has been reported.[8] The resulting linear decrease in K_p values as a function of inhibitor concentration is shown in Fig. 3. Values of K_i at the two OHA concentrations were in good agreement, whereas somewhat more variation was found with K_i values of APHA. The ratios K_m/K_i indicated a quite low order of affinity of the inhibitor for the enzyme–substrate complex.

The mammalian enzyme was used in experiments to assess the capacity of excess pyridoxal phosphate (PyP) to overcome inhibition conferred by OHA and APHA. Double reciprocal plots of the data so obtained in the presence of each inhibitor were consistently nonlinear and were not satisfactorily interpretable. There was evidence, however, that the coenzyme at least partially antagonizes the action of each hydroxamate. OHA is known to bind PyP as evidenced by a marked alteration of the ultraviolet absorption spectrum of the coenzyme;[7] however, no such alteration could be demonstrated in the presence of APHA.

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(3-Bromo-2-oxopropyl) trimethylammonium bromide, an inhibitor of acetylcholinesterase

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WITH the objective of developing an alkylating type of active site reagent for acetylcholinesterase (AChE; acetylcholine acetylhydrolase, EC 3.1.1.7), (3-bromo-2-oxopropyl)trimethylammonium bromide* and several structurally related compounds were examined. These include: 1-(3-bromo-2-oxopropyl)pyridinium bromide, 3-(bromoacetyl)-1-methylpyridinium bromide, (3-bromo-4-oxopentyl)trimethylammonium bromide and 2-[chloro(hydroxyimino)methyl]-1-methylpyridinium chloride. Of the group, BAT showed the most promise. (a) It reversibly inhibits the enzyme if assay is performed rapidly and shortly after mixing. (b) It progressively and irreversibly inhibits the enzyme, upon incubation, in a time-, concentration- and pH-dependent fashion. (c) Progressive inhibition is slowed by the reversible AChE inhibitor, tetramethylammonium bromide (TMA), and is prevented by prior diethyl phosphorylation. These results suggest that BAT may have utility as an active site reagent for AChE.

* Named 3-bromoacetonyltrimethylammonium bromide by Persson *et al.*[1] For convenience, we will refer to this compound as BAT.

Similar substrate-related α-haloketones have been found to react with histidine in α-chymotrypsin[2] and trypsin,[3] and with aspartate in pepsin.[4]

Recently, Persson *et al.*[1] reported that BAT is an irreversible inhibitor of choline acetylase. They observed that BAT, at 5×10^{-4} M, had no inhibitory action on human erythrocyte AChE. Other active site reagents for AChE have been reported by Schaffer *et al.*,[5] Belleau and Tani,[6] Wofsy and Michaeli,[7] and Purdie and McIvor.[8]

BAT (prepared in substantially the same manner as reported by Persson *et al.*[1]), 1-(3-bromo-2-oxopropyl)pyridinium bromide and (3-bromo-4-oxopentyl)trimethylammonium bromide were suggested and synthesized for the purpose of this study by Dr. M. Mednick (these laboratories). 3-(Bromoacetyl)-1-methylpyridinium bromide and 2-[chloro(hydroxyimino)methyl]-1-methylpyridinium chloride were provided by Ash–Stevens, Inc. 1,1-Trimethylenebis(4-formylpyridinium oxime chloride) (TMB-4) was obtained as the hydrate from Aldrich Chemical Company. *O,O*-diethyl S-(2-trimethylammonioethyl)phosphorothioate iodide (Phospholine; Echothiophate) was obtained from Ayerst Labs. Each sterile vial contained 12·5 mg phospholine mixed with 150 mg mannitol. The sugar was not removed prior to use. AChE was "purified" grade eel enzyme from Sigma Chemical Company; the approximate concentration of "active sites" was 2×10^{-5} M in the stock solution.

Reversible inhibition of AChE by BAT. Assays were made with the Radiometer TTT1 titrator at a constant pH of 6·45 with 0·0029 N sodium hydroxide as the titrant. Carbon dioxide absorption was minimized by blowing N_2 over the assay solution in a covered thermostated (25°) titration vessel. Assay solution: AChE, diluted 1/318,000 in 3 ml of 0·15 M KCl containing 0·066% gelatin (KCl/gel);

