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Substituted Benzenes and Phenols as Reversible Inhibitors of Acetylcholinesterase: Polar, Trimethyl, and Synergistic Effects

SAUL G. COHEN,* S. BANO CHISHTI,* HEIDE REESE,* SACHIKO I. HOWARD,* MICHAEL SOLOMON,* AND JONATHAN B. COHEN†

*Department of Chemistry, Brandeis University, Waltham, Massachusetts 02254; and †Department of Anatomy and Neurobiology, Washington University School of Medicine, St. Louis, Missouri 63110

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To characterize the nature of reversible binding to acetylcholinesterase of derivatives of benzene and phenol, including the potent inhibitor 3-trimethylammoniophenol, we have carried out a systematic study of effects of substituents on the inhibition by such compounds of the enzymatic hydrolysis of acetylcholine. A linear relation between competitive binding, log $1/K_{i(com)}$, and substituent *para*–σ values is observed for C_6H_5-Y (Y = HO–, H_2N–, H–, CH_3CONH–, CH_3CO–, and O_2N–) and for the di-substituted 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 correlation, $r = 0.99$; $K_{i(com)}$ decreases to 0.63 mM for nitrobenzene and 0.46 mM for *p*-nitroacetanilide. 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 $(CH_3)_3\overset{+}{N}$–, 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 phenyltrimethylammonium ion. The observed linear relation of binding energies to substituent σ values indicates that the affinity of these benzene derivatives is determined by electron withdrawal by the substituent and that binding arises from a polar interaction of the benzene ring with an amino acid side chain in an aryl-binding site. The hydroxyl group decreases binding in phenol and the nitrophenols, in accord with its negative σ value. However, consistent with the effect of $(CH_3)_nX$– groups in the benzenes, binding of phenols rises linearly with the volume of *meta*-alkyl and alkylamino 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-*tert*-butylphenol. This large effect is attributed to binding of the *meta*-substituent in the trimethyl site, conformational change and synergistic hydrogen bonding of the phenolic hydroxyl. The very strong binding of 3-trimethylammoniophenol ($K_{i(com)} = 0.00033$ mM) is accurately calculated from the K_i of 3-*tert*-butylphenol, $\rho = 1.84$ (above) and the difference in σ values of *tert*-butyl and trimethylammonio, +0.94. The synergism of $(CH_3)_3X$– and hydroxyl is the same in the *tert*-butyl and trimethylammonio phenols. As in trimethylammonio benzene, the positive charge in trimethylammoniophenol 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.

INTRODUCTION

It has recently been proposed that aromatic–aromatic interactions of amino acid side chains within proteins provide a mechanism of stabilization for tertiary and quaternary structures of -1 to -2 kcal/mol per interaction (*1*). Thus, benzenoid

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compounds, interacting with aromatic side chains near an active site in enzymes may affect this stabilization and alter structure and activity. Aromatic compounds, particularly those containing positive charge, i.e., ammonio substituents, are known to affect the reactivity of acetylcholinesterase (AcChE). These include the carbamylating toxic alkaloid physostigmine (*2*), the related synthetic reagent neostigmine (*3*), and the reversible inhibitors phenyltrimethylammonium ion (K_i = 0.08 mM) (*4*) and 3-trimethylammoniophenol (K_i = 0.0003 mM) (*5*). In the latter, primary importance was attributed to the cationic substituent, related to that in acetylcholine (AcCh), and to hydrogen bonding of the phenolic hydroxyl.

Studies of aromatic substituent effects in reactions of AcChE have been reported. Binding of some substituted-phenyl-*N*-methylcarbamates correlated with Hammett σ values, with negative ρ (*6*), possibly indicating charge transfer to the enzyme. Later, multiparameter expressions involving molecular refraction (MR) related to volume (*7*), hydrophobicity, π (*8*), and charge-transfer constants related to σ values were developed to account for such binding. These expressions were applied to small effects arising from substituents with only a limited range of σ values, with strongly withdrawing and donating substituents such as O_2N– and R_2N– unaccounted for (*9, 10*). On the other hand, in a study of benzyl-*N*-methylcarbamates, the O_2N– substituent led to very strong binding, thereby indicating the importance of charge transfer from the enzyme to the substituted benzene ring (*11*). Also, anticholinesterase activity of substituted phenyl phosphates showed a dependence on σ with positive ρ, which was attributed to leaving group activity (*12*). However, in hydrolysis by AcChE of substituted phenyl acetates, the strongest binding and the highest reactivity were found at σ values ~0 (*13*). The aryl group binding site has been termed hydrophobic (*14*), while binding of *N*-methylacridinium ion has been attributed to charge-transfer interaction with a tryptophane residue of the active site (*15*). Thus, combined effects of substituents on binding, on leaving group activity, and on the electrophilic and nucleophilic steps of the hydrolytic process are complex and do not lead to ready interpretation. To characterize the nature of reversible binding by AcChE of benzene derivatives which are powerful inhibitors, it is desirable to study substituents of varying structure in a systematic manner and to assess the contributions of effects on the electronic properties of the benzene ring and of specific interactions within the binding site. We wish to report on such a study.

