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ACETYLCHOLINESTERASE AND ACETYLCHOLINE RECEPTOR

Final Report

Saul G. Cohen, Ph.D.

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Our study of acetylcholinesterase (AcChE) is based on the view that the β-trimethylammonio substituent of acetylcholine binds at an uncharged subsite which is better termed "trimethyl," rather than "anionic," and would be more specifically explored by uncharged reagents. AcChE was isolated from Torpedo nobiliana; polyacrylamide gel electrophoresis and autoradiography showed satisfactory purity and the major band at 70 kDa.

Reversible inhibitors and substrates containing $(CH_3)_3C$, $(CH_3)_3Si$, $CH_3S(O_2)$, $CH_3S(O)$, CH_3S, $(CH_3)_2S^+$, $(CH_3)_2N^+(O^-)$, and Cl_3C substituents were studied.

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Benzene derivatives bind at an aryl group binding site with energies linear with Hammett σ values, ρ +2, and increased by $(CH_3)_2X$ and $(CH_3)_3X$ substituents. tert-Butyl and trimethylammonio groups, binding at the trimethyl site, show strong similar synergism with meta phenolic hydroxyl. Dimethylaminopyridines bind as strongly as N-methylpyridinium ion.

Inactivation of AcChE by styrene oxide, methyl benzenesulfonate, or 1-bromopinacolone (BrPin) prevents subsequent reaction with [^{3}H]-DFP. Inactivation by BrPin is retarded by $C_6H_5N^+(CH_3)_3$, phenyltrimethylammonium ion (PTA), 3-HO-C_6H_4-$N^+(CH_3)_3$, 3-trimethylammoniophenol (TMAP), and $(CH_3)_3N^+CH_2$-$CH_2CH_2COCH_3$, 5-trimethylammonio-2-pentanone (TAP) to extents consistent with K_i values. 1-Bromo-2-[^{14}C]-pinacolone, ([^{14}C]-BrPin) has been prepared; it introduces one ^{14}C per enzyme unit in inactivating. Introduction of ^{14}C is retarded by PTA and TAP. Prior inactivation by [^{3}H]-DFP does not prevent introduction of [^{14}C]-BrPin, and both labels remain. The site of BrPin labeling is being studied.

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SUMMARY

This research seeks information about the properties and amino acid content of the active site of acetylcholinesterase (AcChE). It is based on the view that the subsite at which the positively charged β-substituent of the natural substrate, acetylcholine (AcCh), binds is not anionic, as is generally accepted, but uncharged and complementary to the trimethyl substituted character of that substituent. The subsite would be more specifically explored by uncharged reagents, while positive reagents may react at peripheral negative sites. Thus, uncharged irreversible and reversible inhibitors and substrates are obtained or synthesized and their reactions with the enzyme are studied. Primary emphasis is placed on labeling the active site with 1-bromo-2-[^{14}C]-pinacolone, $(CH_3)_3C^{14}CO$-CH_2Br ([^{14}C]-BrPin); we have found in previous work that BrPin inactivates AcChE with the same kinetics toward hydrolysis of substrates of widely varied structure.

A second line of inquiry derives from the view that the active site is stabilized in part by interactions of aryl side chains which are contiguous with the trimethyl and esteratic subsites. These provide an additional subsite at which aryl compounds may bind and act as labeling agents and as reactivators.

For labeling studies, AcChE is isolated from Torpedo nobiliana by affinity chromatography, characterized by gel electrophoresis and autoradiography after treatment with [^{3}H]-DFP, and assayed by the Ellman procedure based, at present, on k_{cat} for T. californica. It has a major band at about 70 kDa, smaller bands at about 110 and 140 kDa, and very faint bands at lower molecular weights. The ^{3}H is concentrated in the 70-kDa band. For most purely kinetic studies, eel AcChE obtained from Sigma is used. It has much extraneous, possibly stabilizing, protein, and about 4% enzyme of about 50 kDa.

^{14}C-Labeled 1-bromopinacolone, [^{14}C]-BrPin, is prepared reliably on 60-μmole scale by reaction of 1-[^{14}C]-acetyl chloride with a slight (about 3%) deficiency of tert-butylmagnesium chloride in anhydrous ether in the presence of Cu_2Cl_2 catalyst under inert atmosphere, followed by treatment with one equivalent of bromine, and washing to remove Cu^{2+} and residual Br_2, powerful poisons for AcChE. The product solution contains [^{14}C]-pinacolone, [^{14}C]-BrPin, and 1,1-dibromo-2-[^{14}C]-pinacolone ([^{14}C]-Br_2Pin) in an approximate ratio of 1:2:1. The K_i values of the three components are 5.5, 0.18, and 0.8 mM, respectively, and the solution inactivates AcChE similarly to commercial BrPin of like concentration. Br_2Pin does not inactivate AcChE at the level at which it is present.

5-Trimethylammonio-2-pentanone, $(CH_3)_3N^+CH_2CH_2CH_2COCH_3$ (TAP), isosteric with AcCh, is an effective reversible inhibitor. Its effects on the action of inactivators are studied as diagnostic of their active-site-directed properties.

A study of hydrolysis of β-substituted ethyl acetates, β-X-$CH_2CH_2OCOCH_3$, by AcChE was completed. Reactivity, normalized for effect of the β-substituent on intrinsic hydrolytic reactivity toward hydroxide, log $(k_{cat}/K_m)_n$, rises linearly with volume of the substituent, as measured by molar refractivity (MR), for β-X = H, Cl, Br, CH_3CH_2, $(CH_3)_2CH$, $(CH_3)_2S^+$, $(CH_3)_3N^+$, and $(CH_3)_3C$; reactivity levels off with the latter, while the trimethyl site accommodates the larger $(CH_3)_3Si$ group with no further increase in reactivity. β-Substituents with polar surfaces, $CH_3S(O_2)$ and $(CH_3)_2N^+O^-$, show lower reactivity than is consistent with their MR values, possibly because they do not remove H_2O from the active site. The correlation with MR is superior to that with hydrophobicity, Π , which predicts low reactivity for the important positively charged substrates.

The methanesulfonyl group, $CH_3S(O_2)$, is similar in shape to <u>tert</u>-butyl, $(CH_3)_3C$, and leads to similar binding when examined in methylsulfonylethyl acetate, $CH_3S(O_2)CH_2CH_2OCOCH_3$, both as a substrate and as a reversible inhibitor. In methyl methanesulfonate, $CH_3SO_2OCH_3$, it leads to rapid irreversible inhibition, probably by alkylation; methylsulfonyl chloride, CH_3SO_2Cl, inactivates like methylsulfonyl fluoride, probably by acylating the serine-hydroxyl.

The trichloromethyl group in trichloroethanol, Cl_3CCH_2OH, leads to weak binding similar to that of its carbon analogue, neopentyl alcohol, $(CH_3)_3CCH_2OH$, K_i = about 40 and 30 mM, respectively. Chloral hydrate, $Cl_3CCH{=}O{\cdot}H_2O$, K_i = 1 mM, binds more strongly than its carbon analogue, pivalaldehyde, $(CH_3)_3CCH{=}O$.

Derivatives of benzene and phenol have been studied as reversible inhibitors, and the factors leading to the very strong binding of 3-trimethylammoniophenol (TMAP), K_i = 0.00033 mM, have been elucidated. A linear relation between log $1/K_{i(com)}$ and σ_{para} values is observed for C_6H_5-Y (Y = HO, H_2N, H, CH_3CONH, CH_3CO, O_2N) and for the disubstituted compounds p-H_2N - $COCH_3$, m-H_2N - NO_2, m- and p-HO - NO_2, and p-O_2N - $NHCOCH_3$, with ρ = +1.84, r = 0.99; $K_{i(com)}$ decreases to 0.6 mM for nitrobenzene. Dimethylamino and trimethylammonio compounds with substituents $(CH_3)_2N$, p-$(CH_3)_2N$ - $COCH_3$, m-$(CH_3)_2N$ - NO_2, p-$(CH_3)_2N$ - NO_2, and $(CH_3)_3N^+$ bind more strongly relative to σ, with ρ = +2.4, r = 0.99; $K_{i(com)}$ decreases to 0.09 for $(CH_3)_3N^+$ and p-$(CH_3)_2N$ - NO_2. Binding arises from a polar interaction of the benzene ring, probably with aryl amino acid side chains in an aryl binding subsite, and is increased by electron-withdrawing substituents. Binding energies of phenols rise linearly with volume of meta substi-

tuents, H < CH_3 < CH_3CH_2 < $(CH_3)_2CH$ = $(CH_3)_2N$ < $(CH_3)_3C$, r = 0.99. Synergistic binding of these meta substituents in the trimethyl subsite and hydrogen-bonding of the phenolic hydroxyl in the esteratic site occurs. The very strong binding of 3-trimethylammoniophenol is accurately calculated from K_i of 3-tert-butylphenol, $\rho = 1.84$ (above), and the difference in σ values of these charged and uncharged isosteric substituents, +0.94. 4-tert-Butylphenol does not show a synergistic effect of the two substituents, indicating a specific steric relationship between the trimethyl, aryl, and hydrogen-bonding subsites. This is also seen in the trimethylammoniophenols, further showing that tert-butyl and trimethylammonio groups bind at the one trimethyl subsite. The effect of the positive charge in the two trimethylammonio compounds is accounted for by its σ value, and coulombic interactions with anionic charge is not involved. These effects indicate the contiguous relationship of the trimethyl, aryl, and esteratic subsites. Occupation of the aryl subsite alone prevents utilization of the catalytic mechanism, but does not exclude appropriate compounds from the trimethyl subsite. Nitrobenzene is an effective reversible inhibitor, K_i = 0.6 mM in hydrolysis of AcCh, while, like small cations, it accelerates hydrolysis of ethyl acetate.

In an extension of our study of aromatic inhibitors, derivatives of pyridine were found to be very effective. Dimethylaminopyridines, without a permanent positive charge, bind as well as N-methylpyridinium ion. Irreversible inhibitors and dephosphorylating agents derived from dimethylaminopyridines may be useful labeling and medicinal reagents, respectively.

Potential irreversible inhibitors other than BrPin were surveyed briefly in enzymic hydrolysis of AcCh. Chloromethyl pivalate, $(CH_3)_3CCO_2CH_2Cl$, closely related to BrPin, inactivated similarly to BrPin. Chloromethyl acetate, $CH_3CO_2CH_2Cl$, which probably binds at the esteratic or acetyl subsite, appeared to act both as a weak substrate, about 1% as reactive as AcCh, and as an irreversible inhibitor, similar in activity to BrPin. Trimethylammoniomethyl acetate, $(CH_3)_3N^+CH_2OCOCH_3$, the lower homologue of AcCh, acted as a reversible inhibitor, K_i = 1 mM, as a substrate, about 10% as reactive as AcCh, and as a weak irreversible inhibitor, possibly by alkylation or by formation of formaldehyde in the active site. tert-Butyl peracetate, $(CH_3)_3COOCOCH_3$, acted as both a substrate, about 10% as reactive as AcCh, and as an irreversible inhibitor, possibly by generating tert-butyl hydroperoxide, $(CH_3)_3COOH$, in the active site. Methyl benzenesulfonate, $C_6H_5SO_2OCH_3$, K_i = 6 mM rapidly inactivates AcChE irreversibly, and this inactivation is not retarded by tetramethylammonium ion. Styrene oxide, K_i = 10 mM, inactivates AcChE. Phenacyl bromide, $C_6H_5COCH_2Br$, inactivates AcChE more rapidly than phenacyl chloride.

Active-site-directed photoinactivating agents based on phenyldiazirines, for application to AcChE and the AcCh receptor, are being prepared.

Aromatic irreversible inhibitors, N,N-dimethylphenylaziridinium ion, styrene oxide and methyl benzenesulfonate, all prevent subsequent introduction of [^{3}H]-DFP. TAP retards inactivation by styrene oxide and methyl benzenesulfonate.

Prior inactivation of AcChE by BrPin prevents subsequent introduction of [^{3}H]-DFP, inhibiting reaction at the serine-hydroxyl, as it does with substrates. Prior inactivation by DFP does not prevent subsequent introduction of [^{14}C]-BrPin; therefore, BrPin does not react at the serine-hydroxyl. TAP strongly retards introduction of [^{14}C]-BrPin, indicating specific interaction of BrPin at the trimethyl subsite. Phenyltrimethylammonium ion (PTA) also retards introduction of [^{14}C]-BrPin, in both a major band at about 70 kDa and a minor band at about 150 kDa. Inactivation of AcChE by BrPin is retarded by PTA, TAP and TMAP to extents consistent with their K_i values, supporting trimethyl subsite specificity and trimethyl and "anionic" subsite identity. Inactivation of AcChE by [^{14}C]-BrPin introduces about one ^{14}C per enzyme unit, with calculation of enzyme content based on k_{cat} for T. californica.

Cleavage by formic acid of samples of AcChE labeled with [^{3}H]-DFP and [^{14}C]-BrPin leads in both cases to radioactivity in a fragment of 50 kDa. The incorporated pinacolyl group is resistant to hydrolysis, and may not be present as a carboxylate ester; it may alkylate an imidazole.

The inactivation of AcChE by BrPin is acid-catalyzed. The inactivation is subject to a group with $pK_a = 6.0$ with rate constants of 5.2×10^{-4} sec^{-1} and 240×10^{-4} sec^{-1} at pH 7.0 and 5.4, respectively. TAP retards inactivation much less effectively at pH 5.4 than at pH 7.8, both because of the higher rate of the BrPin reaction and weaker binding of TAP, K_i = 1.4 and 0.02 mM at pH 5.4 and 7.8, respectively. PTA is relatively more effective at pH 5.4 since its K_i is not decreased at low pH. The effect of the cationic group in the aromatic compound on the binding of the benzene ring is proportional to its σ value and thus is not affected by change in pH.

Collaboration with Professor J. B. Cohen, Washington University School of Medicine, St. Louis, continues.

FOREWORD

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(1) Statement of the Problem Under Study

Acetylcholinesterase (AcChE) has been widely studied for many years because of its great importance in regulating neurotransmission. This study early led to the serine-hydroxyl as an essential group in the active site of many hydrolytic enzymes. While other serine enzymes have been crystallized and their active sites described in some detail, and the linear sequence of AcChE has recently been established, it may be noted that no other amino acid has been unequivocally characterized in the active site of AcChE.

It has long been accepted, as dogma, that the active site of AcChE has an anionic subsite to which the cationic group of the substrate, acetylcholine (AcCh), is attracted, and this has guided study and interpretation. From study of varied substrates and inhibitors, we have concluded that the binding subsite is uncharged, "trimethyl" rather than "anionic", and that the observed effects of anionic charge are accounted for by the excess negative charges on the enzyme surface, peripheral to the active site, arising in a protein of isoelectric point about pH 5, which is active and studied at pH 7-8. We propose that the active site would be more specifically studied by uncharged reagents and have chosen 1-bromopinacolone, $(CH_3)_3C$-$COCH_2Br$ (BrPin) as such an alkylating reagent, with its <u>tert</u>-butyl group complementary to the trimethyl subsite. We prepare BrPin labeled with ^{14}C and inactivate the enzyme with it, and are engaged in degrading the enzyme to identify the modified active site amino acid. We have prepared and surveyed other uncharged compounds, such as chloromethyl pivalate, $(CH_3)_3CCO_2$-CH_2Cl, and methyl methanesulfonate, $CH_3SO_2OCH_3$ which have potential use, but BrPin is our reagent of choice.

Study of derivatives of benzene and phenol as reversible inhibitors of AcChE has led us to the view that the active site includes an aryl binding subsite, contiguous with the trimethyl and esteratic subsites, possibly comprising aromatic amino acid side chains. This led us to consider uncharged aromatic alkylating agents, derivatives of benzene and pyridine, as reagents to label amino acids at or near this aryl subsite. This view may lead to potentially superior medicinal reagents acting from this subsite.

(2) Background and Review of Appropriate Literature

The structural features involved in the interactions of AcCh, $(CH_3)_3N^+CH_2CH_2OCOCH_3$, with AcChE are the three N-methyl groups, the positive charge, and the ester group. The part of the active site of AcChE at which the trimethylammonium group

binds has generally been depicted as anionic (1-3), with its negative charge increasing enzymic activity by attracting, binding and orienting cationic substrates (4). It has been noted that successive N-methylation of alkylammonium ions, starting with methylamine and ethanolamine, improves binding, and N-methylation of substrates starting with β-aminoethyl acetate increases both binding and reactivity, and the N-methyl groups contribute more to binding than does coulombic attraction (4-6). Thus, it has been proposed that less than 10% of the binding of tetraalkylammonium ions is due to their charge and that there is no negative charge in the "anionic" binding site (7). However, this calculation did not take into account the decrease in solvation of ammonium ions with increasing N-methylation (5), and the presence of a negative charge in the "anionic site" remained widely accepted (8).

Addition of methyl substituents in uncharged analogues of the β-ammonioethyl acetate, from ethyl propionate to 3,3-dimethylbutyl acetate, $(CH_3)_3CCH_2CH_2OCOCH_3$ (DMBAc), the carbon analogue of AcCh, also led to progressively increasing reactivity toward AcChE (1,9). Thus the rate constant for reaction of DMBAc with AcChE is very high, $2 \times 10^6\ M^{-1}sec^{-1}$ (10), though somewhat lower than that for AcCh.

The β-ammonio substituents increase the intrinsic hydrolytic reactivity of such esters toward hydrolysis by hydroxide, as compared with the uncharged carbon analogues. We have deemed it appropriate to apply normalization factors to the enzymic hydrolysis rates, as has been done in studies of chymotrypsin, which also reacts via an acyl-serine intermediate (11). This was done for a series of 14 β-substituted ethyl acetates, $X\text{-}CH_2CH_2OCOCH_3$, with X = the four ammonio groups, H_3N^+ to $(CH_3)_3N^+$, the analogous carbon-centered substituents, and CH_3O, HO, Cl, Br, N≡C and H (Table I) (12). The normalization factors were the ratios of the base-catalyzed hydrolysis rate constant for AcCh to the corresponding rate constants for each of the substrates. We then found that the normalized enzymic reactivities [$\log(k_{2(n)}/K_s)$, where $k_{2(n)}$ is the normalized acylation rate constant and K_s is the binding constant] for these compounds, were proportional to the apparent molal volumes ($\bar{V}^{\circ}_{25}$) of the β-substituents, X (Eq. 1).

$$\log(k_{2(n)}/K_s) = a\,\bar{V}^{\circ}_{25} + C \qquad (1)$$

The normalization factor applies to the acylation rate constant, k_2. Since the reaction rate may be described as either k_2/K_s or k_{cat}/K_m, we have also applied the factor to the latter ratio.

