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THE EFFECT OF CHEMICAL MODIFIERS ON THE INTERACTION OF A SPIN-LABELED LOCAL ANESTHETIC WITH HUMAN ERYTHROCYTE MEMBRANES

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Abstract—The role of electrostatic forces in the interaction between local anesthetics and phospholipid membranes has been well studied previously. The present work tested the hypothesis that the tertiary amine local anesthetics can also interact with charged groups that may be present on membrane proteins.

The rotational mobility of a spin-labeled local anesthetic, 2-[*N*-methyl-*N*-(2,2,6,6-tetramethylpiperidinooxyl)] ethyl *p*-hexyloxybenzoate, in erythrocyte membranes was found to decrease upon addition of chemical modifiers that react covalently with free amino groups of the membrane and decrease the concentration of the membrane's fixed positive charge. Reagents that did not react with free amino groups, or those that did react with amino groups but did not alter the net charge of the membrane, had no effect on the mobility of the spin-labeled local anesthetic. The decrease in the anesthetic's mobility found after reaction with amino-directed reagents was shown to be due to the attraction of the positively charged anesthetic to the modified site. The immobilization of the anesthetics was primarily due to modification of the membrane protein components. The immobilized anesthetics are most probably located at the inner surface of the membrane.

Local anesthetics block the action potential of nerve axons by preventing the transient increases in permeability of sodium and potassium ions to the axonal membrane [1]. Although the detailed molecular nature of the site of anesthetic action is unknown, Narahashi and his coworkers [2, 3] have demonstrated that the tertiary amine local anesthetics exert their effect on the cytoplasmic surface of the axonal membrane. In addition, it has been shown that the tertiary amine local anesthetics are active in their protonated form [2, 3]. The interaction of these positively charged anesthetics with phospholipid model membranes has been thoroughly examined [4–8], and the results of these experiments have indicated a strong correlation between the ability of local anesthetics to interact with negatively charged phospholipids, and their pharmacological action in blocking nerve excitation. However, no information is available concerning what role the charge on membrane proteins may play in the binding of local anesthetics. In the present investigation, we examine how modification of the net membrane protein charge influences the interaction of a local anesthetic with a natural membrane.

The membrane preparation which we have chosen for this series of experiments is the red cell membrane. The well-defined responses of erythrocytes to local anesthetics [9] and the considerable amount of information that is available concerning the structure of the erythrocyte membrane [10], make it an ideal model system for the study of local anesthetic action. Furthermore, the action of a number of chemical modifiers on the red cell membrane has been thoroughly examined. For instance, the covalent reaction of reagents such as 1-fluoro-2,4-dinitrobenzene (FDNB)‡ and 2-methoxy-5-nitrotropone (MNT) with red cells irreversibly decreases anion fluxes and increases cation permeability [11, 12]. These alterations in ion permeabilities have been explained by the reaction of FDNB with membrane amino groups, whose dissociable fixed charges are thought to control the ion permeability properties of the red cell membrane [12]. It is assumed that this reaction with FDNB decreases the concentration of fixed positive charges in the membrane, and for electrostatic reasons, results in an increase in the concentration of diffusable cations and a decrease in the concentration of diffusable anions in the ion-permeable regions of the cell membrane. In this investigation it is demonstrated that chemical modifiers which decrease the net concentration of positive charges in the erythrocyte membrane also reduce the rotational mobility of a population of membrane bound local anesthetics.

The local anesthetic that we have employed for this study is a spin-labeled analog of an intracaine derivative (Fig. 1a), and is a highly active local anesthetic [13]. The spin-labeling technique [14–16] is based

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‡ Abbreviations used are: FDNB, 1-fluoro-2,4-dinitrobenzene; DFDNB, 1,5-difluoro-2,4-dinitrobenzene; TNBS, 2,4,6-trinitrobenzenesulfonic acid; MNT, 2-methoxy-5-nitrotropone; NEM, *N*-ethylmaleimide; PCMBS, *para*-chlormercuriphenyl sulfonic acid; SITS, 4-acetamido-4′-isothiocyano-stilbene-2,2′-disulfonic acid; DMA, dimethyladipimidate dihydrochloride; DMS, dimethylsubcrimidate dihydrochloride; C6SL, 2-[*N*,methyl,*N*-(2,2,6,6-tetramethylpiperidinooxyl)] ethyl *p*-hexyloxybenzoate; C6SLMeI, the methyl iodide salt of C6SL; esr, electron spin resonance.

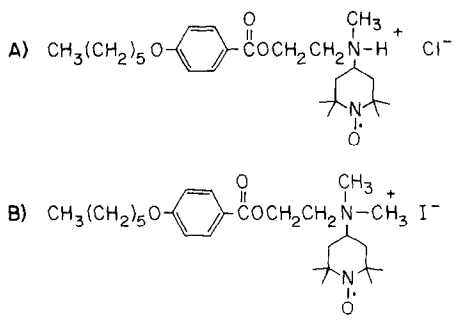

Fig. 1. A) A spin-labeled local anesthetic, 2-[*N*-methyl-*N*-(2,2,6,6-tetramethylpiperidinooxyl)] ethyl *p*-hexyloxybenzoate, abbreviated C6SL, and B) its quaternary amine analog, abbreviated C6SLMeI.

upon line shape changes in the electron spin resonance spectrum of the nitroxide radical, and can provide information concerning the molecular properties of binding sites. In situations where the nitroxide radical undergoes a rapid isotopic tumbling motion as in a nonviscous solvent, three narrow symmetrical lines result. As the molecular motion of the radical decreases, the lines become broad and asymmetrical. In previous studies, we have reported on the interaction of spin-labeled local anesthetics with lobster walking leg nerves [17], erythrocyte membranes [18], and phospholipid model membranes [8].