RESULTS

Benzene and mono- and di-substituted derivatives were examined as reversible inhibitors for hydrolysis of AcCh by AcChE. Rates of hydrolysis of three to six concentrations (0.1–0.6 mM) of AcCh by AcChE (10^{-9}–10^{-10} M) were obtained at three to six concentrations of inhibitor. Typical $1/V$ vs $1/S$ plots for a polar inhibitor, nitrobenzene, and for a phenolic inhibitor, 3-*tert*-butylphenol are given in Figs. 1 and 2. Least-squares slopes and intercepts for $1/V$ vs $1/S$ data were calculated at each inhibitor concentration. Regression analysis of the slopes of the $1/V$ vs $1/S$ data against inhibitor concentrations led to secondary intercepts and

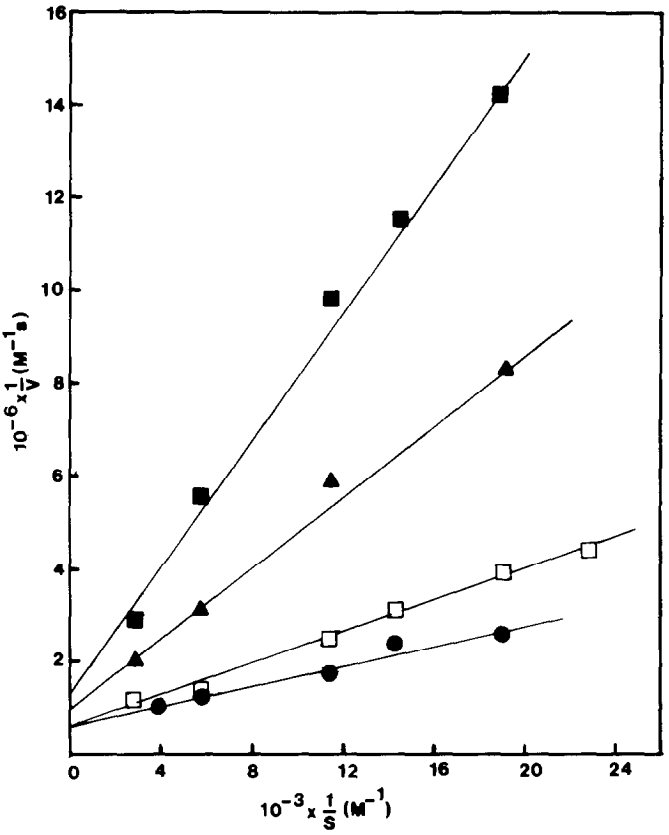


FIG. 1. Inhibition by $C_6H_5NO_2$ of hydrolysis of AcCh by AcChE. ●, no inhibitor; □, 0.59 mM; ▲, 2.3 mM; ■, 4.1 mM.

slopes, the ratios of which led to values of competitive inhibition constants, $K_{i(com)}$. Regression analysis of the intercepts of $1/V$ vs $1/S$ data against inhibitor concentrations led to another set of secondary intercepts and slopes from which noncompetitive inhibition constants were calculated, $K_{i(nonc)}$. Inhibition constants, binding energies, σ values, and molar refraction volumes are given in the tables.

Polar Effects

Competitive binding of the mono-substituted benzenes is increased by electron-withdrawing substituents (Table 1A). Binding of aniline with an electron-donating substituent, like that of phenol (Table 2), is weaker than that of benzene; binding of acetanilide is slightly stronger than that of benzene, and it is stronger still in acetophenone and in nitrobenzene with $K_{i(com)} = 0.63$ mM. Regression analysis of log $1/K_{i(com)}$ vs σ–*para* values for six compounds, C_6H_5–Y (Y = HO–, H_2N–, H–, CH_3CONH–, CH_3CO–, O_2N–), indicates a linear relationship with intercept = 1.64 ± 0.10, slope (ρ) = 1.83 ± 0.08 and correlation coefficient, $r = 0.99$.

