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Interaction between Bisquaternary Ammonium Ligands and Acetylcholinesterase: Complex Formation Studied by Fluorescence Quenching

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SUMMARY

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Complex formation between the purified acetylcholinesterase described in the preceding communication and a series of bisquaternary inhibitors containing a benzoquinone group between two quaternary ammonium moieties maximally positioned 14 A apart was investigated by fluorescence quenching. The benzoquinone group absorbs maximally at 340 nm, providing optimal spectral overlap with the donor tryptophanyl residues in the protein. For the series of bisquaternary ligands, the complexes exhibit 44–49% of the fluorescence of the free enzyme and the dissociation constants vary from 2.8×10^{-8} to 1.2×10^{-5} M, depending on the substituents around the quaternary ammonium group. Uniform quenching of tryptophanyl fluorescence in each of the four binding sites on the tetrameric acetylcholinesterase molecule is observed, which indicates that the interbinding site distance exceeds 40–50 A. Back-titration with a nonquenching inhibitor, decamethonium, effects a return of the fluorescence to that of the free enzyme and permits a calculation of the affinity of a competing ligand. The catalytically participating serine in acetylcholinesterase has been esterified, forming the methanesulfonyl-, ethanesulfonyl-, propanesulfonyl-, diethoxyphosphoryl-, and diisopropoxyphosphoryl-enzymes. The affinity of three bisquaternary ligands possessing different bulk around the ammonium groups has been determined for the modified and catalytically inactive enzymes. These findings have provided a more detailed description of the topographical relationship between the sites of inhibitor binding and the active site. The specificity of binding for the bisquaternary ligand resides largely in the anionic subsite within the active site. In the modified enzymes the sulfone and phosphoryl oxygens can be expected to hydrogen bond in the oxyanion hole of the esteratic site, providing a discrete orientation for the alkoxy groups connected to the phosphorus and two possible orientations for the alkyl group adjoining the sulfonyl moiety. Binding affinity measurements enable a prediction of the favored orientation of the alkyl group with respect to the active center.

INTRODUCTION

The reversible binding of cationic ligands to the enzyme acetylcholinesterase (acetylcholine hydrolase, EC 3.1.1.7) has been a subject of extensive investigation over a number of years (1–5). The cationic group, typically a quaternary ammonium moiety, is known to associate with an anionic subsite within the active center, and the forces conferring stability to the interaction are the

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same as those which stabilize the choline head group of the substrate acetylcholine. In addition, substantial evidence supports the existence of a peripheral anionic site which lies outside the active center (1, 3–5). It has been proposed that the binding of ligands to the peripheral anionic site influences substrate association or catalysis at the active center of acetylcholinesterase (1, 3). However, preferential binding to the peripheral anionic site becomes manifest only at low ionic strength (1, 6, 7), and little is known about the nature of the proposed cooperative behavior between the two sites. Bisquaternary ligands, in which the two quaternary nitrogens are separable by 12–14 A, may span between the anionic subsite within the active center and the peripheral anionic site (3, 5). These ligands, upon binding to the enzyme, appear to adopt an "exo" orientation (3), in which the reactive serine in the active center is not occluded by the inhibitor.

The above interpretations on ligand binding are based largely on two types of experimental approaches: (a) the influence of inhibitory or presumed allosteric ligands on kinetic parameters of acyl ester and carbamoyl ester hydrolysis and (b) the influence of the ligand on the reactivity of the active site serine toward covalent modification by acid-transferring groups which form stable esters. The latter is customarily measured by ascertaining residual enzyme activity following extensive dilution of the reactant molecules.

By examining spectroscopic changes associated with ligand binding (7–9), an alternative experimental approach is available which affords a direct rather than indirect measure of reversible ligand association. This approach permits design of a different type of experiment and carries the additional advantage that the kinetics of the association and dissociation processes may be monitored. We present here an investigation of bisquaternary ligand interactions with a series of modified acetylcholinesterases by following fluorescence quenching of the enzyme tryptophanyl residues. Since catalysis is not required for ascertaining ligand binding, complex formation with a series of covalently modified acetylcholinesterases can be examined and compared with the native enzyme.

METHODS

Materials

Acetylcholinesterase was purified by lytic solubilization of electroplax membranes from *Torpedo californica* and subsequent affinity chromatography as described in the accompanying paper (10). The enzyme appeared homogeneous by criteria of analytical ultracentrifugation and gel electrophoresis and was stored at −20° in polypropylene tubes for periods up to 1 month without appreciable loss of activity or aggregation.

The benzoquinone derivatives were kindly supplied by Dr. F. C. Nachod of the Sterling-Winthrop Research Institute, Rensselaer, N. Y., and used without further purification. These compounds, in 0.01 M Tris-Cl, pH 8.0, showed an absorption maximum at 340 nm ($\epsilon^{M}_{340} = 2.38 \times 10^4$). Diisopropyl fluorophosphate was a product of Calbiochem, while diethyl *p*-nitrophenyl phosphate, pyridine-2-aldoxime methiodide, and decamethonium iodide were obtained from Sigma Chemical Company.

Methanesulfonyl, ethanesulfonyl, and propanesulfonyl esters of 3-hydroxy-*N*-methylpyridinium were synthesized by the procedure described by Ginsburg (11) for the preparation of 1-methyl-3-hydroxypyridinium iodide methanesulfonate, except that triethylamine was used in place of 2,6-lutidine as a proton acceptor. NMR spectra were consistent with the proposed products.

N-Methyl-3-hydroxypyridinium iodide n-propanesulfonate. Recrystallized two times from methanol; m.p. 120–122°.

$C_9H_{14}INO_3S$

Calculated:	C 31.49,	N 4.08,	H 4.08,	I 37.00
Found:	C 31.42,	N 4.22,	H 4.13,	I 36.88

N-Methyl-3-hydroxypyridinium iodide ethanesulfonate. Recrystallized two times from methanol; m.p. 139–142°.

$C_8H_{12}INO_3S$

Calculated:	C 29.19,	N 4.26,	H 3.68,	I 38.53
Found:	C 29.18,	N 4.32,	H 3.69,	I 38.72

N - Methyl - 3 - hydroxpyridinium iodide methanesulfonate. Recrystallized from methanol; m.p. 169–172° (recorded, 173°).

Bis(3-aminopyridinium)-1,10-decane was prepared as the diodide salt and recrystallized from methanol (8); m.p. 179–180.5° (recorded, 180–181.5°).

