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Bis(sulfo-*N*-succinimidyl) Doxyl-2-spiro-5′-azelate: Synthesis, Characterization, and Reaction with the Anion-Exchange Channel in Intact Human Erythrocytes†

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ABSTRACT: We have synthesized and characterized bis(sulfo-*N*-succinimidyl) doxyl-2-spiro-5′-azelate (BSSDA), a membrane-impermeant bifunctional spin-labeling reagent. BSSDA is a nine carbon backbone homologue of bis(sulfo-*N*-succinimidyl) doxyl-2-spiro-4′-pimelate [BSSDP; Beth et al. (1986) *Biochemistry 25*, 3824–3832]. Due to its longer backbone, BSSDA can span longer distances between reactive groups on a protein than can BSSDP. However, the purpose of the bifunctional design of these reagents is to provide a tight motional coupling of the spin labels to the surface of a target protein. To test whether the longer backbone of BSSDA results in a greater local flexibility and thereby undermines the effects of bidentate attachment, we have labeled with BSSDA anion-exchange channels of *intact* human erythrocytes at the same site as we have previously labeled them with BSSDP. Linear and saturation-transfer EPR spectra of BSSDA-labeled anion-exchange channels in intact cells closely approximate the corresponding spectra from BSSDP-labeled channels. Thus, the longer backbone of BSSDA relative to BSSDP does not give rise to significant local flexibility, even when BSSDA is bound to a site that can be spanned by the shorter reagent.

Biophysical techniques including EPR[1] spectroscopy have been utilized to characterize the dynamic properties of membrane proteins in a number of systems over the past decade. In order to carry out EPR measurements, it is ordinarily necessary to introduce a spin-label reporter group into the protein of interest, since most proteins lack endogenous paramagnetic centers. If the experimentalist is interested in the rotational diffusion coefficient of a membrane protein, a parameter that can provide insight into such diverse properties as state of oligomerization, interactions with other membrane proteins, and inherent flexibility, it is necessary to introduce a spin-label probe that will accurately monitor the motion of the protein to which it is covalently attached or noncovalently bound. Thus, the approach to be followed is to design a spin label that will exhibit limited independent motion relative to

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[1] Abbreviations: BSSDA, bis(sulfo-*N*-succinimidyl) doxyl-2-spiro-5′-azelate; BSSDP, bis(sulfo-*N*-succinimidyl) doxyl-2-spiro-4′-pimelate; BS^3, bis(sulfo-*N*-succinimidyl) suberate; $NaDodSO_4$, sodium dodecyl sulfate; DIDS, 4,4′-diisothiocyanostilbene-2,2′-disulfonate; EPR, electron paramagnetic resonance; ST-EPR, saturation-transfer EPR; V_2', second harmonic out-of-phase absorption ST-EPR signal; HPLC, high-performance liquid chromatography; TLC, thin-layer chromatography; EI, electron impact; FAB, fast atom bombardment; NMR, nuclear magnetic resonance; IR, infrared.

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the protein to which it is attached.

One approach to limiting the independent motion of a spin-label reporter group has been to utilize bifunctional spin-labeling reagents that, due to their two-point attachment, have a high probability for immobilization on the target protein (Willingham & Gaffney, 1983; Beth et al., 1986; Anjaneyulu et al., 1988a). In order for the bifunctional strategy to succeed, the reactive groups employed must give rise to covalent adducts with the protein of interest in very high yields, so that the spectra of the modified protein are not dominated by monofunctional derivatives of the probe which have significant independent motion relative to the protein. We have employed sulfo-*N*-succinimidyl esters, reactive groups that combine high yields of reaction with nucleophilic groups in proteins and the useful property of membrane impermeance [reviewed in Staros (1988)], in a membrane-impermeant spin-labeling reagent, BSSDP[1] (Beth et al., 1986), a reagent that we have also prepared in isotopically substituted form (Anjaneyulu et al., 1988a). At low reagent concentrations, BSSDP has been shown to specifically modify the anion-exchange channel of *intact* human erythrocytes at a site that spans the extracytoplasmic chymotryptic cleavage site and overlaps the stilbenedisulfonate binding site (Beth et al., 1986, 1987; Anjaneyulu et al., 1988a). Linear and ST-EPR spectra of BSSDP-labeled erythrocytes suggest that the reagent is tightly motionally coupled to the anion-exchange channel (Beth et al., 1986; Anjaneyulu et al., 1988a). Here we describe the synthesis and characterization of BSSDA, a new member of the BSSDP class of membrane-impermeant bifunctional spin-labeling reagents. The backbone of BSSDA is two methylene groups longer than that of BSSDP but is otherwise identical. In order to test whether the increased backbone length of BSSDA relative to BSSDP will give rise to unacceptable independent motion, we have labeled the anion-exchange channel of *intact* human erythrocytes with BSSDA at the same site that we have previously labeled with BSSDP. A direct comparison of the ST-EPR spectra of intact erythrocytes labeled in parallel with BSSDA or BSSDP suggests that the longer backbone of BSSDA does not give rise to significantly greater local flexibility, even when BSSDA is bound to a site that can be spanned by the shorter reagent.

Experimental Procedures

Melting points were determined on a Thomas-Hoover capillary melting point apparatus and are uncorrected. IR spectra were recorded on a Perkin-Elmer 727 spectrophotometer. Proton NMR spectra were obtained on an IBM/Bruker NR-300 spectrometer. Mass spectra (70 eV) were obtained on a VG 70/250HF GC–MS instrument having extended geometry and equipped with a VG 11/250 data system and capability for fast atom bombardment. FAB mass spectrometry was carried out with an Ion-Tech B-11N gun with xenon as a source of fast atoms at 8 kV and 1 mA. Accurate mass measurements were made on averaged scans in the EI mode with the instrument set to a resolving power of 10000 and scanned at 10 s/decade. TLC analyses were carried out on E. Merck 60F-254 precoated silica gel plates with fluorescent indicator; spots were visualized under ultraviolet light or by iodine vapor. All reagents and solvents were of ACS certified grade or of a comparable or higher grade. Sodium methoxide, ethylene glycol, *p*-toluenesulfonic acid monohydrate, 2-amino-2-methyl-1-propanol, *m*-chloroperbenzoic acid (80–85%), and dicyclohexylcarbodiimide were obtained from Aldrich Chemical Co. Solvents were purified when necessary. *N*-Hydroxysulfosuccinimide was prepared from *N*-hydroxymaleimide as reported previously (Staros, 1982). BSSDP was prepared by modifications of previously published procedures (Beth et al., 1986; Anjaneyulu et al., 1988a).

