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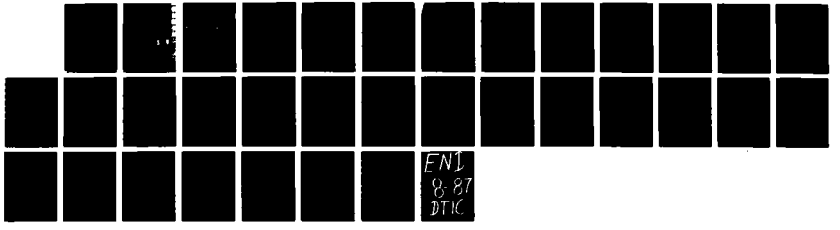

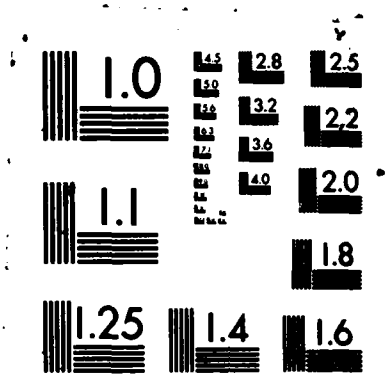

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ACETYLCHOLINESTERASE AND ACETYLCHOLINE RECEPTOR

Annual Report

Saul G. Cohen, Ph.D.

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We are studying the properties of the active site, and the region peripheral to it, of acetylcholinesterase (AcChE), the enzyme which hydrolyzes and thus terminates the action of the important neurotransmitter acetylcholine (AcCh). We have studied the response of the enzyme to synthesized substrates and inhibitors of varied structure for indications about the parts of the enzyme that are complementary to these substrates. From this information radioactive active-site-directed irreversible inhibitors have been designed for use in labeling amino acids in the active site. In this work AcChE was isolated from Torpedo nobiliana. Novel uncharged reversible inhibitors were also prepared; they may have potential application. Compounds were also prepared for study of their action on the AcCh receptor by Professor J.B. Cohen, Department of Anatomy and Neurobiology, Washington University School of Medicine. St. Louis.

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SUMMARY

Appropriate literature and work in our laboratory are reviewed, which have led to our proposal that the part of the active site of AcChE at which the cationic β-substituent of AcCh binds is not anionic, as is generally accepted, but is uncharged and complementary to the spherical shape of the substituent. Since there are anionic charges peripheral to the active site with which cationic reagents, intended to label an amino acid in the active site, may react, we propose to use uncharged active-site-directed labeling reagents. 1-Bromopinacolone, $BrCH_2COC(CH_3)_3$ (BrPin) has been selected as the first such reagent for detailed study.

AcChE was isolated from Torpedo nobiliana, and its inactivation by ^{3}H-DFP and BrPin has been studied. The procedure for synthesis of BrPin on a 60 μmole scale, from (i) reaction of CH_3COCl and $(CH_3)_3CMgCl$ to form pinacolone, $CH_3COC(CH_3)_3$, (ii) followed by bromination is being worked out, for application to synthesis of $BrCH_2{}^{14}COC(CH_3)_3$ (^{14}C-BrPin).

Properties of the binding site for the β-substituent have been examined further by study of reversible inhibitors containing $(CH_3)_3Si$-, $(CH_3)_2N^+(O^-)$-, and $CH_3S(O_2)$- groups. The site binds $(CH_3)_3Si$- and $CH_3S(O_2)$- groups as well as it does $(CH_3)_3C$-, but the $>N^+(O^-)$- group decreases binding. The latter substituent, as well as $CH_3S(O_2)$-, $CH_3S(O)$- and CH_3S- decrease substrate reactivity.

Mono- and di-substituted benzene derivatives bind reversibly to AcChE. Binding is increased by electron withdrawing and $(CH_3)_2N$- substituents, and is described by the Hammett relationship, with a value of ρ of 2. An aryl binding site is proposed adjacent to the trimethyl and esteratic sites. Strong synergism is found in effects of meta-tert-butyl and meta-trimethylammonio groups with phenolic hydroxyl group.

FOREWORD

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(1) Statement of the Problem Under Study

We are studying the properties of the active site, and the region peripheral to it, of acetylcholinesterase (AcChE), the enzyme which hydrolyzes and thus terminates the action of the important neurotransmitter acetylcholine (AcCh). We have studied the response of the enzyme to synthesized substrates and inhibitors of varied structure for indications about the parts of the enzyme that are complementary to these substrates. From this information, radioactive active-site-directed irreversible inhibitors were designed for use in labeling amino acids in the active site. In this work AcChE was isolated from Torpedo nobiliana. Novel uncharged reversible inhibitors were also prepared; such compounds may have permeation property better than that of cationic inhibitors, and potential use as medicinals.

(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 groups. 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 methylation of alkylammonium ions, starting with methylamine and ethanolamine, improves binding, and trimethylation of substrates starting with β-aminoethyl acetate increases both binding and reactivity, and the 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 there is no negative charge in the "anionic" binding site (7). However, this calculation did not take into account decrease in solvation of ammonium ions with increasing 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 β,β-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, 2×10^6 $M^{-1}sec^{-1}$ (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 substituents, and, CH_3O, HO, Cl, Br, N≡C and H, Table I (12). The normalization factors were the ratios of rate constants of base catalyzed hydrolysis of AcCh to 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 (V^*_{25}) of the β-substituents, X, eq. 1, Figure 1.

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

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 for AcCh and DMBAc are similar, indicating no substantial coulombic effect, and the effects on binding in the other cationic and uncharged parts of substrates were even smaller, Table I.