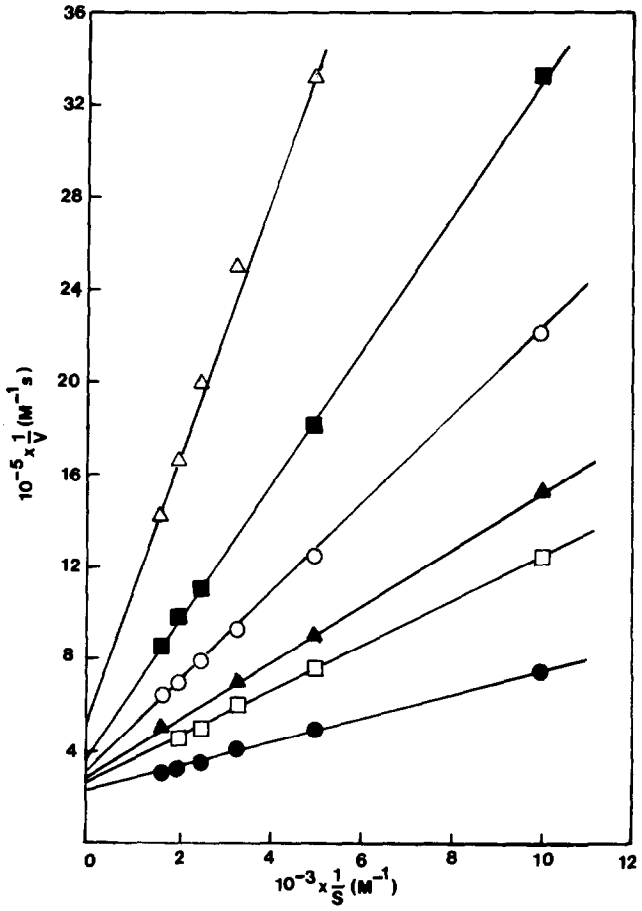


FIG. 2. Inhibition by 3-$(CH_3)_3CC_6H_4OH$ of hydrolysis of AcCh by AcChE. ●, no inhibitor; □, 0.010 mM; ▲, 0.025 mM; ○, 0.050 mM; ■, 0.1 mM; △, 0.20 mM.

These substituents have similar, but not identical, effects in the di-substituted benzenes (Table 1A). Effects of the CH_3CONH– and O_2N– substituents are essentially additive in the 4- and 3-nitroacetanilides, increasing binding over benzene by ~2.4 kcal/mol as compared with 0.2 and 2.1 kcal/mol for the two substituents separately. The H_2N– group decreases binding in 3-nitroaniline and 4-aminoacetophenone, as compared with nitrobenzene and acetophenone, respectively, but does not decrease it in 4-nitroaniline. The HO– group weakens binding in 3- and 4-nitrophenols as it does in phenol. Regression analysis of log $1/K_{i(com)}$ vs σ–*para* and the sum of the σ–*para* values for these mono- and di-substituted benzenes, excluding 3-nitroacetanilide and 4-nitroaniline, leads to intercept = 1.63 ± 0.04, slope (ρ) = +1.84 ± 0.07, and r = 0.99 (Fig. 3, line A). If these two compounds are included, the intercept is 1.63 ± 0.07, ρ rises to 1.98 ± 0.12, and r = 0.98.

Dimethyl and Trimethyl Group Effects

The dimethylamino and trimethylammonio substituents lead to stronger binding than is consistent with this relationship (Table 1B, Fig. 3). Dimethylaniline binds slightly better than benzene despite its electron-donating $(CH_3)_2N$– group and better than aniline, with the same substituent σ value, by −0.8 kcal/mol. 3-Nitro-*N*,*N*-dimethylaniline, 4-nitro-*N*,*N*-dimethylaniline, and 4-*N*,*N*-dimethylamino-acetophenone also bind more strongly than their analogs lacking *N*-methyl groups (Table 1A) by −1.7, −1.1, and −1.0 kcal/mol, respectively. Phenyltrimethylammonium ion binds similarly to the 3- and 4-nitrodimethylanilines, and substantially better than nitrobenzene, with equal substituent σ value, by −1.2 kcal/mol.

Remarkably, the di- and trimethyl compounds (dimethylaniline, 3- and 4-nitrodimethylaniline, 4-dimethylaminoacetophenone, and phenyltrimethylammonium ion) lead to their own linear relation between log $1/K_{i(com)}$ and summed σ values,

TABLE 1

Inhibition by Benzene and Its Derivatives of Hydrolysis of Acetylcholine by Acetylcholinesterase, 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	—
	B. Substituents binding at the trimethyl site				
$C_6H_5N(CH_3)_2$	11. ± 1	1.95	−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
$4\text{-}(CH_3)_2NC_6H_4COCH_3$	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 3,3-dimethylbutyl acetate.
[b] ±0.1 kcal/mol.
[c] ±0.2 kcal/mol.
[d] ±0.3 kcal/mol.
[e] At pH 8.5.

TABLE 2

Inhibition by Phenols of Hydrolysis of Acetylcholine by Acetylcholinesterase 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 (cc)
$3\text{-}(CH_3)_3N^+C_6H_4OH$[a]	3.3 ± 0.5×10^{-4}	6.49	−8.8[c]	4.3 ± 1.1×10^{-3}	—
$3\text{-}(CH_3)_3N^+C_6H_4OH$[a,b]	2.5 ± 0.7×10^{-4}	6.60	−8.9[c]	4.4 ± 2.3×10^{-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	—

[a] At pH 7.0.

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

[c] ±0.1 kcal/mol.

[d] ±0.2 kcal/mol.

[e] ±0.3 kcal/mol.

[f] Studied at one concentration (3 mM) in the 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.

with intercept = 2.35 ± 0.23, slope (ρ) = 2.36 ± 0.16, and r = 0.99 (Fig. 3, line B). For further assessment of the alkyl group effect, inhibition by pyridine, a water-soluble analog of benzene, and 4-*tert*-butylpyridine was examined (Table 1). The *tert*-butyl group increased binding by −0.7 kcal/mol. This occurs despite a σ value of −0.14 which might decrease binding, according to the ρ–σ relation, above, by 0.3 kcal/mol. This indicates an overall contribution of binding of the uncharged trimethyl substituent of −1.0 kcal/mol, an effect consistent with those of the $(CH_3)_2N$– and $(CH_3)_3N^+$– groups.