METHODS

Chemicals

The synthesis and pharmacology of the spin-labeled local anesthetic 2-[*N*-methyl-*N*-(2,2,6,6-tetramethylpiperidinooxyl)] ethyl *p*-hexyloxybenzoate, C6SL (Fig. 1a), has been described previously [13]. Its quaternary amine analog, the methyl iodide salt C6SLMeI (Fig. 1b), was synthesized by addition of a 10-fold excess of methyl iodide to the free base of C6SL dissolved in dichloromethane. The reaction was allowed to proceed for 24 hr at room temperature. The solution was diluted with ether, and the precipitate filtered and washed with ether to give the final product.

FDNB and PCMBS were purchased from Sigma. Glutaraldehyde and SITS were obtained from Ladd Research Laboratories and Polysciences, respectively. DMA and DMS were products of Pierce Chemicals (DMA was a kind gift from Dr. L. Packer). DFDNB, MNT, and NEM were purchased from Calbiochem. TNBS and bovine serum albumin were obtained from Eastman and Miles Laboratories, respectively. The salts utilized were of analytical reagent grade. Chloroform and methanol were of reagent grade. Double distilled water was used throughout.

Membrane preparation

Red blood cell ghosts were prepared by the method of Dodge *et al.* [19] from outdated blood purchased from the San Jose Red Cross. Whole red cells were washed with 310 mOsm phosphate buffer, pH 7.4, and the cells were lysed in the presence of 20 mOsm phosphate buffer, pH 7.4, and washed at least 5 times. Membranes were utilized within 1 day after preparation, or quick-frozen and used within a 2-week period. Identical results were obtained with both membrane samples.

Reaction of erythrocyte membranes with chemical reagents

(*a*) *DFDNB and FDNB.* A procedure similar to that of Marinetti *et al.* [20] was used to react erythrocyte membranes with FDNB and DFDNB. The membrane pellet, containing a total of 15 mg protein as determined by the method of Lowry *et al.* [21], was suspended to a total vol of 17 ml in a Krebs buffer, pH 8.5 (consisting of 100 ml 0.154 M NaCl, 4 ml 0.154 M KCl, 3 ml 0.055 M $CaCl_2$, 1 ml 0.154 M $MgSO_4$, 21 ml 1.3% $NaHCO_3$, and 234 mg glucose to a final vol of 130 ml). To this suspension was added the desired amount of FDNB or DFDNB dissolved in 0.5 ml ethanol. The final concentrations of the nitrobenzene derivatives ranged from 0 to 5.6 mM. For the control, samples were treated as above except DFDNB and FDNB were omitted. After gentle agitation of the mixture at room temperature for a given time period, the reaction was terminated by the addition of cold 10 mM Tris–HCl, pH 7.4, and the sample was spun down and washed twice in this buffer.

(*b*) *Glutaraldehyde.* A membrane pellet containing 15 mg membrane protein was suspended in 5 ml of freshly prepared 2% glutaraldehyde solution in Kreb's buffer. After reaction at room temperature for 1 hr, the sample was diluted with cold 10 mM Tris–HCl, pH 7.4, spun down, and washed twice.

(*c*) *TNBS.* For reaction at higher TNBS concentrations (5 mM), a membrane pellet containing 15 mg protein was suspended to a total vol of 5 ml in 0.1 M phosphate buffer, pH 8.0, and 5 ml of a freshly prepared 10 mM TNBS solution in 0.1 M phosphate buffer, pH 8.0, was added. The mixture was reacted at room temperature for a given time period. At the end of this time, cold 10 mM Tris HCl, pH 7.4, was added and the pellet was spun down and washed twice.

For time-dependent studies at lower TNBS concentrations (1 mM), 2 ml of a 10 mM TNBS solution was added to an 18-ml suspension of red blood cell ghosts containing a total of 75 mg protein in 0.1 M phosphate buffer, pH 8.0. The suspension was gently agitated at room temperature and at various periods in time, 2-ml aliquots were removed and 38 ml of cold 50 mM Tris–HCl, pH 7.4 was added to terminate the reaction. The pellets were quickly spun down and washed twice in 10 mM Tris HCl, pH 7.4, suspended in 10 ml of this buffer, and the absorbance at 335 nm was recorded after the samples came to room temperature.

(*d*) *MNT.* Because of its low solubility in aqueous media, 6 mg of MNT was placed in 40 ml of Krebs buffer and heated in hot tap water at 50–60° for 5 min. This solution was allowed to cool to approximately 30° and the dissolved MNT was added to a membrane pellet containing 7.5 mg protein. The pellet was suspended and the solution allowed to incubate for 3 hr at room temperature. The sample was spun down, the above procedure repeated, and the pellet washed twice in 10 mM Tris–HCl, pH 7.4.

(*e*) *NEM and PCMBS.* For each sample, a membrane pellet containing 15 mg protein was suspended to a total vol of 5 ml in 20 mOsm phosphate buffer, pH 7.4, and the reagents NEM and PCMBS were added in solid form to give final concentrations of 20 mM and 2 mM, respectively. The mixtures were agitated at room temperature for 4 hr, diluted with cold 10 mM Tris–HCl buffer, pH 7.4, spun down, and washed twice.