1,7-Dichloroheptan-4-one (**2**) was synthesized from γ-butyrolactone (**1**) (Figure 1) following the reported procedure (Curtis et al., 1958) in 52% yield, bp 130–133 °C (7 mm). 1,7-Dichloroheptan-4-one (**2**) was converted to 5-ketoazelaic acid (**5**) by the procedure of Hartley (1962), modified as follows:

2,2-Bis(3-chloropropyl)-1,3-dioxolane (**3**). 1,7-Dichloroheptan-4-one (**2**) (164.6 g, 0.9 mol), benzene (500 mL), ethylene glycol (83.7 g, 1.35 mol), and *p*-toluenesulfonic acid (0.3 g) were heated under reflux with a Dean–Stark water trap between the flask and condenser. When no more water separated (35.6 mL collected), the solution was cooled, washed sequentially with water, a saturated sodium bicarbonate solution, and water, and then dried with sodium sulfate. The solution was then filtered and the solvent was removed on a rotary evaporator. The crude material was distilled under vacuum to give 156.4 g (77%) of the ketal **3**, bp 120–126 °C (1 mm).

2,2-Bis(3-cyanopropyl)-1,3-dioxolane (**4**). Sodium cyanide (103 g, 2.1 mol) was added to dimethyl sulfoxide (420 mL), and the slurry was heated to 90 °C with stirring. Heating was stopped and 2,2-bis(3-chloropropyl)-1,3-dioxolane (**3**) (158.5 g, 0.7 mol) was added dropwise. After an induction period, the temperature rose suddenly to 160 °C, at which it was maintained during the rest of the addition. Stirring was continued for 1 h more, and to the cooled mixture was added chloroform (420 mL). The resulting solution was added to saturated sodium chloride, the salt that precipitated was dissolved by adding water, and the chloroform layer was separated. The aqueous layer was extracted repeatedly with chloroform, and the combined extracts were washed with saturated sodium chloride and dried with magnesium sulfate. The chloroform was then removed on a rotary evaporator to give 123.1 g (85%) of the dicyano ketal **4**, mp 38–40 °C.

5-Ketoazelaic Acid (**5**). Hydrogen peroxide (45 mL, 30% solution) was added to a stirred suspension of 2,2-bis(3-cyanopropyl)-1,3-dioxolane (**4**) (104 g, 0.5 mol) in 30% potassium hydroxide solution (700 mL), and a stream of nitrogen was bubbled through the mixture. After 1 h of heating at 40 °C, the reaction mixture was refluxed for 3 h, cooled, and poured into a 40% phosphoric acid solution (700 mL). The resulting mixture was saturated with sodium chloride and was repeatedly extracted with ether. The combined extracts were concentrated by rotary evaporation, and the product was crystallized from ether to give 75.1 g (74%) of 5-ketoazelaic acid (**5**), mp 109–110 °C. The product gave a single peak (retention time = 5.4 min) when subjected to isocratic HPLC on an Alltech no. 6231 1.0 × 25 cm C-18 reverse-phase column with 50:50 (v/v) 10 mM ammonium formate, pH 3.5/methanol, with elution at 3.0 mL/min and detection at 253 nm: IR (Nujol) 2700 (carboxyl), 1710 cm^{-1} (carbonyl); NMR (D_2O) δ 1.65 (quintet, 4 H, 3,7-CH_2), 2.21 (t, 4 H, 2,8-CH_2), 2.43 (t, 4 H, 4,6-CH_2); mass spectrum *m/z* (rel intensity) 202.0836 (0.9) (calcd for $C_9H_{14}O_5$, 202.0841), 184 (3.7), 174 (2.4), 138 (3.7), 115 (100), 87 (63.2), 43 (37.2).

Diethyl 5-Ketoazelate (**6**). This compound was prepared following the general method for esterification of keto dicarboxylic acids (Stetter & Rauhut, 1958). 5-Ketoazelaic acid (**5**) (63.1 g, 0.31 mol), absolute ethanol (150 mL), benzene (300 mL), and concentrated hydrochloric acid (10 mL) were refluxed with stirring for 8 h, azeotropically removing water formed with a Dean–Stark apparatus. The cooled solution was stirred with sodium bicarbonate (30 g), filtered, concen-

trated on a rotary evaporator, and distilled under vacuum to give 71.5 g (89%) of diethyl 5-ketoazelate (**6**), bp 143–145 °C (1 mm). The product gave a single peak (retention time = 37.5 min) when subjected to reverse-phase HPLC as described for 5-ketoazelaic acid, above, and a single spot (R_f 0.56) on TLC in 19:1 benzene/methanol: IR (thin film) 1730 cm^{-1} (ester, carbonyl); NMR ($CDCl_3$) δ 1.20 (t, 6 H, ester CH_3), 1.84 (quintet, 4 H, 3,7-CH_2), 2.27 (t, 4 H, 2,8-CH_2), 2.43 (t, 4 H, 4,6-CH_2), 4.09 (quartet, 4 H, ester CH_2); mass spectrum m/z 258.1463 (calcd for $C_{13}H_{22}O_5$, 258.1467).