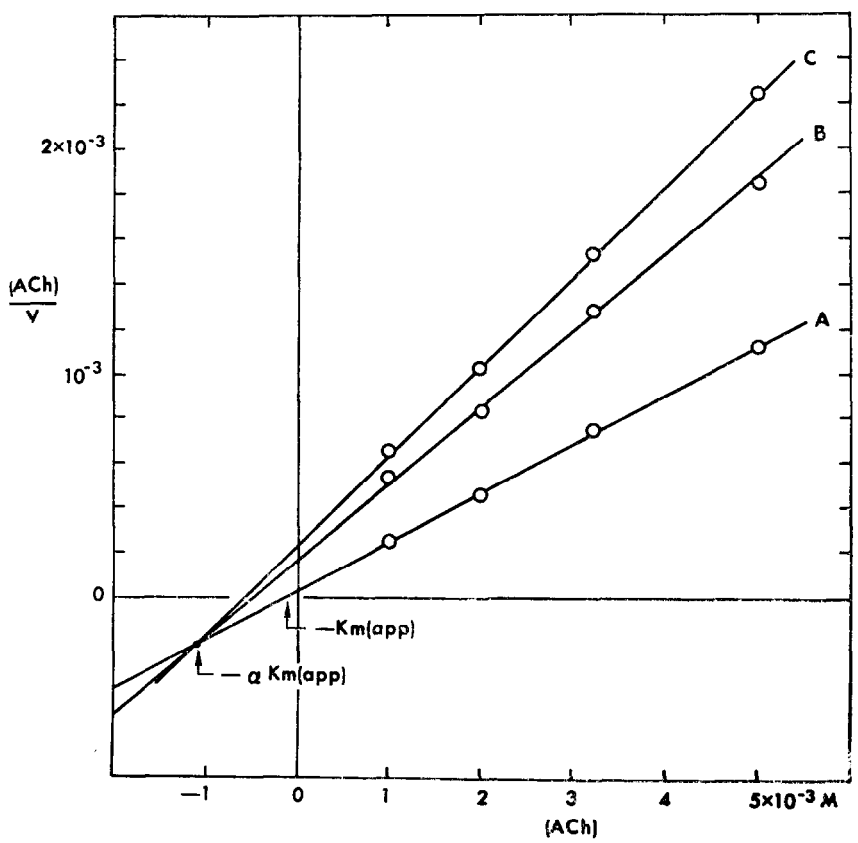

FIG. 1. Reversible inhibition of eel AChE, pH 6·45, 25°. Concentration of BAT: A, 0; B, $2{\cdot}5 \times 10^{-3}$ M; C, $5{\cdot}0 \times 10^{-3}$ M.

acetylcholine bromide (ACh), 1–5 × 10^{-3} M. In each run, a blank value for BAT hydrolysis was measured in the assay mixture for 2–3 min before addition of the enzyme. Initial activity measured for 2–3 min after addition of enzyme gave linear recordings of alkali addition. Lineweaver–Burk[9] plots of the results are presented in the S/V vs. S mode (Fig. 1). The velocity, v, is given in divisions of excursion on the chart paper per minute; 100 divisions = 0·25 ml of alkali titrant.

Irreversible inhibition by BAT. Reaction of BAT and AChE was run in the vessel of a Radiometer Autotitrator TTT1, with pH maintained constant by automatic delivery of 0·0437 N sodium hydroxide, 25°. To 3 ml KCl/gel and the appropriate quantity of BAT at preselected pH values, AChE was added to give a 1/5000 dilution of stock solution. Periodically, 25-μl aliquots were taken for AChE assay on a second Radiometer TTT1. Assay conditions: pH 7·40, 25°, 3 ml KCl/gel, 3·3 × 10^{-3} M ACh; 0·0029 N NaOH. For each assay, a blank value of BAT hydrolysis was measured prior to addition of substrate. In Fig. 2 results are given of progressive inhibition of AChE at several pH values and BAT concentrations. For most of the reactions (all were run in duplicate) there were parallel runs made in the presence of 10^{-2} M TMA. The quantity of TMA carried over into the assay vessel was too small to affect AChE activity (Fig. 2).

