MOLECULAR PHARMACOLOGY, 12, 667-677

Interaction of Spin-Labeled Bisquaternary Ammonium Ligands with Acetylcholinesterase

VICTORIO T. WEE[1] AND BIRANDRA K. SINHA[1,2]

Section on Molecular Pharmacology, Pulmonary Branch, National Heart and Lung Institute, Bethesda, Maryland 20014

PALMER W. TAYLOR

Division of Pharmacology, Department of Medicine, University of California, San Diego, La Jolla, California 92037

COLIN F. CHIGNELL[3]

Section on Molecular Pharmacology, Pulmonary Branch, National Heart and Lung Institute, Bethesda, Maryland 20014

(Received December 22, 1957)

SUMMARY

WEE, VICTORIO T., SINHA, BIRANDRA K., TAYLOR, PALMER W. & CHIGNELL, COLIN F. (1976). Interaction of spin-labeled bisquaternary ammonium ligands with acetylcholinesterase. *Mol. Pharmacol.*, 12, 667-677.

The interaction of a mononitroxide analogue of decamethonium (I) and symmetrical dinitroxide analogues of decamethonium (II) and hexamethonium (III) with *Torpedo californica* acetylcholinesterase has been studied by means of electron spin resonance spectroscopy. The I_{50} values for inhibition of acetylcholinesterase by labels I-III were 0.25 μM, 0.18 μM, and 3.3 μM, respectively. The ESR spectrum of I bound to acetylcholinesterase indicated that the nitroxide group was highly immobilized. Methanesulfonation of the active site serine of acetylcholinesterase resulted in a 6-fold decrease in the affinity of the enzyme for label I. This suggests that, in the unmodified enzyme, label I probably binds with the nitroxide group at the active site. In aqueous solution, at temperatures above 50°, the ESR spectrum of label II showed evidence for intramolecular electron spin-spin exchange, which was abolished by dissolving the label in ethylene glycol and cooling to −30°. When II bound to acetylcholinesterase, both nitroxide groups of the label became highly immobilized and the spin-spin exchange interaction was abolished. These findings indicate that label II binds to acetylcholinesterase in an extended conformation via a 2-point attachment involving both quaternary nitrogens. The observation that the ESR spectra of labels I and II bound to acetylcholinesterase were independent of fractional site occupation suggests that the bisquaternary binding sites are removed from the axis or center of symmetry of this tetrameric enzyme.

This work was supported in part by United States Public Health Service Grant GM-18360-04 to Dr. Palmer W. Taylor.

[1] Visiting Fellow of the National Heart and Lung Institute.

[2] Present address, Microbiological Associates, Bethesda, Maryland 20014.

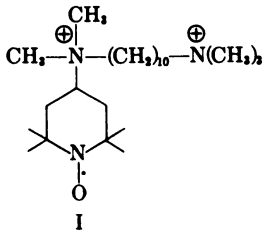
[3] To whom correspondence should be addressed.

INTRODUCTION

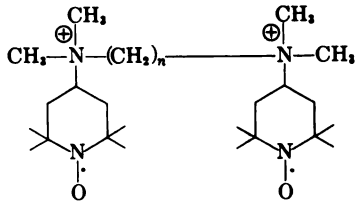
Acetylcholinesterase (acetylcholine hydrolase, EC 3.1.1.7) is inhibited by a wide variety of cationic ligands (1–5). The binding to cholinesterase of bisquaternary ammonium ligands, in which the maximal distance between the 2 quaternary nitrogens is 14.5 A, appears to involve a two-site attachment in which one of the cationic groups interacts with an anionic subsite at the active center, while the other is associated with a peripheral anionic site (3, 5). When these ligands bind to acetylcholinesterase, it is thought that they adopt an "exo" orientation, in which the reactive serine in the active center is not occluded by the inhibitor (3).

Much of the information concerning the binding of bisquaternary ligands to acetylcholinesterase has come either from their inhibitory effect on enzymatic activity or from their influence on the reactivity of the active site serine toward covalent modification by acid-transferring groups. More recently, however, the usefulness of spectroscopic techniques for studying the interaction between inhibitors and acetylcholinesterase has been appreciated (6–8). Mooser and co-workers (6) have employed *N*-methylacridinium and bis(3-aminopyridinium)-1,10-decane as fluorescent probes of acetylcholinesterase from *Electrophorus electricus*. The quenching of tryptophan fluorescence by a series of bisquaternary inhibitors containing the benzoquinone moiety, e.g., 2,5-bis(3-diethyl-*o*-chlorobenzylammonium-*n*-propylamino)- benzoquinone, has been used by Taylor and Jacobs (7) to examine the topography of the active site of *Torpedo californica* acetylcholinesterase. In other studies, the fluorescent dye propidium has been found to be a specific probe for the peripheral anionic site of the same enzyme (8).