Doxyl-2-spiro-5′-azelaic Acid (**8**). Conversion of the keto function of **6** to a doxyl ring was carried out by modifications of previously published procedures (Keana et al., 1967; Hubbell & McConnell, 1971; Beth et al., 1986; Anjaneyulu et al., 1988a). Diethyl 5-ketoazelate (12.9 g, 50 mmol), *p*-toluenesulfonic acid monohydrate (100 mg, 0.53 mmol), 2-amino-2-methyl-1-propanol (45 g, 0.5 mol), and toluene (100 mL) were placed in a 250-mL three-neck flask equipped for anhydrous reflux and fitted with a Dean–Stark water trap between the flask and the condenser. The flask was purged with nitrogen, and the reaction mixture was refluxed for 7 days with constant stirring. After cooling, toluene was removed on a rotary evaporator. The unreacted amino alcohol was removed from the resulting thick orange oil by vacuum distillation. The resulting orange semisolid was dissolved in 200 mL of HPLC-grade methanol, and this solution was stored at room temperature for 2 days over anhydrous sodium carbonate (30 g). The drying agent was removed by filtration, and the solvent was removed on a rotary evaporator. The residue (18 g) was dissolved in diethyl ether (100 mL), and the solution was filtered. To the filtrate was added *m*-chloroperoxybenzoic acid (14 g, 81 mmol) in diethyl ether (50 mL) dropwise at 5 °C. The oxidation was allowed to continue for 36 h, and a bright green color developed. The reaction mixture was washed twice with ice-cold 10% w/v sodium bicarbonate (100 mL each wash), and the ether layer was concentrated by removal of the solvent under reduced pressure. The residue was dissolved in benzene and was subjected to chromatography on a column (3.0 × 20 cm) of silica gel (60–200 mesh). The column was eluted successively with benzene (100 mL), diethyl ether (100 mL), 70:30 diethyl ether/butanol (100 mL), and methanol, and the separation was monitored by C-18 reverse-phase HPLC. The solvent from each of the fractions containing the protected doxyl-2-spiro-5′-azelate **7** was removed under reduced pressure. The residue was saponified by dissolving each fraction in methanol (20 mL), followed by dropwise addition of 20% sodium hydroxide (5 mL) with stirring. After 48 h, the reaction was terminated by removing the solvent under reduced pressure and adjusting the pH of the aqueous layer to 3.5 with HCl. The diacid produced was extracted with ethyl acetate (3 × 25 mL), and the combined extracts were dried over sodium sulfate. After removal of the ethyl acetate, the sample was dissolved in aqueous 10 mM ammonium formate, pH 4.0/methanol (70:30 v/v) and purified by isocratic HPLC in the same buffer, on an Altex no. 256-04 × 25 cm C-18 reverse-phase column, eluting at 2.0 mL/min. The major peak detected at 213 nm (retention time = 20 min) was collected, pooled, and concentrated by lyophilization. The solid (0.125 g) was dissolved in water, the pH was adjusted to 3.0 with dilute HCl, and the solution was extracted with ethyl acetate. The ethyl acetate was removed, and the sample was dried in a desiccator (in vacuo) over phosphorus pentoxide. A positive ion FAB mass spectrum (3-nitrobenzyl alcohol matrix) of the product showed a base peak at m/z 290, corresponding to the protonated hydroxylamine of **8** [$(M + 2H)^+$] (Sweetman & Blair, 1988). Loss of oxygen from this species or loss of CH_3 from the $(M + H)^+$ species resulted in a peak observed at m/z 274, while loss of $CH_2CH_2CH_2COOH$ resulted in a peak at m/z 202.

Bis(sulfo-N-succinimidyl) Doxyl-2-spiro-5′-azelate (**9**). BSSDA was synthesized by the general method for the synthesis of bis(sulfosuccinimidyl) esters of dicarboxylic acids (Staros, 1982; Staros & Anjaneyulu, 1989). Briefly, dicyclohexylcarbodiimide (0.18 g, 0.86 mmol), *N*-hydroxysulfosuccinimide sodium salt (0.17 g, 0.78 mmol), and doxyl-2-spiro-5′-azelaic acid (0.113 g, 0.39 mmol) in *N*,*N*-dimethylformamide (2 mL) were stirred at room temperature overnight and then at 4 °C for 3 h. Dicyclohexylurea was filtered out and washed with *N*,*N*-dimethylformamide (2 mL). The product was precipitated from the combined filtrates by addition of ethyl acetate (200 mL), and the product was collected by filtration and dried in vacuo to give a pale yellow solid, 0.181 g (67%). The positive ion FAB mass spectrum of the product revealed a peak at m/z 710 corresponding to sodiation of (i.e., addition of Na^+ to) the hydroxylamine (M + H) of Na_2BSSDA (calculated for $C_{21}H_{26}O_{16}N_3S_2Na_2$, 686). The corresponding negative ion spectrum showed a peak at m/z 664 corresponding to loss of Na^+ from the hydroxylamine of Na_2BSSDA. The fragmentation pattern was found to be analogous to those of the normal isotope and the isotopically substituted bis(sulfo-*N*-succinimidyl) doxyl-2-spiro-4′-pimelate (BSSDP) (Beth et al., 1986; Anjaneyulu et al., 1988a) and related sulfosuccinimidyl esters examined by FAB mass spectrometry (Anjaneyulu & Staros, 1987). We have also followed the hydrolysis of BSSDA by HPLC (Staros, 1982; Anjaneyulu & Staros, 1987), in which the only hydrolysis products detected were doxyl-2-spiro-5′-azelaic acid and *N*-hydroxysulfosuccinimide in a 1:2 molar ratio, identified by comparison with separately chromatographed standards.

Labeling of Human Erythrocytes with BSSDA with or without Prelabeling with [$^3H_2$]DIDS. Fresh blood from normal volunteers was drawn into heparinized Vacutainer tubes that were then chilled on ice. (All subsequent steps were performed at 0–4 °C, except as noted below.) Plasma proteins and the buffy coat were removed by aspiration after low-speed centrifugation to pellet the erythrocytes. The packed erythrocytes were washed twice with 5 mM sodium phosphate buffer, pH 7.4, containing 150 mM sodium chloride (5PBS7.4). An aliquot of the washed erythrocytes was washed a third time with 106 mM sodium phosphate, pH 7.4. These cells were diluted to 50% hematocrit with an equal volume of 106 mM sodium phosphate buffer containing 100 μM BSSDA (50 μM final concentration) and were incubated for 15 min at room temperature. The reaction was quenched by adding 4 volumes of 106 mM sodium phosphate buffer containing 0.5% bovine serum albumin (BSA) and incubating at room temperature for an additional 30 min. The sample was cooled on ice and centrifuged. The labeled erythrocytes were washed three times with the 106 mM sodium phosphate buffer, and the EPR spectrum was recorded on these packed cells.