The effect of the positive charge in AcCh is to increase the intrinsic reactivity over that of DMBAc by a factor of about 25, 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 hydrobicity (π) of the β-substituents of alkyl esters, II, IV, VI and VIII (10). Hydrophobicity is a parameter derived from 2-octanol/water distribution coefficients; it is related to capacity for hydrophobic interactions and used in drug design (13). For these hydrocarbon substituents, values of π are proportional to volume, but use of this parameter does not allow the non-polar and the water-soluble ammonio derivatives to be correlated on the same scale. The correlation with volume, Figure 1, 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/mol 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 (16).

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-tert-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 and inhibitors; 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 1-bromopinacolone, $BrCH_2COC(CH_3)_3$ (BrPin) (20). This uncharged reagent inhibited AcChE irreversibly with the same rate for hydrolysis of 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.

While we use the term trimethyl for the binding site for β-substituents of substrates and inhibitors related to them, this site also accommodates other groups. As described in the previous Annual Report (21), trichloroethanol (Cl_3CCH_2OH) binds similarly to its carbon analogue, neopentyl alcohol, and chloral binds better than its carbon analogue, pivalaldehyde. In the latter case the effect of the chlorine in rendering the carbonyl electrophilic may be important.

In an extension of the study of the relation of enzymic reactivity to the volume of the β-substituent of β-X-ethyl acetate substrates, we substituted refraction volumes (MR) (22-25) for apparent molal volume ($V_2^*{}_5$) in eq. 1, allowing us to

examine a broader range of substrates containing more hydrophilic and larger β-substituents, Figure 2 (26). The larger silyl compound, $(CH_3)_3SiCH_2CH_2OCOCH_3$, was accommodated in the active site, binding slightly better and with slightly greater k_{cat} than the analogous carbon compound and, possibly, with slightly greater normalized reactivity than AcCh. Essentially, normalized reactivity levels off at the volume of the natural substrate. On the other hand, hydrophilic β-substituents, methylsulfonyl (CH_3SO_2-), dimethylamine oxide [$(CH_3)_2N^+(O^-)$-] and methylsulfoxy (CH_3SO-) led to substantially lower reactivities than were consistent with their MR values. However their reactivities, like those of the reactive $(CH_3)_3N^+$- and $(CH_3)_2S^+$- compounds, were substantially higher than was consistent with hydrophobicity (π) (13,22,27-29), and reactivities of the cationic compounds correlated with MR. Overall, reactivity correlates with volume (V^*_5 or MR) better than with hydrophobicity.

It is noteworthy that the hydrophilic surface of the β-CH_3SO_2- substrate did not decrease its binding as compared with that of the analogous $(CH_3)_3C$- compound (26). The effect was on k_{cat}, indicating that desolvation, removal of water, is, paradoxically, an important aspect of enzymic hydrolytic reactivity (30).

In the last Annual Report (21) we described results on other novel irreversible and reversible inhibitors; these results are summarized briefly here. Chloromethyl pivalate [$(CH_3)_3CCO_2CH_2Cl$] and chloromethyl acetate ($CH_3CO_2CH_2Cl$) may alkylate a nucleophile in the active site; then, if the bound ester group hydrolyzes, the hydroxymethyl substituent may react further to alkylate again and possibly form a cross-link. The two compounds appear to have similar inactivating effectiveness. Trimethylammoniomethyl acetate [$CH_3CO_2CH_2N^+(CH_3)_3$] is a cationic analogue which may behave similarly. It may be hydrolyzed, as a lower homologue of choline, and may eject the potential alkylating group. It seems to be a less effective inactivator than the chloromethyl compounds.

Methyl methanesulfonate and methyl benzenesulfonate apparently act as methylating agents. The benzene derivative is of interest in comparison to styrene oxide, which may introduce an aryl group as it inactivates irreversibly, and also in comparison to a series of substituted-benzene reversible inhibitors, which act largely competitively and may be directed against the active site. These compounds will be discussed in the results section of this report.

(3) Rationale Used in the Current Study

(i) BrPin has properties of an active-site-directed

inactivator, since it is equally effective against substrates of widely varied structure and its own action is retarded by substrate-related reversible inhibitors (20). This year ^{14}C-labeled BrPin [$BrCH_2{}^{14}COC(CH_3)_3$] has been examined as a radioactive label for the active site of AcChE. The enzyme was isolated from Torpedo, or eel enzyme was purchased from Sigma and purified.

(ii) Properties of the subsite at which the β-substituents of β-X-$CH_2CH_2OCOCH_3$ substrates bind have been explored by study of the reactivity of such substrates (16). In the current year they have been studied further with structurally related reversible and irreversible inhibitors.

(iii) Simple benzene derivatives have been found to retard and accelerate hydrolysis by AcChE in a manner comparable to those of common cationic and our newly studied uncharged aliphatic inhibitors. Hence a more detailed study of aromatic inhibitors was performed this year.

(4) Experimental Methods.

Isolation of AcChE from frozen electric organ of Torpedo nobiliana was described in the revious Annual Report (21). Literature procedures were essentially followed for enzyme isolation (16,31), for its purification by affinity chromatography (32,33), for the Ellman assay (34), for enzyme characterization by gel electrophoresis (35,36), and examination of gels labeled with [^3H]diisopropylfluorophosphate (^3H-DFP) [New England Nuclear (NEN) Boston, Massachusetts] by exposure to X-ray film (37). pH stat kinetic studies were carried out as described previously (12,14), the value of k_{cat} of 3.3×10^5 min^{-1} for Torpedo californica was used (38).

Analysis for BrPin when synthesized on micromolar scale was carried out by gas-liquid chromatography (glc) on 10% SE 30 chromosorb WHP 100/120, 110°C, Perkin-Elmer 990 gas chromatograph.