Spin labels are stable free radicals that can be used as probes for biological systems such as enzymes, nucleic acids, and membranes (9). The most useful spin labels are those containing a nitroxide group, since this free radical is stable in aqueous solution over a wide range of temperatures and pH values (9). Spin-labeled ligands have been found extremely useful for examining the topography of hapten-combining sites in immunoglobulins (10) as well as the active sites of several enzymes and other binding proteins, e.g., trypsin, chymotrypsin (11), carbonic anhydrase (12, 13), and avidin (14). While there have been at least two previous studies of acetylcholinesterase that have employed spin labels, both of these have made use of covalently bound analogues of the irreversible inhibitor diisopropyl fluorophosphate (15, 16). We now report a spin label study of *Torpedo californica* acetylcholinesterase employing nitroxide analogues (I–III) of the bisquaternary ligands decamethonium and hexamethonium.



I



II $n = 10$
III $n = 6$

MATERIALS AND METHODS

Materials. Acetylcholinesterase was purified by lytic solubilization of electroplax membranes from *Torpedo californica* and subsequent affinity chromatography as previously described (17). The enzyme appeared homogeneous by criteria of analytical ultracentrifugation and gel electrophoresis and was stored at −20° in polypropylene tubes for periods of up to 1 month without appreciable loss of activity or aggregation. The concentration of acetylcholinesterase was measured by absorption at 280 nm, $E^{1\%}_{1\,cm} = 17.5$ (17). The enzyme exists as a tetramer consisting of four apparently identical subunits, each having a molecular weight of 80,000.

Acetylcholinesterase was esterified,

where indicated, with *N*-methyl-3-hydroxypyridinium iodide methanesulfonate according to the previously described procedure (7), until the activity of the enzyme was reduced to between 93% and 98%. The modified enzyme was then dialyzed at 4° against buffer solution containing 0.01 M Tris-HCl (pH 8), 0.01 M NaCl, and 0.04 M $MgCl_2$.

The synthesis of the spin-labeled analogues (I–III) of decamethonium and hexamethonium has been reported elsewhere (18). Decamethonium iodide was purchased from K & K Laboratories. All other chemicals were of analytical grade and were used without further purification.

Methods. The electron spin resonance spectra were obtained with a Varian E-4 spectrometer operating at 9.5 GHz. Temperatures were maintained with a Varian variable-temperature accessory (E-257) and were measured with a Yellow Springs Instrument Company telethermometer (42SC). The titration studies were carried out by adding increments of a solution containing the spin label to the top of a quartz aqueous sample cell containing 0.5–1.0 ml of enzyme solution by means of a Hamilton microsyringe fitted with a constant-delivery adapter. Mixing of the sample was achieved with the aid of a disposable plastic syringe which was connected to the bottom of the cell by means of an 18-gauge needle embedded in a Teflon stopper. Power saturation studies were carried out in order to determine the maximum allowable modulation and power settings. All spectra were obtained at 20-mW power with a modulation of 2 G. When necessary, the ESR spectra were time-averaged on a Digital Equipment Corporation PDP-8 computer with the aid of the DAQUAN program. The same program was used for double integration of spectra in order to obtain the relative spin concentrations.

The binding parameters governing the interaction of the spin-labeled bisquaternary ammonium ligands with acetylcholinesterase were determined by spectral titrations in which a constant amount of enzyme was titrated with increments of spin label (see above). The ESR spectrum was recorded after each addition, and the concentration of unbound spin label was estimated from the amplitude of the low-field line (Figs. 1, 5, and 6, left arrow). The binding data were plotted according to the Scatchard relationship

$$\frac{r}{C} = nK - rK \qquad (1)$$

where r = number of moles of spin label bound per enzyme subunit, C = molar concentration of unbound spin label, n = number of spin label binding sites per subunit, and K is the association constant (liters per mole). When the Scatchard plots were nonlinear, as in Fig. 4, the data were analyzed in terms of a two-independent-site model and the binding parameters were obtained by means of a nonlinear least-squares regression fit to the data, using the MLAB program run on a Digital Equipment Corporation PDP-10 computer. Hard copies of the Scatchard plots (Fig. 4) were obtained with the aid of a Calcomp plotter.

The affinity of spin labels I–III for acetylcholinesterase was also measured fluorometrically by back-titration against the fluorescent ligand bis(3-aminopyridinium)-1,10-decane (6, 7). The I_{50} values for the inhibition of acetylcholinesterase by labels I–III and decamethonium were determined by previously reported methods (18). The concentration of acetylcholine employed in these measurements (0.25 mM) was close to the K_m value for this substrate. Under these conditions the I_{50} value should approach the true dissociation constant (K_D) for the enzyme-inhibitor complex.