Another aliquot of erythrocytes washed with 5PBS7.4 was washed twice with 150 mM sodium bicarbonate, pH 9.0. These cells were diluted to 50% hematocrit with bicarbonate buffer containing 100 μM [$^3H_2$]DIDS (50 μM final concentration) and incubated for 1 h at 37 °C. Four volumes of bicarbonate buffer containing 0.2% BSA was added to the cell suspension, which was incubated at room temperature for 30 min. The sample was cooled on ice and centrifuged, and the packed cells were washed twice with 106 mM sodium phos-

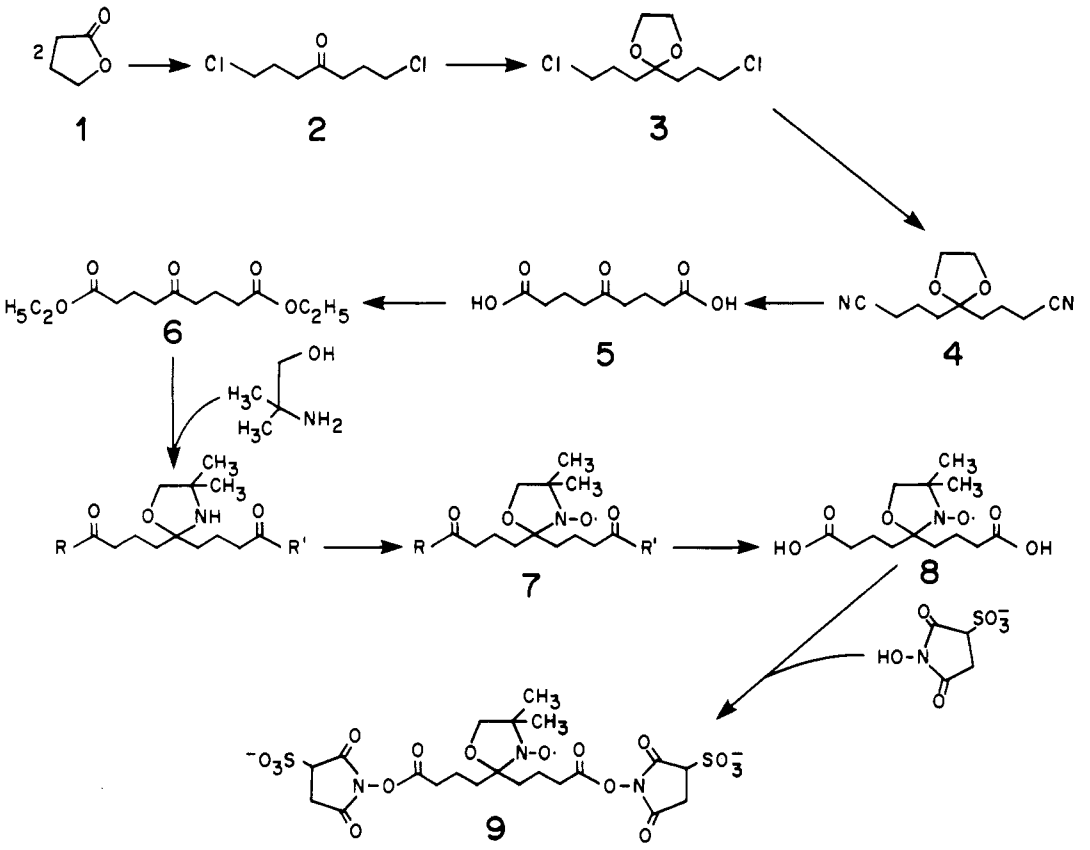

FIGURE 1: Reaction scheme for synthesis of bis(sulfo-*N*-succinimidyl) doxyl-2-spiro-5′-azelate. γ-Butyrolactone (**1**) was converted to 1,7-dichloroheptan-4-one (**2**) by reaction with sodium methoxide followed by hydrogen chloride. The ketone function was protected by forming the ketal **3** with ethylene glycol. The chlorines were displaced by reaction of **3** with sodium cyanide in dimethyl sulfoxide to give **4**. The ketone function was deprotected, and the cyano groups were hydrolyzed by treatment with hydrogen peroxide and 30% aqueous potassium hydroxide to give 5-ketoazelaic acid (**5**). The carboxylic acid groups were protected by formation of the diethyl ester **6**. The doxyl ring was formed by condensation of 2-amino-2-methyl-1-propanol, followed by oxidation of the resulting oxazolidine with *m*-chloroperbenzoic acid. Base-catalyzed hydrolysis of the protected doxyl-2-spiro-5′-azelate **7** led to doxyl-2-spiro-5′-azelaic acid (**8**). BSSDA (**9**) was prepared from **8** by dicyclohexylcarbodiimide-mediated coupling of *N*-hydroxysulfosuccinimide to each of the carboxylate groups.

phate buffer, pH 7.4. The packed cells were labeled with 50 μM BSSDA as described in the preceding paragraph, and the EPR spectrum was recorded.

The BSSDA and [$^3H_2$]DIDS/BSSDA-labeled erythrocytes were lysed by rapid dilution into 15 volumes of ice-cold 5 mM sodium phosphate buffer, pH 8.0 (5P8.0), and the ghost membranes were pelleted by centrifugation. The ghosts were resuspended and washed with 5P8.0 until the pellets were white (three washes). Samples of the pelleted ghosts were analyzed on a 4–12% NaDodSO$_4$–polyacrylamide gradient gel (Laemmli, 1970). HPLC separation of erythrocyte membrane proteins was effected with a TSK-4000-SW column (7.5 × 600 mm) as described previously (Beth et al., 1986).

EPR and ST-EPR Measurements. EPR and ST-EPR spectra were recorded with a Varian E-112 spectrometer equipped with an E-238 high-volume aqueous cavity, as previously described (Beth et al., 1986; Anjaneyulu et al., 1988a).

Results and Discussion

Preparation and Characterization of BSSDA (**9**). The reaction scheme used for the synthesis of BSSDA is shown in Figure 1. γ-Butyrolactone (**1**) was converted to 1,7-dichloroheptan-4-one (**2**) and then to the ethylene ketal **3**. The dichloro ketal **3** on treatment with sodium cyanide gave the dinitrile **4**, which was hydrolyzed to 5-ketoazelaic acid (**5**) and esterified with ethanol to **6**. The purity and identity of 5-ketoazelaic acid (**5**) and diethyl 5-ketoazelate (**6**) were ascertained by analytical TLC, HPLC, IR, NMR, and mass spectrometry. The EI mass spectrum of diethyl 5-ketoazelate (**6**) is shown in Figure 2. The spectrum contained peaks at m/z 143 and 115, corresponding to cleavages α to the carbonyl group. The doxyl ring was formed by condensation of 2-amino-2-methyl-1-propanol with the keto function, followed by oxidation with *m*-chloroperbenzoic acid. HPLC analysis of the mixture indicated that it contained three compounds having an EPR signal. Similar observations have been noted previously in the preparation of the normal isotope as well as the isotopically substituted doxyl-2-spiro-4′-pimelic acid. These products have been identified by spectral methods and individual hydrolysis as doxyl-2-spiro-4′-pimelic acid as the dimethyl ester, a methyl ester amide, and a structurally related amide (Anjaneyulu et al., 1988a), presumably formed by transesterification (Pariza et al., 1983).

