# Binding of Mono- and Bis-$N$-substituted Benzoquinolinium Halides to Butyrylcholinesterase: Fluorescence Measurements

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**Abstract** □ The dissociation constants ($K_{diss}$) of mono- and bis-$N$-substituted benzoquinolinium salts from butyrylcholinesterase were determined by the disappearance of the fluorescence of the free ligand. The $K_{diss}$ value determined by this method agreed closely with the dissociation constants ($K_{I,s}$) of the free enzyme obtained by steady-state kinetic studies. The results indicate that the $N$-substituted benzoquinolinium salts with a strong binding ability to butyrylcholinesterase also are potent inhibitors of the esterolytic properties of the enzyme. Enzyme binding is favored by the coplanar structure and does not require a high concentration of charge on the onium function. The shape of the hydrophobic structures markedly influences binding.

**Keyphrases** □ Benzoquinolinium halides—binding to butyrylcholinesterase, spectrophotofluorometric analysis □ Spectrophotofluorometry—analysis, benzoquinolinium halides, binding to butyrylcholinesterase □ Butyrylcholinesterase—binding to benzoquinolinium halides, spectrophotofluorometric analysis

A series of mono- and bisquaternary ammonium compounds was reported (1) to be reversible inhibitors of butyrylcholinesterase. The most potent inhibitors were characterized by a large nitrogen-containing conjugated heterocycle such as phenanthridine or acridine. Lineweaver–Burk plots of the enzymatic hydrolysis data showed mixed competitive–noncompetitive inhibition kinetics. Since many potential binding sites are present on the enzyme (2), these mono- and bisquaternary onium compounds were studied to determine if their anticholinesterase activity was produced by binding to sites that affect the esterolytic properties of the enzyme.

## EXPERIMENTAL

Horse serum butyrylcholinesterase[1] was purified by gel filtration, using a Sephadex G-200 column (2 × 90 cm) developed with pH 7.5 25 m$M$ sodium phosphate, and by ion-exchange chromatography, using a diethylaminoethyl cellulose column (2 × 85 cm) developed with a 1-liter linear gradient of 1.0 $M$ NaCl in pH 7.5 25 m$M$ sodium phosphate. Protein concentrations (milligrams per milliliter) were determined by the method of Lowry *et al.* (3). Enzyme activities were measured by the continuous automatic titration method, using a pH stat (1). The butyrylcholinesterase solution normality was determined by a titration method using echothiophate iodide (4).

**Table I—Quenching of Hexamethylene-1,6-bisacridinium Diiodide (50 n$M$) Fluorescence by Butyrylcholinesterase**

| $E, N$ | $F$ | $F_0 - F$ | $\alpha$ | $10^{-8} I$, $M$ | $R$ | $10^8 R/I$ |
|---|---|---|---|---|---|---|
| 0 | 40 ($F_0$) | | | | | |
| $5.5 \times 10^{-9}$ | 37.0 | 3.0 | 0.1 | 4.50 | 0.91 | 0.20 |
| $2.0 \times 10^{-8}$ | 29.8 | 10.2 | 0.34 | 3.30 | 0.85 | 0.26 |
| $3.8 \times 10^{-8}$ | 24.0 | 16.0 | 0.53 | 2.35 | 0.70 | 0.30 |
| $5.5 \times 10^{-8}$ | 18.7 | 21.3 | 0.71 | 1.45 | 0.65 | 0.45 |
| $7.5 \times 10^{-8}$ | 16.3 | 23.7 | 0.79 | 1.05 | 0.53 | 0.51 |
| $1.1 \times 10^{-7}$ | 13.3 | 26.7 | 0.89 | 0.55 | 0.40 | 0.73 |
| $2.5 \times 10^{-7}$ | 11.2 | 28.8 | 0.96 | 0.20 | 0.19 | 0.95 |
| $4.5 \times 10^{-7}$ | 10.6 | 29.4 | 0.98 | 0.10 | 0.11 | 1.10 |
| $1.1 \times 10^{-6}$ | 10 ($F_e$) | 30.0 | | | | |

Inhibitors were prepared as described previously (1). The inhibitors, butyrylcholinesterase, and bovine serum albumin were dissolved in 10 m$M$ sodium phosphate buffer containing 100 m$M$ NaCl and 20 m$M$ $MgCl_2$. The enzyme solution was diluted to give concentrations of 50 $\mu N$–5.5 n$N$. For the fluorescence measurements, each solution was mixed with an equal volume of inhibitor solution whose concentration was near its $K_{I,s}$ value. UV absorption spectra of the inhibitor solutions (5 $\mu M$) in the presence of varying amounts of butyrylcholinesterase or bovine serum albumin were measured on a double-beam spectrophotometer[2].