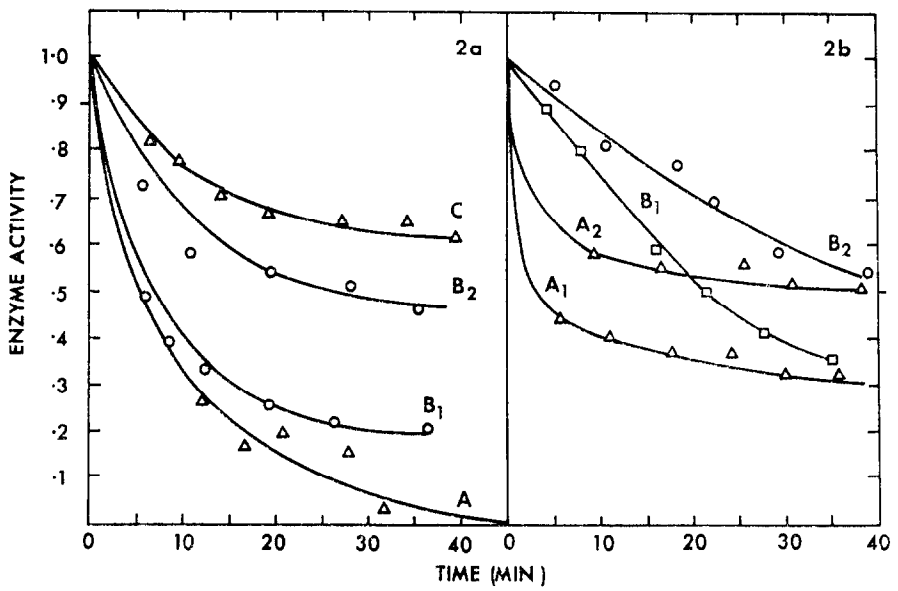

FIG. 2. (a) Progressive inhibition of AChE by BAT, pH 7·0, 25°. Concentration of BAT: A, 10^{-2} M; B, 3·5 × 10^{-3} M (B_1, no TMA; B_2, 10^{-2} M TMA); C, 5 × 10^{-4} M.
(b) Progressive inhibition of AChE by BAT, 3·5 × 10^{-3} M, 25°. A, pH 7·4 (A_1, no TMA; A_2, 10^{-2} M TMA); B, pH 6·0 (B_1, no TMA; B_2, 10^{-2} M TMA).

Phospholine protection. To 3 ml KCl/gel containing AChE at 1/4000 dilution of stock, pH 7·0, 25°, phospholine was added to give a concentration of 10^{-5} M. Assays were made on a second Radiometer as described above. After 1 hr, all of the enzymatic activity had been fully inhibited. Addition of TMB-4 (10^{-4} M) to the assay solution "instantaneously" reactivated the phospholine-inhibited enzyme, giving complete recovery of enzyme activity. After all of the enzyme activity had been inhibited by incubation with phospholine, further incubation with phospholine resulted in no loss of reactivatable activity (Fig. 3, curve A). TMB-4 does not reactivate AChE inhibited by reaction with BAT. After virtually complete inhibition of the enzyme by phospholine, BAT (3·5 × 10^{-3} M) was added at time = 0 (Fig. 3, curve B). Assay solution contained no TMB-4. At t = 12·5 min, TMB-4 (10^{-4} M) was added to the incubation mixture.

Hydrolysis of BAT. Hydrolysis rate studies were performed with the Radiometer Autotitrator at

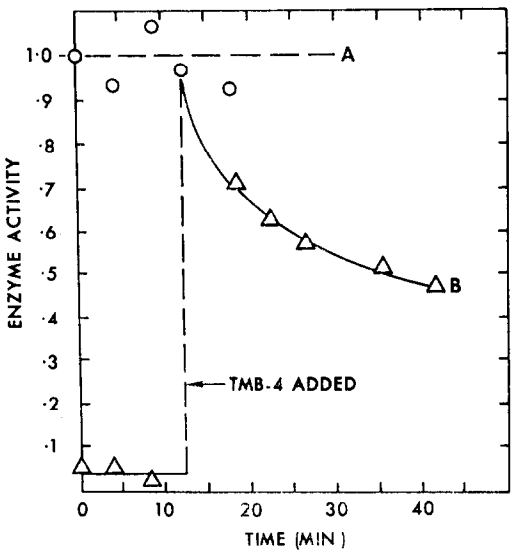

FIG. 3. Protection with phospholine. A, AChE fully inhibited with phospholine, assayed periodically (with TMB-4 in assay mix). B, after complete inhibition with phospholine, BAT ($3{\cdot}5 \times 10^{-3}$ M) was added at t = 0. At t = 12·5 min, TMB-4 (10^{-4} M) was added to the incubation mixture. Assay mix contained no TMB-4.

constant pH, 25°, using KCl/gel solution (Method A) at pH 7·0 and 7·4 and by a colorimetric procedure (Method B)[10] at pH 6·52 in 0·1 M 2-(*N*-morpholino)ethanesulfonic acid (MES). For Method A kinetic analysis was made by both the Guggenheim[11] method and the method of Kezdy *et al.*[12, 13] where a knowledge of the endpoint is not required. At each pH with both methods, the reaction obeyed first-order kinetics with $T_{1/2}$ = 41 min (pH 7·00); 16·5 min (pH 7·40); 161 min (pH 6·52). Bimolecular rate constant for reaction: BAT + OH^- —→ products: $1{\cdot}68 \times 10^5$ M^{-1} min^{-1} (pH 7·0 and 7·4); $1{\cdot}30 \times 10^5$ M^{-1} min^{-1} (pH 6·52).