(*f*) *DMA and DMS.* A membrane pellet (15 mg protein) was suspended to a total vol of 10 ml in a 10 mM NaCl and a 50 mM $NaHCO_3$ solution, pH 8.8. The desired amount of DMA or DMS was added in its solid form, and the pH of the suspension was manually readjusted to pH 8.8 as quickly as possible by addition of 2 N NaOH. The mixtures were incubated at room temperature for up to 5 hr, diluted with cold 10 mM Tris–HCl, pH 7.4, spun down and washed twice.

(*g*) *SITS.* A membrane pellet containing 15 mg protein was suspended to a total vol of 5 ml in a 0.166 M NaCl solution with 20 mOsm phosphate buffer, pH 7.4. SITS was added in its solid form to give a final concentration of 0–4.5 mM. The reaction was allowed to proceed at room temperature for up to 2 hr, and the sample was diluted with Tris–HCl, spun down and washed twice.

Liposomes. Total lipids from erythrocyte ghosts were extracted with 2:1 chloroform–methanol (v/v) by the method of Folch *et al.* [22]. The lipids were reacted with FDNB, DFDNB, and TNBS in a similar manner to that described above for erythrocyte membranes. For instance, 15 mg of red blood cell lipids dissolved in 1 ml of 2:1 $CHCl_3$–CH_3OH were placed in a round bottom flask and the chloroform–methanol was removed by a rotary evaporator. The lipids were then placed under a stream of nitrogen for several minutes to insure complete removal of the solvent. Unsonicated liposomes were prepared by placing the lipids, suspended in 2 ml Krebs buffer, on a vortex mixer for 2 hr. The suspension was diluted with Kreb's buffer to a total vol of 17 ml and 10 mg of DFDNB dissolved in 0.5 ml ethanol was added, giving a final DFDNB concentration of 2.8 mM. The mixture was incubated at room temperature for 4 hr, and the sample was diluted with cold 10 mM Tris–HCl, pH 7.4, spun down at 27 000 *g* for 30 min, and washed twice.

Incorporation of spin labels

The membrane samples were suspended to a total vol of 5 ml in 10 mM Tris–HCl, pH 7.4, and microliter quantities of a concentrated solution of C6SL or C6SLMeI in ethanol were added to give a final spin-labeled local anesthetic concentration of 1.2×10^{-4} M. The samples were allowed to incubate at room temperature for 1 hr, diluted with 10 mM Tris–HCl, spun down, and the e.s.r spectra recorded. For some experiments, erythrocyte membranes were spin-labeled before reaction with the chemical reagent, and identical results were obtained. Most of the spin-labeled anesthetic C6SL remained bound to the membrane preparation even after several washes, which is in agreement with the failure of C6SL to be washed off lobster nerves over a 12-hr period [17].

Spectral measurement

Samples were placed in sealed-tip disposable Pasteur pipettes or sealed capillary tubes for e.s.r study. All spectra were recorded at room temperature with a Varian E-3 spectrometer at a power setting of 5 mW. Experiments were repeated at least three times with consistent readings obtained throughout.

Correlation times

The tumbling times for nitroxides were estimated by the following equation [23]:

$$\tau_0 = 6.5 \times 10^{-10}\, W_0[(h_0/h_{-1})^{1/2} - 1) \qquad (1)$$

where τ_0 is an empirical approximation of the spin label's tumbling time, W_0 is the width of the central line, and h_0 and h_{-1} are the heights of the mid-field and the high-field lines. Strictly speaking, this equation is only valid for rotation correlation times faster than 10^{-9} sec [56]. However, since we are only interested in the relative but not absolute values of the correlation times, the equation serves adequately.

RESULTS

Erythrocyte membranes spin-labeled with C6SL. The e.s.r spectrum of the spin-labeled local anesthetic C6SL with erythrocyte membranes is shown in Figure 2a. In qualitative terms, this spectrum results from a moderately immobilized nitroxide [16], and has a correlation time of 4.7×10^{-9} sec, as calculated from

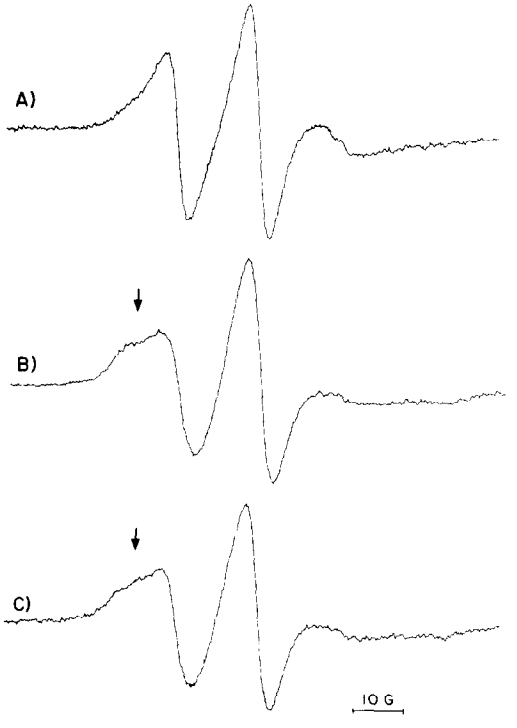

Fig. 2. E.s.r. spectra of C6SL A) in the presence of erythrocyte membranes; B) in the presence of erythrocyte membranes after treatment with 2.8 mM DFDNB for 30 min; C) in the presence of erythrocyte membranes after treatment with 5 mM TNBS for 2 hr. Arrows indicate strongly immobilized components. For details, see Methods.

equation (1). The e.s.r spectrum of C6SL with red cell membranes appears similar to the spectrum obtained with C6SL bound to lobster walking leg nerves [17].