The enzymic reactivity of these acetate esters of widely varied structure was accounted for by two factors: (i) their intrinsic hydroxide-catalyzed reactivity and (ii) the volume of the β-substituent and thus its fit into the active site, and

the resulting effect on placing the ester group at the esteratic site. K_s values for AcCh and DMBAc are similar, indicating no substantial coulombic effect, and the effects of charge on binding in the other cationic and uncharged pairs of substrates appeared even smaller (Table I).

The effect of the positive charge in AcCh is to increase the rate of its hydroxide-catalyzed hydrolysis over that of DMBAc by a factor of about 25 (2.8/0.11 from Table I), and the normalized reactivities are quite similar. No specific effect of anionic charge on the hydrolysis rate and thus no evidence for an "anionic" site is observed. On this basis we propose that the part of the active site at which the trimethylammonio group of AcCh and the β-substituent of other acetate substrates bind is not anionic, and is more accurately considered "trimethyl," complementary to this character of the β-substituent of AcCh rather than to its positive charge.

Others have noted a relation between enzymic reactivity and the hydrophobicity (Π) of the β-substituents of alkyl esters II, IV, VI and VIII (Table I) (10). Hydrophobicity is a parameter derived from 2-octanol/water distribution coefficients; it is related to capacity for hydrophobic interactions and is used in drug design (13). For these hydrocarbon substituents, values of Π are proportional to volume, but use of this parameter, Π, does not allow the non-polar and the water-soluble ammonio derivatives to be correlated on the same scale. The correlation with volume does allow the reactivity of both classes of substrates to be so correlated.

We then examined the effect of positive charge on binding of reversible inhibitors (Table II) (14). The enzyme has isoelectric point of about pH 5 (15,16) and thus excess negative charge on its surface at pH 7-8. Cationic, reversible inhibitors structurally related to AcCh do bind better than their uncharged analogues by small factors (Table II), corresponding to about 1 kcal/mole of binding energy, much less than would be caused by interaction of $(CH_3)_3N^+$ with a "contact" anionic O^- that is implied by a specific anionic site (14). Also, ionic strength effects on binding and on hydrolysis have been interpreted in terms of anionic charges on the enzyme surface, peripheral to the active site (17).

Each of the inhibitors which we studied, structurally related to AcCh, whether neutral or cationic, showed essentially the same binding constant when retarding hydrolysis of AcCh and its uncharged analogue, DMBAc. This indicated that the β-trimethylammonio and β-<u>tert</u>-butyl groups of the two substrates and of the related inhibitors bound at the same subsite (14). The substrate study had indicated that this subsite is uncharged trimethyl, and the nature of this site could then be explored more specifically with uncharged reagents (12). Indeed, arylaziridinium reagents, intended to alkylate the "anionic" site

and prevent substrate access, completely inhibited hydrolysis only of cationic, but not of neutral esters (18,19). We took this to indicate, not that there are separate anionic and neutral subsites, but that cationic, irreversible inhibitors may react with peripheral anionic groups, increasing positive charge and repelling cationic substrates. They modify the active site domain but allow uncharged substrates to bind at the one trimethyl site and react, albeit at reduced rate.

This view was borne out in studies with BrPin (20). This uncharged reagent inhibited AcChE irreversibly. The same rate for inhibition of hydrolysis was observed with a variety of both cationic and neutral substrates, and its inhibiting reaction was retarded by reversible quaternary and trimethylammonio inhibitors to an extent appropriate to their binding constants. These results indicate that both classes of compounds bind at a single trimethyl site (20). BrPin is being used to label the active site.

The study of hydrolysis by AcChE of β-substituted ethyl acetates, $X\text{-}CH_2CH_2OCOCH_3$, was extended to compounds with β-substituents of more varied structure -- to a larger substituent, $X = (CH_3)_3Si$, and to substituents with surface oxygen or sulfur, $X = CH_3SO_2$, $(CH_3)_2N^+O^-$, CH_3SO, CH_3S, and O_2N. The kinetic results (Table III) were obtained prior to the contract of this report. The interpretation and preparation of the manuscript for publication were carried out during this period, and the results of the study are described in Section (5)B.

Cationic aromatic compounds have long been known to affect AcChE. These include the carbamylating toxic alkaloid, physostigmine (21), the related synthetic reagent, neostigmine (22), and the reversible inhibitors phenyltrimethylammonium ion (PTA) (K_i = 0.08 mM) (23) and 3-trimethylammoniophenol (TMAP) (K_i = 0.0003 mM) (24). It seemed of interest to us, by analogy with our studies of uncharged aliphatic substrates and inhibitors, to study substituent effects in uncharged aromatic compounds. Such studies have been reported in some inhibitions of AcChE and the Hammett equation, $\log k/k_o = \sigma\rho$, was applied. This equation has been used to correlate the effects of polar substituents on the rates and equilibria of many reactions of aromatic compounds. Each substituent is characterized by a value, σ, a measure of its electron-withdrawing or donating property, a positive value indicating electron-withdrawal, negative value indicating electron-donation, and higher absolute values corresponding to greater effects. Each reaction is characterized by a value, ρ, that for the ionization of benzoic acids having value 1, positive values indicating processes increased by electron-withdrawal, and negative values for processes increased by electron-donation. For a limited series of phenyl N-methylcarbamates, a Hammett plot led to negative ρ (25); in substituted-benzyl N-methylcarbamates, in contrast, the nitro group led to strong binding (26); in

substituted-phenyl phosphates, positive was observed and attributed to leaving group activity (27); and in hydrolysis of substituted-phenyl acetates, greatest binding and reactivity were observed at σ value of about 0 (28). Since substituent effects in these multi-step reactions are complex, we have examined systematically reversible inhibition by substituted benzenes and phenols. We find three effects which determine binding energies of a wide range of such inhibitors.
(i) Electron-attracting substituents greatly increase binding, for example, for a series of 11 compounds, from $K_{i(com)}$ = 50 mM for phenol and aniline to 0.46 mM for 4-nitroacetanilide;
(ii) dimethylamino and tert-butyl substituents increase binding by interaction at the trimethyl subsite; and (iii) this latter interaction increases binding synergistically in meta-substituted phenols. The strong binding constants of PTA and TMAP are accurately accounted for and may be calculated from parameters determined in the binding of uncharged compounds (29).

(3) Rationale for the Current Study

(i) BrPin has properties of an active-site-directed inactivator, being equally effective against cationic, uncharged and aromatic substrates, and its own action is retarded by substrate-related reversible inhibitors (20). It is being used, as [^{14}C]-BrPin, as a radioactive label for the active site. The enzyme is isolated from Torpedo nobiliana, inactivated by [^{14}C]-BrPin and cleaved into fragments which will be characterized and sequenced.

(ii) Substituents other than $(CH_3)_3C$ and $(CH_3)_3N^+$ bind effectively at the trimethyl subsite, i.e., $CH_3S(O_2)$ and Cl_3C. Aliphatic compounds and benzene derivatives containing such substituents are considered as substrates, reversible inhibitors, and labeling agents and as possible reactivating reagents.

(iii) Study of substituted benzenes and pyridines indicates that they may be effective reversible inhibitors in the absence of permanent positive charge. This may impart superior permeating properties, combined in the pyridines with increased water solubility. Preparation of uncharged benzene- and pyridine-derived labeling and reactivating reagents will be explored.

(4) Experimental Methods

Details are to be found in the 1985, 1986, and 1987 Annual Reports (30-32), which describe the source of electric organ of T. nobiliana and [^{3}H]-DFP; preparation of the affinity ligand

and column, and purification of Torpedo AcChE by affinity chromatography; equipment and procedure for PAGE, and application to impure Sigma eel AcChE, and purified Torpedo AcChE; kinetic procedures for study of reversible inhibitors and irreversible inactivators; and preparation of [^{14}C]-BrPin and its analysis by gas-liquid chromatography. Literature references are given in those reports for these procedures, for the Ellman assay, for pH stat kinetic studies, and for k_{cat} of T. californica.

Sources of substrates and inhibitors with varied β-substituents and references for or details of their synthesis are described in the Annual Reports (30-32) and in our publications (12,14,33,34). Sources and preparation of benzene derivatives studied as reversible inhibitors are described in Reference 29. Sources of pyridine derivatives studied as inhibitors are indicated in Table VII. Sources, preparation, or references for preparation of irreversible inhibitors, chloromethyl pivalate, chloromethyl acetate, trimethylammoniomethyl acetate, tert-butyl peracetate, methyl methanesulfonate, methyl benzenesulfonate, methylsulfonyl chloride, and styrene oxide are given in the 1985 Annual Report (30).

Details of the synthesis of [^{14}C]-BrPin are described in the 1986 and 1987 Annual Reports (31,32). Freshly distilled $CH_3{}^{14}COCl$ is required, and Cu_2Cl_2 catalysis is required for the reaction of $CH_3{}^{14}COCl$ with $(CH_3)_3CMgCl$. Traces of oxygen cause this reaction to fail, and traces of moisture cause the bromination of $(CH_3)_3C^{14}COCH_3$ to fail. A small excess of CH_3COCl is tolerated but a large excess and any excess of Grignard reagent are not, and a mixture of pinacolone and mono- and dibromopinacolones, suitable for labeling studies, is obtained.

(5) Results

A. Preparation of 1-Bromo-[2-^{14}C]-pinacolone, [^{14}C]-BrPin.

A procedure has been worked out for this synthesis on a 60-μmole, 1-mCi scale, in 120 μL of ether. In the process, many experiments were carried out, and difficulties were encountered and resolved. The procedure involves reaction of tert-butylmagnesium chloride with [1-^{14}C]-acetyl chloride in the presence of cuprous chloride catalyst, leading to pinacolone, followed by bromination, washing and drying.

$$(CH_3)_3CMgCl + CH_3{}^{14}COCl \xrightarrow{Cu_2Cl_2} CH_3{}^{14}COC(CH_3)_3 + MgCl_2$$

$$\xrightarrow{Br_2} BrCH_2{}^{14}COC(CH_3)_3 + HBr \qquad (2)$$

If a deficiency of acetyl chloride is used, the bromination fails. If a large excess (about 20%) of acetyl chloride is used, a by-product is formed of unknown structure which inactivates the enzyme instantaneously. In practice, a small excess (about 3%) of acetyl chloride is used. Air and moisture must be rigorously excluded during the reaction as both initial components are destroyed by moisture and the Grignard reagent is sensitive to oxygen, the presence of which leads to tert-butyl acetate. Bromine and cupric ion inactivate the enzyme instantaneously and must be washed out completely.

This synthesis leads to a mixture of [^{14}C]-pinacolone, [^{14}C]-BrPin and 1,1-dibromo-2-[^{14}C]-pinacolone ([^{14}C]-Br_2Pin) in ratio of 1:2:1. The yield of BrPin is relatively insensitive to the amount of bromine used; less than one equivalent leads to less conversion of pinacolone to BrPin, more than one equivalent to more conversion of BrPin to Br_2Pin, with the yield of BrPin relatively unchanged. Pinacolone is not an irreversible inhibitor and Br_2Pin caused no inactivation in the concentration present in the product mixture. Since BrPin is a reactive material and [^{14}C]-BrPin shows some instability, and the preparation was carried out on a small scale, 60 μmole, it was preferable not to attempt a separation of the components and to use the synthesized mixture. Inactivation by the mixture was compared with that by the same concentration of purchased BrPin and no significant difference was found. The mixture was used promptly in the labeling study.

B. Substrates

In an extension of the study of the range of applicability of Eq. 1, the relation of enzymic reactivity to volume of β-substituent, an alternative measure of volume, refraction volume, MR (35-38), which is available for a broader range of structures, was substituted for apparent molal volume $\bar{V}^{*}_{25}$. Results are summarized in Table III and Figure 1. Log of normalized enzyme reactivity for the ethyl acetates, X-CH_2CH_2O-$COCH_3$, (X = H, Cl, Br, CH_3CH_2, $(CH_3)_2CH$, $(CH_3)_2S^+$, $(CH_3)_3N^+$, and $(CH_3)_3C$ rose linearly with refraction volume; slope = 0.117 cm^{-3}, intercept = 5.53, and correlation coefficient, r, = 0.97. The increase in normalized enzymic reactivity with volume leveled off with AcCh and DMBAc. The larger analogous silyl compound, $(CH_3)_3SiCH_2CH_2OCOCH_3$, was accommodated in the active site with no substantial increase in reactivity. The methylsulfonyl, CH_3SO_2, dimethylamine oxide, $(CH_3)_2N^+(O^-)$, methylsulfoxy, $CH_3S(O)$, and methylthio, CH_3S, substituents led to substantially lower reactivity than is consistent with MR values. The first three of these show the greatest deviations from the correlation with MR, by factors of 40, 20, and 10, respectively. While volume is the major parameter affecting reactivity, it may set an upper limit achieved when the surface is hydrophobic, as with hydrocarbon and halogen substituents;

and not achieved with dipolar X-O surface groups. These may retain water in the active site and reduce reactivity (33). This concept of surface hydrophobicity and hydrophilicity is to be distinguished from hydrophobicity (Π) related to water solubility and derived from 2-octanol - water distribution. In correlation with hydrophobicity (Π), a structural parameter widely used in drug design (35,39-41), these substituents (except for methylthio), and the important cationic substituents $(CH_3)_3N^+$ and $(CH_3)_2S^+$, showed substantially greater reactivity than is consistent with Π values (Figure 2). Essentially only the hydrocarbon and halogen substituents lead to correlation with Π values, correlation coefficient = 0.97, and even this we attribute to these values being proportional to volume. Thus volume, with which reactivity due to both these non-polar substituents and the important cationic substituents, $(CH_3)_3N^+$ and $(CH_3)_2S^+$, is correlated, is the relevant structural parameter, affected by surface hydrophobicity. It is noteworthy that binding of the hydrophilic CH_3SO_2 substrate, Compound IV (Table III), is similar to that of the hydrophobic $(CH_3)_3C$ substrate, Compound IIA, K_m = about 6 mM, with substantially lower k_{cat} and greatly lower normalized reactivity (33). Hydrolysis of the CH_3SO_2 substrate is retarded efficiently by TAP, the β-substituent apparently utilizing the trimethyl subsite.

C. Reversible Inhibitors

Varied β-Substituents. In further study of the effects of charge, volume and surface properties on inhibitor binding at the trimethyl site, supplementing our work on substrates (33), a series of related reversible inhibitors of AcChE was tested (34). The results are summarized in Table IV and as follows:

Sterically similar alcohols with tetra-substituted uncharged β-groups, $(CH_3)_3SiCH_2CH_2OH$ (I), $(CH_3)_3CCH_2CH_2OH$ (IA) and $CH_3S(O_2)CH_2CH_2OH$ (VII), bind similarly, K_i = 3-9 mM, and the binding of each is similar to that of the corresponding acetate substrate. Cationic analogues, $(CH_3)_3N^+CH_2CH_2OH$ (IB) and $(CH_3)_2S^+CH_2CH_2OH$ (II), also bind in a similar manner, K_i = 0.4 mM, similar to the K_m values of their acetate substrates, and they bind more strongly than the uncharged alcohols by about 1.5 kcal/mole.

Although the $-COCH_3$ group in esters with strongly binding β-substituents makes essentially no contribution to binding over that of the alcohols, in esters with weakly bound β-substituents, $(CH_3)_2N^+(O^-)$, $CH_3N^+H_2$, $CH_3S(O)$, CH_3CH_2, and CH_3S, binding is dominated by the ester $-COCH_3$ group; these esters have K_m values about 16 mM (Tables I, II, and III).

In comparisons of VII, $CH_3S(O_2)CH_2CH_2OH$, with $CH_3SO_2CH_3$, II, $(CH_3)_2S^+CH_2CH_2OH$, with $(CH_3)_3S^+$, and IB, choline, with

$(CH_3)_4N^+$, hydroxyethyl leads to more favorable binding than methyl by about 0.8 kcal/mole, despite lower hydrophobicity. Two hydrophobic methyl groups (comparison of IA with n-butanol) and two hydrophilic sulfone O atoms, (comparison of VII with 2-methylthioethanol) increase binding similarly, by 1.0 kcal/mole.

In comparison of VIII, $(CH_3)_2SO_2$, with IV, $(CH_3)_3S^+O$, replacement of O by CH_3 and introduction of a positive charge increases binding by 1.8 kcal/ mole. Conversion of $(CH_3)_3S^+$ to $(CH_3)_3SO$ has only a small effect on binding. However, $(CH_3)_3N^+O^-$ does not bind to AcChE, and conversion of 1-dimethylammonio-4-pentanone and 2-dimethylammonioethyl acetate to their N-oxides, changes of $\geqslant N^+H$ to $\geqslant N^+(O^-)$, decrease binding by 1.5 kcal/mole. The oxide, $(CH_3)_3S^+O$ is a monocation, like $(CH_3)_3S^+$, as S expands its octet. There is no decrease in positive charge and the improved binding, about 0.2 kcal/mole, may arise from increase in volume. In contrast, conversion or $\geqslant N^+H$ to $\geqslant N^+O^-$ decreases positive charge and leads to a strongly hydrophilic dipolar bond, decreasing binding.

Although methylsulfonylethanol and its acetate bind to AcChE similarly to the analogous <u>tert</u>-butyl compounds, 1,3-bis-methylsulfonylpropane ($CH_3SO_2CH_2CH_2CH_2SO_2CH_3$), which is soluble only to about 1 mM, does not affect reactivity of AcChE. It seemed possible that the sulfone group might bind at both the trimethyl and acetyl (esteratic) subsites, but this does not appear to occur. Similarly, the bis-methylsulfinyl compound $CH_3S(O)CH_2CH_2CH_2S(O)CH_3$ is a weak inhibitor; at 10 mM it decreases activity by 30%.

