1-Bromopinacolone, an Active Site-directed Covalent Inhibitor for Acetylcholinesterase*

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1-Bromopinacolone, BrPin, acts initially as a reversible competitive inhibitor for acetylcholinesterase, \( K_r = 0.18 \text{ mM} \) in hydrolysis of acetylcholine. Unlike bromoacetone, with time it acts as an irreversible covalent inhibitor. BrPin has a hydrolytic half-life of 30 h at the pH of incubation, 7.8. The enzyme-BrPin complex is 50% inactivated in 2 h. First order kinetics are observed; the rate constant is proportional to the concentration of the complex. Retardation by cationic inhibitors of the inactivation is consistent with inactivation occurring as a result of binding of BrPin to the active site. Efficiency of irreversible inhibition by BrPin is essentially the same for hydrolysis of cationic and uncharged substrates, acetylcholine, 3,3-dimethylbutyl acetate, phenyl acetate, n-butyl acetate, and indophenyl acetate. In contrast, a cationic alkylating agent, \( \text{N},\text{N}-\text{dimethyl-2-phenylaziridinium ion} \), DPA, acts non-competitively; it inactivates completely toward cationic, and partially toward uncharged substrates, and does so slightly more rapidly than BrPin, but less than would be commensurate with its greater intrinsic reactivity. Enzyme first treated with DPA is inactivated by BrPin toward hydrolysis of 3,3-dimethylbutyl acetate. It is proposed that BrPin, and not DPA, binds and reacts in, and may be a useful labeling agent for, the active site.

The active site of acetylcholinesterase has been generally described as containing a specific anionic subsite at which positively charged groups related to the trimethylammonium group of acetylcholine associate, thereby increasing the binding and reactivity of cationic substrates and the binding of cationic inhibitors (1-6). Nevertheless, kinetics of hydrolysis of a series of ethyl acetates, \( \text{XCH}_2\text{COCOCH}_3 \), in which the \( \beta \)-substituents, \( \text{X} \), were nonpolar, polar, and cationic, indicated that the enzymic reaction could be accounted for by the effects of \( \text{X} \) on (i) the intrinsic hydrolytic reactivity of the ester and (ii) the fit of \( \text{X} \) into a subsite deemed complementary to or peripheral to the primary active site, with hydrolytic reactivity altered slightly by the modification (12, 14). Recent study of cationic and uncharged reversible inhibitors, related in structure to acetylcholine and DMBAc, shows that each inhibitor has similar kinetic values to those of hydrolysis of both substrates (10). This strongly supports the view that these cationic and uncharged substrates and inhibitors bind at the same trimethyl subsite. Thus, alternative (ii) is preferred, and uncharged reagents might be more specific in characterizing the active site of acetylcholinesterase.

\( \alpha \)-Halocarbonyl compounds have been used to alkylate nucleophiles in the active site of hydrolytic enzymes, chymotrypsin, trypsin, and pepsin (16-18), and the action of cationic and uncharged compounds of this class on acetylcholinesterase has been explored. (3-Bromo-2-oxopropyl)trimethylammonium ion, (19) \( (\text{CH}_3)_2\text{NCH}_2\text{COCH}_2\text{Br} \), BAT (from the name 3-bromoacetonyltricarbonyltrimethylammonium ion (20)), showed initial mixed competitive inhibition in hydrolysis of acetylcholine, followed by irreversible inhibition, which became complete at \( 10^{-2} \text{ m} \) BAT. Enzymic reactivity toward phenyl and indophenyl acetates was also decreased. However, the closely related compound (3-bromo-4-oxoperoxytrithecachlorotrimethylammonium ion, (21) \( (\text{CH}_3)_2\text{NCH}_2\text{CHBrCOCH}_3 \), which is nearly isosteric with acetylcholine, while a much more efficient reversible inhibitor (\( K_r \sim 10^{-6} \text{ m} \)), did not appear to react covalently with the enzyme (19). It may be noted that bromoacetone did not inactivate acetylcholinesterase (21), and \( \alpha \)-chloroacetoceto-
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phenone (21) showed initial mixed reversible inhibition, $K_i$ ~ 1 mM, similar binding to that of acetylphenone, followed by chemical reaction with a group which was apparently not part of the active site.