After saponification of intermediates **7**, the resulting doxyl-2-spiro-5′-azelaic acid (**8**), which was characterized by positive ion FAB mass spectral analysis, was converted to BSSDA (**9**) by a dicyclohexylcarbodiimide-mediated coupling of the diacid and *N*-hydroxysulfosuccinimide (Staros, 1982). The final product was characterized by positive ion and negative ion FAB mass spectral analysis and by analyzing the products of hydrolysis by HPLC.

EPR Spectrum of BSSDA-Labeled Erythrocytes. In the present study, we have investigated the reaction of BSSDA with intact human erythrocytes under the same conditions used in previous studies with BSSDP (Beth et al., 1986, 1987; Anjaneyulu et al., 1988a). As shown in Figure 3, upper, BSSDA at a concentration of 50 μM reacts with erythrocytes to yield an EPR spectrum characteristic of spin labels bound

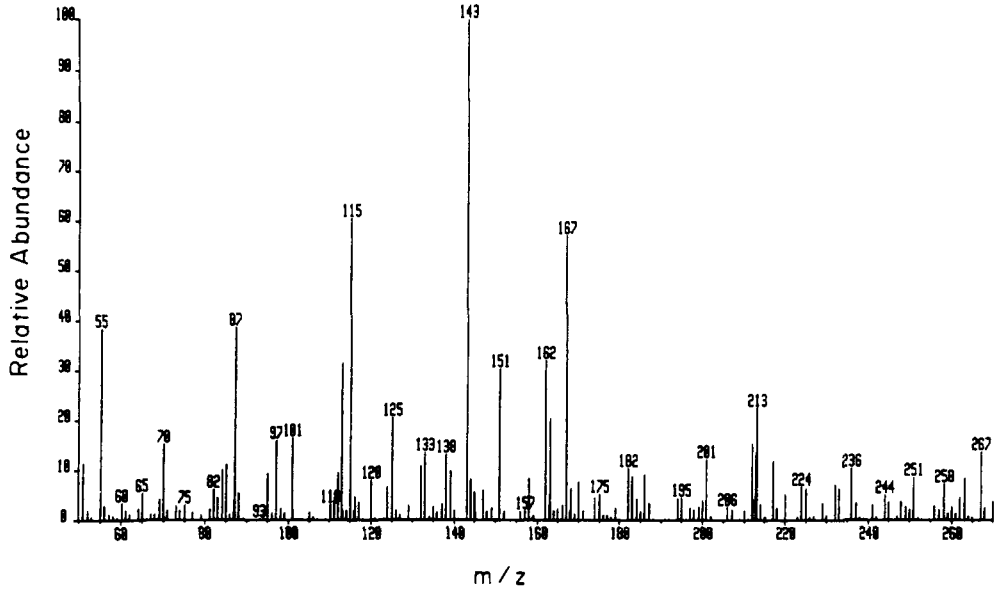

FIGURE 2: High-resolution mass spectrum of diethyl 5-ketoazelate. The spectrum is averaged and reference-subtracted (perfluoroalkane). The peaks at m/z 213, 212, and 167 arise by loss of C_2H_5O, C_2H_5OH, and C_2H_5O plus C_2H_5OH, respectively. The peaks at m/z 143, 115, and 87 are attributed to $[C_2H_5OCO(CH_2)_3CO]^{\cdot+}$, $[C_2H_5OCO(CH_2)_3]^{\cdot+}$, and $[C_2H_5OCOCH_2]^{\cdot+}$, respectively.

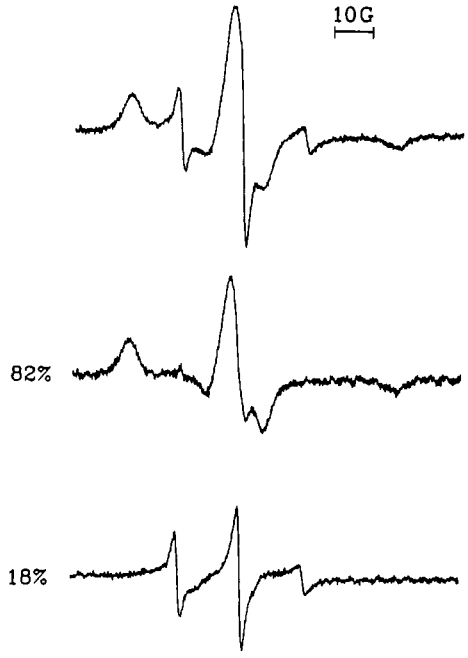

FIGURE 3: EPR spectrum and its components obtained from BSSDA-labeled erythrocytes. Erythrocytes were labeled with 50 μM BSSDA as described under Experimental Procedures. The EPR spectrum from intact erythrocytes (top) was digitally separated into slow (middle) and fast (bottom) rotational motional components. The component spectra were separately numerically integrated to quantitate the contributions of the slow (82%) and fast (12%) components to the composite spectrum.

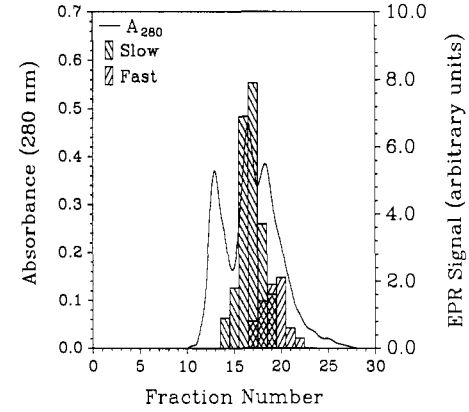

FIGURE 4: HPLC gel permeation chromatogram of erythrocyte ghosts labeled with 50 μM BSSDA. Erythrocyte ghost membranes were prepared from intact cells labeled with 50 μM BSSDA as described under Experimental Procedures. An aliquot of these ghosts ($\approx$3.0 mg of membrane protein) was solubilized in 1% $NaDodSO_4$ and was chromatographed on a 7.5 × 600 mm TSK-4000-SW column equilibrated with 100 mM sodium phosphate/0.3% $NaDodSO_4$, pH 6.8 (1 mL/min flow rate). Fractions (1.0 mL) were collected, and their EPR spectra were recorded by using identical instrument settings. The fast- and slow-motion components were separated by digital subtraction, and the spectra were integrated to obtain values for EPR signal intensity, which are shown as bars superimposed on the A_{280} chromatographic profile (solid line).