RESULTS

Inhibition of acetylcholinesterase by bisquaternary ammonium spin labels. The substitution of the bulky piperidine ring for one or more of the quaternary methyl groups present in either hexamethonium or decamethonium to give spin labels I–III can be expected to produce considerable steric and electronic perturbation of the parent molecule. For this reason it was necessary to demonstrate that the spin labels bound to acetylcholinesterase in the

same fashion as did hexamethonium or decamethonium. All three spin labels were inhibitors of acetylcholinesterase (Table 1). The data in Table 1 are presented in the form of I_{50} values, since it is known that the inhibition of acetylcholinesterase by bisquaternary ammonium ligands is of a mixed (competitive-noncompetitive) type (4, 5). Since there is good agreement between the I_{50} values for the spin labels and their dissociation constants (K_D) measured directly from ESR titrations (see below and Table 1), there seems to be little doubt that the spin labels do indeed bind to the active site of acetylcholinesterase. It is of interest that both decamethonium spin labels (I and II) have greater affinities for acetylcholinesterase than does decamethonium itself. The hexamethonium spin label (III) showed nearly 20-fold less affinity than the symmetrical decamethonium analogue (II). In addition to inhibiting the enzymatic activity of acetylcholinesterase, the spin labels were also directly competitive with the fluorescent inhibitor bis(3-aminopyridinium)-1,10-decane. The K_D values calculated from these experiments are also reasonably close to the I_{50} values determined from the enzyme inhibition measurements (Table 1). Since a number of independent lines of evidence indicate that bis(3-aminopyridinium)-1,10-decane is bound to the active site of acetylcholinesterase (6–8), the competitive titrations provide further confirmation that the spin labels do indeed bind to acetylcholinesterase with one quaternary group at the active center.

TABLE 1

Dissociation constants determined by enzymatic, fluorescence, and electron spin resonance methods

Analogue	I_{50}[a]	K_D[b]	
		ESR titration	Back-titration
	μM	μM	μM
I	0.25[c]	0.19	0.36
II	0.18[c]	0.12	0.20
III	3.3[c]	2.9	5.20
Decamethonium	5.1		5.9[d]

[a] Determined from the inhibition of *Torpedo californica* acetylcholinesterase (see MATERIALS AND METHODS).

[b] ESR titration and back-titration of bis(3-aminopyridinium)-1,10-decane were performed as described in MATERIALS AND METHODS.

[c] Data from ref. 18.

[d] Data from ref. 7.

ESR spectra of bisquaternary ammonium spin labels in ethylene glycol and aqueous solutions. The ESR spectrum of the unsymmetrical decamethonium analogue (I) in dilute aqueous solution at room temperature consisted of three sharp lines with a splitting of 17 G between adjacent peaks. This three-line spectrum results from the anisotropic hyperfine interaction between the unpaired electron and the nuclear spin of the nitroxide nitrogen atom (9). In ethylene glycol at −30°, the ESR spectrum of I became broad and asymmetrical, with a maximal hyperfine splitting ($2T_{\|}$) of 70 G (Fig. 1). This so-called "rigid glass" spectrum is characteristic of a nitroxide radical whose molecular motion is slow on the ESR time scale (i.e., $\tau_c \gg 10^{-8}$ sec, where τ_c is the rotational correlation time). There was no change in signal intensity when an ethylene glycol solution of I was cooled to −30°.

The ESR spectrum of spin label III in dilute aqueous solution at 25° consisted of three fairly sharp lines with a hyperfine splitting of 16.8 G (Fig. 2B). However, the observation that the over-all signal intensity of label III was only about one-third of the expected value suggested that some kind of nitroxide-nitroxide interaction was taking place. When the temperature of an aqueous solution of III was raised above 50°, a five-line spectrum with alternating

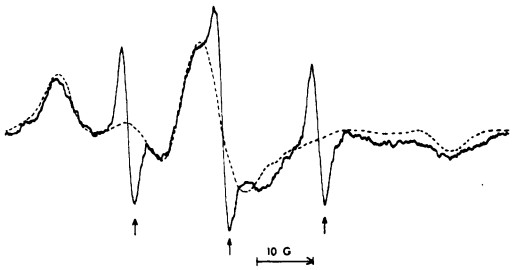


FIG. 1. *ESR spectrum (——) of spin label I (3.8 μM) in the presence of acetylcholinesterase (3.03 μM) at low ionic strength (1 mM NaCl)*

---, ESR spectrum of I (0.1 mM) dissolved in ethylene glycol at −30°. The arrows designate peaks due to free spin label.

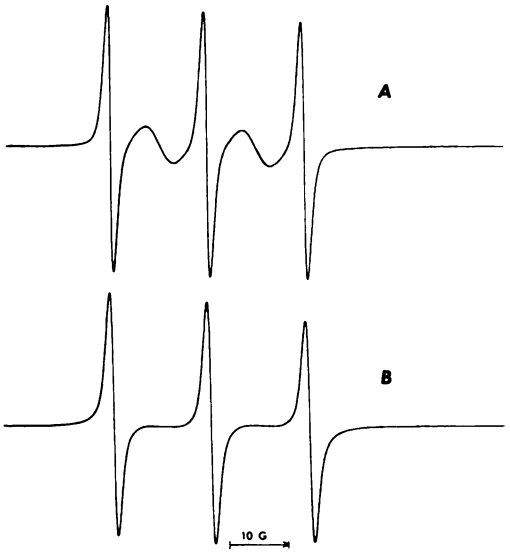


FIG. 2. *ESR spectra of spin label III (0.1 mM) in high ionic strength buffer (0.1 M NaCl, 0.04 M $MgCl_2$, 0.01 M Tris-HCl, pH 8.0) at 75° (A) and 25° (B)*