The effect of DFDNB treatment on the e.s.r spectrum of C6SL spin-labeled red blood cell ghosts is exhibited in Figure 2b. This spectrum is distinctly modified from that obtained with the untreated membranes. The most noticeable difference between the two spectra is the broadening of the low-field peak (the field increasing from left to right) after DFDNB treatment. A strongly immobilized component is now readily apparent (arrow). Such a strongly immobilized component can be assigned a correlation time on the order of 10^{-7} sec [14]. Unfortunately, it is quite complicated to calculate molecular mobilities of spin labels that have correlation times greater than 5×10^{-9} sec, especially for a spectrum such as that shown in Figure 2b which results from superimposed spectra of moderately immobilized and strongly immobilized spin labels. However, we are able to say that treatment of erythrocyte membranes with DFDNB gives rise to a population of spin-labeled local anesthetics with slower rotational mobility than that observed with the unaltered membranes labeled with C6SL. The nature of this strongly immobilized component will be dealt with in the discussion.

It was found that other chemical agents could also produce this effect. For instance, a broadening of the low-field peak and a strongly immobilized component (arrow) is observed in the e.s.r spectrum of C6SL-labeled erythrocyte membranes after reaction with 5 mM TNBS for 2 hr (Figure 2c). This spectrum is very similar to that shown in Figure 2b after DFDNB treatment.

In general, it was found that the degree of broadening of the C6SL e.s.r spectra depended upon the amount of membrane sample present, the concentration of the chemical modifier, and its time of incubation. For instance, Figure 3 shows the reaction of 1 mM TNBS with erythrocyte membranes containing a total of 75 mg protein over a 24-hr period. Most of the label has reacted after 8 hr. The e.s.r spectra of the C6SL in the presence of erythrocyte membranes at various periods in time after 1 mM TNBS treatment are presented in Figure 4. As can be seen from the figure, the e.s.r spectra became broader as more TNBS reacts with the membranes. However, even for the longest reaction times, the broadening was not as great as that observed with the higher TNBS concentration shown in Figure 2c. This is because the TNBS concentration was limiting for this series of experiments.*

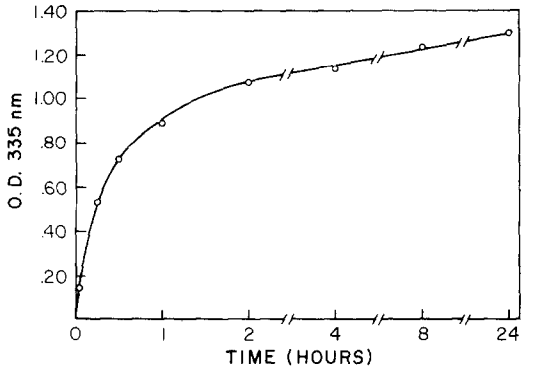

Fig. 3. Time-dependent reaction of 1 mM TNBS with red blood cell ghosts. For details, see Methods. Each point represents the average of three experiments.

Three other reagents were examined which, after reaction with erythrocyte membranes, gave rise to a population of strongly immobilized local anesthetics and e.s.r spectra similar to those shown in Figures 2b and 2c. The monofluoro agent FDNB has essentially the same effect as DFDNB at twice the DFDNB concentration (Fig. 5a, top spectrum). Again, the broadening of the C6SL e.s.r signal was dependent on the concentration and the time of incubation of FDNB. However, the maximal broadening observed was as found in Figures 2b, 2c, and 5a; higher reagent concentrations and long incubation times did not promote any further immobilization of the spin labels. In addition, treatment of erythrocyte membranes with glutaraldehyde or MNT decreased the rotational mobility of a population of spin-labeled local anesthetics in the membrane (Table 1). As will be discussed shortly, the one parameter that all of the above chemical modifiers have in common is their ability to react with free amino groups of the membrane to form uncharged derivatives.

A number of other compounds were examined that had no detectable effect on the e.s.r spectrum of C6SL spin-labeled erythrocyte ghosts (Table 1). The reaction of the sulfhydryl agents PCMBS and NEM, and the diimidoesters DMA and DMS with erythrocyte membranes, even at extremely high concentrations and long reaction times, did not alter the C6SL e.s.r spectra. In addition, the reagent SITS had no detectable effect on the rotational mobility of C6SL in the presence of red cell ghosts (Table 1).

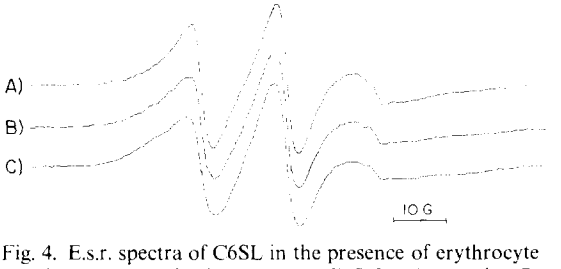

Fig. 4. E.s.r. spectra of C6SL in the presence of erythrocyte membranes treated with 1 mM TNBS for A) 0 min; B) 30 min; C) 4 hr.

* This may be shown by the following calculation (see Methods for exact conditions):

$$\frac{75 \text{ mg protein}}{5{\cdot}4 \times 10^{-10} \text{ mg protein/ghost}} \times 3{\cdot}74 \times 10^{-16} \text{ mole } NH_2 \text{ groups/ghost} = 5{\cdot}2 \times 10^{-5} \text{ mole of amino groups in the reaction mixture.}$$

A total of 2 ml of a 10^{-2} M TNBS solution, or 2×10^{-5} mole of TNBS, was added to the 75 mg protein suspension. Therefore, for this series of experiments, there were over twice as many amino groups as TNBS molecules. (The number of moles of NH_2 groups/ghost was obtained from Knauf and Rothstein [24] and the mg protein/ghost was calculated from the data of Hanahan [25] and Rosenberg and Guidotti [26].)