in two distinct motional environments. In repeated experiments, between 75% and 85% of the erythrocyte-associated spin label reacted with a site that resulted in its complete immobilization on the linear EPR time scale (Figure 3, center; $\tau_r \geq 0.3$ μs), while the remaining spin label reacted with a site that resulted in only partial immobilization (Figure 3, lower; $\tau_r \approx 2$ ns). HPLC separation of erythrocyte membrane proteins by size exclusion on a TSK-4000 column (Figure 4, solid line) indicated that these two signals could be partially resolved and that the slow EPR signal cochromatographed with the anion-exchange channel as revealed by $NaDodSO_4$–polyacrylamide gel electrophoresis of the column fractions (Figure 5). These observations closely parallel those reported previously with the shorter bifunctional reagent BSSDP (Beth et al., 1986).

Demonstration of an Intrachain Reaction Product with BSSDA. Control untreated and BSSDA-treated intact erythrocytes were subjected to mild chymotryptic digestion (Steck et al., 1976) to cleave the anion-exchange channel in its extracellular domain. Chymotrypsin treatment of intact erythrocytes has been shown to result in a unique cleavage of the anion-exchange channel into $M_r \approx 38\,000$ and $M_r \approx 58\,000$ peptides, both of which remain firmly anchored in the membrane (Steck et al., 1976). As shown in Figure 6, the anion-exchange channel is nearly quantitatively cleaved by chymotrypsin in control erythrocytes (lane 3). However, pretreatment of erythrocytes with 50 μM BSSDA results in $\approx$90% of the copies of the anion-exchange channel continuing to migrate

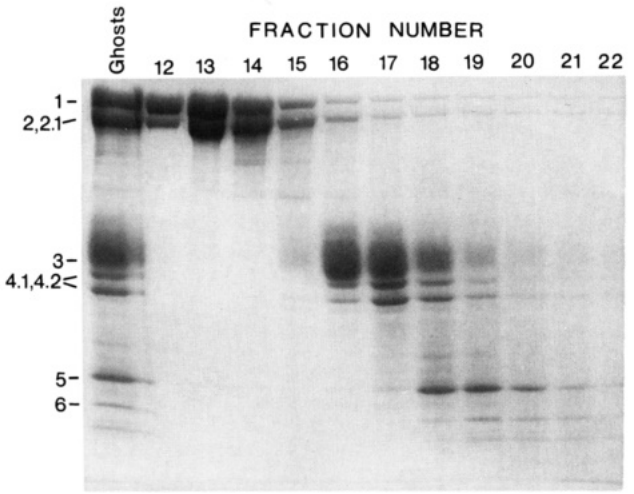

FIGURE 5: $NaDodSO_4$–polyacrylamide gel electrophoresis of HPLC TSK-4000 column fractions. Aliquots (45 μL) of fractions 12–22 from the HPLC TSK-4000 column separation shown in Figure 4 were subjected to electrophoresis in a 4–12% T gel (Laemmli, 1970) BSSDA-labeled ghosts (10 μL, 35 μg of protein) were applied in the left lane, and the protein bands are labeled according to Fairbanks et al. (1971).

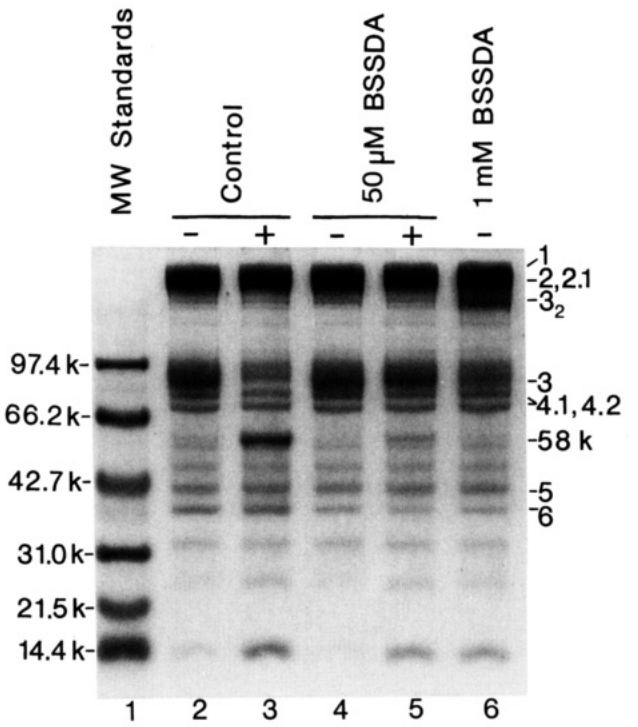

FIGURE 6: $NaDodSO_4$–polyacrylamide gel electrophoresis of BSSDA-labeled and control erythrocyte ghosts with and without α-chymotrypsin treatment. Samples of control and 50 μM BSSDA-labeled intact erythrocytes were treated with 100 μg/mL α-chymotrypsin (Sigma) at 37 °C for 1 h, followed by hemolysis in 5 mM sodium phosphate, pH 8.0. Intact erythrocytes were also labeled with 1 mM BSSDA under identical conditions as employed for the 50 μM BSSDA labeling. The $NaDodSO_4$–polyacrylamide gel electrophoresis procedure was essentially the same as that described by Fairbanks et al. (1971) except that the electrode buffer contained 0.1% $NaDodSO_4$ instead of 1.0% $NaDodSO_4$. Molecular weight standards (Bio-Rad) were run in lane 1, and the bands were labeled with their respective molecular weights. Erythrocyte ghost protein bands are labeled on the right (Fairbanks et al., 1971), with the addition of a band labeled 3_2 corresponding to the BSSDA cross-linked dimer of band 3 (the anion-exchange protein) and a band labeled 58 k corresponding to the $M_r \approx 58\,000$ chymotryptic cleavage product of the anion-exchange protein.

as the $M_r \approx 95\,000$ monomer following chymotryptic cleavage (lane 5), indicating that, like BSSDP (Beth et al., 1986), BSSDA at this concentration forms in high yield an intrachain cross-link that spans the chymotryptic cleavage site.[2] Additionally, BSSDA like BS^3 (Staros & Kakkad, 1983) efficiently forms an intersubunit cross-link of anion channel monomers at high reagent concentrations (lane 6).