linewidths appeared (Fig. 2A). The ratios of the amplitude of the first low-field hyperfine line to the amplitude of the adjacent line were 15, 8.25, 6.45, and 4.97 at 58°, 70°, 78°, and 88°, respectively. Such five-line spectra result from an electron spin-spin exchange interaction, which operates only when the two nitroxides can physically approach one another to within a range of 6 Å or less (19–23). The conformations which a flexible dinitroxide, such as III, must assume in order to achieve intramolecular spin-spin exchange result from torsional motions which bring the $N_{p\pi}$ orbitals of the nitroxide groups into a nearly colinear relationship with each other. Examination of a Corey-Pauling-Koltun model of spin label III readily shows that when the two nitroxide groups of this label are brought near enough to permit spin-spin exchange, the 2 quaternary nitrogen atoms are also brought closer together. The fact that a three-line spectrum was observed at room temperature suggests that electrostatic repulsion between the 2 nitrogens prevented the two nitroxide groups of III from approaching one another with sufficient frequency. At the higher temperatures, the increased kinetic energy of III was probably sufficient to overcome the electrostatic repulsion. Spin label II also underwent intramolecular spin-spin exchange in aqueous solution. At any given temperature, label II showed a more pronounced five-line spectrum than was seen with label III. Thus, at 88°, the ratio between the amplitudes of the first low-field hyperfine line and the adjacent line was 4.97 for III but 3.6 for II. This would suggest that label II has a somewhat faster exchange rate than label III. A comparison of Corey-Pauling-Koltun models of II and III indicates that the additional flexibility provided by the longer methylene chain of II makes it possible for the quaternary nitrogens to remain farther apart when the nitroxide groups are brought close enough for intramolecular spin-spin exchange to occur.

An ethylene glycol solution of III was used to examine the effect of a decrease in molecular motion on the ESR spectrum of this label, since this solvent provided a wider viscosity range than could be achieved with aqueous solutions. At 93°, in ethylene glycol, the ESR spectrum of III (Fig. 3) was similar to that observed in aqueous solution at room temperature (Fig. 2B). As the ethylene glycol solution was cooled, the ESR spectrum of III progressively broadened and finally became highly asymmetrical (Fig. 3). While the ESR spectrum of III in ethylene glycol at −30° (Fig. 3A) resembled the rigid glass spectrum of I under the same conditions (Fig. 1), the hyperfine lines of III were obviously broader. It was also found that the apparent signal intensity of III was 2.24 times greater at −30° (Fig. 3A) than at 93° (Fig. 3F). As previously pointed out, spin-spin exchange results in an apparent loss of the spectral intensity of a dinitroxide ESR spectrum. When the molecular motion of III in ethylene glycol is reduced by cooling, the probability that the nitroxide groups can approach close enough to permit spin-spin exchange decreases so that the over-all intensity of the spectrum increases (Fig. 3).

Since it is probable that electron spin-spin exchange makes only a minor contribution to the over-all line shape of the ESR

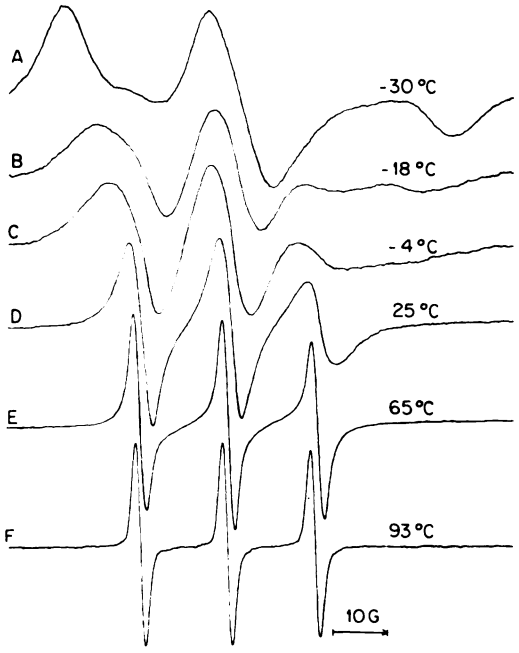


FIG. 3. *ESR spectra of spin label III (0.1 mM) dissolved in ethylene glycol at different temperatures*
The gain settings (relative signal intensities) were: A, 2.5×10^3 (19.06); B, 2.5×10^3 (18.22); C, 2.5×10^3 (15.39); D, 10×10^2 (11.63); E, 6.3×10^2 (10.29); F, 5×10^2 (8.51).