Disappearance of the fluorescence of the inhibitors in the presence of butyrylcholinesterase or bovine serum albumin was measured on a spectrophotofluorometer[3] equipped with a 150-w xenon lamp and a 1P 21 photomultiplier tube. Excitation at 360 nm caused fluorescence maxima of $N$-substituted acridinium halides at 490 nm, of phenanthridinium halides at 420 nm, of 5,6-benzoquinolinium halides at 440 nm, and of 7,8-benzoquinolinium halides at 460 nm. Fluorescence intensity measurements were made at 25° and were reproducible within 0.5%.

The disappearance of the fluorescence spectra of benzoquinolinium salts in the presence of butyrylcholinesterase was used to determine the dissociation constants ($K_{diss}$) of the enzyme–benzoquinolinium salt complex. The binding to butyrylcholinesterase or bovine serum albumin can be expressed according to Scatchard *et al.* (5) by:

$$\frac{R}{[I]} = \frac{n}{K_{diss}} - \frac{R}{K_{diss}} \qquad \text{(Eq. 1)}$$

where $R$ is the number of inhibitor molecules bound per subunit (equivalent to 1 mole of echothiophate iodide) of butyrylcholinesterase or per molecule of bovine serum albumin at a free inhibitor concentration, $[I]$, and $n$ is the number of binding sites on a butyrylcholinesterase subunit or a bovine serum albumin molecule. Both $R$ and $[I]$ were determined by utilizing the disappearance of inhibitor fluorescence intensities while being bound by butyrylcholinesterase (6, 7).

The relative intensities, $F$, of the fluorescence inhibitor were determined for a series of enzyme and inhibitor mixtures of different compositions containing a constant total inhibitor concentration. The expressions for the relative fluorescence intensities in the absence and in an excess of butyrylcholinesterase are $F_0 = r[I]_t$ and $F_e = r'[I]_t$, respectively; $[I]_t$ is the total inhibitor concentration, $r$ is the intensity coefficient of the free inhibitor, and $r'$ is the intensity coefficient of the bound inhibitor ($EI$) whose concentration is equal to the total free inhibitor concentration when an excess of enzyme is added. It is assumed that all inhibitor molecules are bound in the presence of excess enzyme.

The relative fluorescence intensity of butyrylcholinesterase is much less than that of the inhibitor itself and can be neglected while making enzyme blank measurements. Thus, relative fluorescence at varying butyrylcholinesterase concentrations is expressed by $F = r[I] + r'[EI]$. The fraction of total inhibitor bound, $\alpha$, is expressed by:

$$\alpha = \frac{F_0 - F}{F_0 - F_e} = \frac{r[I]_t - r[I] - r'[EI]}{r[I]_t - r'[I]_t} = \frac{[EI]}{[I]_t} \qquad \text{(Eq. 2)}$$

The free inhibitor concentration is expressed by:

$$[I] = [I]_t(1 - \alpha) \qquad \text{(Eq. 3)}$$

and if $[E]_t$ is the amount of butyrylcholinesterase used, then:

$$R = \frac{[EI]}{[E]_t} = \frac{\alpha[I]_t}{[E]_t} \qquad \text{(Eq. 4)}$$

Data for the disappearance of the fluorescence of hexamethylene-1,6-bisacridinium diiodide (I) in the presence of butyrylcholinesterase are shown in Table I. The $K_{diss}$ value is the reciprocal value of the Scatchard

[1] Worthington Biochemical Corp.

[2] Model 15, Cary Instrument Co.

[3] Aminco-Bowman.

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**Table II—Binding of Some Quaternary Ammonium Salts to Butyrylcholinesterase**

| | Compound | Butyrylcholinesterase | |
|---|---|---|---|
| | | $K_{diss}$, $\mu M$ | $K_{I,s}$, $\mu M$[a] |
| I[b] | $N^+—(CH_2)_6—N^+$ · $2I^-$ | 0.0083 ± 0.0002[c] | 0.0073 ± 0.0002 |
| II | $CH_2—N^+(CH_3)_2—(CH_2)_6—N^+(CH_3)_2—CH_2$ · $2Br^-$ | 2.01 ± 0.06[d] | 1.5 ± 0.2 |
| III | $N^+—(CH_2)_6—N^+$ · $2Br^-$ | 210 ± 40[d] | 120 ± 10 |
| IV[b] | $I^-$ $N^+—CH_3$ | 0.14 ± 0.04[c] | 0.24 ± 0.03 |
| V | $I^-$ $N^+—CH_3$ | 21 ± 3[c] | 32 ± 1 |
| VI | $Br^-$ $N^+—CH_3$ | 6.7 ± 0.5[c] | 5.4 ± 0.2 |
| VII | $Br^-$ $N^+—CH_3$ | 1.2 ± 0.5[c] | 1.10 ± 0.02 |