Trichloroethanol (XIII), Cl_3CCH_2OH (Aldrich), $K_{i(com)}$ = 42 mM, $K_{i(nonc)}$ = 110 mM, inhibits similarly to its trimethyl analogue, neopentyl alcohol (XII), $(CH_3)_3CCH_2OH$ (Aldrich), $K_{i(com)}$ = 29 mM, $K_{i(nonc)}$ = 74 mM, and to the ether isomer of the latter, <u>tert</u>-butyl methyl ether (XIV), $(CH_3)_3COCH_3$ (Aldrich), $K_{i(com)}$ = 25 mM, $K_{i(nonc)}$ = 24 mM.

Chloral hydrate (XVI), $Cl_3CCH{=}O{\cdot}H_2O$ (Aldrich), is an effective competitive reversible inhibitor, K_i = 1 mM, binding substantially more strongly than its trimethyl analogue, pivalaldehyde (XV), $(CH_3)_3CCH{=}O$ (Aldrich), K_i = 10 mM.

<u>Benzenes and Phenols</u>. To characterize the nature of reversible binding to AcChE of benzene derivatives, including the potent inhibitor TMAP, we have carried out a systematic study of effects of substituents on the inhibition by such compounds of the enzymic hydrolysis of AcCh.

The Hammett σ values ordinarily reflect effects of substituents on side-chain reactions. In the present case, effects

on electron-density in the benzene ring by both induction and resonance, in turn affecting charge-transfer interaction of the benzene ring are involved. Effects of one substituent on reactions of another, whether in para or ortho relation, are not involved. While substituents might affect the capacity of each other to interact with the ring, this does not seem to be important. Examination of meta and para σ values indicated that para values more truly include resonance effects and these were used. The results are summarized in Tables V and VI and Figures 3 and 4.

In Figure 3, a linear relation between competitive binding, $\log(1/K_{i(com)})$, and substituent σ_{para} values is observed for C_6H_5-Y (Y = HO, H_2N, H, CH_3CONH, CH_3CO, and O_2N) and for the disubstituted compounds, p-H_2N - $COCH_3$, m-H_2N - NO_2, p-HO - NO_2, m-HO - NO_2 and p-O_2N - $NHCOCH_3$, with $\rho = +1.84$ and the correlation coefficient, r, = 0.99; $K_{i(com)}$ decreases to 0.63 mM for nitrobenzene and 0.46 mM for p-nitroacetanilide. Direct resonance interaction in 4-nitroaniline may lead to increased interaction beyond that consistent with the above relationship. The reason for the increased binding of 3-nitroacetanilide is not clear.

Dimethylamino and trimethylammonio compounds, with substituents $(CH_3)_2N$, p-$(CH_3)_2N$ - $COCH_3$, m-$(CH_3)_2$ - NO_2, p-$(CH_3)_2N$ - NO_2 and $(CH_3)_3N^+$, bind more strongly relative to σ values, with $\rho = +2.4$ and r = 0.99; $K_{i(com)}$ decreases to 0.09 mM for p-nitrodimethylaniline and PTA. The observed linear relation of binding energies to substituent σ values indicates that the binding of these benzene derivatives is determined by electron withdrawal by the substituent, and arises from a polar interaction of the benzene ring, probably with an amino acid side chain, in an aryl binding site. This binding is increased by the binding of a $(CH_3)_nX$ substituent in the trimethyl site, contiguous with the aryl site.

The hydroxyl group decreases binding in phenol and the nitrophenols, in accord with its negative σ value, -0.18. However, consistent with the effect of $(CH_3)_nX$ groups in the benzenes, binding of phenols increases linearly with the volume of meta substituents in the order H < CH_3 < C_2H_5 < $(CH_3)_2CH$ = $(CH_3)_2N$ < $(CH_3)_3C$, r = 0.99, with K_i values decreasing from 50 mM for phenol to 0.11 mM for 3-<u>tert</u>-butylphenol (Table I, Figure 4). This large effect is attributed to synergistic binding of the meta substituent in the trimethyl site and hydrogen-bonding of the phenolic hydroxyl. The very strong binding of TMAP ($K_{i(com)}$ = 0.00033 mM) is accurately calculated from the K_i of 3-<u>tert</u>-butylphenol, ρ, as determined for the benzenes (above), and the difference between the σ values of <u>tert</u>-butyl and trimethylammonio, +0.94. The synergism of $(CH_3)_3X$ and hydroxyl is the same in the <u>tert</u>-butyl- and trimethylammonio-phenols (Figure 3). As in PTA, the positive charge in TMAP has no effect beyond that reflected in its σ

value, i.e., in electron withdrawal and in the increase of the polar interaction of the aromatic ring. It is noteworthy that 4-tert-butylphenol, 1.5 mM, essentially at the limit of its solubility, has no inhibitory effect; thus, with $K_i \geq 20$ mM, this isomer shows no synergism, indicating a specific steric relation of the trimethyl, aryl, and hydrogen-bonding subsites. A similar order of binding is found in the isomeric trimethylammoniophenols, for which no synergism is found in the 4-isomer (24); this binding order further supports the view that the trimethyl and "anionic" binding sites are identical.

Nitrobenzene, an effective, reversible inhibitor of hydrolysis of AcCh and also of the uncharged analogue, DMBAc (K_i = 0.8 mM), appears to be slightly less effective in hydrolysis of n-butyl acetate, (K_i = 2.1 mM) and accelerates hydrolysis of 0.1 M ethyl acetate; nitrobenzene at 1 mM doubles the rate, and at 2 and 3 mM increases it four-fold. Acetophenone, 7 mM, increases the rate three-fold. These accelerations are comparable to those caused by small cations (42), but the aromatic compounds do not bind at the trimethyl site, since their binding is increased by tert-butyl and dimethylamino substituents which use that site.

Pyridines. The existence of an aromatic binding site as an integral part of the active site, and the effective binding of uncharged benzene derivatives led us to a study of derivatives of pyridine as materials with potentially useful properties. Some results are summarized in Table VII. Non-polar substituents 4-tert-butyl, 2- and 4-vinyl and 2-oxiryl, with $K_{i(com)}$ values of 1-4 mM, improve binding over that of pyridine by about 1 kcal/mole, possibly by interaction at the trimethyl site. The increase due to the acetyl substituent, about 1 kcal/mole, may arise from its electronegativity, and is similar to that in the acetophenone-benzene pair. Dimethylaminopyridines, interacting with the trimethyl site, bind strongly, by 3 kcal/mole more than pyridine, and by 1.5 to 2 kcal/mole more than aminopyridines. Remarkably, they bind as well as the permanently cationic N-methylpyridinium ion.

D. Survey of Irreversible Inhibitors

Earlier studies had indicated that BrPin would be an active site inactivator (20). While the synthesis of [^{14}C]-BrPin was being developed, other reagents were examined briefly. Study of reversible aromatic inhibitors indicated that irreversible aromatic inhibitors might also be found. The effects of such irreversible and reversible inhibitors on AcChE binding of [^{3}H]-DFP were also surveyed (see Section E).

Potential Suicide Inhibitors. Chloromethyl acetate, chloromethyl pivalate, trimethylammoniomethyl acetate, and

tert-butyl peracetate were examined. They are potential suicide inhibitors, substrates acting as alkylating agents or hydrolyzing to form formaldehyde or a peroxide in the active site (Table VIII):

Chloromethyl acetate, $CH_3CO_2CH_2Cl$, was synthesized from formaldehyde and acetyl chloride. At 4.4 mM it led to 90% inactivation after 4 hr when the incubation solution was allowed to become acidic (pH 5), and to 70% inactivation when pH was maintained at pH 7.8. The increased inactivation by the chloromethyl esters at acidic pH may be due to enzyme instability, but acid-catalysis of alkylation by α-haloacyl compounds is also possible. This and related effects in inactivation by BrPin require further study. As a substrate it showed k_{cat}/K_m about 10^4 sec^{-1}/16 mM = 6 x 10^5 $M^{-1}sec^{-1}$, or 4 x 10^5 $M^{-1}sec^{-1}$ when normalized for its intrinsic hydrolytic reactivity; $k_{(OH^-)}$ = 4.4 $M^{-1}sec^{-1}$.

Chloromethyl pivalate, $(CH_3)_3CCOOCH_2Cl$ (Aldrich), 3.5 mM, which is structurally analogous to BrPin, led to complete inactivation after 3 hr when the incubating solution was allowed to become acidic (pH 5); 50% inactivation was observed when pH was maintained at 7.8. This compound, examined as a substrate, appeared to hydrolyze slowly.

No reactivation occurred in either case on standing or on treatment with 2-methylpyridinium aldoxime (PAM).

Trimethylammoniomethyl acetate, $(CH_3)_3N^+CH_2OCOCH_3$, examined as a reversible inhibitor, showed mixed inhibition, $K_{i(com)}$ = 1.4 mM, $K_{i(nonc)}$ = 4.8 mM. As a substrate it had k_{cat}/K_m = 3.9 x 10^3 sec^{-1}/0.8 mM = 4.5 x 10^6 $M^{-1}sec^{-1}$, 3.8 x 10^6 $M^{-1}sec^{-1}$, when normalized for intrinsic reactivity; $K_{(OH^-)}$ = 3.3 $M^{-1}sec^{-1}$. After hydrolysis of this compound, initially present at 3 mM, reactivity of the enzyme was reduced about 30%.

tert-Butyl peracetate, $(CH_3)_3COOCOCH_3$ (Pfaltz and Bauer), is hydrolyzed by AcChE, k_{cat}/K_m = 3.4 x 10^3 sec^{-1}/2.8 mM = 1.2 x 10^6 $M^{-1}sec^{-1}$, or 1.7 x 10^6 $M^{-1}sec^{-1}$ when normalized for its intrinsic reactivity; $k_{(OH-)}$ = 2.0 $M^{-1}sec^{-1}$. The measured enzymic activity with this substrate was greater than that with its carbon non-peroxidic analogue neopentyl acetate, $(CH_3)_3C$-CH_2OCOCH_3, k_{cat}/K_m = 1.9 x 10^2 sec^{-1}/8.6 mM = 2.2 x 10^4 $M^{-1}sec^{-1}$, but normalized activities were similar, 8.8 x 10^5 $M^{-1}sec^{-1}$ for the neopentyl compound. Enzymic hydrolysis (4 nM enzyme) of 3 mM peracetate was complete in 1 hr, and enzymic reactivity was decreased about 30%. Further additions of two portions of 3 mM peracetate led to 50 and 30% residual activity. Similar treatments with 2 mM tert-butyl hydroperoxide (Aldrich) led to smaller decreases in reactivity, 10, 15 and 25%.

Alkylating and Acylating Inactivators. Other potential active-site-directed alkylating agents examined were methyl methanesulfonate, methyl benzenesulfonate, phenacyl bromide, phenacyl chloride, epoxides of styrene and vinylpyridine, and a sulfonylating agent, methylsulfonyl chloride (Table IX):

Phenacyl bromide, $C_6H_5COCH_2Br$, is an irreversible inhibitor and potential labeling agent at the aryl binding site. At 3 mM, it led to 21% inactivation in 2 hr, 32% in 3 hr, 55% in 4.2 hr, 73% in 5.25 hr and complete inactivation when incubated overnight. Phenacyl chloride (3 mM) was less effective, leading to a linear decrease in activity, 30% in 6.5 hr, then to a slower decrease, yielding 45% residual activity after 25 hr. Reaction of the chloride with the enzyme at this concentration may be too slow to compete with its hydrolysis.

Styrene oxide, a weak reversible inhibitor (Table V), is a moderately effective irreversible inactivator and also has potential as a labeling agent at the aryl site. At 3 mM, it caused progressive inactivation, 21, 46, 69 and about 100% inactivation after incubation for 1.0, 3.0, 4.5, and 22 hr, respectively. Inactivation by styrene oxide is retarded by TAP.

2-Pyridyloxirane, $K_{i(com)}$ = 1.5 mM (Table VII), binds more strongly than its carbon analogue, above, but does not appear to be an irreversible inhibitor. At 3 mM for 23 hr, pH 7.8, room temperature, it led to no inactivation.

tert-Butyl isothiocyanate and trichloromethylethylene oxide are not effective irreversible inhibitors.

Methyl benzenesulfonate (MBS), $C_6H_5SO_2OCH_3$ (Pfaltz and Bauer), showed mixed reversible inhibition, $K_{i(com)}$ = 6 mM, $K_{i(nonc)}$ = 21 mM, and irreversible inactivation. At 3 mM, there was 65% inactivation in 1 hr and 95% in 2 hr, largely unaffected by 30 mM tetramethylammonium ion. The active site accommodates MBS and $(CH_3)_4N^+$ simultaneously. At 0.6 mM MBS there was no inactivation after 1 hr, 15% after 3.3 hr, 33% after 5 hr and 93% after 24 hr, with similar effects on hydrolysis of both AcCh and DMBAc. Inactivation by MBS is retarded by TAP.

Methyl methanesulfonate (MMS), $CH_3SO_2OCH_3$, (Aldrich), at 9 mM, inactivated AcChE rapidly, 40% in 1.6 hr, 100 % in 4 hr, with respect to hydrolysis of both AcCh and DMBAc. At 1 mM MMS, no inhibition was observed for 4 hr, then 50% inactivation was found after 6 hr. At 3 mM, there was 30% inactivation in 1 hr and 70% in 2 hr. Tetramethylammonium ion afforded complete protection for 1 hr and then inactivation proceeded. As a reversible inhibitor, MMS acted purely non-competitively, K_i = 96 mM.

Methylsulfonyl chloride (MSC), CH_3SO_2Cl (Aldrich), at 3 mM, inactivated AcChE completely in 10 min; at 0.3 mM, it inactivated 54% in 10 min, and inactivation was complete after 19 hr. At 0.03 mM, there was 38% inactivation after 10 min, 72% after 14 hr. MSC appears as active as methylsulfonyl fluoride (MSF), CH_3SO_2F.

3-Phenyl-3-trifluoromethyldiazirine and 3-(m-iodophenyl)-3-trifluoromethyldiazirine are being prepared as potential photo-inactivating agents.

E. Effects of Inhibitors on Incorporation of [^{3}H]-DFP

Since the uncharged inactivators are novel, a reported (19) cationic inhibitor, N,N-dimethyl-2-phenylaziridinium (DPA), was first examined. Incorporation of [^{3}H]-DFP was determined after 4 days of dialysis against 2 L of water, with 3 changes. [^{3}H]-DFP alone showed about 7% retention of radioactivity in the dialysis tube as compared with that present in Sigma enzyme fully inactivated by [^{3}H]-DFP. Sigma AcChE, 47% and 100% preinactivated by DPA, then treated with [^{3}H]-DFP, showed 48% and 7% of the counts in enzyme fully inactivated by [^{3}H]-DFP. Torpedo AcChE, 97% inactivated by DPA, showed 13% of the counts in enzyme inactivated by [^{3}H]-DFP alone. Inactivation by DPA largely prevents incorporation of [^{3}H]-DFP in both enzymes.

Sigma AcChE and Torpedo AcChE, 100% inactivated by styrene oxide, and then treated with [^{3}H]-DFP, appeared to incorporate counts equivalent to 22% of those due to 100% inactivation by [^{3}H]-DFP. This inhibitor introduces a hydroxyl near the active site, which may react slowly with DFP.

Sigma AcChE, inactivated 83% and 100% by MBS, and then treated with [^{3}H]-DFP, appeared to incorporate counts equivalent to 35% and 18% of those due to 100% inactivation by [^{3}H]-DFP. Torpedo AcChE, 79% inactivated by MBS, appeared to incorporate counts equivalent to 14% of those due to 100% inactivation by [^{3}H]-DFP. Inactivation by these inhibitors appears to exclude subsequent incorporation of [^{3}H]-DFP. Greater accuracy is desirable.

F. Action of BrPin and [^{14}C]-BrPin

Effect of pH on Inactivation by BrPin. Inactivation of Torpedo AcChE by BrPin was studied as a function of pH over the range 7.5 - 4.6. The second-order rate constant, k_2/K_i (where k_2 is the unimolecular rate constant for the inactivating reaction of enzyme-inhibitor complex, and K_i is the binding constant) increases, in the form of a sigmoid, with decreasing pH, crossing an ionization of pK_a of 6.0. The values of k_2 are (i) 5.2×10^{-4} sec^{-1} at pH 7.0 and (ii) 240×10^{-4} sec^{-1} at pH

5.4, with K_i = 0.14 mM at both values of pH. The reaction of a nucleophile with BrPin is acid-catalyzed. This may involve protonation or hydrogen-bonding by a carboxyl to the carbonyl oxygen of BrPin, facilitating attack of a nucleophile on the α-carbon. The alkylated product is stable to treatment with nucleophiles in the gel electrophoresis experiments and thus may not be an α-ketoester. The latter might be formed as an intermediate which transfers the alkyl group to another nucleophile, i.e., an imidazole of histidine. It may be noted that chloromethyl pivalate and acetate, above, also inactivated more rapidly when the solution became acidic.

Protection by TAP and PTA was examined for evidence as to whether inactivation at pH 5.4 is specific. No protection against 0.2 mM BrPin was found for these reversible inhibitors when they were used at their pH 7.8 K_i values, 0.02 and 0.08 mM, respectively, or at 10 times their pH 7.8 K_i values. TAP at 2.0 mM, 100 times its K_i at pH 7.8, decreased the initial rate of inactivation at pH 5.4 by 0.2 mM BrPin to about 10% of the unprotected rate; PTA at 40 times its K_i at pH 7.8 decreased the initial rate of inactivation to about 25% of the unprotected rate. The higher rate of inactivation by BrPin at pH 5.4 and possibly higher K_i values of the cationic reversible inhibitors at low pH may lead to their lower protecting effectiveness. Evidence of specificity is found in the effects at high concentration of the reversible inhibitors.

K_i values for these reversible inhibitors were then determined at pH 5.4 by a modification of the Ellman procedure. Hydrolysis of acetylthiocholine was allowed to proceed for 2 min, at the low pH in the presence of varying concentration of the inhibitors, 1.0 to 9.0 mM TAP or 0.125 to 1.0 mM PTA. Aliquots were added to the Ellman reagent at high pH, and the single rate values at varying inhibitor concentrations were determined. The value for K_i for TAP at pH 5.4 was 1.4 mM as compared with 0.02 mM at pH 7.8. The value for the aromatic cationic inhibitor, PTA, was 0.04 mM, similar to that at pH 7.8. This is consistent with our previous observation that the effect of the cationic substituent in the aromatic compound is accounted for by its value, i.e. its effect on binding of the aromatic nucleus, and by trimethyl effect, with no effect due to coulombic interaction. Further, this result indicates that this interaction is not with a carboxylate anion donor, which would be protonated at low pH, and probably is with an aromatic side chain.