spectrum of III in ethylene glycol at −30° (Fig. 3A), it would appear that the observed line broadening must be due to some other mechanism. This mechanism is termed the electron spin-spin dipole interaction, which, unlike the spin-spin exchange interaction, is enhanced by a reduction in molecular motion. The dipole interaction is dependent upon the spatial relationship between the two nitroxide groups and has been observed to operate at distances up to 17 A (14, 24, 25). When molecular motion is essentially absent, the net result of the spin-spin dipole interaction is to split each of the major hyperfine peaks into two components (14, 19). The magnitude of this splitting is given by the relationship

$$|2D| = \frac{5.56 \times 10^4}{r^3} \qquad (2)$$

where r is the distance between the nitroxides (A) and $|2D|$ is the splitting in gauss (26). In the derivation of Eq. 2 it has been assumed that the observed splitting is derived from an orientation in which the dipolar axis (i.e., the axis between the two nitroxides) is perpendicular to the z axes of the nitroxides. Since it is highly unlikely that, at −30° in ethylene glycol, the nitroxide groups at each end of label III will maintain a preferred spatial relationship to one another, the net result of the dipole interaction is line broadening (cf. Fig. 3A and Fig. 1). The ESR spectrum of II in ethylene glycol at −30° was similar to that of III. The apparent increase in signal intensity on cooling an ethylene glycol solution of II from 92° to −30° was 2-fold.

ESR spectra of bisquaternary ammonium spin labels bound to acetylcholinesterase. At low ionic strength, in the presence of acetylcholinesterase, the ESR spectrum of the unsymmetrical decamethonium label I became broad and asymmetrical (Fig. 1). This spectrum was similar to the rigid glass spectrum of the same label dissolved in ethylene glycol at −30° (Fig. 1). The maximal hyperfine splitting ($2T_{\parallel}$) was 70 G, which meant that the molecular motion of enzyme-bound I was very slow on the ESR time scale. Indeed, the magnitude of the splitting would suggest that the nitroxide group of I had little or no motion relative to the enzyme. Double integration of the ESR spectrum of I bound to acetylcholinesterase revealed no change in the signal intensity of the spin label.

While increasing the ionic strength did not affect the over-all shape of the ESR spectrum of I bound to acetylcholinesterase, it did dramatically reduce the affinity of the label for the enzyme (Table 2). At high ionic strength, the Scatchard plot for the binding of I to acetylcholinesterase was nonlinear, indicating the presence of more than one set of sites (Fig. 4). The binding data have been analyzed in terms of a two-site model in which there is no site-site interaction (Table 2). There can be little doubt that the high-affinity site represents the binding of I to the active site of acetylcholinesterase, since there is good agreement between the dissociation constant for this site as determined from ESR titration and the I_{50} value determined from enzyme inhibition studies (Table 1). The affinity of the secondary binding sites

TABLE 2

Binding parameters determined from ESR measurements with native and modified acetylcholinesterase

Serine modification[a]	Ligand	Buffer ionic strength[b]	Binding parameters			
			N_1[c]	K_1	N_2[c]	K_2
				μM^{-1}		μM^{-1}
–	I	High	0.8	5.20	3.4	0.02
–	II	High	0.8	8.49	2.4	0.02
–	III	High	1.1	0.34		
–	I	Low	1.3	30[d]		
–	II	Low	1.3	285[d]		
–	III	Low	1.1	8.59		
+	I	High	1.0	0.81		
+	II	High	1.1	0.54		

[a] Enzyme was allowed to react with *N*-methyl-3-hydroxypyridinium iodide methanesulfonate according to the procedure decribed in MATERIALS AND METHODS.

[b] High ionic strength buffer was 0.1 M NaCl, 0.04 M $MgCl_2$, and 0.01 M Tris-HCl (pH 8.0); low ionic strength buffer was 1 mM NaCl.

[c] Moles of ligand bound per 80,000 mol wt subunit.

[d] These values can only be considered estimates, because of the inherent inaccuracy of the method (see the text).

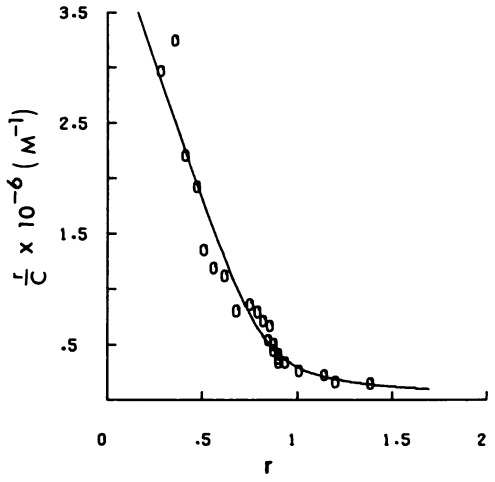


FIG. 4. *Scatchard plot of binding of spin label I to acetylcholinesterase at high ionic strength (0.1 M NaCl, 0.04 M $MgCl_2$, 0.01 M Tris-HCl, pH 8.0)*

r = number of moles of I bound per 80,000 mol wt subunit; *C* = molar concentration of unbound I.