(5) Results

A. Inactivation of AcChE

Acetylcholinesterase, about 10 mg, was isolated from electric organ of Torpedo, purified as described in the previous Annual Report (21), and stored in pH 8 buffer at -70°C. It hydrolyzes AcCh with K_m of 0.06 mM, compared with 0.33 mM for Sigma eel enzyme. 1-Trimethylammonio-4-pentanone (TAP) has K_i

of 0.05 mM as compared with 0.02 mM for the eel enzyme. BrPin, has K_i of 0.2 mM, the same as its K_i for eel enzyme.

Gel electrophoresis at high loading, >11 μg of protein, showed small bands at 133,000 D, 107,000 D, and 95,000 D, and a major band at 70,000 D comprising about 75% of the total. Treatment with 1.3 μM ^{3}H-DFP for 1 hr and autoradiography showed tritium incorporation at 70,000 D and 133,000 D, the latter band apparently consisting of a dimer. Pretreatment with 1 mM carbamyl choline for 1 hr prevented incorporation of ^{3}H-DFP.

Inactivation by ^{3}H-DFP was studied along with that by other inactivators, BrPin, methyl sulfonates, and styrene oxide, for information as to their mode of action, while the synthesis of ^{14}C-BrPin was being worked out. The course of inactivation of Sigma AcChE by ^{3}H-DFP was followed at 0° and 25°C for guidance in study of the purer but less readily available Torpedo enzyme, Table III. Since the ^{3}H-DFP was obtained in propylene glycol, the stability of DFP in the hydroxylic solvent and thus its true concentration was investigated. The correct concentration would be important in labeling and counting experiments if the radioactive reagent was diluted with unlabeled DFP. A solution of unlabeled DFP in propylene glycol was obtained from NEN for use in model experiments and found to have no inhibiting activity. We turned to neat DFP from Sigma and made our own dilutions. Concentrations and times for half-inacitvation ($t_{1/2}$) of Sigma eel AcChE for this DFP were: 48 μM, 2 min; 10 μM, 4 min; 2 μM, 18 min; 1 μM 38 min; and 0.5 μM, 78 min. The value of $t_{1/2}$ for a nominal concentration of 2 μM ^{3}H-DFP was 30 min, corresponding to an effective concentration of 1.4 μM, 30% less than that indicated. This was confirmed by inactivation of Torpedo AcChE by ^{3}H-DFP and 3:1 DFP:^{3}H-DFP, with ^{3}H-DFP content based on the effective concentration, 70% of nominal. Observed counts after dialysis were in the ration of 3.9:1.

Sigma AcChE was inactivated by 7 μM and 1.4 μM ^{3}H-DFP for 1 hr and subjected to gel electrophoresis and autoradiography. The gels were streaky, with many bands and much low molecular weight material. The 7 μM ^{3}H-DFP sample showed autoradiograph bands at 57,000, 53,000, 34,000 and 31,000 D; the 1.4 μM sample gave labeled bands only at 57,000 and 53,000 D.

Sigma AcChE samples that (i) were 98% inactivated by 20 hr treatment with 3.8 mM BrPin or (ii) were not pretreated with BrPin were 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 55,000 and 50,000 D.

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 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 64,000 cpm is due to non-specific reaction of impure Sigma AcChE and incomplete dialysis, subtraction leads to 162,000 cpm for (ii), 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) 16,5000 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 the remaining 47% inactivation by DFP. 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 this 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 6.15×10^{-11} moles of Torpedo AcChE used in these experiments, 100% inactivated by ^{3}H-DFP, was 183,000 cpm, to be compared with 204,000 cpm observed, and 188,000 cpm corrected, and in the enzyme inactivated about 69% by BrPin, 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 or with other complete or partial inactivators 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 that in part totally inactivated and in part totally active. This may be evidence for the specificity of action of BrPin.

B. Synthesis of BrPin, 60 μmole Scale

Many experiments were carried out on the micromole scale synthesis of BrPin that would be required for synthesis of the radioactive material. Preliminary large-scale experiments were carried out on 500 μmole of CH_3COCl and 400 μmole of 2 M $(CH_3)_3CMgCl$ in 200 μL of tetrahydrofuran (THF), to form

pinacolone. A substantial by-product was detected, which was identified as tert-butyl acetate by its retention time and conversion by 6 M HCl to tert-butyl alcohol and tert-butyl chloride. It is formed from CH_3COCl and $(CH_3)_3COH$, the latter being formed from the Grignard reagent and O_2 or peroxide present in THF solvent. Air or peroxide and moisture must be rigorously excluded in the small-scale synthesis by this procedure.

Other possible syntheses of pinacolone were examined: treatment of $(CH_3)_3CMgCl$ with ethyl and methyl acetate, or with $CdCl_2$ prior to treatment with CH_3COCl, failed to form pinacolone in good yield. Then it was found that CH_3COCl and $(CH_3)_3CMgCl$, 60 μmole, in the presence of about 0.5 mg Cu_2Cl_2 catalyst, led reproducibly to pinacolone in >80% yield (39). However, Cu^{++} was found to inactivate AcChE instantaneously. Complexing with ethylenediaminetetraacetic acid (EDTA) retarded but did not stop this inactivation; Cu^{++}, formed from Cu^{+} by air oxidation or in the subsequent reaction with Br_2, will have to be removed completely from synthesized ^{14}C-BrPin.

$CH_3{}^{14}COCl$ obtained from NEN in a sealed ampoule, was transferred by pipette, with rinsing, to a Reacti-vial, where it was treated with the Grignard reagent. Unlabeled CH_3COCl in THF in the standard sealed ampoule, obtained from NEN for use in a model experiment, failed to yield the desired product. We decided to forego the NEN dilution with solvent and make our own dilutions.