Styrene oxide (Table 1B), an alkylating agent, binds less strongly than its ketone isomer acetophenone. In a preliminary experiment, AcChE was incubated with 3 mM styrene oxide; enzymatic activity fell to 56% in 1.3 h, to 30% in 6 h, and to 10% of initial activity in 23 h.

The Alkyl–Hydroxyl Synergism

In agreement with the reported value (*5*), 3-trimethylammoniophenol binds very strongly—by −4.9 kcal/mol more than is consistent with the σ relationship of Fig. 3, line A—as indicated directly by comparison with 3-nitrophenol of similar σ values. Since di- and trimethyl-bearing substituents increase binding relative to σ

(comparison of lines A and B, Fig. 3), this effect was examined in 3-*tert*-butylphenol. This compound also bound very strongly (Fig. 3), by −4.8 kcal/mol more than consistent with the σ relationship, as calculated from the value for phenol and the Hammett equation with $\rho = 1.85$, and σ for *tert*-butyl = −0.14. The trimethyl substituent leads to the same large effect in the charged and uncharged phenols.

The effect of the volume of such substituents in binding of phenols was then examined (Table 2). Binding of phenol, the *meta*-alkyl phenols with substituents CH_3–, C_2H_5–, $(CH_3)_2CH$–, and $(CH_3)_3C$–, and 3-dimethylaminophenol rises lin-

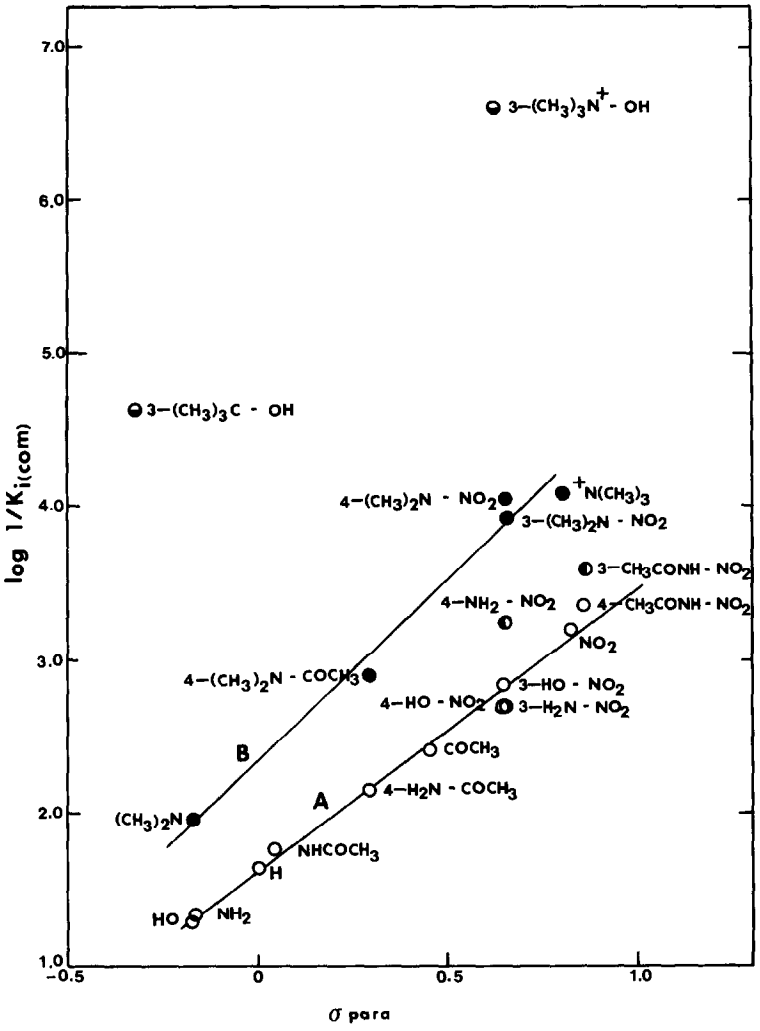


FIG. 3. Polar effects in competitive inhibition by benzene derivatives of hydrolysis of AcCh by AcChE. Line A (–○–), substituents without specific interaction; line B (–●–) substituents binding at the trimethyl site; ◐, 4-nitroaniline and 3-nitroacetanilide; ◒, 3-*tert*-butylphenol and 3-trimethylammoniophenol.