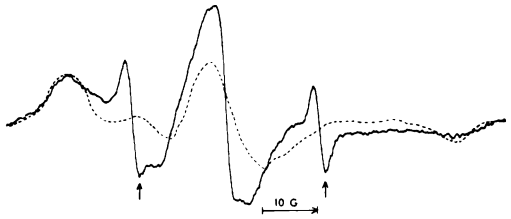


FIG. 5. *ESR spectrum (——) of spin label II (3.56 μM) in the presence of acetylcholinesterase (6.7 μM) at low ionic strength (1 mM NaCl)*

– – –, ESR spectrum of II (0.1 mM) dissolved in ethylene glycol at −30°. The arrows designate peaks due to free spin label.

for I was at least 2 orders of magnitude less than the affinity of the primary binding site (Table 2). The nature of these secondary binding sites is at present unknown. The high affinity of I for acetylcholinesterase at low ionic strength made it difficult to estimate the association constant with any degree of accuracy, since it was impossible to make precise binding measurements at enzyme concentrations below 100 nM because of the inherent insensitivity of the ESR method.

When the symmetrical decamethonium label (II) bound to acetylcholinesterase at low ionic strength, its ESR spectrum became broad and asymmetrical, with a maximal hyperfine splitting ($2T_{\parallel}$) of 70 G between outer extrema (Fig. 5). When compared with the spectrum of enzyme-bound I, the spectrum of bound II showed evidence of pronounced line broadening. Also, in contrast to label I, when label II bound to acetylcholinesterase, the apparent intensity of its ESR spectrum increased 2-fold. While the affinity of II for acetylcholinesterase decreased when the

ionic strength was increased (Table 2), the over-all shape of its ESR spectrum was unchanged. At high ionic strength, there was some evidence for the presence of low-affinity sites for II (Table 2). Once again, the dissociation constant for the high-affinity site agreed quite well with the I_{50} value calculated from inhibition of enzymatic activity (Table 1).

Spin label III also became highly immobilized when bound to acetylcholinesterase (Fig. 6), with a $2T_{\parallel}$ value of 70 G. The spectrum of enzyme-bound III was much broader than that of enzyme-bound I. Furthermore, there was an apparent 3-fold increase in the signal intensity of III bound to acetylcholinesterase. As with labels I and II, there was good agreement between the dissociation constant calculated from ESR titration (Table 2) and the I_{50} value determined from enzyme inhibition studies (Table 1).

When the serine hydroxyl at the active site of acetylcholinesterase is methanesulfonated, the affinity of the enzyme is reduced for bisquaternary ammonium ligands containing bulky substituents on the nitrogen atoms, but not for the bistrimethonium or triethonium analogues (7). Preparation of the methanesulfonyl ester of acetylcholinesterase reduced the affinity of the enzyme for labels I and II by 6-fold and 16-fold, respectively (Table 2). Esterification of acetylcholinesterase did not appear to change the ESR spectrum of enzyme-bound labels I and II. The affinity of methanesulfonated acetylcholinesterase for label III was so low that it proved impossible to detect any bound label by means of ESR.

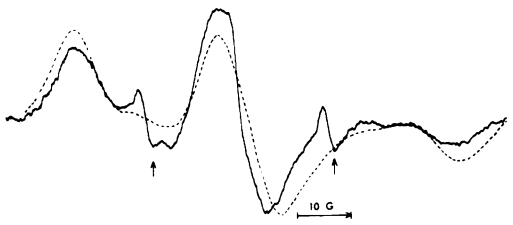


FIG. 6. *ESR spectrum (——) of spin label III (0.51 μM) in the presence of acetylcholinesterase (3.02 μM) at low ionic strength (1 mM NaCl)*

---, ESR spectrum of spin label II (0.1 mM) dissolved in ethylene glycol at −30°. The arrows designate peaks due to free spin label.

DISCUSSION

A comparison of the dissociation constants shown in Table 1 clearly shows that the replacement of a single quaternary methyl group in decamethonium by the nitroxide-bearing piperidine ring, to give spin label I, results in a 16–20-fold increase in the affinity of this ligand for acetylcholinesterase. The observation by Hubbell and McConnell (27) that 2,2,6,6-tetramethylpiperidine-1-oxyl preferentially partitions into regions of low polarity in nerve membranes suggests that this spin label may undergo hydrophobic interactions fairly readily. Thus it would appear that dispersion forces involving the piperidine ring probably confer stabilization energy on the complex between label I and acetylcholinesterase. It is of interest that the substitution of a second quaternary methyl group in label I by the piperidine moiety, to give spin label II, results in only a 1.4–1.8-fold increase in affinity (Table 1). In previous studies Taylor and Jacobs found (7) that dispersion forces are of much less importance in stabilizing that portion of the bisquaternary ligand that is at the peripheral anionic site. If label I binds to acetylcholinesterase with the piperidine ring located predominantly at the active site, then it is perhaps not surprising that the introduction of a second piperidine ring produces a rather small increase in affinity. Evidence for such a preferential orientation of label I was obtained from methanesulfonation studies (see below).