The view that the $\beta$-X subsite is trimethyl rather than anionic has led us to study inhibition of acetylcholinesterase by BrPin (22), $(CH_3)_2COCH_2Br$, as possibly a more specific site-directed alkyating agent. It may be noted that uncharged trimethyl compounds may be powerful anticholinesterase agents: methylcarbamyl oximes of pinacolone derivatives are insecticides (23) and O-1,2,3-trimethylpropyl methylphosphonofluoridate (agent GD or Soman) is a poison gas (24).

MATERIALS AND METHODS

Acetylcholinesterase (EC 3.1.1.7), acetylcholine, 3,3-dimethylbutyl acetate, n-butyl acetate, 4-oxo-N,N,N-trimethylanilinio oxide, (CH$_3$)$_3$N$^+$CH$_2$CH$_2$CH$_2$COCl$^-$, 3-oxo-(N-tert-butyl)buraniminium chloride, (CH$_3$)$_3$N$^+$CH$_2$CH$_2$CH$_2$COCl$^-$, were available from previous studies (7, 10). Phenyl acetate, Aldrich, was redistilled. 1-Bromopinacolone, Parsh, indophenol acetate, Sigma, and 1,1-trimethyl-4-hydroximinomethylpyridinium bromide, Aldrich, were used directly. N,N-Dimethyl-2-chloro-2-phenylethylamine hydrochloride (12) was synthesized and crystallized from methanol-ethanol, m.p. 299-210°C decomposition. Assay procedure and pH stat equipment were as described previously (10), except that enzyme stock solutions were in 0.18 M NaCl.

Hydrolysis of BrPin—The medium (distilled water, 0.18 m NaCl, or 0.18 M NaBr), 25 ml, was brought to pH 7.8, 9, or 10, BrPin, 10 µl, was added, leading to 3 mM solutions, and hydrolysis was followed in the pH stat. Pseudo-unimolecular rate constants, $K_{\text{cat}}$, were calculated from the rates, and from the expression, $k_{\text{cat}} = k_{\text{cat, dep}} + k_{\text{cat, dep, HO}}$, values of the rate constants for hydrolysis by water and by hydroxide, $k_{\text{cat, dep, HO}}$, and $k_{\text{cat, dep}}$, respectively, were calculated.

Reversible Inhibition by BrPin—Stock solutions of substrate and 3 x 10^{-4} M BrPin in 0.18 M NaCl were prepared. Aliquots were added to 0.18 M NaCl and brought to pH 7.8, 9, or 10, BrPin, 10 µl, was added, leading to 3 mM solutions, and hydrolysis was followed in the pH stat. Pseudo-unimolecular rate constants, $K_{\text{cat}}$, were calculated from the rates, and from the expression, $k_{\text{cat}} = k_{\text{cat, dep}} + k_{\text{cat, dep, HO}}$, values of the rate constants for hydrolysis by water and by hydroxide, $k_{\text{cat, dep, HO}}$, and $k_{\text{cat, dep}}$, respectively, were calculated.

Reversible Inhibition by DPA—Aliquots of stock solutions of 0.010 M N,N-dimethyl-2-chloro-2-phenylethylamine hydrochloride, substrate, and salt solution were diluted appropriately and brought to pH 7.8. Enzyme solution was added, 3 x 10^{-10} M in the assay, and the hydrolysis was followed. Concentration of acetylcholine was 2.0 x 10^{-9} M in the stock solution, 2.6 x 10^{-9} M in the assay; five concentrations of DPA were used, 0.10-10^{-5} M DMBAc, 7.6 x 10^{-9} M phenyl acetate, and 5.0 x 10^{-9} M n-butyl acetate.

Reversible Inhibition by DPA—Aliquots of stock solutions of 0.010 M N,N-dimethyl-2-chloro-2-phenylethylamine hydrochloride, substrate, and salt solution were diluted appropriately and brought to pH 7.8. Enzyme solution was added, 3 x 10^{-10} M in the assay, and the hydrolysis was followed. Concentration of acetylcholine was 2.0 x 10^{-9} M in the stock solution, 2.6 x 10^{-9} M in the assay; five concentrations of DPA were used, 0.10-10^{-5} M DMBAc, 4.3 x 10^{-9} M DMBAc, 7.6 x 10^{-9} M phenyl acetate, and 5.0 x 10^{-9} M n-butyl acetate.