<u>BrPin and [^{3}H]-DFP</u>. <u>Torpedo</u> AcChE, when dilute (about 10^{-8} M), is very sensitive to BrPin; 0.2 mM [^{14}C]-BrPin inactivates this enzyme with $t_{1/2}$ of about 10 min. In contrast, for Sigma AcChE, $t_{1/2}$ is about 5 hr at 0.6 mM. At higher concentrations, about 10^{-6} M, <u>Torpedo</u> AcChE is more resistant to BrPin. It may be that the additives (protein in character) in Sigma AcChE, apparently used to stabilize the enzyme in

storage, also protect against BrPin, and possibly against other inhibitors.

In four runs, Sigma AcChE that was 100% inactivated by BrPin, and then treated with [^{3}H]-DFP incorporated counts equivalent to 15 ± 2% of those due to 100% inactivation by [^{3}H]-DFP alone. In two runs, Torpedo AcChE that was 100% inactivated by BrPin incorporated 8% and 21% of the radioactivity due to 100% inactivation by [^{3}H]-DFP. In one run, 70% inactivation by BrPin led to 30% of maximum incorporation of [^{3}H]-DFP. BrPin inactivation appears to exclude DFP.

Sigma AcChE samples (i) were 98% inactivated by 20 hr treatment with 3.8 mM BrPin or (ii) were not pretreated with BrPin; the samples were then treated with 51 μM 5:1 DFP:[^{3}H]-DFP, containing 6.3 μM [^{3}H]-DFP. Electrophoresis and autoradiography showed no [^{3}H]-DFP in the BrPin-pretreated AcChE, and incorporation of [^{3}H]-DFP in untreated enzyme at 55 and 50 kDa.

Sigma AcChE was first exposed to (i) complete inactivation by treatment with 3.7 mM BrPin for 36 hr; (ii) 48% inactivation by 2.5 mM BrPin treatment for 3.5 hr; and (iii) no preinactivation by BrPin. Then incorporation of [^{3}H]-DFP into the AcChE by 2:1 DFP:[^{3}H]-DFP, 46 μM, containing 12 μM [^{3}H]-DFP, for 36 hr was examined. After dialysis, samples showed (i) 64,000 cpm, (ii) 226,000 cpm and (iii) 355,000 cpm. If incorporation of 64,000 cpm is due to non-specific reaction of impure Sigma AcChE and incomplete dialysis, subtraction leads to 162,000 cpm for (ii) and 291,000 cpm for (iii), giving a ratio of 0.56, consistent with 52% residual activity.

In a parallel experiment, Torpedo AcChE was subjected to (i) complete inactivation by treatment with 1.5 mM BrPin for 16 hr, (ii) 53% inactivation by treatment with 0.88 mM BrPin for 60 min, and (iii) no preinactivation by BrPin. Then incorporation of [^{3}H]-DFP into Torpedo AcChE by 2:1 DFP:[^{3}H]-DFP, 49 μM, containing 13 μM [^{3}H]-DFP, for 36 hr was examined. After dialysis, samples showed (i) 16,500 cpm, (ii) 58,000 cpm and (iii) 204,000 cpm. Torpedo AcChE was more rapidly inactivated by BrPin than was Sigma AcChE. The time course of inactivation by 49 μM DFP indicated that in (ii), 30 min would be required for DFP inactivation of the remaining 47% activity. In this period the BrPin would contribute about 35% of the remaining inactivation, about 16% of total inactivation, leading to about 69% total inactivation due to BrPin and about 31% due to [^{3}H]-DFP. Counts in (ii) are 29% of the counts in (iii), consistent with is calculation. However, if the counts in (i) are subtracted, 42,000 cpm are left in (ii), 22% of the 188,000 cpm left in (iii), a poorer but not unsatisfactory correspondence in view of the difficulty arising from comparable rates of inactivation due to BrPin and [^{3}H]-DFP in this case. The total number of counts calculated for 6.15 x 10^{-11} moles of Torpedo AcChE used in these experiments, 100% inacti-

vated by [^{3}H]-DFP, was 183,000 cpm, to be compared with 204,000 cpm observed, and 188,000 cpm corrected. In the enzyme inactivated about 69% by BrPin, the corresponding values are 57,000 cpm calculated, 58,500 cpm observed, and 42,000 cpm corrected.

Our isolated Torpedo enzyme appears to be rather pure, but very easily inactivated by BrPin. Pretreatment with BrPin leads to appropriate exclusion of [^{3}H]-DFP. This indicates that partial inactivation does not lead to enzyme that is modified peripherally to yield decreased activity and slower [^{3}H]-DFP incorporation. Instead, these results are consistent with enzyme of which part is totally inactivated and part is totally active. This may be evidence for the specificity of action of BrPin.

Reversible Inhibitors and [^{14}C]-BrPin. Three samples of 0.73 μM Torpedo enzyme were prepared, in 0.1 M phosphate, pH 8: (i) containing 0.68 mM [^{14}C]-BrPin, which led to 30% inactivation in 10 min, at which time inactivation was stopped by 0.1 M sodium azide; (ii) containing 0.68 mM [^{14}C]-BrPin, as in (i), plus 22 mM TAP, which led to no inactivation after 10 min; (iii) 98% preinactivated by 0.053 mM Sigma DFP for 1 hr before addition of [^{14}C]-BrPin as in (i). All samples were put on polyacrylamide gel. One-half was placed in contact with X-ray film for 1 week at -70°C. Stain and radioactivity were found at Rf 0.26-0.30, 72-75 kDa, quite similarly in absence and presence of pretreatment with DFP, but with significantly lower exposure in presence of TAP (Figure 5). Higher molecular weight bands, quite small, were found at Rf 0.07 and 0.14; radioactivity was observed in the absence of additive and in the presence of DFP, but not in the presence of TAP. It should be noted that pretreatment with BrPin largely prevents subsequent introduction of [^{3}H]-DFP, as it prevents reaction of substrates, by preventing utilization of the enzymic mechanism. However, pretreatment with DFP, phosphorylating the serine-hydroxyl, did not appear to prevent [^{14}C]-BrPin from binding at the trimethyl site and alkylating the enzyme. Thus, BrPin does not alkylate the serine-hydroxyl. Decreased introduction of [^{14}C]-BrPin in the presence of TAP indicates some specificity in this alkylation.

Inactivation of Torpedo AcChE by 2 mM BrPin was studied alone and in the presence of reversible, site-specific protecting agents, TAP, PTA and TMAP. Assays were carried out by the Ellman procedure. When BrPin and the reversible inhibitor were each used at concentrations of 10 x K_i, the $t_{1/2}$ values for inactivation increased two- to three-fold. These three reversible inhibitors, containing trimethylammonio substituents and occupying the trimethyl site, protect AcChE from BrPin to extents consistent with K_i values, indicating that BrPin acts via binding at that site.

Inactivation was then examined in the presence of (i) PTA and (ii) nitrobenzene. (i) Inactivation by 1 mM [^{14}C]-BrPin, alone and in the presence of 17 mM PTA for 120 min, led to 46% and 11% inactivation, respectively, while the enzyme alone showed about 6% loss of activity. Gel electrophoresis showed, as before, a major band at 70 kDa and minor bands at 90 and 140 kDa. Autoradiography showed ^{14}C at 70 and 150 kDa in the absence of PTA and substantially less ^{14}C in the presence of PTA, about 3000 and 1000 cpm, respectively (Figures 6 and 7). (ii) The presence of 4 mM nitrobenzene (K_i = 0.6 mM) had a small effect on inactivation by 1 mM [^{14}C]-BrPin, 32% inactivation as compared with 41% in its absence.

Torpedo AcChE was treated with 1 mM [^{14}C]-BrPin alone and in the presence of 20 mM TAP, at room temperature, pH 7.0, I = 0.2. After 120 min, there was about 50% inactivation in the absence of TAP, and essentially complete protection in the presence of TAP. The solutions were made 0.1 M in sodium azide and subjected to gel electrophoresis. Stain bands were found at 70 kDa (major) and at 90 and 140-150 kDa (minor). Part of the gel was cut for scintillation counting and another part was autoradiographed. A major band of film exposure was found at 70 kDa in the absence of TAP and much less exposure in its presence. This is reflected in the scintillation counting (Figures 8 and 9). The gel track contained 101 μg, 1.44 nmoles of enzyme, based on k_{cat} for T. californica of which 50% was inactivated. The counts, 22,000 cpm at 70% efficiency, correspond to 31,000 dpm in the 70 kDa slice, or 0.84 nmoles of ^{14}C, based on [^{14}C]-BrPin specific activity of 16.7 mCi/mmol, 36.7 x 10^9 dpm/mmol, and 1.2 ^{14}C per enzyme unit inactivated.

DFP and [^{14}C]-BrPin. Unexpectedly, prior inactivation by DFP, leading to a group bound to the esteratic serine-hydroxyl, did not appear to retard subsequent introduction of [^{14}C]-BrPin, as indicated by autoradiography (Figure 5). This was confirmed by scintillation counting. Aliquots of 1.4 μM Torpedo AcChE (0.30 mL, 0.42 nmoles) were (i) completely inactivated at 25°C by 5 μM DFP, and aged overnight at 4°C, or (ii) allowed to stand as in (i) without DFP, and both were made 1.0 mM in [^{14}C]-BrPin. When activity in (ii) fell to 36% of its initial value (3 hr), the two samples were made 0.1 M in sodium azide. The samples, 0.19 nmoles of enzyme, based on k_{cat} for T. californica, 0.12 nmoles inactivated, were subjected to PAGE and the 70 kDa bands were digested and scintillation-counted; yielding 4750 cpm after inactivation by DFP and 4930 cpm in the absence of treatment with DFP (Figures 10 and 11). Phosphorylation of the serine-hydroxyl had no effect on action of [^{14}C]-BrPin at the trimethyl site. The counts corresponding to 0.13 nmoles of label, or 0.18 nmoles of label after correction for 70% counting efficiency, indicated 1.5 [^{14}C]-BrPin per enzyme unit inactivated.

The failure of inactivation by DFP to prevent introduction of [^{14}C]-BrPin was examined by double-labeling, dialysis and scintillation counting. An aliquot inactivated by [^{3}H]-DFP had a count of 4050 cpm in the ^{3}H plus ^{14}C counting channel and 3800 cpm in the ^{3}H counting channel. An aliquot treated with [^{14}C]-BrPin to 43% inactivation had 440 cpm. A third aliquot inactivated first by [^{3}H]-DFP and treated in the same way with [^{14}C]-BrPin showed 4960 cpm in the ^{3}H plus ^{14}C channel and 4560 cpm for ^{3}H alone. Thus, treatment with BrPin did not remove the phosphoryl group, and phosphorylation did not prevent introduction of ^{14}C. Occupation of the esteratic subsite does not prevent reaction of BrPin, but occupation of the trimethyl site does.

G. Formic Acid Cleavage of Labeled Enzyme

Procedures for cleaving the enzyme after labeling are being worked out. An initial experiment was carried out with [^{3}H]-DFP. A solution of equal concentrations, about 5 μM, of enzyme and DFP was allowed to react for 100 min, about 70% inactivation, and aged for 12 hr, and then was made 75% in formic acid and kept for 48 hr. The samples were dried, reconstituted in buffer and subjected to gel electrophoresis and autoradiography. Enzyme, largely of m.w. 70 kDa initially, was converted to fragments of 20, 35, and 50 kDa. The radioactive label was associated with the 50 kDa fragment, in agreement with predictions from the primary amino acid sequence and the position of the Asp-Pro bond susceptible to formic acid cleavage (43). Radioactivity also appeared in the unfragmented 70 kDa monomer. The 35 kDa band may arise from the 110 and 150 kDa components, which are present to low extent and showed no label.

This experiment was repeated in parallel with an inactivation by 0.1 mM [^{14}C]-BrPin, samples being taken at intervals for assay of residual activity. After 230 min, 77% and 42% inactivation by DFP and BrPin, respectively, were observed. The BrPin incubation was made 0.1 M in sodium azide and both solutions were aged for 12 hr, made 75% in formic acid, and left for 48 hr at 37°C. They were subjected to gel electrophoresis and autoradiographed. The BrPin and DFP labels appeared on the 50 kDa fragment (Figures 10 and 11).

H. Gel Filtation of Trypsin Digestion

Sephadex G-50 columns were examined, for their capacity to separate peptide fractions from trypsin cleavage of [^{14}C]-BrPin labeled <u>Torpedo</u> AcChE. A 1.5 x 45 cm column failed to separate Sigma myoglobin fractions, 2.5-17 kDa, offered as gel electrophoresis markers. Cytochrome C also appeared not to be retained. Bromophenol Brilliant Blue, m.w. 670, was retained longer than methylene blue, m.w. 374, although the reverse

would be expected. Similarly, a mixture of oxidized glutathione, m.w. 612; bradykinin, m.w. 1240; and somatostatin, m.w. 1638; showed greater retention of the higher molecular weight components.

A 1.5 x 170 cm column successfully separated a peptide fragment from a trypsin digestion of [^{3}H]-DFP-labeled Torpedo AcChE. The enzyme, 0.54 mg, based on k_{cat} for T. californica, was inactivated by [^{3}H]-DFP, aged, dried, resuspended in a solution of 0.1 M Tris and 6 M guanidine hydrochloride, pH 8, treated with 1.65 x 10^{-6} moles of dithiothreitol (3 hr, 50°C), then with 2.7 x 10^{-6} moles of iodoacetic acid (1 hr, 25°C) and finally with excess dithiothreitol. The solution was dialyzed against 1 L of 50 mM NH_4HCO_3, pH 8.3, overnight, then treated with 0.04 mg of trypsin, 37°C, 48 hr. The product was applied to the Sephadex G-50 column and 3-mL fractions were collected. Absorbance at 219 nm was followed and scintillation counts of 0.2 mL aliquots in 10 mL of scintillation liquid were taken. One peak of radioactivity was found (Figure 12).

(6) Discussion and Conclusions

A more useful view has been developed in our studies of the properties of the active site of AcChE. The long and generally accepted view describes the active site as containing (i) an active serine-hydroxyl which is acetylated and deacetylated during the hydrolysis (44); and (ii) an anionic site at which the cationic β-substituent of AcCh binds, leading to a correct orientation for reaction at the serine (1-4). Hydrophobic domains at which aryl groups may bind (7) and peripheral binding sites which may have allosteric function were added to this model, and the presence of six to nine peripheral anionic groups has been proposed (17), appropriate in an enzyme of isoelectric point about pH 5 (15), functioning at pH 7-8.

Our view deals with the sites at which the β-substituent of acetate substrates and aryl groups bind. We conclude that the first is better considered to be an uncharged and non-polar site complementary to the trimethyl character of the substrate's β-substituent rather than to its positive charge. We term it "trimethyl" and not "anionic" (12). We conclude, further, that the forces affecting binding of aryl groups are polar in character, charge transfer or hydrogen-bonding, and not hydrophobic (29). These contrary views lead to alternate interpretations of previous results, to new results, and to new and, we believe, superior suggestions for reagents useful for study of the amino acid content of the active site, for reversible modifiers of activity of the enzyme and for inactivating and reactivating agents. These views arise from study of substrates, reversible inhibitors and irreversible inactivators acting from the trimethyl site, the aryl site or both (29, 33).

The basis for our views is given in the body of this report. In summary, with respect to the trimethyl site, (i) the effects of cationic, uncharged, and non-polar β-substituents on substrate reactivity are satisfactorily accounted for by the volume of the substituents and their effects on intrinsic hydrolytic reactivity, and there is no need to call on the presence of a specific orienting anionic site (12); (ii) cationic and uncharged reversible inhibitors structurally related to AcCh each show the same K_i values in inhibiting enzymatic hydrolysis of AcCh and its isosteric uncharged analogue, DMBAc, indicating the presence of a single trimethyl binding subsite for the cationic and uncharged β-substituents (14); and (iii) the effects of positive charge in increasing binding may be attributed to the presence of peripheral anionic charges.

These data indicate that uncharged tetra-substituted reagents will bind more specifically to the trimethyl site, and be more specific labeling agents, while cationic ones, such as N,N-dimethylphenylaziridinium ion, may interact at peripheral anionic sites. The substituents of uncharged reagents may have varied surface properties, i.e., hydrocarbon in tert-butyl, oxygen in methylsulfonyl, chlorine in trichloromethyl, and thus show varied permeating and solubility characteristics, as compared with cationic groups. They may have varied size, with a core Si atom instead of C or N^+ (33). Such compounds have been surveyed briefly, but emphasis is placed on the tert-butyl group at present. BrPin is proving to be a specific labeling agent. It binds competitively and inactivates similarly for all substrates examined -- uncharged, cationic, alkyl and aryl (20). Now prepared with ^{14}C label, it introduces about one ^{14}C per unit inactivated, and this inactivation coupled with labeling is appropriately retarded by an active-site-directed reversible inhibitor isosteric with AcCh, i.e., $(CH_3)_3N^+CH_2CH_2$-CH_2COCH_3, TAP. Some uncertainty remains in the figure, one ^{14}C, since we isolate and study AcChE from Torpedo nobiliana and are using k_{cat} from T. californica to determine enzyme quantity; k_{cat} for T. nobiliana will be determined.

Inactivation by BrPin is catalyzed by an acid of pK_a about 5.5, but a carboxylate is not the labeled group, since the label is retained under conditions which would hydrolyze an ester. The active serine is not the labeled group, since prior inactivation by DFP does not affect introduction of [^{14}C]-BrPin. However, inactivation by BrPin prevents reaction of [^3H]-DFP, as it prevents reactions of substrates. It may be noted that despite the long and widespread study of AcChE it is only this serine which has been unequivocally characterized in the active site, while other serine enzymes have been crystallized and their active sites have been studied in detail (11, 45). Even the imidazole nucleus, common to these enzymes, has not been identified in AcChE. The amino acid sequence of AcChE is known

(46), but the X-ray structure is not, and identification of amino acids labeled from a specified binding site may lead to inferences about the protein folding. We are now engaged in cleaving Torpedo AcChE that has been inactivated by [^{14}C]-BrPin to identify the labeled amino acid.