Effect of $[^3H_2]$DIDS Prelabeling on BSSDA Reaction with BSSDA. Previous work with non-spin-labeled BS^3 (Staros & Kakkad, 1983; Jennings & Nisknish, 1985) as well as with the seven carbon backbone spin-labeling reagent BSSDP (Beth et al., 1986) indicated that these membrane impermeant reagents react with high affinity with the extracellular domain of the anion-exchange channel in intact human erythrocytes to form an intrasubunit cross-link that spans the unique extracellular chymotryptic cleavage site. This reaction site for BS^3 (Jennings & Nicknish, 1985) and for BSSDP (Beth et al., 1987) is the same as, or overlapping with, the reaction site for 4,4′-diisothiocyanostilbene-2,2′-disulfonate (DIDS), a potent inhibitor of anion transport in human erythrocytes (Cabantchik & Rothstein, 1974). In order to test whether BSSDA reacts with the DIDS site on the anion channel, we labeled intact erythrocytes with 50 μM $[^3H_2]$DIDS, washed away the unreacted label, and then labeled them with 50 μM BSSDA. The EPR spectrum obtained (Figure 7, upper) indicated that the highly immobilized signal observed in the absence of $[^3H_2]$DIDS prelabeling (Figure 3, center) was greatly diminished relative to the partially immobilized signal. Integration of the slow-motion EPR signal (Figure 7, center) following spectral subtraction indicated that this signal was reduced by 88% relative to labeling in the absence of $[^3H_2]$-DIDS, while the partially immobilized signal was approximately the same. $NaDodSO_4$–polyacrylamide gel electrophoresis of membrane proteins indicated that $[^3H_2]$DIDS labeling did not alter the mobility of membrane proteins, and a fluorograph of the dried gel indicated that band 3 was the only protein detectably labeled under these conditions (data not shown). Likewise, HPLC separation of membrane proteins indicated an elution profile similar to that observed in the absence of $[^3H_2]$DIDS labeling (Figure 8), and the ^3H cochromatographed with the anion channel, with very little slow-motion EPR signal recovered in the column fractions containing the anion channel. Thus, BSSDA labeling of the intrasubunit reaction site is effectively blocked by the anion channel specific stilbenedisulfonate inhibitor, suggesting that the two covalent probes are competing for the same, or an overlapping, binding site.

The observation that BSSDA competes for a site that is the same as, or overlaps, the stilbenedisulfonate site, together with the previous observation that BSSDP competes with stilbenedisulfonates in the same manner (Beth et al., 1987; Anjaneyulu et al., 1988a), suggests that BSSDA and BSSDP compete for the same site. Further, the absence of dipolar interactions (Figure 3) between BSSDA-modified sites under conditions in which ≈90% of all anion-exchange channel subunits are modified (see Figure 6 and text above) suggests

[2] It could be argued that modification of the intrasubunit site sterically blocks subsequent chymotryptic cleavage of anion-exchange channel subunits in intact cells. However, previous experiments, in which the intrasubunit site was cross-linked with 3,3′-dithiobis(sulfosuccinimidyl propionate), a cleavable analogue of BS^3, and was then treated with chymotrypsin, have demonstrated that cross-linking at this site does not block subsequent chymotryptic cleavage (Staros & Kakkad, 1983). Further, we have carried out a separate control experiment in which modification with BSSDA *followed* chymotryptic digestion and have observed that BSSDA ligates the two chymotryptic fragments (data not shown).

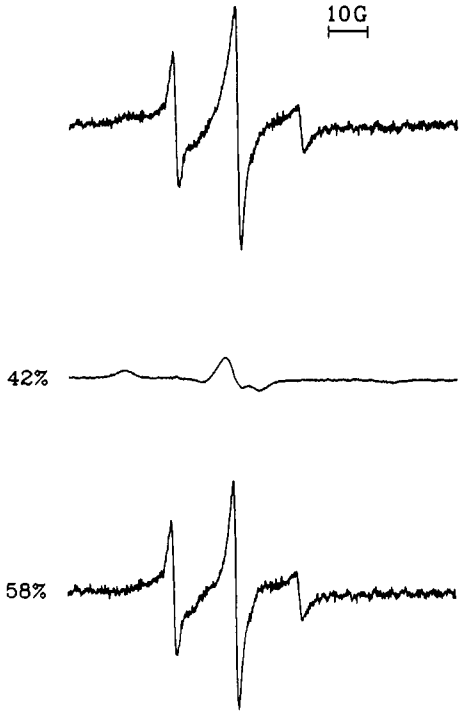

FIGURE 7: EPR spectrum of BSSDA-labeled erythrocyte ghosts prelabeled with [$^3H_2$]DIDS. Intact erythrocytes were labeled with 50 μM [$^3H_2$]DIDS in bicarbonate buffer, followed by labeling with 50 μM BSSDA in sodium phosphate buffer as described under Experimental Procedures. The resulting EPR spectrum (top) was digitally separated into slow (middle) and fast (bottom) rotational motional components, which were integrated to quantitate the contributions of the slow (42%) and fast (58%) components to the composite spectrum.

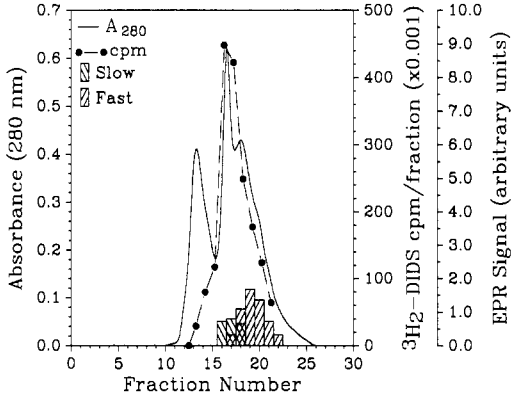

FIGURE 8: HPLC TSK-4000 gel permeation chromatogram of BSSDA-labeled erythrocyte ghosts prelabeled with [$^3H_2$]DIDS. An aliquot of $NaDodSO_4$-solubilized [$^3H_2$]DIDS/BSSDA-labeled ghosts (4.0 mg of protein; see Figure 7 and Experimental Procedures) was chromatographed on a TSK-4000-SW column under the same conditions as described in the legend to Figure 4. Fractions were collected and their EPR spectra recorded, the slow and fast motion components separated by digital subtraction, and the resulting spectra numerically integrated to obtain values for EPR signal intensity. The [$^3H_2$]DIDS was detected by liquid scintillation counting of aliquots of the fractions.

that the nitroxide moieties of the bound doxylazelate probes are >14 Å apart, as was shown previously for BSSDP-modified anion-exchange channels (Beth et al., 1986).[3]

BSSDA as a Probe for Anion Channel Rotational Mobility. Since the backbone of BSSDA is two methylene groups longer than that of BSSDP, it is important to ascertain whether the

[3] Studies with isotopically substituted [^{15}N,$^2H_{16}$]BSSDP suggest that the separation between nitroxide moieties bound to adjacent subunits is >16 Å.

