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# APROPHEN: A SUBSTRATE AND INHIBITOR OF BUTYRYLCHOLINESTERASE AND CARBOXYLESTERASES

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**Abstract**—Aprophen, α-methyl-α-phenylbenzeneacetic acid-2-(diethylamino) ethyl ester, is a potent reversible inhibitor and a poor substrate of human serum butyrylcholinesterase (BuChE). Complex mixed competitive noncompetitive inhibition kinetics were observed; an apparent competitive inhibition constant was estimated to be $3.7 \times 10^{-7}$ M. BuChE hydrolysis of aprophen to diphenylpropionic acid and diethylaminoethanol did not appear to follow Michaelis–Menten kinetics. The BuChE turnover number for aprophen was $2.0 \times 10^{-3}$ sec$^{-1}$. Rabbit liver oligomeric and monomeric carboxylesterases (CE) also hydrolyzed aprophen with a similar turnover number that varied from $1.4 \times 10^{-3}$ sec$^{-1}$ to $4.3 \times 10^{-4}$ sec$^{-1}$ respectively. Comparison of the catalytic rate of aprophen hydrolysis with butyrylthiocholine (BTC) and the neutral aromatic substrate, phenylthiobutyrate (φTB), indicated that BuChE hydrolyzed BTC and φTB $3.2 \times 10^5$ and $3.1 \times 10^5$ times more rapidly than aprophen respectively. Similarly, the CEs also hydrolyzed BTC and φTB 17.6 and $1.9 \times 10^5$ times more rapidly than aprophen. Acetylcholinesterases from bovine erythrocyte and electric eel were not inhibited by aprophen nor was aprophen hydrolyzed by these enzymes. The hydrolysis and inhibition reactions may best be described by a complex reaction scheme involving multiple binding sites for both the substrate and the inhibitor as well as positive cooperative ligand binding.

Benzilates are parasympatholytic drugs which exhibit antispasmodic and anticonvulsant pharmacological effects. Specific benzilates, including aprophen, adiphenine and benactyzine, inhibit horse and human serum butyrylcholinesterase (BuChE† EC 3.1.1.8) [1]. Similarly, benzilates bind to muscarinic receptors derived from rat brain [2] and from neuroblastoma cell cultures [3]. High concentrations of adiphenine and other benzilate analogs weakly inhibit both membrane bound (red cell) and electric eel acetylcholinesterase (AChE, EC 3.1.1.7) [4]. Tissue extracts and homogenates also hydrolyze benzilates [5]. Pharmacodynamic studies on the absorption, distribution and metabolic fate of benzilates have been reported [6, 7]. In general, studies on the metabolism of benzilates have been carried out with impure enzyme preparations or with tissue extracts [5, 8]. Thus, the relationships between purified enzymes and the benzilates have not been delineated. We have therefore studied the interaction between highly purified enzymes, including both carboxylesterases (CE, EC 3.1.1.1) and cholinesterases (BuChE and AChE), and the benzilate aprophen. Aprophen is a potent reversible inhibitor of pure human serum BuChE, but it is also hydrolyzed by BuChE as well as by rabbit liver CEs. However, commercial preparations of bovine erythrocyte and electric eel AChE did not hydrolyze aprophen nor did this drug inhibit AChE activity at the concentrations employed.

Aprophen hydrolysis by highly purified human serum BuChE does not appear to follow Michaelis–Menten kinetics. The substrate and inhibition kinetics are complex and may involve allosteric and/or cooperative interactions. Inhibition appears to follow a hyperbolic inhibition reaction pattern which implicitly involves multiple inhibitor binding sites.

## MATERIALS AND METHODS‡

Aprophen was synthesized as previously described [9]. Labeled aprophen [ethyl ester-1,2-$^{14}$C] was custom synthesized by New England Nuclear, Boston, MA. 2,2-Diphenylpropionic acid and diethylaminoethanol were from the Aldrich Chemical Co., Milwaukee, WI. Silica gel (13181) thin-layer chromatography sheets with fluorescent indicator were from Eastman Kodak, Rochester, NY. All other reagents were obtained from normal sources and were of the highest purity available.