Reversible Inhibition by BrPin—Stock solutions of substrate and 3 x 10^{-4} M BrPin in 0.18 M NaCl were prepared. Aliquots were added to 0.18 M NaCl and brought to pH 7.8, 9, or 10, BrPin, 10 µl, was added, leading to 3 mM solutions, and hydrolysis was followed in the pH stat. Pseudo-unimolecular rate constants, $K_{\text{cat}}$, were calculated from the rates, and from the expression, $k_{\text{cat}} = k_{\text{cat, dep}} + k_{\text{cat, dep, HO}}$, values of the rate constants for hydrolysis by water and by hydroxide, $k_{\text{cat, dep, HO}}$, and $k_{\text{cat, dep}}$, respectively, were calculated.

Irreversible Retardation—Solutions (0.18 M NaCl) containing (i) 2.6 x 10^{-6} M enzyme and 1.0 x 10^{-4} M BrPin, alone, and with 2.0 x 10^{-4} (CH$_3$)$_3$N$^+$CH$_2$CH$_2$COOH$^-$, and (ii) 4 x 10^{-4} M enzyme and 1.0 x 10^{-4} M BrPin, alone and with 1.9 x 10^{-3} M (CH$_3$)$_3$N$^+$CH$_2$CH$_2$COOH$^-$, were used, and residual enzymic activity was assayed after periods of time by 1:20 dilution into 2 x 10^{-4} M acetylcholine.

Inhibition by DPA—(i) A solution of 2.5 ml of 0.01 M N,N-dimethyl-2-chloro-2-phenylethylamine hydrochloride, 0.4 ml of 7 x 10^{-8} M enzyme, and 17 ml of distilled water was maintained at pH 7.8 for 6 h, and aliquots, 3 ml, were added to 17 ml of 0.18 NaCl containing 2 x 10^{-4} M acetylcholine or 2 x 10^{-5} M DMBAc, and hydrolysis was followed.

Results

The intrinsic hydrolytic reactivity of BrPin under the conditions of its use as an irreversible inhibitor was examined briefly. The observed pseudo-unimolecular rate constants for its hydrolysis in 0.18 M NaCl were 5.1 x 10^{-9} s^{-1} at pH 10, 5.3 x 10^{-9} s^{-1} at pH 9. These data lead to rate constants 5.1 M^{-1} s^{-1} for reaction with hydroxide and 2 x 10^{-6} s^{-1} with water, and to a calculated constant at pH 7.8 of our studies of 5 x 10^{-6} s^{-1}. The observed constant at pH 7.8 was 6 x 10^{-6} s^{-1}. Presence of 0.18 M NaCl had the same effect as that of NaBr, decreasing the hydrolysis rate slightly as compared with that in the absence of added salt.

In studies of BrPin as a reversible inhibitor, enzyme was added to solutions of substrate and BrPin and initial rates of hydrolysis were measured. Linear plots of $1/v$ versus $1/s$ at varying concentrations of inhibitor were obtained. Acetylcholine, phenyl acetate, n-butyl acetate, and DMBAc, Fig. 1, A-D, respectively, show largely competitive inhibition, and Dixon plots lead to values of $K_i$, 0.18, 0.32, 0.24, and 0.57 mM, respectively. Estimated uncertainty in these values is ~30%.

Results of irreversible inhibition arising from incubation of enzyme with BrPin in 0.18 M NaCl are summarized in Fig. 2. Values of $V_o/V_i$, the ratios of enzymic rate of substrate hydrolysis after periods of incubation, $V_i$, to the rate at the start of the incubation, were plotted against time of incubation. In these experiments 1 or 2-ml aliquots of incubation solution were added to 19 or 18 ml of acetylcholine. Incubation times for 50% inhibition were 2.8 ± 0.5 h at 1 x 10^{-6} M BrPin (75% at 5.5 h), 4.8 ± 0.5 h at 3 x 10^{-5} M BrPin, and 9.1 ± 1 h at 1 x 10^{-4} M BrPin. Inhibition was also observed at 3 x 10^{-6} M BrPin, ~10% in 6 h, while the effect at 3 x 10^{-5} M was within experimental error. After incubation for 24 h, inhibition was essentially complete with 10^{-5} M BrPin, ~90% with 3 x 10^{-4} M, >80% with 1 x 10^{-3} M, only 23% with 3 x 10^{-3} M. Incubation in distilled water led to effects similar to that in 0.18 M NaCl.
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3 × 10⁻⁴ M BrPin led to 50% inhibition in 4 h. Inactivation by 3 × 10⁻⁵ M BrPin, studied in detail with acetylcholine and DMBAc, was equally effective with the two substrates, Fig. 2. Enzyme inactivated by 3 × 10⁻⁵ M BrPin behaved similarly toward other substrates. In hydrolysis of a much less reactive ester, n-butyl acetate, 50% inhibition occurred after incubation for 4.5 h, 75% inhibition after 12 h; with phenyl acetate 50% inhibition was observed after 3.5 h, 75% after 8 h, 94% after 23 h. In one experiment with indophenylacetate, enzyme incubated with 1.5 × 10⁻⁷ M BrPin for 5 and 10 h showed 30 and 10% residual reactivity, respectively. The α-haloketone structure was essential for covalent inhibition. When 3 × 10⁻⁴ M BrPin was hydrolyzed to completion at pH 10 and brought back to pH 7.8, and enzyme was incubated in this solution, no time-dependent change in activity was observed.