Table 1. The effect of chemical reagents on the e.s.r spectrum of the spin-labeled local anesthetic C6SL in the presence of human erythrocyte membranes.

Reagent	Concentration*	Strongly immobilized component†
FDNB	5.6 mM	+
DFDNB	2.8 mM	+
Glutaraldehyde	2%	+
TNBS	5 mM	+
MNT	~0.8 mM§	+
SITS	4.5 mM	−
NEM	20 mM	−
PCMBS	2 mM	−
DMA	29 mM	−
DMS	26 mM	−

* Maximum concentration that was tested; for details, see Methods.

† A + indicates that the action of the chemical reagent on red blood cell membranes caused a strongly immobilized component to appear in the e.s.r spectrum of the C6SL as is shown in Figures 2b) and 2c). A − indicates that the action of the chemical reagent on red blood cell membranes caused no detectable change in the e.s.r spectrum of C6SL.

§ Because of MNT's low solubility in aqueous media, the membrane suspension was treated twice with a MNT solution (see Methods).

Effect of pH. The e.s.r spectrum of C6SL in the presence of erythrocyte membranes after treatment with 5.6 mM FDNB for 2 hr is shown in Figure 5a. The top spectrum is that of the sample in a pH 7.4 buffer. Again, a strongly immobilized component is readily apparent. However, when the sample is placed in a pH 9.0 buffer (Fig. 5a, bottom spectrum) much of the strongly immobilized component disappears; the low-field becomes noticeably sharper and the spectrum reverts towards the moderately immobilized spectrum of the C6SL spin label in the presence of unmodified erythrocyte membranes (Fig. 2a).

The above observation may be explained by (1) a change in the net charge of the membrane (and possibly membrane structural changes) or by (2) a change in the amount of C6SL in its positively charged or uncharged form as the pH is brought from 7.4 to 9.0. The pK_a of the C6SL is approximately 8.5–9.0.* Therefore, at pH 7.4 most of the spin-labeled local anesthetic will be positively charged whereas at pH 9.0 at least half of the anesthetic molecules will be in the form of the uncharged free base. In order to distinguish between the effect of pH on the membrane and on the anesthetic, we employed the quaternary amine analog C6SLMeI (Fig. 1b), the methyl iodide salt of C6SL. This compound has a permanent positive charge independent of the solution's pH. The e.s.r spectrum of C6SLMeI in the presence of red blood cell membrane is shown in Figure 6.

The e.s.r spectrum of C6SLMeI in the presence of red blood cell membrane after treatment with 5.6 mM FDNB for 2 hr is shown in Figure 5b. The top spectrum is that of the sample in a pH 7.4 buffer and the bottom spectrum represents the sample in a pH 9.0 buffer. The two spectra are essentially the same; the strongly immobilized component is apparent in both. Thus it seems that the removal of the strongly immobilized component of the spin label C6SL at pH 9.0 (Fig. 5a), is primarily due to the removal of the charge on the anesthetic molecule. The average pK_a of the red cell amino groups is also thought to be approximately 9 [12], but after treatment of the membranes with FDNB, it is likely that uncharged dinitrophenyl derivatives would have formed with the majority of the membrane amino groups, and that the charge on these groups would no longer be susceptible to the pH of the solution.

* Since only a limited supply of the C6SL spin label is available, we could not use a substantial amount of the C6SL for an accurate pK_a reading. However, the 2-(*N,N*-diethylamino) ethyl *p*-alkoxybenzoates, a series of local anesthetics from which C6SL is derived, have pK_a values of 8.95 [27]. In addition, the commonly used local anesthetics procaine, tetracaine, and dibucaine all have pK_a's in the range of 8.5–9.0 [28]. It is very probable that the pK_a of C6SL lies in this region.

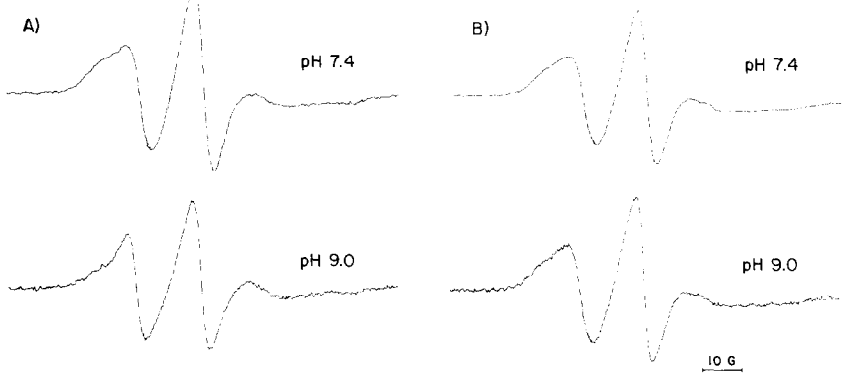

Fig. 5. A) E.s.r spectra of C6SL in the presence of erythrocyte membranes treated with 5.6 mM FDNB for 2 hr. Top spectrum was taken in 10 mM Tris–HCl, pH 7.4. For the bottom spectrum, the spin-labeled pellet was spun down, suspended in 40 ml of 10 mM Tris–HCl, pH 9.0, and incubated at room temperature for 30 min. The sample was spun down and the e.s.r. reading taken. B) E.s.r. spectra of the quaternary amine analog C6SLMeI in the presence of erythrocyte membranes after treatment with 5.6 mM FDNB for 2 hr. Top spectrum was taken in 10 mM Tris–HCl, pH 7.4, and the bottom spectrum in 10 mM Tris–HCl, pH 9.0.