In a series of brominations of pinacolone on 20-400 μmole scale it was difficult to achieve reproducibility. Room light had no effect. Added acetic acid and $AlCl_3$ had no effect. Aqueous HCl prevented bromination. It was determined that small amounts of water led to low or no yields of BrPin. After washing to remove copper and magnesium salts present in the pinacolone synthesis, it proved difficult to dry THF, the solvent in which $(CH_3)_3CMgCl$ was obtained; this problem led to irreproducible results. The Grignard reagent was then obtained in ether and the bromination proceeded well. Bromination with equivalent quantities of bromine and pinacolone leaves residual bromine, which inactivates AcChE instantaneously and must be washed out. After the period covered by this report it was found that the mixture formed in the reaction of CH_3COCl with the Grignard reagent could be brominated directly without washing. Residual bromine, Cu^{++} and other salts can be washed out readily after the bromination. Successful micromolar synthesis of BrPin, suitable for inactivation and labeling AcChE, was achieved. The use of $CH_3{}^{14}COCl$ was worked out after the period covered by this report.

C. Sulfonyl, Sulfonate and Related Compounds

In further study of the effects of charge, volume and surface properties on binding at the trimethyl site, supplementing our work on substrates (26), a series of related reversible inhibitors of AcChE was tested (40). 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), bind similarly, K_i = 0.4 mM, have K_m values similar to those of their acetate substrates, and bind more strongly than the uncharged alcohols by about 1.5 kcal/mol. In comparisons of VII with $CH_3SO_2CH_3$, II with $(CH_3)_3S^+$, and IB with $(CH_3)_4N^+$, hydroxyethyl leads to more favorable binding than methyl by about 0.8 kcal/mol, 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/mol. Conversion of $(CH_3)_3S^+$ to $(CH_3)_3S^+O^-$ also improves 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/mol. 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, with values of K_m about 16 mM.

Inhibitors containing the methanesulfonyl group (CH_3SO_2-), a polar analogue of the tert-butyl group, are being studied. The effectiveness of methanesulfonyl chloride (MSCl) is similar to that of methanesulfonyl fluoride (CH_3SO_2F; MSF). These compounds introduce methanesulfonyl groups. Irreversible inhibition by MSF is not retarded efficiently by TAP. It is accelerated by small quaternary ammonium compounds (41). Irreversible inhibition by methyl methanesulfonate ($CH_3SO_2OCH_3$; MMS), a methylating agent, is not retarded efficiently by $(CH_3)_4N^+$. Hydrolysis of methylsulfonylethyl acetate ($CH_3SO_2-CH_2CH_2OCOCH_3$; MSAc) by AcChE is retarded efficiently by TAP. The small CH_3SO_2- inhibitors may not utilize the trimethyl subsite and thus their action may not be retarded, and may be accelerated, by small cations. It is difficult to account for the inefficiency in retardation by TAP of inhibition by methanesulfonyl compounds.

Methyl benzenesulfonate ($C_6H_5SO_2OCH_3$; MBS), a methylating agent, at 3 mM inactivated AcChE 70% in 1 hr and 90% in 2 hr;

this inactivation was not retarded by 29 mM $(CH_3)_4N^+$. Benzene derivatives related to MBS are reversible inhibitors with substantial competitive activity. It appears that the active site can accommodate the $(CH_3)_4N^+$ at the trimethyl subsite, and the benzenesulfonyl group at another site.

While simple tert-butyl and trichloromethyl compounds were moderately effective reversible inhibitors, tert-butyl isothiocyanate and trichloromethylethylene oxide were not effective irreversible inhibitors, unlike the related compounds BrPin and styrene oxide.

Although methylsulfonylethanol and its acetate bound to AcChE similarly to the analogous tert-butyl compounds, 1,3-bis-methylsulfonylpropane ($CH_3SO_2CH_2CH_2CH_2SO_2CH_3$), which is soluble only to about 1 mM, did 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 decreasing activity by about 30%. 2-Methylsulfinylethanol will be examined.

D. Reversible Inhibition by Derivatives of Benzene and Phenols

An aspect of a study of inhibition of AcChE by benzene and some mono- and di-substituted derivatives was completed. In this study effects of substituents are satisfactorily interpreted in terms of the parameters of the Hammett equation (42), in which σ-values are measures of the electron withdrawing (positive values) and electron donating (negative values) power of the substituents, and ρ is a measure of the sensitivity of the interaction to the electronic effect, positive values arising when effects are increased by electron withdrawal. Results are summarized in Table V and as follows:

Benzene and 14 mono- and di-substituted derivatives, and styrene oxide, pyridine and 4-tert-butylpyridine were examined as inhibitors for hydrolysis of AcCh by AcChE. Linear relations between binding, log $1/K_i$, and summed σ-values were observed as follows: (i) In competitive inhibition, binding of C_6H_5-Y, where Y = H_2N (IX), H (I), CH_3CONH (II), CH_3CO (III), and O_2N (IV), and binding of di-substituted compounds para-O_2N,$NHCOCH_3$ (XI), meta-O_2N,NH_2 (XII), and para-H_2N,$COCH_3$ (XVI) led to ρ of 1.85 ± 0.09, correlation coefficient (r) of 0.99, with K_i values from 46 mM for IX to 0.63 mM for IV, and 0.46 mM for XI. Inclusion of meta-O_2N,$NHCOCH_3$ (X) gave K_i of 0.26 mM, and para-O_2N,NH_2 (XIII) led to ρ of 2.03 ± 0.15 and r of 0.98. (ii) The di- and trialkylamino compounds with substituents $(CH_3)_2N$ (VIII), $(CH_3)_3N^+$ (VI), meta-$(CH_3)_2N$,NO_2 (XIV), para-$(CH_3)_2N$,NO_2 (XV), and para-$(CH_3)_2N$,$COCH_3$ (XVII) bound with

higher values of ρ, 2.36 ± 0.16, and r, 0.99; K_i values ranged from 11 mM for VIII to 0.091 mM for XV and 0.077 mM for VI. The cationic charge in VI had no effect beyond that of its σ-value. The fact that binding was increased by electron withdrawal is attributed to charge-transfer interaction. CH_3 groups of $(CH_3)_3N$ increase competitive binding by interaction in the trimethyl site. The effect is consistent with that of CH_3 groups in the pyridine, tert-butylpyridine pair. The CH_3CO- substituent leads to enhanced noncompetitive binding. The substituent pairs meta- and parar-$(CH_3)_2N,NO_2$ and para-H_2N,NO_2 are mutually reinforcing; para-$(CH_3)_2N,COCH_3$ interaction is small. Styrene oxide inhibits irreversibly.