Fluorescence Titrations

Equilibrium affinity constants for complex formation were determined from titration of the acetylcholinesterase tryptophan fluorescence (λ excitation = 290 nm/λ emission = 335 nm). A Farrand SPL spectrofluorometer was adapted with a rotating four-cell turret enclosed in a thermostated chamber. All measurements were made in 0.01 M Tris-Cl buffer, pH 8.0, containing 0.1 M NaCl and 0.04 M $MgCl_2$, with the temperature regulated at 25° ± 0.2°. Small relative volumes of ligand were added from a Gilmont micrometer syringe to 2.0 ml of acetylcholinesterase solution in a 1.0 cm^2 cuvette. For the less affine ligands, a 0.3 cm^2 cuvette containing 0.2 ml of sample was employed in order to minimize internal light absorption at high inhibitor concentrations. To determine the affinity of a nonquenching inhibitor, a competitive back-titration was employed and the affinity constant could be calculated from the return of the fluorescence and affinity of the quenching ligand (12). The enzyme concentration was 0.02–0.5 μM; the higher concentrations were used in the semimicrocuvette. The fluorescence signal from a standard, either tryptophan or human carbonic anhydrase B, placed in one of the turret positions was used to correct for changes in excitation energy or inner filter effects when high concentrations of titrant were used. The recorded values represent means of at least three separate titrations. Individual titrations were found to vary by less than 10% from the mean except in the case of the low-affinity complexes (K_D > 10 μM), where the variation tended to be somewhat higher.

The same titration procedure was adopted when fluorescence of bis(3-aminopyridinium)-1,10-decane was monitored, except that the excitation and emission wavelengths were 322 nm and 405 nm, respectively (8).

Chemically Modified Acetylcholinesterase

The purified acetylcholinesterase (2–5 μM) was allowed to react with the phosphorylating or sulfonylating agents in 0.01 M Tris-Cl buffer, pH 8.0, containing 0.1 M NaCl and 0.04 M $MgCl_2$. The following concentrations were employed: diisopropyl fluorophosphate, 50 μM; diethyl *p*-nitrophenyl phosphate, 50 μM; methanesulfonyl fluoride, 10 mM; *N*-methyl-3-hydroxypyridinium methanesulfonate, 0.5 mM; *N*-methyl-3-hydroxypyridinium ethanesulfonate, 2.0 mM; *N*-methyl-3-hydroxypyridinium *n*-propanesulfonate, 10 mM. The degree of irreversible inactivation was ascertained by diluting an aliquot from the reaction mixture 1:2000 and assaying for acetylcholinesterase activity. When the activity was reduced by 96–99%, the samples were cooled to 4° and passed over a 0.9 × 25 cm Sephadex G-25 column to separate the enzyme from the low molecular weight reaction products. In some cases following the gel separation step, samples were also dialyzed against the buffer used for titration.

RESULTS

Figure 1 shows the titration of acetylcholinesterase fluorescence upon addition of 2,5-bis(3-diethyl-*o*-chlorobenzylammonium-

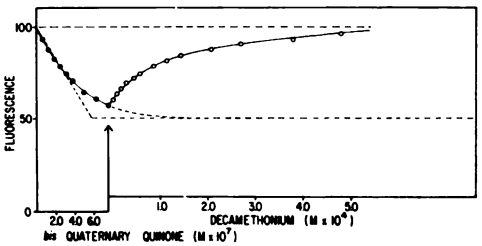


FIG. 1. *Fluorescence titration of 1.25 × 10^{-7} M Torpedo acetylcholinesterase with 2,5-bis(3-diethyl o-chlorobenzylammonium-n-propylamino) benzoquinone and back-titration with decamethonium*

Increments of the benzoquinone derivative were added, and fluorescence was monitored at 335 nm (excitation, 290 nm). At the discontinuity increments of decamethonium were added, and the increase in fluorescence was followed. The complete benzoquinone titration curve is shown by the dashed line extension. The buffer was 0.01 M Tris-Cl, pH 8.0, with 0.1 N NaCl and 0.04 M $MgCl_2$, at 25° ± 0.2°.

n-propylamino)benzoquinone. The inhibitor, whose absorption maximum is 340 nm, quenches the protein fluorescence arising primarily from the 19 tryptophanyl residues in acetylcholinesterase, and from the titration curve the affinity of the complex may be calculated. Back-titration with decamethonium, which possesses no spectral overlap with tryptophan fluorescence, competitively displaces the original ligand from the complex, resulting in a return of the fluorescence to that exhibited by the free enzyme (Fig. 1). The affinity of the nonquenching ligand may, in turn, be calculated from the profile of the return of the fluorescence and the affinity of the quenching inhibitor. Table 1 lists the dissociation constants (K_D) for a series of bisquaternary ligands. There is good agreement between K_D for the benzoquinone inhibitors determined from fluorescence and the relative I_{50} values determined by measuring inhibition of erythrocyte acetylcholinesterase-catalyzed acetylcholine hydrolysis (13). Agreement between direct measurements of inhibitor binding and indirect determinations which measure substrate catalysis can be expected when all inhibitors in the series exhibit the same kind of inhibition, i.e., competitive or noncompetitive (14).

Owing to the close overlap between the fluorescence emission spectrum of the protein and the absorption spectrum of the benzoquinone inhibitors, the decreased quantum yield of the complex (Table 1) will be a consequence primarily of radiationless energy transfer from the excited-state tryptophanyl donors to the benzoquinone acceptor. The aminopyridinium derivative, because it has an absorption maximum at 322 nm and a lower extinction coefficient ($\epsilon^{M}_{322} = 7.15 \times 10^3$), would be expected to show less fluorescence quenching upon complexation (Table 1). The efficiency of the dipolar energy transfer, T, can be expressed by the following equation (15–17):

$$T = 1 - \frac{F}{F_0} = \frac{(R_0/R)^6}{(R_0/R)^6 + 1} \qquad (1)$$

where R is the distance between donor and acceptor, F and F_0 are fluorescence yields in the presence and absence of energy transfer, and R_0 is the critical transfer distance at 50% efficiency. Also

$$R_0 = 9.79 \times 10^3 (J\kappa^2 n^{-4} Q)^{1/6} \qquad (2)$$

where Q is the quantum yield in the absence of transfer, J the overlap integral, n the refractive index, and κ a dipole orientation factor. Owing to the sixth-power dependence on distance between donor-acceptor pairs (Eq. 1), the efficiency of energy transfer falls off markedly as the critical transfer distance is exceeded (16, 17).