[a] Determined by steady-state enzyme kinetic studies (1). [b] The $K_{diss}$ values of I and IV *versus* bovine serum albumin, determined by the disappearance of the free ligand fluorescence, were 15 ± 4 and 87 ± 5 $\mu M$, respectively. [c] Determined by the disappearance of the free ligand fluorescence. [d] Determined by a fluorescence probe technique (8).

plot slope of $R/[I]$ *versus* $R$ (Fig. 1) and was calculated by fitting the values of $R/[I]$ and $R$ to the least-squares computer program.

The same procedure was used for measuring the binding of the nonfluorescent inhibitor, hexamethylene-1,6-bis-*p*-phenylbenzyl ammonium dibromide (II), with butyrylcholinesterase by the addition of a fluorescent probe ($A$) such as I (1). The dissociation constant ($K_B$) of the nonfluorescent inhibitor ($B$) which competes with $A$ for sites on the enzyme can be determined according to a literature method (8) and expressed by:

$$K_B = (K_A)\left(\frac{\alpha}{1-\alpha}\right)\left(\frac{[B]_t - S}{S}\right) \quad \text{(Eq. 5)}$$

where $K_A$ is the dissociation constant of the fluorescent probe, $[B]_t$ is the total concentration of the nonfluorescent inhibitor, and $S$ is the bound nonfluorescent inhibitor in moles per liter. The value of $S$ can be calculated by:

$$S = n[E]_t - \alpha[A]_t - \frac{\alpha K_A}{1-\alpha} \quad \text{(Eq. 6)}$$

where $[A]_t$ is the total fluorescent probe concentration. A Scatchard plot for the probe–enzyme complex in the presence of a nonfluorescent inhibitor is shown in Fig. 2.

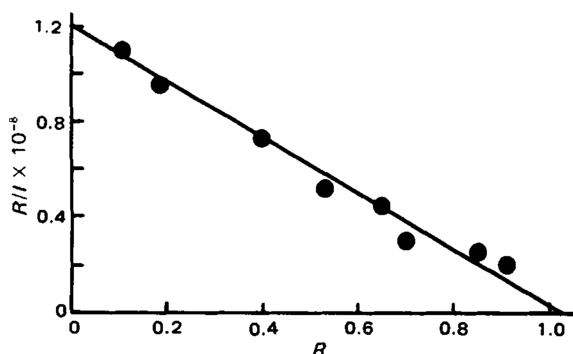


**Figure 1**—*Scatchard plot for I (50 nM) binding to various concentrations (5.5 nN–1.1 µN) of butyrylcholinesterase by spectrofluorometry.*

## RESULTS AND DISCUSSION

The addition of butyrylcholinesterase to aqueous solutions of mono- and bisquaternary ammonium salts that are potent reversible enzyme inhibitors caused a hypochromic shift in their UV spectrum. The UV absorbance of a 5 $\mu M$ solution of I was altered from maxima at 251 and 258 nm to a single maximum at 253 nm with a decrease of the extinction coefficient as the butyrylcholinesterase concentration was increased from 2.2 to 18 $\mu N$ (Fig. 3). Similar mixtures of I solutions and bovine serum albumin in concentrations from 0.29 to 72 $\mu M$ did not cause any significant change in the UV absorption spectrum of I. The acridinium compound spectral changes, which occurred following enzyme addition but not following addition of the same or even higher bovine serum albumin concentrations, suggest a specific and extensive binding by the inhibitor to butyrylcholinesterase.

Dissociation constants ($K_{diss}$) of mono- and bisonium salts complexed with butyrylcholinesterase were measured by disappearance of the heterocyclic moiety fluorescence spectra in the presence of butyrylcholinesterase (Table II). The $K_{diss}$ of I from butyrylcholinesterase was 8.3 ± 0.2 n$M$ and compared favorably with the inhibition constant ($K_{I,s}$ = 7.3 ± 0.2 n$M$) determined by the kinetics of the hydrolysis rate inhibition under Michaelis–Menten conditions (1). The dissociation constant of I from bovine serum albumin determined by free ligand fluorescence disappearance showed less affinity by at least $10^3$.