The strongly immobilized ESR spectrum of enzyme-bound II (Fig. 5) provides convincing evidence that both ends of this label are firmly attached to the surface of acetylcholinesterase. If spin label II interacts with acetylcholinesterase in the same fashion as decamethonium, it follows that one of the piperidine rings must probe the active site while the other must interact with the peripheral anionic site. A close examination of the spectrum of enzyme-bound II (Fig. 5) reveals that the low-field line is somewhat asymmetrical while the intensity of the high-field line is

low. These features can be seen more clearly if the spectrum of enzyme-bound II (Fig. 5) is compared either with the spectrum of the same label dissolved in ethylene glycol at −30° (Fig. 5) or with the spectrum of enzyme-bound label III (Fig. 6). One possible explanation for these spectral differences is that the ESR spectrum of enzyme-bound II consists of two bound species attributable perhaps to nitroxide labels at the active site and peripheral sites. An alternative explanation could be that when label II binds to acetylcholinesterase the nitroxides at each end may assume some preferred spatial relationship which permits a spin-spin dipole interaction to occur.

Additional evidence for the 2-point attachment of II to acetylcholinesterase is provided by the increase in signal intensity which is seen when this label binds to the enzyme. In order for intramolecular spin-spin exchange to occur, it is necessary for the two nitroxide groups of II to come within 6 A of each other. When this happens, there is an apparent loss of signal intensity. The increase in signal intensity observed when label II interacts with acetylcholinesterase strongly suggests that this ligand binds in an extended conformation with both ends firmly anchored so that they cannot approach close enough for spin-spin exchange to occur.

Since it appears probable, from the data with label II, that both the active and peripheral sites can interact with the nitroxide-bearing piperidine ring, the question arises as to which site interacts with the piperidine ring of label I. A possible clue to the orientation of enzyme-bound I is provided by the binding data obtained with acetylcholinesterase esterified by the methanesulfonyl group (Table 2). Fluorescence studies by Taylor and Jacobs have shown (7) that while the binding of 2,5-bis(3-trimethylammonium-*n*-propylamino)-benzoquinone to acetylcholinesterase is not affected by such modification, the affinity of 2,5-bis(3-diethyl-*o*-chlorobenzylammonium-*n*-propylamino)benzoquinone decreases 5-fold. The 16-fold lower affinity of label II for esterified acetylcholinesterase suggests that the steric interaction between the bulky piperidyl group of II and the methanesulfonyl function is greater than that observed with the chlorobenzylbenzoquinone analogue. The affinity of spin label I for acetylcholinesterase was also reduced by methanesulfonation, but only by a factor of 6 (Table 2). This reduction in stabilization energy would suggest that the quaternary nitrogen atom of label I that bears the nitroxide function probably binds to the active center of the native enzyme. Methanesulfonation of the active site serine undoubtedly places additional steric constraints on the binding site, which in turn may increase the distance between the quaternary ammonium group and its binding surface and lead to a reduction in both the coulombic and hydrophobic contributions to binding. In the case of the spin label I, methanesulfonation of acetylcholinesterase may result in preferential binding of the quaternary nitrogen bearing the nitroxide at the peripheral anionic site. The availability of this alternative binding orientation to spin label I could well account for the finding that methanesulfonation affects the binding energetics of this label to a lesser extent than that of spin label II.

The observation that the affinity of acetylcholinesterase for label III is also decreased by methanesulfonation would suggest that 1 of the quaternary nitrogen atoms of this label must bind at the active site. This finding is in contrast to kinetic evidence, which indicates that hexamethonium may bind exclusively to the peripheral site of erythrocyte carbonic anhydrase (28, 29). The reduction in affinity on going from label II to III probably reflects a reduction in hydrophobic interactions as well as coulombic interactions, or a redistribution of the relative contributions of these two forces. However, even though there is a reduction in affinity, the ESR spectrum of enzyme-bound III (Fig. 6) clearly shows that both ends of the inhibitor are firmly anchored to the surface of acetylcholinesterase. In addition, the increase in spectral intensity observed when III interacts with acetylcholinesterase provides evidence that this ligand binds to the enzyme in an extended conformation. A

comparison of the ESR spectra of III bound to acetylcholinesterase and III dissolved in ethylene glycol at −30° reveals certain qualitative differences both in the linewidths and in the over-all line shapes (Fig. 6). The broadness of the hyperfine lines of label III in ethylene glycol at low temperature undoubtedly results from both spin-spin dipole and exchange interactions, although it seems likely that the dipole mechanism would predominate. Since enzyme-bound III cannot undergo spin exchange, it must be assumed that the broadness of the hyperfine lines results from a spin-spin dipole interaction. If this assumption is indeed correct, the observation that the spin-spin dipole interaction caused line broadening but not discrete splitting would imply that when label III binds to acetylcholinesterase the two nitroxide groups do not adopt any preferred orientation to each other. Since 1 quaternary nitrogen of label III is bound to the active site, it would appear that there is probably a locus of sites available between the active site and peripheral site with which the other quaternary nitrogen and its associated piperidine group can interact.