A corrected acetylcholinesterase tryptophanyl spectrum and quantum yield were obtained from a comparative spectral method using tryptophan and bovine carbonic anhydrase B as standards (18, 19). With the corrected emission spectrum, overlap integrals, J, were calculated according to the formula

$$J = \frac{\int F_d(\lambda)\epsilon_A(\lambda)\lambda^4 \, d\lambda}{\int F_d(\lambda) \, d\lambda} \qquad (3)$$

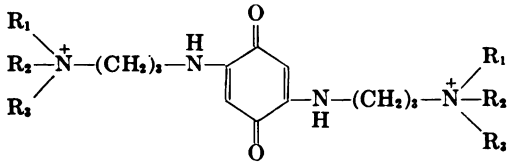
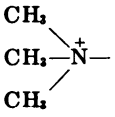
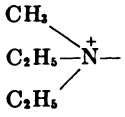
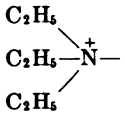
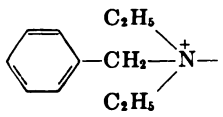
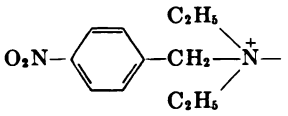
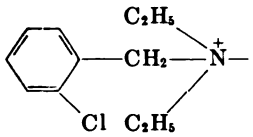
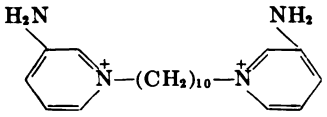
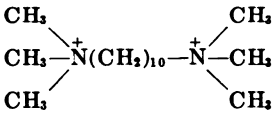
where $F_d(\lambda)$ is the donor emission and $\epsilon_A(\lambda)$ the molar extinction coefficient of the acceptor ligand at wavelength λ. For the benzoquinone ligands which have a relatively high molar extinction, $J = 18.8 \times 10^{-15}$ cm^6/mole, while for bis(3-aminopyridinium)-1,10-decane $J = 4.21 \times 10^{-15}$ cm^6/mole. In turn, the critical transfer distances, R_0, for the two complexes have been calculated to be 24.2 A and 18.9 A,[1] respectively. In the latter calculations a random dipole factor, $\kappa^2 = \frac{2}{3}$, was employed and the acetylcholinesterase quantum yield was estimated to be 0.121. A refractive index value of 1.6 (18) was selected, assuming that transfer occurs through the protein matrix. If the refractive index value for the solvent ($n = 1.36$) is employed in these calculations, R_0 will be increased by 10%.

Since each subunit in acetylcholinesterase contains 19 tryptophanyl residues, only a mean transfer distance between donor-acceptor pairs, represented by $(R_0^{-6})^{-1/6}$, can

[1] The aminopyridinium compound has two acceptor moieties which are separable by 12–14 A. No attempt has been made to calculate R_0 for the case of random donors and two acceptor chromophores (20).

TABLE 1

Dissociation constants, K_D, determined from ligand or protein fluorescence changes associated with complex formation at 25°

Bisquaternary ligand	K_D	Quenching of protein fluorescence
	M	%
		
	1.2×10^{-5}	52
	3.40×10^{-6}	51
	3.1×10^{-6}	53
	1.9×10^{-7}	50
	2.9×10^{-6}	56
	2.8×10^{-8}	53
	3.1×10^{-7a} 2.6×10^{-7b}	35
	6.1×10^{-6c} 5.9×10^{-6d}	<3

[a] Determined from quenching of protein fluorescence.

[b] Determined from ligand fluorescence.

[c] Determined by back-titration of the *o*-chlorobenzyldiethyl BQ.

[d] Determined by back-titration of bis(3-aminopyridinium)-1,10-decane.

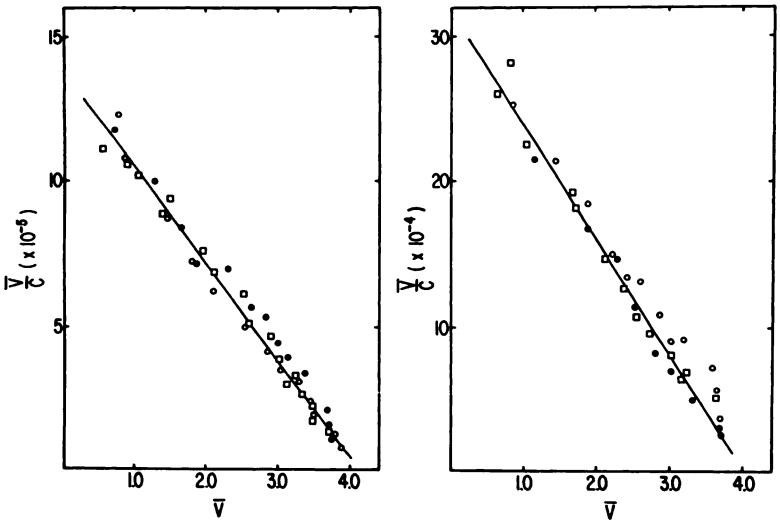


FIG. 2. *Scatchard plots of fluorescence titrations of Torpedo acetylcholinesterase and various bisquaternary benzoquinone inhibitors*

Conditions were the same as in Fig. 1. $\bar{v}$ is moles of ligand bound per mole of enzyme. c is the free ligand concentration: Left-hand panel, bis-triethyl BQ; right-hand panel, bis-trimethyl BQ. $\bar{v}$ was determined from the fractional approach to maximum quenching. At maximum quenching, which was determined from reciprocal plots, it is assumed that 4 ligand molecules are bound per mole of acetylcholinesterase. The different symbols denote separate titrations.

be calculated. While this value in itself is not particularly informative, an additional consideration arises with oligomeric proteins containing multiple binding sites. If the distance between the four binding sites on the tetrameric acetylcholinesterase molecule is less than twice the critical transfer distance (approximately 48 A for the benzoquinonium derivatives), ligand association with the first and in some cases the second unoccupied subunit site could produce proportionally greater contributions to quenching than would association with the third and fourth sites. The relative quenching of each of the four combining ligands on the tetramer would be dependent upon the distance between the acceptor chromophores and the degree of subunit or binding site symmetry. Equalization of the quenching contributions would become evident as the distance between binding sites exceeds 40–50 A, since the configuration space where efficient energy transfer occurs would no longer be conjoint with that of an adjacent subunit.

As shown in Fig. 2, Scatchard plots of fluorescence titrations approximate a straight line, which indicates uniform quenching per subunit. Consistent with this behavior is the observation that a similar fraction of bis(3-aminopyridinium)-1,10-decane is calculated to be bound when protein fluorescence quenching (λ_{ex} = 290 nm/λ_{em} = 335 nm) and ligand fluorescence (λ_{ex} = 322 nm/λ_{em} = 405 nm) are compared (Fig. 3). The reduction of ligand fluorescence upon binding presumably results from a change in effective polarity or ligand mobility within the macromolecular site (8), but does not involve longer-range energy transfer with residues in the macromolecule. Accordingly, quantum yield changes in the bis(3-aminopyridinium) ligand should be independent of inter-binding site distances.