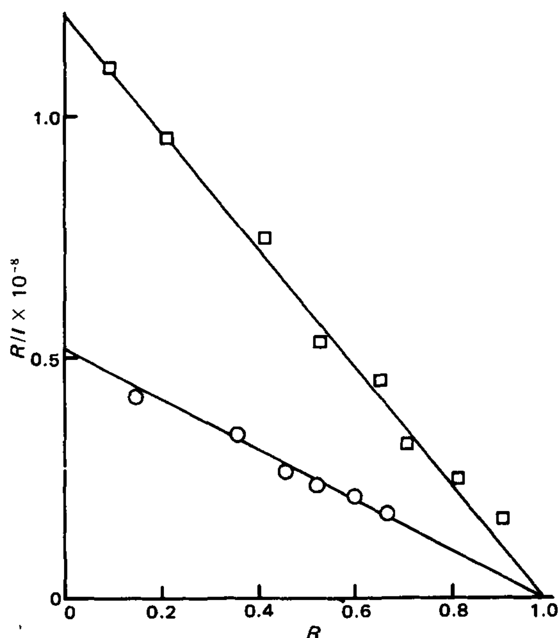


**Figure 2**—*Scatchard plots for the probe-enzyme complex of I (50 nM) and varying concentrations (5.5 nN–1.1 μN) of butyrylcholinesterase in the absence (□) and presence (○) of hexamethylene-1,6-bisbiphenyl ammonium dibromide (1.0 μM).*

$N$-Methylacridinium (IV) had a smaller binding affinity than I for butyrylcholinesterase when calculated from fluorescence measurements ($K_{diss} = 0.14 \pm 0.04\ \mu M$) or enzyme kinetic data ($K_{I,s} = 0.24 \pm 0.03\ \mu M$). Binding of IV to bovine serum albumin was also weaker than that of I. The additional acridinium moiety in I undoubtedly contributes to the stronger binding properties on both butyrylcholinesterase and bovine serum albumin. Since butyrylcholinesterase has a single anionic site at the active center (9–12), the enhancement of the binding force of the bis compound to both butyrylcholinesterase and bovine serum albumin could result from the hydrophobic binding of the additional onium moiety to the hydrophobic area adjacent to the anionic site of the enzyme.

The other $N$-methyl benzoquinolinium salts, 7,8-benzoquinolinium (V), 5,6-benzoquinolinium (VI), and 3,4-benzoquinolinium (VII), gave $K_{diss}$ values that were comparable to the $K_{I,s}$ values obtained from enzyme kinetic data. A parallel can be seen between the data from the dissociation constants as determined by fluorescence measurements and by enzyme kinetics. All compounds appeared to bind in a similar manner to the enzyme with binding energies depending on their lipophilic and steric properties. The Scatchard plots showed that both mono- and bis-$N$-substituted benzoquinolinium compounds bound stoichiometrically to the enzyme (*i.e.*, one molecule of inhibitor bound per subunit of enzyme). The esterolytic function was affected proportionately to the intensity of inhibitor binding.

Among these compounds, the onium group probably serves primarily as a carrier to deliver the hydrophobic structure to the active site. The positive charge in these heterocyclic structures may be delocalized by either internal resonance or pseudobase formation (1). Together with the hydrophobic nature of the hydrocarbon mass and the lack of hydrogen bonding substituents, these compounds would be unfavorable to solvation but would probably influence the structure of neighboring water, either in bulk solution or as the inhibitor–enzyme complex.

The effects of hydrocarbon-substituted ammonium salts on water structure were reported to vary with the hydrophobic mass of the substituents, the concentration, and the temperature (13–16). It would be of interest to determine the effect of structure of these isomeric monoquaternaries on the structure of the surrounding water. The hydrophobic mass structure may contribute to potency by influencing the difficulty with which other molecules and ions in solution can displace the inhibitor from the inhibitor–enzyme complex.

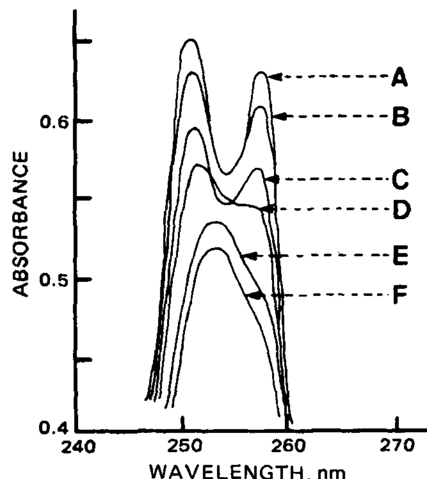


**Figure 3**—*Hypochromic effect of the UV spectra of I (5.0 μM) in the presence of different butyrylcholinesterase concentrations: A, 0; B, 2.3 μN; C, 4.6 μN; D, 9.2 μN; E, 14 μN; and F, 18 μN.*

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