It is noteworthy that the ESR spectra of the enzyme-bound spin labels were independent of the fractional occupation of binding sites. In the case of biotin binding to the tetrameric avidin molecule, Chignell and co-workers (14) observed dipole-dipole coupling between spin-labeled biotin analogues bound to adjacent subunits. From the observed splitting constant ($|2D|$), they were able to calculate, by means of Eq. 2, the distance between adjacent bound labels up to a distance of 17 A. On the basis of tryptophan fluorescence quenching titrations with acetylcholinesterase, it was previously suggested that the bisquaternary binding sites are probably far removed from the axis or center of symmetry of the tetrameric enzyme (7). The absence of intermolecular spin-spin interactions among spin labels I, II, and III bound to adjacent subunits is entirely compatible with the fluorescence measurements.

These studies have shown that bisquaternary spin labels I–III are useful tools for studying the mechanism whereby these ligands interact with *Torpedo californica* acetylcholinesterase. Since decamethonium also depolarizes nicotinic receptors, it is hoped that labels such as I and II will also prove to be useful probes of the acetylcholine receptor isolated from the same tissue.

REFERENCES

1. Changeaux, J.-P. (1966) *Mol. Pharmacol.*, 2, 369–392.
2. Belleau, B. & DiTullio, V. (1970) *J. Am. 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. Mooser, G., Schulman, H. & Sigman, D. S. (1972) *Biochemistry*, 11, 1595–1602.
7. Taylor, P. & Jacobs, N. (1974) *Mol. Pharmacol.*, 10, 93–107.
8. Taylor, P. & Lappi, S. (1975) *Biochemistry*, 14, 1989–1997.
9. Jost, P. & Griffith, O. H. (1972) in *Methods in Pharmacology* (Chignell, C. F., ed.), Vol. 2, pp. 222–276, Appleton-Century-Crofts, New York.
10. Hsia, J. C. & Piette, L. H. (1969) *Arch. Biochem. Biophys.*, 129, 296–307.
11. Berliner, L. J. & Wong, S. S. (1974) *J. Biol. Chem.*, 249, 1668–1677.
12. Chignell, C. F., Starkweather, D. K. & Erlich, R. H. (1972) *Biochim. Biophys. Acta*, 271, 6–15.
13. Erlich, R. H., Starkweather, D. K. & Chignell, C. F. (1973) *Mol. Pharmacol.*, 9, 61–73.
14. Chignell, C. F., Starkweather, D. K. & Sinha, B. K. (1975) *J. Biol. Chem.*, 250, 5622–5630.
15. Morrisett, J. D., Broomfield, C. A. & Hackley, B. E. (1969) *J. Biol. Chem.*, 244, 5758–5761.
16. Hsia, J. C., Kosman, D. J. & Piette, L. H. (1969) *Biochem. Biophys. Res. Commun.*, 36, 75–78.
17. Taylor, P., Jones, J. W. & Jacobs, N. M. (1974) *Mol. Pharmacol.*, 10, 78–92.
18. Sinha, B. K. & Chignell, C. F. (1975) *J. Med. Chem.*, 18, 669–673.
19. Ferruti, P., Gill, D., Klein, M. P., Wang, H. H., Entine, G. & Calvin, M. (1970) *J. Am. Chem. Soc.*, 92, 3704–3713.
20. Hsia, J. C., Kosman, D. J. & Piette, L. H. (1972) *Arch. Biochem. Biophys.*, 149, 441–451.
21. Ferruti, P., Gill, D., Klein, M. P. & Calvin, M.

(1969) *J. Am. Chem. Soc.*, **91**, 7765–7766.
22. Calvin, M., Wang, H. H., Entine, G., Gill, D., Ferruti, P., Harpold, M. A. & Klein, M. P. (1969) *Proc. Natl. Acad. Sci. U. S. A.*, **63**, 1–8.
23. Luckhurst, G. R. (1966) *Mol. Phys.*, **10**, 543–550.
24. Glarum, S. H. & Marshall, J. H. (1967) *J. Chem. Phys.*, **47**, 1374–1378.
25. Lamaire, H. (1967) *J. Chim. Phys.*, **64**, 559–571.
26. Rohde, O., Van, S. P., Kester, W. Z. & Griffith, O. H. (1974) *J. Am. Chem. Soc.*, **96**, 5311–5318.
27. Hubbell, W. L. & McConnell, H. M. (1968) *Proc. Natl. Acad. Sci. U. S. A.*, **61**, 12–15.
28. Wombacher, H. & Wolf, H. U. (1971) *Mol. Pharmacol.*, **7**, 554–566.
29. Lüllman, H., Ohnesorge, F. K., Tonner, H. D., Wasserman, O. & Ziegler, A. (1971) *Biochem. Pharmacol.*, **20**, 2579–2586.