The Stokes radius of *Torpedo* acetylcholinesterase has been estimated to be 76 A, and the hydrodynamic measurements also indicate a degree of dimensional asymmetry in the 335,000 molecular weight macromolecule (f/f_0 = 1.65) (10). These physical constants do not allow one to infer dimensional characteristics to the four individual subunits; however, a globular protein subunit of molecular weight 82,500 would have an effective radius of about 36 A. Since the through-space

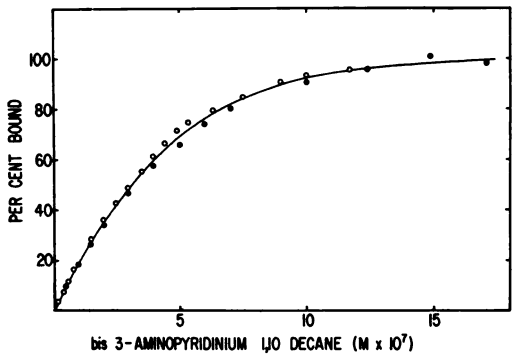


FIG. 3. *Association of bis(3-aminopyridinium)-1,10-decane with acetylcholinesterase*

The percentage of enzyme in the bound state was measured by quenching of protein fluorescence (λ_{ex} = 290 nm/λ_{em} = 335 nm) (●——●) and by the decrease in ligand fluorescence (○——○) (λ_{ex} = 322 nm/λ_{em} = 405 nm). The percentage decrease in ligand fluorescence was determined from the minimum quantum yield obtained in the presence of excess acetylcholinesterase.

distance between subunits should be greater than 40–50 A when fluorescence quenching is uniform, some constraints can be placed on the location of binding sites in acetylcholinesterase. For example, our observations would argue against the presence of the sites at the interfaces between subunits positioned near the geometric center of the molecule or near an axis of symmetry separating individual subunits. Linear Scatchard plots would also be indicative of a lack of homotropic cooperativity for the binding of bisquaternary ligands.

Complex Formation with Chemically Modified Acetylcholinesterase

Extensive studies over a number of years have clearly established that phosphorylating and sulfonylating agents inhibit acetylcholinesterase by forming relatively stable ester bonds with the active site serine (see refs. 21, 22). While hydrolysis of these bonds occurs relatively slowly in H_2O, stronger nucleophiles promote cleavage, forming an active enzyme (21, 22). More rapid rates of phosphorylation and sulfonylation can be achieved by site-directing the reactive group 4–5 A from a quaternary nitrogen (21–23). Similarly, nucleophilic attack on the serine ester is enhanced by site-directing the hydroxamate or oxime nucleophile (21, 22). In Fig. 4 the purified acetylcholinesterase has been phosphorylated by diisopropyl fluorophosphate and subsequently dephosphorylated with *N*-methylpyridine-2-aldoxime to regenerate active enzyme. Prior to the reaction and following each reactive step and removal of the reactants, the enzyme was titrated with chlorobenzyldiethyl BQ.[2] Both the regenerated and original acetylcholinesterase yield the same titration profile. This observation indicates that the chemical modification procedures are attended by minimal denaturation or side reactions that could influence the interpretation of our findings.

Space-filling models of the acid-transferring groups that have been linked to the active site serine are shown in Fig. 5. In the case of the phosphoryl-enzymes, titrations were conducted directly after reaction and separation of the enzyme from the reactants in order to minimize aging effects (22). All the sulfonyl-enzymes were prepared from the reaction with the corresponding sulfonyl ester of *N*-methyl-3-hydroxypyridinium, which could afford an additional degree of specificity by site direction toward the catalytic serine. Nevertheless, we have found that modification by methanesulfonyl fluoride and the corresponding sulfonyl ester yields methanesulfonyl-enzymes with identical ligand binding profiles.

As shown in Fig. 6, the rate of inactivation of acetylcholinesterase by the sulfonyl esters decreases with increasing chain length of the acid-transferring group. At low substrate concentrations differences in the rates of sulfonylation appear to correlate more closely with the intrinsic reactivity, k_3, rather than the apparent substrate affinity, K_s (Fig. 6). Kinetic constants for the methanesulfonyl derivative are in reasonable accord with those observed previously for the *Electrophorus* enzyme (23). However, deviations from the expected bimolecular substrate association and subsequent unimolecular enzyme sulfonylation kinetics (23) are ob-

[2] The abbreviations used are: chlorobenzyldiethyl BQ, 2,5-bis(3-diethyl-*o*-chlorobenzylammonium-*n*-propylamino)benzoquinone; trimethyl BQ, 2,5-bis(trimethylammonium-*n*-propylamino)-benzoquinone: triethyl BQ, 2,5-bis(triethylammonium-*n*-propylamino)benzoquinone.

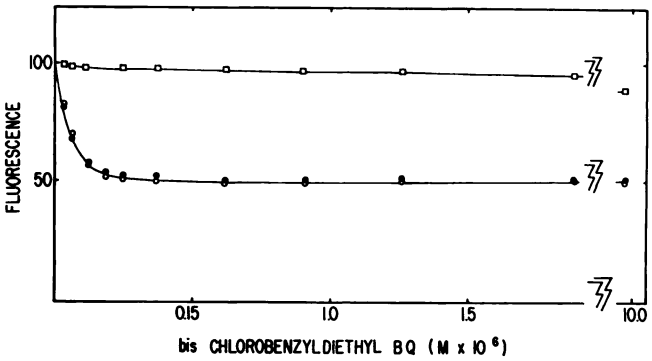


FIG. 4. *Titration of acetylcholinesterase fluorescence by 2,5-bis(3-diethyl—o-chlorobenzylammonium-n-propylamino)benzoquinone after phosphorylation by diisopropyl fluorophosphate and subsequent regeneration of active enzyme by pyridine-2-aldoxime methiodide*

O——O, native enzyme; □——□, diisopropylphosphoryl-enzyme; ●——●, aldoxime-regenerated enzyme. Details of the titration procedure are described in the text.

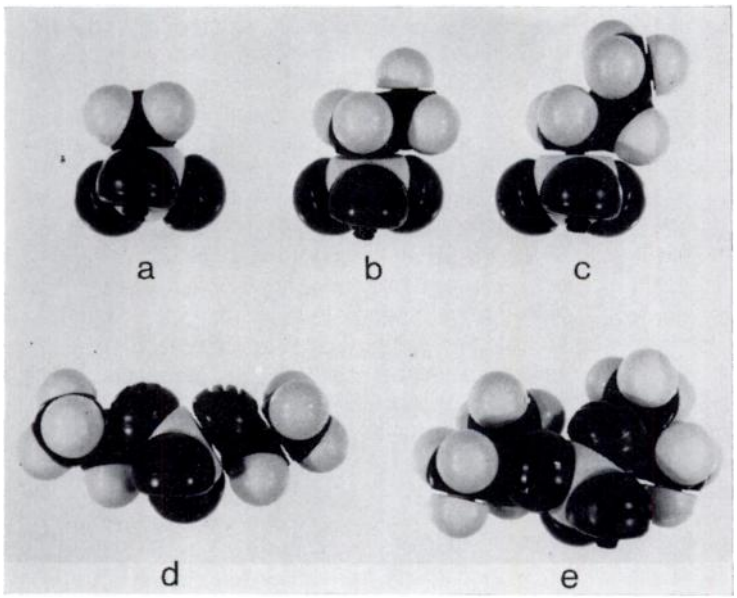


FIG. 5. *Corey-Pauling-Koltun models of the acid-transferring portion of serine-modifying groups*

The position of attachment to the serine is marked with the connecting link: (a) methanesulfonyl, (b) ethanesulfonyl, (c) propanesulfonyl, (d) diethoxyphosphoryl, or (e) diisopropoxyphosphoryl.

served at high substrate concentrations of the ethane and propanesulfonyl esters. While this behavior may reflect secondary binding of the substrate to a peripheral anionic site, it should be noted that at these high substrate concentrations the sulfonyl esters contribute significantly to the ionic strength of the medium.