Benzene derivatives have long been important in the study of AcChE, from the use of physostigmine to prove the function of AcCh (21), to neostigmine (22) and the very strongly binding TMAP (24). These compounds contain cationic charge, and binding at the "anionic" site was generally postulated. On the other hand, binding by aromatic compounds at hydrophobic sites has also been proposed (7). Study of aromatic substrates and inactivators is complicated by differing effects of structure on the several steps in such processes -- binding, acylation and deacylation (25,27,28).

To avoid complication, we have studied an extensive series of reversible inhibitors, mono- and disubstituted derivatives of benzene (29) and pyridine, containing cationic, uncharged polar, and non-polar substituents. We find that the binding of benzene derivatives rises, in accordance with the Hammett equation (47), with high positive ρ; i.e., binding is increased by electron withdrawal by the substituent, with cationic and uncharged substituents following the same $\sigma-\rho$ relation. This indicates that there is no direct coulombic interaction of positively and negatively charged groups, but a charge-transfer or hydrogen-bonding interaction of the benzene ring with the enzyme -- probably with amino acid side chains. Further, we find that those interactions are enhanced by the presence of tert-butyl, dimethylamino and trimethylammonio substituents, presumably binding at the trimethyl site, and that binding of these disubstituted compounds, cationic and uncharged, also follows a single $\sigma-\rho$ relation (29). Thus, the aryl and trimethyl binding sites are contiguous, and appropriate compounds may occupy either or both.

The phenols are of particular interest since the positive σ value of the hydroxyl weakens binding but meta-alkyl substituents (methyl, ethyl, etc.), also with positive σ, increase binding substantially (29). This binding enhancement rises with volume to that of the tert-butyl substituent; all meta-alkyl groups bind in the trimethyl site. This effect is less in the ortho isomer and absent in the para, and apparently is absent in the anisoles. Thus there is a specific steric relation between the trimethyl site and that where the phenolic hydroxyl hydrogen binds. Further, the effect of the tert-butyl is the same as that of trimethylammonio in the very strongly binding TMAP, when correction is made for the difference in their σ values (29). That is, the same $\sigma-\rho$ relation is observed and the effect of the cationic charge in the strongly binding TMAP is not coulombic but arises from its effect on the benzene ring.

Our study of pyridines is not as far advanced but raises interesting possibilities. The dimethylaminopyridines, in which cationic and uncharged forms are in equilibrium, bind as strongly as N-methylpyridinium ion, with its permanent charge. N-Methylpyridinium ion, thought to occupy the anionic site, may occupy only the aryl site. Derivatives of the dimethylaminopyridines would show both water and lipid solubility and superior permeating properties. Occupying both the trimethyl and aryl binding sites, they might lead to more effective reactivating agents.

The relations among compounds acting from or at the several parts of the active site are of particular interest. The action of inhibitors is generally observed as preventing reaction of the serine-hydroxyl. Thus BrPin, acting from the trimethyl site, and styrene oxide, DPA, and MBS, acting from the aryl site, prevent subsequent introduction of [^{3}H]-DFP, which acts by acylating the serine. Partial inactivation of AcChE by BrPin leads to reduction of subsequent introduction of [^{3}H]-DFP to an appropriate extent, indicating that a portion of the enzyme is inactivated and the remainder is active, and not merely of reduced activity. This is also borne out by the extent of introduction of ^{14}C in partially inactivated samples. MMS, which may act from and at the esteratic site itself, also excludes [^{3}H]-DFP. MBS and MMS may alkylate the serine-hydroxyl, and like DFP, may also not preclude subsequent introduction of [^{14}C]-BrPin, but this has not yet been examined.

PTA and TMAP, reversible inhibitors occupying the trimethyl in addition to the aryl site, retard introduction of [^{14}C]-BrPin, which acts from the trimethyl site, but nitrobenzene, occupying only the aryl site, does not appear to do so. Thus, the two sites are contiguous (or a single site has two parts) and may be occupied by a single compound, as by PTA and TMAP, or by different compounds simultaneously, as by BrPin and nitrobenzene. This will be examined further with nitropyridine, which may be studied at higher concentration than nitrobenzene. Similarly, inactivation by MBS, acting from the aryl site, is not retarded by $(CH_3)_4N^+$, which simultaneously occupies the trimethyl site. Inactivation by MMS is also not retarded by $(CH_3)_4N^+$, but MMS may be reacting at the esteratic site without occupying either the aryl or the trimethyl site. TAP, isosteric with AcCh, occupies the trimethyl and esteratic sites and retards inactivation by styrene oxide and MBS. The oxide may direct its functional group toward the trimethyl site, whereas MBS may direct its functional group to the esteratic site. It will be of interest to ascertain whether TAP retards the action of the small inhibitors MMS and methylsulfonyl halides.

Nitrobenzene, K_i = 0.6 mM, acting from the aryl site, inhibits hydrolysis of AcCh at least as effectively as

$(CH_3)_4N^+$, acting from the trimethyl site, and it accelerates hydrolysis of ethyl acetate just as $(CH_3)_4N^+$ does. Since nitrobenzene does not retard inactivation by BrPin, the benzene ring, in the aryl site, does not appear to overlap with either the β-substituent or the $-CH_2CH_2OCOCH_3$ moiety of acetate substrates. However, it prevents access of substrates containing both a large β-substituent and this moiety. The aryl-trimethyl binding site complex may have considerable flexibility. With respect to the binding of benzene derivatives, it may be noted that protein structures are stabilized by aromatic-aromatic interactions of appropriate amino acid side chains (48). The aryl binding domain may contain such structures with which benzene derivatives interact, binding and altering the protein structure.

From these viewpoints, it may be possible to devise labeling agents acting from or in the trimethyl site, and agents acting in or from the aryl site and directed toward the trimethyl or the esteratic site. Styrene oxide and phenacyl bromide and their derivatives, and related pyridine compounds may be designed for reaction with enzyme nucleophiles. Derivatives of dimethylaminopyridines have potential as effective reversible activity modifiers, labeling agents and reactivators. Pivalophenone and phenyldiazirines may be examined as photoinactivators.

Table I

Hydrolysis of $X\text{-}CH_2CH_2OCOCH_3$ by AcChE,

in 0.18 M NaCl, pH 7.8, 25 °C, and by Hydroxide

Substrate										
Compound Number	X	$10^{-2} \times k_{cat}$ sec^{-1}	K_m mM	$10^{-4} \times k_{cat}/K_m$ $M^{-1}sec^{-1}$	$10^{-2} \times k_1$ sec^{-1}	K_s mM	k_{OH^-} $M^{-1}sec^{-1}$	$10^{-2} \times k_{2(n)}$ sec^{-1}	$10^{-4} \times k_{2(n)}/K_m$ $M^{-1}sec^{-1}$	$\bar{V}^o_{25}$ ml/mol
I	$(CH_3)_3\overset{+}{N}-$	160	0.33±0.07	4800	1100	1.2[a]	2.8			62.8
II	$(CH_3)_3C-$	31±3	2.6±0.3	120	37	3.1	0.11	950	3100	73.6
III	$(CH_3)_2\overset{+}{N}H-$	48±2	1.1±0.1	440	64	1.4	2.7[b]			45.9
IV	$(CH_3)_2CH-$	12±2	2.6±0.7	46	13	2.8	0.11	330	1200	58.4
V	$CH_3\overset{+}{N}H_2-$	22±1	20±1	11	24	22				27.5
VI	CH_3CH_2-	13±2	12±2	11	14	13	0.11	350	270	42.4
VII	$\overset{+}{N}H_3-$	19±2	24±3	8.0	21	27				9.3
VIII	CH_3-	2.6±0.3	33±5	0.80	2.6	33	0.11	67	20	26.4
IX	H-	3.7±0.4	220±29	0.17	3.8	230	0.11	97	4.3	10.4
X	HO-	1.6±0.3	22±4	0.71	1.6	22	0.46	9.5	4.3	12.2
XI	CH_3O-	8.3±1.2	4.7±0.7	18	8.6	4.9	0.38	64	130	31.9
XII	Cl-	15±0.9	32±2	4.8	16	35	0.31	150	43	19.0
XIII	Br-	9.6±0.5	9.9±0.6	9.7	10	10	0.42	68	65	26.6
XIV	$N\equiv C-$	11±1	17±2	6.2	11	18	1.3	25	14	21.6

[a] Estimated value
[b] Determined at pH 7.8

Table II

Reversible Inhibition of Hydrolysis by AcChE of AcCh and DMBAc, in 0.18 M NaCl, pH 7.8, 25°C

Inhibitor			K_i	
No.	Compound	Concentration (mM)	Acetylcholine (mM[a])	DMBAc (mM[a])
I[b]	$(CH_3)_3{}^+NCH_2CH_2CH_2COCH_3I^-$	0.1-0.3	0.022	0.024
II[b]	$(CH_3)_3C^+NH_2CH_2CH_2COCH_3Cl^-$	0.1-1	0.16	0.14
III[b]	$(CH_3)_3CSCH_2CH_2COCH_3$	1-3	0.40	0.33
IV[b]	$(CH_3)_3C^+NH_3Cl^-$	0.1-2	0.43	0.44
V[b]	$(CH_3)_2{}^+NHCH_2CH_2CH_2COCH_3Cl^-$	1-5	0.77	0.71
VI[c]	$(CH_3)_3{}^+NCH_2CH_2OHCl^-$	0.1-2	1.0	0.63
VII[c]	$(CH_3)_3{}^+NCH_3Cl^-$	0.4-2	1.5	1.0
VIII[b]	$(CH_3)_3COCH_2CH_2COCH_3$	0.4-3	1.6	1.0
IX[b]	$(CH_3)_2CH^+NH_2CH_2CH_2COCH_3Cl^-$	1-5	2.0	1.5
X[b]	$(CH_3)_2CHOCH_2CH_2COCH_3$	2-9	4.8	3.1
XI[c]	$(CH_3)_3CCH_2CH_2OH$	2-16	7.5	5.7

[a] ±25%.

[b] Showed competitive inhibition.

[c] Mixed or noncompetitive inhibition.

Table III

Hydrolysis of $X\text{-}CH_2CH_2OCOCH_3$ by AcCHE, in 0.18 M NaCl, pH 7.8, 25 °C
Molar Refractivity (MR), Hydrophobicity (Π), and Kinetic Constants

Cpd	β-Substituent				$10^9 x [E]$	$10^{-2} x k_{cat}$	K_m	$10^{-4} x (k_{cat}/K_m)$	$k_{(OH-)}$	$10^{-4} x (k_{cat}/K_m)_n$
No.	X-	MR (cm^3)	Π	Conc (mM)	M	sec^{-1}	(mM)	$M^{-1}sec^{-1}$	$M^{-1}sec^{-1}$	$M^{-1}sec^{-1}$
I	$(CH_3)_2S^+$-	16.4	-0.50	0.2-1.2	0.6	77	0.33	2300	3.1	2100
IA	$(CH_3)_3N^+$-	17.2	-1.24	0.1-0.6	0.1	160	0.33	4800	2.8	----
II	$(CH_3)_3Si$-	25.0	+2.59	0.1-3.0	0.4	57	3.5	160	0.06	7600
IIA	$(CH_3)_3C$-	19.6	+1.98	0.7-5.3	0.2	66	5.3	125	0.07	5000
III	$(CH_3)_2N^+(OH)$-	15.0	-3.0	1.5-9.0	0.3	45	18	25	0.82	86
IIIA	$(CH_3)_2CH$-	15.0	+1.53	1.0-5.0	1.1	34	3.6	93	0.11	2400
IV	$CH_3S(O_2)$-	13.9	-1.9	6-21	0.6	11	6.2	18	1.4	36
IVA	$CH_3S(O)$-	14.1	-1.85	7-22	0.5	38	16	24	0.45	150
IVB	O_2N-	6.7	-0.85	7-22	1.1	--	---	(5)	----	----
V	CH_3S-	13.3	+0.45	3-21	0.6	35	15	23	0.28	230
VA	CH_3CH_2-	10.3	+1.02	10-17	0.2	36	13	26	0.11	660
VI	Cl-	5.93	+0.39	4-40	0.2	34	14	25	0.31	220
VIA	Br-	8.80	+0.60	1-12	0.1	45	7.5	59	0.42	400

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Table IV

Reversible Inhibition of Hydrolysis of AcCh by AcChE, in 0.18 M NaCl, pH 7.8, 25°C

No.	Compound	$K_{i(com)}$ (mM)	$K_{i(nonc)}$ (mM)	K_m [a] (mM)
I	$(CH_3)_3SiCH_2CH_2OH$	3.3		3.5
IA	$(CH_3)_3CCH_2CH_2OH$	7.5	19	5.3
IB	$(CH_3)_3N^+CH_2CH_2OHCl^-$	0.4	7.6	0.33
II	$(CH_3)_2S^+CH_2CH_2OHI^-$	0.4	13	0.33
III	$(CH_3)_3S^+I^-$	2.0	7.2	
IV	$(CH_3)_3S^+OI^-$	1.3		
V	$(CH_3)_3N^+O^-$	>>200		18
VI	$(CH_3)_2N^+(O^-)CH_2CH_2CH_2COCH_3$	14		18
VII	$CH_3S(O_2)CH_2CH_2OH$	8.7	100	6.2
VIIA	$CH_3S(O_2)CH_2CH_2OCOCH_3$	6.4	11	6.2
VIII	$(CH_3)_2SO_2$	28	260	
IX	$(CH_3)_2SO$	25		16
X	$CH_3SCH_2CH_2OH$	40		15
XI	$CH_3CH_2CH_2CH_2OH$	47		13

[a] $K_{m(app)}$ of corresponding acetate, Ref. 39.

Table V

Inhibition by Benzene and its Derivatives of Hydrolysis of AcCh by AcChE, in 0.18 M NaCl, pH 7.8, 25 °C

Inhibitor	$K_{i(com)}$ (mM)	log $1/K_{i(com)}$	$\Delta G_{(com)}$ kcal/mol	$K_{i(nonc)}$ (mM)	$\Sigma\sigma_{para}$
A. Substituents with no specific interaction.					
C_6H_6	23±3	1.64	-2.2[b]	48±8	0
$C_6H_5NH_2$	46±14	1.33	-1.8[c]	67±5	-0.17
$C_6H_5NHCOCH_3$	17±3	1.76	-2.4[b]	-----	0.04
$C_6H_5COCH_3$	4.1±1.2	2.39	-3.2[c]	3.3±0.2	0.46
$C_6H_5NO_2$	0.63±0.17	3.19	-4.3[c]	3.0±0.4	0.82
$C_6H_5NO_2$[a]	0.80±0.19	3.10	-4.2[c]	2.5±0.5	0.82
$3\text{-}CH_3CONHC_6H_4NO_2$	0.26±0.025	3.59	-4.8[b]	1.2±0.2	0.86
$4\text{-}CH_3CONHC_6H_4NO_2$	0.46±0.05	3.34	-4.5[b]	0.53±0.01	0.86
$3\text{-}H_2NC_6H_4NO_2$	2.0±0.4	2.69	-3.6[b]	3.3±1.5	0.65
$4\text{-}H_2NC_6H_4NO_2$	0.57±0.12	3.24	-4.4[b]	0.83±0.13	0.65
$4\text{-}H_2NC_6H_4COCH_3$	7.4±1.7	2.13	-2.9[c]	3.6±2.1	0.29
$C_6H_5CH{-}{-}CH_2$ (O)	10±1.5	2.00	-2.7[b]	33±1.5	----
C_5H_5N[e]	12±3	1.92	-2.6[c]	110±50	----

Table V (Cont.)

Inhibition by Benzene and its Derivatives of Hydrolysis of AcCh by AcChE, in 0.18 M NaCl, pH 7.8, 25 °C

Inhibitor	$K_{i(com)}$ (mM)	log $1/K_{i(com)}$	$\Delta G_{(com)}$ kcal/mol	$K_{i(nonc)}$ (mM)	$\Sigma\sigma_{para}$
B. Substituents binding at the trimethyl site.					
$C_6H_5N(CH_3)_2$	11±1	1.94	-2.6[b]	74±27	-0.17
$C_6H_5N^+(CH_3)_3$	0.086±0.008	4.07	-5.5[b]	0.44±0.04	0.80
$3\text{-}(CH_3)_2NC_6H_4NO_2$	0.12±0.02	3.92	-5.3[b]	0.079±0.003	0.65
$4\text{-}(CH_3)_2NC_6H_4NO_2$	0.091±0.005	4.04	-5.5[b]	0.29±0.04	0.65
$4\text{-}(CH_3)_2NC_6H_4OCH_3$	1.3±0.6	2.90	-3.9[d]	3.8±0.7	0.29
$4\text{-}(CH_3)_3CC_5H_4N$[e]	3.7±0.6	2.43	-3.3[b]	13±3	-0.14

[a] In hydrolysis of DMBAc.

[b] ± 0.1 kcal/mol.

[c] ± 0.2 kcal/mol.

[d] ± 0.3 kcal/mol.

[e] At pH 8.5.