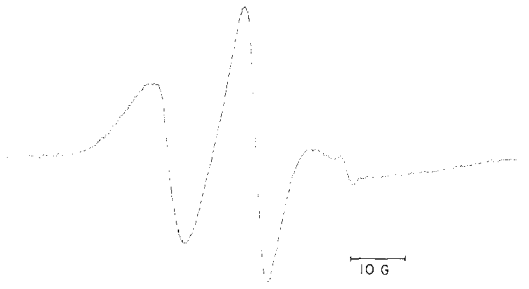

Fig. 6. E.s.r spectrum of the quaternary amine analog C6SLMeI in the presence of erythrocyte at pH 7.4.

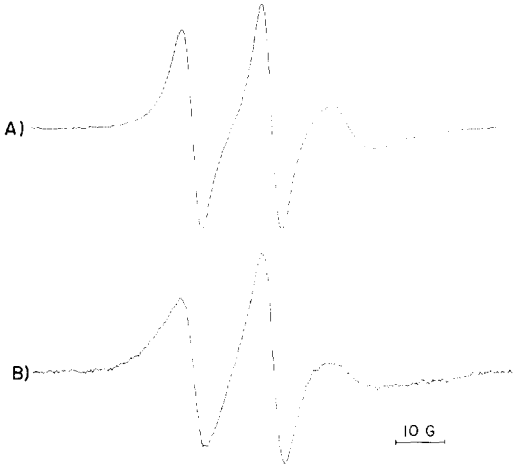

Fig. 7. E.s.r spectra of C6SL A) in the presence of liposomes prepared from the total lipids of the red cell membrane, and B) in the presence of liposomes prepared from the total lipids of the red cell membrane after treatment with 2.8 mM DFDNB for 4 hr. For details, see Methods.

Liposomes. The spectrum of C6SL-labeled liposomes prepared from the total lipids of red blood cell ghosts is presented in Figure 7a. The correlation time for C6SL in the presence of liposomes is 3.3×10^{-9} sec; approximately 2/3 the value obtained with C6SL in the presence of red blood cell ghosts. Upon reaction of the liposomes with the reagent DFDNB (Fig. 7b), only relatively small alterations occurred in the spectrum. The spin-labeled local anesthetic is somewhat more immobilized in the DFDNB treated than in the untreated liposomes, and from equation (1) its correlation time is estimated at 5.4×10^{-9} sec. However, no strongly immobilized component was apparent after reaction with DFDNB as was found with the intact erythrocyte membranes (Fig. 2b). Similar results were obtained when liposomes were reacted with FDNB and TNBS. Even at very high reagent concentrations (up to 10 mM) and long reaction times (up to 24 hr), the e.s.r signal of C6SL in the presence of chemically modified liposomes did not show any further decrease in mobility from that shown in Figure 7b.

DISCUSSION

Of the 10 chemical modifiers examined, most of them have a known effect on the passive ion permeabilities of the erythrocyte membranes. However, only the action of 5 of these reagents result in a strongly immobilized component in the e.s.r spectra of C6SL spin-labeled erythrocyte membranes. The other 5 reagents showed no effect whatsoever on the mobility of the spin-labeled local anesthetic (Table 1). In order to understand the nature of this strongly immobilized component, we shall consider the action of each chemical modifier on erythrocyte membranes.

FDNB and DFDNB. It was discovered by Berg *et al.* [29] that treatment of erythrocytes with nitrobenzene derivatives caused a large increase in the Na^+ and K^+ permeability of erythrocytes. Furthermore, it was found that reaction with DFDNB induced a greater increase in cation permeability than reaction with FDNB. In a later study it was shown that the anion permeability of red cells reacted more sensitively to FDNB treatment than cation permeability [30]. For a given FDNB concentration, the inhibition of anion permeability developed faster than the facilitation of cation permeability.

In the present investigation, it was found that reaction of red cell membranes with FDNB and DFDNB reduced the mobility of a population of spin-labeled local anesthetics. The action of FDNB was qualitatively similar to but smaller than that of DFDNB.

To explain the decrease in anion permeability, increase in cation permeability, and decrease in the mobility of the spin-labeled local anesthetic C6SL after treatment with nitrobenzene derivatives, we must look at the mode of action of these reagents. FDNB reacts by displacement of fluorine and formation of a covalent bond with free amino, sulfhydryl, tyrosyl, and histidyl groups to form stable dinitrophenyl derivatives [31]. One important consequence of this reaction is the removal of uncharged amino groups from the equilibrium

$$R{-}NH_3^+ \rightleftharpoons R{-}NH_2 + H^+ \qquad (2)$$

by the formation of $(NO_2)_2$-C_6H_3-NH-R, where R represents a membrane component. This decrease in the concentration of fixed positive charges in the membrane has been invoked to explain the observed inverse changes of the penetration rates for cations and anions after FDNB treatment [30].

We believe the decrease in rotational mobility of the anesthetic C6SL in the erythrocyte membrane after reaction with FDNB (Fig. 2b) or DFDNB (Fig. 5a) can also be explained by the removal of the membranes positively charged amino groups. This supposition is strengthened as we consider the action of the other chemical modifiers.

TNBS. The main reactions of TNBS are with sulfhydryl and unprotonated amino groups [32, 33]. As with the nitrobenzene derivatives, the reaction of TNBS with free amino groups results in a decreased concentration of the membrane's fixed positive charge and inhibits anion permeability and enhances cation movements [30]. In the present study, reaction of erythrocyte membranes with TNBS reduced the rotational mobility of a population of spin-labeled local anesthetics (Figs. 2c, 4).