Purification of aprophen was conducted by a slight modification of the procedure of Eck *et al.* [10] using high performance liquid chromatography. The modifications were as follows: (1) a Radial Pac B 10 μm bead size silica column, 8.0 mm I.D. (Waters, Inc.), was used in place of the Whatman Partisil 5 column; (2) the column effluent was monitored at 254 nm and with a flow through radioactive detector

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† Abbreviations: BuChE, butyrylcholinesterase; CE, carboxylesterase; AChE, acetylcholinesterase; BTC, butyrylthiocholine; $\phi_{TB}$, phenylthiobutyrate; DEAE, diethylaminoethanol; and $k_{cat}$, maximum turnover rate.

‡ The manufacturer's name and products are given as scientific information and do not constitute an endorsement by the United States Government.

(Flo-One DR) with Flo-Scint II scintillant (1.0 ml/min).

Enzymes employed in this study were electrophoretically pure unless specifically mentioned. The purification procedures are described elsewhere [11–13]. Commercial bovine erythrocyte and electric eel AChEs (Sigma) were used without further purification.

Inhibition studies utilized the colorimetric assay of Ellman *et al.* [14] as described by Rush *et al.* [15].

Substrate studies employed [$^{14}$C]aprophen and were conducted as follows. [$^{14}$C]Aprophen was maintained in methanol, and working solutions were made through evaporation of the solvent followed by resuspension in a solution of 0.05 M sodium phosphate buffer, pH 8.0, and 5% methanol. Routine assays employed the isotopically labeled aprophen (sp. act. 0.0346 $\mu$Ci/$\mu$mole) at a concentration of 0.838 mM. Approximately 10 $\mu$g of enzyme was employed for each assay in a final volume of 0.025 ml. Mixtures were incubated at 25° for specified time periods in capped Eppendorf tubes. The reactions were started by the addition of enzyme and stopped by spotting four repetitive 2.5-$\mu$l aliquots directly on the silica gel TLC sheets. Each assay was overlaid with 125 nmoles of pure, standard [$^{12}$C]aprophen. Enzymatic activity was determined from the radioactive decay of the labeled diethylaminoethanol; each activity measurement was corrected for nonenzymatic hydrolysis. Mass balance of radioactive material applied to the TLC plate was determined by counting both the [$^{14}$C]aprophen spot in addition to the diethylaminoethanol spots. Control reactions contained no enzyme, heat-inactivated enzyme, or diisopropylfluorophosphate-inactivated enzyme.

Reaction products were determined by thin-layer chromatography on silica gel plates with a solvent system composed of ethylacetate–acetonitrile–water–triethylamine (50:50:5:0.01, by vol.). The relative mobilities of the substrate and the reaction products were as follows: aprophen, 0.75; 2,2-diphenylpropionic acid, 0.42; and diethylaminoethanol, 0.0. Aprophen and 2,2-diphenylpropionic acid were visualized by short wave ultraviolet illumination; diethylaminoethanol and aprophen were quantitated by cutting out the corresponding spot and counting in a Searle Mark III liquid scintillation spectrophotometer.

## RESULTS

*Inhibition of human serum butyrylcholinesterase.* Pure human serum BuChE exhibited complex kinetics characterized by activation at concentrations in excess of 0.3 mM *n*-butyrylthiocholine iodide (BTC). Four concentrations of BTC substrate were therefore employed to determine the $I_{50}$ values for aprophen inhibition of BTC hydrolysis; these substrate concentrations were 0.025, 0.075, 0.75 and 1.0 mM. The two higher concentrations (0.75 and 1.0 mM) caused BuChE activation, whereas the two lower concentrations (0.025 and 0.075 mM) were in the low substrate region where Michaelis–Menten type kinetics are presumed to be approximated. The $I_{50}$ values for aprophen inhibition of BuChE hydrolysis were determined from Fig. 1; the $I_{50}$ values were

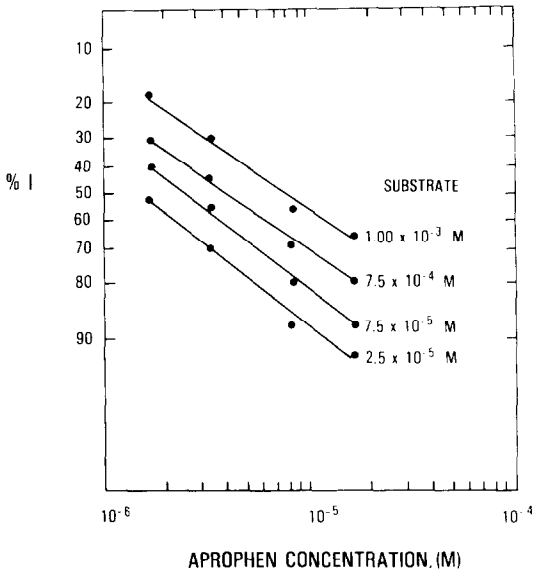


Fig. 1. $I_{50}$ inhibition of human serum BuChE by varying the concentration of aprophen at four fixed concentrations of substrate, BTC. The concentrations of BTC employed are shown on the graph. The ordinate depicts the percent inhibition and the abscissa the aprophen concentration.

