily by the ability of the former and the inability of the latter to hydrolyze choline esters (2, 20). The present work indicates that this distinction is relative rather than absolute, particularly in view of the intermediate properties of the monomeric esterases, mCE and mBuChE I. The relationship between the monomeric esterases, the oCE, and human serum oligomeric butyrylcholinesterase (oBuChE) with respect to hydrolysis of BuSch and inhibition by $10^{-4}$ m eserine is shown in Table II. As would be expected, eserine had little effect on the oCE activity and almost completely inhibited the oBuChE. Inhibition by mCE was significant, 41%, while mBuChE I was inhibited over 92%. The degree of inhibition by eserine was paralleled by the ability to hydrolyze and bind BuSch. What is seen is not a sharp, all-or-nothing demarcation between carboxylesterases and cholinesterases, but a graduated increase in ability to react with BuSch and eserine. Differences in the substrate specificities of carbonyl- and cholinesterases appear then to be relative rather than absolute, and taken with their common kinetic behavior, these results indicate that the four esterases considered in Table II are interrelated kinetically. Their similar subunit size, amino acid compositions, and, in the case of horse cholin- and carboxylesterase, identical sequence about the active serine, sug-
suggests that they share common structural features which may be reflected in their kinetic behavior.

REFERENCES
Carboxylesterase Hydrolysis of Choline Esters

EXPERIMENTAL PROCEDURES

**Materials**

Enzyme preparations (electrophoretically pure preparations of rabbit liver CEs and porcine liver CEs were obtained by the method of Weiss et al. (22) Electro-potentiality of preparations of which CEs 1 and 2 are known to be the two major isoenzymes. The isoenzymes CEs 3 and 4 were obtained by the method of Hecht et al. (21).)

Methods of Enzyme Preparations: Enzyme preparations, enzymatic conversion of choline esters were performed as described elsewhere (21).

Procedures: For the preparation of choline esters, the enzymes were incubated at 37°C for 4 hours. The resulting solutions were then analyzed for the production of free choline and glycine by colorimetric methods.

**Results**

The results obtained indicate that the enzymes hydrolyze choline esters at different rates. The rate of hydrolysis decreases with increasing substrate concentration. The enzymes also show a preference for free choline and glycine over their respective esters.

**Discussion**

The data obtained suggest that the enzymes hydrolyze choline esters through a mechanism involving the formation of an enzyme-substrate complex. The complex undergoes a conformational change, resulting in the release of free choline and glycine.

**Conclusion**

The enzymes studied hydrolyze choline esters at different rates, with a preference for free choline and glycine. The mechanism involves the formation of an enzyme-substrate complex, which undergoes a conformational change, resulting in the release of free choline and glycine.
Fig. 5. Showing the fit of the theoretical lines calculated from equation (2), derived from the data in Table 1, to the experimental points in Fig. 4. The points are shown by symbols and the lines by the solid curve (A) and the dotted curve (B). The conditions were identical with those described in the legend to Table 1.

TABLE I

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<tr>
<th>Enzyme</th>
<th>(k_{\text{cat}})</th>
<th>(V_{\text{max}})</th>
<th>(K_{\text{m}})</th>
<th>(V_{\text{max}}/K_{\text{m}})</th>
<th>(k_{\text{cat}}/K_{\text{m}})</th>
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<td>1.3</td>
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<tr>
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<td>0.6</td>
<td>2.9</td>
<td>0.2</td>
<td>0.03</td>
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</table>

Note: The values for \(k_{\text{cat}}\), \(V_{\text{max}}\), and \(K_{\text{m}}\) were determined in the presence of 100 mM substrate, 100 mM acetyl-CoA, and 100 mM ATP, as described in the text.

Fig. 5. Plots of the hydrolysis of (A) butyrylcholine by Acetylcholinesterase and (B) phosphorylcholinesterase by acetylcholinesterase. The conditions were identical with those described in the legend to Table 1.

Note: The values for \(k_{\text{cat}}\), \(V_{\text{max}}\), and \(K_{\text{m}}\) were determined in the presence of 100 mM substrate, 100 mM acetyl-CoA, and 100 mM ATP, as described in the text.

Fig. 6. Plots of the hydrolysis of (A) butyrylcholine by acetylcholinesterase and (B) phosphorylcholinesterase by acetylcholinesterase. The conditions were identical with those described in the legend to Table 1.

Note: The values for \(k_{\text{cat}}\), \(V_{\text{max}}\), and \(K_{\text{m}}\) were determined in the presence of 100 mM substrate, 100 mM acetyl-CoA, and 100 mM ATP, as described in the text.