Glutaraldehyde. When glutaraldehyde reacts with macromolecules the solution slowly becomes more

acidic [34]. This phenomenon is explained by the reaction of glutaraldehyde with free amino groups removing the uncharged amino groups from the equilibrium between the uncharged and protonated amino groups, again resulting in a decreased concentration of positive charges in the membrane. Although free amino groups are the primary targets for glutaraldehyde, reaction also occurs to some extent with guanidinium, secondary amino, and hydroxyl groups [34].

Upon treatment of red cell membranes with glutaraldehyde, an immediate leakage of K^+ ions was observed [35]. This increase in passive K^+ diffusion out of the cells was explained by the blocking of amino groups by glutaraldehyde. In our experiments (Table 1), glutaraldehyde treatment of red blood cell membranes resulted in a strongly immobilized component of the C6SL e.s.r signal and a reduced mobility of local anesthetics in the membrane.

MNT. All of the reagents discussed above react with free amino groups, but they may also exhibit certain side reactions. In contrast, MNT is thought to be specific for free amino groups of biomolecules and does not react with sulfhydryl, hydroxyl, or imidazole groups [36]. The reaction of MNT with intact red cells has also been found to lower anion permeability and accelerate cation efflux [11]. In addition, MNT treatment of erythrocyte membranes resulted in a reduced rotational mobility of a population of the spin-labeled local anesthetics (Table 1).

Thus, the one common property of the reagents FDNB, DFDNB, TNBS, glutaraldehyde, and MNT is that they are all able to react with free amino groups and decrease the concentration of the membrane's fixed positive charge. The result is a decreased anion and increased cation permeability of the red blood cell, and a decrease in the rotational motion of the anesthetic C6SL. However, the other 5 chemical modifiers did not alter the e.s.r spectra of C6SL labeled erythrocyte membranes (Table 1). We now consider the action of each of these reagents.

NEM. The reaction of NEM is fairly specific for sulfhydryl groups at neutral pH, but reaction may occur with imidazole and amino groups under certain conditions [37]. This reagent has been found to increase red cell permeability to K^+ ions [38]. However, even at concentrations up to 20 mM, NEM had no effect on the C6SL e.s.r signal. (Table 1).

PCMBS. PCMBS is another reagent which is specific for sulfhydryl groups. The reaction of intact red cells with PCMBS increased the cation permeability but had no effect on anion permeability [24]. It is interesting to note that although both FDNB and PCMBS increased cation permeability, PCMBS did not protect against the effects of FDNB [24]. It was concluded that PCMBS and FDNB increased cation permeability by different mechanisms. In our experiments, PCMBS (2 mM) had no detectable effect on the e.s.r spectrum of C6SL spin-labeled membranes.

SITS. This reagent was first employed by Maddy [39] as a non-penetrating label of the erythrocyte's outer surface. Its effect on intact red cells is rather unusual in that it decreases anion permeability but has no effect on cation permeability [24]. At first, it was thought that the isothiocyanate group of the SITS molecule reacted covalently with amino or sulfhydryl groups of the membrane, because washing the SITS treated cells caused little reversal of binding or of inhibition [24, 39]. However, this was found not to be the case. Washing the cells with a buffer containing albumin reversed the inhibitory effect on anion permeability and showed that only a small fraction of the SITS was covalently bonded [40]. The decrease of anion permeability by SITS could then be explained by an increase in the membrane's net negative charge due to noncovalent bonding of the disulfonic SITS. SITS had no effect on the rotational mobility of the spin-labeled local anesthetic C6SL in the presence of erythrocyte membranes (Table 1).

DMA and DMS. Imidoesters are known to react specifically with amino groups of proteins [41]. However, unlike the amino reactive reagents FDNB, DFDNB, TNBS, MNT, and glutaraldehyde, imidoesters do not alter the net charge of the biomolecule after reaction. In addition, the action of imidoesters on the red cell is relatively mild compared to the other amino reagents. Red cells are capable of maintaining K^+ ion selectivity even after treatment with 5 mM DMA, although higher DMA concentrations resulted in K^+ loss from the cell [42]. Both DMA and DMS, at extremely high concentrations and for reaction periods up to 5 hr, had no detectable effect on the e.s.r spectrum of C6SL labeled erythrocyte membranes (Table 1).

From the above discussion, we conclude that the strongly immobilized component ($\tau_0 \sim 10^{-7}$ sec) of the C6SL spin-labeled anesthetic after treatment with chemical modifiers results from the removal of the positively charged amino groups. Those reagents which do not react with free amino groups, or react with amino groups but do not alter their net charge, have no effect on C6SL mobility.

One might argue that reagents such as DFDNB and glutaraldehyde decrease the mobility of C6SL by crosslinking membrane components. In fact, since these were the first compounds we tested this was our initial interpretation. We are now able to exclude this notion on the following grounds: (1) The monofunctional agents FDNB, TNBS, and MNT also give rise to a strongly immobilized population of spin-labeled anesthetics after reaction with red cell membranes at approximately twice the bifunctional reagent concentration, and (2) DMA and DMS have no effect on the e.s.r spectra of C6SL even though diimidoesters are known to make red cells resistant to hypotonic hemolysis [42, 43] and crosslink erythrocyte membrane components [20, 44–46].