Figures 7 and 8 illustrate titration curves for a series of modified enzymes. A reduction in quantum yield of the bound ligand and similar titration behavior of bis(3-aminopyridinium)-1,10-decane has been reported by Mooser *et al.* (8) with the *Electrophorus* enzyme. We find that the bound ligand exhibits about 9% of the fluorescence of the free ligand. A comparison of the affinity of this aromatic bisquaternary ligand for the diisopropoxyphosphoryl- and native enzyme shows that a substantial reduction in affinity

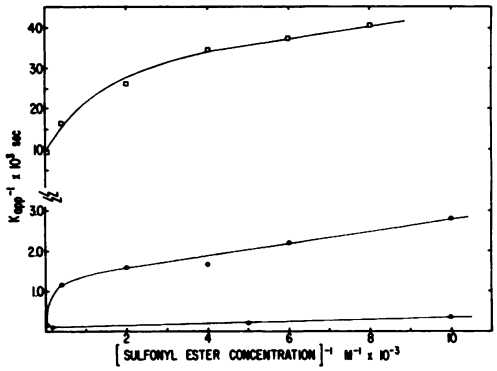


FIG. 6. *Reciprocal plots of rate constant for inactivation of Torpedo acetylcholinesterase (AChE) as a function of concentration of sulfonyl ester*

The reaction was carried out as previously described (23), except that a pH 8.0 Tris-Cl buffer containing 0.1 M NaCl and 0.04 M $MgCl_2$ was used. Data for the methanesulfonyl ester are in good agreement with previous findings on the *Electrophorus* enzyme. O——O, *N*-methyl-3-hydroxypyridinium methanesulfonate; ●——●, *N*-methyl-3-hydroxypyridinium ethanesulfonate; □——□, *N*-methyl-3-hydroxypyridinium *n*-propanesulfonate. Using the scheme developed in ref. 23, where AChE + RSO_2O-pyridinium

$$\underset{}{\overset{K_s}{\rightleftharpoons}} \text{AChE}\cdots RSO_2O\text{-pyridinium} \xrightarrow{k_3} RSO_2O\text{-AChE} + \text{pyridinium}$$

k_3 is estimated by extrapolation of the linear portion of the graph to the ordinate intercept and K_s is obtained from the slope and k_3. The values are: methanesulfonyl, $K_s = 4.5 \times 10^{-4}$ M, $k_3 = 100 \times 10^{-4}$ sec^{-1}; ethanesulfonyl, $K_s = 1.3 \times 10^{-4}$ M, $k_3 = 8.3 \times 10^{-4}$ sec^{-1}; propanesulfonyl, $K_s = 0.5 \times 10^{-4}$ M, $k_3 = 0.35 \times 10^{-4}$ sec^{-1}.

accompanies modification of the active site serine. However, when ligand fluorescence changes are monitored, a precise determination of affinity requires concentrations of enzyme which are equivalent in magnitude to the affinity constant. Relatively low enzyme concentrations may be employed for the benzoquinone derivatives when protein fluorescence is monitored. Furthermore, chemical modification of acetylcholinesterase, in addition to affecting the affinity of the complex, may alter fluorescence quantum yields of the protein or the bound ligand. This is less apt to be an influencing factor when one is monitoring protein fluorescence changes which are dependent on longer-range energy transfer rather than on environmental differences localized to the binding

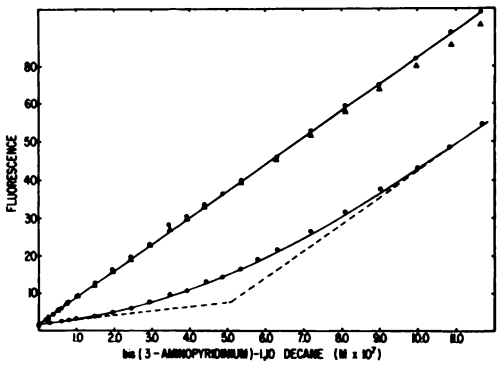


FIG. 7. *Fluorescence titrations with bis(3-aminopyridinium)-1,10-decane*

O——O, no enzyme; ●——●, 1.25×10^{-7} M enzyme; △——△, 1.2×10^{-7} M diisopropoxyphosphoryl-enzyme in 0.01 M Tris-Cl, pH 8.0, containing 0.1 M NaCl and 0.04 M $MgCl_2$ (λ_{ex} = 322 nm/ λ_{em} = 405 nm).

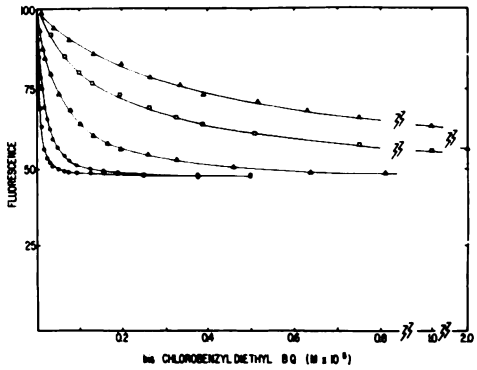


FIG. 8. *Fluorescence titration of acetylcholinesterase esterified at the active site serine by various modifying agents*

●——● native enzyme; O——O, methanesulfonyl-enzyme; ▲——▲, ethanesulfonyl-enzyme; □——□, propanesulfonyl-enzyme; △——△, diethoxyphosphoryl-enzyme. The enzyme concentration was 2.5×10^{-6} M; other conditions were the same as in Fig. 1.

site. Reciprocal plots of the data in Fig. 8 show essentially the same degree of quenching for the modified and the native enzyme at saturation. Thus the benzoquinone derivatives appear to afford the more suitable system for examining the influence of serine modification on ligand binding. Table 2 lists the dissociation constants, K_D, and free energy of association, ΔF, between three bis-quaternary inhibitors and five covalently modified acetylcholinesterase derivatives.