It seemed possible that the progressive inhibition by BrPin might involve formation of a relatively labile covalent bond, which could be broken in part in the dilution of the incubating solution into the substrate assay solution. Therefore, some experiments were carried out in which enzyme and BrPin in 200–300 ml of 0.18 M NaCl were incubated, and 20-ml aliquots were treated on the titrimer with 0.1-ml portions of acetylcholine. Results were very similar to those found by the dilution techniques. Times for half-inhibition of the enzyme were 2 h at 3 × 10⁻⁵ M BrPin, and 5 h at 3 × 10⁻⁴ M BrPin, and the higher concentration led to essentially complete inhibition in 10 h.

Possible reactivation was examined in dialysis and attempted regeneration experiments. Enzyme inactivated to varying degrees by incubation with 10⁻⁷–10⁻⁴ M BrPin for varying periods of time showed no recovery of activity on dialysis under conditions which removed free BrPin. Also, enzyme strongly inactivated by 3 × 10⁻⁴ M BrPin showed no recovery when treated with 1,1-trimethylenebis(4-hydroximi-
M, confirmed that enzyme inactivated by treatment with inhibitor. At M, acetylcholine, and dialysis did not regenerate activity. Treatment of the enzyme from the titrimeter, and thus could be studied for its effect as an inactivator of each substrate. Linear plots of inhibition by substrate, acetylcholine, (CH₃)₃N+(CH₂)₃COCH₃, competitive, BrPin, acetylcholine substrate; examined briefly. Reversible inhibition, reported to be noncompetitive, was studied at one concentration of each substrate. Linear plots of V/V₀ as in Fig. 2. Fig. 2. Irreversible inactivation of acetylcholinesterase by incubation with BrPin: V₀, rate of hydrolysis after incubation for time, t; V₀, rate at t₀, corrected for decay of enzyme. ○—□, 1 x 10⁻⁵ M BrPin, acetylcholine substrate; △—△, 1.5 x 10⁻⁴ M BrPin, indophenyl acetate substrate; ○—○, 3 x 10⁻⁴ M BrPin, acetylcholine substrate; △—△, 3 x 10⁻⁴ M BrPin, acetylcholine substrate. Inset, irreversible inactivation of acetylcholinesterase by incubation with BrPin, acetylcholine substrate, first order plots. ○—□, 1 x 10⁻⁵ M BrPin; ○—○, 3 x 10⁻⁴ M BrPin.

nomenethylpyridinium bromide, an effective pyridinium oxime reactivator against phosphorylase inactivation (19).

Previously studied (10) competitive reversible inhibitors for hydrolysis by acetylcholinesterase retarded the inactivation by BrPin. These inhibitors are ketone analogues of the natural substrate, acetylcholine, (CH₃)₃N(CH₂)₃COCH₃, Kᵢ = 0.022 ± 0.006 mM and (CH₃)₃CN⁺H₂CH₂CH₂COCH₃, Kᵢ = 0.16 ± 0.04 mM. The latter, a secondary amine but largely protonated at the pH of the study, 7.8, showed no reaction with BrPin in the titrimeter, and thus could be studied for its effect as an inhibitor. At 1.9 x 10⁻⁴ M, it increased time for half-inactivation of the enzyme from 3 to 13 h; the quaternary compound, (CH₃)₃N⁺CH₂CH₂CH₂COCH₃, 2.0 x 10⁻⁴ M, increased t₁/₂ to 6 h, Fig. 3.