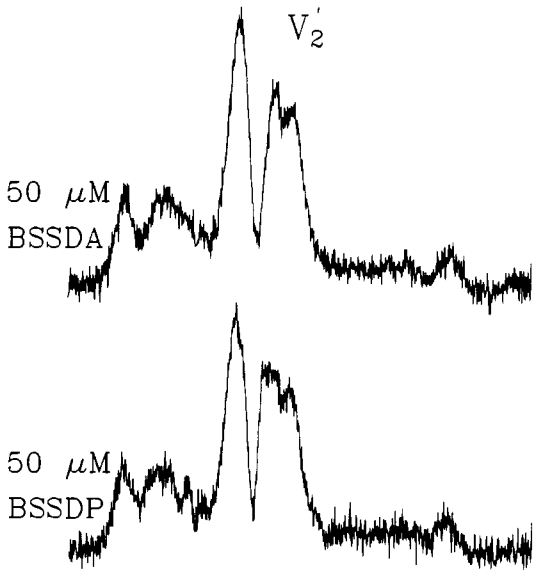

FIGURE 9: Saturation-transfer EPR spectra of BSSDA- and BSSDP-labeled erythrocytes. Intact erythrocytes were labeled with 50 μM BSSDA (upper) or 50 μM BSSDP (lower) as described under Experimental Procedures. The V_2' saturation-transfer EPR spectra shown were recorded at 20 °C as described previously (Beth et al., 1986). The spectral contribution from the partially immobilized signal has been digitally substracted from each spectral display in order to clearly visualize the V_2' signal from the spin-labeled anion-exchange protein.

longer reagent is motionally coupled to the anion channel and, thus, is useful as a probe for its rotational dynamics. To address this question, we carried out parallel ST-EPR measurements on BSSDA- (Figure 9, upper) and BSSDP- (Figure 9, lower) labeled intact erythrocytes. Overall, the two spectra are remarkably similar, indicating that any differences in effective mobility of the spin labels bound to the anion channel are small. The subtle differences in line shapes, which are most noticeable in the center of the ST-EPR spectrum, could be due to a slightly different orientation of the spin label relative to the membrane normal axis of this transmembrane protein (Beth & Robinson, 1989). However, on the basis of observed results, it appears that the additional length of BSSDA relative to BSSDP does not result in significant mobility of the label relative to its anion channel reaction site and, therefore, it should also serve as a reliable probe for anion channel rotational dynamics.

Applicability to Other Proteins. These results also have important implications for other applications of bifunctional spin-labeling reagents such as BSSDA and BSSDP. It would appear from the data shown in Figure 9 that tight motional coupling of spin label to protein can be achieved, even when the experimentalist employs a reagent with a slightly longer backbone than is necessary to span the distance between the two nucleophilic residues at the site of labeling. This flexibility in labeling geometry should help increase the variety of proteins for which BSSDA and BSSDP could be applied as useful spin labels.

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Interaction of Melittin with Mixed Phospholipid Membranes Composed of Dimyristoylphosphatidylcholine and Dimyristoylphosphatidylserine Studied by Deuterium NMR

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ABSTRACT: The interaction of bee venom melittin with mixed phospholipid bilayers composed of dimyristoylphosphatidylcholine deuterated in the α- and β-methylenes of the choline head group (DMPC-d_4) and dimyristoylphosphatidylserine deuterated in the α-methylene and β-CH positions of the serine head group (DMPS-d_3) was studied in ternary mixtures by using deuterium NMR spectroscopy. The changes in the deuterium quadrupole splittings of the head-group deuteriomethylenes of DMPC-d_4 induced by DMPS in binary mixtures [DMPC-d_4:DMPS (80:20 mol/mol)] were systematically reversed by increasing concentrations of melittin, so that at a melittin concentration of 4 mol % relative to total lipid the deuterium NMR spectrum from DMPC-d_4 in the ternary mixture was similar to the spectrum from pure DMPC-d_4 bilayers. This concentration of melittin is sufficient to neutralize the excess negative charge from DMPS. The absence of deuterium NMR signals arising from melittin-bound DMPS in ternary mixtures containing DMPS-d_3 indicates that the reversal by melittin of the effects of DMPS on the quadrupole splittings of DMPC-d_4 results from the response of the choline head group to the net surface charge rather than from phase separation of melittin–DMPS complexes. In mixtures containing deuterated DMPS [DMPC:DMPS-d_3 (50:50 mol/mol)] melittin caused systematic changes in the quadrupole splittings of the DMPS head-group deuterons that closely matched effects observed for a cationic transbilayer polyleucyl peptide ($K_2GL_{20}K_2A$) in similar ternary mixtures [Roux, M., Neumann, J. M., Hodges, R. J., Devaux, P. F., & Bloom, M. (1989) *Biochemistry 28*, 2313-2321]. The similarity in the effects of the two cationic but otherwise dissimilar peptides indicates that the DMPS head group responds to the surface charge resulting from the presence in the bilayer of charged amphiphiles, in a manner analogous to the response of the choline head group of phosphatidylcholine to the bilayer surface charge. The presence of DMPS greatly stabilized DMPC bilayers with respect to melittin-induced micellization, indicating that the latter effect of melittin may not be important for the hemolytic activity of the peptide.

Bee venom melittin (Habermann & Jentsch, 1967) is a basic, hydrophobic peptide of 26 amino acids that binds to model and cell membranes, causing their lysis through as yet unknown mechanisms. Melittin associates with membranes as an amphiphilic α-helix; the aggregation state of the peptide and the orientation of the helix with respect to the membrane remain undetermined [see Altenbach and Hubbel (1988) for a recent discussion]. In model membranes, melittin induces

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