Table VI

Inhibition by Phenols of Hydrolysis of AcCh by AcChE, in 0.18 M NaCl, pH 7.8, 25 °C

Inhibitor	$K_{i(com)}$ (mM)	log $1/K_{i(com)}$	$\Delta G_{(com)}$ kcal/mol	$K_{i(nonc)}$ (mM)	MR (cm^3)
$3\text{-}(CH_3)_3N^+C_6H_4OH$[a]	$3.3\pm0.5\times10^{-4}$	6.49	-8.8[c]	$4.3\pm1.1\times10^{-3}$	----
$3\text{-}(CH_3)_3N^+C_6H_4OH$[a,b]	$2.5\pm0.7\times10^{-4}$	6.60	-8.9[c]	$4.4\pm2.3\times10^{-3}$	----
C_6H_5OH	50±5	1.30	-1.8[c]	105±10	1.03
$3\text{-}O_2NC_6H_4OH$[a]	1.4±0.5	2.84	-3.9[d]	2.8±1.0	----
$4\text{-}O_2NC_6H_4OH$[a]	2.0±0.5	2.70	-3.6[d]	0.43±0.13	----
$3\text{-}(CH_3)_3CC_6H_4OH$	0.024±0.003	4.62	-6.2[c]	0.17±0.02	19.6
$2\text{-}(CH_3)_3CC_6H_4OH$	1.0±0.15	2.98	-4.0[c]	1.7±0.16	----
$3\text{-}(CH_3)_2CHC_6H_4OH$	0.09±0.04	4.05	-5.5[e]	0.21±0.03	15.0
$4\text{-}(CH_3)_2CHC_6H_4OH$	4.2±1.0	2.38	-3.2[d]	9.5±1.0	----
$3\text{-}C_2H_5C_6H_4OH$	1.8±0.5	2.74	-3.7[d]	48±14	10.3
$3\text{-}CH_3C_6H_4OH$	10.4±2.9	1.98	-2.7[d]	40±4.5	5.65
$3\text{-}(CH_3)_2NC_6H_4OH$	0.11±0.055	3.96	-5.3[e]	0.24±0.06	15.6
$C_6H_5OCH_3$	53±2.4	1.28	-1.7[c]	38±5	----
$3\text{-}(CH_3)_3CC_6H_4OCH_3$[f]	5.6	2.25	-3.0	-----	----
$3\text{-}O_2NC_6H_4OCH_3$	0.39±0.02	3.41	-4.6[c]	0.51±0.07	----

Table VI (Notes)

[a] At pH 7.0.

[b] In hydrolysis of DMBAc.

[c] ± 0.1 kcal/mol.

[d] ± 0.2 kcal/mol.

[e] ± 0.3 kcal/mol.

[f] Studied at one concentration, 3 mM, in presence of 0.05 M acetonitrile. $K_{i(com)}$ for 3-*tert*-butylphenol = 0.076 mM, studied at 0.03 mM, 0.05 M acetonitrile.

Table VII

Reversible Inhibition by Pyridine Derivatives of Hydrolysis of AcCh by AcChE, pH 7.8[a]

Compound No.	Compound Substituent	pK_a	$\%AH^+$	$K_{i(com)}$ (mM)	$K_{i(nonc)}$ (mM)	$\Delta G_{(com)}$ kcal/mol
I	H-	5.25	0.06[b]	12[b]	110[b]	-2.6[b]
II	4-$(CH_3)_3C$-	5.3	0.06[b]	3.7[b]	13[b]	-3.3[b]
III	2-CH_2=CH-	4.98	0.15	1.4	10	-3.8
IV	4-CH_2=CH-	5.62	0.07	2.0	6.6	-3.6
V	2-CH_2-CH- (O)	3.84	0.01	1.5	14	-3.8
VI	3-CH_3CO-	----	----	2.4	-----	-3.5
VII	$\overset{+}{N}$-O^-	0.79		11	63	-2.6
VIII	2-H_2N-	6.86	10	2.2	3.6	-3.6
IX[c]	3-H_2N-	5.98	1.5	0.82		-4.2
X[d]	2-H_2N - 4-CH_3-	7.8	50	0.26	1.6	-4.8
XI	2-$(CH_3)_2N$-	6.99	13	0.08	1.3	-5.5
XII[e]	3-$(CH_3)_2N$-	6.46	4.4	0.10	0.82	-5.4
XIII	4-$(CH_3)_2N$-	9.7	99	0.05	0.12	-5.8
XIV	$\overset{+}{N}CH_3$ - 3-$(CH_3)_2N$-	----	(100)	0.02	0.06	-6.3
XV	$\overset{+}{N}$-CH_3		(100)	0.1[f]		-5.4

Table VII (Notes)

[a] Values at other pH's are noted.

[b] At pH 8.5.

[c] At pH 9, $K_{i(com)}$ = 10 mM, $K_{i(nonc)}$ = 11 mM, $\%AH^+$ = 0.1, $\Delta G_{(com)}$ = -2.6.

[d] At pH 9, $K_{i(com)}$ = 1.1 mM, $K_{i(nonc)}$ = 6.0 mM, $\%AH^+$ = 5.9, $\Delta G_{(com)}$ = -4.0.

[e] At pH 9, $K_{i(com)}$ = 0.79 mM, $K_{i(nonc)}$ = 1.2 mM, $\%AH^+$ = 0.3, $\Delta G_{(com)}$ = -4.2 and at pH 6.5, $K_{i(com)}$ = 0.067, $K_{i(nonc)}$ = 0.25 mM, $\%AH^+$ = 48, $\Delta G_{(com)}$ = -5.6.

[f] Ref. 49.

Pyridine derivatives were obtained from following sources:

I from J. T. Baker; II, III, IV, VI, VIII, IX X, XI, and XIII from Aldrich; V (50), VII (51), and XII (52) were prepared by the literature methods; XIV and XV (53) were prepared by the standard method from corresponding pyridines.

Table VIII

Esters as Substrates and Inhibitors of AcChE, pH 7.8

	As Inhibitor			As Substrate				
Compound	conc mM	time hr	inact. %	k_{cat} $10^{-3}sec^{-1}$	K_m mM	k_{cat}/K_m $10^{-4}M^{-1}sec^{-1}$	$k_{(OH^-)}$ $M^{-1}sec^{-1}$	$(k_{cat}/K_m)n$ $10^{-4}M^{-1}sec^{-1}$
$ClCH_2OCOCH_3$	4.4	4	70[a]	10	16	60	4.4	38
	4.4	4	90[b]					
$ClCH_2OCOC(CH_3)_3$	3.5	3	50[a]					
	3.5	3	100[b]					
$(CH_3)_3N^+CH_2OCOCH_3$	3	0.5	30	3.9	0.8	450	3.3	380
$(CH_3)_3COOCOCH_3$	3	1	30	3.4	2.8	120	2.0	170
$(CH_3)_3CCH_2OCOCH_3$	--	--	--	0.19	8.6	2.2	0.07	88

[a] pH 7.8.

[b] pH 5.

Table IX

Potential Active-Site-Directed Inactivating Agents

Compound	conc mM	time hr	inact. %
$BrCH_2COC_6H_5$	3	2	21
	3	3	32
	3	4.2	55
	3	5.25	73
	3	20	100
$ClCH_2COC_6H_5$	3	6.5	30
	3	25	55
$CH_2-CH-C_6H_5$ (O)	3	1	21
		3	46
		4.5	69
		22	100
2-$CH_2-CH-C_5H_5N$ (O)	3	23	0
$CH_3OSO_2C_6H_5$[a]	3	1	65
	3	2	95
	0.6	1	0
	0.6	3.3	15
	0.6	5	33
	0.6	24	93
$CH_3OSO_2CH_3$[b]	9	1.6	40
	9	4	100
	3	1	30
	3	2	70
	1	4	0
	1	6	50
$ClSO_2CH_3$	3	0.16	100
	0.3	0.16	54
	0.3	19	100
	0.03	0.16	38
	0.03	14	72

[a] $K_{i(com)}$ 6 mM; $K_{i(nonc)}$ 21 mM; unaffected by 30 mM TMA, retarded by TAP.

[b] $K_{i(nonc)}$ 96 mM.

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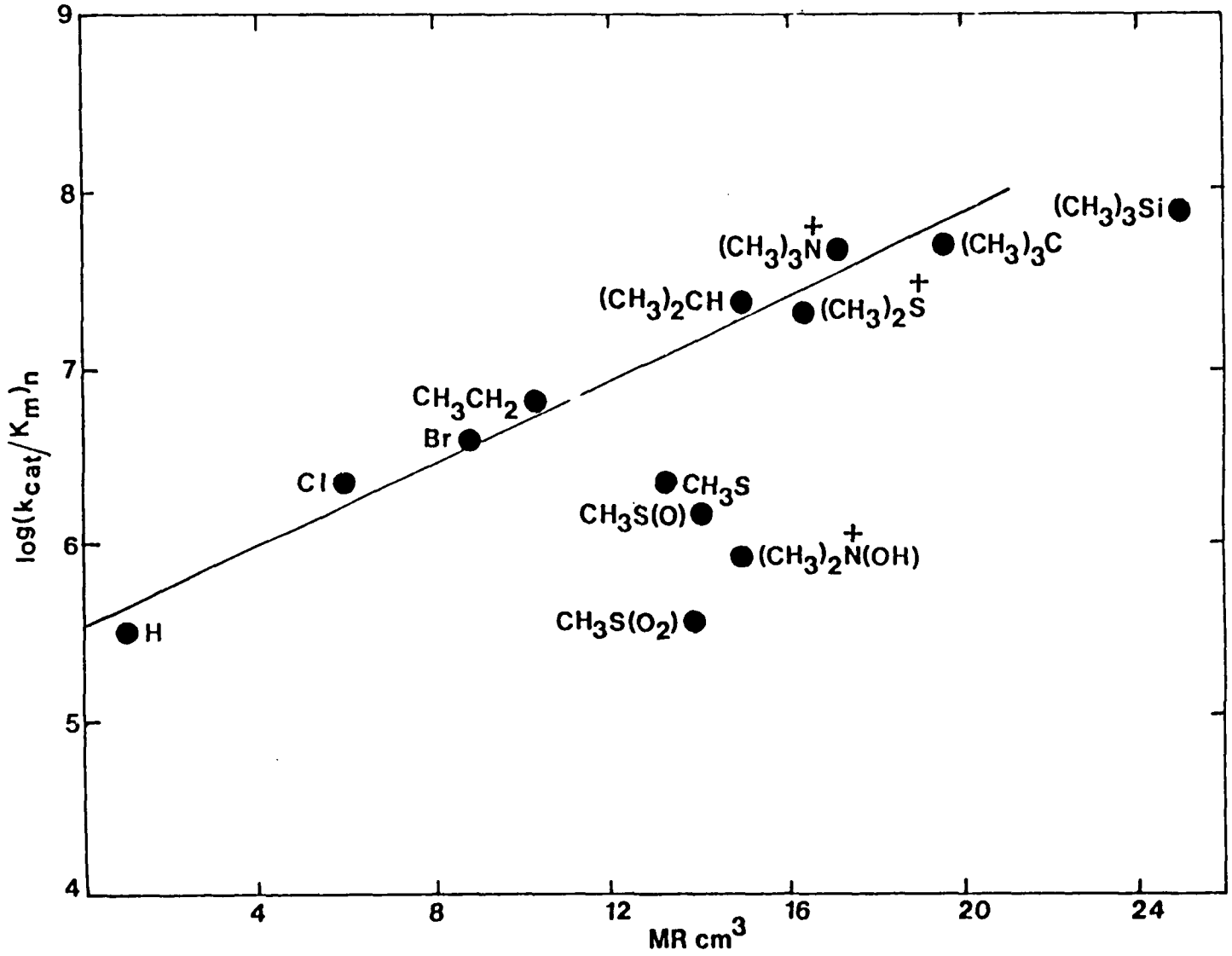

Figure 1. Normalized Reactivity of $X\text{-}CH_2CH_2OCOCH_3$, $\log (k_{cat}/k_m)_n$ and Molar Refractivity, $MR_{(X-)}$.

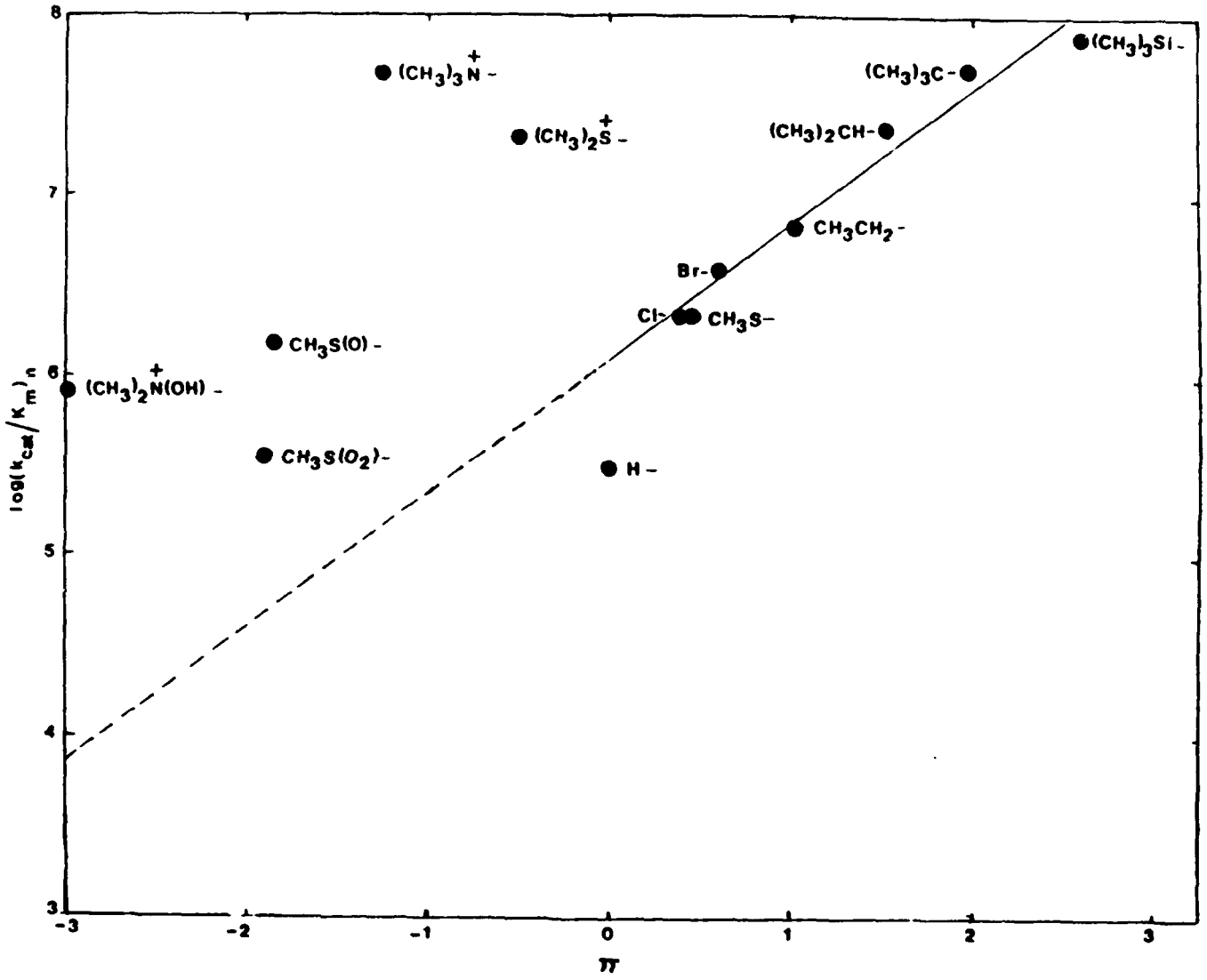

Figure 2. Normalized Reactivity of $X\text{-}CH_2CH_2OCOCH_3$, $\log(k_{cat}/k_m)_n$, and Hydrophobicity $(\pi)_{(X-)}$.

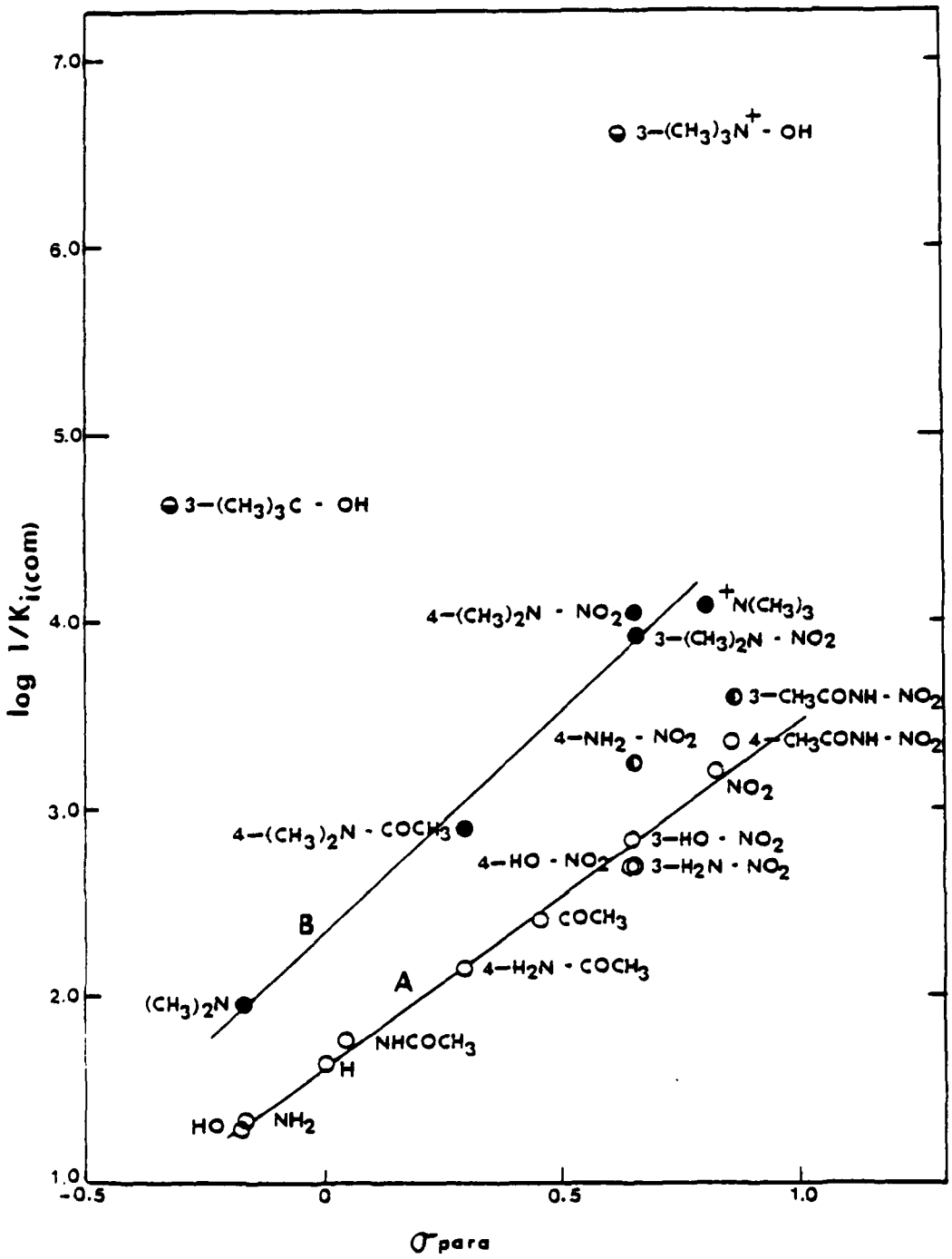

Figure 3. Relation of Binding Energies to σ Values in Inhibition by Substituted Benzenes and Phenols of Hydrolysis by AcChE.