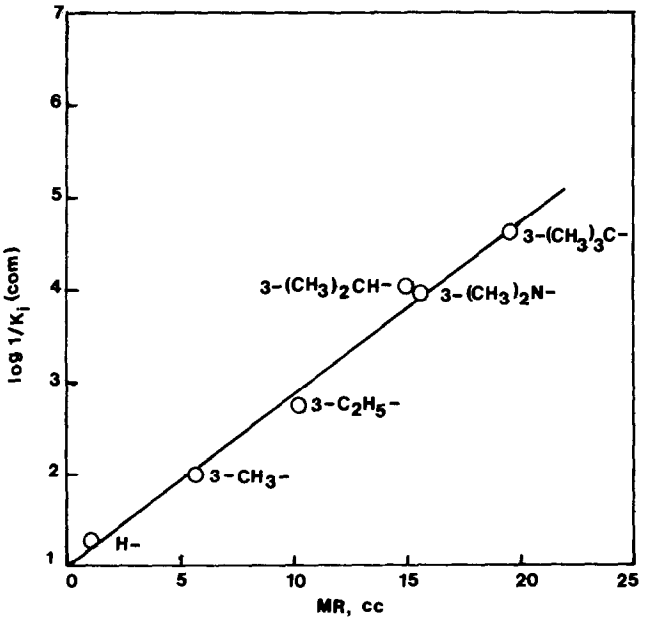


FIG. 4. Effects of volume of *meta*-alkyl substituents in competitive inhibition by phenols of hydrolysis of AcCh by AcChE.

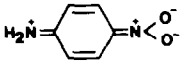
early with volume, measured by molar refraction (MR) (Fig. 4). Regression analysis leads to intercept 1.01 ± 0.16, slope (ρ) = 0.187 ± 0.013, and r = 0.995. The *meta* orientation is superior to *ortho* or *para;* 2-*tert*-butylphenol and 4-isopropylphenol bind less than their *meta* isomers by 2.2 kcal/mol; 1.5 mM 4-*tert*-butylphenol does not inhibit. 4-*tert*-Butylanisole binds more weakly than the phenol by 3.2 kcal/mol; binding of anisole and 3-nitroanisole is similar and slightly stronger than that of phenol and 3-nitrophenol, respectively.

Noncompetitive binding energies are generally slightly less than competitive ones. Regression analysis of log $1/K_{i(\text{nonc})}$ vs σ for C_6H_5–Y (Y = H–, O_2N–, $(CH_3)_2N$–, H_2N–, and HO–) and the di-substituted compounds 3-nitroaniline, 3-nitroacetanilide, and 3-nitrophenol, leads to intercept = 1.37 ± 0.05, slope (ρ) = +1.67 ± 0.10, and r = 0.99. Data for a second set comprising of acetophenone, phenyltrimethylammonium ion, 4-nitroacetanilide, 4-nitroaniline, 4-aminoacetophenone, and 4-dimethylaminoacetophenone with higher bindings relative to σ values, lead to intercept = 1.88 ± 0.14, slope (ρ) = +1.72 ± 0.23, and r = 0.97. 3- and 4-nitrodimethylanilines and 4-nitrophenol have higher noncompetitive binding relative to their σ values than consistent even with this relation. Inhibition by acetanilide is purely competitive and that of 3-trimethylammoniophenol is largely so.

Nitrobenzene (Table 1A) and 3-trimethylammoniophenol (Table 2) were also examined as inhibitors of hydrolysis of 3,3-dimethylbutyl acetate (DMBAc). In each case, both competitive and noncompetitive binding constants were the same as in inhibition of hydrolysis of acetylcholine.

DISCUSSION

That effects of substituents of different size and structure, namely, H–, CH_3CONH–, CH_3CO–, O_2N– and H_2N, and HO– in phenol and the nitrophenols, lead to a linear relation of binding energy to substituent σ values (Fig. 3, line A) indicates that specific interactions of these substituents with groups on the enzyme are not important to the competitive binding of these mono- and di-substituted benzenes. In particular, the O_2N– group in nitrobenzene converts weakly binding benzene to an inhibitor at least as strong as choline or tetramethylammonium ion (*16*). In contrast, the O_2N– group leads neither to appreciable binding nor to enhanced enzymatic reactivity in hydrolysis of an aliphatic ester, β-nitroethyl acetate (*17*), nor does it cause detectable inhibition by nitromethane, examined up to 200 mM (*18*). Electron withdrawal from the benzene ring increases interaction with protein aromatic groups and strongly increases binding. Similarly, pyridine, essentially neutral and isosteric with benzene, binds better than benzene despite its water miscibility and, thus, much lower hydrophobicity. This effect also may arise from the ring being relatively electron depleted, in this case by the electronegative heteroatom. Thus, polar rather than hydrophobic interactions are involved in binding of these aromatic compounds. The H_2N– group weakens competitive binding in aniline and 3-nitroaniline, consistent with its negative σ. However, decrease is not observed in 4-nitroaniline. The quinonoid structure,



with an electron-depleted aromatic ring, may be a favored acceptor group, as in quinone itself (*19*) and the flavins (*20a*).

The binding energies (Table 1A) are larger than for isolated tryptophanamide–acceptor complexes (*20b*), but consistent with the higher values of related protein-associated complexes (*20c*) and with widely occurring aromatic–aromatic interactions in proteins (*1*). X-ray diffraction studies of the latter indicate nearly perpendicular orientation of associating aromatic rings (*1*). This may reflect a hydrogen-bonding interaction of aryl-hydrogen to aromatic orbital electrons, which would also increase with electron depletion in the hydrogen donor, rather than electron or charge transfer of parallel ring systems. The value of $\rho \sim +2$, observed in this study, is high, similar to values for charge formation of weakly dissociating species described in Table 2.2 of Wells (*21*).