Inhibition by DPA and its relation to that by BrPin were examined briefly. Reversible inhibition, reported to be noncompetitive, Kᵢ = 0.06 mM (13), was studied at one concentration of each substrate. Linear plots of V/(V – V₀), where V and V₀ are rates in absence and presence of inhibitor, I, respectively, against 1/[I] were obtained. Values of Kᵢ were 0.030 and 0.028 mM in hydrolysis of acetylcholine and DMBAc, respectively. In study of irreversible inhibition it was confirmed that enzyme inactivated by treatment with 1.25 x 10⁻³ M DPA for 6 h had essentially no reactivity toward acetylcholine, and dialysis did not regenerate activity. Treatment of enzyme with 2.4 x 10⁻³ M DPA for 7.5 h, followed by dialysis, led to modified enzyme which hydrolyzed DMBAc 10% as rapidly as unmodified enzyme, consistent with the reported effect on Kᵢ for uncharged substrates (12). Then, addition of 3 x 10⁻⁴ M BrPin caused progressive decrease in reactivity, 50% loss in 6 h as compared with 2 h with enzyme which had not been treated previously with DPA.

Discussion

α-Bromoketones are reactive alkylating agents, ~10⁴ times more reactive toward nucleophiles than normal alkyl bromides (22). It may be noted that the three methyl groups of BrPin do not cause significant inductive retardation or steric hindrance, since they do not decrease its reactivity in SN₅ reactions, as compared with bromoacetone, by more than a factor of 3 (25). Nevertheless, these rates are such that BrPin is relatively stable under our experimental conditions, pH 7.8, with a hydrolytic half-life of approximately 30 h at pH 7.8. Also the chloride ion in the medium does not appear to interfere with its use by conversion of the bromo- to the chloroketone. The aziridinium compound, DPA, is more readily hydrolyzed, with a half-life of 1.5 h at pH 7.4 (13), and the quaternary ammonio-bromoketone, BAT, is still more reactive with a hydrolytic half-life of 17 min at pH 7.4 (19).

Initially, BrPin is an effective irreversible inhibitor and its action is primarily competitive. Values of Kᵢ may vary somewhat with substrate, in the range 0.2-0.6 mM. Binding, thus, is similar to that of cationic compounds related to acetylcholine, tetramethylammonium ion, and choline, Kᵢ = 1 mM. It is also similar to that of BAT, Kᵢ = 0.6 mM, which shows mixed inhibition (19), but less effective than the noncompetitive inhibition by DPA. Since bromoacetone and α-chloroacetophenone do not inactivate acetylcholinesterase irreversibly, we conclude that inactivation by BrPin results from reversible binding due to its tert-butyl group at the trimethyl subsite, followed by covalent bonding of the α-C to a nucleophilic group of the active site. Displacement of the α-Br is essential, since prior hydrolysis of BrPin eliminates time-dependent inhibition. The covalent bonding is firm, and is not reversed by dialysis or attempted regeneration.

Irreversible inhibition by BrPin is indicated in Equation 1, with rate proportional to the concentration of the complex, E · BrPin.

\[ E + BrPin \rightleftharpoons [E \cdot BrPin] \rightarrow E \cdot Br + Br^- \] (1)
Loss of enzymic activity would show first order kinetics at high enough concentration of BrPin that inactivation is fast relative to loss of BrPin by hydrolysis. Consistent with this, plots of \( \log V/V_0 \) versus time for the data at \( 3 \times 10^{-3} \) and \( 1 \times 10^{-3} \) M BrPin are linear over the 70–80% inactivation, which was followed kinetically, Fig. 2, inset. The fractions of complexed enzyme, \( E\cdot BrPin \), at \( 3 \times 10^{-3}, 1 \times 10^{-3}, 3 \times 10^{-4}, \) and \( 1 \times 10^{-4} \) M BrPin, based on \( K_r = 0.18 \pm 0.05 \) mm in the reversible inhibition of hydrolysis of acetylcholine, are 0.94 ± 0.02, 0.85 ± 0.04, 0.63 ± 0.06, and 0.36 ± 0.06, respectively. A plot of time for half-inactivation against inverse of complexed fraction, Fig. 4, indicates \( t_{1/2} \) of 2 h for fully complexed enzyme, corresponding to a rate constant \( k_z = 1 \times 10^{-6} \) s\(^{-1}\) for conversion of \( E\cdot BrPin \) to \( E \cdot Pin + Br^- \). Equation 1, 17 times greater than that for the solvolysis of BrPin in the medium. DPA, on the other hand, undergoes solvolysis at a rate comparable to that at which it inactivates the enzyme (13). Its hydrolysis rate constant, \( 1.2 \times 10^{-4} \) s\(^{-1}\), is 20 times that of BrPin, while \( t_{1/2} \) for its inactivation of enzyme at concentrations comparable to its \( K_r \) was 2–3 h (13). Thus, the enzyme-DPA complex may inactivate somewhat more rapidly than the enzyme-BrPin complex, but not with a rate commensurate with the greater intrinsic reactivity of DPA.