$1.43 \times 10^{-6}$ M, $2.75 \times 10^{-6}$ M, $4.3 \times 10^{-6}$ M, and $7.5 \times 10^{-6}$ M respectively. Assuming competitive inhibition, the apparent $K_i$ for aprophen inhibition can be calculated from equation 1.

$$I_{50} = ([S]/K_m + 1)\, K_i \qquad (1)$$

Employing this equation, an average apparent $K_i$ of $3.7 \times 10^{-7} \pm 1.4 \times 10^{-7}$ M was determined from the above $I_{50}$ data using a $K_m$ of $20 \times 10^{-6}$ M (Fig. 2A).

Aprophen inhibition of BTC hydrolysis by human serum BuChE appears to be largely competitive as judged by the slope changes for fixed inhibitor concentrations depicted in Fig. 2A. However, the inhibition was also complex, that is, a significant noncompetitive component was indicated by changes in the ordinate intercept values (see Fig. 2A). Assuming Michaelian competitive kinetics, the apparent competitive component can be estimated from the slope changes in the low substrate region. The following $K_i$ values were calculated from the four fixed concentrations of aprophen used: $1.2 \times 10^{-6}$ M, $1.2 \times 10^{-6}$ M, $1.8 \times 10^{-6}$ M, and $2.8 \times 10^{-6}$ M. A replot of the slope versus the inhibitor concentration in the low substrate region (Fig. 2B) indicates hyperbolic inhibition kinetics [16]. A similar replot in the high substrate region (Fig. 2C) depicts linearity, i.e. competitive kinetics. No simple interpretation is possible in either region because the equilibrium binding constants are not independent of each other or of modifying coefficients. The apparent competitive $K_i$ of aprophen inhibition, determined from either the slope of the Hofstee plot (Fig. 2A) in the low substrate region or from the tangent of the replot (Fig. 2B), indicates a value of about $1.2\ 10^{-6}$ M. Hyperbolic inhibition produces an apparent inhibition constant that is not a true equilibrium binding