There is evidence that protein tertiary and quaternary structure is stabilized by aromatic-aromatic interactions of amino acid side chains, contributing 1 to 2 kcal/mol per interaction (44). The effects found in this study indicate that added aromatics may compete with these stabilizing actions, affecting structure and, in the case of AcChE, deceasing reactivity. This may be a general phenomenon that is found with other enzyme which, like AcChE, have no apparent involvement of aromatic groups in their normal substrate interactions. In the present case the added aromatics act as electron acceptors, with enzyme groups as donors; in others the reverse may be true.

This study is now being extended to derivatives of phenol. Some data are summarized in Table VI.

In phenol the hydroxyl group led to binding weaker than benzene, and similar to that of aniline, consistent with the hydroxyl σ-value. Effects of the tert-butyl group are remarkable. In the meta position (III), it increased competitive binding over that of phenol (I) by 4.5 kcal/mol and noncompetitive binding by 3.8 kcal/mol, far greater than its effects on pyridine (Table V). However, when tert-butyl was in the para position (IV) no inhibition was observed up to 0.65 mM, a limit set by its low solubility. In 4-tert-butylcatechol (VII) 25% of the binding energy of the 3-tert-butylphenol was lost in both binding modes, the para-hydroxyl causing this decrease. Binding of tert-butyl in the trimethyl site led to a specific interaction of the meta-hydroxyl, but not of the para. However, in the nitrophenols different effects were observed. In the meta compound, the hydroxyl decreases binding, consistent with its positive σ, thus not affecting the nitrophenyl binding per se. But in the para position it removes all competitive binding and retains the noncompetitive binding characteristic of nitrobenzene. Those are remarkable effects and will be studied further.

The effect of the meta-tert-butyl in III indicates that it fits into the trimethyl site, and that that the benzene ring itself does not utilize this part of the site. This is

consistent with the observation that tetramethylammonium ion does not retard inactivation by methyl benzenesulfonate. However, the benzene ring is in the active site and the benzenesulfonate remains an interesting labeling compound. The non-synergistic action of O_2N and HO in V supports the view that O_2N makes no specific steric interaction in the active site and acts only by electron withdrawal, as indicated by the σ relation.

The strong binding of meta-tert-butylphenol (III) leads to an interesting application of the ρ-σ relation determined in the study of benzene derivatives (Table V). meta-Trimethylammoniophenol, the cationic analogue of III, was reported (45) to bind strongly, with K_i of 0.00031 mM and ΔG of -8.8 kcal/mol; III, with K_i of 0.024 mM, has ΔG of -6.24 kcal/mol. The difference in the σ-values of $(CH_3)_3C-$ and $(CH_3)_3N^+-$, 0.94 (Table V) and application of the Hammett relationship, $\log K - \log K_o = \rho\Delta\sigma$, where $\log K_o$ refers to meta-tert-butylphenol and has the value of 4.62, and ρ has the value of 2.36, for polymethylamino compounds, above, leads to the value of log K for meta-trimtheylammoniophenol, 6.84. This corresponds to ΔG = -9.2 kcal/mol for the binding energy of the cationic compound, the same, within the error of such measurements (Table V), as the value reported in 1958! (45). It will be interesting to check this measurement. This is an important compound, which we will prepare. Our study of aromatic inhibitors leads to a reliable correlation of inhibitory power to structure which has predictive value for the effects of hitherto unstudied compounds.

(6) Discussion and Conclusions

We were able to isolate and purify AcChE from Torpedo nobiliana, and to purify commercially available eel AcChE. We can now prepare ^{14}C-BrPin on a 60 μmole scale, and we will study the incorporation of this labeled inactivator into the enzyme, and the effects of other active-site-directed reversible and irreversible inhibitors on this incorporation. We will undertake to identify the group or groups labeled by this compound.

We studied the effects of novel irreversible alkylating inhibitors -- BrPin, methyl methanesulfonate, methyl benzenesulfonate and styrene oxide -- on subsequent incorporation of the inhibitor ^3H-DFP, which acylates the active site serine. Partial inactivation of Sigma eel AcChE by BrPin leads to a proportional decrease in subsequent incorporation of ^3H-DFP. A similar result is obtained with Torpedo AcChE, but this is somewhat more difficult to assess quantitatively because of the very rapid inactivation of this enzyme by BrPin. The partially inactivated material appears to comprise fully inactivated and

uninactivated material, supporting the specificity of action of this inhibitor. This study will be extended to the sulfonate- and benzene-related inhibitors.

We explored the active site of AcChE by studying the reactivity of β-X-$CH_2CH_2OCOCH_3$ substrates, and reversible and irreversible inhibitors related to these substrates. We have termed the subsite at which the β-X substituent binds trimethyl rather than anionic because of the relative reactivity and binding of analogous compounds in which X is $(CH_3)_3C$- and $(CH_3)_3N^+$-. We found that $CH_3S(O_2)CH_2CH_2$- compounds bind as well as analogous $(CH_3)_3CCH_2CH_2$- compounds, indicating that trimethyl may also be too specific a name. Small CH_3SO_2- compounds, CH_3SO_2Cl and $CH_3SO_2OCH_3$, inactivate AcChE as they acylate and alkylate, respectively. These processes are not retarded by $(CH_3)_4N^+$ ion, indicating that they do not utilize the trimethyl subsite, unlike the corresponding substrate, $CH_3SO_2CH_2CH_2OCOCH_3$.