Dimethylamino, trimethylammonio, and *tert*-butyl substituents increase, to similar extents (~1 kcal/mol), competitive binding (Table 1B) over that expected from substituent σ values; this is independent of the nature of the core atom, N or C, and of the presence or absence of cationic charge. We attribute this to specific binding of these substituents in the trimethyl subsite (*16*), generally and inappropriately termed the anionic site, via nonpolar interaction of their surface methyl substituents. Thus, the aromatic ring binds at a subsite so located that $(CH_3)_nX$– substituents may bind simultaneously at the trimethyl subsite in the free enzyme. Further, the correlation of Fig. 3, line B indicates that the effect of the important $(CH_3)_3N^+$– substituent on binding is accounted for completely by this interaction

of the methyl groups at the trimethyl subsite and electron withdrawal from the benzene ring by the positive charge, as reflected in its σ value. The latter increases the polar interaction of the benzene ring at the aryl binding subsite, and Coulombic attraction to anionic charge on the enzyme need not be called upon.

Interaction of the methyl groups in the trimethyl subsite is essential to the very strong binding of 3-trimethylammoniophenol and increases binding of the 3-alkylphenols. While the hydroxyl substituent in phenol decreases binding of already weakly bound benzene and in the 3- and 4-nitrophenols decreases that of relatively strongly bound nitrobenzene, consistent with its small negative σ, *meta*-alkyl substituents, despite negative σ, lead to synergistically increased binding in phenols. This is particularly evident in 3-*tert*-butylphenol which binds more strongly than phenol by -4.4 kcal/mol, while the *tert*-butyl group increases binding in the pyridine–*tert*-butylpyridine pair by only -0.7 kcal/mol, and dimethylamino increases binding over amino in benzenes by ~ -1 kcal/mol. Binding of the *tert*-butyl group in the trimethyl site fixes the hydroxyl for effective hydrogen bonding with a group in the enzyme, just as has been proposed for the trimethylammonio group (*5*). The synergism of the 3-*tert*-butyl and 3-trimethylammonio substituents in the uncharged and cationic phenols is identical, indicating that both substituents bind at the one trimethyl subsite. The stronger overall binding of the cation arises from its withdrawal of electron density from the benzene ring. Thus, from the observed binding of 3-*tert*-butylphenol (log $1/K_{i(\mathrm{com})} = 4.62$), $\rho = +1.84$, (Fig. 3, line A) and the summed σ values of *tert*-butyl and trimethylammonio (0.94), the Hammett equation leads to a calculated value of the binding of 3-trimethylammoniophenol (log $1/K_{i(\mathrm{com})} = 6.36$, $\Delta G = -8.6$ kcal/mol), as compared with the observed values (log $1/K_{i(\mathrm{com})} = 6.49$, $\Delta G = -8.8$ kcal/mol). The line that would connect the data points of *tert*-butyl- and trimethylammoniophenols (Fig. 3) has the same slope as the line in the figure which correlates compounds affected only by nonspecific polar factors. Thus, again in the phenols as in the benzenes, the increase in binding due to the trimethylammonio group is accounted for by the trimethyl interaction and electron withdrawal from the benzene ring, and evidence for an additional Coulombic interaction is not found.

Further, the overall effect of the phenolic group is the same in the binding of *tert*-butyl- and trimethylammoniophenols. From the observed effect of *tert*-butyl on the binding in the pyridine–*tert*-butylpyridine pair (-0.7 kcal/mol) and the binding energy of benzene (-2.2 kcal/mol), we estimate binding of *tert*-butylbenzene at -2.9 kcal/mol. Then, from the binding of 3-*tert*-butylphenol (-6.2 kcal/mol), the effect of the hydroxyl is -3.3 kcal/mol, which is identical to that observed in the 3-trimethylammoniophenol–phenyltrimethylammonium ion pair. Hydrogen bonding is important in the 3-*tert*-butyl and 3-trimethylammoniophenols since conversion to the anisoles by methylation of the hydroxyl greatly decreases the binding in the uncharged compound (Table 2) and in the previously studied cationic compound (*5*). In phenol and the nitrophenols, where a hydrogen-bonding effect is not observed, replacement of hydroxyl by methoxyl does not decrease binding. Strong binding of nitrophenol in the aryl site does not lead to

synergistic hydrogen bonding in the nitrophenols. An additional interaction in the trimethyl site is needed to bring the phenolic hydroxyl and a group of the enzyme into suitable relation, possibly by a conformational change. The failure of the O_2N– group to enhance binding also in nitroethyl acetate and nitromethane (*17*) indicates that this group cannot interact with the trimethyl site.