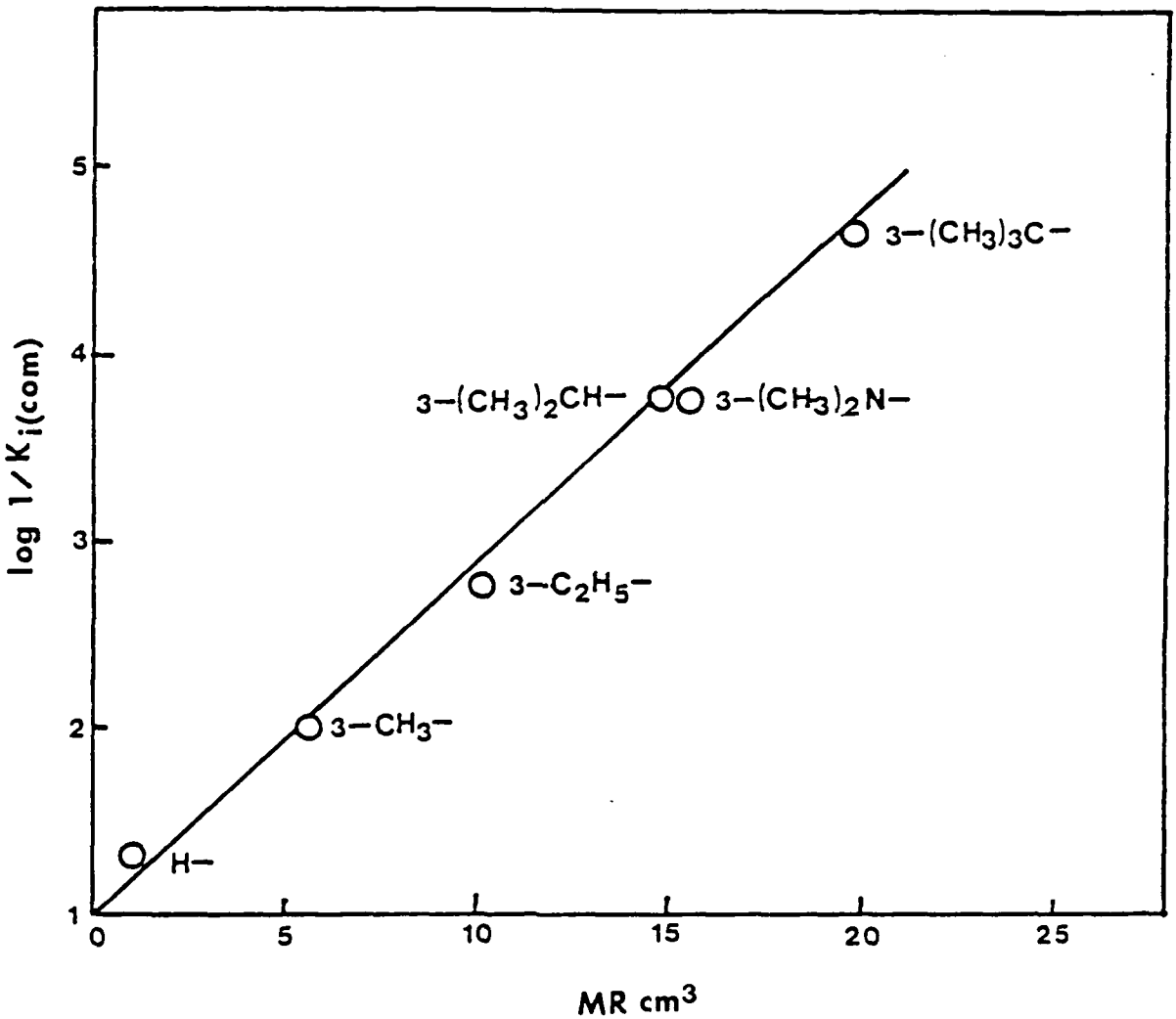

Figure 4. Effect of Volume of meta-Substituents on Binding Energies of Phenols in Inhibition of Hydrolysis by AcChE.

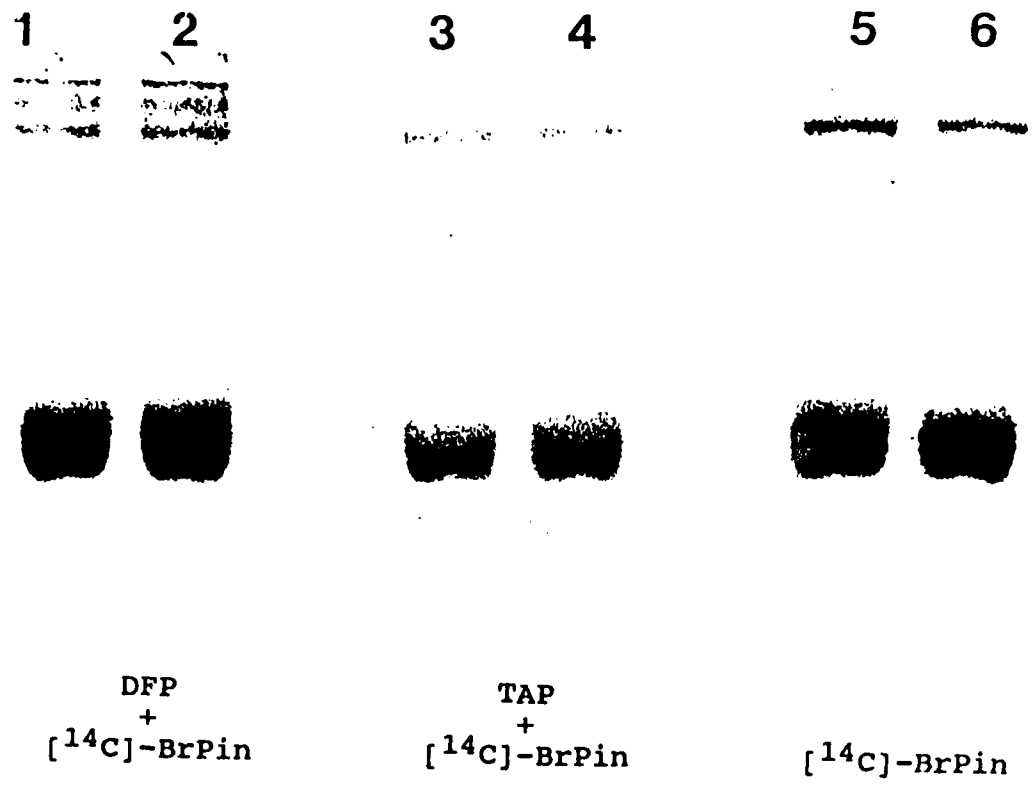

Figure 5. Radiography: Effect of DFP and TAP on Reaction of AcChE with [^{14}C]-BrPin.

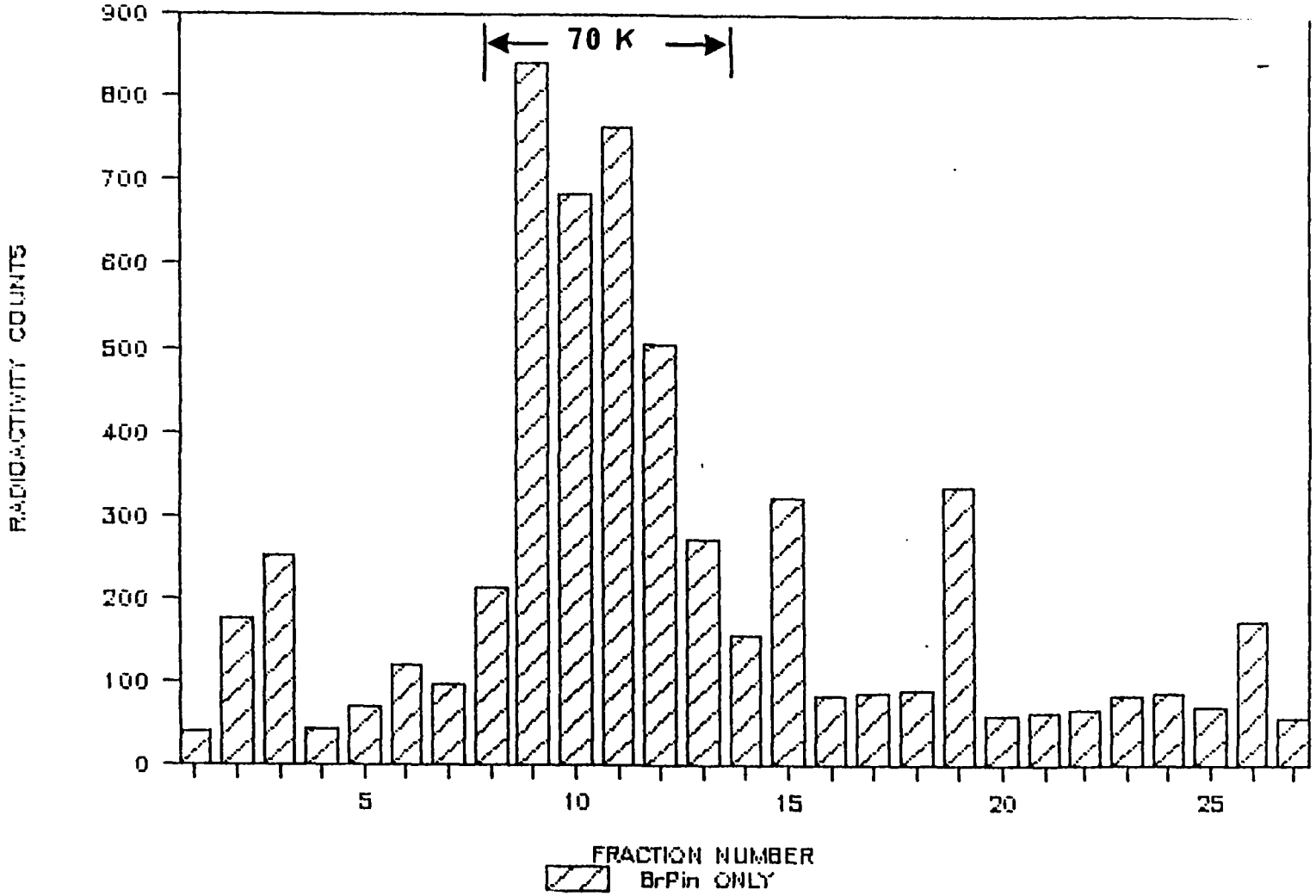

Figure 6. Histogram: Reaction of [^{14}C]-BrPin with AcChE in Absence of PTA. Radioactivity in Sequential Segments of Electrophoresis Gel.

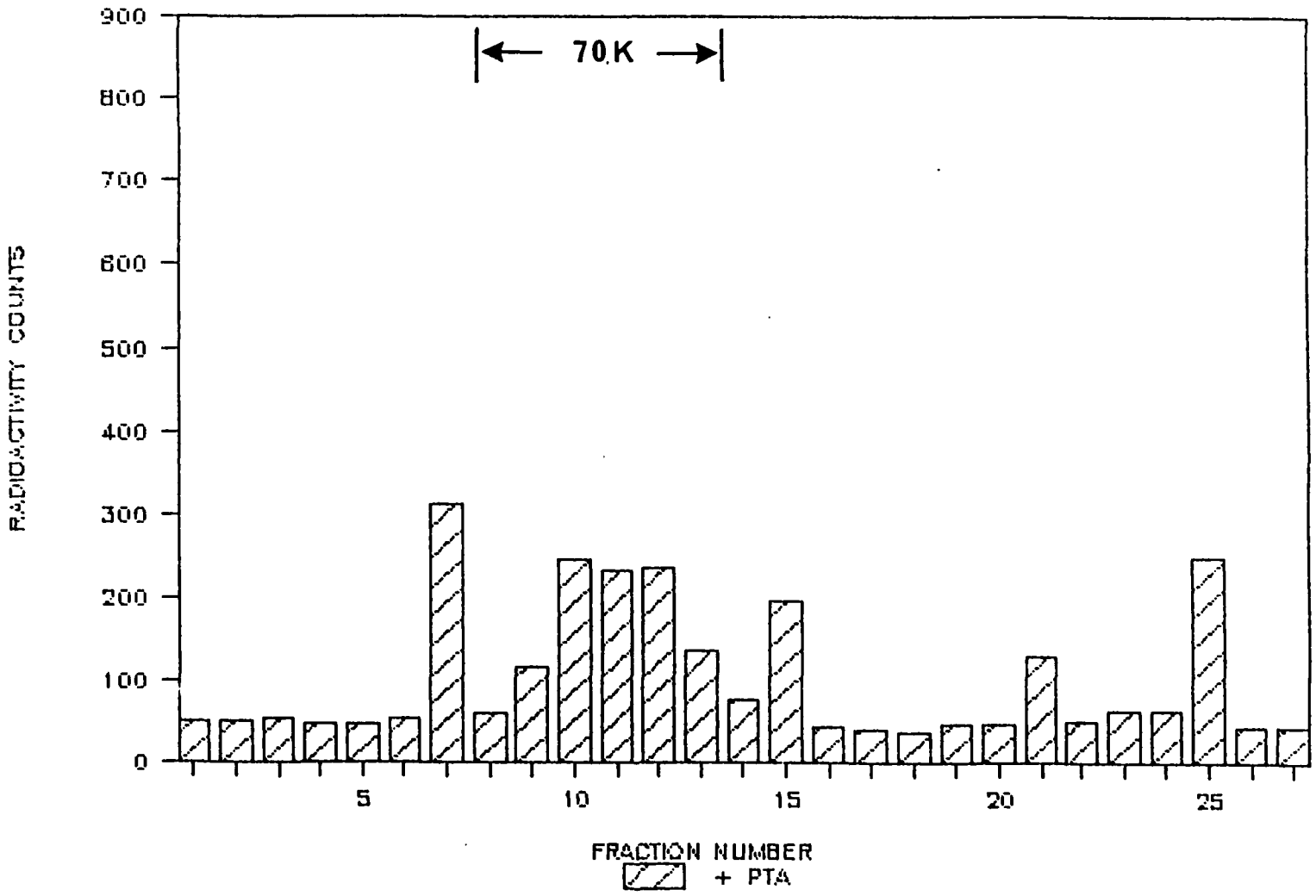

Figure 7. Histogram: Effect of PTA on Reaction of [^{14}C]-BrPin with AcChE. Radioactivity in Sequential Segments of Electrophoresis Gel.

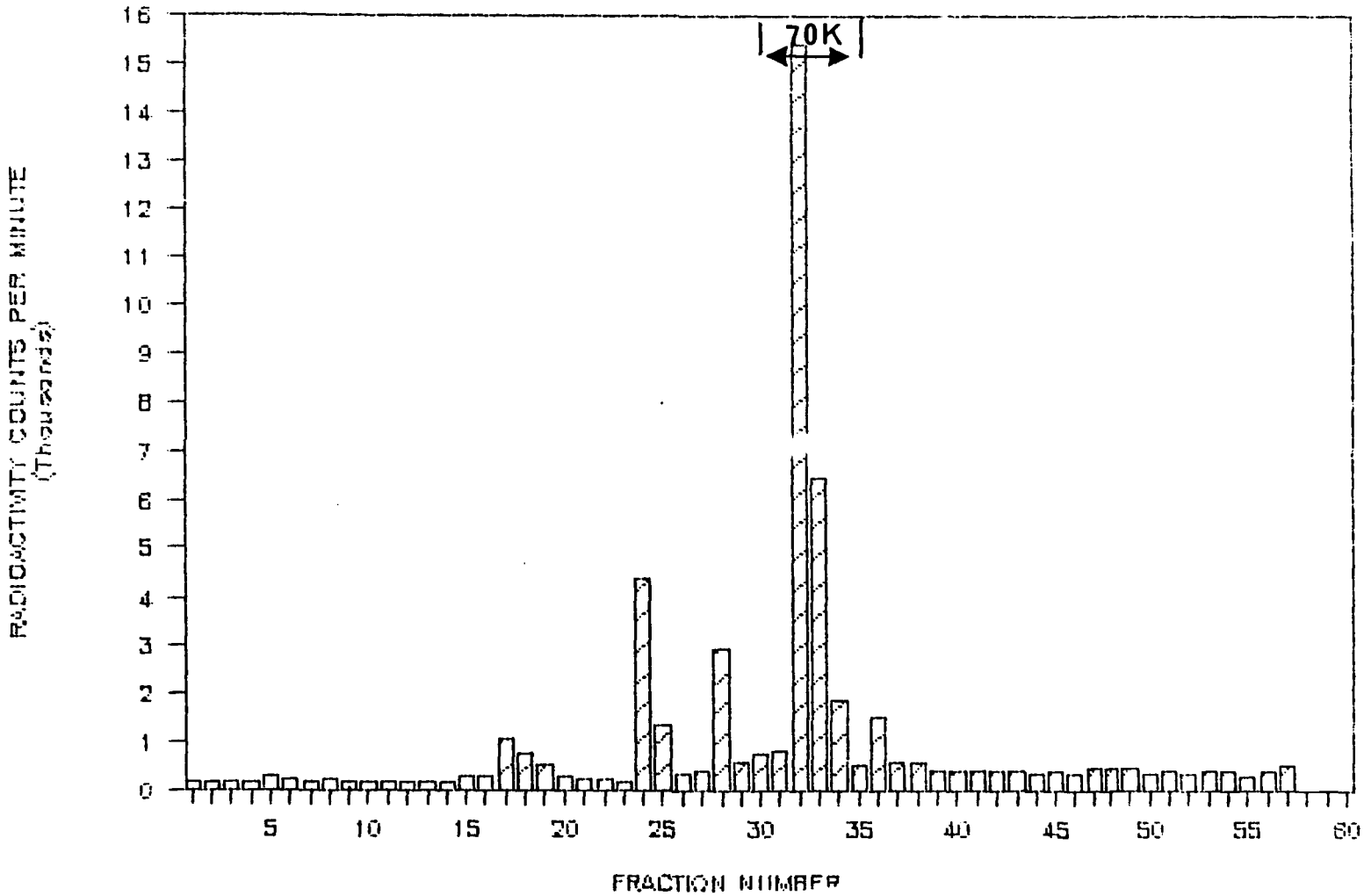

Figure 8. Histogram: Reaction of [^{14}C]-BrPin with AcChE in Absence of TAP. Radioactivity in Sequential Segments of Electrophoresis Gel.