Simple mono- and di-substituted benzene compounds, containing as substituents CH_3CONH-, CH_3CO-, O_2N-, H_2N-, $(CH_3)_2N$-, and $(CH_3)_3N^+$-, act as reversible inhibitors for AcChE, in which inhibiting efficiency increases with electron attraction by the substituent as indicated by correlations with σ-values. K_i values fall from about 50 to 0.1 mM. Binding is also increased by $(CH_3)_2N$- amd $(CH_3)_3C$- substituents, indicating that these occupy the trimethyl site and that the benzene ring itself does not. The meta-$(CH_3)_3C$- and $(CH_3)_3N^+$- groups greatly increase the binding of phenol, from K_i of 50 mM to 0.024 mM and 0.0003 mM, respectively, as the substituents bind in the trimethyl site. The difference between the neutral and charged substituents can be calculated accurately from their σ-values and ρ obtained in the study of benzene derivatives.

Methyl benzenesulfonate is an effective methylating inactivating agent, and it is not retarded by $(CH_3)_4N^+$ ion, supporting the view that the benzene ring does not occupy the trimethyl site. Styrene oxide is also an alkylating inactivator, and effects of these aromatic compounds on subsequent introduction of ^{3}H-DFP will be examined. Effects of $(CH_3)_3C$-, $(CH_3)_2N$- and O_2N- substituents on inactivation by methyl benzenesulfonate and styrene oxide may be examined and appropriate ^{14}C-labeled materials prepared for study on AcChE.

Table I

Hydrolysis of $X-CH_2CH_2OOCCH_3$ by Acetylcholinesterase

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

Substrate Compound Number	X	$10^{-2} \times k_{cat}$ s^{-1}	K_m mM	$10^{-4} \times k_{cat}/K_m$ $M^{-1}s^{-1}$	$10^{-2} \times k_1$ s^{-1}	K_x mM	k_{OH^-} $M^{-1}s^{-1}$	$10^{-2} \times k_2(n)$ s^{-1}	$10^{-4} \times k_2(n)/K_y$ $M^{-1}s^{-1}$	V_{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≡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 Acetylcholinesterase of Acetylcholine and 3,3-Dimethylbutyl Acetate, in 0.18 M NaCl, pH 7.8, at 25°C

Inhibitor No.	Compound	Concentration (mM)	K_i Acetylcholine (mM)	K_i DMBAc (mM[a])
I[b]	$(CH_3)_3{}^+NCH_2CH_2CH_2COCH_3\ I^-$	0.1-0.3	0.022	0.024
II[b]	$(CH_3)_3C^+NH_2CH_2CH_2COCH_3\ Cl^-$	0.1-1	0.16	0.14
III[b]	$(CH_3)_3CSCH_2CH_2COCH_3$	1-3	0.40	0.33
IV[b]	$(CH_3)_3C^+NH_3\ Cl^-$	0.1-2	0.43	0.44
V[b]	$(CH_3)_2{}^+NHCH_2CH_2CH_2COCH_3\ Cl^-$	1-5	0.77	0.71
VI[c]	$(CH_3)_3{}^+NCH_2CH_2OH\ Cl^-$	0.1-2	1.0	0.63
VII[c]	$(CH_3)_3{}^+NCH_3\ Cl^-$	0.4-2	1.5	1.0
VIII[b]	$(CH_3)_3COCH_2CH_2COCH_3$	0.4-3	1.6	1.0
IX[b]	$(CH_3)_2CH^+NH_2CH_2CH_2COCH_3\ Cl^-$	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

Inactivation of Sigma AcChE by NEN ^{3}H-DFP

^{3}H-DFP (μM)	T (°C)	Time (min)	Inactivation (%)
1	0	10	20
10	0	60	55
10	25	10	55
10	25	30	88
10	25	60	94
48	25	5	75
48	25	15	84
48	25	30	97

Table IV

Reversible Inhibition of Hydrolysis of Acetylcholine by Acetylcholinesterase, pH 7.8, 25°C, 0.18 M NaCl

No.	Compound	$K_{i(com)}$[a] (mM)	$K_{i(nonc)}$[a] (mM)	K_m[b] (mM)	$MR_{(\beta)}$[c] (cc)
I	$(CH_3)_3SiCH_2CH_2OH$	3.3		3.5	25.0
IA	$(CH_3)_3CCH_2CH_2OH$	7.5[d]	19[d]	5.3	19.6
IB	$(CH_3)_3N^+CH_2CH_2OHCl^-$	0.4[e]	7.6[e]	0.33	17.2
II	$(CH_3)_2S^+CH_2CH_2OHI^-$	0.4	13	0.33	16.4
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	16.4
VII	$CH_3S(O_2)CH_2CH_2OH$	8.7	100[f]	6.2	13.9
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	14.1
X	$CH_3SCH_2CH_2OH$	40		15	13.3
XI	$CH_3CH_2CH_2CH_2OH$	47		13	10.3

[a] ±20%; com = competitive; nonc = noncompetitive.

[b] $K_{m(app)}$ of corresponding acetate, Ref. 26.

[c] Refraction volume of β-substituent, $(CH_3)_3Si-$, $(CH_3)_3C-$, $(CH_3)_3N^+-$, $(CH_3)_2S^+$, $(CH_3)_2N^+(O^-)-$, $CH_3S(O_2)-$, $CH_3S(O)-$, CH_3S-, CH_3CH_2, Ref. 12.

[d] Ref. 14.

[e] D. Bell, unpublished result.

[f] This value has high uncertainty.