The synergism of 3-alkyl and hydroxyl groups also functions with the smaller substituents, to the extent that they interact at the trimethyl site, and the effects are reflected in the binding-volume relationship (Fig. 2). Correlation of binding effects of hydrocarbon substituents with volume may not be distinguished from correlation with hydrophobicity, π (*22*). However, the good fit to volume of the effect of the $(CH_3)_2N$– substituent, equal to that of isopropyl despite very different hydrophobicity, $\pi = 0.18$ and 1.53, respectively (*23*), rules out hydrophobicity, as does the relation, above, based on σ values alone, between contributions of the hydrocarbon *tert*-butyl- and water-soluble trimethylammonio substituents. Volume, leading to fit in the trimethyl subsite, restriction of motion, and appropriate location of the hydrogen bonding hydroxyl are the determining factors. The *meta* relation is important and the hydrogen-bonding effects in the 2-*tert*-butyl and 4-isopropyl phenols are reduced to about two-thirds of that for the respective *meta* isomers. In the trimethylammoniophenols (*5*), an *ortho*-hydroxyl led to about one-half the increase of a *meta*, but the *para*-hydroxyl led to little, if any, increase in binding. A similar situation in 4-*tert*-butylphenol would account for our failure to observe inhibition by 1.5 mM of this compound, the maximum concentration which we could conveniently study.

The nature of the noncompetitive inhibition was examined briefly by comparison of effects in hydrolysis of the reactive substrate, AcCh, and the less reactive uncharged analog, 3,3-dimethylbutyl acetate, in which high and low steady-state concentrations of acetylenzyme would be present, respectively. This was done with nitrobenzene, which binds only at the aryl site, and 3-trimethylammoniophenol, which binds in addition at the trimethyl and a hydrogen-bonding site. In both cases, the same binding was observed with the two substrates, indicating that binding to the acetylenzyme, affecting deacylation, is not involved. Binding to the enzyme–substrate complex appears to be responsible for noncompetitive inhibition by nitrobenzene, indicating that the substrates need not use the aryl site, while its occupation by the inhibitor affects reactivity. Since inhibition by 3-trimethylammoniophenol is largely competitive, further study would be needed for analysis of its noncompetitive mode.

Consideration of the structural features affecting binding at an aryl subsite, which is strongly affected by varied substituents, indicates that potent inhibitors of AcChE may be devised without emphasis on a cationic substituent. It will also be of interest to ascertain whether factors considered in this report have general relevance: whether benzene derivatives will interfere with aromatic–aromatic interactions in other enzymes and affect their reactivity, whether substituents related to those of the natural substrates of these enzymes will enhance these effects, and whether in other serine enzymes an appropriately placed phenolic hydroxyl will further increase the effects by a hydrogen-bonding interaction, possibly at the esteratic site, interfering with action of the serine hydroxyl.

MATERIALS AND METHODS

Benzene, acetanilide, acetophenone, nitrobenzene, phenyltrimethylammonium iodide, dimethylaniline, aniline, 4-nitroacetanilide, 3-nitroaniline, 4-nitroaniline, 3-nitrodimethylaniline, 4-aminoacetophenone, 4-*tert*-butylpyridine, styrene oxide, 3-*tert*-butylphenol, 2-*tert*-butylphenol, 3-isopropylphenol, 4-isopropylphenol, 3-ethylphenol, 3-methylphenol, 3-nitroanisole, and acetylcholine iodide were obtained from Aldrich. 4-Nitrodimethylaniline, 4-aminoacetophenone, 3-nitrophenol, and 4-nitrophenol were from Eastman Kodak; anisole was from Fisher Scientific. Phenol, Special Absolute, was from Natural Biological Stains. 4-Dimethylaminoacetophenone was from Frinton Laboratories; pyridine was from J. T. Baker. Solids were crystallized from water, ethanol, or aqueous ethanol (1–3 times) to give sharp melting points in agreement with literature values. Liquids were distilled under reduced pressure.

3-Dimethylaminophenol (Aldrich) was converted to the hydrochloride by HCl in ethanol and crystallized from ethanol–ether.

3-Nitroacetanilide was prepared from 3-nitroaniline and acetic anhydride in benzene following a literature procedure (*24*).

3-Trimethylammoniophenol iodide was prepared from 0.10 mol each of 3-dimethylaminophenol and methyl iodide in 32 ml of methanol under reflux for 6 h. Solvent was removed and the residue was washed with acetone, leaving purple crystals. The solid was dissolved in ethanol, treated three times with Norit, concentrated, and crystallized from ethanol–ether, yielding white crystals, mp 182–184°C, lit. 181°C (*4*).

3-*tert*-Butylanisole was prepared by treatment of a solution of 0.090 mol each of potassium hydroxide and 3-*tert*-butylphenol in 50 ml of ethanol with 0.135 mol of methyl iodide under reflux for 10 h. The mixture was filtered, the filtrate was concentrated, and the oil residue was dissolved in ether, stirred with 5% potassium hydroxide for 3 h, washed with water, dried over magnesium sulfate, concentrated, and distilled. It showed satisfactory C and H analyses and NMR spectrum.

Values of molecular refraction and hydrophobicity, π, were taken from a compilation (*23*). σ values were taken from Table 2.3, 2.4, and 2.5 of Wells (*21*). "Normal" values (σ^n), derived from well-correlated series not involving direct conjugative effects, were used where available, i.e., for $(CH_3)_3N^+$–, $(CH_3)_2N$–, H_2N–, $(CH_3)_3C$–, and HO–; the value of the latter is -0.18. σ^0 values were used for CH_3CO– and O_2N–, and an ordinary σ value was used for CH_3CONH–. *Para* values were chosen as being less affected by direct field effects than *meta*. Algebraic addition of the values was used for the di-substituted compounds; σ^+ and σ^- values, derived from special cases and having little predictive use (*21*, p. 29), were not found useful.