TABLE 2

Dissociation constants and free energy of association for complexes between bisquaternary benzoquinones and esters of acetylcholinesterase

Quaternary substitution on ligand	Serine modification of acetylcholinesterase											
	None HO		Methanesulfonyl $CH_3-S(=O)_2-O-$		Ethanesulfonyl $CH_3-CH_2-S(=O)_2-O-$		Propanesulfonyl $CH_3-CH_2-CH_2-S(=O)_2-O-$		Diethoxyphosphoryl $(C_2H_5O)_2P(=O)-O-$		Diisopropoxyphosphoryl $(CH_3)_2CHO)_2P(=O)-O-$	
	K_D	$-\Delta F$	K_D	$-\Delta F$	K_D	$-\Delta F$	K_D	$-\Delta F$	K_D	$-\Delta F$	K_D	$-\Delta F$
	M	*kcal/mole*	*M*	*kcal/mole*	*M*	*kcal/mole*	*M*	*kcal/mole*	*M*	*kcal/mole*	*M*	*kcal/mole*
$(CH_3)_3\overset{+}{N}-CH_3$	1.21×10^{-5}	6.70	1.3×10^{-5}	6.66	1.32×10^{-5}	6.65	4.4×10^{-5}	5.95	4.0×10^{-5}	5.99	5.9×10^{-5}	5.76
$(C_2H_5)_3\overset{+}{N}-C_2H_5$	3.12×10^{-6}	7.51	3.55×10^{-6}	7.43	3.50×10^{-6}	7.43	1.23×10^{-5}	6.70	4.5×10^{-5}	5.93	5.3×10^{-5}	5.82
$(C_2H_5)_3\overset{+}{N}-CH_2$-(2-Cl-phenyl)	2.8×10^{-8}	10.3	1.1×10^{-7}[a] 1.22×10^{-7}[b]	9.49 9.43	4.8×10^{-7}	8.60	1.7×10^{-6}	7.87	2.4×10^{-6}	7.66	2.9×10^{-5}	6.18

[a] Ester formed methanesulfonyl fluoride.

[b] Ester formed with *N*-methyl-3-hydroxypyridinium methanesulfonate.

The observation that the active site serine remains exposed and, in fact, becomes more reactive, when succinylcholine or decamethonium is bound to acetylcholinesterase led Belleau *et al.* (3) to propose that these bisquaternary inhibitors bind in an "exo" orientation in which the active center serine is not occluded. The magnitude of the reduction in ligand binding affinity, which correlates roughly with the size of the acid-transferring group in the esterified enzyme and substituents on the quaternary ammonium (Table 2), provides independent evidence supporting this proposed orientation.

Bisquaternary inhibitors exhibit a higher affinity for acetylcholinesterase than various monoquaternary analogues, indicating the involvement of an accessory site in stabilizing the complex (24, 8). Moreover, competitive kinetic studies suggest that one of the complementary sites lies within the active center while the other end of the bisquaternary ligand may bind in the vicinity of the peripheral anionic site, presumably some 10–12 A away from the active center (3, 5). The simplest explanation for the observed reduction in affinity which results when the active center serine is esterified is that the insertion of the acid-transferring group renders the fit at the anionic subsite within the active center less favorable, while the peripheral anionic site is unaffected. Steric interference with the active center fit would diminish dispersion forces as well as increase the internuclear distance between charged centers, reducing the coulombic attraction.

An examination of the dissociation constants of the bisquaternary ligands for native acetylcholinesterase (Table 1) indicates that an increase in substituent bulk around the quaternary nitrogens tends to enhance binding affinity. This observation, when considered with analogous previous findings for other mono- and bisquaternary congeners (25, 26), reinforces the argument that hydrophobic forces play a dominant role in stabilizing the complex. Groups which tend to interposition between charged centers on the ligand and macromolecule limit the approaching distance between surfaces of opposing charge. Thus any enhancement of binding energy acquired from hydrophobic interactions when such groups are interposed must occur at the expense of reducing coulombic forces, which show an approximate dependence on the inverse square of the internuclear distance in aqueous systems. For example, the difference in ionic radii of tetramethyl- and tetraethylammonium is 0.63 A. If we take 4.0 A as a reasonable equilibrium internuclear distance between ionic centers, the coulombic contribution toward stabilization of the complex will be decreased by at least 26 %. Thus the 0.8–0.9-kcal increase in free energy of stabilization for the bis-triethyl over the bis-trimethyl ligand (Table 2) is the result of an even greater enhancement of dispersion forces.

In contrast to native acetylcholinesterase, binding of the three bisquaternary inhibitors to the diisopropoxyphosphoryl-enzyme shows a convergence in affinity (Table 2). Thus, when the largest acid-transferring group is linked to the active site serine, little structural specificity toward these ligands can be discerned. Selective modification of the active site serine diminishes the contribution of the anionic subsite with the active center relative to the peripheral anionic site in conferring stability to the complex. Thus, in the modified enzyme complexes, the virtual absence of a dependence of binding energy on bulky substituents around the quaternary ammonium group suggests that dispersion forces are of less importance in stabilizing the portion of the bisquaternary ligand at the site peripheral to the active center.

DISCUSSION

A close inspection of the degree of reduction in affinity of the bisquaternary ligands resulting from esterification by acid-transferring groups of various sizes reveals distinct differences among the three bisquaternary ligands. Chlorobenzyldiethyl BQ shows an incremental decrease in affinity with each increase in dimension of the acidic group of the ester. We might infer, since this ligand exhibits the highest affinity for the native enzyme, that the aromatic ring system contributes additional stabilization energy to the complex. It therefore seems probable that the chlorobenzyl group overlaps with a large portion of the active site, and perturbation of the surface of the active site by esterifying the serine diminishes the extent of aromatic

ring interaction. If the perturbing acid transferring group were large enough, the optimal binding orientation might necessitate that the aromatic group be directed away from the surface of the enzyme.

The progressive reduction in binding energy for the chlorobenzyldiethyl derivative can be contrasted with triethyl BQ and particularly trimethyl BQ, with which a very abrupt transition is observed between ethanesulfonyl- and propanesulfonyl-acetylcholinesterase. This would suggest that overlap of the acid transferring group in the esterified enzyme with the anionic subsite becomes appreciable at the ethane-propane juncture. To further understand this structural dependence necessitates a proposal for the preferred orientation of the sulfonyl or phosphoryl groups within the active center of the enzyme. Substantial evidence has accumulated over the years to support the contention that acetylcholinesterase exhibits catalytic behavior typical of a large class of serine hydrolases. Kinetic studies have indicated that this class of enzymes share common transacylation and deacylation mechanisms (21, 22). Moreover, hemisubstrates, which effectively transfer an acidic group to the reactive serine, forming a relatively stable ester bond, are inhibitory through the series of enzymes (21, 27, 28). Finally, considerable sequence homology exists in the active site peptides (29). It should be recognized, however, that the similarities apply to the actual transesterification mechanism rather than to substrate specificity.