Table V

Competitive and Uncompetitive Inhibition by Benzene and Substituted Benzenes of Hydrolysis of Acetylcholine by Acetylcholinesterase, in 0.18 M NaCl, pH 7.8, 25°C.

No.	Compound	$K_{i(com)}$ (mM)	log $1/K_{i(com)}$	ΔG kcal/mol com	$K_{i(nonc)}$ (mM)	log $1/K_i$ nonc	ΔG kcal/mol nonc	$\Sigma\sigma$ para
I	C_6H_6	23±3	1.64	-2.2[d]	48±8	1.32	-1.8[d]	0
II	$C_6H_5NHCOCH_3$	17±3	1.76	-2.4[d]			~0	0.04
III	$C_6H_5COCH_3$	4.1±1.2	2.39	-3.2[e]	3.3±0.2	2.48	-3.4[d]	0.46
IV	$C_6H_5NO_2$	0.63±0.17	3.19	-4.3[f]	3.0±0.4	2.52	-3.4[d]	0.82
V[a]	$C_6H_5NO_2$	0.80±0.19	3.10	-4.2[f]	2.5±.5	2.60	-3.5[f]	0.82
VI[b]	$C_6H_5N^+(CH_3)_3$	0.077	4.11	-5.6	0.53	3.28	-4.4	0.80
VII[b]	$(CH_3)_4N^+$	2.9	2.54	-3.4	56	1.25	-1.7	
VIII	$C_6H_5N(CH_3)_2$	11+1	1.95	-2.6[d]	74±27	1.13	-1.5[e]	-0.17
IX	$C_6H_5NH_2$	46±14	1.33	-1.8[e]	67±5	1.17	-1.6[d]	-0.17
X	$3\text{-}CH_3CONHC_6H_4NO_2$	0.26±0.025	3.59	-4.8[d]	1.2±0.2	2.91	-3.9[d]	0.86
XI	$4\text{-}CH_3CONHC_6H_4NO_2$	0.46±0.05	3.34	-4.5[d]	0.53±0.014	3.28	-4.4[d]	0.86
XII	$3\text{-}H_2NC_6H_4NO_2$	2.0±0.3	2.69	-3.6[d]	3.3±1.5	2.48	-3.3[g]	0.65
XIII	$4\text{-}H_2NC_6H_4NO_2$	0.57±0.12	3.24	-4.4[d]	0.83±0.13	3.08	-4.2[d]	0.65
XIV	$3\text{-}(CH_3)_2NC_6H_4NO_2$	0.12±0.02	3.92	-5.3[d]	0.079±0.002	4.10	-5.5[d]	0.65
XV	$4\text{-}(CH_3)_2NC_6H_4NO_2$	0.091±0.005	4.04	-5.5[d]	0.29±0.04	3.54	-4.8[d]	0.65
XVI	$4\text{-}H_2NC_6H_4COCH_3$	7.4±1.7	2.13	-2.9[f]	3.6±2.1	2.44	-3.3[h]	0.29
XVII	$4\text{-}(CH_3)_2NC_6H_4COCH_3$	1.3±0.6	2.90	-3.9[g]	3.8±0.7	2.42	-3.3[d]	0.29
XVIII[c]	C_5H_5N	12±3	1.92	-2.6[f]	110±50	0.95	-1.3[g]	
XIX[c]	$4\text{-}(CH_3)_3CC_5H_4N$	3.7±0.6	2.43	-3.3[d]	13±3	1.88	-2.5[d]	-0.14
XX	$C_6H_5CH\text{-}CH_2$ (O)	10±1.5	2.00	-2.7[d]	33±1.5	1.48	-2.0[d]	

Table V (Cont.)

[a] In hydrolysis of 3,3-dimethylbutyl acetate.

[b] Data from Krupka (Ref. 43).

[c] Studied at pH 8.5.

[d] ± ≤0.1 kcal/mol.

[e] ± 0.2 kcal/mol.

[f] ± 0.15 kcal/mol.

[g] ± 0.3 kcal/mol.

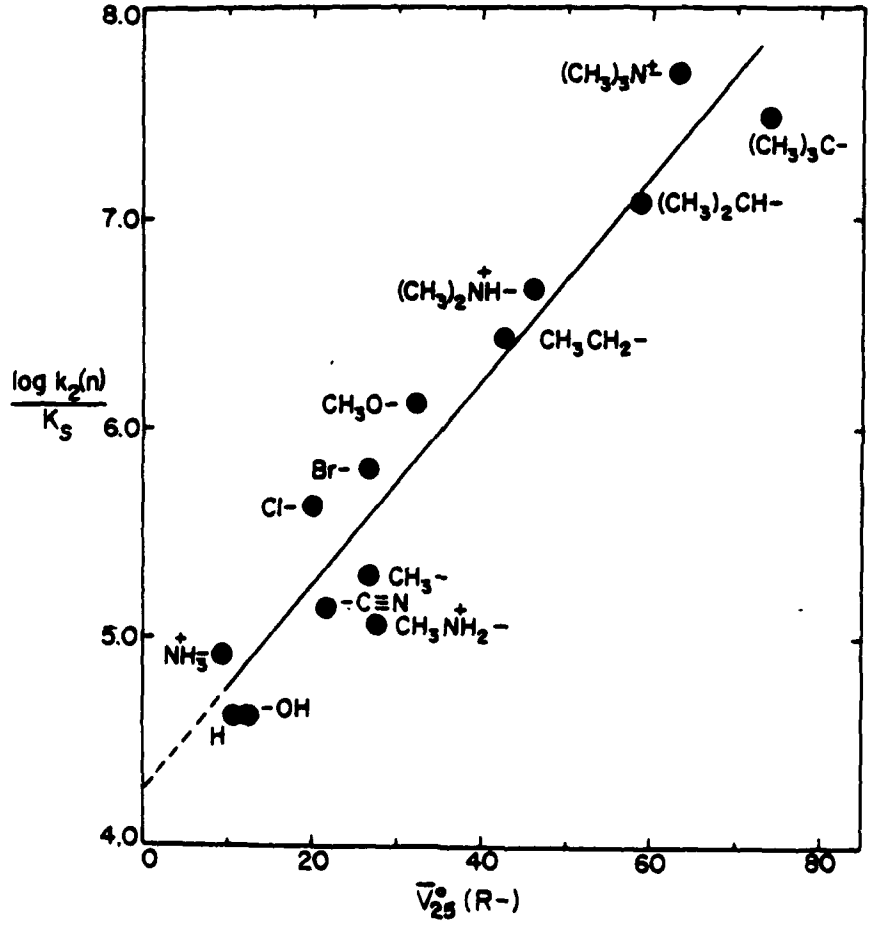
[h] ± 0.4 kcal/mol.