Effect of pH. When the pH of the solution was raised to 9.0 after FDNB treatment, much of the strongly immobilized component in the e.s.r spectrum of C6SL disappeared (Fig. 5a). However, the change in pH did not alter the e.s.r spectrum of the C6SLMel labeled membranes after FDNB treatment (Fig. 5b). Thus it appears that upon removal of certain positively charged amino groups in the membrane, a population of the positively charged C6SL molecules are able to approach these sites and become immobilized. If the positive charge is removed from the anesthetic molecules, they are no longer attracted to the modified site and the strongly immobilized component disappears. Presumably, a similar type of attraction and immobilization at these modified sites could occur with cations such as Na^+ and K^+, result-

ing in an increased concentration of cations at these sites and an increased cation permeability, as predicted by the fixed-charge hypothesis [12].

Involvement of lipid and protein components. The interaction of C6SL with liposomes prepared from the total lipids of erythrocyte membrane exhibits at τ_0 value of 3.3×10^{-9} sec (Fig. 7a). After treatment with DFDNB, C6SL spin-labeled liposomes show an apparent correlation time of 5.4×10^{-9} sec (Fig. 7b). This was the maximal effect; treatment at higher reagent concentrations and for longer periods in time did not promote any further decrease in rotational mobility of C6SL. Thus the lipids seem to play at best a minor role in the immobilization of the C6SL spin labels in erythrocyte membranes after treatment with amino reactive agents. The lipids involved would be one or both of the amino phospholipids, phosphatidylserine and phosphatidylethanolamine, which together compose approximately 28 per cent of the lipids of the human erythrocyte membrane [25]. However, reaction with lipids alone cannot explain the strongly immobilized component ($\tau_0 \sim 10^{-7}$ sec) in the e.s.r spectra of C6SL spin-labeled erythrocyte membranes reacted with amino attacking compounds (Figs. 2b, 2c, 5a). It therefore appears that reaction of protein amino groups with reagents such as FDNB and TNBS plays a primary role in immobilizing the spin-labeled local anesthetics in the red cell membrane. The reaction of these chemical modifiers with membrane proteins has been further exemplified by the ability of these reagents to alter the e.s.r spectra of two protein spin labels covalently bound to red cell membrane sulfhydryl groups [47].

One other point of interest is that the rotational correlation time of C6SL in liposomes prepared from the total lipids of the red cell membrane (3.3×10^{-9} sec) is approximately 2/3 of the value obtained with C6SL in the presence of red blood cell ghosts (4.7×10^{-9} sec). A plausible explanation for the higher mobility of C6SL in the isolated lipids than in the erythrocyte membranes is that a small population of the spin-labeled local anesthetics are strongly bound to the membrane proteins. In fact, nanosecond lifetime measurements have shown a direct interaction of local anesthetics with membrane proteins [48]. Thus the e.s.r. spectrum of C6SL in the presence of erythrocyte membranes may be represented by two different components. The first component, containing a vast majority of the spin labels, is represented by a moderately immobilized signal arising from C6SL bound to the lipid components of the membrane. The second component, containing a small population of the spin labels, is represented by a strongly immobilized e.s.r signal arising from C6SL bound to the protein components of the membrane. Presumably then, the reaction of free membrane protein amino groups with reagents such as DFDNB, TNBS, and FDNB, increases the population of spin-labeled anesthetics bound to membrane proteins, and the strongly immobilized component in the e.s.r spectrum of C6SL becomes readily apparent (Figs. 2b, 2c and 5a).

Asymmetrical location of the strongly immobilized component. As was mentioned above, phosphatidylserine and phosphatidylethanolamine are the lipids which are capable of reaction with amino agents. A number of studies have shown that these phospholipids are distributed asymmetrically across the red cell membrane, residing almost completely in the inner half of the bilayer [49–53]. Therefore, any broadening of the C6SL e.s.r signal due to the action of FDNB, DFDNB, TNBS, MNT, or glutaraldehyde on these lipids most likely results from the action of these compounds on the inner surface of the membrane.

In addition, in a recent study the positions of protein amino groups in the red cell membrane were explored with the aid of two radioactive imidoesters [54]. One imidoester was isethionyl acetimidate which was unable to penetrate the erythrocyte membrane but had the same specificity for amino groups as ethyl acetimidate, which was able to penetrate the membrane. Double labeling experiments with these compounds showed that there were more than ten times as many reactive amino groups in protein on the inner surface than on the outer surface of the membrane. Thus it seems probable that the strongly immobilized component in the C6SL e.s.r signal that develops after reaction of red cell membranes with amino reagents, whether due to reaction with the lipids or the proteins, arises from the inner surface of the membrane. This interpretation may be compared with the finding by Sheetz and Singer [55] that the tertiary amine local anesthetics are cup-formers of the intact erythrocyte. They proposed that local anesthetics and other cationic drugs bound preferentially to the inner half of the red cell bilayer and attributed the differential distribution of the local anesthetics to interactions with acidic phosphatidylserine molecules concentrated in the cytoplasmic half of the membrane. Our results, combined with those of Whitely and Berg [54], indicate that after treatment of red cell membranes with amino reagents, a population of spin-labeled anesthetics also bind strongly to proteins located at the cytoplasmic membrane surface. It is tempting to speculate that the strongly immobilized C6SL molecules bound to proteins of the unmodified erythrocyte membrane are also found at the cytoplasmic surface. The asymmetric distribution of local anesthetics in the nonexcitable red cell membrane may be directly compared to the asymmetric action of local anesthetics in nerve membranes [2, 3], and suggests that the red cell membrane, whose lipid and protein composition are very well characterized, will prove very useful in future studies for determining the exact molecular nature of local anesthetic binding sites.

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