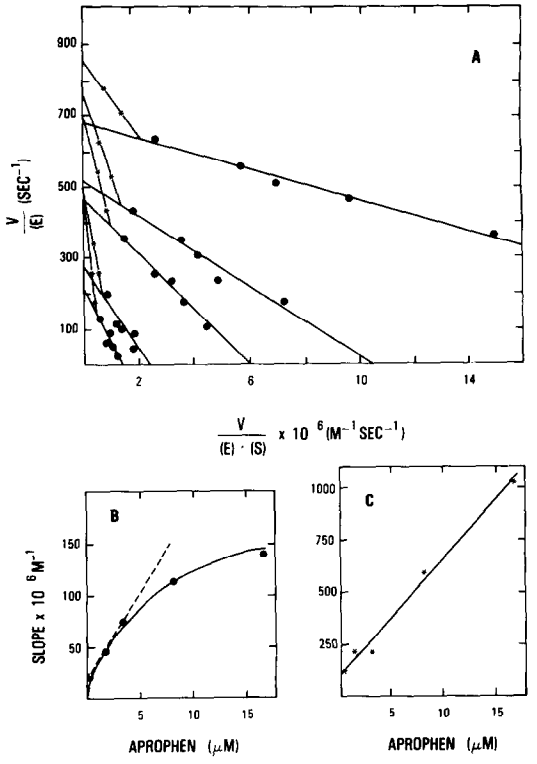


Fig. 2. (A) Hofstee plot depicting aprophen inhibition of pure human serum BuChE at various fixed concentrations of aprophen. The upper curve is the control reaction, no aprophen; the inhibition curves were constructed with the following concentrations of aprophen: $1.66 \times 10^{-6}$ M, $3.33 \times 10^{-6}$ M, $8.30 \times 10^{-6}$ M, and $1.66 \times 10^{-5}$ M respectively. The corresponding slopes of the inhibited curves in the low substrate region (from which an apparent $K_i$ value can be calculated) are $-48 \times 10^{-6}$ M, $-75 \times 10^{-6}$ M, $-112 \times 10^{-6}$ M and $-140 \times 10^{-6}$ M respectively. The assay of Ellman *et al.* [14] was used. The reaction was started by adding a fixed concentration of enzyme ($3.12 \times 10^{-10}$ M active sites) to the reaction mixture. The inhibition was immediate and did not change with time. Linear regression analysis was employed to draw the solid lines. (B) Replot of the slope of the Hofstee plot in the low substrate region, Fig. 2A filled circles (●), against the concentration of aprophen. (C) Replot of the slope of the Hofstee plot in the high substrate region, Fig. 2A asterisks (*), against the concentration of aprophen.

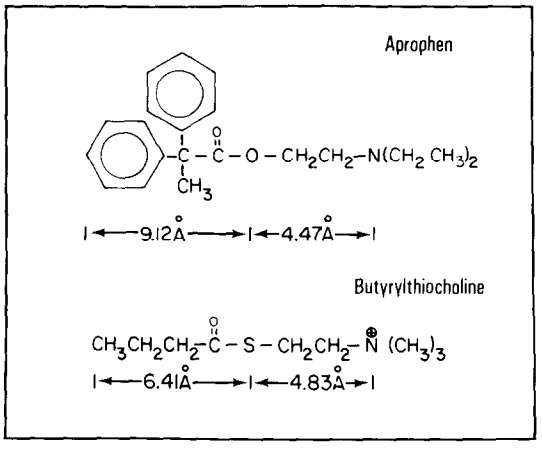


Fig. 3. Structure and estimated bond distances of aprophen and BTC. The bond distance from the aryl portion of the aprophen molecule to the labile esteratic bond was estimated to bridge a single aromatic ring.

constant, but instead is a composite constant composed of modifying coefficients and frequently inhibitor concentration terms as well [16]. The fact that the difference in estimated $K_i$ values determined from a Hofstee plot and that estimated from $I_{50}$ data only differed by a factor of 3.2 implies rather close correspondence between the two methods of calculation. It also indicates that the assumptions implicitly involved in estimating the $K_i$ from these plots are not extreme. The inhibition appeared largely competitive with a noncompetitive component attributable to the inhibitor binding to a secondary or modifier site on the BuChE.

*Hydrolysis of aprophen by human serum butyrylcholinesterase.* Aprophen and BTC are structurally related (Fig. 3) since both compounds contain an ester linkage. Additionally, both compounds contain a tertiary and/or quaternary nitrogen as well as an ethylene bridge between the nitrogen and the respective ester bonds. The distance from the terminal nitrogen to the labile bond in both compounds is approximately the same, i.e. 4.47Å and 4.83 Å respectively. The bond lengths were estimated according to the Schomaker–Stevenson relationship [17]. It is therefore reasonable that aprophen could also be a substrate for human serum BuChE.

The progress curves obtained for the hydrolysis of aprophen by human serum BuChE were linear with respect to time and enzyme concentration (Fig. 4A and B). Similarily, crude esterase preparations derived from either horse serum or a rabbit liver homogenate hydrolyzed aprophen linearly (Fig. 4B). The hydrolysis of aprophen could be blocked by either heat inactivation of the enzyme or by inhibition of the enzyme with diisopropylfluorophosphate prior to reaction with aprophen.

The hydrolysis of aprophen by BuChE was saturable, as indicated by Fig. 5A. At saturable levels, it was estimated that 10% of the aprophen was converted to products (diethylaminoethanol and diphenylpropionic acid) during a 4-hr incubation. The hydrolysis of aprophen did not appear to follow Michaelis–Menten kinetics in the lower substrate region (see Fig. 5A). The saturation curve depicts an intermediate plateau region. At concentrations above 0.030 mM, the velocity versus substrate plot approached Michaelian kinetics (Fig. 5A). Data shown in this figure were used to construct the Hofstee plot (Fig. 5B) of the velocity × enzyme$^{-1}$ ($V/E$) against the velocity × enzyme$^{-1}$ × substrate$^{-1}$ ($V/E \cdot [S]$). Complex substrate kinetics are further shown by the nonlinear portion of the Hofstee plot in the lower substrate region. Hooked-shape Hofstee plots have been interpreted to indicate allosterism [18]. The slope of the linear portion of the Hofstee plot depicts an apparent binding constant of $11.3 \times 10^{-6}$ M and the extrapolated maximal velocity was estimated to be $2.92 \times 10^{-3}$ sec$^{-1}$. The deviation from Michaelian kinetics is emphasized by comparison of the observed velocity substrate data with