Table VI

Reversible Inhibition by Phenols

No.	Compound	$K_{i(com)}$ (mM)	$K_{i(nonc)}$ (mM)
I	C_6H_5OH	50	110
II	$3\text{-}CH_3C_6H_4OH$	10	40
III	$3\text{-}(CH_3)_3CC_6H_4OH$	0.024	0.17
IV	$4\text{-}(CH_3)_3CC_6H_4OH$		
V	$3\text{-}O_2NC_6H_4OH$	0.92	1.7
VI	$4\text{-}O_2NC_6H_4OH$		
VII	$1,2\text{-di(HO)-}4\text{-}(CH_3)_3CC_6H_3$	0.35	1.8

Figure 1. Normalized Reactivity of $XCH_2CH_2OCOCH_3$, log $(k_{2(n)}/K_s)$, and Apparent Molal Volume (V^o_{25}).



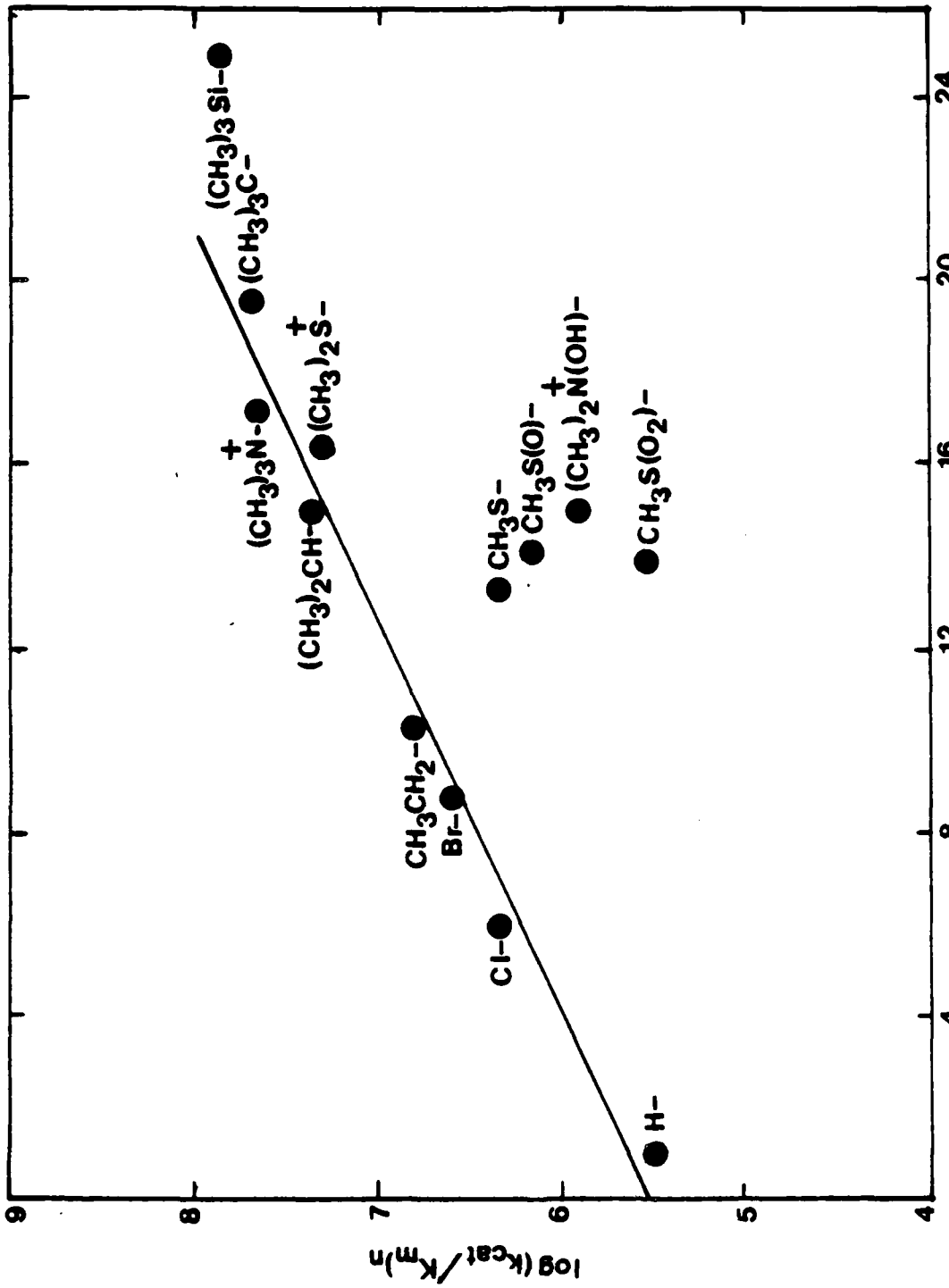


Figure 2. Normalized Reactivity of $XCH_2CH_2OCOCH_3$, log (k_{cat}/K_m)n, and Molar Refractivity (MR).

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