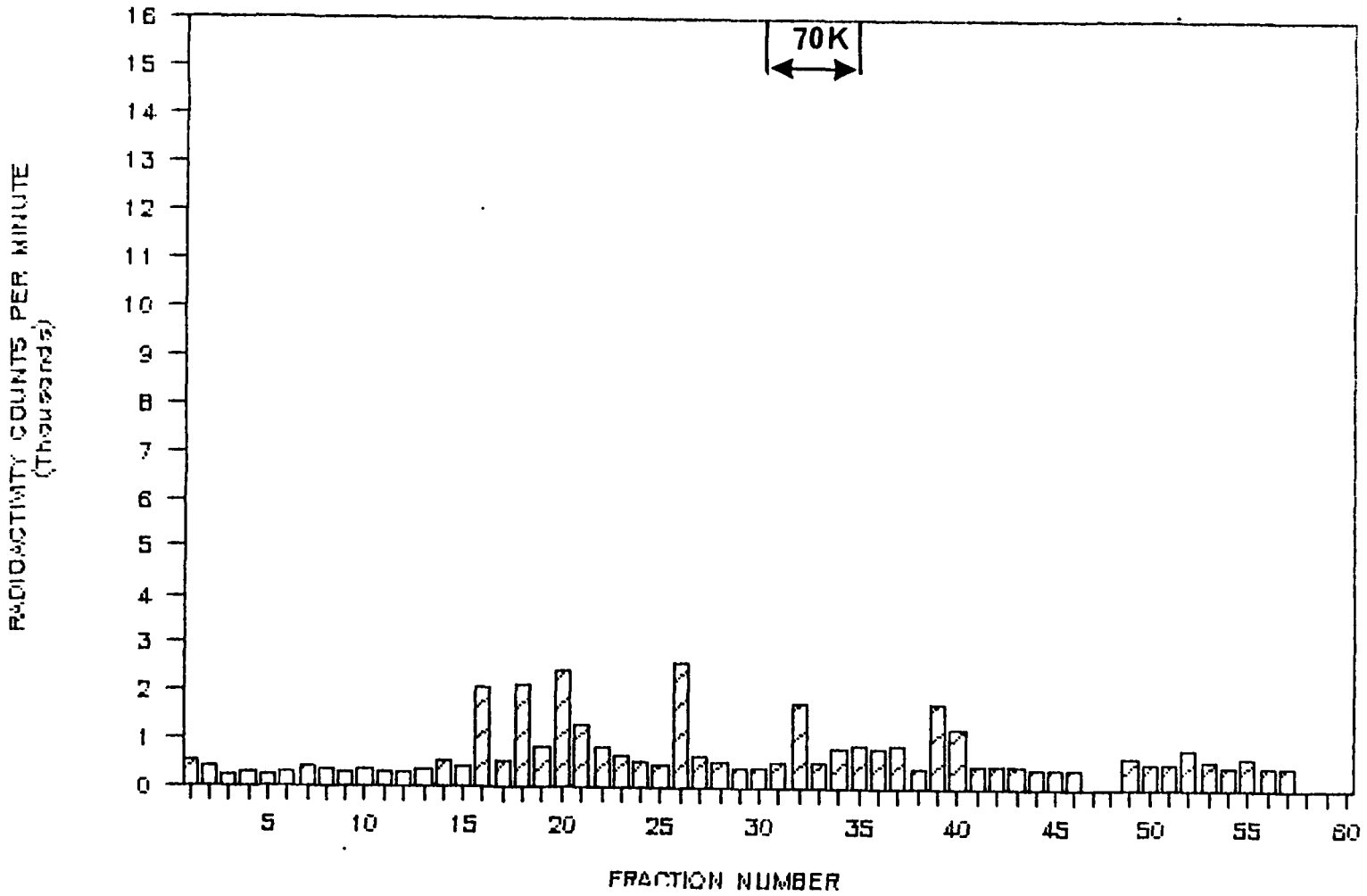

Figure 9. Histogram: Effect of TAP on Reaction of [^{14}C]-BrPin with AcChE. Radioactivity in Sequential Segments of Electrophoresis Gel.

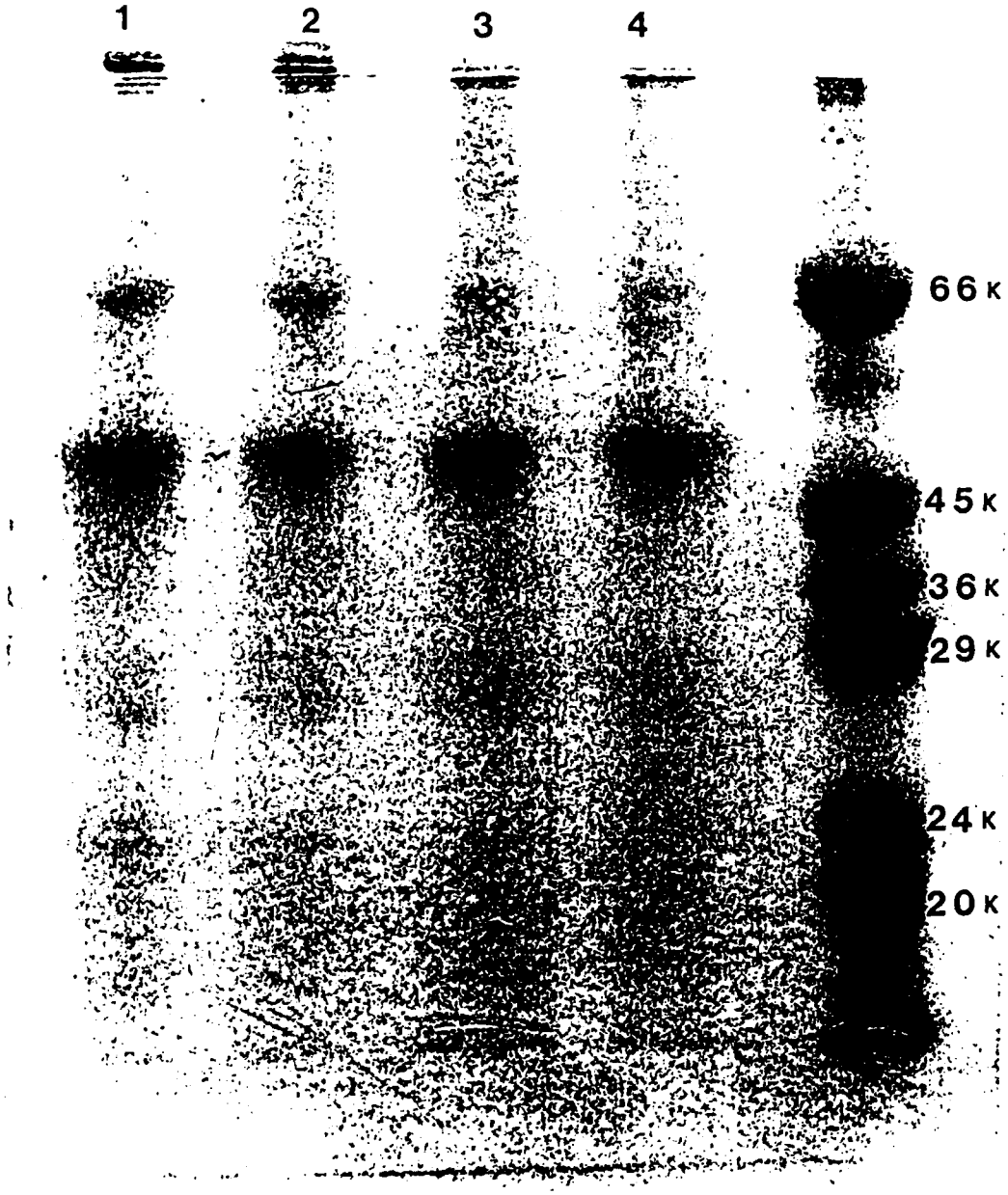

Figure 10. PAGE of [^{14}C]-BrPin and [^{3}H]-DFP labeled AcChE after formic acid cleavage: Lanes 1 and 2, [^{3}H]-DFP; lanes 3 and 4 [^{14}C]-BrPin.

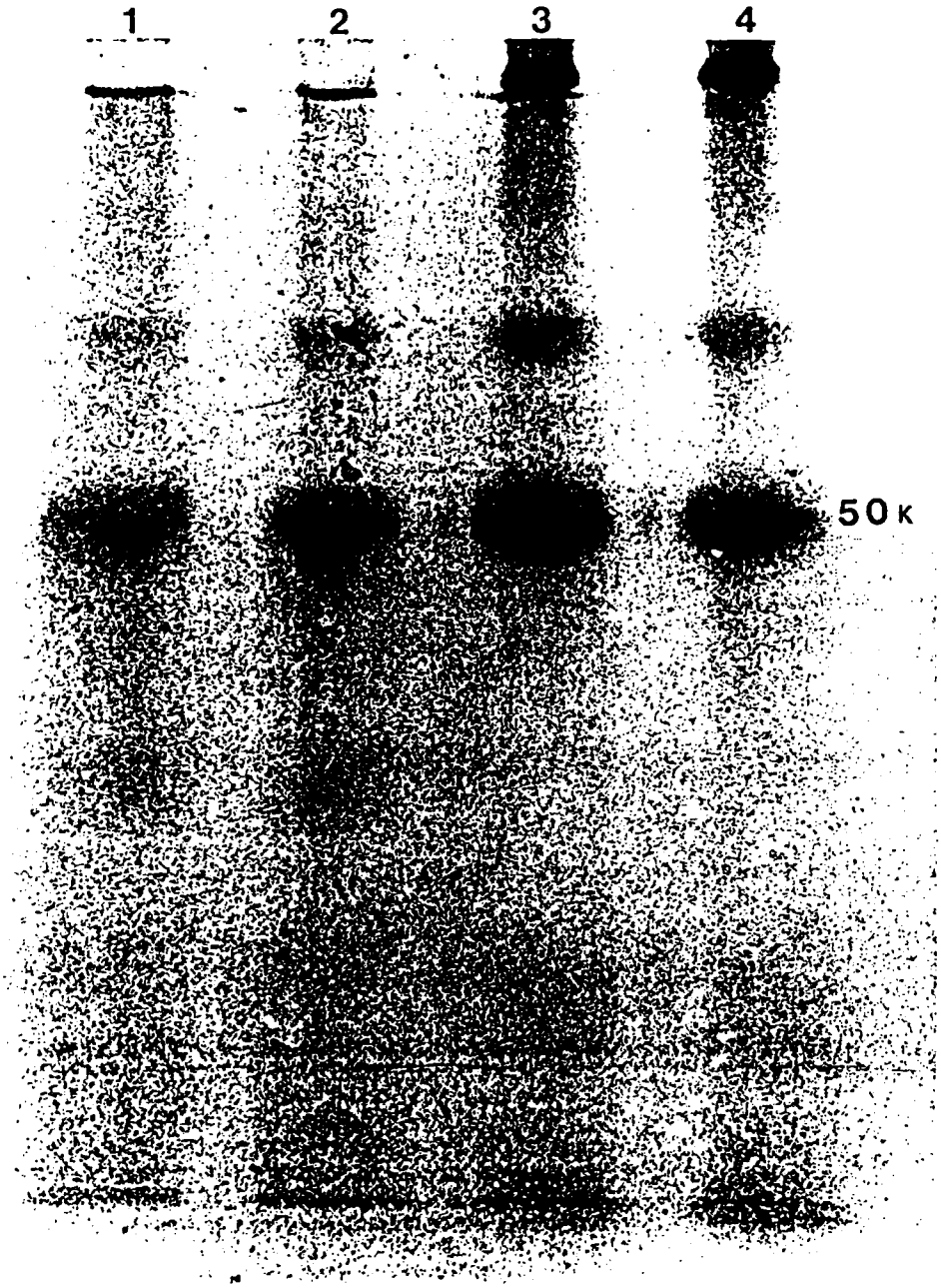

Figure 11. Autoradiograph of PAGE of Figure 10: Lanes 1 and 2, [^{14}C]-BrPin; lanes 3 and 4, [^{3}H]-DFP.

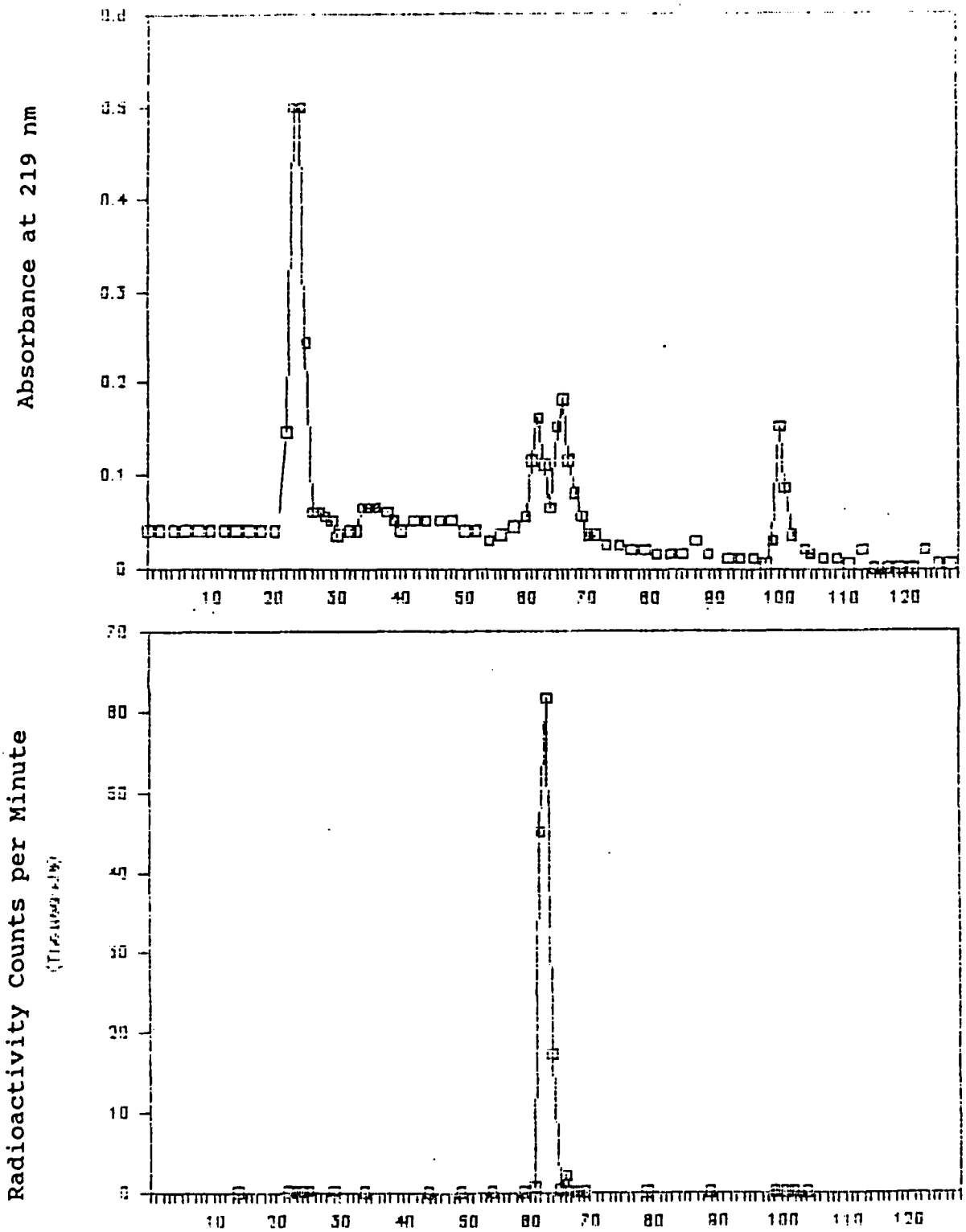

Figure 12. Gel Filtration of Peptide Fractions.

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LIST OF PUBLICATIONS

"Substituted Benzenes and Phenols as Reversible Inhibitors of Acetylcholinesterase: Polar, Trimethyl, and Synergistic Effects," Cohen, S. G., Chishti, S. B., Reese, H., Howard, S. I., Solomon, M., and Cohen, J. B. (1987) Bioorg. Chem. **15**, 237-249.

LIST OF PERSONNEL

1.	Dr. Saul G. Cohen, PI	
2.	Dr. Zeev Tashma	September 1983 - August 1984
3.	Dr. S. Bano Chishti	September 1983 - June 1987
4.	Dr. Michael Solomon	February 1984 - January 1986
5.	Dr. Sachiko Howard	November 1984 - June 1987
6.	Dr. Erdjan Salih	April 1986 - June 1987
7.	Mrs. Ethel Crain, Secretary	

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GLOSSARY OF ABBREVIATIONS

AcChE	Acetylcholinesterase
AcCh	Acetylcholine
BrPin	1-Bromopinacolone
[^{14}C]-BrPin	1-Bromo-2-[^{14}C]-pinacolone
Br_2Pin	1,1-Dibromopinacolone
[^{14}C]-Br_2Pin	1,1-Dibromo-2-[^{14}C]-pinacolone
DFP	Diisopropyl fluorophosphate
[^{3}H]-DFP	1,3-[^{3}H]-Diisopropyl fluorophosphate
DMBAc	3,3-Dimethylbutyl acetate
DPA	N,N-Dimethyl-2-phenylaziridinium ion
MBS	Methyl benzenesulfonate
MMS	Methyl methanesulfonate
MR	Molar refractivity
MSC	Methylsulfonyl chloride
MSF	Methylsulfonyl fluoride
PAGE	Polyacrylamide gel electrophoresis
PAM	2-Methylpyridinium aldoxime
PTA	Phenyltrimethylammonium ion
TAP	5-Trimethylammonio-2-pentanone
TMAP	3-Trimethylammoniophenol

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