The cationic bromoketone BAT hydrolyzes 100 times more rapidly than BrPin, and at high concentration, \( 10^{-2} \) M, it inactivated the enzyme with \( t_{1/2} \approx 6 \) min, 20 times more rapidly than BrPin, \( k_z = 2 \times 10^{-3} \) s\(^{-1}\) (19). The high concentration of BAT was required because much of it was lost by hydrolysis. Thus, with \( K_r \) similar to that of BrPin, \( 5 \times 10^{-4} \) M BAT inactivated only 40% of the enzyme, as compared with 90% by this concentration of the more stable, less reactive BrPin. As with DPA, the higher intrinsic reactivity is reflected partially in the inactivation rate. In all three cases, the inactivation steps are slow and do not utilize the catalytic mechanism of the enzyme.

The irreversible inhibitors, \((CH_3)N^+CH_2CH_2CH_2COCH_3\) and \((CH_3)CN^+H^+_CH_2CH_2CH_2COCH_3\), which retarded the irreversible inhibition by BrPin, are isosteric with the natural substrate acetylcholine, have acetyl and trimethyl groups in the corresponding positions, act competitively in retarding hydrolysis of acetylcholine and DMBAc, and fit into the active site as these substrates do, binding at the trimethyl and acetyl subsites (10). If BrPin also binds in the active site, their effect on the fraction of enzyme present as \( E\cdot BrPin \) complex may be estimated from Equation 2, an expression for substrate-inhibitor competition in which BrPin is treated as the substrate,

\[
\frac{E \cdot BrPin}{E_0} = \frac{[BrPin]}{[BrPin] + K_s \left( 1 + \frac{[I]}{K_i} \right)}
\]

Thus, with \( K_s = 0.18 \pm 0.05 \) mm. At \( 1.0 \times 10^{-3} \) M BrPin, and \( 2.0 \times 10^{-4} \) M \((CH_3)N^+CH_2CH_2CH_2COCH_3\) and \( 1.9 \times 10^{-3} \) M \((CH_3)CN^+H^+_CH_2CH_2CH_2COCH_3Cl^-\) the fractions of the enzyme present as \( E\cdot BrPin \) are \( 0.36 \pm 0.11 \) and \( 0.30 \pm 0.11 \), respectively. The uncertainties in values of \( K_i \) for BrPin and the reversible inhibitors lead to uncertainties in the fractions of \( E\cdot BrPin \) and the values of \( t_{1/2} \). The latter, estimated from Fig. 4, are 9 ± 4 and 12 ± 5 h. The observed values, 6 and 13 h, respectively, Fig. 3, are consistent with the assumption of Equation 2.

That reversible inhibition by DPA is noncompetitive has suggested that this cationic compound does not bind in the active site. Since acetylcholine and DMBAc utilize the same site (10), the great difference in reversible effects of DPA in hydrolysis of these substrates (complete inactivation toward acetylcholine and less effect on DMBAc) further supports this view. DPA would introduce positive charge near the active site and inhibit binding of cationic substrates and inhibitors. Indeed, labeling has indicated incorporation of two DPA moieties/enzyme unit, at chemically different sites (14). In the present study, inactivation by BrPin toward hydrolysis of DMBAc, albeit retarded, of enzyme previously inactivated by DPA toward acetylcholine gives further cogent evidence that DPA may not specifically label an anionic group within the active site.

That the reversible inhibition by BrPin is largely competitive, and the subsequent irreversible inactivation is essentially the same toward hydrolysis of the cationic and uncharged substrates of varied structure acetylcholine, DMBAc, phenyl acetate, n-butyl acetate, and indophenyl acetate support the proposal that the active site may be more specifically characterized by such uncharged reagents. The cationic bromoketone, BAT, is reported to decrease reactivity toward cationic and uncharged substrates, and may well enter the primary binding site. However, it is also recognized that it may react at peripheral anionic sites (19). Further study of BrPin and related compounds may lead to identification of the group(s) with which they react, while study of DPA may characterize peripheral groups.

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