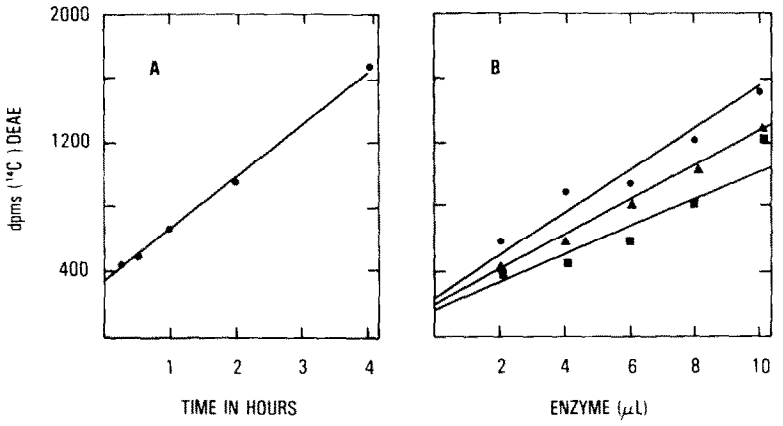


Fig. 4. (A) Time course of aprophen hydrolysis by human serum BuChE. The reaction system contained 0.050 M sodium phosphate buffer, pH 8.0, 54,000 dpm of [$^{14}$C]aprophen at a concentration of 0.838 mM, and approximately 10 μg of enzyme in a reaction volume of 0.025 ml. (B) Enzyme concentration curve for the hydrolysis of aprophen by pure human serum BuChE (●), horse serum (▲), and a rabbit liver homogenate (■). Reaction conditions are given in Fig. 4A except that the incubation time was 1 hr.

the theoretical Michaelian curve (dashed line, Fig. 5A). The theoretical values were calculated employing the kinetic constants from the Hofstee plot.

Additional complexities of cooperativity may also be involved. The measured cooperativity index ($R_x$), as defined by Taketa and Pogell [19], is 26.5. Another index of cooperativity is the Hill ($h$) coefficient. The calculated cooperativity index was found to agree with the measured value, i.e. 26.1. The Hill coefficient ($h$) was 1.35 and was determined from the slope of Fig. 5C.

*Hydrolysis of aprophen by other enzymes.* Carboxylesterases (CE), bovine erythrocyte and electric eel AChE were primarily tested for their abilities to hydrolyze aprophen rather than for their sensitivities towards the drug. Progress curves for aprophen hydrolysis were linear with respect to time and to the concentration of the respective enzymes, i.e. rabbit liver oligomeric and monomeric carboxylesterases (*o*CE and *m*CE respectively), and horse serum monomeric carboxylesterase (hsCE) (data not shown). The turnover numbers for the hydrolysis of aprophen by these CEs and human serum BuChE are summarized in Table 1. There was no apparent difference between these enzymes when the turnover numbers were normalized for the number of active sites per enzyme, i.e. $4.7 \times 10^{-4}$ sec$^{-1}$ per active site. Commercial preparations of bovine erythrocyte and electric eel AChEs failed to hydrolyze aprophen (0.838 mM) nor was the AChE activity inhibited by $1.0 \times 10^{-4}$ M aprophen. These investigations have been restricted to specific serine hydrolases, and it is possible that other enzymes may also hydrolyze aprophen.