Kinetic studies were carried out on a pH stat as described previously (*22*), in 0.18 M NaCl, at 25°C, under a nitrogen atmosphere and at pH 7.8, except for inhibition by pyridine and 4-*tert*-butylpyridine which were studied at pH 8.5. Eel acetylcholinesterase (Sigma, V-S, lyophilized), ~1000 units, was dissolved in 0.18 M NaCl and stored at 4°C. An aliquot was assayed before each inhibitor run by

hydrolysis of acetylcholine iodide with $k_{cat} = 1.6 \times 10^4$ s^{-1} (*25*). In the study of inhibitors sufficiently soluble in 0.18 M NaCl, appropriate aliquots of concentrated solutions of inhibitor and AcCh were added to 0.18 M NaCl (total volume = 19.9 ml) and the enzyme solution was then added. For less soluble inhibitors, large aliquots of dilute solutions were used to which small volumes of 0.18 M NaCl, AcCh, and enzyme were added. Still less soluble inhibitors, *N,N*-dimethylaniline, styrene oxide, and anisole, were injected directly into the titration cup containing 19.9 ml of 0.18 M NaCl and AcCh and stirred until clear, and then enzyme was added.

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REFERENCES

1. BURLEY S. K., AND PETSKO, G. A. (1985) *Science* **229,** 23–28.
2. STEDMAN, E., AND BARGER, G. (1925) *J. Chem. Soc.* **127,** 247–258.
3. AESCHLIMANN, J. A., AND REINERT, M. (1931) *Pharmacol. Exp. Ther.* **43,** 413–444.
4. KRUPKA, R. M. (1965) *Biochemistry* **4,** 429–435.
5. WILSON, I. B., AND QUAN, C. (1958) *Arch. Biochem. Biophys.* **73,** 131–143.
6. KOLBEZEN, M. J., METCALF, R. L., AND FUKUTO, T. R. (1954) *J. Agric. Food Chem.* **2,** 864–870.
7. VOGEL, A. I. (1948) *J. Chem. Soc.*, pp. 1820–1854.
8. LEO, A., HANSCH, C., AND ELKINS, D. (1971) *Chem. Rev.* **71,** 525–616.
9. HETNARSKI, B., AND O'BRIEN, R. D. (1975) *J. Med. Chem.* **18,** 29–33.
10. SU, C.-T., AND LIEN, E. J. (1980) *Res. Commun. Chem. Pathol. Pharmacol.* **29,** 403–415.
11. HETNARSKI, B., AND O'BRIEN, R. D. (1973) *Biochemistry* **12,** 3883–3887.
12. FUKUTO, T. R., AND METCALF, R. L. (1956) *J. Agric. Food Chem.* **4,** 930–936.
13. SUSZKIW, J. B. (1973) *Biochem. Biophys. Res. Commun.* **54,** 417–425.
14. KENLEY, R. A., CLIFFORD, D., BEDFORD, C. D., DAILEY, O. D., HOWD, R. A., AND MILLER, A. (1984) *J. Med. Chem.* **27,** 1201–1212.
15. SHINITZKY, M., DUDAI, Y., AND SILMAN, I. (1973) *FEBS Lett.* **30,** 125–128.
16. HASAN, F. B., ELKIND, J. L., COHEN, S. G., AND COHEN, J. B. (1981) *J. Biol. Chem.* **256,** 7781–7785.
17. COHEN, S. G., ELKIND, J. L., CHISHTI, S. B., GINER, J.-L., REESE, H., AND COHEN, J. B. (1984) *J. Med. Chem.* **27,** 1643–1647.
18. COHEN, S. G., CHISHTI, S. B., ELKIND, J. L., REESE, H., AND COHEN, J. B. (1985) *J. Med. Chem.* **28,** 1309–1313.
19. ANDREWS, L. J., AND KEEFER, R. M. (1964) *in* Molecular Complexes in Organic Chemistry, Chap. 1, Holden–Day, San Francisco.
20. SLIFKIN, M. A. (1971) Charge-Transfer Interactions of Biomolecules, (a) Table 7.1; (b) Table 3.6; (c) p. 170, Academic Press, London.
21. WELLS, P. R. (1968) Linear Free Energy Relationships, Academic Press, London.
22. HASAN, F. B., COHEN, S. G., AND COHEN, J. B. (1980) *J. Biol. Chem.* **255,** 3898–3904.
23. HANSCH, C., AND LEO, A. (1979) Substituent Constants for Correlation Analyses in Chemistry and Biology, pp. 50, 74, 85, 95, 105, 115. Wiley, New York.
24. KAUFMAN, A. (1909) *Ber.* **42,** 3480–3483.
25. ROSENBERRY, T. L. (1975) *Adv. Enzym. Relat. Areas Mol. Biol.* **43,** 103–218.