The reaction of BAT both with AChE and with aqueous solvent (OH^-) increases in rate as the pH is raised. The leveling off in AChE inhibition (Fig. 2) is probably caused by the combined effects of loss of BAT through aqueous hydrolysis and inhibition of reaction by the product of hydrolysis* (probably $(CH_3)_3\overset{+}{N}CH_2COCH_2OH$ Br^-). Optimum pH for reaction with AChE would probably be at 6 or a bit lower. Reversible inhibition by BAT (Fig. 1) is of the mixed type:[14, 15] $K_I = 5{\cdot}8 \times 10^{-4}$ M, $\alpha = 8{\cdot}7$, where K_I and αK_I are the dissociation constants, respectively, for the competitive and the noncompetitive components. Krupka[15] attributes the latter to inhibition of the deacetylation step. The magnitude of K_I is quite low compared to that of other simple quaternary ammonium compounds [i.e. choline (RBC ChE) $K_I = 1{\cdot}3 \times 10^{-3}$ M].[15] The reversible inhibitor of AChE, TMA, slows the reaction of BAT with AChE at all pH values which were examined.

In Fig. 3, we see that inhibition of the enzyme with phospholine (diethylphosphorylation of serine at the active site) completely stopped reaction with BAT for the experimental period of 12·5 min. In the absence of phospholine protection, there would have been 65–70 per cent inhibition of the enzyme. Upon removal of the diethylphosphoryl group with TMB-4, BAT inhibition proceeded in the normal fashion.

Thus, BAT gives kinetic evidence of reaction with the active site of eel AChE. Whether it reacts exclusively with the active site remains to be ascertained. The results of the protection experiment (Fig. 3) suggest that even if BAT shows some degree of random reaction, it may be possible to label differentially the active site by reacting phospholine-protected enzyme with "cold" BAT prior to reactivation and reaction with labeled BAT. No attempt was made to repeat the observation of

* Approximate value of K_I, 6×10^{-4} M; pH 7·4, 0·1 M KCl, $1{\cdot}65 \times 10^{-3}$ M ACh.

Persson *et al.*[1] that 5×10^{-4} M BAT fails to inhibit human RBC AChE. He performed the reaction at 0° for 60 min at an unspecified pH. Our studies were performed at a higher temperature, with a different source of enzyme and possibly at a different pH. Under our conditions, at the specified concentration of BAT, reaction is slow (Fig. 2). Hence we do not consider the difference in results to be incompatible. Purdie and McIvor[8] found that the AChE active site reagent, 1,1-dimethyl-2-phenylaziridinium ion, reacts with bovine erythrocyte AChE to inhibit the enzyme when assayed with several substrates including phenyl acetate. However, they observed that reaction increases catalytic action of the enzyme toward indophenyl acetate. With BAT and eel AChE, we have found that reaction reduces enzymatic activity toward both phenyl acetate and indophenyl acetate.

The related compounds received less detailed examination. The corresponding 1-(3-bromo-2-oxopropyl)pyridinium bromide and 3-(bromoacetyl)-1-methylpyridinium bromide progressively inhibited the enzyme at pH 7·0, but more slowly and less completely than BAT. (3-Bromo-4-oxopentyl)trimethylammonium bromide did not react covalently with the enzyme.* However, it has high affinity for the enzyme (K_I = approx. $1{\cdot}5 \times 10^{-6}$ M). 2-[Chloro(hydroxyimino)methyl]-1-methylpyridinium chloride reacts progressively with eel AChE. At 10^{-4} M, it inhibits more rapidly than BAT does at 5×10^{-3} M. However, protection with phospholine (or DFP) is incomplete. Reaction is slowed, not stopped. This difference from BAT in the failure for phosphorylation to afford protection suggests multiple site inhibition of the enzyme. An alternate possibility (suggested by Dr. M. Bender) is that the hydroximino compound (or a hydrolysis product; the corresponding pyridinium hydroxamic acid is found in part upon aqueous hydrolysis) reactivates the phosphorylated enzyme, thus liberating the enzyme's active site.

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* No irreversible loss of enzymatic activity occurred when incubated at 10^{-3} M for 83 min.

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