## DISCUSSION

The anticholinergic properties of benzilates, in par-

Table 1. Turnover constants and substrate ratios*

| | Substrate | | | | |
|---|---|---|---|---|---|
| | APR | φTB | BTC | φTB/APR | BTC/APR |
| Enzyme | $k_{cat}$ (sec$^{-1}$) | $k_{cat}$ (sec$^{-1}$) | $k_{cat}$ (sec$^{-1}$) | | |
| Human BuChE | $1.97 \pm 0.15 \times 10^{-3}$ (5) | 600 | 632† | $3.05 \times 10^5$ | $3.21 \times 10^5$ |
| Rabbit *o*CE | $1.42 \times 10^{-3}$ (2) | 276† | 0.025† | $1.94 \times 10^5$ | 17.6 |
| Rabbit *m*CE | $4.28 \pm 0.06 \times 10^{-4}$ (3) | 195† | 1.13† | $4.56 \times 10^4$ | 264 |
| Horse hsCE | $4.70 \pm 0.42 \times 10^{-4}$ (3) | 4.25‡ | | $9.05 \times 10^3$ | |

* Turnover numbers ($k_{cat}$) were determined for pure human serum butyrylcholinesterase (BuChE), rabbit liver oligomeric and monomeric carboxylesterase (*o*CE and *m*CE) and from horse serum carboxylesterase (hsCE) at a saturating concentration of aprophen (APR) (0.838 mM). Turnover numbers are also reported for butyrylthiocholine (BTC) and phenylthiobutyrate (φTB) at 1.0 mM concentration (as referenced) for comparative purposes. The $k_{cat}$ values for aprophen are reported ± the standard error of the mean with the number of experiments given in parentheses.

† From Ref. 18.

‡ R. S. Rush, A. R. Main and J. S. Ralston (unpublished observations).