X-ray crystallographic studies on the structure of certain serine proteases have revealed that within this group of enzymes the potential nucleophilic character of the catalytic serine could be enhanced through a charge-relay system, which involves both an aspartic acid and a histidine residue from noncontiguous portions of the amino acid sequence (30). A second common structural feature of these enzymes, which has more recently emerged from the crystallography of α-chymotrypsin (31, 32) and subtilisin (33), is the presence of an oxyanion hole, where the opportunity for hydrogen bonding is afforded to the carbonyl oxygen of the acyl substrate. For hydrogen bonding to be optimal, the trigonal carbonyl carbon in the acyl substrate must distort to a tetrahedral disposition, thus favoring the apparent transition state for transesterification relative to the substrate ground state (33). A tetrahedral addition product has been proposed as the acyl transition state, where the active site serine oxygen attacks the carbonyl carbon of the susceptible ester or amide bond and the carbonyl oxygen becomes stabilized within the oxyanion hole. The latter process involves a rotation of the carbonyl oxygen by about 50°. The apparent transition state adduct then collapses to form the acyl-enzyme (33). Acid-transferring substrates, such as the sulfonyl or phosphoryl esters, which maintain a tetrahedral disposition could be expected to adopt an orientation similar to the acyl-substrate transition state within the active center, as has been suggested by crystallographic studies on diisopropyl fluorophosphate and chloromethyl ketone derivatives of chymotrypsin and subtilisin (34, 35). That such mechanistic arguments would apply directly to acetylcholinesterase is reinforced by the recent observation that a borinic acid analogue of acetylcholine possesses an affinity for acetylcholinesterase that exceeds the K_m for acetylcholine by four orders of magnitude. Borinic acid tends to form tetrahedral adducts that can be regarded as transition state analogues for the serine hydrolyases.[3]

The above considerations suggest that the phosphoryl oxygen and one of the two sulfone oxygens would be associated with the oxyanion hole in the case of the phosphoryl or sulfonyl esters of acetylcholinesterase. A satisfactory model for the orientation of the acid-transferring groups in the active site can be developed from the crystal structure of the active site of α-chymotrypsin. At the active center of this enzyme serine-195 becomes esterified, and the hydrogens located on the amide backbone nitrogens of serine-195 and glycine-193 serve as the hydrogen bond donors in the oxyanion hole (31) (see Fig. 9). If the phosphoryl oxygen in the hemisubstrate serves as a suitable hydrogen bond acceptor, as we would anticipate with the approximate tetrahedral configuration

[3] G. P. Hess, unpublished observations, cited by Lienhard (36).

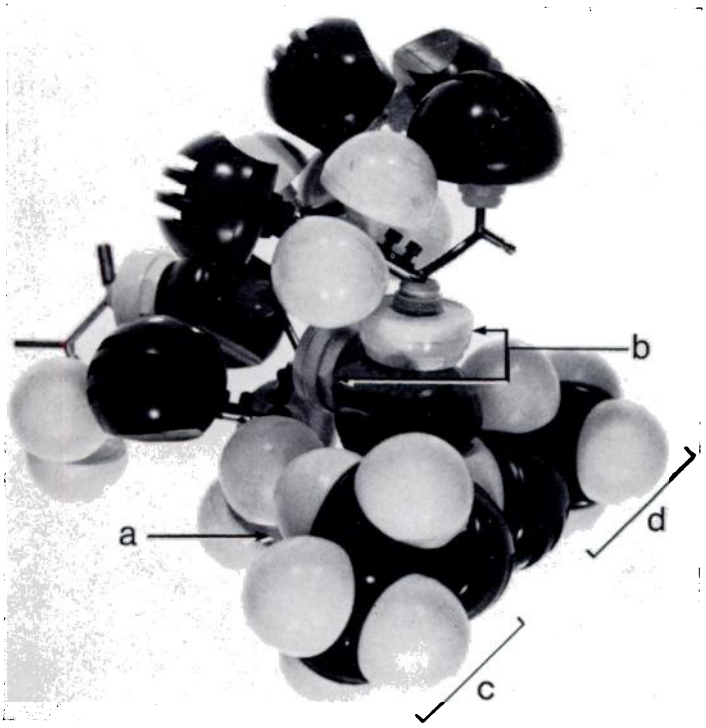


FIG. 9. *Proposed orientation of the acid-transferring group in the active center of acetylcholinesterase, using the chymotrypsin model*

The residues glycine-193, aspartate-194, and serine-195 have the orientation and bond angles shown in the crystallographic structure (31). Space-filling hydrogens and oxygens are added. The diethoxyphosphoryl group is linked through the side chain oxygen of serine-195 (designated by *a*), and the phosphoryl oxygen is hydrogen bonded to the two donor hydrogens on the amide nitrogen backbone of glycine-193 and serine-195 (*b*). This area has been deemed the "oxyanion" hole (33). Hydrogen bonding at this position would fix the orientation of the alkoxy groups with respect to the active site surface. One of the alkoxy groups (*c*) would be oriented toward the anionic subsite, while the other (*d*) would be directed into the acyl pocket. Accordingly *d* would have the same orientation as the acyl-CH_3 group in its transition state configuration. The chymotrypsin model was constructed by Dr. J. J. Birktoft, Department of Chemistry, University of California, San Diego.

for the phosphorus, the two alkoxy groups will assume distinct orientations. One alkoxy group would be directed toward the anionic subsite where the quaternary nitrogen of choline would bind, while the other group would assume the same geometry as the acetyl-CH_3 moiety when in its transition state configuration (i.e., as the tetrahedral addition product). With this orientation one would expect, as is observed, considerable interference by the alkoxy group with the binding of bisquaternary inhbitors at the anionic subsite when the active center serine is phosphorylated (Table 2). The diethoxyphosphoryl ester of the residue 193–195 tripeptide of chymotrypsin depicts the geometric relationship between the alkoxy groups and the active site (Fig. 9).

For the alkanesulfonyl-enzymes an analogous situation would be predicted, since sulfur-oxygen and phosphorus-oxygen bond distances differ by only 0.1–0.15 A. However, with the two oxygens available as potential hydrogen bond acceptors, two orientations of the alkyl group are possible; that is, directed toward either the anionic subsite or the acyl pocket. If we assume that steric considerations will dictate which orientation of the alkyl group prevails, the dimensions of the acyl pocket represent the critical consideration. Some inference on this can be obtained by examining substrate specificity. Com-

pared with acetylcholine, the acetylcholinesterase-catalyzed ester hydrolysis rate is slightly diminished with propionylcholine and markedly lower with butyrylcholine (37). Analogous structural specificity is evident when the relative rates of serine sulfonylation by the alkanesulfonylpyridinium esters are compared (Fig. 5). The reduction in intrinsic reactivity of the ethanesulfonyl and particularly the propanesulfonyl ester relative to the methane congener would suggest that steric factors also place constraints upon the fit within the acyl pocket of the tetrahedrally disposed alkanesulfonyl group.

In the alkanesulfonyl-enzymes, if the alkyl group cannot be accommodated favorably within the acyl pocket, an alternative configuration can be achieved simply by rotation around the serine β-carbon–oxygen–sulfur bond and association of the opposing sulfone oxygen within the oxyanion hole. If the alkyl group were oriented toward the acyl pocket, no direct steric overlap with the bound bis-trimethyl BQ and bis-triethyl BQ would be predicted. However, upon rotation of 109° the alkyl group assumes an orientation directed in the vicinity of the anionic subsite, and distinct overlap with the quaternary alkyl groups would be anticipated. The marked difference in free energy values which is observed when proceeding from the ethanesulfonyl- to the propanesulfonyl-enzyme for *both* trimethyl and triethyl BQ complexes (Table 2) would indicate that a change in orientation of the alkanesulfonyl group prevails with this substitution change.