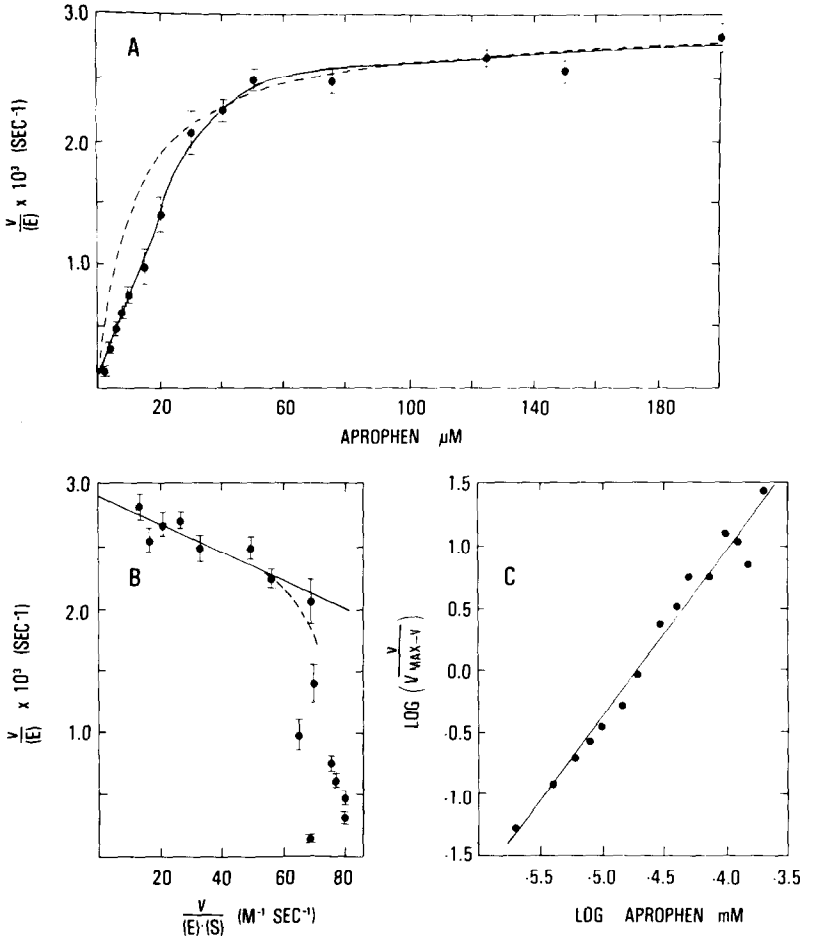


Fig. 5. (A) Saturation curve for the hydrolysis of aprophen by a partially purified preparation of human serum BuChE. Each experimental point was generated with 0.005 ml BuChE (1.74 mg/ml) with a specific activity of 363 BTC units/mg protein. The enzyme preparation was thus 66–76% pure (specific activity of pure BuChE with 1.0 mM BTC has been reported to vary from 479 to 546 units/mg protein [13]). The enzyme preparation was totally resolved from any contaminating esterase activity as evidence by electrophoretic profiles stained for enzymatic activity with indoxyl acetate. Identical substrate kinetics were observed with this preparation compared to pure human serum BuChE with BTC, $\phi$TB and aprophen. Each point was replicated eight times and the mean value plotted ± standard error of the mean. The saturation curve was constructed over an aprophen concentration range of 100 ($2.0 \times 10^{-6}$ M to $200 \times 10^{-4}$ M). The observed maximal velocity ($V$) was $2.92 \times 10^{-3}$ sec$^{-1}$. The observed velocity data have been approximated by the Michaelis–Menten equation using an apparent $K_m$ of $11.3 \times 10^{-6}$ M and $V_{max}$ of $2.92 \times 10^{-3}$ sec$^{-1}$. (B) Replot of velocity ($v$) agsinst $v/[S]$, i.e. a Hofstee plot, clearly indicating nonlinearity in the lower substrate region, closed circles (●). The solid line (——) approximates the Michaelian type kinetic portion of the plot whereas the dashed line (– – –) shows the deviations from Michaelian kinetics. Linear regression analysis was employed to draw the solid line. (C) Replot of the log $v/(V_{max} - v)$ against the log of the substrate concentration, i.e., a Hill plot, indicating a slightly positive cooperative binding of aprophen. Best fit to the experimental data points was calculated by linear regression analysis.

ticular adiphenine, benactyzine, and quinuclidinyl derivatives, have been widely recognized [2, 3, 20]. In general, these compounds have been studied as muscarinic receptor antagonists. The major metabolic products derived from adiphenine metabolism are diphenylacetic acid, diethylaminoethanol, and diphenylacetic acid glucuronide [7]. Aprophen is a structural analogue of adiphenine. We have shown that BuChE and CEs hydrolyze the ester bond of aprophen forming the reaction products diphenylpropionic acid and diethylaminoethanol *in vitro*. Furthermore, we have shown that the crude biological samples, horse serum and rabbit liver homogenates, also hydrolyze aprophen. It is noteworthy that aprophen, a good muscarinic antagonist, is also a potent reversible inhibitor of the BuChE but not AChE. Structural relationships between the enzymes hydrolyzing aprophen and the muscarinic receptors remain to be defined. It is known, however, that there is structural and kinetic homology between the BuChE and the CEs [18, 21].

The results of this investigation establish that apro-

phen is both a substrate and a potent reversible inhibitor of BuChE. As a substrate, it was possible to induce nearly complete hydrolysis by prolonged incubation or increased enzyme concentrations. As a reversible BuChE inhibitor, aprophen is similar to other reversible inhibitors like the phenothiazines, acridines, and related drugs [15]. The apparent $K_i$ of aprophen binding to BuChE is $3.7 \times 10^{-7}$ M, 1 to 2 orders of magnitude greater than for the phenothiazine drugs [15, 22]. These drugs also exhibit mixed inhibition patterns composed of competitive and noncompetitive components which have been tentatively interpreted as an indication of the presence of multiple binding sites. This hypothesis is further supported by the hyperbolic inhibition kinetics. However, unlike the neuroleptic drugs, aprophen is also a substrate for BuChE.

Aprophen is a poor substrate as evaluated by catalytic efficiency. The catalytic efficiency ($k_{cat}/K_s$ or $K_m$) of BuChE hydrolysis of aprophen, BTC, and $\phi$TB is 174 $M^{-1}$ $sec^{-1}$, $3.2 \times 10^7$ $M^{-1}$ $sec^{-1}$, and $1.2 \times 10^7$ $M^{-1}$ $sec^{-1}$ respectively. BTC and $\phi$TB are hydrolyzed approximately $1.8 \times 10^5$ and $6.9 \times 10^4$ times more rapidly than aprophen with BuChE. The kinetic mechanism by which human serum BuChE hydrolyzes aprophen remains to be delineated. Intermediate plateau regions in the saturation curves have been interpreted as evidence for cooperative binding [23] of ligands to regulatory proteins. However, it should be noted that, in these special cases, multiple binding sites involving substrate and cofactors have been involved. Curved Hofstee plots have been observed and interpreted as indicating the presence of allosteric or modifier sites [18]. These curves have also been observed for horse serum BuChE [24] and have been interpreted as indicating homotropic cooperative interactions. A detailed kinetic investigation of the deviations observed in the hydrolysis of aprophen by human serum BuChE is beyond the scope of the present paper. We suspect that the intermediate plateau may be the result of positive cooperativity and that a functional allosteric site may also bind aprophen.

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