The stabilization energy of a ligand-macromolecule complex where two discrete positions on the ligand interact with topologically distinct sites on the macromolecule cannot be treated simply in terms of a linear summation of free energy contributions at each of the sites. Association at one of the sites enhances the likelihood of the second interaction occurring, since its entropic demands are reduced (38, 39). Thus a statistical advantage in the direction of complex formation is imparted to the reaction.

By analogy with chelation of bidentate ligands (38), one might expect that the free energy of association for a bisquaternary inhibitor would be greater than the sum of interaction energies at the two individual quaternary nitrogens. This lack of additivity of the free energy of individual interactions in contributing energy of stabilization of the complex, while it may be of general importance in specificity of drug action (40), adds an inherent complexity to consideration of bisquaternary ligand–acetylcholinesterase interactions. Nevertheless, selective modification of one of the sites of interaction for the bisquaternary ligands provides one means, although somewhat limited by the above considerations, of delineating the binding specificity and force contributions in the complex. We believe that the over-all problem can be approached in more depth by investigating the effects of ionic strength on ligand association with the free and modified enzymes. Furthermore, the information to be derived can be enhanced by investigating individual association and dissociation steps, and we are currently investigating ligand binding to acetylcholinesterase by stopped-flow and temperature-jump methods.

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REFERENCES

1. Changeux, J.-P. (1966) *Mol. Pharmacol.*, **2**, 369–392.
2. Belleau, B. & DiTullio, V. (1970) *J. Amer. Chem. Soc.*, **92**, 6320–6325.
3. Belleau, B., DiTullio, V. & Tsai, Y.-H. (1970) *Mol. Pharmacol.*, **6**, 41–45.
4. Kitz, R. J., Braswell, L. M. & Ginsburg, S. (1970) *Mol. Pharmacol.*, **6**, 108–121.
5. Roufogalis, B. D. & Quist, E. E. (1972) *Mol. Pharmacol.*, **8**, 41–49.
6. Crone, H. D. (1973) *J. Neurochem.*, **20**, 225–236.
7. Mooser, G. & Sigman, D. S. (1972) *Biochem. Biophys. Res. Commun.*, **48**, 559–564.
8. Mooser, G., Schulman, H. & Sigman, D. S. (1972) *Biochemistry*, **11**, 1595–1602.
9. Kato, G. (1972) *Mol. Pharmacol.*, **8**, 582–588.
10. Taylor, P., Jones, J. W. & Jacobs, N. M. (1974) *Mol. Pharmacol.*, **10**, 78–92.
11. Ginsburg, S. (1962) *J. Med. Chem.*, **5**, 1364–1367.
12. Taylor, P. W., King, R. W. & Burgen, A. S. V. (1970) *Biochemistry*, **9**, 2638–2645.

13. Hoppe, J. O., Funnel, J. E. & Lape, H. (1955) *J. Pharmacol. Exp. Ther.*, **115**, 106–119.
14. Webb, J. L. (1963) *Enzyme and Metabolic Inhibitors*, Vol. 1, pp. 182–183, Academic Press, New York.
15. Förster, T. (1956) in *Modern Quantum Chemistry*, Part 3 (Sinanoglu, O., ed.), pp. 93–99, Academic Press, New York.
16. Stryer, L. (1968) *Science*, **162**, 526–533.
17. Latt, S. A., Cheung, H. T. & Blout, E. R. (1965) *J. Am. Chem. Soc.*, **87**, 995–1003.
18. Chen, R. F. & Kernohan, J. C. (1967) *J. Biol. Chem.*, **242**, 5813–5823.
19. Chen, R. F., Edelhoch, H. & Steiner, R. F. (1969) in *Physical Principles and Techniques in Protein Chemistry*, Part A, (Leach, S. J., ed.), pp. 171–240, Academic Press, New York.
20. Gennis, R. B. & Cantor, C. R. (1972) *Biochemistry*, **11**, 2509–2517.
21. Froede, H. C. & Wilson, I. B. (1971) in *The Enzymes*, (Boyer, P., ed.), Vol. 5, pp. 87–114, Academic Press, New York.
22. Cohen, J. A. & Oosterbaan, R. A. (1963) in *Handbuch der experimentellen Pharmakolgie* (Koelle, G. B., ed.), Vol. 15, pp. 300–362' Springer, Berlin.
23. Kitz, R. J. & Wilson, I. B. (1962) *J. Biol. Chem.*, **237**, 3245–3249.
24. Bergmann, F. & Segal, R. (1954) *Biochem. J.*, **58**, 692–698.
25. Bergmann, F. & Wurzel, M. (1953) *Biochim. Biophys. Acta*, 11, 440–441.
26. Wilson, I. B. (1952) *J. Biol. Chem.*, **197**, 215–225.
27. Gold, A. M. (1967) *Methods Enzymol.*, **11**, 706–711.
28. Cohen, J. A., Oosterbaan, R. A. & Berends, F. (1967) *Methods Enzymol.*, **11**, 686–702.
29. Schaffer, N. K., Michel, H. O. & Bridges, A. F. (1973) *Biochemistry*, **12**, 2946–2950.
30. Blow, D. M., Birktoft, J. J. & Hartley, B. S. (1969) *Nature*, **221**, 337–340.
31. Henderson, R. (1970) *J. Mol. Biol.*, **54**, 341–362.
32. Henderson, R., Wright, C. S., Hess, G. P. & Blow, D. M. (1971) *Cold Spring Harbor Symp. Quant. Biol.*, **36**, 63–71.
33. Robertus, J. D., Kraut. J., Alden, R. A. & Birktoft, J. J. (1972) *Biochemistry*, **11**, 4293–4303.
34. Steitz, T. A., Henderson, R. & Blow, D. M (1969) *J. Mol. Biol.*, **46**, 337–348.
35. Robertus, J. D., Alden, R. A., Birktoft, J. J., Kraut, J., Powers, J. C. & Wilcox, P. E. (1972) *Biochemistry*, **11**, 2439–2449.
36. Lienhard, G. E. (1973) *Science*, **180**, 149–154.
37. Augustinsen, K. (1949) *Arch. Biochem.*, **23**, 111–126.
38. Eigen, M. & Wilkens, M. (1965) *Adv. Chem. Ser.*, **49**, 55–72.
39. Margerum, D. W., Rohrabacher, D. B. & Clark, J. F. G. (1967) *Inorg. Chem.*, **2**, 667–675.
40. Burgen, A. S. V. (1970) in *Ciba Found. Symp. Molecular Properties of Drug Receptors*, 263–269, Churchill, London.